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Original Research Article

The Effect of Sambiloto (*Andrographis Paniculata*) Leaf Extract In Combination With Phosphomycin On The Germ Number, Urine Leukocyte Esterase, And Urine Procalcitonin Levels In Wistar Rats (*Rattus Norvegicus*) Urinary Tract Infection Model

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Abstract

Background: Bacterial resistance to antibiotics is still common due to unwise use. Urinary Tract Infections (UTI), 80% of which are caused by *E. coli* and other bacteria such as *Enterobacter sp, Klebsiella sp, S. aureus*. Fosfomycin is a first-line antibiotic for UTI. Combining natural compounds with antibiotics is one treatment strategy to increase the effectiveness of anti-bacterial therapy. *Andrographis paniculata* has been reported to have strong anti-infective activity. This study aimed to prove the differences in the germ number, leukocyte esterase levels, and urine procalcitonin levels in *Rattus norvegicus* UTI model given the fosfomycin, Sambiloto leaf extract, and Sambiloto leaf extract-fosfomycin combination.

Methods: Thirty *Rattus novergicus* rats were divided into five groups. All groups were induced 50 µl of *E. coli* bacterial inoculum for 7 days, followed by standard feed (negative control), fosfomycin (Monuril®) 54 mg (positive control), Sambiloto leaf extract (S1 [100 mg/BW], S2 [200 mg/BW], Sambiloto leaf extract-fosfomycin combination (FS1 [sambiloto 100 mg/BW and fosfomycin 54 mg], and FS2 [sambiloto 200 mg/BW and fosfomycin 54 mg]) for the next 7 days orally. The germ number, leukocyte esterase, and urine procalcitonin were measured after all rats were given treatment.

Results: The largest average reduction in the germ number, levels of leukocyte esterase, and urinary procalcitonin (4.80 ± 3.70 CFU/ml [$p<0.05$], 3,00 ± 6,71 cells/µL [$p<0.05$], 4,66 ± 1,35 ng/L [$p<0.05$] respectively) was observed in the combination of 200 mg/BW Sambiloto leaf extract-fosfomycin combination group.

Conclusion: A combination of Sambiloto leaf extract and fosfomycin reduced germ number, levels of leukocyte esterase and urinary procalcitonin in rat model of UTI.

Keywords: *Sambiloto (Andrographis paniculata); fosfomycin; E. coli; leukocyte esterase; procalcitonin*

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INTRODUCTION

Urinary Tract Infection (UTI) is marked by bacterial growth and proliferation within the urinary system, extending from the kidneys down to the bladder, often accompanied by notable levels of bacteria in the urine. The prevalence of UTI increases with age, but the peak prevalence is in the 14-24 year age group.[1] The global prevalence rate for uncomplicated UTIs is 11%.[2] The prevalence of healthcare-associated urinary tract infection (HAUTI) is around 24% in several developing countries.[3,4,5]

