Phaleria macrocarpa Leaves Extract Reduce Tumors Growth and Improve Histological Changes of Liver and Kidney on 4T1 Breast Cancer Mice Model

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Abstract

Breast cancer is a type of cancer that highly occurs globally and causes death cases. Of the many ways of treating breast cancer, chemotherapy is the most recommended, even though it causes various disturbing side effects. Therefore, alternative medicine using bioactive compounds of medicinal plants has begun to be widely used, for example, *Phaleria macrocarpa*, a plant native to Indonesia with anticancer and antioxidant activity. The liver and kidneys are important organs that function to maintain body homeostasis. The use of crude extracts of medicinal plants often causes damage to those organs at inappropriate doses. This research aimed to get an effective dose for reducing breast cancer growth and is safe for the liver and kidneys. A total of 36 mice were divided into six groups, including healthy control, cancer control, cisplatin, and three doses of *P. macrocarpa extract* (58.9, 117.8, and 235.6 mg.kg^{-1}). The experimental animals were injected using a 4T1 cell line and treated orally using *P. macrocarpa* leaf ethanol extract for two and three weeks. The tumor volume of mice was measured periodically. At the end of treatment, mice were sacrificed, and their liver and kidney organs were isolated. Both organs were then prepared for H&E staining and observed using a microscope. The results showed that a dose of 58.9 mg.kg^{-1} and 117.8 mg.kg^{-1} of *P. macrocarpa* extract could reduce tumor volume by more than 90%, and the 117.8 mg.kg^{-1} dose is the safest dose to use because it does not affect the kidney and cause chronic damage to liver tissue.

Keywords: extract, kidney, liver, tissue damage, tumor.

INTRODUCTION

Worldwide, breast cancer comprises 10.4% of all cancer incidences among women, making it the fifth most common cause of cancer death. Breast cancer refers to the abnormal growth and proliferation of cells originating in breast tissue due to disturbed and unregulated cell cycles [1]. Indonesia alone has more than 300,000 new breast cancer cases in 2020, most of them in women [2]. This problem needs to be solved because it metastasizes into several organs such as lungs, bones, and liver [3]. The efforts to treat and cure breast cancer depend on tumor grade, hormone receptor status, metastatic potential, patient profile, and many other things. Several treatments can be used to treat breast cancer, including surgery, radiation therapy, and chemotherapy. Chemotherapy is the most recommended treatment, but like other therapeutic agents, chemical drugs for chemotherapy can be toxic to normal tissue and have fairly obvious side effects. Up to 80% of the patients have a risk to went through vasomotor syndrome [4] and other symptoms, including nausea, vomiting, diarrhea, fatigue, hair loss, and psychological stress [5].

Indonesia is a country that has the second-largest biodiversity in the world and has many potential plants used in traditional medicine but has no scientific evidence [6]. *Phaleria macrocarpa* is an original plant from Indonesia, precisely in the tropical region of Papua. This plant has been widely reported to have medical activities such as anti-tumor, anti-hyperglycemic, anti-inflammatory, antioxidant, and anti-microbial. The leaves of *P. macrocarpa* have been used to treat various types of cancer, including breast cancer. The methanol extract from *P. macrocarpa* can work as an anti-proliferative, anti-angiogenic, and apoptotic inducer due to its main compounds, phalerin, and gallic acid [7]. Based on in vitro research conducted by Christina *et al.* [8], ethanol extract of *P. macrocarpa* leaves demonstrated cytotoxic activity of 50% at a dose of 97 µg mL-1 in a breast cancer cell line. In addition, the high content of phenolic and flavonoid compounds makes this extract can be used as a natural anticancer and antioxidant to replace or complement modern treatment methods.

The high interest in using herbal medicine as an alternative drug demands a scientific evaluation to assess the toxicity of these medicinal drugs. Using crude extract containing

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various phytochemicals is feared to have side effects if used at inappropriate doses [9]. The liver is a crucial organ of the human body that detoxifies various xenobiotics such as drug metabolites and helps maintain homeostasis. Liver cells frequently experience stress during this detoxification process due to oxidative damage from free radicals [10]. However, the kidney maintains metabolism and homeostasis in the body as well. The kidneys have a crucial role in excretion by forming urine by filtering harmful or excessive substances for the body [11].

