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Comparison of Antifungal Susceptibility Basil Leaves Extract (*Ocimum sanctum* Linn.), Eugenol, and Nystatin against Isolates of *Candida* spp. as Important Agent causing Oral Candidiasis in HIV/AIDS Patient

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ABSTRACT

Background: Oral candidiasis is an infection caused by *Candida* spp. in areas of the oral mucosa that are often found in HIV/AIDS patients. Due to increased antifungal resistance, it was important to find new antifungal candidates, especially from natural ingredients. One example was basil leaf extract (*Ocimum sanctum* Linn.), which had a major compound of eugenol that had an antifungal effect in inhibiting *Candida* spp. **Purpose:** To evaluate the comparison of the antifungal susceptibilities of nystatin, basil leaf extract (*Ocimum sanctum* Linn.), and eugenol against isolates of *Candida* spp. **Methods:** This study examined the comparison of the antifungal susceptibility of nystatin suspension at the concentration of 100 IU, basil leaf extract (*Ocimum sanctum* Linn.) with doses equivalent to 800 µg/mL and 800 µg/mL and 400 µg/mL eugenol, and eugenol 800 µg/mL and 400 µg/mL against 40 stored isolates of *Candida* spp. from the oral cavity of HIV/AIDS patients which were reactivated. **Result:** The mean inhibition zone of nystatin for all isolates was 22.98 mm, while the mean inhibition zones of eugenol with doses of 800 µg/mL and 400 µg/mL were 17.07 mm and 15.89 mm, and the mean inhibition zone of basil leaf extract (*Ocimum sanctum* Linn.) with doses equivalent to 800 µg/mL and 400 µg/mL eugenol were 14.87 mm and 14.01 mm. The inhibition zone of basil leaf extract (*Ocimum sanctum* Linn.) and eugenol was significantly lower than nystatin ($p = 0.001$). **Conclusion:** Basil leaf extract (*Ocimum sanctum* Linn.) and eugenol have antifungal effects by the inhibition zone. The inhibition zone of nystatin was significantly higher compared to basil leaf extract (*Ocimum sanctum* Linn.) and eugenol against *Candida albicans* and non-*albicans* isolates.

Keywords: Basil leaf extract (*Ocimum sanctum* Linn.), eugenol, nystatin, oral candidiasis, HIV/AIDS.

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BACKGROUND

Oral candidiasis is an opportunistic infection of the oral mucosa caused by an overgrowth of *Candida* spp. Oral candidiasis infection is an opportunistic infection that is influenced by host factors' predisposition.[1] One of the predisposing factors for this opportunistic infection is the occurrence of immune disorders or immune failures which are most often found in patients with HIV/AIDS.[2,3]

Oral candidiasis therapy can be given according

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to the severity of the disease. Oral candidiasis is treated with the oral antifungal drug nystatin and/or fluconazole as systemic antifungal.[2] An in vitro study in India that examining the sensitivity of nystatin to *Candida* spp. in HIV patients found 2.8% of *Candida* spp. isolates were resistant to nystatin.[4] Resistance to nystatin is reported to be rare and has been associated with changes in fungal cell membranes and biofilm formation.[5] One of the problems of resistance and the formation of antifungal drug biofilms can be overcome with new antifungal drugs.[6]

Several antifungal candidates, one of them is basil leaf extract (*Ocimum sanctum* Linn.), which has the main component, such as eugenol, which from several studies has a role in inhibiting the growth of *Candida* spp. This compound is effective against the adaptive mechanism of *Candida albicans* biofilm.[7,8]. Based on some of these data, the researchers aimed to conduct an in vitro study of basil leaf extract (*Ocimum sanctum* Linn.) and eugenol against stored isolates of *Candida* spp. and compare them with standard drug therapy in oral candidiasis, which is nystatin, which currently has been reports of resistance.

