Trop J Nat Prod Res, May 2024; 8(5):7244-7248 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

In Vitro and *In Vivo* Nephrotoxicity Evaluation of the Methanol Extract of *Ficus deltoidea Jack* Leaf

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ARTICLE INFO

Article history:
Received 19 October 2023
Revised 28 April 2024
Accepted 30 April 2024
Published online 01 June 2024

ABSTRACT

Ficus deltoidea Jack leaves extract (FDLE) is rich in phytochemicals like alkaloids, phenolics, flavonoids, and steroids. These phytochemicals possess an excellent potential for medicinal applications. This study aimed to assess the kidney toxicity of FDLE through *in vitro* and *in vivo* methods. FDLE was obtained by maceration in methanol at room temperature for 24 h. The *in vitro* toxicity of FDLE at 10 – 100 µg/mL was evaluated in Baby Hamster Kidney (BHK)-21fibroblast cells using the MTT assay method. The *in vivo* toxicity was assessed by histopathological examination of the kidneys of Wistar rats following oral acute administration of FDLE at 1.250 mg/kg, 2.500 mg/kg, and 3.750 mg/kg twice daily for 14 days. *In vitro* toxicity tests showed no toxic effect of FDLE on BHK-21 fibroblast cells. The percentage cell viability of BHK-21 fibroblast cells was higher than 60% at all the concentration of FDLE tested, and the IC_{50} value was 71.63 µg/mL. The histopathological analysis revealed no significant difference in the haemarrage and necrosis observed in the tissues of the kidneys of rats in the FDLE treated groups compared to the control group. Therefore, it could be concluded that FDLE demonstrated no toxic effects *in vitro* and *in vivo*, and may be relatively safe when administered to humans.

Keywords: *Ficus deltoidea* Jack, Phytochemicals, Toxicity, Kidneys.

Introduction

Ficus deltoidea Jack is a plant belonging to the Moraceae family, it is widespread in Southeast Asia, including Indonesia.[1,2] Rural communities in Kalimantan utilize *Ficus deltoidea* Jack leaves as a medicinal plant for the treatment of variety of diseases.[3] The plant has been scientifically proven to possess numerous biological activities, including antioxidant, anti-inflammatory, antimicrobial, antihypertensive, and antidiabetic activiries.[1,2,3] Phytochemical investigation has revealed that *Ficus deltoidea* Jack leaves contain alkaloids, phenolics, flavonoids, and steroids at concentrations of 154.31 mg/mL, 99.689 mg/mL, 62.917 mg/mL, and 49.036 mg/mL, respectively.[4]

Due to its antibacterial activity, *Ficus deltoidea* Jack leaves have been used as mouthwash as it has been found to possess both bactericidal and bacteriostatic effects on harmful oral microorganisms.[5] The study revealed that the leaves of *Ficus deltoidea* Jack demonstrated inhibitory effects on the growth of gram-positive bacteria, specifically *Staphylococcus aureus* with inhibition zone diameter of 15.67 mm, and minimum inhibitory concentration of 3.125 mg/mL. Additionally, the leaves were found to inhibit the growth of *Bacillus subtilis* with minimum inhibitory concentration of 25 mg/mL. Furthermore, the study indicated that *Ficus deltoidea* Jack leaves also inhibited the growth of gram-negative bacteria, namely *Escherichia coli* and *Pseudomonas aeruginosa*.[6]

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Citation: Apriasari ML, Nadia H, Hanifa N, Utami JP, Firdaus IWAK. *In Vitro* and *In Vivo* Nephrotoxicity Evaluation of the Methanol Extract of *Ficus deltoidea Jack* Leaf. Trop J Nat Prod Res. 2024; 8(5):7244-7248. https://doi.org/10.26538/tjnpr/v8i5.28

