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Paper Title: **Evaluation of the antifungal effects of rosemary oil and comparison with nystatin on the growth of isolate Candida Species from HIV/AIDS patients with Candidiasis oral**

Authors: **Dwi Murtiastutik; Afif Nurul Hidayati; Septiana Widyantari; Astindari; Bernadya Yogatri Anjuwita Saputri; Evy Ervianti; Damayanti; Maylita Sari**

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Paper Title: Evaluation of the antifungal effects of rosemary oil and comparison with nystatin on the growth of isolate Candida Species from HIV/AIDS patients with Candidiasis oral

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RESEARCH ARTICLE

Evaluation of the Antifungal effects of Rosemary Oil and comparison with Nystatin on the Growth of *Candida* species isolates from HIV/AIDS patients with Oral Candidiasis

Dwi Murtiastutik[1]*, Afif Nurul Hidayati[1,2], Septiana Widyantari[1], Astindari[1], Bernadya Yogatri A. Saputri[1], Lunardi Bintanjoyo[1], Evy Ervianti[1], Damayanti[1], Maylita Sari[1]

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ABSTRACT:

Background: Oral candidiasis is an infection due to the activity of *Candida albicans* in the oral cavity. Oral candidiasis is one of the most common opportunistic infections occuring among Human Immunodeficiency Virus (HIV)/Acquired immune deficiency syndrome (AIDS) patients. Due to increasing resistance and adverse effects to commonly used antifungal drugs, many recent studies have examined the use of herbal essential oils as antifungal agents. In this study, essential oil of *Rosmarinus officinalis (Lamiaceae)* and nystatin were examined for in vitro antifungal activity against *Candida* species. **Aim:** To evaluate antifungal activity of essential oil of *Rosmarinus officinalis (Lamiaceae)* and nystatin by comparing inhibition zone diameters. **Methods:** This study was an experimental laboratory study with a posttest only design conducted in Dr. Soetomo General Academic Hospital, Surabaya. Forty isolates consisted of 20 isolates of *Candida albicans* and *Candida* non-*albicans* were subjected to test for antifungal activity using the diffusion disk method using paper discs or blank discs and inhibitory zones were recorded. **Results:** Diffusion test results revealed stronger antifungal effect of nystatin against all analyzed *Candida* strains. This study showed the mean diameter of the inhibitory zone for *Candida albicans* formed by rosemary essential oil is 2.25 mm and the average inhibition zone formed by rosemary essential oil for *Candida* non-*albicans* is 1.5 mm. **Conclusion:** The antifungal activity of nystatin is stronger when compared to rosemary essential oil as seen from a greater inhibition zone than rosemary essential oil in the diffusion method.

KEYWORDS: Antifungal activity, nystatin, *Rosmarinus officinalis*, HIV, *Candida albicans*, *Candida* non-*albicans.*

INTRODUCTION:

Candida species are opportunistic yeasts and the etiologic agent of candidiasis, the most frequent fungal infection in humans. In the oral cavity, *Candida albicans* (*C. albicans)* is the most abundant *Candida* species in health and disease state. As an opportunistic pathogen, it usually causes infection in immunosuppressed or immunodeficient individuals such as HIV/AIDS patients.[1]

Various strategies are being used to manage candida infections, including the use of topical or systemic fungistatic or fungicidal agents. Antifungal agents including polyenes (nystatin, amphotericin B), azoles (fluconazole, itraconazole and miconazole), and antimetabolites agents (5-fluorocytosine) are most common medications used to treat candidiasis. However, occasionally consumption of the effective dose cannot be tolerated due to side effects.[2]

Resistance to commonly-used antifungals has been recently reported. Therefore, new methods to control fungal infection with improved efficacy should be developed. Latest studies had shown evidence for

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antimicrobial compounds of botanical extracts, in the form of crude extracts, essential oils and molecules, including flavonoids and terpenes. Recent studies have also addressed the association between conventional medications and natural products to achieve better treatment efficacy.[3]

Essential oils are defined as volatile and hydrophobic aromatic natural substances extracted from various parts, such as flowers, leaves, peels, barks, roots, seeds, resins, of aromatic plants, containing highly concentrated effective compounds. Rosemary (*Rosmarinus officinalis L.*), of the *Lamiaceae* family, is a perennial shrub with pleasant scent that grows in several regions globally. Most of these are used in the local folk tradition for many purposes.[4] Historically, rosemary has been used as a medicinal agent to treat renal colic and dysmenorrhea. It has also been applied for relief of respiratory symptoms, stimulation of hair growth, as aromatherapy for anxiety treatment and increasing alertness.

