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The effect of Callina papaya (*Carica papaya* L. var. Callina) leaves extract on histopathology of kidney and liver in cigarette smoke-exposed rats (*Rattus norvegicus* Berkenhout, 1769)

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ABSTRACT. Cigarette smoke is a source of free radicals that can cause oxidative stress that damages organ systems in the body. The study aimed to determine the protective effect of Callina papaya leaves ethanol extract on the histopathology of the kidneys and liver in cigarette smoke exposed rats. The study used 25 male rats divided into five groups, consisting of control (distilled water), negative control (exposed to cigarette smoke/ECS), P1 (ECS and given 100 mg/kg body weight (BW) extract), P2 (ECS and given 200 mg/Kg BW extract), and P3 (ECS and given 300 mg/kg BW extract) for 21 days. On the 22nd day, rats were sacrificed to take kidneys and liver for histopathological preparation. Observation parameters consists of organ index, and histopathology structure. All data were then analyzed using one-way ANOVA with Duncan's pos hoc test ($P<0.05$). The results showed no significant differences in organ index, glomerular number, and liver central vein area ($P>0.05$). Inflammation area, necrosis cells number, diameter, and area of glomerulus in kidney at doses of 200 mg/Kg BW and 300 mg/Kg BW showed improvement compared to other treatments ($P>0.05$). Hepatocyte area, sinusoid diameter, inflammatory area, number of hydropic degeneration cells, and number of necrosis cells in the liver also showed improvement at doses of 200 mg/kg BW and 300 mg/kg BW compared to other treatments ($P>0.05$). The conclusion shows that the Callina papaya leaves ethanol extract can protect against kidney and liver damage in rats exposed to cigarette smoke.

Keywords: Callina papaya leaf; cigarette smoke; histopathology structure; kidney and liver damage; *Rattus norvegicus*

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INTRODUCTION

Indonesia is one of the countries with the largest cigarette consumers and producers in the world (Kementerian Kesehatan, 2022). The death rate from smoking in Indonesia reaches 225700 people per year (WHO, 2019). The organs that experience the most disturbance due to exposure to cigarette smoke are the liver and kidneys which function as an excretion system and detoxify toxins in the body (Itoh *et al*., 2014; Arifa *et al*., 2017). Each cigarette contains 4000 chemicals harmful to the health of the body, including arsenic, carbon monoxide, ammonia, cadmium, acetone, urea, and other toxic materials (Csordas & Bernhard, 2013; Horinouchi *et al*., 2016). Toxic compounds like carbon monoxide, tar, and nicotine can increase the production of free radicals, lead to oxidative stress, and trigger an inflammatory response in the human cells (Papathanasiou *et al*., 2014; Kopa & Pawliczak, 2020; Mocniak *et al*., 2022).

Toxic substances and free radicals that reach the body are filtered and neutralized by the kidney and liver. Cigarettes can damage blood vessels in the kidneys by increasing the production of free radicals that can trigger inflammatory oxidative reactions, oxidative stress, increased blood pressure, and vasoconstriction in blood capillaries (Milnerowicz *et al*., 2015; Vlachopoulos *et al*., 2016; Synn *et al*., 2019; Zhang *et al*., 2019). Cigarette smoke can also trigger chronic liver disease, resulting in fibrosis in the liver tissue (Jensen *et al*., 2013; Han *et al*., 2021), necrosis (Heijink *et al*., 2015), and swelling in liver hepatocytes (Battah *et al*., 2016; Marti-Aguado *et al*., 2022).

Damage caused by cigarette smoke-free radicals can be overcome by protective efforts with antioxidants in the body. Consuming a variety of sources of antioxidants, such as herbal plants, can help receive exogenous antioxidants from outside the body (Palipoch, 2013; Jarisarapurin *et al*., 2019). The utilization of herbal materials in papaya can function in preventing, slowing down, inhibit the release of pro-inflammatory, and repairing the negative effects of free radicals caused by exposure to cigarette smoke (Rahman, 2013; Heung *et al*., 2023). However, studies about cigarette smoke exposure on organ histology by utilizing Callina papaya leaves are yet relatively limited.

