Antibacterial, Antifungal And Subchronic Toxicity Test Of *Ficus Deltoidea* Jack Leaves Extract

Maharani Laillyza Apriasari[*], Geyanina Melda Adhiya[**], Yusrinie Wasiaturrahmah[***], Juliyatin Putri Utami[***], I Wayan Arya Krishnawan Firdaus[****], Selviana Rizky Pramitha[*]

[*]Department of Conservative Dentistry, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia
[*]Department of Oral Medicine, Dentistry Faculty, University of Lambung Mangkurat, Banjarmasin, Indonesia
[**]Dentistry Faculty, University of Lambung Mangkurat, Banjarmasin, Indonesia
[***]Department of Biomedicine, Dentistry Faculty, University of Lambung Mangkurat, Banjarmasin, Indonesia
[****]Department of Oral Biology, Dentistry Faculty, University of Lambung Mangkurat, Banjarmasin, Indonesia

Correspondence: maharaniroxy@gmail.com

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ABSTRACT

Background: *Ficus deltoidea Jack is a plant that is often used as herbs medicine because of secondary metabolites such as alkaloids, phenolics, flavonoids, and steroids. This study aims to determine the activity of antibacterial, antifungal and toxicity effect of Ficus deltoidea Jack leaves (FDLE) extract.*
Method: *Pure experimental study with a posttest-only design with a control group design. The in vitro test by dilution methods using Staphylococcus aureus and Candida albicans. The toxicity test used 16 male Rattus novergicus divided into four group consist a control group and three treatment group given FDLE doses with 1,250, 2,500, and 3,750 mg/kgBW. The treatment applied 2x1 ml FDLE every 24 hours orally for 28 days.*
Result: *The MIC value and the MBC value of FDLE againts S. aureus were 8% and 10%, respectively. While, MIC and MBC value on C. albicans has similar value about 25%. The mean results of kidney toxicity test were still in the normal range. There was a significant difference in the results of the Kruskall Wallis test for urea ($p<0.05$), while there was no significant difference in the results of urea and creatinine level ($p>0.05$).*
Conclusion: There was antibacterial and antifungal efficacy of FDLE. Also, there was no toxic effect of giving FDLE at doses of 1,250, 2,500, and 3,750 mg/kgBW was showed on urea and creatinine normal level.

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INTRODUCTION

Ficus deltoidea Jack is proven to have some biological activities, such as antibacterial, antioxidant, anti-inflammatory, antimicrobial, antihypertension, and antidiabetic.[1-3] Firdaus et al, research showed that levels of the phytochemical of *Ficus deltoidea Jack* extract (FDLE) contained alkaloids of 154.31 mg/ml, phenolics of 99.689 mg/ml, flavonoids of 62.917 mg/ml, and steroids of 49.036 mg/ml. There are two secondary metabolites with the highest levels, such as alkaloids and phenolics.[4]

The presence of antibacterial content in FDLE can be used as a mouthwash, because the content of mouthwash contains antibacterial properties which have bactericidal and bacteriostatic properties that can suppress harmful bacteria in the mouth.[5] Most of the commercial mouthwashes contain alcohol and other chemicals such as chlorhexidine gluconate and triclosan that causing various side effects ranging from taste disturbance to allergic contact stomatitis.[6] To overcome such side effects, nontoxic herbal mouthwashes using plant extracts such as FDLE has been introduced.

It was found FDLE inhibited the growth of mouth gram-positive bacteria, *Staphylococcus aureus* with an inhibition zone of 15.67 mm and a minimum inhibitory concentration of 3.125 mg/ml and *Bacillus subtilis* with the highest minimum inhibitory concentration of 25 mg/ml, and can inhibit growth gram-negative bacteria, namely *Esherichia coli* and *Pseudomonas aeroginosa*.[7] This antibacterial efficacy has to be confirmed using other methods and other bacteria and fungi samples. The ingredients contained in FDLE have a good potential to be used as medicine and every ingredient used in humans must not be harmful, but the presence of secondary metabolites in plants can cause plants to have toxic potential.[8,9] Previously, there is no evidence about toxicity subchronic test of FDLE in kidney. So, it needs a determination of the safety of a compound, because a compound still has a probability of toxicity in the body at certain doses.[10] Toxicity test is one of the preclinical tests to meet quality standards as one of the requirements for herbal plants.[11] Toxicity testing needs to be done to evaluate a dental material before it is used by humans.[12]