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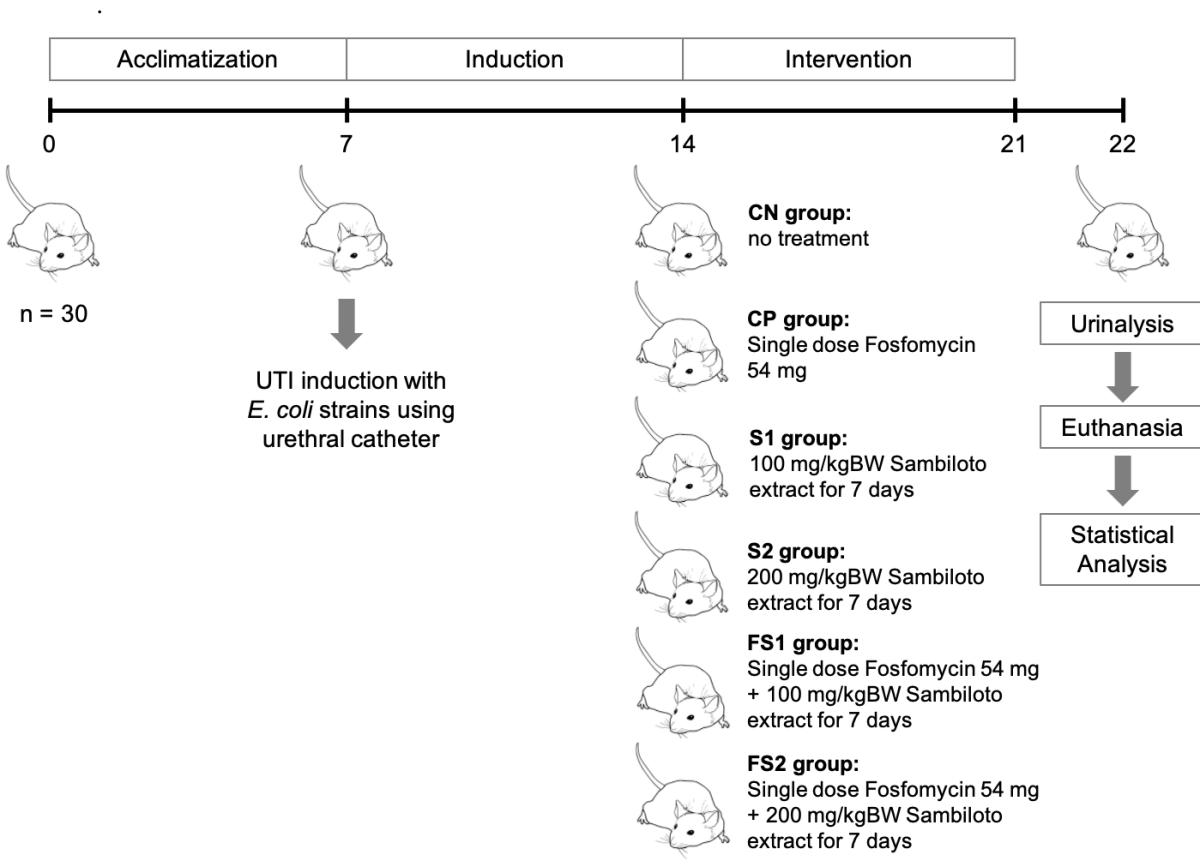

Figure 1. Study timeline

The Global Prevalence Study on Infections in Urology (GPIU) in 2003-2010 `reported that of 19,756 patients, 9.4% were diagnosed with HAUTI, and 70.4% were female.[6,7] According to the data obtained by the Ministry of Health of the Republic of Indonesia in 2018, the prevalence of UTI stands at 90-100 cases per 100,000 population annually, equating to roughly 180,000 cases per year.[8]

Various indicators can be employed to identify inflammation in the urinary tract, including counts of polymorphonuclear (PMN) leukocyte type in urethral swabs, prostate secretions, ejaculate fluid, urine sediment, and levels of PMN elastase.[9] Additionally, markers such as leukocyte esterase, C-reactive protein, and different interleukins can also be utilized for UTI detection.[10] Urinary biomarkers such as leukocyte esterase is useful for identifying UTIs, although it has low specificity. Interestingly, procalcitonin has become increasingly popular over the past decade for enhancing the diagnosis of bacterial infections.[11]

Treatment for UTI involves administering antibiotics. Fosfomycin is one of the most widely used antibiotic. However, the widespread and often imprudent use of numerous antibiotics has resulted in the development of antibiotic resistance. Consequently, microorganisms have evolved diverse resistance mechanisms due to the absence of new antibiotics for enhanced therapy.[12,13] Multiple studies have noted a rising trend of resistance to fosfomycin in E. coli, the most prevalent pathogen responsible for UTIs. Resistance to fosfomycin increased from 14.3% in 2013 to 20% in 2021.[14] Additionally, another study found fosfomycin resistance in 38.5% of E. coli isolates.[15]