Because of those functions, the kidney and liver are target organs often used to observe the effect or toxicity of drugs or other chemical substances. Histopathology is the most appropriate screen evidence for the kidney and liver because this method is convenient enough to detect diseases that occur in a short time in experimental animals in the laboratory [12]. *Phaleria macrocarpa* anticancer effect has been proven for breast cancer cells, but it still has limited study to confirm the effect on the liver and kidneys. Thus, this research aimed to examine the impact of various *P. macrocarpa* leaf extract doses to find an effective dose to treat breast cancer while not having side effects on other vital organs.

MATERIAL AND METHODS

Plant Material and Extraction

The leaves of the *P. macrocarpa* plant were dried and grounded to a powder form. The powder was then macerated with 70% ethanol for 24 hours while stirring several times. The ethanol extract obtained concentrated using a vacuum pump evaporator at a low pressure of 50°C until it forms a paste. The extract will be dissolved in water and given for 28 days through oral injection.

Cell Line

4T1 cell line derived from mice breast cancer and a type of TNBC was obtained from Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, University of Gadjah Mada, Yogyakarta. Cells were then cultured on DMEM complete media (DMEM, 10% FBS, and 1% penicillin-streptomycin) and incubated at 37°C with 5% CO_2 content. The culture cells will be split 2-3 times a week to reach confluency (60%-80%).

Breast Cancer Induction

The experimental animal (*Mus musculus*) was obtained from the Laboratory of Animal Physiology, Department of Biology, State Islamic University of Malang, aged 5-6 weeks old. The animal research protocol has been reviewed and approved by Animal Care and Use Committee, Brawijaya University, Indonesia (Approval number 025-KEP-UB-2021).

Induction of breast cancer was done by 4T1 cell injection dissolved in PBS at 100µl. 4T1 cell lines (3×10^6 cell.mL^{-1}) was injected into the subcutaneous part of the mammary glands of mice three times in two weeks (4-5 days interval into the next injection). Then we observed whether they experienced toxic symptoms such as weight loss, changes in appetite, or other clinical signs in the body. It is our modification of Pulaski [13]. The bulge that appears at the injection point was observed, and if the tumor bulge has reached a volume of 100-300 mm^3, mice are ready to be given further treatment [14].

Experimental Design

A total of 18 female BALB/C mice were divided into six groups with three animals each. The mice were acclimatized for seven days before being given treatment. Mice were then divided into several treatment groups, as follows:

Control - : Mice were not induced by breast cancer and were not given the extract.

Control + : Mice induced by breast cancer and not given the drug/extract.

Cisplatin : Mice induced by breast cancer and injected intraperitoneally with 4 mg.kgBW^{-1} of cisplatin [15].

Dose 1 : Mice induced by breast cancer and treated with 58.9 mg.kg^{-1} of *P. macrocarpa* leaves extract.

Dose 2 : Mice induced by breast cancer and treated with 117.8 mg.kg^{-1} of *P. macrocarpa* leaves extract

Dose 3 : Mice induced by breast cancer and treated with 235.6 mg.kg^{-1} of *P. macrocarpa* leaves extract.

Tumor Volume Measurement

Tumor length (L) and width (W) were measured three times per week using a calliper. The data is then calculated using a formula based on the Faustino-Rocha [16] reference:

$$V = \frac{(W^2 \times L)}{2}$$

Description:

V = Tumor Volume
W = Tumor width
L = Tumor length

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Histopathology Analysis

After the treatment, mice were sacrificed by dislocation to isolate several organs, including breast, liver, and kidney, for histopathology analysis. The organs obtained were then immersed in a fixative solution (10% formalin) and prepared using paraffin wax. The preparations were cut with a thickness of 5-7 mm and then stained using hematoxylin and eosin (H&E) staining. After that, the samples were observed using an Olympus BX51 microscope and photographed using OptiLab 3.0 software.

