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# Anti-inflammation effect of *Apium graveolens* Extract against lead-acetate-induced brain injury in rats

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## ABSTRACT

**Purpose:** The current study investigated the protective potential of *Apium graveolens* extract (APE) against lead-induced brain injury in rats by exploring anti-inflammatory and antiapoptotic mechanism. **Methods:** Twenty male Wistar rats were randomly allocated into four groups (n=5). The control group was orally administrated with distillate water. The second group received lead acetate 200mg/kg body weight orally for 14 days, the third group were orally administered lead acetate 200 mg lead acetate/kg body weight and vitamin E 50IU/kg body weight for 14 days. The fourth group was administrated with leas acetate like second group and APE 300mg/kg body weight for 14 days. The TNF-a levels and caspase-3 expression was analyses under ELISA and flow cytometry assay, respectively. **Results:** The phytochemical analysis of APE indicated the presence of alkaloids, flavonoids, tannins, saponins, and steroids. Leads acetate increased the serum levels of TNF-α and caspase-3 expression, as well as altering the brain tissue architecture. **Conclusion:** In conclusion, the presence of APE inhibited the lead acetate toxicity by inhibition of TNF-α proinflammation protein and caspase-3 proapoptosis protein.

**Keywords:** *Apium graveolens* extract, TNF-α, Caspase-3, Lead acetate, brain injury

## INTRODUCTION

Lead (Pb) is a toxic metal that induces a wide range of behavioral, biochemical and physiological effects in humans (1). Lead exposure is estimated to account for 143 000 deaths per year with the highest burden in developing regions (2). Importantly, no lowest safe concentration exists for lead acetate, which contributes to 0.6% of the global burden of disease (3,4). Furthermore, lead exposure tends to be sub-acute, produces only subtle clinical symptoms (4). In addition, chronic exposure cases are more common than acute toxicity (5,6). Lead via gastrointestinal absorption is first taken up by the red blood cells and is distributed to all vascular organs including brain (7,8). Previous study reported that lead cause oxidative stress by generating the release of reactive oxygen species (ROS) leading to secretion of inflammatory cytokine such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNF-α) (9).

Overexpression TNF-α induces the apoptosis of brain cells by Fas-associated proteins with death domain (FADD) signaling, resulting in caspase-3 activation and promote cell death (10,11). Previous study reported that, antioxidant compound could prevent the brain injury due to chronic lead exposure (12,13). The *Apium graveolens* herbs have several secondary metabolites that have significant anti-inflammatory activity (14). Therefore, treatment with natural compound such as *Apium graveolens* extract may be the potential strategy for inhibiting brain injury due to lead acetate exposure.

A number of natural compounds produced from vegetation offer alternative healthcare options that are more effective and safe (15–17). *Apium graveolens* is a species of herb that has several common names, including celery, parsley, and leek. *Apium graveolens* contains a several of phytochemical compounds, such as flavonoids and phenolic acids (18,19). Flavonoids in *Apium graveolens* inhibits the activity of proinflammatory cytokine including IL-6 and TNF-a by blocking ROS expression (20). Previous study also reported that the antioxidant activity of *Apium graveolens* extract prevent cell death through inhibition pro-apoptotic protein (21,22). However, no evidence supporting a favourable role of *Apium graveolens* extract (APE) in the regulation of TNF- α level and caspase-3 expression in the lead-acetate-induced brain injury still unclear. Therefore, in this present study aims to evaluate the effect of APE on the regulation of TNF- α level and caspase-3 expression in the lead-acetate-induced brain injury in rats models.

## MATERIAL AND METHODS

### *Material and study design*

This post-test only control group study design was conducted in Stem Cell and Cancer Research (SCCR) Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang from June – August 2022.

The study was approved by the Ethic Committee of Sultan Agung Islamic University (No. 290/VIII/2022/Komisi Bioetik).

### *Extraction of Apium graveolens extract*

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples collected from healthy *Apium graveolens* herbs were collected from Tawangmangu in Central Java Indonesia in August 2022 (Latitude -7.665158 and Longitude 111.129500). They were rinsed with tap water followed by distilled water to remove the dirt on the surface. The dried *Apium graveolens* herbs was blended until small pieces and sieved with a mesh size of 120 mesh. The 100 g of *Apium graveolens* herbs was extraction in a maceration apparatus with 1000 mL 98% ethanol for 24 h. The filtrated was then evaporated under rotary vacuum evaporator (IKA) and the crude extract west kept in refrigerator 4 $^{\circ C}$ (17,23). The *Apium graveolens* crude extract was dissolved in CMC-Na suspension based until the extract concentration was 60mg/mL. The formulations were stored at 4 $^{\circ C}$ until further analysis (24–26).