Rosemary essential oils have varying biological activities according to their chemical compositions. Around 13 chemotypes of rosemary essential oil have been recognized, which differ in their relative amounts of camphor, borneol, verbenone, pinene, 1, 8-cineole, and bornyl acetate. Nature-derived compounds which are effective against resistant *Candida* species are necessary options to control oral candidiasis. Fungistatic properties of *Rosmarinus officinalis* essential oil has been studied in several researches.[5] Fontenelle et al. stated that at low concentrations rosemary essential oil produced antifungal activity against *Candida*, but the anti-adherent activity of this oil has not been studied. Thus, further investigation of the antifungal activity of these oils is necessary to substantiate the use of essential oils clinically. The mechanism of antifungal action of rosemary essential oils has not been fully eluciated. Hammer et al. proposed that interaction of essential oils with lipid components results in changes in the cell membranes.[6] Other studies found that *Rosmarinus officinalis L.* possesses several antifungal mechanisms. It has been shown to alter cellular structure and fungal membrane pemeability thus inhibiting the adhesion of *Candida albicans*. A study even showed that bionanosystem consisting of nanoparticles coated with rosemary essential oil was able to reduce the adhesiveness and to prevent the development of highly resistant biofilms of *Candida*.[7]

Various in vitro studies have examined the antifungal activity of rosemary essential oil against *C. albicans*. Hosein Nejati et al. studied topical rosemary for wound caused by *C. albicans* in mice, and found that topical rosemary has antifungal and antiinflammatory properties in the wound due to its high terpenoids, limonene, 1.8 cineol content.[8] Other studies examined the effect of rosemary essential oil against *C. albicans* isolated from vaginal smears, and found that rosemary essential oil has a minimum inhibition concentration (MIC) of of 0.1 mg/ml.[4] Cavalcanti et al. compared rosemary essential oil with nystatin against *Candida* species isolates and showed that rosemary essential oil has a MIC value of 2.25mg/ml which was better than 100,000 IU/ml nystatin.[6] This study evaluated the antifungal activity of rosemary essential oil compared with synthetic nystatin against isolates of *Candida* species.

MATERIAL AND METHODS:

This is an experimental laboratory study with a posttest only design conducted in Dr. Soetomo General Academic Hospital, Surabaya. Forty isolates used in this study, which consisted of 20 isolates of *Candida albicans* and 20 isolates of *Candida* non-*albicans*, were obtained from forty HIV/AIDS patients with oral candidiasis. This research has been reviewed and approved by the Ethics Committee of the Dr. Soetomo General Academic Hospital (1614/KEPK/XI/2019). Analyzed essential of rosemary (*Rosmarinus officinalis L.*), were commercial product produced by Young Living USA, according to the producer's notification, characterized as 100% natural essential oil. This essential oil were analyzed by GC containing 45.12% *1.8-cineol*, 12.78% *alpha-pinene*, 10.79% *camphor*, 3.50% *borneol*. Nystatin used as susceptibility test. The isolates were tested for antifungal activity using the disc diffusion method. The colonies were mixed in 0.85% sterile normal saline (5ml volume). Mueller Hinton agar with 2% glucose and 0.5µg/ml of methylene blue was used. Strain suspensions (10^6 CFU/ml) were swabbed on Saboraoud Dextrose Agar. Filter paper disc (6 mm diameter) were placed on the agar surface and added with 10µl of 100% concentrate *rosemary* essential oil and 100% concentrate of nystatin. Plates were incubated at 37°C for 48 hours. The zone diameters were interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines. The obtained results (mean and standard deviation) were analyzed using SPSS. The data for diffusion methods were analyzed by Mann-Whitney test. Statistical significance was determined at $p \leq 0.05$.

RESULTS:

The isolates used in this study consisted of 20 isolates of *Candida albicans* and 20 isolates of *Candida* non-*albicans* taken from HIV/AIDS patients with oral candidiasis. The investigation on antimycotic activity of rosemary essential oils and nystatin against *Candida* species was done by agar diffusion technique. The zone of inhibition in 100% concentrations of essential oils and nystatin against *Candida albicans* is recorded and tabulated in Table 1.

Table 1: Inhibitory zones (mm±sd) of rosemary essential oils and nystatin in disc-diffusion assay

Species	Concentration	n	Means±SD (mm)	p value
Candida albicans	Nystatin 100%	20	16.50 ±4.7	< 0.001*
	Rosemary 100%	20	2.25 ± 4.1	
Candida non-*albicans*	Nystatin 100%	20	12.45±4.1	< 0.001*
	Rosemary100%	20	1.5±3.1	

*Significant differences (p<0.05), SD: Standard deviation

Table 1 shows the inhibition zone formed by rosemary essential oil is smaller when compared to the inhibition zone formed by nystatin. The mean value of inhibition zone formed by nystatin is 16.5mm which indicates that nystatin is still sensitive against *Candida albicans*. Statistically, the p value obtained from this study is p <0.001, showing a significant difference between inhibition zones formed by nystatin and inhibitory zones formed by rosemary essential oil. This study also showed inhibition zones formed by rosemary essential oil on the growth of *Candida* non-*albicans* were smaller when compared to inhibition zones formed by nystatin. The average value of inhibition zone formed by nystatin is 12.45mm, where this value indicates that nystatin is not sensitive but is included in the intermediate classification against *Candida* non-*albicans*. The statistical value obtained from this study is the value of p<0.001 where there is a significant difference between the inhibition zone formed by nystatin and the inhibition zone formed by rosemary essential oil.