RESULT AND DISCUSSION

Breast Tumor Volume

Figure 1 demonstrated a decrease in tumor volume after treatment with *Phaleria macrocarpa* ethanol extract. The reduction in tumor volume until the third week in the control treatment occurred by 77%, while treatment with cisplatin decreased by 98%. However, the tumor volume for doses 1, 2, and 3 seems to be reduced by 100%, 94%, and 87% in order. A 58.9 $mg.kg^{-1}$ *P. macrocarpa* was the most effective dose to treat breast cancer in vivo. The active compounds of *P. macrocarpa* leaves extract effectively inhibit the growth of cancer cells, characterized by a decrease in tumor volume. Based on the in silico study, *P. macrocarpa* extract has bioactive compounds such as the lignan group that stimulate Caspase 3 and Bax proteins. In addition, the flavonoid group can also act as a Bcl-2 inhibitor so that it can be used as an apoptotic agent [8].

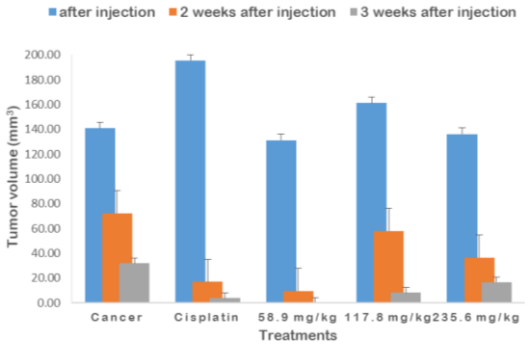

Figure 1. The decrease of breast tumor volume in all groups after two weeks and three weeks of treatment.

The results showed that *P. macrocarpa* extract groups worked as effectively as the cisplatin groups. Cisplatin or cis-diamminedichloroplatinum (CDDP) is a chemotherapy drug used to treat various types of cancer. Cisplatin compound consists of two chloro and two ammine ligands. This complex compound reacts in vivo, binds, and causes DNA crosslinks, triggering apoptosis [17]. Cisplatin could induce apoptosis and trigger cell death by DNA damage through many pathways, including reactive oxygen species and binding to the N7 reactive center on purine residues [18].

Several bioactive compounds are found in P. macrocarpa, and gallic acid is the most widely isolated and studied natural antioxidant [19]. Gallic acid is a polyhydroxy phenolic compound that can be found in various natural ingredients in nature. Various studies showed that it has an anticancer activity that has been tested in vitro and in vivo by inhibiting cell proliferation and inducing apoptosis [19,20]. Gallic acid can cause cell cycle perturbation in the G1 phase and interfere with the mitotic phase in cancer cells. Another study suggested that gallic acid induces DNA fragmentation through caspase activation and cell cycle arrest via decreased Cdks and cyclin protein levels [20].

Histology of Liver

The liver and renal organs was observed to determine the effect of *P. macrocarpa* on other crucial organs. Liver histology of control mice that were not exposed to the *P. macrocarpa extract* appeared mainly in normal structure (Fig. 2). The hepatic cells have normal nuclei and are arranged toward the central vein. Meanwhile, cisplatin and *P. macrocarpa* extract treatment can change the normal structure and cause abnormalities characteristics. Figure 3 showed the presence of sinusoidal dilatation, blood vessel dilatation, and congestion in cisplatin treatment [21]. Cisplatin has several toxicities and side effects, including hepatoxicity and nephrotoxicity. Because cisplatin can accumulate in the liver and kidney cells, enhancing the production of reactive oxygen species [17]. Cisplatin induces liver damage such as cytoplasmic changes, especially in cells around the central vein. A higher dose of cisplatin can cause hepatocellular vacuolization and sinusoidal dilatation [22].

The histopathology changes on three doses of *P. macrocarpa* extract (Fig.4). Figure 4 represents treatment with dose 1 of *P. macrocarpa* extract, showing various liver abnormalities. The most visible part is the hydropic degeneration which makes the hepatocyte structure looks not solid. Hydropic degeneration is cellular swelling, an acute reversible hepatocyte change. Cells with hydropic degeneration will look enlarged, with clear cytoplasm but with a normal nucleus [23].

