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The Effect Of Combination Fosfomycin And Sambiloto *(Andrographis paniculata)* Leaf Extract On The Leukocytes Count, Nitrite Levels, And Lactoferrin Levels In Wistar Rats *(Rattus norvegicus)* Urinary Tract Infection Model

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ABSTRACT

Background: Fosfomycin is the first-line of antibiotic for urinary tract infection (UTI). To prevent antibiotic resistance, antibiotics and herbal medicines combination was developed. Studies have shown that *Andrographis paniculata* has antimicrobial and anti-inflammatory agent.

Objective: To investigate the effect of *A. paniculata* extract for treating UTI in animal model.

Methods: Using post test only control group design, thirty females *Rattus norvegicus* rats were induced by 1 cc transurethral inoculation of *E. coli* and then randomly grouped into negative control (NC) received no treatment, positive control (PC) that had 54 mg fosfomycin, and treatment groups which were divided into T1 (A. paniculata 100 mg/kgBW), T2 (A. paniculata 200 mg/kgBW), T3 (fosfomycin 54 mg with A. paniculata 100 mg/kgBW), T4 (fosfomycin 54 mg with A. paniculata 200 mg/kgBW). Urinary samples were utilized to count leukocytes under microscope, nitrite levels with a dipstick, and lactoferrin using ELISA.

Results: Combination of fosfomycin and *A. paniculata* led to a reduction in the number of leukocytes and nitrite levels compared to the single therapy group, particularly in T4 group ($p<0.001$). There were no significant differences in lactoferrin levels among all study groups ($p>0.05$).

Conclusion: Combination of *A. paniculata* with fosfomycin resulted in a more pronounced reduction in the number of leukocytes and nitrite levels compared to single therapy, while lactoferrin levels were unaffected.

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1. Introduction

Urinary tract infection (UTI) is a bacterial infection of the urinary tract. Under normal circumstances, urine does not contain bacteria, viruses or other microorganisms. Approximately 80% of UTIs are caused by Escherichia coli.1 Empiric antibiotics can be given while waiting for the results of culture and sensitivity tests for the pathogen that causes UTI. According to the World Health Organization (WHO), UTI ranks as the second most common infectious disease, with a total of 8.3 million reported cases annually.[2,3] The Basic Health Research in 2018 reveals that in Indonesia, the incidence of UTI is approximately 90-100 cases per 100,000 population per year, totaling around 180,000 cases annually.[4]

One of the main problems of UTI is difficult diagnosis in asymptomatic cases and delays in treatment which often adds to the complexity of the disease. The management of UTI according to the Association of Indonesian Urologists in 2020 is the administration of 3 mg of fosfomycin trometamol which is known to be a first-line empirical antibiotics for treating UTI's.[5] Fosfomycin was chosen because it has a sensitivity of 76% against bacteria that causes UTI's, namely Escherichia coli, (80%), Klebsiella pneumonia (24%), and Pseudomonas aeruginosa (38%). However, inappropriate administration of antibiotics can cause resistance. In the search for alternative solutions to this predicament, one alternative considered was the use of herbal medicines in combination with standard antibiotics.[6]

Sambiloto (*Andrographis paniculata*) is a medicinal plant that has been extensively studied for its medicinal properties. Various preparations of A. paniculata are currently available in Indonesia, either as a standalone or in combinations with other herbal ingredients. It possesses anti-inflammatory properties due to the presence of andrographolite and isoandrographolite, potential

antimicrobial effects from 14-deoxyandrographolide, and the ability to enhance the immune system against infection.[7-9] A. paniculata has successfully passed the experimental stage in vitro and in vivo in mice, demonstrating therapeutic benefits. In a study by Bao, et al, the extract showed the ability to reduce inflammatory cells in asthma model rat. Previous research has also highlighted the efficacy of A. paniculata in treating prostate cancer and its effectiveness against pathogens causing urinary tract infections.[10,11] Combining A. paniculata with fosfomycin may enhance its effectiveness in reducing inflammatory effects and acting as an antimicrobial agent.[12] However, to our knowledge there has been no research investigating the connection between A. paniculata extract and urinary tract infections in rats, specifically assessing the number of leukocytes, nitrite levels, and urine lactoferrin levels. Therefore, this study aims to determine differences in the number of urinary leukocytes, nitrite, and lactoferrin levels in the urine of Rattus norvegicus UTI model rats treated with antibiotics, A. paniculata extract, and their combination.