### *Phytochemical screening of Apium graveolens extract*

The *Apium graveolens* crude extract (APE) were tested for the presence of flavonoids, alkaloids, tannins, steroids, terpenoids, and saponins. The qualitative results are expressed as (+) for the presence and (−) for the absence of phytochemicals. The flavonoids were test using Wilstater's test according to (27). Briefly, 2 mg of APE was mixed with HCl 500µL and 0.02 mg magnesium. The presence of flavonoids is characterized by the occurrence of discoloration. The presence of alkaloids indicated with a brown colored precipitate that determined under Wagner's test, 15 mg of APE was stirred with 1% HCl (6 mL) on water bath for 5 minutes and filtered. The filtrate was added with few drops of Wagner solution (2 gram of Potassium iodide and 1,27 g of Iodine in 95 mL of distilled water) (28). Furthermore, Tannins content was analysis the APE with 1% ferric chloride, the black or blue coloration was taken a

positive result of tannins (29). Liebermann-Burchard test was used to determine the presence of steroids and terpenoids, briefly 100 mg of APE was shaken with chloroform and added the few drops of acetic anhydride was added to the test tube and boiled in a water bath and rapidly cooled in iced water. Concentrated $H_2SO_4$ (2 mL) was added alongside of the test tube. Formation of a brown ring at the junction of two layers and turning the upper layer to green shows the presence of steroids while formation of deep red color indicates the presence of triterpenoids (30). The saponin presence was analysis under Forth's test, 500 mg of APE was shaken with 10 mL of distilled water. The formation of frothing, which persists on warming in a water bath for 5 min, shows the presence of saponins (30).

### *Total flavonoid content of Apium graveolens extract*

Total flavonoid content was determined using the aluminum colorimetric method (31) with some modifications using quercetin as the standard. A calibration curve of quercetin was prepared in the range of 200 - 700µg/mL. Briefly, extract (0.5 mL) and standard (0.5 mL) were placed in different test tubes and to each 10% aluminum chloride (0.1 mL), 1 M potassium acetate (0.1 mL), 80% methanol (1.5 mL) and distilled water (2.8 mL) were added and mixed. A blank was prepared in the same manner where 0.5 mL of distilled water was used instead of the sample or standard, and the amount of aluminum chloride was also replaced by distilled water. All tubes were incubated at room temperature for 30 min. The absorbance was taken at 415 nm. The concentration of flavonoid was expressed as mg quercetin equivalent (QE) per gram of extract (32,33).

### *Lead acetate poisoning animal model.*

Twenty male Wistar rats weighing (200-250) g were used for this study. The animals were housed in a temperature (25 ± 1 °C), humidity controlled room and a 12-h light-dark cycle. Rats were allowed free access to tap water and standard pellet diet. The institutional Animal Ethics Committee approved all experimental protocols. The animals were classified into 4 groups, each of 5 as follows:

Control groups (C): Rats received distilled water

Lead acetate-treated group (LA): Rats were orally administered lead acetate at a dose of 200 mg lead acetate/kg body weight, by stomach tube once daily for 14 days.

Lead acetate and vitamin E 50IU/kg body weight (reference drug) treated group (LA + 50IU/kg BW Vitamin E): Rats were orally administered lead acetate at a dose of 200 mg lead acetate/kg body weight and vitamin E 50IU/kg body by stomach tube once daily for 14 days.

Lead acetate and 300mg/kg body weigh APE treated group (LA + 300mg/kg BW APE): Rats were orally administered lead acetate at a dose of 200 mg lead acetate/kg body weight and 300 mg/kg APE/body weight by stomach tube once daily for 14 days.