METHODS

This study used experimental research design with the disk diffusion method. The aim was to evaluate the comparison of the antifungal susceptibilities of basil leaf extract (*Ocimum sanctum* Linn.) with doses equivalent to 800 µg/mL and 400 µg/mL eugenol, eugenol 800 µg/mL and 400 µg/mL, and then compared with nystatin at the concentration of 100 IU against 40 stored isolates of *Candida* spp., which consisted of 20 *Candida albicans* and 20 *Candida* non-*albicans* isolated from the oral cavity of HIV/AIDS patients. The patients were hospitalized in the Infectious Disease Intermediate Treatment Unit (UPIPI) Dr. Soetomo General Hospital Surabaya during April 2019 – July 2019. The stored isolates were taken from gargle wash cultures of HIV/AIDS patients with oral candidiasis. Identification of *Candida* species was carried out by examining *Candida* cultures on CHROM agar. Colonies of each *Candida* species will give different colony colors after being grown for 36-48 hours. The examination was continued with VITEK 2 to confirm species that could not be identified by the CHROM agar method.

The antifungal activity susceptibility was tested with the disk diffusion method using paper discs or blank discs on Saboroud Dextrose Agar (SDA) media. The dosage form of basil leaf extract (*Ocimum sanctum* Linn.) in this study was processed by the Faculty of Pharmacy, Airlangga University, Surabaya. In this study, the dosage of eugenol was 800 g/mL and 400 g/mL, while the dosage of basil leaf extract (Ocimum sanctum Linn.) was adjusted to the dosage of eugenol. In accordance with a study conducted by Sharifzadeh and Shokri in 2020, which examined the minimal inhibitory concentration (MIC) on eugenol, the MIC for Candida growth was found at a dose of 400-800 µg/mL.[9]

The nystatin used in this study was nystatin analytical disk with a dose of 100 IU. The data were analyzed using non-parametric statistical methods (Anova) because the data were not normally distributed and not homogeneous. These data are then entered into a data collection sheet and analyzed with the Statistical Package for Social Sciences (SPSS). This research has obtained ethical approval from the Ethics Committee of Dr. Soetomo General Academic Hospital Surabaya (No 0522/LOE/301.4.2/VII/2021).

RESULT

This study used the disk diffusion method. To compare against *Candida* spp., the standard antifungal drug nystatin 100 IU, against the mean results of the inhibition zone test results of basil leaf extract (*Ocimum sanctum* Linn.) with doses equivalent to 800 µg/mL and 400 µg/mL eugenol, and eugenol 800 µg/mL and 400 µg/mL.

In all *Candida* spp., the mean of nystatin 100 IU, eugenol 800 µg/mL, eugenol 400 µg/mL, basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 800 µg/mL eugenol, and basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 400 µg/mL eugenol inhibition zone were 22.98, 17.07 mm, 15.89 mm, 14.87 mm, and 14.01 mm. The comparison of the mean of inhibition zone results for all *Candida* species can be observed in Table 1 below.

In *Candida albicans*, the mean of nystatin 100 IU, eugenol 800 µg/mL, eugenol 400 µg/mL, basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 800 µg/mL eugenol, and basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 400 µg/mL eugenol inhibition zone were 23.08 mm, 16.76 mm, 15.52 mm, 14.41 mm, and 13.62 mm. A comparison of the mean results of the inhibition zone against *Candida albicans* can be observed in Table 2 below.

In *Candida* non-a*lbicans*, the mean of nystatin 100 IU, eugenol 800 µg/mL, eugenol 400 µg/mL, basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 800 µg/mL eugenol, and basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 400 µg/mL eugenol inhibition zone were 22.88 mm, 17.38 mm, 16.25 mm, 15.34 mm, and 14.39 mm. The comparison of the mean inhibition zone results against *Candida* non-*albicans* can be observed in Table 3 below.