DISCUSSION:

This study was conducted to examine the in vitro antifungal activity of rosemary essential oils and nystatin against *Candida albicans* and *Candida* non-*albicans*. When fungal isolate is given substances with antifungal properties, its growth will be inhibited. In disc diffusion method, inhibition of fungal growth can be observed as inhibitory clear zones found around paper discs on media that have been inoculated with *Candida albicans* and *Candida* non-*albicans*.[9] The formation of inhibition zones around disc paper depends on the presence or absence of active compounds in the extract, while the size of the inhibition zone correspond to the level of activity of the substances in the extract. Hermawan et al. in 2007 stated that interpretation of inhibition zones refers to the general standard issued by the Ministry of Health in 1988 in which microbes are defined to be sensitive to active compounds of a plant if the measurement of power diameter resistance is 12 to 24mm.[10] Disc diffusion method is easier to perform, can be interpreted within 24hours and can be used for diagnostic purposes on daily basis whereas broth microdilution method is more cumbrous and requires more practical skills.[9]

In this study, the mean diameters of the inhibitory zone formed by nystatin and rosemary essential oil against *Candida albicans* are 16.5mm and 2.25mm respectively. *Candida* non-*albicans* were also included in this study which consisted of 9 isolates of *Candida krusei*, 4 isolates of *Candida glabrata*, 2 isolates of *Candida dubliniensis*, 2 isolates of *Candida parapsilosis*, 2 isolates of *Candida tropicalis* and 1 isolate of *Candida lipolytica*. The mean diameters of the inhibitory zone formed by nystatin and rosemary essential oil against *Candida* non-*albicans* are 12.45mm and 1.5mm respectively. The largest inhibitory zone formed by nystatin was observed against two isolates of *Candida dubliniensis* which was 20mm and 18mm respectively. The largest inhibitory zone formed by rosemary essential oils were observed against *Candida krusei* with a diameter of 8mm.

Diameter of inhibitory zone more than 15mm indicates sensitivity to nystatin.[11] This study showed that *Candida albicans* is still sensitive to nystatin. Carvalhinho et al. in 2012 examined the sensitivity of nystatin and essential oils, one of which was rosemary, showing that all 40 isolates of *Candida albicans* species were still sensitive to nystatin and rosemary essential oils with respective inhibitory zone's diameters of more than 15 mm and 12mm.[5] Murtiastutik et al. in 2019 studied the sensitivity of *Candida albicans* and *non-albicans* isolated from HIV/AIDS patients at Dr. Soetomo General Academic Hospital to nystatin by the diffusion disk method, and showed that all isolates was still sensitive to nystatin.[12] This study also showed also that *Candida* non-*albicans* has intermediate sensitivity to nystatin, except *Candida krusei* which showed to be sensitive to nystatin. The result of this study slightly differs from study by Kengne et al. in 2017 on sensitivity of *Candida* species isolated from vulvovaginal candidiasis patients which showed that nystatin was the most sensitive antifungal against *Candida* non-*albicans*.[11] The mechanism of antifungals that causes inhibition of fungal growth is damage to cell membrane. This will disrupt the integrity of cellular components and impair the process of fungal cellular respiration. The end result is insufficient energy to transport active substances so that fungal growth is disrupted.[13,14]

In this study, the formation of inhibitory zone by rosemary essential oil although smaller than those formed by nystatin indicated that rosemary essential oil has antifungal activity against both *Candida albicans* and *Candida* non-*albicans*, also that the antifungal

activity of nystatin was better than rosemary essential oil. This is possibly attributed to nystatin which could diffuse well on agar media and which was the first choice drug for infections by *Candida*. This also could be due other factors including the content of antifungal compound within rosemary essential oil that is not too high, diffusion power, types of fungi and the concentration of the extract.[15]

Periodic antifungal resistance surveillance protocols by susceptibility testing of all *Candida* isolates is essential to provide crucial information about the resistance pattern in a particular region. This also will guide in choosing the empiric or prophylactic drug before the antifungal resistance test result is available.[16–20] In conclusion, plants with medicinal properties are considered to be important source of novel potentially therapeutic compounds. The present study has shown the antimycotic activity of rosemary oil on *Candida* species. However, the antifungal activity of nystatin is stronger when compared to rosemary essential oil as seen from a greater inhibition zone than rosemary essential oil in the diffusion method.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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