Effects of Golden Sea Cucumber Extract (Stichopus hermanni) on Hyphae, Neutrophils and TNF-α in BALB/c Mice Inoculated with C. albicans Intravaginally

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ABSTRACT

Introduction: Candidal vaginitis is an inflammatory disease that caused mainly by Candida albicans. Yeast transitions to filamentous hyphae considered the most important virulence factor. Neutrophils are the first line of defense of the immune system, but in patients with Candidal vaginitis the recruitment of neutrophils into the vaginal lumen is positively correlated with symptoms of the disease. This is supported by the release of proinflammatory cytokines such as TNF-α. Standard treatment is considered less effective in relieving symptoms, so other alternative/adjunctive treatments are needed. Golden sea cucumber (Stichopus hermanni) extract has been widely studied, especially for anti-fungal and anti-inflammatory. This study aims to analyze the mechanism of decreasing number of hyphae and neutrophils, and pro-inflammatory cytokine TNF-α in BALB/c mice inoculated intravaginally with C. albicans after administration of golden sea cucumber extract (S. hermanni). Methods: Experimental research uses a post-test only control group design. The experimental unit consisted of 36 BALB/c mice that were inoculated intravaginally with C. albicans and divided into 4 groups, group that did not receive treatment (K-), group that received standard treatment fluconazole (K+), group that received treatment with golden sea cucumber extract (S. hermanni) (P1) and group that received standard treatment with fluconazole plus extract of golden sea cucumber (S. hermanni) (P2). The hyphae and neutrophils number were seen microscopically on vaginal mucosal tissue. Cytokine levels of TNF-α were seen from the ELISA blood samples. Results: Results showed from the vaginal mucosal tissue of mice, there was significant difference in the number of hyphae (p = 0.001) between groups and no significant difference in the number of neutrophils (p = 0.070) between groups. From the blood serum of mice, there were significant differences in TNF-α levels (p=0.001) between groups. From the path analysis obtained a significant relationship from the number of hyphae to number of neutrophils (β = 0.034) and the number of neutrophils to TNF-α levels (p = 0.021). The strength of the pathway from number of hyphae to number of neutrophils (β = 0.354) and number of neutrophils to TNF-α levels (β = 0.382) with positive interactions all. Conclusion: In summary, the administration of S. hermanni extract was able to reduce the number of hyphae, neutrophils and TNF-α levels through the hyphae, neutrophil and TNF-α pathway. Key words: Stichopus hermanni extract, Candida vaginitis, Hyphae, Neutrophils, TNF-α.

INTRODUCTION

Worldwide, recurrent vulvovaginal candidiasis (RVVC) has a global annual prevalence around 4 per 100,000 women and at least 400 million women are affected by recurrent vulvovaginal candidiasis during their lifetime. Abnormal vaginal discharge is the main feature and the first sign of VVC that often causes women to seek health care from a gynecologist. Various risk factors for VVC include douching, antibiotic therapy, elevated estrogen and diabetes. Many factors that increase estrogen levels are risk factors for the development of this disease. The association between increased estrogen and the incidence of C. albicans colonization or vaginal disease is due to the effects of estrogen on the host, on fungal cells, or both. The morphological transition of the budding yeast to the form of filamentous hyphae is considered to represent the most important virulence factor of C. albicans. Hyphae are more invasive and contribute to host tissue damage. Pathogen recognition by the host immune system broadly involves pattern recognition receptors (PRR). Vaginal epithelial cells “detect” the danger signal generated by C. albicans and respond by activating immune cells, secreting inflammatory immune mediators and eliciting an immune system response. Neutrophils are the main innate immune cells that are recruited at the site of infection in response to epithelial cells that secrete immune mediators. Neutrophils further release TNF-α which consequently regulates TLR4 expression in epithelial cells. The recruitment of polymorphonuclear neutrophils (PMNs) into the vagina is strongly associated with symptoms of vaginal inflammation. This vaginal inflammation was observed to decrease with a decrease in PMN. Neutrophils do not always play a protective role, as uncontrolled influx of neutrophils to the vagina provokes and amplifies pathogenic inflammation. Damage to the vaginal epithelium occurs by a series of mechanisms as above as well as other factors such as hyphae elongation and the simultaneous release of fungal peptide toxin secretion (Candidalysin). Most women with RVVC (71%) require long-term antifungal treatment as maintenance therapy to

control symptoms. Standard treatment is considered less effective and causes many side effects, for example, topical antifungals cause local hypersensitivity reactions (itching or burning), oral antifungals cause systemic side effects, especially gastrointestinal effects and drug interaction toxicity. With standard treatment, especially the azole group, relapses often occur and treatment is only a temporary solution for patients with RVVC. In addition to specific antimicrobial treatment, anti-inflammatory therapy, both systemic and topical (vaginal douche), is usually added. Most used are non-steroidal anti-inflammatory drugs (NSAIDs). Side effects that often occur are the occurrence of plateau aggregation and digestive tract disorders. Problems associated with the management of Candida infections require discovery and innovation, where the need for herbal medicines is increasing and natural plant-derived products may offer more amelioration of the existing infections.