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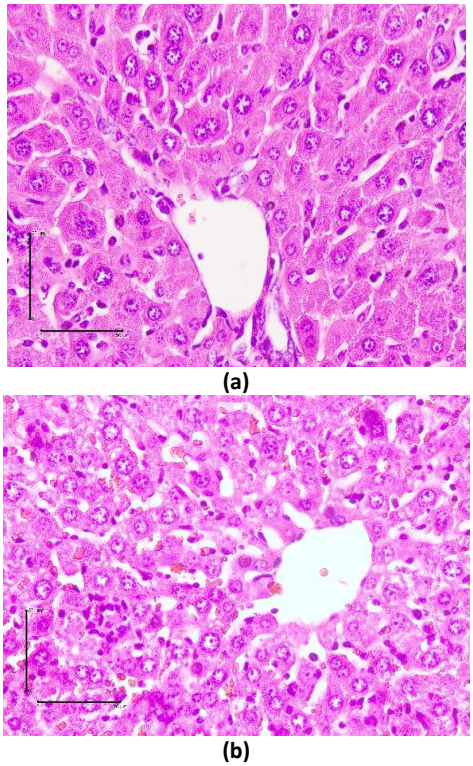

Figure 2. Normal liver histology on: control (a) and cancer (b) treatment at 400× magnification. The bar at the bottom left represent 50 µm.

These swollen cells can end up releasing the cell content into the ECM and causing necrotic cell death. Therefore, the area with this kind of cell injury is also likely to show a necrotic area [24]. Hydropic degeneration increases intracellular water by ion and fluid homeostasis [25]. It also indicates mild dilatation and congestion of the existing central vein. In addition, infiltration of inflammatory cells was also found at several points.

Figure 5 is a representative picture of treatment with dose 2 of *P. macrocarpa* extract. The structure of hepatocytes in the second dose did not appear to have a severe hydropic degeneration as in the first dose. Although infiltration of inflammatory cells at some points and dilatation in the central veins and blood vessels, the congested blood vessels were not as many as the previous dose. Figure 6 is a representative picture of treatment with dose 3 of *P. macrocarpa* extract. Observations at 100x magnification showed less organ damage than the previous doses. There was mild dilatation in the portal vein and cloudy swelling surrounded by hydropic degeneration, also categorized as mild. There were no infiltration of inflammatory cells and no Kupffer cells activation seen in the picture. These results agree with the research conducted by Sundari *et al.* [26] that *P. macrocarpa* extract has hepatoprotective activity because it reduces the level of liver tissue damage, including necrosis and degeneration of liver cells. Flavonoids in *P. macrocarpa* act as antioxidants that eliminate free radicals by releasing the hydrogen atoms from their hydroxyl groups. The hydroxyl group of flavonoids can accommodate superoxide radicals and prevent the damage of membrane lipids that damage tissues [27].

Damaged cell membranes and proteins result from oxidative stress caused by free radicals. Oxidative stress is a condition in which there is an imbalance between reactive oxygen species (ROS) such as hydrogen peroxide and antioxidative compounds such as SOD. ROS, such as hydrogen peroxide, superoxide, hydroxyl radical, etc., can cause oxidative damage such as lipid peroxidation, amino acid oxidation, cross-links protein formation, to DNA strands ruptured. However, SOD is an enzyme that is very important in converting superoxide into H_2O_2 to prevent damage at the cellular level because H_2O_2 is less reactive [28].