2. Methods

This study was limited to only a post-test control group design. Thirty *Rattus Norvegicus* rats aged 2 months and weighes 150-200 grams were procured from an animal Laboratory operated by the Faculty of Medicine from the University of Sebelas Maret in Solo, Indonesia, and housed in a controlled environment. The subjects were provided with standard rodent chow and water ad libitum for 7 days of acclimation. UTI was induced using E. coli bacteria provided by the Microbiology Laboratory from the faculty of Medicine from Diponegoro University. The culture of the bacteria were made into a 0.5 MacFarland standard suspension with a dose of 50 µl for each sample. The rats were given 0,5 mL ketamine hydrochloride (Dexa Medica, Cikarang, Indonesia) intramusculary as anaesthesia. Subsequently, transurethral instillation was performed by placing a catheter into the rat's urethra and 50 µl of E. coli inoculum was instilled slowly to minimize vesicourethral reflux.

Fosfomycin (Monuril®, Zambon, Jakarta, Indonesia) was given in 54 mg doses, which was originally a suitable dose for humans but then converted to doses suitable for rats. While the A. paniculata extract (Jamu Iboe, Jakarta, Indonesia) was given in 100 and 200 mg/kgBW doses. Thirty rats were randomly divided into six groups, each consist of five rats who had previously been inducted with UTI: (Negative Control) NC and was given no treatment; (Positive Control) PC was given 54 mg fosfomycin; group T1 was given 100 mg/kgBW A. paniculata extract; group T2 was given 200 mg/kgBW A. paniculata extract; group FT1 was given 100mg/kgBW A. paniculata extract and 54 mg fosfomycin; and group FT2 was given 200 mg A. paniculata extract and 54 mg. Fosfomycin and A. paniculata extract was given once daily with an oral gastric tube (7 gauge feeding tube) inserted daily and taken off after supplementation, for 14 days. The subjects were terminated using a lethal dose of ketamine after 15 days of UTI-induction.

A total of 1 mL of urine was extracted from each test subject and stored in a specimen container for analysis. Leukocyte count, nitrite levels, and urinary lactoferrin were ascertained from the study's outcomes. The measurement of urine leukocytes involved examining the urine sediment obtained from the sample centrifugation process under a microscope. The evaluation of urine nitrite levels was conducted using a strip dipped into the urine samples, with visual readings performed based on the manufacturer's inspection procedures. Meanwhile, lactoferrin levels were measured using the ELISA method, employing a Biotinylated detection antibody, horseradish peroxide conjugate, and Reagan substrate. The calculated values were then determined using the standard curve.

Data was expressed as mean and median. Statistical analysis of differences in leucocyte counts and urinary nitrite levels from each group was carried out using the Mann-Whitney test, while differences in lactoferrin levels were analyzed using the Post Hoc Games-Howell test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 27.0 for Windows (IBM Corportaion, Amonk, NY, USA). A $p<0.05$ was considered significant

3. Result

All subject animals were alive until the end of the study with no drop-out criteria.

The results of the Mann-Whitney test shows that there were differences between each group. Mice treated combination fosfomycin 54 mg with 200 mg/kgBW A. paniculata produced the lowest number of leukocytes compared to other groups (0.60 ± 0.55 vs 92.60 ± 4.67 in CN, p = 0.008; vs 21.20 ± 4.55 in CP, p=0.008; vs 75.40 ± 2.70 in T1, p=0.008; vs 54.20 ± 4.55 in T2, p=0.008; vs 38.80 ± 12.80 in FT1 , p=0.008) (Figure 1).