One of the varieties developed in Indonesia is the Callina (California) variety with several advantages for instance faster harvesting time and short stem size ± 1.5 m (Sujiprihati *et al*., 2010). Our previous studies also show that Callina papaya leaves can protect against damage to the respiratory organs of Wistar rats exposed to cigarette smoke (Setiawan *et al*., 2022). Based on this background, information is needed on the protective effect of Callina papaya leaves to prevent the adverse effects of free radicals on the excretory organs. This study aims to determine the protective effect of ethanol extract of Callina papaya leaves on the kidney and liver organs of Wistar rats exposed to cigarette smoke. This study establishes that Callina papaya leaves can function naturally as an antioxidant against the free radicals brought on by cigarette smoke.

MATERIALS AND METHODS

This study is a preclinical research model using test animals (Wistar rats) in testing the effectiveness of Callina papaya leaf extract as a potential source of antioxidants by looking at the repair of tissue damage in the liver and kidney organs of rats exposed to cigarette smoke. The research was conducted from January to June 2022 at the Laboratory of Animal Structure and Physiology, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan (UAD) Yogyakarta. This research was also conducted based on procedures approved by the UAD Ethics Committee with number 012101004. Callina papaya (*Carica papaya* L. var. Callina) leaves were obtained from Druwo vegetable plantation, Bangunharjo, Bantul, Yogyakarta.

Preparation of papaya leaves ethanol extract. Callina papaya leaves (7 kg and dark green) was made simplisia (wind dry method/ by drying without direct exposure to sunlight for 5 days), then mashed and extracted by maceration method using 96% ethanol solvent (for 3 days). The filtrate was then filtered and concentrated using a rotary evaporator (temperature 60°C, speed 85 rpm) until the extract preparation was obtained. The extract was then stored in the refrigerator (-4°C).

Test animal treatments. The study used 25 Wistar rats (*Rattus norvegicus* Berkenhout, 1769) (Male, age ± 3 months, weight ± 200 g) acclimatized for 14 days. The rats were then grouped into 5 groups (one group consisted of 5 rats) consisting of control (distilled water), negative control (exposed to cigarette smoke), P1 (exposed to cigarette smoke and given 100 mg/kg BW extract), P2 (exposed to cigarette smoke and given 200 mg/kg BW extract), and P3 (exposed to cigarette smoke and given 300 mg/kg BW extract). Determination of the number of replicates of test animals using Federer's formula. The cigarettes used were *kretek* cigarettes (34-65 mg tar, 1.9-2.6 mg nicotine, and 18-28 mg CO), which were given as many as 3 cigarettes for 21 days. Cigarette smoke was administered using a smoking chamber specifically designed as a cage for experimental subjects (made of a plastic box; size 50cm×35cm×30cm) by placing a pipe on one side of the cage to flow cigarette smoke. The smoking chamber has three holes to connect with the smoking pump and 20 holes to exchange air from outside. The process of exposure to cigarette smoke begins with the introduction of cigarette smoke into the smoking chamber using a smoking pump until it fills the smoking chamber. The smoking pump was made from a modified aquarium air pump (Fig. 1.3.). Cigarette smoke exposure was carried out on all treatment groups and negative controls with 3 cigarettes per group for 21 days. Cigarette smoke exposure is done every morning at 08.00 WIB. After giving cigarette smoke, a pause of 10 minutes was given so that male Wistar strain white rats could inhale the remaining cigarette smoke and then returned to their respective cages. Cigarette giving is done by burning the cigarette until it runs out then leaving it for 2 h. Ethanol extract from

papaya leaves dissolved in 1ml of distilled water was given orally using a roundabout for 21 consecutive days. Body Weight (BW) was measured every day to-0,7,14, and 21 (Setiawan *et al.*, 2022).