At the end of experiment, fasting blood samples were withdrawn from the retro-orbital vein of each animal using a glass capillary tube after fasting period of 12 h. The blood samples allowed to coagulate and then centrifuged at 3000 rpm for 20 min. The separated sera were used for the estimation of serum protein of TNF- α by using ELISA kits (Invitrogen, USA). Total serum protein was evaluated using kits from Biodiagnostic, Egypt. The brains was excised and

### *Analysis of TNF- α levels*

The blood of rats was harvested via periorbital venous plexus bleeding under general anaesthesia on days 14 and the serum was collected by centrifugation at 4 C. We measured TNF-α concentrations by enzyme-linked immunosorbent assay (ELISA) kits, based on the manufacturer's instructions (Invitrogen, USA) and according to a standard curve constructed for each assay. The colorimetric absorbance was recorded at a wavelength of 450 nm.

### *Analysis of Caspase-3 expression*

The immunohistochemistry processes were carried out with the Starr Trek Universal-HRP Detection Kit (Biocare Medical; cat.no BRR 700 AH, AL10) manufacturer's protocols. Briefly, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in ethyl alcohol with different concentrations. Non-enzymatic antigen retrieval was performed by heating the sections to 92-95 °C in citrate buffer (10 mM, pH 6.0) for 15 min and washed three times with PBS for 5 min. The samples were soaked with 3% H2O2 (Sigma-Aldrich) in PBS for 5 min to block endogenous peroxidase and subsequently treated with Background Sniper for 20 minutes for suppressing nonspecific binding. Afterwards, the slides were incubated with mouse anti-rat caspase-3 antibody (dilution, 1:200) at 4◦C overnight. The slides were washed the next day with Trekkie Universal Link incubation for 60 minutes. Then, the samples were incubated with Trek-avidin HRP Label for staining development. The samples were developed with diaminobenzidine (DAB) and counterstained with hematoxylin. After the slides were washed with distilled water and dehydrated, they were made transparent and mounted under a microscope for examination. Image-J software was employed to evaluate the mean optical density value of the images after immunohistochemical analysis.

### *Statistical analysis*

Statistical analyses were accomplished with software SPSS 26.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). Data analysis used Kruskal-Wallis and continued with the Man-Whitney test with p-value <0.05. P < 0.05 was considered to be statistically significant.

## RESULTS

### *Phytochemical screening of Apium graveolens extract*

The phytochemical screening of crude ethanolic extract of *Apium graveolens* revealed the presence of some secondary metabolites such as alkaloids, saponins, tannins, flavonoids, and steroids (Table 1). The total flavonoid content of APE was 14.30 ± 0,55 QE/g. These values are higher than total flavonoid content of the same family with methanol solvent (34).

**Table 1. Phytochemical screening of secondary metabolites from *Apium graveolens* extract**

| Chemical componenet | Name of the test | CTFE |
|---|---|---|
| Alkaloids | Wagner test | ++ |
| Flavonoids | Wilstater test | +++ |
| Tannins | Braemer's test | + |
| Saponins | Forth test | + |
| Steroids | Lieberman Burchardt test | + |
| Terpenoids | Lieberman Burchardt test | - |

### *The effect of Apium graveolens extract on TNF- α levels*

Lead toxicity caused a significant increase in proinflammatory cytokine TNF- α in blood when compared to the corresponding controls, $P<0.05$. Treatment of APE as well as vitamin E to lead-toxicity rats revealed a significant decrease in TNF- α levels as compared to lead treated animal, $P<0.05$ (Figure 1).