An approach to tackle this resistance is through combination therapy, which involves administering two or more drugs simultaneously. These combinations often include multiple active ingredients, which can be natural compounds, a mix of natural substances, or a combination of natural and conventional antibiotics. The goal of combination therapy is to prevent the emergence of resistant bacterial strains. It also allows for lower doses of drugs to be used, reducing toxicity, while potentially enhancing treatment efficacy.[16] Currently, alternative UTI treatments other than chemical drugs are increasingly being used.[17] Traditional medicines that have antibacterial effects are increasingly common in developing countries.[18] Based on World Health Organization (WHO) data in 2004, herbal medicines often used as a traditional treatment, accounting approximately 65% of the population in developed countries and 80% in developing countries.[19]

One of the medicinal plants used in traditional medicine is Sambiloto (*Andrographis paniculata*) which is known to have beneficial compositions and properties. Based on the category of traditional medicines, Sambiloto is classified as a herbal medicine.[8] The Sambiloto plant is potential in regulating inflammatory responses and possesses antibacterial properties that can mitigate the side effects associated with chemical treatments for various inflammatory conditions, including lung infections and sepsis.[20–25] Previous research showed the ability of the Sambiloto plant to prevent the growth of biofilm isolates of *E. coli* in vitro.[9] Sambiloto also showed antibacterial properties in the

Table 1. Subjects Characteristics (Bacterial Count)

Group	Bacterial Count (CFU/ml)	
	Mean ± SD	p§
CN	858.60 ± 135.86*	<0,001•
CP	26.00 ± 10.27*	
S1	156.60 ± 56.06*	
S2	78.40 ± 21.48	
FS1	19.40 ± 7.64*	
FS2	4.80 ± 3.70*	

*Normal distribution (p > 0.05); §Kruskal-Wallis; •Significant (p < 0.05)

Table 2. Subjects Characteristics (Leukocyte Esterase)

Group	Leukocyte Esterase (cells/uL)	
	Median (min-max)	p§
CN	125 (125 -500)	<0.001•
CP	15 (0 - 15)	
S1	70 (70-125)	
S2	70 (15-70)	
FS1	0 (0 -15)	
FS2	0 (0 -15)	

*Normal distribution (p > 0.05); §Kruskal-Wallis; •Significant (p < 0.05)

Table 3. Subject Characteristics (Procalcitonin)

Group	Procalcitonin (ng/ml)	
	Mean ± SD	p⸸
CN	783.77 ± 56.40*	<0.001•
CP	28.40 ± 7.28*	
S1	239.29 ± 82.00*	
S2	110.74 ± 13.66*	
FS1	25.29 ± 6.24*	
FS2	4.66 ± 1.35*	

*Normal distribution (p > 0.05); ⸸One Way Anova; •Significant (p < 0.05)

urine of UTI patients tested in vitro.[10] Therefore, this research aims to assess the efficacy of Sambiloto leaf extract in conjunction with first-line antibiotics against infectious microorganisms to offer an alternative therapy that enhances the effectiveness of antibiotics. In addition, very little data is available on Sambiloto leaf extract in combination with standard antibiotic drugs as infection prevention therapy, especially in UTI.

METHODS

Design and Subject

The study adopted a post-test only control group design. Thirty healthy Rattus norvegicus rats, aged between 12 and 16 months, weighing 150-200 g, were sourced from the Laboratory of Experimental Animals at the Faculty of Medicine, Universitas Sebelas Maret. This study was conducted at September 2023. Treatment consisted of administering the fosfomycin, sambiloto leaf extract, as well as Sambiloto leaf extract-fosfomycin combination with variable measurement parameters, namely the germ number, leukocyte esterase levels, and urine procalcitonin levels.

Preparation of *A. paniculata* leaf extract

Sambiloto (*A. paniculata*) leaf extract was acquired from Sambiloto Herbal Supplement produced by Jamu Iboe brand. Each capsule of the supplement contains 500 mg of Sambiloto leaf extract. The capsule was opened to obtain the powdered form of Sambiloto leaf extract, which was then dissolved in a 1% Na-CMC (sodium carboxymethylcellulose) solution. The extract was orally administered according to the respective dose of each treatment group.