Table 1. Comparison of the mean diameter of the inhibition zone of basil leaf extract (*Ocimum sanctum Linn.*), eugenol, and nystatin in all *Candida* species (*albicans* and non-*albicans*)

No.	Antifungal type	Number of isolates	Mean of inhibition zone diameter (mm)	p
1	Nystatin 100 IU	40	22.98	0.001
2	Eugenol 800 µg/mL	40	17.07	
3	Eugenol 400 µg/mL	40	15.89	
4	Basil leaf extract equivalent to 800 µg/mL eugenol	40	14.87	
5	Basil leaf extract equivalent to 400 µg/mL eugenol	40	14.01	

Table 2. Comparison of the mean diameter of the inhibition zone of basil leaf extract (*Ocimum sanctum* Linn.), eugenol, and nystatin in *Candida* albicans

No.	Antifungal type	Number of isolates	Mean of inhibition zone diameter (mm)	p
1	Nystatin 100 IU	40	23.08	0.001
2	Eugenol 800 µg/mL	40	16.76	
3	Eugenol 400 µg/mL	40	15.52	
4	Basil leaf extract equivalent to 800 µg/mL eugenol	40	14.41	
5	Basil leaf extract equivalent to eugenol 400 µg/mL eugenol	40	13.62	

Table 3. Comparison of the mean diameter of the inhibition zone of basil leaf extract (*Ocimum sanctum* Linn.), eugenol, and nystatin in *Candida* non-*albicans*

No.	Antifungal type	Number of isolates	Mean of inhibition zone diameter (mm)	p
1	Nystatin 100 IU	40	22.88	0.001
2	Eugenol 800 µg/mL	40	17.38	
3	Eugenol 400 µg/mL	40	16.25	
4	Basil leaf extract equivalent to 800 µg/mL eugenol	40	15.34	
5	Basil leaf extract equivalent to 400 µg/mL eugenol	40	14.39	

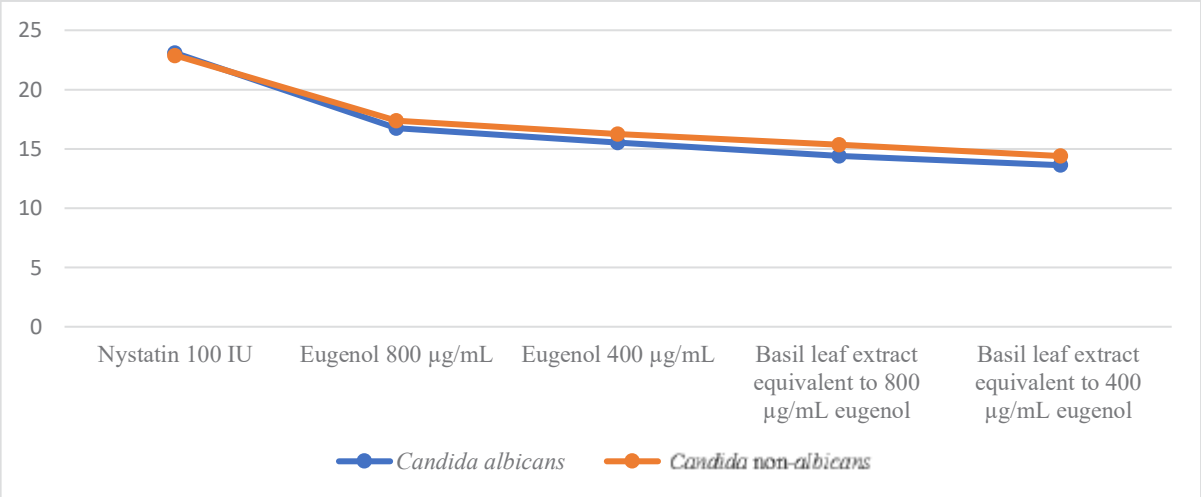

Figure 1. The distribution of inhibition zone results for nystatin, eugenol 800 µg/mL, eugenol 400 µg/mL, basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 800 µg/mL eugenol, and basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 400 µg/mL eugenol.