Toxicity tests can be carried out in vitro and in vivo.[13] In the previous study, a cytotoxicity test was carried out on FDLE concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% against BHK-21 fibroblast cells in vitro and proved to have no toxic effect at all concentrations, so it is necessary to continue with in vivo toxicity tests FDLE.[14] In vivo toxicity test is a test to detect toxic effects carried out on test animals or laboratory animals by administering certain doses. Subchronic in vivo toxicity tests are usually carried out for 28 or 90 days with the test preparation at several dose levels.[15]

The organ that is the main target of toxic effects is kidney. This is because the kidneys are one of the vital organs that function to filter, excrete waste products from the body's metabolism such as producing urine, and have a high blood volume.[16] One indication of impaired kidney function is increased levels of urea and creatinine. Urea is the end product of amino acids and protein, while creatinine is a metabolic waste product produced by muscle creatinine which can be toxic in the body if the levels are too high.[17] Normal urea levels in rat are 10-50 mg/dL and normal creatinine levels in rat are 0.2-1.00 mg/dL.[18] Therefore, this study aims to analyze Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) through in vitro antibacterial test againts *Staphylococcus aureus*, antifungal test

againts *Candida albicans* which become pathogen cause oral infection, and also conduct in vivo toxicity test of FDLE using urea and creatinine levels marker in the kidneys of wistar rats *Rattus norvegicus*.

RESEARCH METHODS

Experimental design

This research was carried out at the Biochemistry Laboratory, Medicine Faculty, Lambung Mangkurat University in February-April 2023. The research protocol was approved by the Ethics Committee for Health Research no 012/KEPKG-FKGULM/EC/II/2023, Dentistry Faculty, Lambung Mangkurat University. The toxicity test used male wistar *Rattus norvegicus* with weight 200-250 grams and aged 8-12 weeks in good health. The sampling technique was carried out by simple random sampling using the unpaired numerical comparative analytic research formula with a 20% probability of failure according to Higgins and Kleinbaum.[19] Based on the sampling technique, the minimum number of samples per group is 4, so that the total number of samples is 16. There are 4 treatment groups, namely 1 negative control group with the administration of distilled water, 3 treatment groups with the administration of FDLE 1250 mg/kgBW, 2500 mg/kgBW and 3750 mg/kgBW given 2x1 ml every 24 hours for 28 days. Wistar rats were given food and drink ad libitum during the adaptation period in the laboratory.

Preparation of FDLE

Ficus deltoidea Jack leaves were obtained from the Center Development of Food Crops and Horticulture Seeds, Banjarbaru City, South Kalimantan, Indonesia. *Ficus deltoidea Jack* leaves amount about 12 kg. It was wet sorting, then the leaves are washed and chopped into smaller shapes and dried at room temperature and put in the oven with a temperature of 40-50 ^{0}C for 4 hours.[14] After that, dry sorting was carried out, then pulverized with a blender and sieved until 1.2 kg of simplicia powder was formed. Next, the maceration process was carried out and 98% methanol was added for 3x24 hours, interspersed with stirring with the help of a shaker and the solvent was changed every day, then the solution was filtered through WH-40 filter paper. The solution was evaporated using a vacuum rotary evaporator with a temperature of 50-60 ^{0}C for 4-6 hours and then heated over a water bath to obtain a 100% viscous extract of ± 210 g.[20] The methanol-free test can be dissolved by dissolving FDLE with H_2SO_4 reagent and adding acetic acid then covering it with cotton, heating it until it boils. The extract is free from methanol if there is no ester odor. Preparation of FDLE concentration by dilution using distilled water as a solvent to obtain a homogeneous mixture of solvents and test materials. FDLE is stored at a low temperature of 10 ^{0}C.[21]

