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Protective effect of olive leaf (*Olea europaea* L.) extract against chronic exposure of liver and kidney tissues of Wistar rats to aluminum chloride

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ABSTRACT

Introduction: The liver and kidney are the main sites of aluminum (Al) accumulation. Lifetime exposure to significant amounts of Al is inevitable, hence its toxicity on the liver and kidney should be a health concern. Natural antioxidants have been proven to alleviate pathologies in various liver and kidney injuries. However, the effect of olive leaf extract (OLE) on Al-exposed animals is yet to be confirmed. This study aimed to investigate the OLE effect against $AlCl_3$ chronic exposure in rats' liver and kidneys.

Methods: Thirty-two male Wistar rats were divided into four groups (n=8), including the control group, the $AlCl_3$ group treated with 128 mg/kg $AlCl_3$ solution, as well as $AlCl_3$+OLE50, and $AlCl_3$+OLE100 groups (Other than $AlCl_3$ they received 50 and 100 mg/kg of OLE, respectively, 2 hours after $AlCl_3$ administration). All treatments were given orally for 12 weeks. All groups were evaluated for liver and kidney histopathological features, then scoring was performed.

Results: $AlCl_3$ administrations produced histopathological lesions in the liver and kidney, indicated by increased liver necro-inflammatory grades, ballooning scores, and renal inflammatory cell infiltration ($P<0.05$). OLE100 mg/kg significantly reduced liver necro-inflammatory grade, ballooning score, and kidney inflammatory cell infiltrations. The dose of 50 mg/kg also reduced these parameters ($P<0.05$), except for the liver necro-inflammatory grade. There was a significant correlation between OLE dose and liver necro-inflammatory grade and ballooning score amelioration.

Conclusion: OLE ameliorates liver and kidney histopathological features induced by oral Al chronic exposure in a dose-dependent manner.

Implication for health policy/practice/research/medical education:
Our findings justified the potential benefit of the leaf extract of the olive plant cultivated in Indonesia for protecting liver and kidney tissues against insults resulting from inevitable chronic exposure to aluminum chloride.

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Introduction

Aluminum (Al) is the 3rd most abundant metal on earth (1-3). Al from various sources, such as the surrounding environment, food, and workplaces, can enter the human body (4). Al is widely used in everyday life, such as vaccines, dialysis fluids (3), cooking utensils, cosmetics, and pharmaceuticals (4). Therefore, humans are at high risk of exposure to Al daily (5). Excessive Al exposure in the body causes toxicity to various organs, including the liver and kidneys (1). Al toxicity leads to physiological, biochemical, and structural damage to the liver and kidneys (1,6,7). It can provide diseases such as liver steatosis, while in the kidney, Al toxicity causes nephrotic syndrome and nephrotoxicity (3).

The underlying mechanism is still unclear, but many studies have demonstrated that ROS and oxidative stress

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are the leading causes of such damage (1,6-9). AI that enters the circulation can accumulate in various organs, especially the liver and kidney, which are the main sites for Al accumulation (6,10). It causes a functional and biochemical imbalance in the liver and kidneys, impacting their morphological composition and structure. A histopathological study is needed to evaluate the effect of treatment on tissue (11). Previous studies showed that Al toxicity causes histopathological damage to the liver and kidney (1,6,12,13).

Aluminum toxicity can be prevented by natural antioxidants, such as propolis (13), curcumin (14), resveratrol (15), garden cress (6), *Premna odorata* (7), and pursley (9). Antioxidants may prevent ROS formation and break up oxidative chains (16). The antioxidant benefits of bioactive substances in olive plants, notably the leaf, have been the subject of several investigations, (17-22). Olive leaves are rich in flavonoids and other polyphenols. Oleuropein is a polyphenol constituent found in the highest content in the leaves (23). Oleuropein has excellent antioxidant activity and can prevent metal poisoning (19). However, the effect of olive leaf (*Olea europaea* L.) extracts on liver and kidney toxicities due to Al exposure is yet to be confirmed. Therefore, this study was aimed at investigating the effect of olive leaf extract (OLE) on the histopathological features of the liver and kidney of the male Wistar rats chronically exposed to $AlCl_3$.