Histopathological preparation and observation. On day 22, rats were anesthetized using ether 10%, then sacrificed using neck dislocation. Rats were then dissected and kidney and liver organs were taken to be weighed and calculated organ index (%), and histology preparations were made. The kidney and liver were washed using NaCl (0.9 %) and then fixed using Buffer Neutral Formalin (BNF)10%. Organs were then prepared using the paraffin method. The kidney and liver were sliced using a scalpel with a size of ± 1 cm, then the organs were put into a cassette and put into an automatic tissue processor (dehydrated process with alcohol concentrations of 70%, 80%, 90%, 95%, and absolute). After that, the tissue was put in xylol to dissolve the alcohol contained in the tissue for easy infiltration by paraffin. The next stage is the embedding process by embedding the tissue into liquid paraffin, then the paraffin block is stored in a refrigerator 5℃. Each paraffin block containing tissue is sliced using a microtome with a thickness of 4-6 μm. Very thin pieces of tissue are placed on the surface of warm water so that the tissue does not shrivel, and placed on an object glass to be incubated for 24 h so that the tissue adheres to the object glass. In making histopathology preparations, hematoxylin eosin was used (Setiawan *et al.,* 2022).

The preparations were observed using an Olympus CX23 microscope and images were taken using an Optilab Camera in 5 views at 400× magnification. Parameters observed in kidney tissue are the area of inflammation, the number of cells that undergo necrosis in the glomerular and tubular areas, and the diameter, area, and number of glomeruli. The parameters observed in the liver tissue were hepatocyte area, central vein area, sinusoid diameter, inflammatory cell area, number of cells undergoing hydropic degeneration, and number of necrosis cells (Padmiswari *et al*., 2020; Rusman *et al*., 2021). All histology observations were measured using the Image Raster application.

Data analysis. All observed data were then analyzed using the One-Way ANOVA test using SPSS ver. 26 and continued with the Duncan test ($P < 0.05$) to see differences between treatments.

RESULTS AND DISCUSSION

The results of the ANOVA test showed that on day 21 there was a significant difference in the body weight of rats treated with negative control and P1, which was lower than the other treatments ($P<0.05$), presented in Table 1.

Table 1. Body weight of rats in treatment

Variable	Body Weight (g)/Day			
	0	7	14	21
Control	191.25 ± 23.47a	191.75 ± 23.34a	192.50 ± 23.70a	196.50 ± 22.25b
Negative control	199.25 ± 9.00a	178.25 ± 13.43a	165.50 ± 7.42a	154.25 ± 9.91a
P1	186.75 ± 22.25a	181.75 ± 22.23a	171.75 ± 11.67a	170.75 ± 8.10a
P2	169.25 ± 17.52a	177.50 ± 20.40a	188.50 ± 16.34a	205.25 ± 15.20b
P3	178.50 ± 2.65a	182.00 ± 1.63a	191.50 ± 6.95a	201.50 ± 12.78b

Notes: Control (aquadest 1 ml), negative control (exposed to cigarette smoke), p1 (papaya leaf ethanol extract (plee) 100 mg/kgbb and exposed to cigarette smoke), p2 (PLEE 200 mg/kgbb and exposed to cigarette smoke), P3 (PLEE 300 mg/kgBB and exposed to cigarette smoke). Mean ± SD. superscript a-b = a similar notation on numbers followed by different letters in the same column indicates there is a significant difference ($P<0.05$).

This is due to the effect of exposure to cigarette smoke which causes a decrease in body weight. The nicotine content in cigarette smoke has a suppressive effect on appetite, so food nutrients in rats are not well supplied (Huriyati & Amareta, 2020). Free radicals caused by cigarette smoke such as CO also cause a decrease in the physiological function of the body which results in a decrease in body weight (Unitly *et al*., 2022). Continuous exposure to cigarette smoke causes emphysema in the lungs of rats so that the amount of energy (ATP) released becomes more. This causes a decrease in body weight in rats (Kamiide *et al.,* 2015). Giving the extract for 21 days has not shown a significant difference in body weight in P1 ($P>0.05$), but in P2 and P3 there has been a significant difference

(P<0.05) which is higher than negative control. This is thought to be due to the influence of Callina papaya leaves such as the flavonoid content which acts as an antioxidant in protecting against tissue damage due to exposure to cigarette smoke and also plays a role in increasing appetite (Santoso *et al*., 2020).