The distribution graphic of the comparison inhibition zone results of nystatin 100 IU, eugenol 800 µg/mL, eugenol 400 µg/mL, basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 800 µg/mL eugenol, and basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 400 µg/mL eugenol can be seen in figure 1.

In Table 1, 2 and 3, the data were analyzed using non-parametric statistical methods (Anova) because the data were not normally distributed and homogeneous. The results of the non-parametric statistical test showed that the data significance value was 0.001. As it is < 0.05, which means that there was a significant difference between the mean inhibition zone of nystatin as a standard antifungal drug compared to the inhibition zone of eugenol 800 µg/mL, eugenol 400 µg/mL, basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 800 µg/mL eugenol, and basil leaf extract (*Ocimum sanctum* Linn.) equivalent to 400 µg/mL eugenol for *Candida* growth in all species, *Candida albicans* and non-*albicans*.

DISCUSSION

In this study, nystatin sensitivity criteria were assessed based on the standards according to the Clinical and Laboratory Standards Institute (CLSI). The sensitivity criteria for basil leaf extract (*Ocimum sanctum* Linn.) and eugenol do not yet have a standard according to the CLSI, so it cannot be concluded that the sensitivity value of basil leaf extract and eugenol are the same. The value of the inhibition zone that can be compared in this study is the average diameter of the drug inhibition zone in the disk diffusion method in millimeter.

The result of this study found that the mean inhibition zone of nystatin was greater than the mean inhibition zone of eugenol and basil leaf extract (*Ocimum sanctum* Linn.). Both eugenol and basil leaf extract (*Ocimum sanctum* Linn.) have antifungal effects with an inhibition zone from the formation of a clear zone that were able to inhibit the growth of *Candida* spp., although the inhibition zones of both eugenol and basil leaf extract (*Ocimum sanctum* Linn.) were not better than those of nystatin as a standard antifungal drug.

In another similar study, nystatin had a low minimum inhibitory concentration (MIC) and was still sensitive to *Candida* spp. isolates. Nystatin is an antifungal drug that is still effective in vitro against *Candida* spp.[10] Research by Lydiawati and colleagues in 2020 using the disk diffusion method found that nystatin is a standard antifungal drug that is still sensitive and none is resistant to the growth of *Candida* spp., both in *Candida albicans* and non-*albicans*.[11] Resistance to nystatin is reported to be rare and has been associated with changes in fungal cell membranes.[5]

Nystatin is a topical antifungal from the polyene group that works by binding to membrane sterols found in *Candida* spp. The components of the polyene molecule will form irreversible bonds to increase membrane permeability. This causes intracellular leakage and induces fungal death. Nystatin functions as a fungistatic agent at low concentrations and fungicidal at higher concentrations.[2]

Research by Silva and colleagues in 2017 found that nystatin and eugenol have an antifungal effect, but when compared, nystatin has an antifungal effect by inhibiting the growth of *Candida* spp. better than

eugenol.[12] A similar study by Khan and colleagues in 2010 found that nystatin inhibited the growth of *Candida* sp. more than basil (*Ocimum sanctum* Linn.).[13]

When referring to the study of Nenoff and colleagues in 2016, the MIC of nystatin was in the range of 3.7 – 7.4 IU/mL (0.625 – 1.25 µg/mL) for *Candida* spp. Nystatin showed excellent in vitro activity against *Candida* spp., and had a low MIC against *Candida* spp., so the antifungal activity of nystatin was still very good and sensitive to *Candida* spp.[14] In several previous studies, basil leaf (*Ocimum sanctum* Linn.) and eugenol have antifungal activity by having the ability to inhibit *Candida* spp., but have a high MIC, thus the antifungal activity of both basil leaf (*Ocimum sanctum* Linn.) and eugenol were still considered low. One of the constituents of basil leaf (*Ocimum sanctum* Linn.) that is suspected to be effective in inhibiting *Candida* spp. is eugenol.[13,15]