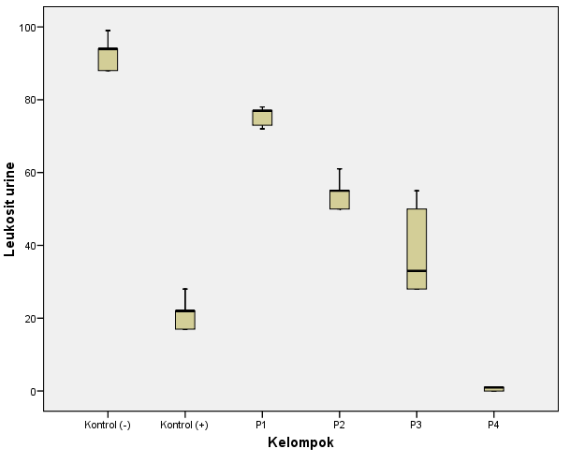

Figure 1. Fosfomycin and A. paniculata extract on urinary leukocyte count.

Urine nitrite is an examination carried out to assess the levels of nitrite reductase metabolites produced by microorganisms that infect the urinary tract. Analysis of nitrite levels showed that there were significant differences

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between CN and treatment groups (vs T2, p=0.003; vs FT1, p=0.003; vs FT2, p=0.003). We found that the addition of A. paniculata extract to fosfomycin antibiotic therapy could result in a significant reduction in nitrite (T1 vs T2, p=0.050; vs FT1, p=0.050; vs FT2, p=0.050). Meanwhile, between combination administration with different doses of A paniculata, no differences were found (FT1 vs FT2, p=1,000).

Table 1. Urinary Nitrate Analysis in Each Group

Group	Nitrit		p
	Positive	Negative	
CN	5 (100%)	0 (0%)	<0,001*
CP	0 (0%)	5 (100%)	
T1	3 (60%)	2 (40%)	
T2	0 (0%)	5 (100%)	
FT1	0 (0%)	5 (100%)	
FT2	0 (0%)	5 (100%)	

* p<0,05 is considered as significant

Lactoferrin is an acute-phase protein secreted by kidney cells and has been found to be associated with the immune response to urinary tract infections. The analysis showed that the mean lactoferrin level in CN was 17.58 ± 4.54 pg/mL; CP was 12.91 ± 3.69 pg/mL; T1 was 13.64 ± 3.84 pg/mL; T2 was 10.70 ± 0.81 pg/mL; FT1 was 10.74 ± 2.26 pg/mL; and FT2 was 9.11 ± 0.49 pg/mL. We did not find any significant differences in lactoferrin levels between all groups (p>0.05) (Figure 2).

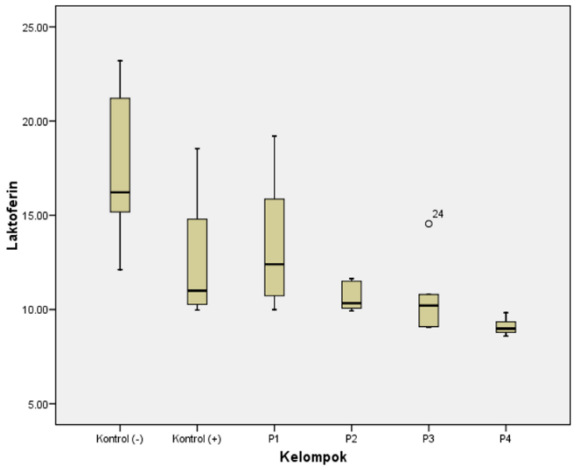

Figure 2. Fosfomycin and *A. paniculata* extract on lactoferrin level.