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

The phytochemicals contained in *Ficus deltoidea* Jack leaves have been shown to have excellent medicinal potential. All substances to be used in humans must be non-toxic, and safe. However, some secondary metabolites in plants have toxic potential.[7,8] Therefore, prior to assessing the safety of a substance, it is imperative to conduct a toxicity test, as the compound may pose a potential risk of toxicity to the body at certain doses.[9] Toxicity testing is a preclinical assessment to ensure that herbal plants satisfy quality standards. It aims to identify the potential harm of the test herbal preparation on human consumption, and to establish a safe dose for human usage.[10-12] *In vitro* and *in vivo* studies can be conducted to assess toxicity.[13] In the present study, *in vitro* toxic effect of *Ficus deltoidea* Jack leaves was assessed on baby hamster kidney-21 (BHK-21) fibroblast cells using the MTT assay. Hamster fibroblast cells are commonly utilized in assessing the cytotoxicity of plant materials due to their resemblance to human fibroblast cells in terms of form and ability to produce growth factors. Moreover, these cells are highly stable, sensitive, and easy to culture.[14] The toxic effect of an extract is usually reported as the half maximal inhibitory concentration (IC_{50}) which indicates the presence or absence of toxic potential of the extract.[15]

The *in vivo* toxicity test is a procedure used to assess toxic effects of an extract on test laboratory animals by administering specific doses.[16] Excessive administration of an extract dose might lead to metabolic toxicity in the kidneys. The kidneys are one of the body's excretory organs and the target of toxicants besides the liver.[17] As per the principle of drug excretion, when the amount of a drug administered surpasses the normal dosage, it can become a nephrotoxic chemical.[18] High blood flow to the kidneys causes various drugs and chemicals in the systemic circulation to be transported in significant quantities to the kidneys. The accumulation of these toxic substances will result in kidney damage.[19,20] Ultimately, changes occur in the kidneys as the body responds to toxic substances, such as bleeding and necrosis, which can be proven by the acute *in vivo* tests for 14 days.[21] Previously, anti-nephrolithiasis activity of aqueous plant extracts (*Paronychia argentea*, *Teucrium polium*, *Alhagi maurorum*, *Crataegus aronia*, *Varthemia iphionoides*) has been

conducted by evaluating inhibition of calcium oxalate (CaOx) nucleation and aggregation.[22] To ensure the safety of FDLE administration, it is important to assess its effect on the kidney as the vital organ of the excretory system. Therefore, this study aimed to assess the *in vitro* toxic effect of *Ficus deltoidea* Jack leaves extract using the MTT assay, and the *in vivo* toxic effect based on acute oral toxicity test in rats.

Materials and Methods

Plant collection and drying

Ficus deltoidea Jack leaves were collected from the Center for the Development of Food Crops and Horticulture Seeds (BBPTH) Banjarbaru City, South Kalimantan, Indonesia on March 2023. The leaves were picked and carefully selected. About 12 kg of the leaves were washed and sliced into smaller pieces. The leaves were dried at room temperature and thereafter put in an oven at 40-50°C for 4 hours.

Preparation of Extract

The dried leaves were pulverized with the aid of a mechanical blender and then sieved. The powdered leaves (1.2 kg) were macerated in methanol (98%) three times for 24 h each, with intermittent agitation using a shaker. The extract was filtered using WH-40 filter paper. The extract was concentrated in a vacuum rotary evaporator (EYELA Rotary Vacuum Evaporator) at 50-60°C for 4-6 h and then heated over a water bath to obtain a 100% viscous extract coded FDLE of approximately 210 g. The methanol-free extract was removed by dissolving FDLE in a mixture of sulphuric acid and acetic acid. The mixture was covered with a cotton wool, and heated until boiling. A homogenous solution of the methanol-free FDLE at various concentrations was prepared by dissolving FDLE with distilled water. The different concentrations of aqueous solution of FDLE were then stored at 10°C until ready for use.