The study showed that the mean kidney weight and kidney/body weight ratio of rats in all treatments did not show significant differences (P>0.05), illustrated in Table 2. These results indicate that the administration of cigarette smoke in the treatment did not reduce the organ weight and the ratio of kidney weight/body weight of rats macroscopically for 21 days. Kidney organ weights that show no difference are thought to be due to exposure to cigarette smoke for 21 days not being able to damage all parts of the kidney organ but can damage cellularly (microscopically). The damage caused by exposure to cigarette smoke is more visible in histopathological observations, which found several forms of tissue damage such as necrosis and inflammation (Fig. 1). This is supported by the same weight in all treatments but has a different cell tissue structure after the administration of cigarette smoke such as the appearance of inflammation and necrosis (Mahmoud *et al.*, 2014).

Table 2. Histopathological structure of kidney tissue in the treatments

Variable	Control	Negative control	P1	P2	P3
Kidney weight (g)	0.80 ± 0.04a	0.68 ± 0.10a	0.69 ± 0.06a	0.72 ± 0.12a	0.76 ± 0.10a
Kidney index (%)	0.41 ± 0.03a	0.38 ± 0.04a	0.43 ± 0.05a	0.38 ± 0.10a	0.38 ± 0.02a
Inflammatory area (mm^2)	3.34 ± 0.44ab	5.04± 0.51c	4.58 ± 0.66c	3.72 ± 0.37b	2.96 ± 0.16a
Number of glomerular necrosis cells	0.15 ± 0.19a	1.20 ± 0.28b	0.95 ± 0.19b	0.45 ± 0.19a	1.00 ±0.16b
Number of tubular necrosis cells	9.10 ± 0.26a	26.15 ± 1.77d	25.90 ± 0.66d	17.60 ± 0.94b	22.60 ± 0.54c
Glomerular diameter (µm)	91.83 ± 6.57c	62.71 ± 4.04a	69.35 ± 8.35ab	83.99 ± 0.85c	72.22 ± 5.36b
Glomerular area (mm^2)	6.87 ± 0.35c	4.77 ± 0.72a	5.25 ± 0.89ab	5.34 ± 0.45ab	5.75 ± 6.76b
Number of glomerulus	49.40 ± 11.32a	59.40 ± 9.07a	55.80 ± 4.65a	48.80 ± 6.57a	57.25 ± 8.84a

Notes: Control (aquadest 1 ml), negative control (exposed to cigarette smoke), P1 (papaya leaf ethanol extract (PLEE) 100 mg/KgBB and exposed to cigarette smoke), P2 (PLEE 200 mg/kgBB and exposed to cigarette smoke), P3 (PLEE 300 mg/kgBB and exposed to cigarette smoke). Mean ± SD. superscript a-d = a similar notation on numbers followed by different letters in the same column indicates there is a significant difference (P<0.05).

Observation of the histopathological structure of the kidney (Fig. 1) shows that the kidney is composed of glomeruli enveloped by Bowman's capsule. The glomerulus can be damaged and shrunk as a result of free radicals caused by cigarette smoke. The administration of cigarette smoke also causes infiltration of inflammatory cells to the center of damage, resulting in the formation of an inflammatory area. Tissue areas with small glomerular structures, inflammatory areas, and large numbers of necrosis cells are indicators of microscopic organ damage in the kidney (Wijayanti, 2016). Fig. 2 also shows that P2 and P3 have small inflammatory areas compared to negative control and show the same shape and glomerular area as the control.