Sea cucumbers in the form of extracts have been shown to have inhibitory activity against pathogenic fungi. The most abundant and most important secondary metabolites of sea cucumbers are triterpene glycosides (saponins). Saponin compounds are able to inhibit the formation of biofilms from fungi, increase hyphal permeability and inhibit hyphal growth. Sea cucumbers reduce levels of inflammatory markers interleukin-1β, interleukin-6, nitric oxide, and matrix metalloproteinase 9 in cancer cells. A study of liver anti-tumor and immunomodulatory effects showed that sea cucumbers decreased serum levels of ALT, AST, GGT and TNF-α and increased serum IL-2. Thus, the tested extracts could be explored as new marine sources for novel anti-fungal and anti-inflammatory agents. The purpose of this study was to analyze the effects of golden sea cucumber (S. hermanni) extract on the number of hyphae and neutrophils, and pro-inflammatory (TNF-α) levels in BALB/c mice inoculated with C. albicans intravaginally.

**MATERIALS AND METHODS**

The design of this study was a true experimental study using a post-test only control group design to prove the relationship between effect on number of hyphae and neutrophils, and pro-inflammatory cytokine (TNF-α) on BALB/c mice inoculated with C. albicans intravaginally after administration of golden sea cucumber (S. hermanni) extract (ethanolic extract).

The units of examination in this study were 36 female BALB/c mice aged 6-8 weeks that were inoculated with C. albicans intravaginally. The experimental unit will be divided into 4 groups as follows: the first group was a group that received standard feed and drink and inoculated with C. albicans but did not receive gold sea cucumber extract (K- / negative control); the second group was a group that received standard feed and drink and was inoculated with C. albicans and received 0.25 mg/kgBW of azole drug (fluconazole) orally (K+ / positive control); the third group was a group that received standard feed and drink and was inoculated with C. albicans and received treatment with 17 mg/kgBW orally administered gold sea cucumber extract (P1 / treatment 1); the fourth group was a group that received standard feed and drink and was inoculated with C. albicans and received treatment with 17 mg/kgBW orally administered gold sea cucumber extract along with azole class drugs (fluconazole) (P2 / treatment 2).

The study was started by observing C. albicans colonization (hyphae) and neutrophil infiltration in the infected group (on the fourth day). Then the golden sea cucumber (S. hermanni) extract was administered to the treatment group (P1 and P2) and azole (fluconazole) to the control group (K+ and P2) for seven days. The parameters studied were examined then, which were the number of hyphae, neutrophils, and TNF-α levels.

**Extraction of Stichopus hermanni**

*S. hermanni* weighing 100-250 grams was taken from the sea waters of Sumenep in Madura, Indonesia. The sea cucumbers were cleaned, cut into pieces with a size of 3-10 cm, weighed by wet weight after drying on a solar drying rack for samples until they looked dry (3-4 days) to reduce the moisture content. The sea cucumber samples were dried, cut into ± 1 cm pieces, crushed in a blender. The extraction process was carried out by a maceration process, by immersing 250 grams of dry sample in 500 mL of fine methanol solvent until all samples were submerged and left at room temperature for 24 hours. After filtering with filter paper to separate the filtrate and residue, then soaked again with 500 mL of methanol solvent for 24 hours. After being filtered with filter paper to separate the filtrate and residue, the filtrate will be obtained with a sample ratio of 250 grams / 1000 mL of solvent (1: 4 w/v). The methanol filtrate (polar) was homogenized with hexane solvent (non-polar) and 1,000 mL was performed by partitioning a separating funnel, then each layer of the methanol filtrate solvent and hexane solvent was separated. The methanol filtrate was homogenized again with chloroform solvent (non-polar) and 1,000 mL was carried out with a separate funnel partition, then each layer of the methanol filtrate solvent and chloroform solvent was separated. Each filtrate was then separated from the solvent using a rotary evaporator to obtain the extract.