Experimental Design

The rats were fed with a standard feed and water ad libitum seven days prior to treatment. Thirty Rattus norvegicus rats were induced to develop urinary tract infection by administering 50 µl of *E. coli* bacterial inoculum for 7 days. The sample was then divided into six treatment groups. The negative control group (CN) consisted of rats that had not been given any treatment. The positive control group (CP) received a single dose of 54 mg of the antibiotic fosfomycin (Monuril®, Zambon, Jakarta, Indonesia). The Sambiloto 100 group (S1) was given 100 mg/kgBW of Sambiloto leaf extract for 7 days. The Sambiloto 200 group (S2) was given 200 mg/kgBW of Sambiloto leaf extract for 7 days. The combination group 100 (FS1) was given a combination of the fosfomycin 54 mg single dose and Sambiloto leaf extract at dose of 100 mg/kgBW for 7 days. The combination group 200 (FS2) was given a combination of the fosfomycin 54 mg single dose and Sambiloto leaf extract at dose of 200 mg/kgBW for 7 days. All treatment was given orally and the combination of Sambiloto leaf extract-fosfomycin was given simultaneously. Then, on the 22nd day after the UTI appeared, urinalysis was performed to measure the growth of the bacterial count, leukocyte esterase values, and procalcitonin levels in each group. The study timeline is shown at Figure 1.

Table 4. Comparative analysis of bacterial count, leukocyte esterase, and procalcitonin in all treatment groups

Group		p-value		
		Bacterial Count§	Leukocyte Esterase§	Procalcitonin⸸
CN	CP	0.009*	0.006*	<0.001*
	S1	0.009*	0.042*	<0.001*
	S2	0.009*	0.005*	<0.001*
	FS1	0.009*	0.006*	<0.001*
	FS2	0.009*	0.005*	<0.001*
CP	S1	0.009*	0.007*	0.025*
	S2	0.009*	0.014*	<0.001*
	FS1	0.248	0.549	0.973
	FS2	0.009*	0.221	0.010*
S1	S2	0.012*	0.093	0.126
	FS1	0.009*	0.007*	0.024*
	FS2	0.009*	0.006*	0.018*
S2	FS1	0.009*	0.011*	<0.001*
	FS2	0.009*	0.007*	<0.001*
FS1	FS2	0.009*	0.513	0.009*

*Significant ($p < 0.05$); §Mann-Whitney; ⸸Post Hoc Games-Howell

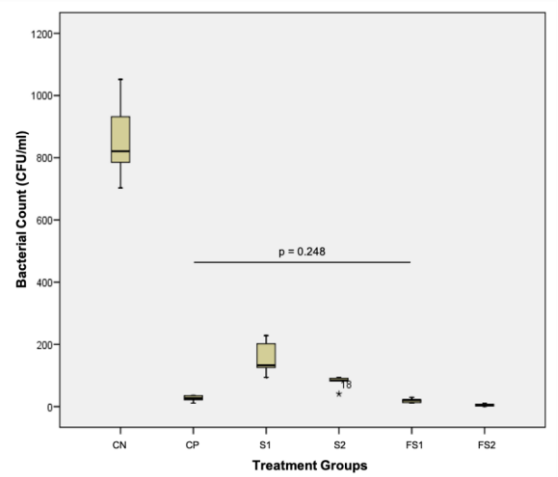

Figure 2. Comparison of bacterial count in all treatment groups analyzed by Mann-Whitney analysis. The differences in each group are statistically significant ($p < 0.05$) other than group CP vs FS1 ($p = 0.248$).