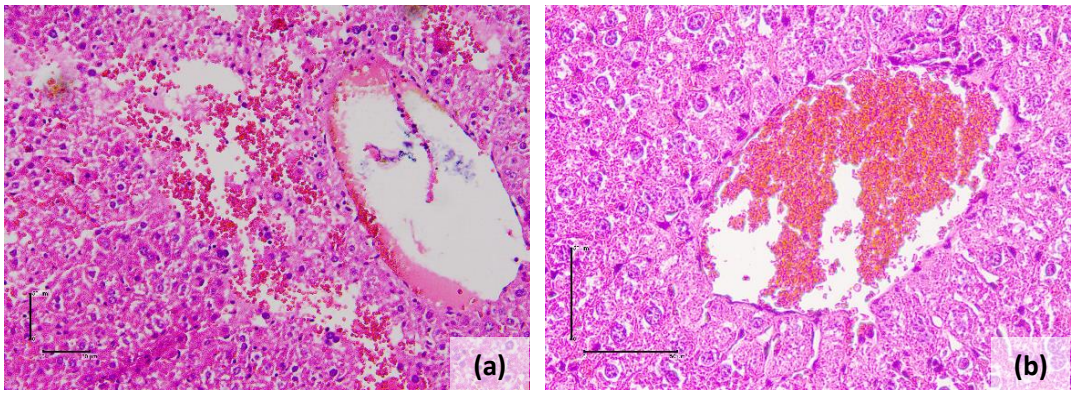

Figure 3. Liver histology on cisplatin treatment at 200× (a) and 400× magnification (b). The bar at the bottom left represents 50 µm.

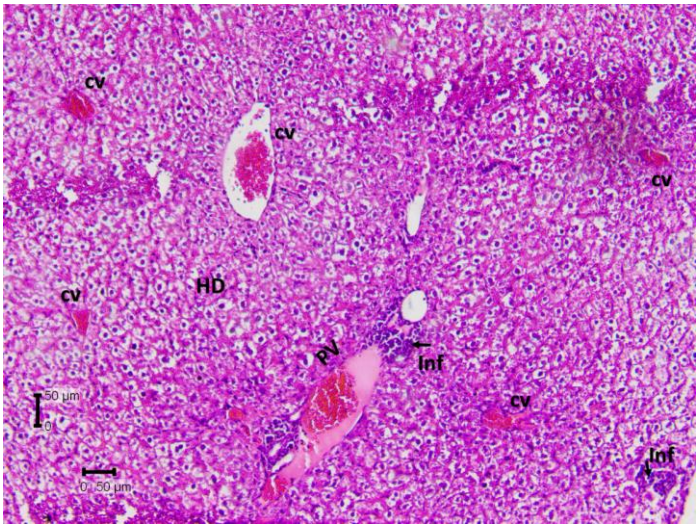

Figure 4. Liver histology on dose 1 of *Phaleria macrocarpa* extract treatment at 100× magnification. The bar at the bottom left represent 50 µm. (**Notes:** cv= central vein, pv= portal vein, hd= hydropic degeneration, inf= inflammation).

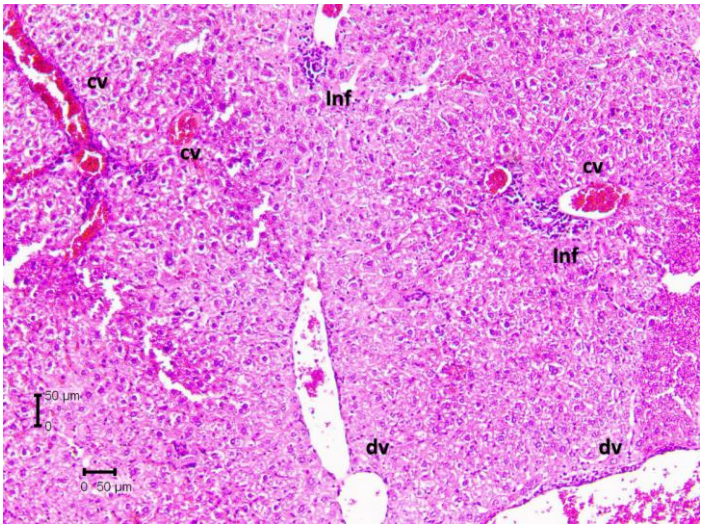

Figure 5. Liver histology on dose 2 of *Phaleria macrocarpa* extract treatment at 100× magnification. The bar at the bottom left represent 50 µm. (**Notes:** cv= central vein, dv= dilated vessel, inf= inflammation).