In vitro cytotoxicity screening

Cell line and cell culture

Baby Hamster Kidney (BHK)-21 Fibroblast Cell line were obtained from the Central Laboratory of Veterinary Farma Surabaya, Indonesia. The cells were cultured in Eagle's minimum essential medium (EMEM) with 10% fetal bovine serum (FBS) in Roux culture bottles. Cell culture was incubated at 37°C for 48 h. The medium was replaced every 48 h, and cell culture was continued until the BHK-21 fibroblast cells reached a density of 2.4 x 10^4 cells/mL after which the culture medium was removed from the Roux bottle, and the cells were washed three times with 15 mL phosphate buffered saline (PBS) to remove residual serum. Trypsin EDTA solution (1 mL) was added to detach adherent cells and inhibit cell aggregation. The bottle was tapped gently to release the cells from the wall until the Roux bottle wall became clean. Cells (100 µL each) were transferred to a 96-well microplate using a multichannel micropipette.

Treatment with FDLE and MTT assay

BHK-21 fibroblast cells in 96-well microplate were treated with FDLE at various concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/mL). The cells were incubated in a CO_2 incubator at 37ºC for 24 h. Subsequently, the culture medium was discarded, and the microplate was rinsed three times with PBS to eliminate residual serum. Fresh culture medium and MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] reagent were added to each well, and then incubated again in a CO_2 incubator at 37ºC for 4 h. The MTT solution was discarded, and DMSO was added to stop the reaction between the MTT and the cells. The microplate was shaken for 5 to 10 min to allow the cells to excrete formazan. Cell viability was accessed by measuring the absorbance of the formazan using an ELISA reader at a wavelength of 620 nm. Afterward, the percentage of cell viability and IC_{50} were calculated. Cells without extract treatment and the culture medium without cells and extract were used as controls.

In vivo toxicity screening

Acute oral toxicity test

Animals

Sixteen (16) male Wistar rats, aged 8 - 12 weeks with a weight range of 200 to 250 g were used for the study. All rats were in a state of good health, and they were fed with rodent feed (BR2) and drank distilled water *ad libitum*. The animals were acclimatized to the laboratory conditions for a period of two weeks.

Ethical approval

The research protocol was approved by the ethics committee of the Faculty of Dentistry, University of Lambung Mangkurat, Indonesia, with registration number 011/KEPKG-FKGULM/EC/II/2023.

Grouping of animals and administration of FDLE

Animals were grouped by simple random sampling and divided into four groups of 4 rats each. Group 1 served as the control group and were given distilled water only, while groups 2, 3, and 4 were used as the treatment groups, and were administered FDLE at doses of 1,250 mg/kg bw, 2,500 mg/kg bw, and 3,750 mg/kg bw, respectively. The doses were administered orally with a gastric probe twice a day for 14 days. Prior to administration of the extract, the rats in the treatment group were fasted for 6 h.

Histopathological examination of the kidneys of treated rats

On the 15th day, the rats were sacrificed under ketamine anaesthesia (0.5 mL i.p.). The kidneys were surgically removed; they were washed with normal saline (0.9% NaCl) and soaked in 10% BNF (Neutral Buffer Formalin) solution for tissue fixation and processing. Bleeding and necrotic lesions of the kidney tissues were analyzed after haematoxylin-eosin (HE) staining. Histopathology data were presented microscopically using an Olympus XC43 light microscope with 100x and 400x magnification in 5 fields of view. The number of lesions was assessed by the percentage of lesion area in each field of view and averaged based on bleeding and necrosis criteria. Bleeding lesions were characterized by red blood cells migration from the blood vessels into the tissues. Meanwhile, necrotic lesions were characterized by changes in the shape of the cell nucleus. Lesions on kidney cells were counted and scored.

Statistical analysis

Data were processed using the Statistical Product and Service Solutions (SPSS) 26.0 for Windows programme. Data were presented as mean ± standard deviation (SD), and differences between means were analysed by *Kruskal-Wallis* test. Statistical significance between means was indicated at $p\text{-value} < 0.05$.

