Bay Leaf Extract Worsens Oral Candidiasis in Rat Model: An In Vivo Study

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ABSTRACT

Background: *The types of antifungal drugs are still very limited, and those that can be given systemically have significant side effects. Numerous in vitro studies have demonstrated that bay leaves have antifungal properties. This study aimed to evaluate the effect of bay leaf extract in vivo on the oral candidiasis rat model.*

Method: *The rat model for oral candidiasis was created by adding Tetracycline HCl to the rat's drinking water at a 500 mg/L concentration. Then, the rats were injected with methylprednisolone one day before and after inoculating 0.3 ml of Candida albicans (9.4 × 10^7 cells/ml) into the oral cavity of the rats. The bay leaf extract was given to the oral candidiasis rat model by intragastric tube at 250 mg/kgBW/day (P1) and 500 mg/kgBW/day (P2) for 5 days. Rats were given solvent (NaCMC) as a negative control group, while the positive control group received fluconazole. Oral candidiasis infection was evaluated on the last day by counting the number of colonies from swabs of the oral mucosa cultured on SDA-C.*

Result: *After 5 days of treatment, the number of fungal colonies x 10^4/mL of rat group P2 was 180,3 ± 35,12, P1 (2,5 ± 1,4), positive 0,3 ± 0,2, and negative control 11,8 ± 2,3. Non parametric test test resulted P2 was significantly higher than P1, negative control and positive control (P≤0.05)*

Conclusion: *This study revealed that bay leaf extract aggravated oral candidiasis in vivo. The hypothesis is that flavonoids in bay leaves may inhibit the production of pro-inflammatory cytokines, which could diminish the rat's immune response.*

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INTRODUCTION

The prevalence of oral candidiasis has been increasing due to various predisposing factors.[1] One of the major challenges in developing antifungal drugs stems from the evolutionary closeness of fungi to humans: both are eukaryotes with similar cellular machinery, so identifying targets that kill fungi without harming human cells is difficult and limits options for specific, non-toxic therapies.[2] Compounding this, antifungal resistance is rising—species such as *Candida auris* and *Aspergillus fumigatus* show resistance to azoles, echinocandins, and even amphotericin B through mechanisms like target mutations, efflux pumps, and biofilm formation.[3] Moreover, the antifungal development pipeline is under-resourced: only three drug classes exist, just one new class emerged in over 30 years, and funding remains far below levels for antibacterials, with fewer novel compounds progressing in clinical trials.[4] These intersecting obstacles, target scarcity, toxicity risks, resistance trends, and limited R&D investment, significantly impede progress in creating effective antifungals.

Indonesian bay leaves (Syzygium polyanthum (Wight) Walp) are commonly found in Indonesia and Malaysia. These leaves have been extensively studied as anti-pathogenic microorganisms. Numerous in vitro studies have demonstrated that the extract of bay leaves possesses antifungal properties.[5–9] The bay leaf component penetrates the fungal cell membrane to destroy and inhibit cell growth.[9] Therefore, the authors conducted an in vivo study of the antifungal effects of bay leaves on the oral candidiasis rat model.

RESEARCH METHOD

Six-week-old female Wistar rats (Iwan Farm, Pakis Aji, Malang) were housed in cages that received adequate sunlight, with the ambient temperature maintained between 26-29°C. Three rats were kept in each cage and had free access to food and water. Before the study, it was confirmed that the female rats were non-pregnant, as they had been separated from the male rats since they were 15 days old. Two randomly selected rats were examined and confirmed healthy and non-pregnant by a veterinarian at Klinik Hewan, Dinas Peternakan, Jember. All research procedures received approval from the Health Research Ethics Committee of the Faculty of Dentistry at the University of Jember, under approval number 2067/UN25.8/KEPK/DL/2023.

The oral candidiasis rat model was made by following previous studies.[10] Tetracycline HCL (Sanbe Farma, Indonesia) was added 500 mg per liter into the rat's drinking water from the first day until the end of the study. On the second day, the rats were injected with Methylprednisolone sodium succinate (MP) 40 mg/kg BW subcutaneously. On the third day, the rats were sedated with an injection of Chlorpromazine HCl (Phapros Ltd, Indonesia) 5 mg/kg BW IM, then 0.3 ml, 9.4 x 10^7 cells/m C. albicans ATCC 10231 suspension was inoculated into the mouths of the mice. The rats were re-injected with MP at the same dose on the day 4^{th} and 11^{th}.