Materials and Methods

Chemicals and sample preparation

Olive leaves (*O. europaea* L.) were collected from Bogor, Indonesia and given a certificate of identification by Purwodadi Plant Conservation Center, Indonesia Science Institute, East Java, where the plant specimens were deposited in (No: B-32401III/KS.Ol.03/4/2021). OLE was made by the maceration method. The powdered dried olive leaves were steeped in 80% ethanol for 24 hours at room temperature and stirred periodically. Afterward, the solution was filtered and evaporated in a water bath to obtain the extract residue. The OLE was then refrigerated at 2-8°C until used (24). Anhydrous $AlCl_3$ was provided from Merck (KGsA, Darmstadt, Germany). $AlCl_3$ and OLE were dissolved in aquadest and made fresh every day.

Animal and experimental design

Thirty-two male Wistar strain rats of 6-7 weeks old (200 – 230 g) were kept at 23- 24 ºC on 12:12 hours light-dark cycle. They were fed and drank *ad libitum* for 2 weeks of adaptation in the animal house facility of Pharmacological Laboratory, Medical Faculty of Universitas Airlangga. Rats were randomly divided into four groups (n=8); control group was administered with 2 mL of aquadest, $AlCl_3$ group received 128 mg/kg BW $AlCl_3$ solution, $AlCl_3$+OLE50 group received 50 mg/kg BW of OLE and 128 mg/kg BW $AlCl_3$, and $AlCl_3$+OLE100 group received 100 mg/kg BW of OLE and 128 mg/kg BW $AlCl_3$. AlCl3 dose was determined according to a previous study (25). OLE was administered 2 hours after $AlCl_3$ administration. $AlCl_3$ and OLE were given orally through the gastric tube for 12 weeks.

Organ specimen preparation

After being given anesthesia with a ketamine + xylazine cocktail, the rats were terminated by cervical dislocation at the conclusion of the 12th week. Afterward, abdominal dissection was carried out to collect the rat's liver and kidney. All treatments were carried out carefully to keep the liver and kidneys from being damaged. Organs taken were fixed in 10% neutral buffer formalin for 24 hours. Tissue preparations were continued with dehydration, clearing, infiltration, and embedding, to make formalin fixed paraffin embedded (FFPE) tissue. Then, they were 4µ cut and stained with H&E (26).

Histopathological examination

Histopathological slides of the liver and kidneys were observed using Olympus BX53F2 and cellSens software at 200x and 400x magnification. Observations in a blinding way were performed in ten fields of view.

Scoring the damages

Liver damage was assessed using the METAVIR score for necro-inflammatory and NAFLD score for ballooning (27). Necro-inflammatory grading was scored as A0 (none), A1 (mild), A2 (moderate), and A3 (severe) based on the finding of the portal and parenchymal injuries. The ballooning was scored as 0 (none), 1 (few), and 2 (many). According to (28), the kidney damage was also scored. Kidney histopathology scoring was based on the findings of inflammatory cell infiltration, degeneration, and necrosis in cortical and medullary areas. The scores were 0 (none), 1 (0-10%), 2 (11-25%), 3 (26-45%), 4 (46-75%), and 5 (76-100%) for each finding.

Statistical analysis

For liver scoring, data were presented in a frequency graph. Statistical analysis was performed for liver necro-inflammatory grading and liver ballooning scoring using a nonparametric test of Kruskal-Wallis. The Mann-Whitney-U test was employed to compare between independent groups. Spearman's test was performed to analyze the correlation between the administration of OLE with liver score improvement. For kidney scoring, data were presented in mean ± SD. They were tested for normality and homogeneity with Shapiro Wilk and Lavene's tests. Since the data were not normal, Kruskal-Wallis and Mann-Whitney-U tests were performed for analysis. Statistical analysis was performed for kidney inflammatory cell infiltration parameter. The statistical result was considered significant if $P < 0.05$.

Results

Liver histopathology evaluation

The liver tissue of rats was evaluated to see any damage caused by chronic oral $AlCl_3$ exposure and the effect of OLE administration on histopathological features (Figure 1).