Results and Discussion

In vitro cytotoxicity of FDLE

Cell viability was assessed based on the absorbance of the purple formazan crystals formed on the reaction between MTT reagent and the mitochondrial enzymes of metabolically active cells (living cells). Living cells transforms the succinate dehydrogenase enzyme into purple formazan crystals. The intensity of the purple colour is directly proportional to the number of living cells. Cytotoxicity test results of FDLE using MTT reagent on a 96-well microplate showed that the purple formazan solution after addition of MTT was less intense for lower concentrations of the extract, and became more intense as the concentration of the extract increased (Figure 1). This indicates that the BHK-21 fibroblast cells were more viable at higher concentrations of the extract. Table 1 displays the percentage viability of BHK-21 fibroblast cells treated with FDLE. The percentage cell viability at all the concentration of the extract tested was higher than 60%, which indicate that the extract is non-toxic. The IC_{50} was found to be 71.63 mg/mL, indicating that FDLE had no toxic effects on BHK-21 fibroblast cells.

In this study, BHK-21 fibroblast cells maintained their viability after treatment with FDLE at concentrations of 10 – 100 µg/mL. This can be attributed to the presence of phytochemicals *such as* alkaloids, flavonoids, phenolics, and steroids which has been found in copious

amount in *Ficus deltoidea* Jack leaves. These phytoconstituents possess antioxidant activity, and can combat free radicals and enhance cell viability.[21] For example, alkaloids can contribute to the neutralization of free radicals by donating single electron to free radicals to prevent cell damage and promote cell viability. The result from this study is consistent with the findings from a previous study, which found that the alkaloid content of FDLE correlated positively with percentage cell survival.[20]

In vivo acute nephrotoxicity of FDLE

The histopathological features (bleeding and necrosis) of kidney tissues of the control and treated rats are presented in Table 2. There were no bleeding or necrotic features in the kidneys of rats in group 1 (control) and group 2 (FDLE 1250 mg/kg). Therefore, both groups were assigned mean percentage bleeding and necrosis of 0, and a corresponding score of 0. For group 3 (FDLE 2500 mg/kg) animals, the mean percentage bleeding and necrosis were 1.245 ± 1.589% and 0.415 ± 0.83%, respectively. While the group 4 (3750 mg/kg) animals had an average percentage of 2.905 ± 2.834% for bleeding, and 0.83 ± 0.958% for necrosis. Therefore, both groups 3 and 4 received a score of 1, indicating a histopathological feature of mild bleeding and necrosis of less than 25% in each group.

The histomicrographs following microscopic observations of the kidney tissues in the control and treatment groups are shown in Figures 2 and 3. There were no histopathological changes in the bleeding of the kidney tissues, and blood vessels appeared normal in both group 1 (control) and group 2 (FDLE 1250 mg/mL) (Figures 2A and 2B, respectively). Meanwhile, in groups 3 and 4 that is the FDLE 2500 mg/kg and 3750 mg/kg groups, mild bleeding (< 25%) occurred, and were characterized by vascular extravasation causing red blood cells to leak and migrate from the blood vessels into the tissue between the tubule and glomerular spaces such that there appeared a feature of red blood cells penetrating the bowman's capsule wall, covering the glomerulus in group 3 animals (Figure 2C)) and covering the tubule space in group 4 animals (Figure 2D). Similarly, no necrotic features were observed in the histopathological examination of the kidneys of both groups 1 and 2 animals. The kidney tissues in these groups presented normal proximal tubules, and glomerular cells were visible (Figures 3A and 3B). These observations may be due to the absence of toxic substances and the absence of cell repair following administration of the extract.[23]

However, in groups 3 and 4, mild necrosis of less than 25% was found in the glomerulus in the group 3 rats, and in the proximal tubule in the group 4 rats (Figure 3C and 3D).

The presence of bioactive secondary metabolites in FDLE may have influenced the histological characteristics of the kidneys. These metabolites include alkaloids, phenolic, and flavonoids.[23] These compounds possess antioxidant properties that effectively minimize necrosis and kidney cell damage, acting as nephroprotectors.[26] Alkaloids for example, possess antioxidant properties that function as scavengers of hydroxyl radicals (OH) by interrupting the lipid peroxidation process. They achieve this by donating a hydrogen atom from the ring OH group to free radicals, thereby stabilizing them. It prevents excessive cell damage that surpasses the cell's repair capacity, thereby averting cell death or necrosis.[26] In addition to alkaloids, phenolic compounds and flavonoids at low concentrations also function as antioxidants because they are easily oxidized by donating hydrogen atoms to free radicals. This process prevents cell oxidation and protein denaturation, ensuring cell activity remains undisturbed and prevents cell damage.[27] However, at high concentrations, these phytochemicals has the potential to be transformed into prooxidants, which can trigger oxidative stress and continue to cause mitochondrial dysfunction, resulting in cell necrosis.[24]