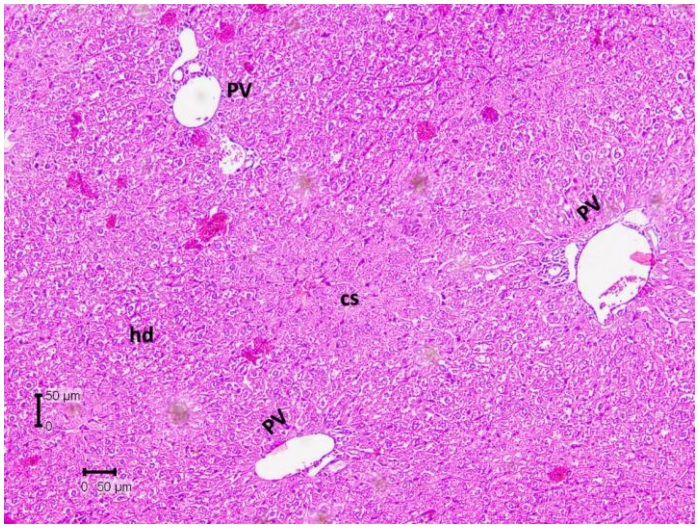

Figure 6. Liver histology on dose 4 of *Phaleria macrocarpa* extract treatment at 100× magnification. The bar at the bottom left represent 50 µm. (**Notes:** cs= cellular swelling, hd= hydropic degeneration, pv= portal vein).

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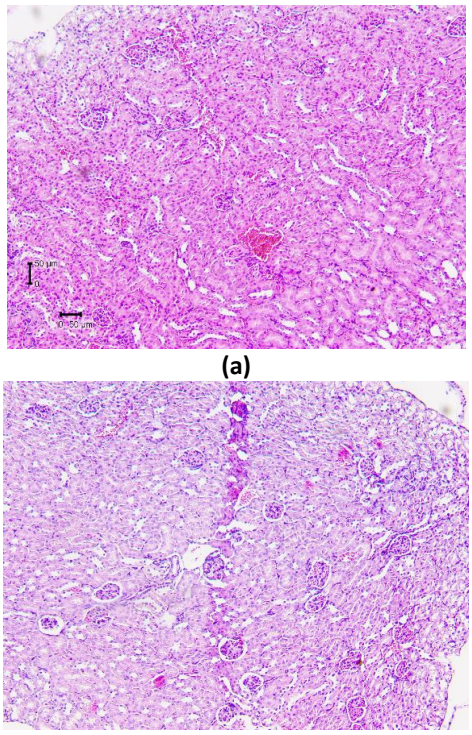
This study showed that *P. macrocarpa* extract has a side effect on liver tissue and hepatoprotective activity at a higher dose. The fruit and leaves of *Phaleria macrocarpa* contain flavonoids and phenolics, which are antioxidant agents. The antioxidant activity of this extract is associated with free radical scavenging activity [7] and induces the production of superoxide dismutase [29]. Protective actions against ROS are performed by several enzymes, including superoxide dismutase (SOD), catalase and glutathione peroxidase. A nonenzymatic compound, such as tocopherol, vitamin E, beta-carotene, and ascorbate, has the same function [30].

Histology of Renal

The next results are the gross examination of renal histology. Figure 7 shows the kidney histological observations on healthy control and cancer without any significant differences. Both control treatments showed a fairly large area without any major damage to either the tubules or the glomerulus. There was only a small amount of bleeding in the dilated blood vessels and mild inflammatory cells. Inflammation is the body responds to external and internal stimuli. Inflammation caused by tissue damage can be characterized by increased blood flow and vascular permeability, accompanied by the accumulation of leukocytes and other inflammatory mediators such as cytokines [31]. Figure 8 showed vascular bleeding in the glomerulus and between the tubules in the cisplatin treatment. Mild tubular necrosis, especially in proximal tubules marked by pyknotic nuclei also shown in the picture. The accumulation in kidney tissue cells is the basis for cisplatin-induced nephrotoxicity.