**Administration of Stichopus hermanni extract**

Treatment groups 1 and 2 (P1 and P2) were given a golden sea cucumber (S. hermanni) extract at a dose of 17 mg/kgBW using a feeding tube. Treatment group 2 and positive control (P2 and K+) were given fluconazole 0.25 mg/kgBW using a feeding tube on the 7th day after the treatment, the mice were killed.

**Vaginal inoculation of C. albicans**

Mice were held to expose the stomach so that 100 mL of sesame oil containing 0.1-0.5 mg estradiol could be injected intraperitoneally (three days before inoculation). The needle is inserted about 5 to 10 mm lateral to the skin to minimize leakage from the injection site. The injections were repeated once a week during the study period. Inoculum was prepared by adding a full circle of *C. albicans* blastoconidia subcultured on Sabouraud-Dextrose Agar (SDA) into 10 mL of Phytone-peptone medium supplemented with 0.1% glucose. The Phytone-peptone medium mixture containing *C. albicans* was incubated for 18 h at 25°C in a vibrating water bath. After incubation, culture medium was collected into 15 mL conical tubes and centrifuged at 800 x G for 5 min. The pellets were washed twice using sterile PBS. Blastocandia were counted using a hemocytometer. The cell concentration was adjusted to 2.5 x 106/ml (or desired inoculum concentration) in sterile PBS. Mice were stabilized by holding the base of the tail with two fingers and lifting the hips up so that the vagina was facing the examiner. The inoculum suspension was taken using a pipette as much as 20 L (or the desired volume did not exceed 20 L). The inoculum suspension was inserted by inserting the tip of the pipette about 5 mm into the vaginal lumen.

**Mice vaginal fluid sampling for examination of hyphae and neutrophils (PMN)**

Mice were anesthetized using Ketamine. The mice were held at the base of the tail with two fingers so that the vaginal opening was opened. The vaginal lumen was rinsed using 100 L of sterile PBS and repeated aspiration was performed with the tip of the pipette. The rinsing fluid was collected into a microcentrifuge tube.
Hyphae and Neutrophil (PMN) count examination

Wet preparations were made by transferring 10 μL of vaginal rinse fluid to a glass slide. Cell and nuclear morphology examination (hyphae and neutrophils/PMN) were carried out by staining the cellular fraction of the rinsing fluid. Hyphae and neutrophil count were observed with a light microscope at 400-1000x magnification.36

Serum sampling for examination of TNF-α levels

The mice were anesthetized using Ketamine. To take blood, surgery is done first. Then the needle is inserted directly into the heart and aspirated slowly. The blood obtained was used for the purposes of examining TNF-α levels. The mice were then euthanized.35

Measurement of TNF-α levels using ELISA technique

TNF-α levels were examined quantitatively using ELISA with the following examination principles: reagents, standard solutions, and sample solutions were prepared according to the manufacturer’s instructions. All reagents were placed at room temperature before use. This test was carried out at room temperature. The number of strips required is determined for the test. The strip is inserted in the appropriate place. Unused strips should be stored at 2-8 °C. 50 μL of substrate solution A was added to each well, 50 μL of substrate solution B was added to the sample well and standard well. The plate was closed with a sealer and incubated for 60 minutes at 37 °C. The sealer was removed and the plate was washed 5 times with a rinsing fluid. Hyphae and neutrophil count were observed with a light microscope at 400-1000x magnification.36

Data analysis

The selection of statistical tests referred to the type of data from the independent variables. Data analysis was carried out on the number of hyphae, neutrophils, and TNF-α levels in each group by analyzing the mean and standard deviation, then testing for the normality of the distribution in all groups. The homogeneity of variance test between groups was carried out. The Anova comparison test for data with normal distribution and Kruskal Wallis test for data that were not normally distributed.

RESULTS

Mean value and standard deviation of the examination of variables in each group

The results of the mean and standard deviation of the examination of the variables (hyphae count, neutrophils count, and TNF-α levels) are presented in table 1.

Research data normality test

The results of the normality distribution of examination data for variables (hyphae count, neutrophils count, and TNF-α levels) are presented in table 2.