After being weighed according to the rat's body weight, all test material was dissolved in 2 ml of 0.5% Na CMC. Bay leaf extract was given daily with a gastric tube on the 7^{th} to 17^{th} day. Group T1 was given 250 mg/kg BW/day, and T2 was given 500 mg/kg BW/day bay leaf extract. The negative control group C (-) was given the solvent, and C (+) was given Fluconazole 9,24 mg/kgBW/day.

Table 1. The sequence of procedures in research

Day											
1	2	3	4	5	6	7	8	9	10	11	12
Start 1st day adding 500 mg/L Tetracyclin HCL in rat's dringking water											
Formation of oral candidiasis rat model						Giving bay leaf extract					
Injection 6 mg MP/SC	injection CPZ 0,7 mg IM +innoculation of C. albicans 9,4 x 107	Injection 6 mg MP/SC	Evaluation of infection						Injection 6 mg MP/SC	Evaluation of infection	

The infection was evaluated before and after the treatment by swabbing the entire oral cavity surface with sterile cotton. The tip of the sterile cotton swab was inserted into a test tube containing 2 mL of Potato Dextrose Broth (PDB) solution with a concentration of 2,4 gr/L. Then, the tube was vortexed to release all the microorganisms attached to a cotton swab. Then, the tube was incubated at 37°C for 24 hours. Furthermore, the suspension was diluted 4 times using sterile distilled water, then 0.1 mL of C. albicans suspension was cultured on Sabouroud Dextrose Agar–Chloramphenicol (SDA-C) plate media and incubated at 37℃ in an incubator for 24-48 hours. The petri dish was placed in an inverted position so that the condensation water fell on the lid of the petri dish so as not to interfere with the growth of C. albicans. After 24-48 hours, the number of colonies in each petri dish was counted. All procedures are shown in table 1. The number of colonies on each group's petri dish was tabulated and analyzed using the Statistical Package for the Social Sciences (SPSS) program, which includes preliminary tests with normality and homogeneity tests.

RESULT

The fungal colonies on SDA-C media are smooth, yellowish-white spheres with a uniform diameter, as seen in Figure 1

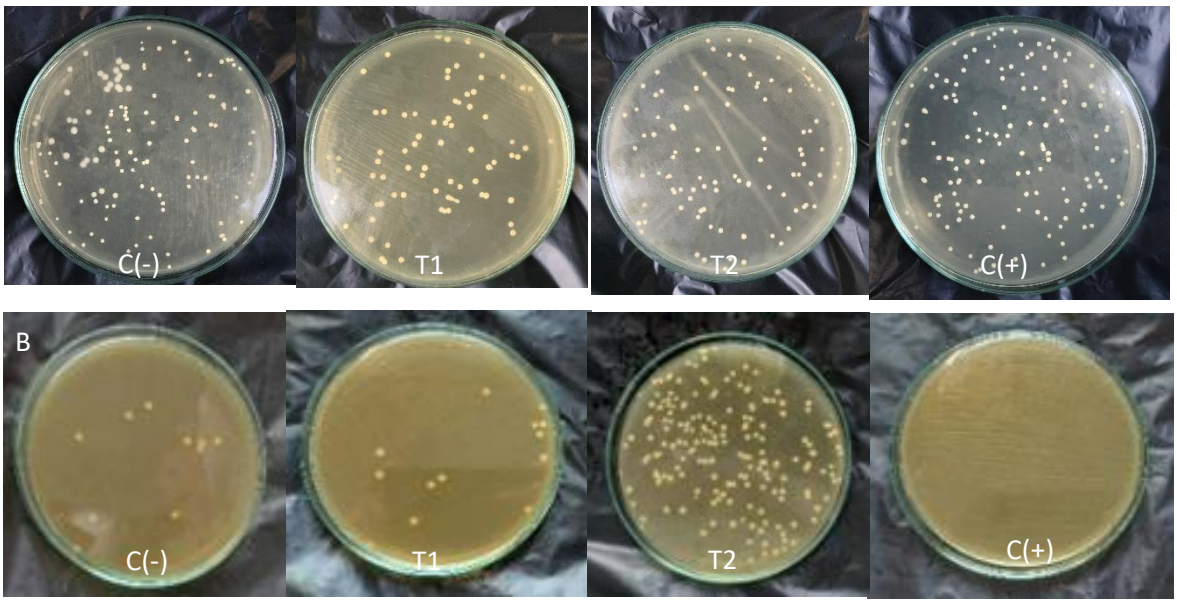

Figure 1. Fungal colonies in culture on SDA-C from oral cavity swab results (A) before and (B) after treatment for 5 days.Group C(-) given placebo, T1 given 250 mg/kgBW/day, T2 given 500 mg/kgBW/day and C(+) given 9,24 mg/kgBW/day

