ORIGINAL ARTICLE

Antifungal Activity of *Rosmarinus Officinalis* Essential Oil and Nystatin on Store Isolate of C*andida species* from HIV/AIDS Patients with Oral Candidiasis

Bernadya Yogatri Anjuwita Saputri[1], Iskandar Zulkarnain[1], Muhammad Yulianto Listiawan[1], Evy Ervianti[1], Rahmadewi[1], Pepy Dwi Endraswari[2], Damayanti[1], Dwi Murtiastutik[1]

[1]*Department of Dermatology and Venereology, Faculty of Medicine, Universitas Airlangga, Dr. Soetomo General Academic Teaching Hospital, Surabaya, Indonesia*

[2]*Department of Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia*

ABSTRACT

Background: Oral candidiasis is caused by the mycotic activity of *Candida albicans* present in the oral cavity, and it is one of the most common opportunistic infections found in patients with Human Immunodeficiency Virus (HIV)/acquired immune deficiency syndrome (AIDS). The growing resistance and side effects to common antifungal drugs have promoted herbal essential oils as antifungal agents in recent years. In this study, essential oils (EO) of *Rosmarinus officinalis* (*Lamiaceae*) were examined for in vitro antifungal activity against *Candida* species. **Purpose:** To evaluate the antifungal activity of essential oils of *Rosmarinus officinalis* (*Lamiaceae*) and nystatin using the microdilution technique by determining the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of *Candida spesies*. **Methods:** This was an experimental laboratory study with a post-test-only design conducted in Dr. Soetomo General Academic Teaching Hospital, Surabaya. Forty isolates consisted of twenty isolates of *Candida albicans* and twenty isolates of *Candida non-albicans* were collected. The isolates were tested for antifungal activity using the microdilution on 96-well plates. **Result:** There was a significant difference from the results of the MIC concentration of rosemary essential 100% to 6.25% microdilution method between nystatin and rosemary essential oil ($p < 0.05$). **Conclusion:** The antifungal activity of rosemary essential oil was better than nystatin that the lowest MIC value, which was 6.25%, has been obtained the microdilution method. The minimum fungicidal concentration of rosemary essential oil was 25%, while the minimum fungicidal concentration nystatin was higher than 100%.

Keywords: Antifungal activity, *Candida albicans*, *Candida non-albicans*, essential oil, nystatin, *Rosmarinus officinalis.*

Correspondence: Dwi Murtiastutik, Department of Dermatology and Venereology Faculty of Medicine, Universitas Airlangga / Dr. Soetomo General Academic Hospital, Surabaya, Jl. Mayjen Prof. Dr. Moestopo No. 6-8 Surabaya 60131, Indonesia. Phone: (031) 5501609, e-mail: dwimurtiastutik@yahoo.co.id.

BACKGROUND

Candida species are opportunistic microorganisms involved in the most prevalent fungal infection in humans, known as candidiasis. In the oral cavity, among various *Candida* species, *Candida albicans* (*C. albicans)* comprises the most prevalent one in health and disease. It is an opportunistic pathogen as it usually causes infection in individuals with low or suppressed immunity such as HIV/AIDS patient.[1]

Many strategies are known to fight candidal infections, including antifungals with systemic or topical effects, showing a fungistatic or fungicidal action. Antimycotic agents such as azoles, polyenes, and antimetabolites agents (i.e., nystatin, amphotericin B, fluconazole, miconazole, itraconazole, and 5-fluorocytosine) are recognized as the drug of choice for candidiasis treatment.