Hyphae examination in the vaginal tissue of Balb/c mice

The results of microscopic examination of the number of C. albicans hyphae in the vaginal tissue of Balb/c mice are presented in table 3.

| Table 1: The results of the mean and standard deviation (SD) of the examination of variables for each group. |
|---|---|---|---|---|---|---|---|
| No. | Variable | Groups | K- | K+ | P1 | P2 |
| | | | Means | SD | Means | SD | Means | SD |
| 1. | Hyphae Count | 2.78 | 0.23 | 1.69 | 0.63 | 1.8 | 0.87 | 1.18 | 0.78 |
| 2. | Neutrophils Count | 2.13 | 0.52 | 1.78 | 0.67 | 1.38 | 1.07 | 1.11 | 1.07 |
| 3. | TNF-α levels | 357.11 | 129.56 | 575.04 | 137.9 | 121.65 | 142.08 | 199.66 | 179.66 |

| Table 2: Test results for the normality distribution of research data. |
|---|---|---|---|---|---|---|---|
| No. | Variable | p Value |
| | | K- | K+ | P1 | P2 |
| | | (n = 9) | (n = 9) | (n = 9) | (n = 9) |
| 1. | Hyphae Count | 0.041 | 0.026 | 0.220 | 0.303 |
| 2. | Neutrophils Count | 0.029 | 0.069 | 0.261 | 0.058 |
| 3. | TNF-α levels | 0.247 | 0.290 | 0.412 | 0.052 |

| Table 3: Number of C. albicans hyphae in the vaginal tissue of Balb/c mice of each group. |
|---|---|---|---|---|
| No. | Groups | n | Median (min – maks) | p Value |
| 1. | K- | 9 | 2.8 (2.4 – 3) | < 0.001 |
| 2. | K+ | 9 | 1.8 (1 – 2.4) | < 0.001 |
| 3. | P1 | 9 | 2 (0.8 – 3) | < 0.001 |
| 4. | P2 | 9 | 1.2 (0.2 – 2.2) | < 0.001 |
were different. The results of the Mann Whitney test showed that the number of hyphae in the K- group was significantly different from the other three groups, while the number of hyphae in the K+, P1 and P2 groups was not significantly different.

Figure 1 shows a significant decrease in the number of hyphae between the K- (without treatment) compared to the K+ (fluconazole), P1 (S. hermanni extract) and P2 (fluconazole + S. hermanni extract) groups. Figure 2 shows images comparison of hyphal elements on the vaginal mucosa in several treatment groups.

Neutrophil examination in the vaginal tissue of Balb/c mice
The results of microscopic examination of the number of neutrophils in the vaginal tissue of Balb/c mice are presented in table 4.

The results of the Kruskal Wallis test showed that there was no significant difference in the number of neutrophils between groups (p=0.070; p>α; α=0.05). Figure 3 shows a decrease in the number of neutrophils in the K+ (fluconazole), P1 (S. hermanni extract) and P2 (fluconazole + S. hermanni extract) groups compared to the K- group (without treatment). Figure 4 shows images comparison of vaginal mucosal inflammation (neutrophils infiltration) in several treatment groups.

TNF-α ELISA examination in the serum of Balb/c mice
The results of the ELISA examination of TNF-α levels in the blood serum of Balb/c mice can be seen in table 5.

Analysis of variance showed that there were significant differences in TNF-α levels between groups (p<0.001; p<α; α=0.05), so further testing was necessary to determine which groups were different. The results of the LSD test showed that the levels of TNF-α in the K- and K+ groups were significantly different from those in the P1 and P2 groups, while the P1 group was not significantly different from the P2 group. Figure 5 shows TNF-α levels in the blood serum of Balb/c mice in all groups. The K+ group (fluconazole) had the highest levels of TNF-α compared to all groups. The P1 group (S. hermanni extract) had the lowest levels.
Sea cucumbers are rich in glycosides, especially triterpene glycosides (saponins) both holostane (Stichlorosides, Stichosaposides and Holotoxins) and non holostane. The biological activity of holothurian saponins occurs through their membrane-lytic function after a certain threshold concentration is reached. Triterpene glycosides cause membrane disruption; alter membrane permeability, loss of barrier function, and rupture of cell membranes. The interaction of glycosides (agalactone moiety) with membrane D5(6)-sterols (ergosterol in fungi) creates a glycoside-sterol complex in the membrane, modifying microviscosity, ion permeability and activity of membrane proteins. The strong membrane-lytic function of D5-sterol-containing biological membranes due to the formation of single ion channels and larger pores is the basis of the antifungal features of this substance. The glycoside activity of sea cucumbers in sub-cytotoxic doses disrupts specific membrane transport proteins in fungal cells and alters their activity.