In previous studies, administration of high dose of FDLE affects the ventricles' thickness due to mucosal irritation. This was attributed to the toxic effect of the alkaloids and flavonoids at high concentrations.[28] However, alkaloids do not significantly cause toxic effects,[29] whereas, flavonoids at relatively high concentrations trigger ROS formation, causing toxic effects on cells.[30] However, the flavonoids detected at a high concentration in *Acacia rugata* L had no effect on rat kidney.[31]

Prolonged exposure to toxic substances can impose an excessive load on the kidneys, especially in the Bowman's capsule area. In the kidneys of humans and rodents, cell surface and soluble complement regulatory proteins related to the immune response are expressed by various microanatomic kidney compartments. These compartments include glomerular capillaries, peritubular capillaries, proximal tubules, collecting ducts, medullary interstitium, and various types of glomerular cells (endothelial, epithelial, and mesangial). Cellular insults in intrinsic acute kidney injury (AKI) can lead to various cell death modalities such as apoptosis, autophagy, or regulated and genetically controlled cell death (necroptosis). [25]

Increased production of *Reactive Oxygen Species* (ROS), a factor that causes tissue damage, stimulates lipid peroxidation in the kidneys. Oxidative stress alters the structure and function of the tubules because ROS affects mesangial cells and endothelial dysfunction of capillaries.[26] Bleeding occurs due to the infiltration of toxic substances into the kidney tissue, resulting in lesions on endothelial cells of the blood vessel wall. Anti-platelet activity is impaired in the presence of endothelial cell injury, leading to platelet adhesion to the endothelial cells of the blood vessels. At the site of an endothelial cell lesion, blood is released into the interstitial tissue, leading to the attachment of platelets to the endothelium of blood vessels. This attachment causes a reduction in the diameter of the blood vessels, leading to the obstruction of blood flow and, ultimately, the rupture of the blood vessels. Vascular damage triggers a mediator response, causing pathological changes in capillary endothelial vasodilation, developing into bleeding in the glomerulus.[23]

Table 1: Percentage Viability BHK-21 Fibroblast Cells after treatment with FDLE

Concentration of FDLE (µg/mL)	Percentage cell viability
100	69%
200	81.81%
300	100%
400	100%
500	100%
600	100%
700	100%
800	100%
900	100%
1000	100%

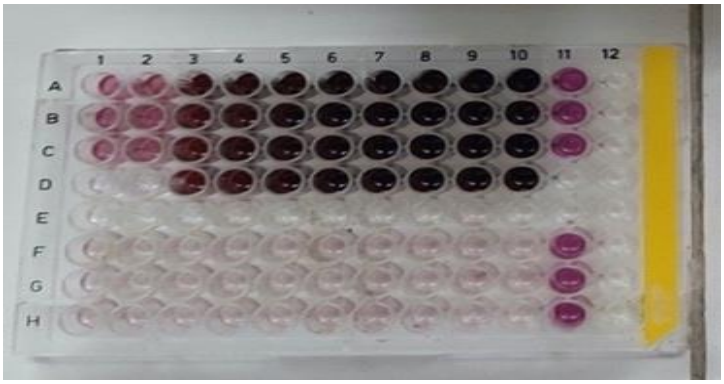

Figure 1: 96-well microplate showing the colour intensity of the formazan solution after addition of MTT reagent to BHK-21 fibroblast cells.