Control group: Figure 1A shows the typical liver histopathological feature of the healthy control group. It shows the normal central vein, radially arranged hepatocytes, and clearly defined hepatocyte plates. The cytoplasm of the cell is clear and pink in color, and round shaped, purple stained-nucleus is obviously seen. No inflammatory cell infiltration was found in this group.

$AlCl_3$ group: Figure 1B shows that $AlCl_3$ chronic exposure caused hepatic portal inflammatory cell infiltration, and hepatic parenchymal inflammatory cell infiltration.

$AlCl_3$+OLE50 group: Figure 1C shows that the $AlCl_3$+OLE50 group having histopathological features of mild inflammatory cell infiltration and vascular congestion. It shows pink and clear hepatocyte cytoplasm.

$AlCl_3$+OLE100 group: Figure 1D shows the histopathological feature of the $AlCl_3$+OLE100 group with normal central vein and clearly defined hepatocyte plates. It shows pink and clear cytoplasms and round shaped, purple stained cell's nuclei.

Based on the results of statistical analysis, the frequencies of liver's necro-inflammatory grading and ballooning scoring was shown in Table 1. There were significant differences in the necro-inflammatory grade and ballooning score of rat liver ($P<0.05$; Table 1). The comparison between two groups is shown to Table 2. There were significant differences in the necro-inflammatory grade and ballooning score between the control and $AlCl_3$ groups. The ballooning score, but not the necro-inflammatory grade between control and $AlCl_3$+OLE50 group was significantly lower than that of $AlCl_3$ group ($P<0.05$). $AlCl_3$+OLE100 group indicated a significantly lower necro-inflammatory grade and ballooning score compared to the $AlCl_3$ group ($P<0.05$). Additionally, increased dose of OLE was correlated with improved necro-inflammatory grade and ballooning score. $AlCl_3$ chronic exposure was associated with an increase in necro-inflammatory grade and ballooning score in the histopathological features of rat liver ($P<0.05$; r = -0.542, r = -0.607, Spearman's correlation test).

Kidney histopathology evaluation

The kidney slides were evaluated to observe any pathology induced by chronic exposure of oral $AlCl_3$ and any effect produced by OLE administration. The pathological