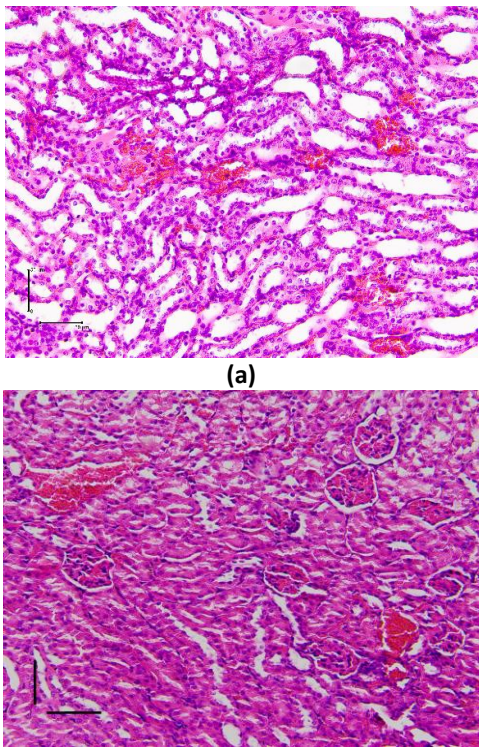
Oxidative stress was implicated in kidney injury and liver injury by cisplatin. These histological changes after cisplatin treatment confirm irreversible kidney injury, which develops from inflammation and oxidative stress that cause vascular damage. Cisplatin causes the injury by interfering with mitochondrial function and maintaining calcium homeostasis [32]. Cisplatin injures multiple renal compartments, including blood vessels, glomeruli, and tubules. Chloride on cisplatin is one of the molecules that promote kidney injury. The chloride goes through the cell, increases intracellular concentrations, and triggers intracellular injury pathways, including caspase activation, cyclin-dependent kinases, mitogen-activated protein kinase activation, and p53 signaling [33].



(a)

(b)

Figure 7. Normal renal histology on control (a) and cancer (b) treatment at 100× magnification. The bar at the bottom left represent 50 µm.



(a)

(b)

Figure 8. Renal histology on cisplatin treatment at 200× (a,b) magnification. The bar at the bottom left to represent 50 µm.

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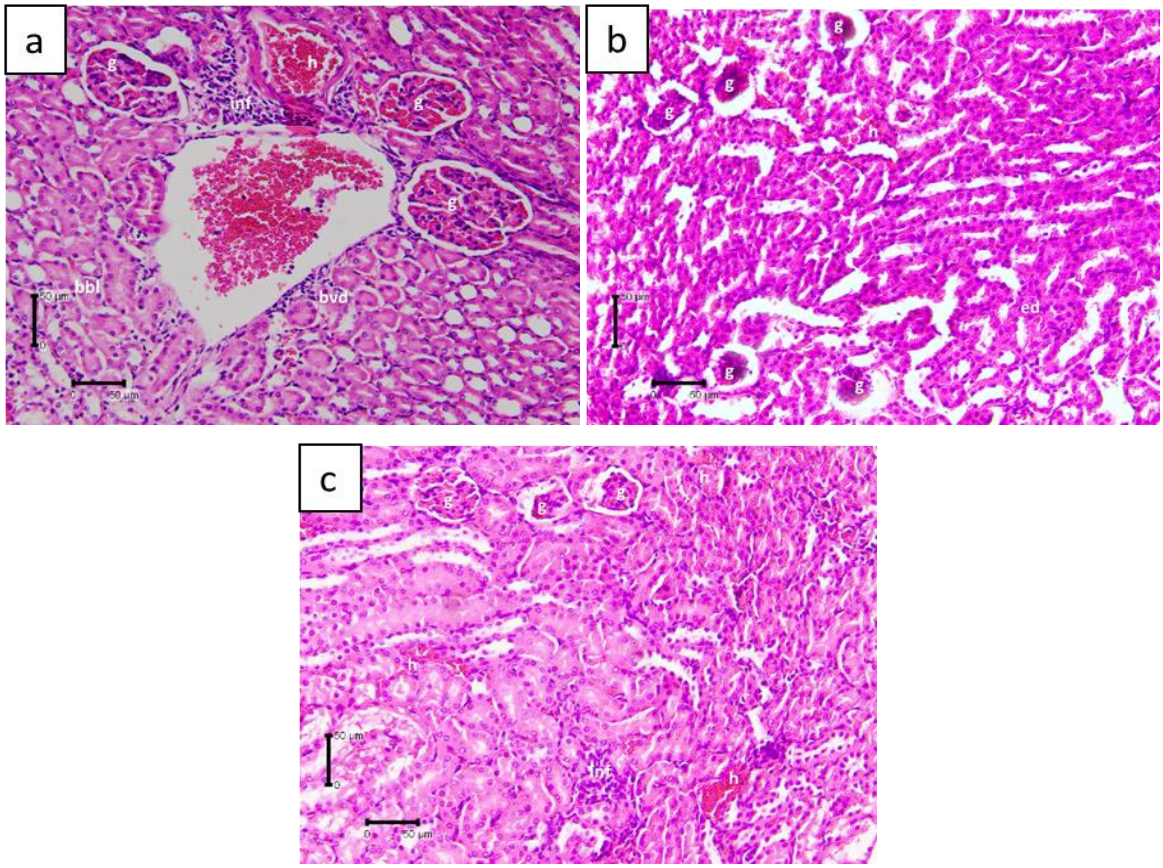