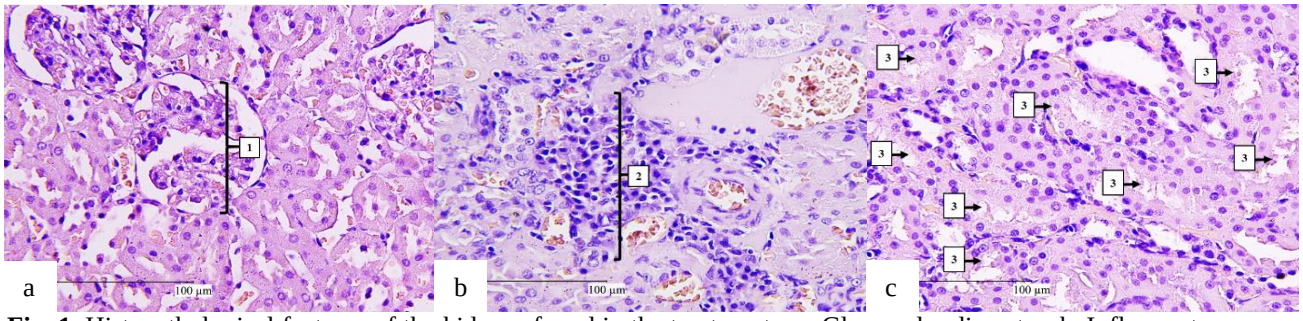

Fig. 1. Histopathological features of the kidneys found in the treatments: a. Glomerular diameter; b. Inflammatory area; c. Necrotized cells; hematoxylin-eosin staining. Scale bar 100µm

The results of observations on the glomerulus showed that there were significant differences in the diameter and area of the glomerulus (P < 0.05). Glomerular diameter showed the smallest size in negative control, while the highest was in P2. The glomerular area showed that P3 was higher than the other treatments, and the smallest glomerular area was found in negative control. The number of glomeruli did not show significant differences between treatments (P<0.05). The results of the observation of the histopathological structure of the rat kidney are that there are significant differences in the inflammatory area in the kidney tissue (P < 0.05) (Table 2). The most extensive inflammatory area was found in the negative control and P1 treatments. Inflammatory areas in P3 and P2 have the lowest results compared to other treatments, it is also seen in the distribution of inflammatory areas in P3 tends to be less than the other treatments (Fig. 3). The observation of necrosis cells also showed that the number of necrosis cells in the glomerular area and renal tubules had significantly different results (P < 0.05). The number of necrosis cells was found in negative control and P1, then significantly different in P2. This indicates that tissue repair due to cigarette smoke exposure to cell necrosis is higher in the P2 treatment. The most effective group for cell damage repair is P2 because it contains antioxidant compounds that can reduce the amount of damage to cells. These observations show that P2 or ethanol extract of Callina papaya leaves at a dose of 200 mg/kg BW influences protecting free radicals that damage the renal glomerulus, characterized by the same size as the control. The small and narrow glomerulus shows necrosis in the cells that make up the tissue which is thought to be due to exposure to cigarette smoke.