1A-1C: FDLE 10 µg/mL; 2A-2C: FDLE 20 µg/mL; 3A-3C: FDLE 30 µg/mL; 4A-4C: FDLE 40 µg/mL; 5A-5C: FDLE 50 µg/mL; 6A-6C: FDLE 60 µg/mL; 7A-7C: FDLE 70 µg/mL; 8A-8C: FDLE 80 µg/mL; 9A-9C: FDLE 90 µg/mL; 10A-10C: FDLE 100 µg/mL; 11A-11C: Control (Cells); 12A-12C: Control (Media).

Table 2: Histopathological features (bleeding and necrosis) of kidneys of Wistar rats treated with FDLE

Group	Percentage Bleeding (Mean ± SD)	Bleeding Score	Percentage Necrosis (Mean ± SD)	Necrosis Score
1 (Control)	± 0.0	0	0.0 ± 0.0	0
2 (FDLE 1250 mg/kg)	0.0 ± 0.0	0	0.0 ± 0.0	0
3 (FDLE 2500 mg/kg)	1.245 ± 1.589	1	0.415 ± 0.83	1
4 (FDLE 3750 mg/kg)	2.905 ± 2.834	1	0.83 ± 0.958	1

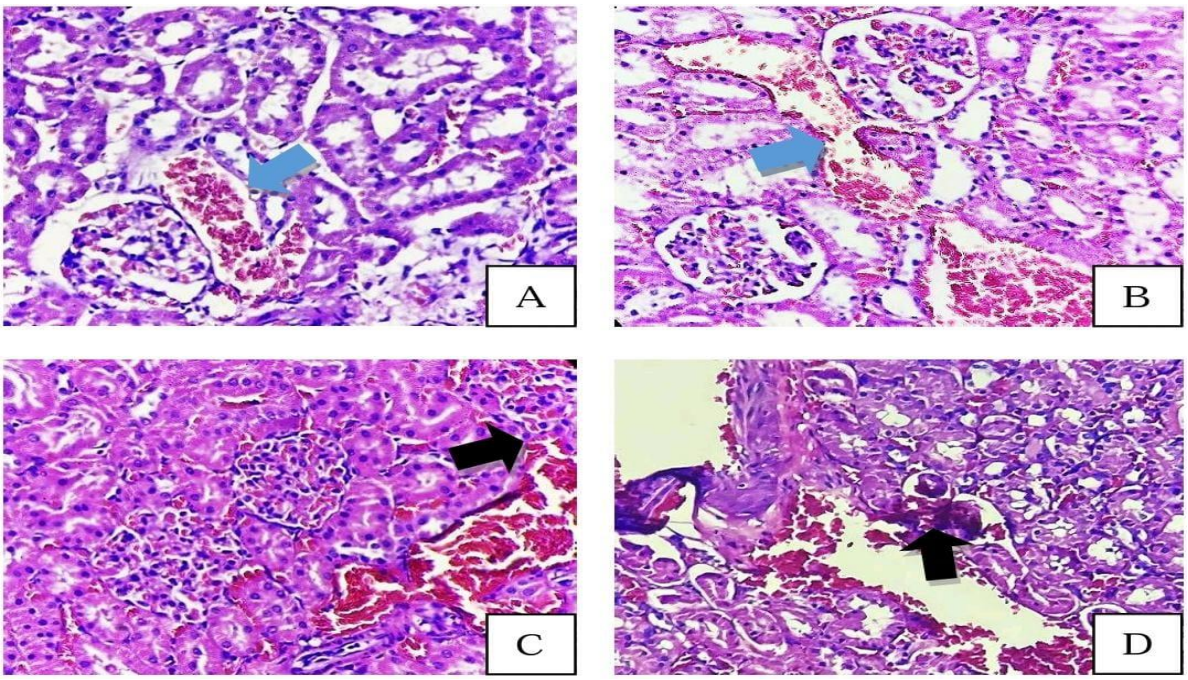

Figure 2: Histomicrograph of rats' kidneys showing the effect of FDLE on kidney tissues (bleeding). A. (Control group), B. (FDLE 1,250 mg/kg bw), C. (FDLE 2,500 mg/kg bw), D. (FDLE 3,750 mg/kg bw). Blue arrows indicate normal blood vessels, black arrows indicate blood vessels extravasation (bleeding seeping into the proximal and glomerular tubules).