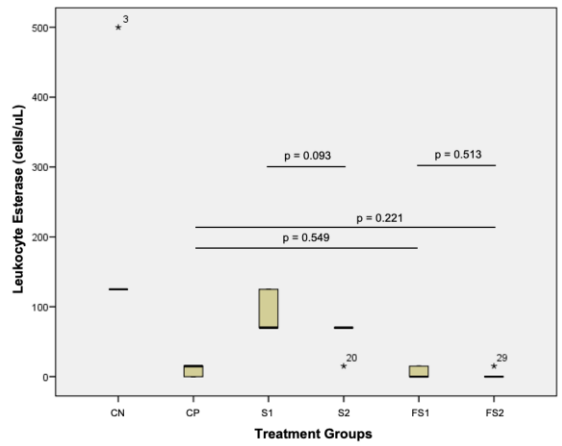

Figure 3. Comparison of leukocyte esterase levels in all treatment groups analyzed by Mann-Whitney analysis. The differences in each group are statistically significant ($p < 0.05$) other than group CP vs FS1 ($p = 0.549$); CP vs FS2 ($p = 0.221$); S1 vs S2 ($p = 0.093$); FS1 vs FS2 ($p = 0.513$).

Induction of Urinary Tract Infection

E. coli strains were retrieved from a storage facility maintained at -80°C in the Microbiology Laboratory, Faculty of Medicine, Universitas Diponegoro. The bacteria were cultured in Brain-Heart Infusion Broth (BHI) media and incubated for 24 hours at 37°C. From the BHI media, cultures were transferred to MacConkey agar plate media. A 0.5 Standard McFarland suspension was prepared, equivalent to 1.5 x 10^8 CFU/ml. This inoculum at a dose of 50 µl was then administered into the urinary tracts of experimental animals using urethral catheter.[26]

Determination of Bacterial Concentration

To assess the bacterial count, the procedure involved a microscopic examination. A technician will enumerate the number of bacteria per field of view (expressed in CFU/ml) with respective steps. The urine sample was diluted to a concentration of 1:1000, and then 0.1 ml of each dilution was pipetted and inoculated on the surface of the plate count agar (PCA) media. Using a sterile spreader, the sample was evenly distributed across the entire surface of the PCA media until it appeared dry. The plates were then inverted and incubated at 37˚C for 24 hours. After incubation, bacterial colonies were counted using a colony counter (Quebec Manual Darkfield Colony Counter, Reichert, USA).

Measurement of Leukocyte Esterase

When assessing leukocyte esterase levels, a dipstick (HEALGEN URS-10 T Reagent Strips for Urinalysis, Zhejiang Orient Gene Biotech Co., Ltd, China) was dipped into the urine sample obtained from the rats. Any color change on the dipstick was observed and the color of the sample's dipstick was compared to the standard color provided by the dipstick manufacturer.

Measurement of Procalcitonin Levels

Urine samples are collected from the experimental animals and then subjected to centrifugation with a speed of 2,000 rpm for 5 minutes. Following centrifugation, the supernatant is discarded, then the urine was furtherly analyzed using a procalcitonin immunoassay (Rat Procalcitonin Enzyme-Linked Immunosorbent Assay

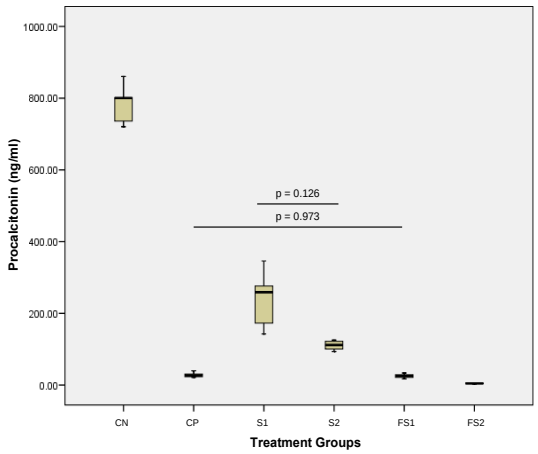

Figure 4. Comparison of procalcitonin levels in all treatment groups analyzed by Post Hoc Games-Howell analysis. The differences in each group are statistically significant ($p < 0.05$) other than group CP vs FS1 ($p = 0.973$); S1 vs S2 ($p = 0.126$).