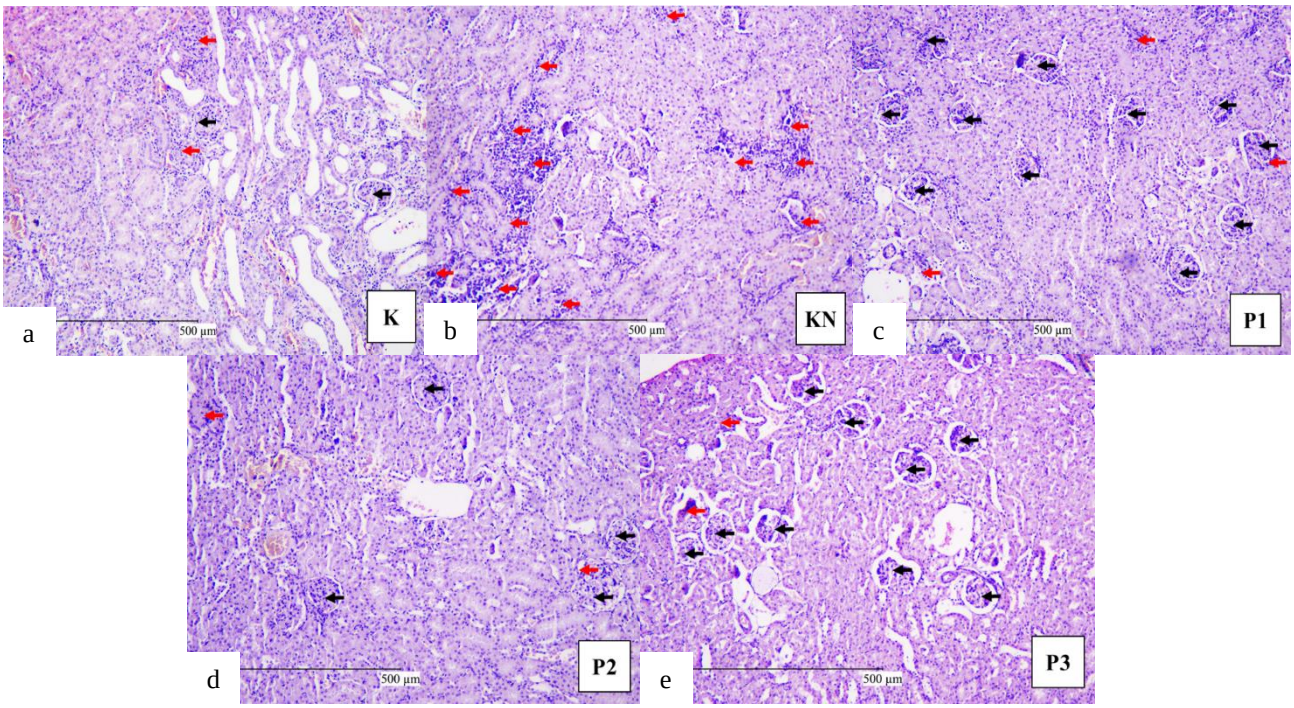

Fig. 2. Histopathological features of the kidneys found in the treatments: a. K (control; aquadest 1 ml); b. KN/negative control (exposed to cigarette smoke); c. P1 (papaya leaf ethanol extract (PLEE) 100 mg/kgBB and exposed to cigarette smoke); d. P2 (PLEE 200 mg/kgBB and exposed to cigarette smoke); e. P3 (PLEE 300 mg/kgBB and exposed to cigarette smoke). Red arrows indicate the inflammatory area, black arrows indicate the glomerulus, hematoxylin-eosin staining. Scale bar 500µm

The results showed that there was no macroscopic damage due to exposure to cigarette smoke on the liver. This is shown in the weight of the liver and liver organ index does not show significant differences between treatments (P>0.05) (Table 3). The observation of liver tissue structure in Wistar rats showed that cigarette smoke damaged the liver of rats microscopically. This is shown in the area of inflammation and cells that experience high hydropic degeneration found in negative control (P <0.05) (Table 3). Nicotine, tar, carbon monoxide, and volatile organic compounds in cigarette smoke stimulate free radicals (ROS) in liver cells/hepatocytes. This causes damage to cell membranes,

proteins, and DNA, leading to cell death. This triggers inflammation by reducing the dead cells by activating inflammatory signaling pathways. Aldehyde compounds in cigarette smoke can damage liver cells and trigger the release of pro-inflammatory cytokine molecules such as TNF-α, IL-1, and IL-6. These molecules stimulate the activation of macrophage cells, NK cells, and lymphocyte cells in liver tissue (Morris *et al.,* 2021). Activation of these cells will trigger the release of more pro-inflammatory cytokine molecules, increasing the number of inflammatory cells in liver tissue.