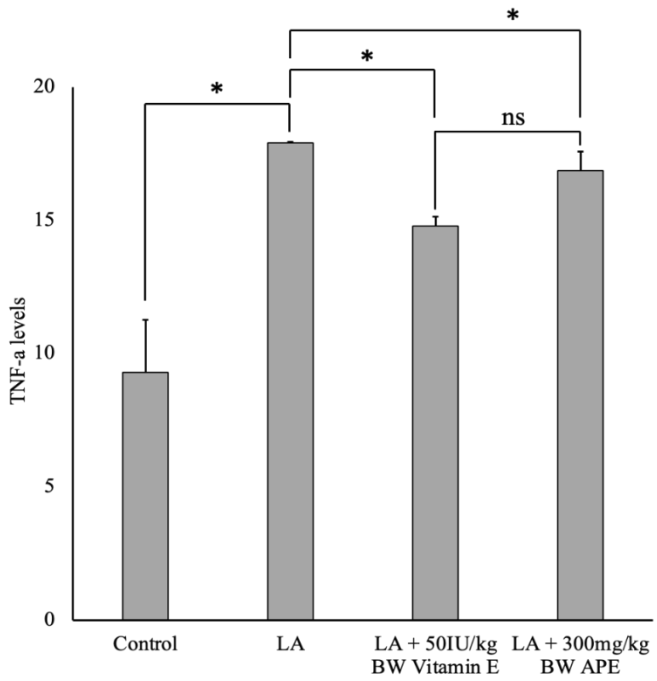


**Figure 1.** Effect of APE on blood TNF- α level in control (C), lead acetate (LA), LA + 50IU/kg BW Vitamin E, and, LA + 300mg/kg BW APE, treated groups. Data represent the mean ± S.E.M (n = 5). *: Differences were considered statistically significant at $p < 0.05$. ns: were considered statistically non-significant different at $p > 0.05$.

### *The effect of Apium graveolens extract on caspase-3 expression*

Lead toxicity caused a significant increase in pro-apoptotic protein caspase-3 in brain as compared to the corresponding controls, $P<0.05$. The LA groups showing severe immunopositivity in brain and the treated group with vitamin E and APE groups showing low immunopositivity in brain (Figure 2A). Treatment of APE as well as vitamin E to lead-toxicity rats revealed a significant decrease in caspase-3 expression as compared to lead treated animal, $P<0.05$ (Figure 2B).

## DISCUSSION

The brain is considered one of the target organs affected by lead toxicity owing to its storage in the brain after lead exposure (4,35). Lead exposure is affected in produces oxidative damage in the brain by generating reactive oxygen species leading to increase of pro-inflammatory cytokine and induced cell death (36,37). The present study was planned to investigate the anti-inflammatory and anti-apoptotic effects of APE against lead-induced brain injury in rats. Lead acetate and its metabolites induce the redox cycle, with the generation of superoxide radicals and hydrogen peroxide (38), which subsequently increases lipid peroxidation and decreases antioxidant enzyme activities, resulting in brain cell destruction, the activation of innate immunity by producing pro-inflammatory cytokines such as TNF-α (1,39). Lead damages many tissues through the induction of oxidative stress which induces inflammatory processes (40,41). Moreover, occupational exposure of humans to lead increases serum levels of some pro-inflammatory cytokines such as TNF- α. TNF-α is produced at the site of inflammation by activated macrophages and lymphocytes and participates with IL-1β and IL-6 to induce systemic inflammatory reactions (1,42). On the other hand, increased oxidative stress caused disturbances in mitochondrial membrane permeability, causing leakage of free radicals and cytochrome-c from the mitochondria to the cytosol (43). Once cytochrome-c is released into the cytosol, it binds to

another protein, Apaf-1, and promotes activation of the caspase cascade especially caspase-3, leading to cell death (26,44).