Subchronic toxicity test of FDLE

The toxicity test was carried out by divided all samples into four groups containing FDLE groups with various concentrations namely 1,250 mg/kgBW, 2,500 mg/kgBW, 3,750 mg/kgBW and control group.[14] The doses are given as much as 2x1 ml orally for 28 days for each treatment. On the 29th day, the mice are anesthetized intraperitoneally by giving a mixture of 91 mg/kg ketamine and 9.1 mg/kg xylazine given as much as 10 ml/kg, then blood was taken intracardially using injection syringe. The method for examining

urea is Glutamate dehydrogenase and the method for examining creatinine is colorimetry using UV-Vis single beam spectrophotometry.[22]

Bacterial and Candida strains and growth conditions

Staphylococcus aureus (ATCC 6538) was obtained from Thermoscientific (Lenexa, KS) while *Candida albicans* (ATCC 10231) was obtained from MBRIO Food Laboratory (Bogor, Indonesia). Both of them were cultured on Brain Broth (BHIB) media and then incubated for 24 hours under anaerobic conditions. Bacteria and fungi suspension was made by adding NaCl 0.9% and then homogenised until the turbidity was equivalent to Mc Farland 0.5 standard ($1.5x10^8$ CFU/ml).[22,23]

Dilution method

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values were determined using liquid and solid dilution methods. Each 1 ml of FDLE doses of 1%, 1,25%, 1,5625%, 2%, 2,5%, 3,125%, 4%, 5%, 6,25%, 8%, 10%, 12,5%,16%, 20%, 25%, 32%, 40%, 50%, 64%, 80% and 100% was mixed with 1 ml of *Staphylococcus aureus* bacterial suspensions and *Candida albicans* fungal suspensions.[23] After mixing, the FDLE antibacterial activity was tested by liquid dilution to obtain MIC and solid dilution for MBC. This test was also carried out on a positive control in the form of chlorhexidine gluconate 0.2% and distilled water as a negative control. MIC is assed from the measurement of the difference in absorbance values after and before incubation for 24 hours using a UV-Vis spectrophotometer with a wavelength of 440 nm. MBC is the smallest concentration that has a negative absorbance value from the difference in absorbance values after and before incubation. The results of the liquid dilution were transferred into petri dishes containing NA media using micropipettes and then incubated for 24 hours. MBC was determined from the smallest concentration with no bacterial colonies after incubation on NA media for 24 hours.[22,23]

RESULTS

Ficus deltoidea Jack extract (FDLE) contained alkaloids of 154.31 mg/ml, phenolics of 99.689 mg/ml, flavonoids of 62.917 mg/ml, and steroids of 49.036 mg/ml (4). The results of examining the levels of urea and creatinine in the Wistar rats after being given FDLE can be seen in Table 1. Based on research results, it can be seen that the lowest average urea level was in group P1 which was given a dose of 1,250 mg/kgBW with a value of 29.556 mg/dL, while the highest average urea level was in group P3 which was given a dose of 3,750 mg/kgBW with a value of 38.785 mg/dL. The lowest average creatinine level was in the negative control group with a value of 0.568 mg/dL, while the highest average creatinine level was found in the P3 group which was given a dose of 3,750 mg/kgBW with a value of 0.909 mg/dL.

Table 1. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of FDLE against *S. aureus* and *C. albicans*

No	Type of Microorganism	MIC	MBC

1	*Staphylococcus aureus*	8 %	10 %
2	*Candida albicans*	25 %	25 %

Note:

Not turbid
Turbid

Kruskal-Wallis test showed a significance value of 0.006 for urea content ($p<0.05$), meaning that H0 was rejected, it can be concluded that there were a differences in urea levels of wistar rats between groups. The Mann-Whitney test obtained significant differences in the negative control group with the P2 group, the negative control group with the P3 group, the P1 group with the P2 group, the P1 group with the P3 group, and the P2 group with the P3 group, while for the negative control group with the P1 showed that there was no significant difference.