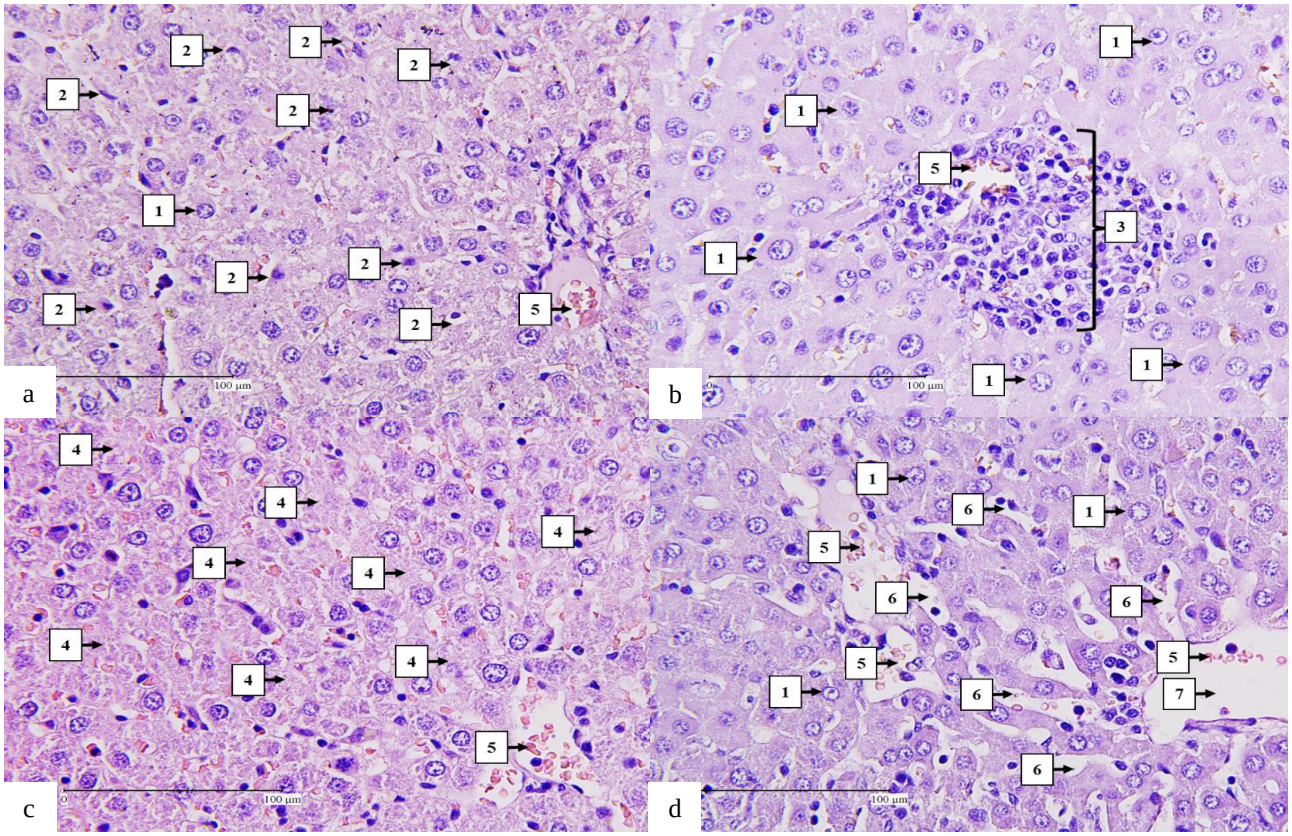

Fig. 3. Histopathological features of the kidneys found in the treatments: a. K (control; aquadest 1 ml); b. KN/negative control (exposed to cigarette smoke); c. P1 (papaya leaf ethanol extract (PLEE) 100 mg/kgBB and exposed to cigarette smoke); d. P2 (PLEE 200 mg/kgBB and exposed to cigarette smoke); e. P3 (PLEE 300 mg/kgBB and exposed to cigarette smoke). Red arrows indicate the inflammatory area, black arrows indicate the glomerulus, hematoxylin-eosin staining. Scale bar 500µm

In addition, exposure to cigarette smoke can also stimulate the release of nuclear factor kappa B (NF-κB) in liver cells. NF-κB is a transcription factor that is critical in the activation of inflammatory signaling in liver tissue. When NF-κB is activated, it triggers the production of more proinflammatory cytokine molecules, such as interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). Persistent activation of inflammatory signaling in liver tissue can lead to chronic inflammation and damage to liver cells (Wang *et al*., 2018; Alharbi *et al.,* 2021). The area of inflammation was found to be lowest in control, P2, and P3 ($P<0.05$), and the amount of hydropic degeneration was lowest in P2 and control ($P<0.05$). This shows that the ethanol extract of papaya leaves in doses of 200 mg/kg BW and 300 mg/kg can reduce the inflammatory area and cells that experience hydropic degeneration due to free radicals from exposure to cigarette smoke. Fig. 3 shows the structure of liver tissue composed of hepatocytes, sinusoids, and central veins. The characteristics that appear in liver tissue due to cigarette smoke exposure are areas of hydropic degeneration, areas of inflammation, and areas of cell necrosis. The presence of hydropic degeneration and cell necrosis is an indicator of microscopic organ damage in the liver (Syed & Shangloo, 2020). Fig. 3 also shows that the area of hepatocyte cells is getting smaller due to exposure to cigarette smoke, also characterized by a larger sinusoid diameter. The results also showed that the hepatocyte area and