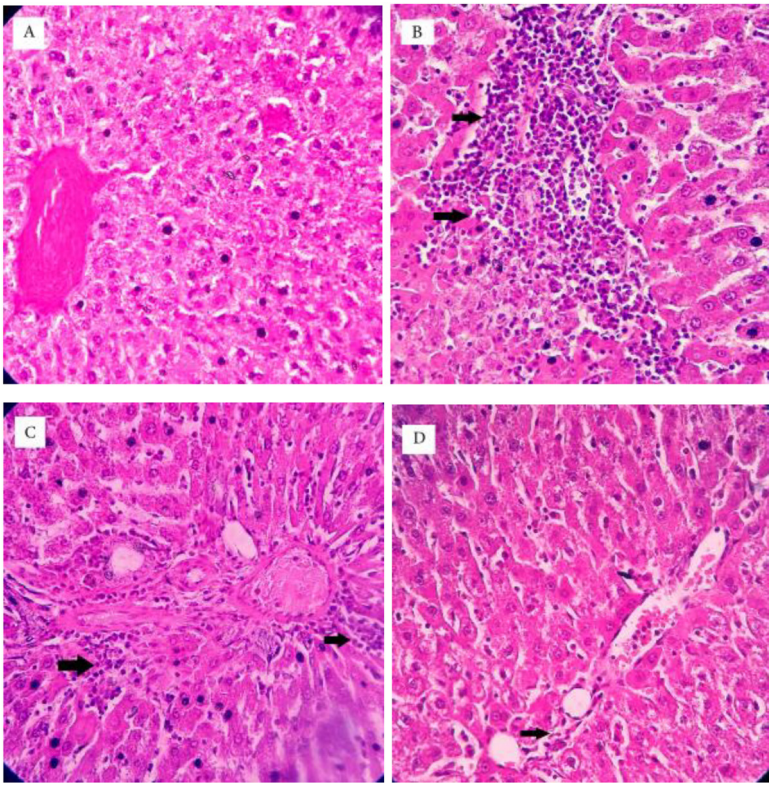

Figure 1. Effects of olive leaf extract (OLE) on liver histopathological features exposed to Al chloride ($AlCl_3$). A: Control group with a normal central vein, radially arranged hepatocytes, and clearly defined hepatocyte plates (No inflammatory cell infiltration was found in this group; B: $AlCl_3$ group with hepatic portal inflammatory cell infiltration and hepatic parenchymal inflammatory cell infiltration (Arrows); C: $AlCl_3$+OLE50 group with hepatic portal and mild inflammatory cell infiltration (Arrows); D: $AlCl_3$+OLE100 group with minimal inflammatory cell infiltration and clearly defined hepatocyte plates (Arrows) (Hematoxylin-Eosin: 400x).

Table 1. Statistical test results which compare scoring and grading liver histopathological feature of all the groups after $AlCl_3$ chronic exposure and olive leaf extract administration

Parameter	Group	Grading/Scoring	Frequency	*P* value#
Necro-inflammatory grading	Control	A0	7	0.009**
		A1	1	
	$AlCl_3$	A0	1	
		A1	6	
		A3	1	
	$AlCl_3$+OLE50	A0	3	
		A1	5	
	$AlCl_3$+OLE100	A0	6	
		A1	2	
Ballooning scoring	Control	0	6	0.006**
		1	1	
		2	1	
	$AlCl_3$	1	3	
		2	5	
	$AlCl_3$+OLE50	0	2	
		1	5	
	$AlCl_3$+OLE100	2	1	
		0	3	
		1	5	

**indicates the significant differences among all groups in each grading and scoring parameter (The Kruskal-Wallis test).
METAVIR scores: A0= none; A1= mild; A3= severe (portal and parenchymal injuries observed from liver histological sections). NAFLD scores: 0= none; 1= few; 2 = many (finding of ballooning hepatocytes observed from liver histological sections). OLE: olive leaf extract; $AlCl_3$+OLE50 and $AlCl_3$+OLE100: groups, which other than olive oil received olive leaf extract (50 and 100 mg/kg, respectively).
#For two-by-two comparison refer to Table 2.

Table 2. Results of statistical comparisons on necro-inflammatory grading and ballooning score of liver histological sections between two groups

Parameter	Group	Compared Group	*P* value
Necro-inflammatory grading	Control	$AlCl_3$	0.004**
		$AlCl_3$+OLE50	0.046**
		$AlCl_3$+OLE100	0.535
	$AlCl_3$	$AlCl_3$+OLE50	0.175
		$AlCl_3$+OLE100	0.013**
	$AlCl_3$+OLE50	$AlCl_3$+OLE100	0.143
Ballooning scoring	Control	$AlCl_3$	0.005**
		$AlCl_3$+OLE50	0.105
		$AlCl_3$+OLE100	0.256
	$AlCl_3$	$AlCl_3$+OLE50	0.028**
		$AlCl_3$+OLE100	0.005**
	$AlCl_3$+OLE50	$AlCl_3$+OLE100	0.424

** indicates the significant differences between two groups in each grading and scoring parameters
The Mann Whitney test was used to determine the *P* values.
OLE: olive leaf extract; $AlCl_3$+OLE50 and $AlCl_3$+OLE100: groups, which other than olive oil received olive leaf extract (50 and 100 mg/kg, respectively).

findings in kidney slides were milder than those of the liver, since $AlCl_3$ group showed inflammatory cells infiltration only, without any distinctive cells destruction or death. The OLE threated groups showed decreased inflammatory cells infiltration (Figure 2). Figure 2A (Control group) shows the typical kidney histopathological feature of the healthy control group. The inflammatory cell infiltration was rarely found in this group. Figure 2B ($AlCl_3$ group) shows the histopathological feature of the kidney with massive inflammatory cell infiltration. Figure 2C ($AlCl_3$+OLE50 group) and Figure 2D ($AlCl_3$+OLE100 group) show the histopathological feature of the kidney with slight inflammatory cell infiltration.