The results of the Mann-Whitney test of creatinine levels obtained significant differences in the negative control group with the P2 group, the negative control group with the P3 group, the P1 group with the P2 group, the P1 group with the P3 group, and the P2 group with the P3 group, while for the negative control group with the P1 showed that there was no significant difference. The results of the analysis from the Kruskal-Wallis test showed a significance value of 0.382 for creatinine levels ($p>0.05$)

MIC of *Staphylococcus aureus* was used to assess the antibacterial activity. The MIC test after incubation revealed that the value to inhibit bacteria growth starting at a concentration 8% of FDLE (Table 2). Whereas, the bactericidal activity of FDLE was initiated in 10% concentration with MBC value. Antifungal activity was depicted through *Candica albicans* growth inhibition (Table 2) with MIC dan MBC value was 25%. MBC is more accurately determined by the absence of growth on solid medium, rather than observation on the turbidity of the treated inoculum.[24]

Table 2. Results of examination of urea and creatinine levels in the kidneys of wistar rats after being given FDLE

Group	Mean ± Std. Deviation (mg/dL)	
	Urea	Creatinine
Control	30.373 ± 0.763	0.568 ± 0.227
P1	29.556 ± 0.884	0.682 ± 0.262
P2	32.944 ± 0.603	0.795 ± 0.227
P3	38.785 ± 0.539	0.909 ± 0.371

Note:

C : Negative control was given aquadest

P1 : Treatment group 1 dose of 1,250 mg/kg BW

P2 : Treatment group 2 dose of 2,500 mg/kg BW

P3 : Treatment group 3 dose of 3,750 mg/kg BW

DISCUSSION

The antimicrobial and antifungal efficacy of the FDLE were determined by the standard dilution methods. Different concentrations of FDLE were prepared, and MIC was calculated by tube macro-dilution method. The MBC was decided by the lowest concentration that kills 99.9% of the initial *S. aureus* and *C. albicans* population, respectively. According to the result, MIC of FDLE towards *S. aureus* was 8% and *C. albicans* was 25%. It means FDLE have potential properties that stimulate antibacterial and antifungal mechanism of pathogen microorganism. The active compounds contains alkaloids of 154.31 mg/ml, phenolics of 99.689 mg/ml, flavonoids of 62.917 mg/ml, and steroids of 49.036 mg/ml have versatile pharmacological effect to disrupt some cellular mechanism of bacteria cell. One of the targets is cell membrane quality which can be measured by cell membrane integrity, membrane permeability, membrane depolarisation, and morphological and ultrastructural changes. In addition, flavonoid is well-known to inhibit bacterial growth via nucleic acid synthesis supression and energy metabolism, adhesion and biofilm formation reduction. Alkaloid can make a bond with porin that affects the nutrient transport in bacteria cell. The antifungal effect of FDLE on *C. albicans* is influenced by alkaloids and flavonoids derivate as well. Previous study showed the biofilm of *C. albicans* can be influenced by berberine alkaloids and flavonoids from *Tinospora crispa* L stem.[23,24]

Regarding to the results of the study it was found FDLE at doses of 1,250, 2,500, and 3,750 mg/kgBW was not toxic to the kidneys of Wistar rats, because normal urea levels were in the range of 10-50 mg/dL and normal creatinine levels were in the range of 0.2 -1.0 mg/dL.[32-34] This sub-chronic toxicity studies showed that FDLE does not have general toxic effects in renal function. The investigation of major toxic affect in kidney by monitoring creatinine and urea level in all animal groups in these study revealed that there were not difference compare to control group.

In the P2 group which was given a dose of 2,500 mg/kg BW and P3 which was given doses 3,750 mg/kg BW, the results showed higher ureum levels than the control group and shows significant result statistically. It is match with previous one that is no signs of toxicity and mortality in diabetic mice model after treament with FDLE up to a dose 2000 mg/kg BW for 14 days toxicity test. The subchronic test of FDLE that given by oral administration for male rat does not induce clinical symptoms of toxicity or mortality.[25] The level of urea in the rat blood serum was also influenced by several factors, including the presence of acute kidney damage and chronic, shock, samples of urea levels experienced lysis during measurement after centrifugation, as well as a state of dehydration of body fluids.[26,27] Elevated levels of urea in the blood can be considered as a sign of kidney damage only when accompanied by urine examination and clinical symptoms that support the diagnosis of kidney failure.[28]