sinusoid diameter had a normal size (equivalent to control) after the administration of ethanol extract of Callina papaya leaves in the P2 treatment compared to other treatments ($P < 0.05$). Liver cells have a mechanism to neutralize radical load using endogenous antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase. However, due to continuous exposure to cigarette smoke, the production of ROS can exceed the ability of liver cells to neutralize them, so oxidative stress on liver cells will continue to increase. Callina papaya leaf extract acts as an additional exogenous antioxidant that can help counteract liver-damaging cigarette smoke-free radicals by binding to and removing them from the body before they damage liver cells (Sharma *et al.*, 2022, Setiawan *et al.*, 2022).

Papaya leaves contain several phytochemical compounds such as flavonoids, saponins and alkaloids that act as antioxidants in capturing free radicals so that damaged cells get the opportunity to regenerate themselves (Himaniarwati *et al.*, 2019). Free radicals and nicotine caused by exposure to cigarette smoke can increase reactive oxygen species (ROS), causing oxidative stress. The impact arising from oxidative stress in cell and tissue damage (Caponnetto *et al.*, 2013; Karoline *et al.*, 2022). Papaya leaf ethanol extract plays a role in stopping the chain reaction carried out by cigarette smoke free radicals and returning oxidants to harmless forms. Antioxidants can help reduce and neutralize free radicals by providing electrons to unstable molecules, thereby preventing kidney and liver tissue damage (Bursal *et al.*, 2019). In addition, antioxidants in papaya leaves can also reduce the production of proinflammatory cytokines such as IL-6, IL-1β, and TNF-α, which act as key mediators in inflammation and can exacerbate inflammatory conditions (Roy *et al*., 2023). Our study showed a decrease in inflammatory areas in kidney tissue and rats after administration of Callina papaya leaf ethanol extract. Flavonoids in papaya leaves can prevent damage caused by free radicals by reacting with radical compounds so that the reactivity of flavonoid hydroxyl groups becomes high and free radicals become inactive in cells (Panche *et al*., 2016). Flavonoids also play a role in inhibiting the reaction of excess inflammatory cells (macrophages and neutrophils) in triggering inflammatory reactions as our previous studies (Setiawan *et al*., 2021). The compound can inhibit inflammation by neutralizing free radicals and other oxygen-reactive molecules formed during oxidative reactions in cells involved in inflammation. Papaya leaves also contain saponin compounds that can trigger several immunostimulators (TNF-a, IL-1 β, and IL-2) in accelerating the inflammatory process and repairing cells in the tissue (Briggs *et al*., 2020). Alkaloid found in papaya leaves can stop the lipid peroxidation process in protecting damaged cells (Adedayo *et al*., 2021). Based on our observations of kidney and liver tissue structure, it can be concluded that the effective dose that can repair cell and tissue damage in both organs is a dose of 200 mg/kg BW (P2) and 300 mg/kg BW. This dose can effectively repair cells damaged by free radicals from exposure to cigarette smoke such as reducing the area of inflammation, the number of necrosis cells, and maintaining tissue structure in kidney glomeruli and liver hepatocyte cells.

CONCLUSION

Ethanol extract from Callina papaya leaves does not affect kidney and liver index, while ethanol extract from Callina papaya leaves at a dose of 200 mg/Kg BW and 300 mg/Kg BW is effective on rat body weight and can repair damage to the structure of kidney and liver tissues of Wistar rats exposed to cigarette smoke. Callina papaya leaves can act as a natural antioxidant in counteracting free radicals caused by cigarette smoke.

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