The statistical test results of kidney histopathological

scoring on inflammatory cells infiltration parameters are shown in Figure 2. The score of inflammatory cells infiltration in the $AlCl_3$ group was considerably higher than that of the control group. However, the scores in the groups given OLE were lower compared to the $AlCl_3$ group ($P<0.05$). The post hoc analysis showed significant differences in inflammatory cell infiltration between the control and $AlCl_3$ groups, $AlCl_3$ and $AlCl_3$+OLE50 groups, and $AlCl_3$ and $AlCl_3$+OLE100 groups ($P<0.05$). The scores of $AlCl_3$+OLE50 and $AlCl_3$+OLE100 groups were not significantly different with those of the control group. In addition, there was no significant difference between OLE50 and OLE100 groups ($P>0.05$) (Figure 3).

Discussion

$AlCl_3$ chronic exposure for 12 weeks produced damage to the liver tissue as indicated by increased necro-inflammatory grade and ballooning score. However, it resulted in a mild pathology to kidney tissue with the finding of inflammatory cell infiltration without any cellular damage. This study found that it can cause inflammatory cell infiltration in the portal and parenchymal areas, cells ballooning, sinusoidal congestion, and apoptosis in the liver. These are in line with previous studies whereby exposure to $AlCl_3$ could cause hepatocyte membrane damage, necrosis (29), portal area distortion, sinusoidal congestion (14), inflammatory cell infiltration (13,14,30), ballooning and focal necrosis with inflammatory cell infiltration (31). Exposure to $AlCl_3$ causes irregular cell arrangement, and unclear cell boundaries of hepatocytes (32). Al-Hazmi et al reported that, in contrast to our study, intraperitoneal administration of $AlCl_3$ for 45 days at a dose of 1.5 mg/kg in Wistar rats with an average body weight of 120 g resulted in pathological features on the kidneys, including inflammatory cell infiltration between the renal tubules, epithelial cells degeneration, and Bowman's space dilatation (33). Similar results were demonstrated by Othman et al that intraperitoneal injection of $AlCl_3$ at a dosage of 34 mg/kg body weight to Sprague Dawley rats weighing 200–220 g for 8 weeks resulted in tubular cell degeneration and glomerular collapse in the kidneys (1). The difference between these results with ours is most likely due to higher systemic AI levels related to intraperitoneal administration leading to higher Al concentration distributed to the kidney.

Aluminum readily accumulates in the liver, particularly in the tissue macrophages residing in liver sinusoids 'Kupffer cells', and cell organelles, such as lysosomes (34). In this regard, the macrophages produce prostaglandins, especially PGE2, as well as ROS, cytokines, and proteases, to mediate inflammation, and cause an imbalance of cyclooxygenase-2 (COX2) and E2 signaling pathways. Al leads to the activation of the MAPK pathway, then caspase-1, caspase-3, and caspase-11, thereby increasing the production of TNF-α, IL-1, IL-6, and IL-8 in parenchymal cells and macrophages (8,35). It also causes inflammatory cell infiltration (35). Al accumulation also induces the formation of ROS, leading to oxidative damage to proteins, DNA, and the imbalance of Ca^{2+} ions in the cell (3,34). In addition, oxidative stress reduces the integrity of cell membranes and triggers cell damage (36). As a consequence, it increases cell permeability and disintegration and promotes lipid peroxidation (37,38), which contributes to cell ballooning or hydropic