In the P1 group, which was given a dose of 1,250 mg/kgBW, P2, which was given a dose of 2,500 mg/kgBW, and P3, which was given a dose of 3,750 mg/kgBW, showed higher creatinine levels than the control group. Creatinine diffuses throughout the body's water at a slower rate than urea, and takes about four hours to equilibrate; therefore, serum creatinine concentration changes more slowly compared to urea. This is the reason why creatinine levels are still higher than urea levels. In the kidney, creatinine is filtered

through the glomerulus without experiencing reabsorption, because the creatinine is not reabsorbed by the tubules, the creatinine level in the blood can reflect the ability of the glomerulus to process GFR or glomerular filtration.[29] The mean creatinine result was higher in the treatment group than in the negative control group due to the high dose of the test preparation which resulted in the accumulation of metabolites in the kidney which could injure the nephron epithelial cells. If the kidney nephron cells are damaged, the serum creatinine will re-enter the body and enter the bloodstream where the serum creatinine should be disposed of.[17] Eventhough creatine is a product of the arginine biosynthesis pathway in vivo, is stored in skeletal muscles and metabolizes into creatinine, but some researchers found that food, age, gender, activity level, and diet have no proportional effect on blood serum creatinine levels. Whole creatinine is excreted into the urine through kidney filtration in the glomerulus, so that if there is damage to the kidneys, the creatinine will accumulate in the blood.[29,30]

The reason for the higher levels of urea and creatinine than the negative control group is one of the reasons for the presence of excess free radicals in the body. These free radicals can come from alkaloids which are one of the highest contents in FDLE. This is supported by the research of Firdaus et al (2023) that FDLE contain several active substances such as alkaloids, phenolics, flavonoids and steroids.[4] Alkaloids in high doses can undergo changes to pro-oxidants. Unbalanced pro-oxidants and antioxidants in the body will cause the formation of free radicals. These excess free radicals and Reactive Oxygen Species (ROS) can cause oxidative stress in the kidneys. Increased oxidative stress can cause cell death, causing the released cell contents to bind the protein fibronectin in the tubular lumen .This condition can cause a cylindrical blockage, therefore urea and creatinine cannot be excreted properly, urea and creatinine levels increase even though creatinine does not exceed normal levels.[31-33]

According to research by Aryani *et al* (2022) the ingredients contained in FDLE have a toxic effect when used in high doses.[31] In animal toxicity tests, it was found that administration of alkaloids could cause damage and even necrosis of renal tubular epithelial cells in rats. Inhibition of organic anion transporters by alkaloids may be one mechanism of nephrotoxicity. Phenolics undergo metabolism and transform into various forms of reactive intermediates, especially the quinone group, which can easily form covalent bonds with proteins, so that phenolics are able to exert toxic effects on humans.[32,33] Flavonoids are one of the compounds derived from phenol which have activity as antioxidants, but in certain circumstances these flavonoids can become pro-oxidants. Antioxidants in plant extracts at high concentrations can be cytotoxic by inducing severe oxidative stress.[10]

The average urea level in the P1 group was lower than the negative control group due to the presence of compounds that could inhibit the formation of lipid peroxide by increasing the concentration of intracellular antioxidants and preventing free radicals. The contents of secondary metabolites contained in FDLE have a renoprotective effect which can help clean up free radicals and protect the kidneys from injury.[31] Kenta's research (2019) showed that the results of giving purple leaf extract had activity that could reduce urea and creatinine levels in Wistar rats which was also due to its resemblance to FDLE which contained secondary metabolites in the form of alkaloids, flavonoids and steroids.[34] Compounds that have activity as antioxidants are excellent free radical reducing agents and can directly or indirectly inhibit the

ability of endogenous antioxidant expression. Antioxidants are known to be able to repair damaged cells so that they can improve kidney performance by lowering urea and creatinine levels in the body.[29]

CONCLUSION

Antibacterial activity was presented through MIC value of FDLE was on 8% concentration and MBC value was on 10% concentration of FDLE, while antifungal activity was on 25% concentration. There are no toxic effects in the kidney of FDLE at doses of 1,250 mg/kg BW, 2,500 mg/kgBW, and 3,750 mg/kgBW which was depicted by normal range of urea and creatinine level.

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CONFLICTS OF INTEREST

There is no conflict of interest in the study.

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