A study conducted by Sharifzadeh and Shokri in 2020 showed that eugenol was able to inhibit the growth of *Candida* spp., but the mechanism by which eugenol could induce *Candida* cell death was not fully understood.[9,12] The researchers conducted a study on the mechanism of action of eugenol against fungi using scanning electron microscopy (SEM) to show that eugenol causes ultrastructural morphological changes in the envelope of *Candida* spp. Eugenol caused a significant decrease in the number of *Candida* cells followed by a significant increase in the number of damaged and disturbed cells, with the surface of *Candida* cells becoming rough and wrinkled. Eugenol is also a lipophilic compound with the ability to penetrate the lipid bilayer membrane which is composed of fatty acid chains, by changing the fluidity and permeability of the cell membrane so that the cell loses its structure and function, which results in cell lysis.[16]

Eugenol works as an antifungal with the mechanism of disruption of the fungal cell membrane components. Eugenol also functions by inhibiting the synthesis of ergosterol and the formation of free radicals. Eugenol also has an anti-inflammatory effect. Research on the anti-inflammatory effect of eugenol has found that this compound is able to suppress the expression of the cyclooxygenase II enzyme. The eugenol dimer can inhibit the expression of cytokines in macrophages, which are stimulated by polysaccharides. Eugenol also has an inhibitory effect on cell proliferation through suppression of NF-kB. Eugenol can also modulate the expression of NF-kB target genes that are responsible for the regulation of cell proliferation and cell survival.[17]

In this study, the mean inhibition of eugenol was greater than that of basil leaf extract (*Ocimum sanctum* Linn.). This could be due to the content of basil leaf extract (*Ocimum sanctum* Linn.), besides eugenol there were other compounds. De Ornay and colleagues in 2017 found that besides of 0.31% eugenol, the chemical content of basil leaf (*Ocimum sanctum* Linn) also contains other compounds, including flavonoids, essential oils, alkaloids, and tannins.[7] The concentration of the extract, the content of antifungal compounds, the type of fungus inhibited, and the diffusion power all affect the antifungal activity of a compound. The concentration of the extract can also affect the inhibition zone formed, with the higher the concentration, the greater the clear zone. The more active compounds present, the more focused the concentration, thus affecting the diameter of the inhibition zone formed on the growth of herbal medicine.[18] The major component of basil leaf extract (*Ocimum sanctum* Linn.), namely eugenol, is highly volatile, so that after treatment and storage of eugenol and basil leaf extract (*Ocimum sanctum* Linn.) were very important.[19]

Basil leaf (*Ocimum sanctum* Linn.) has antifungal activity by inhibiting germ tube and biofilm formation from *Candida* spp. Basil leaf (*Ocimum sanctum* Linn.) can damage the composition of the exposed *Candida* biofilm, as visible by electron microscopy, suppressing the expression levels of *Candida albicans* HWP1 and ALS3 adhesin genes.[20] This mechanism causes shrinkage of *Candida* fungal cell walls, which results in disrupted living cell activity, inhibited growth, and, at certain doses, can cause fungal death.[7,21] In a study on isolates of *Candida* spp. given essential oil of basil leaf (*Ocimum sanctum* Linn.), the result showed that essential oil of basil leaf (*Ocimum sanctum* Linn.) with a concentration of 10% showed an inhibition zone of 9 mm, and the inhibition zone increased to 13 mm when the concentration was increased to 30%.[13,15]

Further in vivo studies in animal models to assess their therapeutic efficacy, formulations for topical application, as well as their toxicity, are needed to assess the potential of basil leaf (*Ocimum sanctum* Linn.) and eugenol for therapeutic applications, given the evolving treatment failure and antifungal resistance in *Candida* spp., and suggest treating resistant *Candida* infections through a combination drug approach. The synergistic interaction between basil leaf (*Ocimum sanctum* Linn.) and eugenol with antifungal drugs also needs to be evaluated and investigated in the further research.[16,22]

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