Figure 9. Renal histology on doses 1 (a), 2 (b), and 3 (c) of Phaleria macrocarpa extract treatment at 200× magnification. The bar at the bottom left to represent 50 µm.
(**Notes:** inf= inflammation, h= hemorrhage, g= glomerulus, bvd= blood vessel dilatation, bbl= brush border loss)

In dose 1, even though the glomerulus did not undergo structural changes, tubule inflammation, haemorrhage, and dilation of blood vessels were seen. In doses 2 and 3, there was not much damage to the tubules area but more to the glomerulus. Renal impairment may occur as a direct adverse effect of a metabolite or xenobiotic, specifically in the glomerular area of the tubules (Fig. 9). The cellular mechanism of renal pathogenesis is varied as the wide variety of agents that induce it, including oxidative stress, effect on ion homeostasis, cytoskeletal and mitochondrial injury, lysosomal accumulation and breakdown, and inactivation of signalling kinase [34]. The kidney histology results showed that the plant extract dose does not affect the tissue.

In the glomerular region of the kidney, several glomeruli showed atrophic changes, widening Bowman's space with obvious degeneration of cells. It loses the prominent glomerular structure suggesting apoptotic cell death [35]. Dilation of Bowman's space may occur as a consequence of increased hydrostatic pressure within Bowman's capsule due to glomerular hyperfiltration or as a consequence of shrinkage of the capillary tufts due to atrophy [34].

Hemorrhage often accompanies acute injury and can occur in the kidney as a primary lesion associated with nephrotoxicants without significant degeneration or necrosis. The presence of luminal hemorrhage implies either damage to the interstitial vascular supply and epithelial basal lamina or damage to the glomeruli as intact erythrocytes do not pass functioning glomerular filtration barriers. The inflammatory reaction is a vital body mechanism to transfer fluid from plasma protein and leukocytes to the tissues in response to injury. This injury can be caused by many factors, including excreted toxic substances or pathogen infection [36]. However, inflammatory cell infiltrates are extremely common in rats and mice and often have no toxicologic significance. The number of inflammatory cell foci increases with age and/or with the presence of chronic nephropathy characteristics [34].

CONCLUSION

Phaleria macrocarpa leaf extract at a dose between 58.9 $mg.kg^{-1}$ to 117.8 $mg.kg^{-1}$ BW is optimal for breast cancer treatment because it can reduce tumor volume by more than 90% after three weeks of treatment. Histopathology analysis showed these doses does not cause significant damage to the kidneys tissue, while liver tissue damage can be minimized because it has hepatoprotective activity.

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