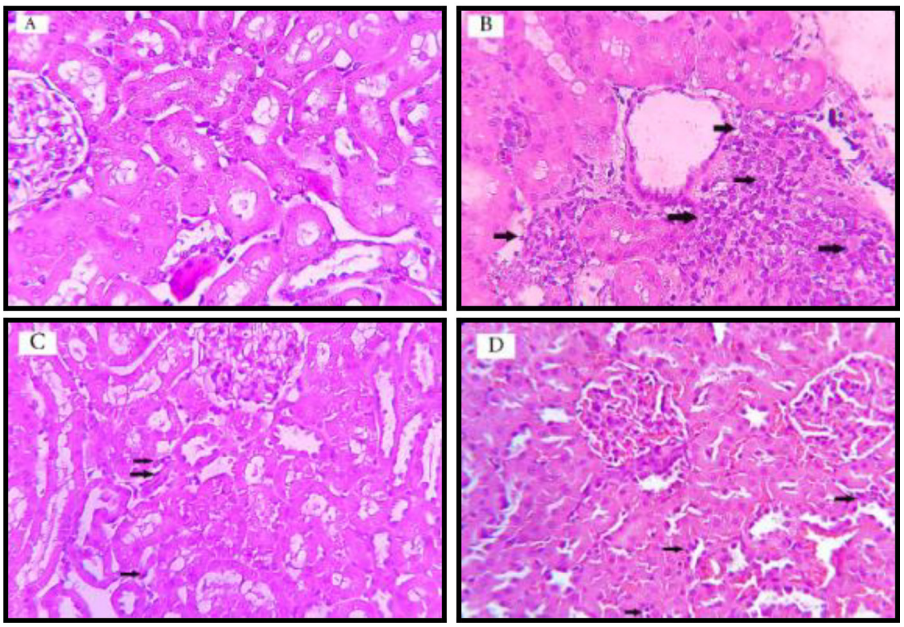

Figure 2. Effects of olive leaf extract (OLE) on kidney histopathological features exposed to aluminum chloride ($AlCL_3$). Kidney histological features and inflammatory cells infiltration (black arrows). The cell shape is normal in all groups, with pink color and obvious cell nuclei. No cell degeneration or cell necrosis was found (Hematoxylin-Eosin, 400x, A; Control group, B; $AlCl_3$ group, C; $AlCl_3$+OLE50 group, D; $AlCl_3$+OLE100 group).

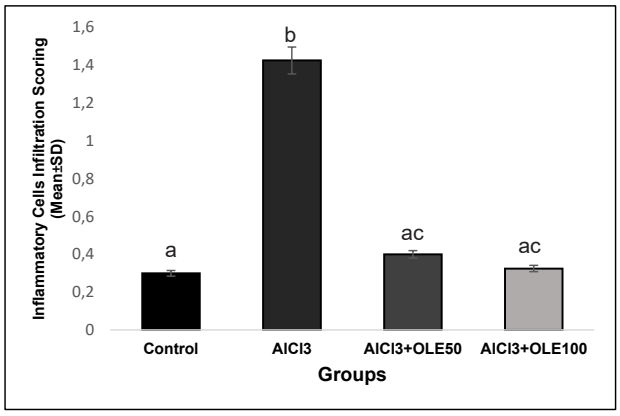

Figure 3. Statistical test results of kidney histopathological scoring on inflammatory cells infiltration parameters. Each value is represented as mean ± SD (n=8). Different letters indicate statistically significant difference ($P \leq 0.05$). $P<0.05$ control group compared with $AlCl_3$ group; $P<0.05$ $AlCl_3$ group compared with $AlCl_3$+OLE50 group; $P<0.05$ $AlCl_3$ group compared with $AlCl_3$+OLE100 group (Kruskal–Wallis & Mann-Whitney U tests). $AlCl_3$: Aluminum chloride; OLE: Olive leaf extract.

degeneration. The pathogenesis of Al toxicity in the liver is also related to apoptosis mechanisms. The expression of bcl-2 protein regulation decreases in $AlCl_3$-induced rats, causing apoptosis (1). This condition is also associated with increased p53 gene expression (3). The increase in ROS due to $AlCl_3$ exposure causes changes in the intracellular structure of hepatocytes and induces apoptosis (39).

Kidney is the main route for the excretion of toxic materials, including Al, leading to their accumulation in the kidney (15). Nevertheless, only a few animal studies reporting its deleterious effect on kidney. Long term dietary exposure of Al in dog caused mild tubular glomerulonephritis without impaired renal function (40). It is parallel with our finding of inflammatory cells infiltration in tubulointerstitial. Al induces high levels of free radicals and causes oxidative stress leading to increased inflammatory cell infiltration (41). Furthermore, $AlCl_3$ exposure increases pro-inflammatory cytokines, such as TNF-α and IL-6 (15).