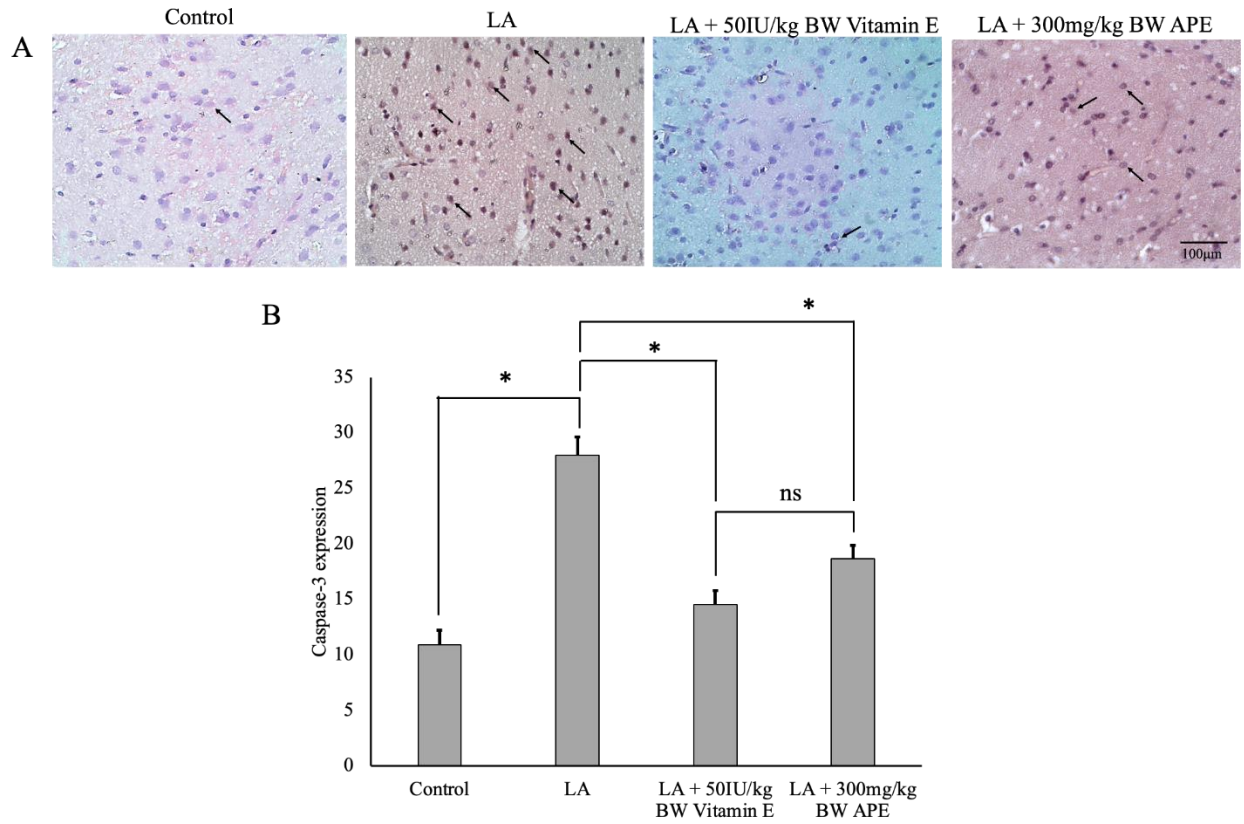


**Figure 2.** Effect of APE on brain caspase-3 expression in control (C), lead acetate (LA), LA + 50IU/kg BW Vitamin E, and, LA + 300mg/kg BW APE, treated groups. (A) Caspase-3-positive cells in brain were stained and visualized by immunohistochemistry. Black arrow indicated the caspase-3 positive. (B) Bar graph represents the number of caspase-3-positive cell indicating caspase-3 expression at 14 days after the lead acetate exposure. Data represent the mean ± S.E.M (n = 5). *: Differences were considered statistically significant at $p < 0.05$. ns: were considered statistically non-significant different at $p > 0.05$.

In the present study, the toxicity of rats with lead acetate in the current study increased the serum level of the pro-inflammatory cytokine TNF-α and pro-apoptosis protein caspase-3. Interestingly, the treatment with APE significantly decreased the TNF-α level in blood and caspase-3 expression in brain. Previous study reported that component of APE is flavonoids and alkaloids encourages antioxidant activity (20). Flavonoid compounds induced the release of antioxidant enzyme such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase through induction of nrf2 (45). Flavonoids scavenge free radicals effectively by forming semiquinone radicals which bind to free radicals to form a stable quinone structure (46). Flavonoid also inhibits the activation of phosphoinositide 3-kinase (PI3K) and activates protein kinase (MAPK) to induce the expression of antioxidant enzymes (47). The ability of flavonoid compounds as antioxidants has been shown to reduce oxidative stress conditions leading to inhibition of TNF-α levels (48,49). The low level of TNF-α inhibits the apoptosis of brain cell by inhibition Fas-associated proteins with death domain (FADD) signaling, resulting in caspase inactivation (11). Previous study also reported that the blocking of TNF-α production or TNF-α signaling pathways with caspase inhibitors reduces TNF-α-induced hepatic injury (50–52). Taken together, the presence of APE inhibited the lead acetate toxicity by inflammation and apoptosis inhibition.

## CONCLUSIONS

In this study, we concluded that the APE have ability to prevent brain injury in lead acetate toxicity by antiinflammation and antiapoptotic activity. Overall, the finding of this study could be beneficial for research on accelerating and directing the screening of possible targets and identifying the molecular mechanism of APE to prevents lead acetate toxicity.

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## CONTRIBUTORS

SS, HS, TS, and AP contributed to the conception of the work. NDA, HAS, FEH, ST, and C contributed to the acquisition of the work. SS and NDA contributed to the analysis and interpretation of data. AP, SS and NDA contributed to drafting the work. HS and TS contributed to revising the work critically. NDA contributed to the revising of the manuscript. SS and AP is responsible for giving the final approval of the manuscript.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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