Kit, Elabscience Biotechnology Co., Ltd., Wuhan, China; Detection range = 97-109%; Sensitivity = 9.38 pg/mL)

Statistical Analysis

The data were analyzed by using SPSS version 26.0. The data normality test was carried out using the Shapiro-Wilk test. Data that were not normally distributed was analyzed by using non-parametric statistics. Data with a ratio scale and not normally distributed were subjected to the Kruskal-Wallis test, followed by the post-hoc Mann-Whitney U test. The p-value was considered significant if $p < 0.05$ with a 95% confidence interval.

RESULTS

The subjects of this study included the germ number, leukocyte esterase, and urine procalcitonin levels. Based on the results of univariate analysis, the characteristics of the research subjects can be identified as shown in tables 1, 2 and 3.

The germ number data was normally distributed with $p > 0.05$ in CN, CP, S1, FS1, and FS2 groups. found to be not normally distributed, whereas the procalcitonin data were normally distributed. Therefore, Kruskal-Wallis test was employed to compare the germ number and leukocyte esterase data, while the One-Way ANOVA test was used for comparing the procalcitonin data. Based on these two tests, all data showed significant results with $p < 0.001$. The Mann-Whitney test was then carried out on the data on the germ number and leukocyte esterase and the Post Hoc Games-Howell test on the procalcitonin data to determine the differences in numbers between treatment groups. The results of the Mann-Whitney and Post Hoc analysis are shown in table 4 and figure 2, 3, and 4.

Among all treatment groups, the administration of Sambiloto leaf extract alone at a dose of 100 mg/kgBW showed the highest number of bacteria (156.60 ± 56.06 CFU/ml), as well as the highest level of leukocyte esterase (92.00 ± 30.13 cells/uL) and procalcitonin (239.29 ± 82.00 ng/ml). In contrast, the combination of Sambiloto leaf extract and conventional antibiotics, specifically fosfomycin in this study, resulted in reduced germ number and lower levels of leukocyte esterase and procalcitonin as compared to the negative control group. A combination of Sambiloto leaf extract and fosfomycin at 100 mg/kgBW reduced the germ number to 19.40 ± 7.64 CFU/ml. This combination also lower the leukocyte esterase and procalcitonin levels to 6 ± 8.22 cells/uL and 25.29 ± 6.24 ng/ml, respectively. A higher dose of fosfomycin at 200 mg/kgBW in combination with Sambiloto leaf extract exhibited an enhanced effect in lowering the germ number, leukocyte esterase, and procalcitonin levels to 4.80 ± 3.70 CFU/ml, 3.00 ± 6.71 cells/uL, and 4.66 ± 1.35 ng/ml, respectively, representing the lowest levels of the measured parameters in this study, even when compared to the positive control group.

The comparative analysis using Mann-Whitney test, as shown in Figure 2 and 3, demonstrated a significant difference ($p < 0.05$) of germ number in all treatment groups except for the positive control group and FS1 group ($p = 0.248$), and the differences of leukocyte esterase levels in each group are also statistically significant ($p < 0.05$) except for group CP vs FS1 ($p = 0.549$), CP vs FS2 ($p = 0.221$), S1 vs S2 ($p = 0.093$), and FS1 vs FS2 ($p = 0.513$). The Post Hoc analysis of procalcitonin levels, as shown in Figure 4, revealed a significant difference ($p < 0.05$) among all treatment groups other than CP vs FS1 ($p = 0.973$) and S1 vs S2 ($p = 0.126$).

DISCUSSION

The results show that 100 mg/BW of Sambiloto leaf extract, as an antimicrobial agent, proves to reduce the germ number to 156.60 ± 56.06 CFU/ml. In addition, administering 200 mg/BW reduces the germ number to 78.40 ± 21.48 CFU/ml. The findings of this study indicate that a higher dosage of Sambiloto extract displayed superior efficacy in reducing the germ number in the UTI rat model. Therefore, it can be concluded that increasing the dose of Sambiloto leaf extract is directly proportional to decreasing the number of germs. This aligns with research by Mishra et al., who find that sambiloto has the main compound in the form of andrographolide. Andrographolide can fight disease due to its strong antibacterial properties and its ability to activate B lymphocyte cells to produce antibodies.[27]