Aluminum buildup is affected by age, exposure time, and exposure method (41,42). Although oral bioavailability Al is low, its detrimental effects should be of concern. We used oral route to reflect daily lifetime oral exposure to Al through foodstuffs, food additives, food contact materials, and pharmaceuticals like antacids and buffered analgesics (40,43). A study on German population, which might be extrapolated to general population, showed a significant exceedance of the tolerable weekly intake of Al may occur, increase health risk (43). Daily exposure to Al is inevitable and its toxicity has been confirmed by considerable studies particularly in susceptible groups such as children, elderly, and chronic kidney disease patients (40). To prevent the oxidative damage and diseases caused by exposure to Al, exogenous antioxidants are necessary. OLE is one of the greatest naturally occurring antioxidant sources for reducing oxidative stress (20,44). It has been demonstrated to lessen oxidative damage produced by exposure to toxic metals like cadmium (19,45) and lead (46,47). Olive trees are rich in phenol content, which is particularly concentrated in the leaves (23). The most abundant polyphenol content in olive leaf is oleuropein (48). Olive leaf also contains flavonoid compounds, catechins, and luteolin, which offer potent antioxidant activity, that can be greater than vitamins C and E (49).

This study found that OLE ameliorated the liver tissue damage produced by $AlCl_3$ chronic oral exposure. Vidičević et al in a study showed the beneficial effect of oleuropein rich standarized dry OLE in reducing CCl_4-induced hepatinc lessions in male Wistar rats (50). Oleuropein and hydroxytyrosol in OLE play an essential role in preventing inflammation and apoptosis by decreasing the COX2 and p53 expression and increasing the Bcl-2 expression (19,20,22). In addition, oleuropein and hydroxytyrosol in OLE also reduce TNF-α expression, thereby reducing the inflammatory process due to exposure to oxidative stress (22). These mechanisms might contribute to the reduced liver necro-inflammatory grade in $AlCl_3$ exposed animal. Furthermore, OLE can prevent congestion, sinusoidal dilation, and bleeding in hepatocytes by reducing microvascular leakages, leukocyte adhesion, and ROS formation (51). Therefore, OLE might be utilized as a hepatoprotective agent (20).

Based on the results of this study, the administration of OLE decreased the inflammatory feature of renal histology in rats induced by chronic oral exposure of $AlCl_3$. However, we did not find any cell degeneration and necrosis. Our result confirmed the protective effect of OLE in improving renal histopathological alterations as shown by previous studies (45,52,53). It might be related to its antioxidant property (54,55). This study provided valid quantification and detailed description on histological features of the pathological processes in the liver and kidney induced by oral $AlCl_3$ chronic exposure, along with OLE protective effect upon these insults. However, several limitations should be taken into consideration, as this study did not include examination of oxidative stress biomarkers, as well as liver and renal function parameters. Further studies are necessitated to examine those parameters and to unravel the mechanisms of OLE protective effects on liver and kidney tissues.

Conclusion

Chronic oral exposure to $AlCl_3$ leads to the impaired histopathological appearance of the liver, increased liver necro-inflammatory grade, and liver ballooning score. In addition, this exposure also causes mild changes to the histopathological appearance of the kidney, signified by increased inflammatory cell infiltration. OLE provides protective effects in ameliorating the histopathological features of the liver and kidney induced by chronic oral

exposure to aluminium, whereby OLE dose correlates to reduced hepatic lesions.

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Authors' contributions

Conceptualization: Nurina Hasanatuludhhiyah, Ahila Meliana, Arifian Hardi Putri Ratnani.
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Writing–review & editing: Nurina Hasanatuludhhiyah, Ahila Meliana, Arifian Hardi Putri Ratnani.

Conflict of interests

The authors declared that they did not have any conflict of interest.

Ethical considerations

The Ethics Committee on Health Research, Faculty of Medicine, Universitas Airlangga, Indonesia, reviewed and approved this study (No. 175/EC/KEPK/FKUA/2021 and No. 209/EC/KEPK/FKUA/2021).

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