The histogram of the average number of colony-forming units (CFU) per milliliter, as seen in Figure 2

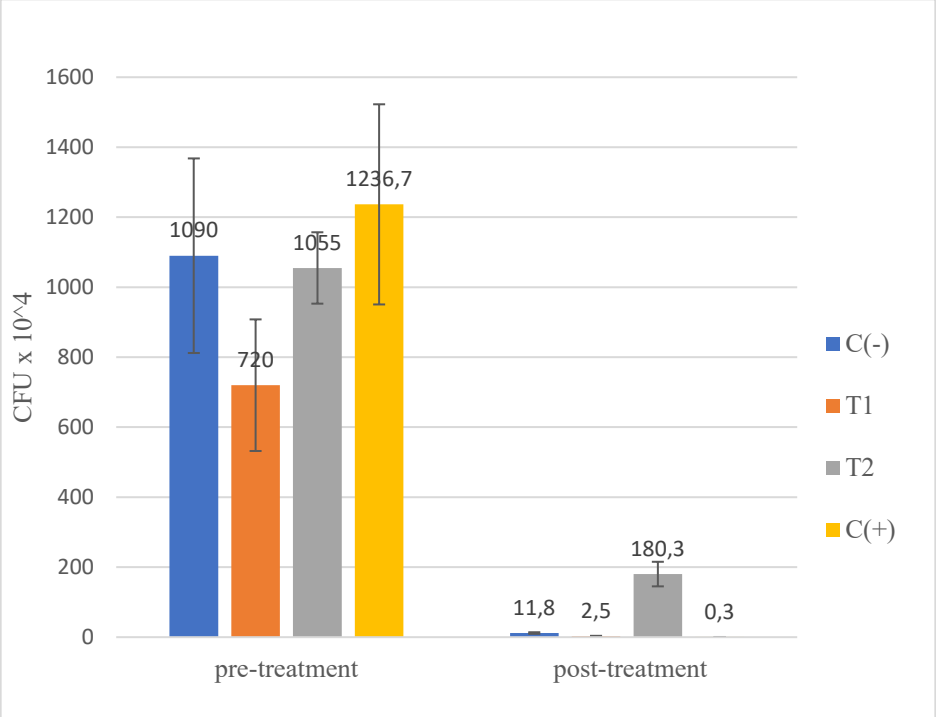

Figure 2. Histogram of the average ± 0,5 SD colony-forming unit pre-treatment and post-treatment Group C(-) given placebo, T1 given 250 mg/kgBW/day, T2 given 500 mg/kgBW/day, and C(+) given 9,24 mg/kgBW/day.

The results of the normality and homogeneity tests indicate that the data are not normally distributed and lack homogeneity. Therefore, the Kruskal-Wallis non-parametric test was used to differentiate between the groups, and the Mann-Whitney test was then used to conduct pairwise comparisons. The statistical analysis results before treatment showed no difference in the number of fungal colonies between the negative control group, treatment 1, treatment 2, or the positive control.These results showed that the creation of the oral candidiasis rat model was successful, and the infection level was the same in all groups.

After 5 days of treatment, treatment group 2 (T2), which was given 500 mg/KgBW/day of bay leaf extract, showed greater CFU than the other groups. There were no differences between the control negative (C-), which was given 0.5% NaCMC, the group given 250 mg/KgBW/day (T1) of the bay leaf extract, and the positive control group ($p< 0.05$).

DISCUSSION

The selection of experimental animals that can represent the actual disease condition in humans is very important in research on therapy. Mistakes in selecting experimental animal models can waste resources. Candida infections in humans can be triggered by a decrease in the immune system and the use of antibiotics, which reduce the normal bacterial flora in the oral cavity. The rat model of oral candidiasis that can represent the patient's condition has been successfully developed and proven microbiologically and histopathologically in our previous study.[10] The steps for creating this rat model were extrapolated from research using mice.[11] The

statistical analyses found that after administering bay leaf extract at 250 mg/kg.BW/day for 5 days, there was no decrease in the number of fungal colonies, and at a dose of 500 mg/kg BW/day, there was an increase in the number of fungal colonies. Systemic administration of bay leaf extract cannot overcome fungal infections in the oral cavity, and at higher doses, the infection becomes more severe. These results are contrary to those of in vitro research.