The results of the study also revealed a further reduction in germ number, even lower than the positive control group which received fosfomycin alone, following the administration of Sambiloto leaf extract administration at 100 mg/kgBW and 200 mg/kgBW with fosfomycin combination. Fosfomycin is a broad spectrum antibiotic which is effective against gram-positive and gram-negative bacteria, especially gram-negative bacteria that are the most common causes of urinary tract infections, including *Escherichia coli*, *Klebsiella* sp., *Enterobacter* sp. and *Proteus* sp.[28] Previous studies showed a synergistic effect between the combination of andrographolide with conventional antibiotics, including fosfomycin, was observed against *Pseudomonas aeruginosa* strains. A significant increase in the anti-infection property and anti-biofilm activity was observed when the antibiotics were combined with andrographolide.[29] Some benefits associated with

combining antimicrobial agents like antibiotics with andrographolide include heightened antibacterial activity, mitigation of side effects, reduction in the duration of long-term antimicrobial therapy, and prevention of the emergence of resistant microorganisms.[30] In normal rats, as observed in humans, the urine is sterile, with no bacteria found in microscopic examinations.[31] However, in UTI model rats, consistent with the study findings, administering a combination of fosfomycin and Sambiloto leaf extract at dose of 200 mg/kgBW resulted in a greater reduction in bacterial count compared to the combination of fosfomycin and Sambiloto leaf extract at dose of 100 mg/kgBW.

The reduction in the germ number when administering a combination of fosfomycin and Sambiloto leaf extract is also directly proportional to the decrease in leukocyte esterase and procalcitonin, which are used as markers for detecting UTI. It shows a decrease in leukocyte esterase with the greatest results after being given a combination of Sambiloto leaf extract 200 mg/BW and fosfomycin. This is caused by the anti-inflammatory effect of andrographolide, a compound found in Sambiloto leaf extract. Andrographolide can inhibit neutrophil adhesion/transmigration by suppressing MAC-1 upregulation. Studies have shown that andrographolide possesses anti-inflammatory properties by inhibiting the initial phase of neutrophil infiltration. This inhibition effectively reduces the release of esterase enzymes caused by leukocyte membrane lysis. As a result, the release of esterase enzymes due to inflammation-induced leukocyte lysis can be diminished.[32,33]

The largest reduction in procalcitonin levels is also obtained after being given a combination of the Sambiloto leaf extract 200 mg/BW and fosfomycin. Thus, it can be concluded that increasing the dose of Sambiloto leaf extract combined with fosfomycin can reduce the germ number, leukocyte esterase, and calcitonin levels higher than using Sambiloto leaf extract or fosfomycin alone. Based on research conducted by Li et al., andrographolide can significantly inhibit the expression of TNF-α, IL-6, and IL-1β due to LPS stimulation.[34] In the context of inflammation, heightened procalcitonin levels correlate with the presence of bacterial endotoxins and inflammatory cytokines.[35] Therefore, indirectly, andrographolide is able to reduce procalcitonin levels because it inhibits the expression of proinflammatory cytokines and bacterial endotoxin, both of which are procalcitonin activators.[34]

This research still presents several limitations. It is necessary to identify the synergistic interaction mechanism observed from the combination of fosfomycin and Sambiloto extract as a basis for conducting direct evaluations in humans with UTI. Such evaluations would provide valuable insights into the potential of this combination therapy as a novel alternative for UTI treatment in the future. Further research is recommended to investigate the interactions between other antimicrobials and Sambiloto leaf extract, determining whether these interactions are synergistic or antagonistic.

CONCLUSION

A combination of Sambiloto leaf extract and fosfomycin reduced the germ number, levels of leukocyte esterase, and procalcitonin in Rattus norvegicus UTI models. The greatest decrease in the number of germs, leukocyte esterase levels, and procalcitonin levels occurred when the combination of Fosfomycin and Sambiloto leaf extract was administered at a dose of 200 mg/kg BW. The combination of the antibiotic and Sambiloto leaf extract improved the antimicrobial effectiveness compared to using either the conventional antibiotic or the Sambiloto extract alone.

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