4. Discussion

Andrographis paniculata has been identified to possess two main bioactive compounds: andrographolide and flavonoid derivatives. Andrographolide (C20H30O5) plays an anti-inflammatory role by reducing cyclooxygenase (COX)-2 and regulating inflammatory mediators such as IL-1β, IL-6, and TNF-α. Andrographolide's antimicrobial role has also been proven against several gram-positive bacteria, suspected to occur due to the inhibition of bacterial RNA protein synthesis, disrupting DNA formation, and cell wall construction. Flavonoids also support antimicrobial activity by suppressing cytoplasmic membrane function, reducing adhesion and biofilm formation, porin on the cell membrane, membrane permeability, and pathogenicity crucial for bacterial growth.[13,14]

Our research results indicate that the combination of A. paniculata and the antibiotic fosfomycin produces the best levels of leukocytes, nitrites, and lactoferrin among other treatment groups. This finding aligns with Zeng et al., who reported a synergistic antimicrobial effect between andrographolide and conventional antibiotics such as fosfomycin, streptomycin, and erythromycin against P. aeruginosa bacterial infections.[15] Fosfomycin works by inhibiting the early stage of bacterial cell wall formation through phosphoenolpyruvate transferase enzyme. Additionally, fosfomycin inhibits the production of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and IL-1α, increases IL-10 production, and enhances neutrophil phagocytic killing of attacking pathogens.[16] Ativui et al. reported that the combination of antibiotics and andrographolide yields many benefits by enhancing antibacterial activity, minimizing side effects, reducing the time needed for long-term antimicrobial therapy, and preventing the emergence of resistant microorganisms.[17]

The influence of the antibiotic combination supported by A. paniculata extract that we discovered on the decrease in leukocyte levels may be caused by the presence of andrographolide and flavonoids, which can reduce inflammation and bacterial activity in the bladder. Both accelerate the inflammatory process, resulting in minimal recruited leukocyte numbers, as evidenced by the recorded urine leukocyte counts in urinalysis.[18,19] We also found the lowest nitrite levels in rats given combination therapy, indicating the ability of this antibiotic and herbal combination to suppress the activity of pathogenic bacteria. Additionally, the highest lactoferrin levels were found in the negative control group, and the lowest were obtained in the treatment group given combination therapy. Patras et al. reported that uropathogenic E. coli infection would increase lactoferrin release in urine, making this parameter one of the indicators of urinary tract infections.[20] Although no significant difference in lactoferrin levels was found in our study among treatment groups, it can be demonstrated that a positive effect resulted from combination therapy.

This research has several limitations. First, we only used one type of antibiotic (fosfomycin) with one dosage form combined with A. paniculata, so this study could not determine the effects and interactions of other antimicrobial agents when combined with A. paniculata. Second, our study also could not explain the pharmacological interactions that occur between A. paniculata and fosfomycin antibiotics that lead to a decrease in urine leukocyte counts, nitrite levels, and lactoferrin. Third, although our research supports the use of a combination of A. paniculata and fosfomycin in urinary tract infections, this study was conducted on rats, and further research is needed to test the safety and effectiveness of sambiloto and fosfomycin antibiotics in humans.

5. Conclusion

Combination of fosfomycin 54 mg with A. paniculata extract 200 mg/kgBW shows greater levels of efficacy compared to their single use in managing urinary tract infection (UTI) cases.

Ethical Approval

This research was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Diponegoro (Protocol Numbers: 123/EC-H/KEPK/FK-UNDIP/X/2023).

Conflicts of Interest

The authors declare no conflict of interest.

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Author Contributions

Conceptualization, Sutriono, Endang, Eriawan, Yora, Sofyan; Methodology, Sutriono, Endang, Yora; software, Sutriono; validation, Sutriono, Endang; formal analysis, Sutriono; investigation, Sutriono; resources, Sutriono; data curation, Sutriono, Endang; writing—original draft preparation, Sutriono; writing—review and editing, Sutriono, Endang, Eriawan, Yora, Sofyan; visualization, Sutriono; supervision, Sutriono, Eriawan, Sofyan; project administration, Sutriono; funding acquisition, Sutriono".

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