At a dose of 250 mg/kg body weight (BW) per day, the number of *Candida* colonies did not increase to the same extent as observed at a dose of 500 mg/kg BW per day. These findings indicate that administration of 250 mg/kg BW per day may not significantly suppress the immune response in mice. Based on the results, it is hypothesized that immunosuppression is dose-dependent; however, further studies are needed to confirm this relationship.

The cause of this increase in infection level was a decrease in the rat's immune system. One of the components that reduces the immune system is flavonoids found in bay leaves. Bay leaves are known to contain flavonoids, essential oils, sesquiterpenes, triterpenoids, phenols, steroids, citral, lactones, saponins, carbohydrates, selenium, and vitamins in bay leaves such as A, C, and E. Bay leaves also contain tannins, saponins, and niacin.[6]

In vivo outcomes frequently diverge from in vitro findings because living organisms introduce complexities that lab models can't replicate, drug absorption, distribution, metabolism by liver enzymes (such as CYP3A4), protein binding, and immune responses alter drug concentration and activity at the infection site; moreover, tissue-specific penetration varies greatly.[12] Drugs may be rapidly converted to inactive metabolites in vivo, as seen with albendazole transforming into inactive albendazole sulphoxide, nullifying effects seen in vitro.[13] Additionally, while in vitro MIC-based assays provide static snapshots, in vivo efficacy depends on dynamic pharmacokinetic-and pharmacodynamic relationships and immune-mediated clearance, which in vitro models cannot simulate.[14]

Various research results have proven the efficacy of flavonoids from various natural ingredients in lowering the immune system. Twelve types of flavonoids from Scutellaria baicalensis (herbal ingredients from China) have been shown to inhibit the proliferation of cytotoxic T cells, thereby lowering the immune system [15] Flavonoids in breadfruit leaves (Artocarpus sp.) have an in vivo immunosuppressive effect, as evaluated in male Swiss albino mice. The decrease in humoral antibody titer test, delayed-type hypersensitivity, and phagocytosis index in mice given breadfruit leaf extract and fraction indicated an immunosuppressive effect, possibly due to flavonoid content.[16] Quercetin, the most abundant flavonoid derivative, can inhibit various transcriptional factors, which modulate the differentiation, proliferation, and activation of immune cells and enhance the formation of Treg cells.[17,18] Flavonoids in bay leaves can provide anti-inflammatory effects by inhibiting NFκB and NLRP3 inflammasomes and inhibiting the production of pro-inflammatory cytokines IL-1β, IL-2, IL-6, TNF, and IL-17A, downregulating chemokines, and reducing reactive oxygen and nitrogen species.[19] IL-17A, often called IL-17, is the main cytokine in the host's resistance to fungal infections. Decreased IL-17 can cause decreased recruitment of inflammatory cells to infected tissue.[20,21] IL-17 is also essential for regulating microbial colonization of tissue barriers. Congenital disabilities in IL-17 production suggest a role for IL-17 in protective immunity against the opportunistic fungal pathogen C. albicans.[22] Beside flavonoids, bay leaves contain approximately 17% essential oils.[6] The highest essential oil content of bay leaves is -4-*decanal* (43.489%), 1-

decyl aldehyde (19.752%), and *capryl aldehyde* (14.092%).[23] Aldehydes have been shown to inhibit the inflammatory response to LPS by reducing the gene expression and levels of pro-inflammatory cytokines such as IL-6 and TNFα.[24]

In the positive control group, the rats treated with fluconazole for five days demonstrated the lowest number of fungal colonies, outperforming the other groups significantly. This clear outcome indicates there were no false negatives, affirming the reliability of the results. Furthermore, the increased fungal colonies in the given bay leaf extract group can be thoroughly justified. The level of infection observed in the group administered 250 mg/kg BW/day of bay leaf extract was statistically similar to that of the negative control group receiving a placebo. This observation likely stems from the insufficient dosage, which may fail to adequately reduce the immune response in the rats.

It's important to recognize a limitation in this study: the post-treatment data were found to be neither normal nor homogeneous. This variability can be attributed to the use of non-certified heterogeneous rats in terms of genotype and phenotype, resulting in inconsistent responses among individual subjects.

CONCLUSION

Systemic administration of bay leaf extract at a dose of 500mg/kg BW/day increased the number of colonies in swab culture results throughout the oral candidiasis rat model's oral mucosa. This fact indicates that the infection worsens after giving the leaf extract.

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