Activity of Stichopus hermanni extract on neutrophil count

Neutrophils are the most abundant circulating leukocyte, and have been considered the first line of defense of the immune system. Neutrophils also act as inflammatory mediators. However, in patients with *Candidal vaginitis*, as well as in some studies using mice, recruitment of neutrophils into the vaginal lumen is positively correlated with disease symptoms. Many studies have targeted neutrophil removal as a treatment for *Candidal vaginitis.* From Figure 3 and Table 4, the administration of *S. hermanni* extract decreased the number of neutrophils more than the K- group (without treatment) and the K+ group (fluconazole) but slightly higher than the P2 group (fluconazole + *S. hermanni* extract), although this decrease in decrease was not significant in statistics (p=0.070; p>α; α=0.05). The administration of *S. hermanni* extract decreased the number of neutrophils more than the administration of fluconazole alone. And when combined with fluconazole further increase the effectiveness of reducing the number of neutrophils.

This decrease in the number of neutrophils may be because of the activity of *Stichopus hermanni* of fucosylated chondroitin sulfate (FucCS) substances. This polysaccharide isolated from sea cucumbers is a strong inhibitor of P- and L-selectin. FucCS reduces neutrophil recruitment to inflamed tissues. Activation, migration, and aggregation of neutrophils can also be inhibited by PGE2 where PGE2 can be produced by host cells or from Candida cells themselves.

Activity of Stichopus hermanni extract on TNF-α levels

Tumor necrosis factor α (TNF-α) exhibits a large number of functions and is associated with the development of pain, cell infiltration, and tissue structural damage. From figure 5 and table 5, the administration of *S. hermanni* extract decreased TNF-α levels compared to the K- group (without treatment), this decrease was statistically significant (p=0.001; p<α; α=0.05). The administration of *S. hermanni* extract also significantly reduced TNF-α levels compared to the administration of fluconazole alone (p=0.001; p<α; α=0.05). Likewise, the combined administration of *S. hermanni* extract and fluconazole also reduced TNF-α levels, where the decrease in TNF-α levels was not significantly different from the administration of *S. hermanni* extract alone (p=0.221; p>α; α=0.05). Some of the things that can reduce TNF-α levels from some types of sea cucumbers may be fucosylated chondroitin sulfate and PGE2.

DISCUSSION

Activity of Stichopus hermanni extract against Candida albicans hyphae

The pathogenicity of *C. albicans* in causing *Candidal vaginitis* is caused by a change in the shape of yeast cells into hyphae. The formation of these hyphae will trigger an inflammatory reaction that causes symptoms. So that efforts to inhibit the formation of candida hyphae will relieve symptoms. From Figure 1, the administration of *S. hermanni* extract reduced the number of hyphae significantly (p=0.011; p<α; α=0.05) compared to the K- group (without treatment). Administration of *S. hermanni* extract reduced the number of *C. albicans* hyphae almost as much as fluconazole alone, where statistically the difference between the administration of *S. hermanni* extract and administration of fluconazole was not significant (p=0.894; p>α; α=0.05). When given concurrently with fluconazole, it was very effective in reducing hyphae of *C. albicans*, which is more hyphae reduction than the administration of *S. hermanni* extract and fluconazole alone, although the difference was not statistically significant.

Sea cucumbers are rich in glycosides, especially triterpene glycosides (saponins) which have been shown to have antifungal activity. *S. hermanni* also produces triterpene glycosides (saponins) both holostane (Stichlorosides, Stichosaposides and Holotoxins) and non-holostane. The strength of the pathway of hyphae number to neutrophils number and activity of membrane proteins is the basis of the antifungal features of this substance. The glycoside activity of sea cucumbers in sub-cytotoxic doses disrupts specific membrane transport proteins in fungal cells and alters their activity.
This study analyzed the effect of *S. hermanni* extract on the pathway of *C. albicans* hyphae, neutrophil recruitment and production of cytokine TNF-α. This study did not see the relationship with other immune cells, especially dendritic, macrophage and lymphocyte cells.

CONCLUSION

In summary, the administration of *S. hermanni* extract was able to reduce the number of *C. albicans* hyphae. The combination of fluconazole – *S. hermanni* extract decreased the most of *C. albicans* hyphae. The administration of *S. hermanni* extract was able to reduce the number of neutrophils. The combination of fluconazole – *S. hermanni* extract was able to reduce the number of *C. albicans* hyphae. The combination of *S. hermanni* extract alone was the largest.

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