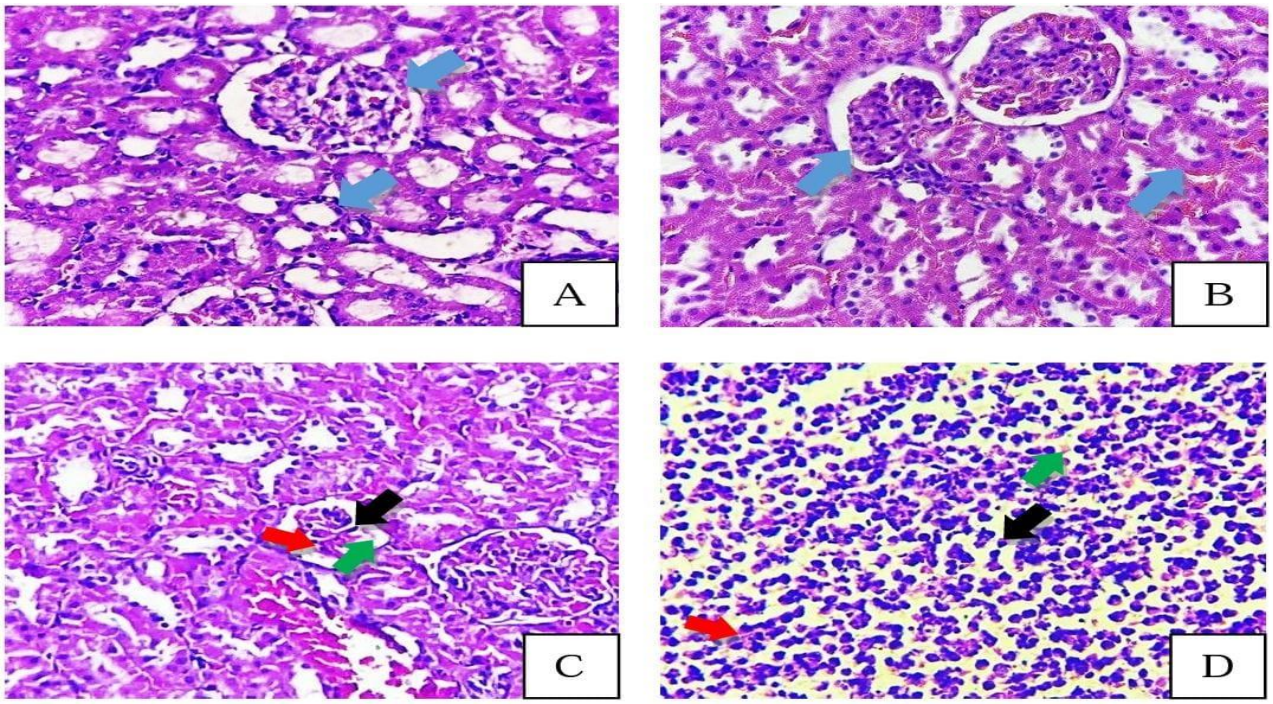

Figure 3: Histomicrograph of rats' kidneys showing the effect of FDLE on kidney tissues (necrosis). A. (Control group), B. (FDLE 1,250 mg/kg bw), C. (FDLE 2,500 mg/kg bw), D. (FDLE 3,750 mg/kg bw).. Blue arrows indicate normal proximal tubules and normal glomerulus, black arrows indicate pyknosis, red arrows indicate cariorrexis, green arrows indicate karyolysis.

Conclusion

Ficus deltoidea Jack leaves extract (FDLE) showed no toxic effect on Baby Hamster Kidney (BHK)-21fibroblast cells *in vitro*, and no significant changes were observed in the kidney tissues of male Wistar rats treated with FDLE. From the above results, it could be concluded that FDLE have no toxic effect on the kidneys both *in vitro* and *in vivo*.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors are grateful to the Institute of Research and Community Service, University of Lambung Mangkurat, Indonesia for the financial support from the research grant number 615/UN8/PG/2023.

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)