However, occasional consumption of the effective dose cannot be tolerated due to side effects.[2] Resistance to some of the widely used antifungals has been recently reported in the literature. Therefore, new strategies for fungal infection control are necessary to improve the efficacy of such treatments. Furthermore, recent studies have evidenced antimicrobial compounds extracted from plants in the form of essential oils, crude extracts, and molecules, such as flavonoids and terpenes. Recent studies have also focused on the association between natural products and conventional pharmacological agents in order to achieve better efficacy of treatments.[3]

Essential oils are natural materials extracted from flowers, leaves, peels, barks, roots, seeds, resins of aromatic plants, and are volatile and hydrophobic aromatic substances containing effective compounds in high density. Rosemary (*R. officinalis L.*), belonging to the *Lamiaceae* family, is a pleasant smelling perennial shrub that grows in several regions all over the world. Most of these are used in folk traditions for many purposes.[4] Historically, rosemary has been used as a medicinal agent to treat renal colic and dysmenorrhea. It has also been used to relieve symptoms caused by respiratory disorders and to stimulate the growth of hair. Extracts of rosemary are used in aromatherapy to treat anxiety related conditions and to increase alertness.[5]

The biological activities of *R. officinalis* essential oils doubtless depend on the chemical compositions and at least 13 different rosemary oil chemotypes have been previously identified, based on the relative percentages of pinene, 1,8-cineole, camphor, borneol, verbenone, and bornyl acetate. Natural products that provide effective antifungal activity against resistant microorganisms are a necessary alternative for the control of oral candidiasis. The fungistatic activity of *R. officinalis* essential oil has been reported by several studies.[6] Fontenelle et al. reported that *R. officinalis* essential oil has antifungal activity against *Candida* at low concentrations. However, there were no studies on the anti-adherent activity of this natural product. Therefore, further investigation of the antifungal activity of these products is needed to justify and validate the clinical use of essential oils. The mechanism of action by which essential oils might be effective has not been fully described. However, Hammer et al. suggested that essential oils can interact with lipid structures and cause changes in cell membranes.[5] *Rosmarinus officinalis L.* has several antifungal mechanisms and its essential oil has been shown to inhibit the adhesion of *C. albicans* by changing cellular structure and the permeability of fungal membranes. A study reported that rosemary could even prevent the development of highly resistant fungal biofilms by coating nanoparticles with rosemary essential oil; the bio nano system is produced significantly by inhibiting adherent power and the development of biofilm strains of *Candida*.[7]

Several in vitro studies have tested antifungal activity against *C. albicans*. Research conducted by Hosein Nejati and colleagues mentioned that topical application of rosemary for wound healing had provided anti-inflammatory and antifungal effects as it contains high terpenoids, limonene and 1,8-cineol.[8,9] Other studies have also tested the antifungal activity of rosemary essential oil against *C. albicans* isolates obtained from vaginal smears. The study reported that the Minimum Inhibition Concentration (MIC) of rosemary essential oil was 0.1 mg / ml.[4] Comparison study conducted by Calvanti and colleagues between rosemary essential oil and nystatin against *Candida* species isolates reported that the MIC value of rosemary essential oil was 2.25 mg/ml, which was better compared to the 100,000 IU/ml MIC of nystatin.-

This study evaluated the antifungal activity of rosemary essential oil and compared it with synthetic nystatin against *Candida* species.

METHODS

This was an experimental laboratory study with a post-test-only design conducted in Dr. Soetomo General Hospital, Surabaya. Forty isolates consisted of twenty isolates of *Candida albicans*, and *Candida non-albicans* were obtained from forty HIV/AIDS patients with oral candidiasis. The isolates were tested for antifungal activity using the microdilution method to determine MIC in the culture of 96-well cell plates. The rosemary (*Rosmarinus officinalis* L.) essential oil was a commercial item produced by Young Living USA, claimed to have 100% natural essential oil. The Gas Chromatography analysis showed that the essential oil contained 45.12% *1.8-cineol*, 12.78% *alpha-pinene*, 10.79% *camphor* and 3.50% *borneol*. Nystatin used as a susceptibility test. We used disposable 96-well microtiter plates. Its row represented decreased concentration, where the first row had the highest drug concentration, and the tenth row had the lowest concentration. The microtiter plates were incubated at 37^0C for 24–48 hours. The MIC was determined by observing at concentration the solution in the well. Clear well without deposits indicates that the growth of *Candida* is inhibited. The minimum fungicidal concentration (MFC) was tested by taking 10 µL from the well with the MIC determined by each inoculated well. The MIC endpoint was observed and interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines. The MFC means the lowest concentration without growth that serves as the endpoint for the fungicidal effect. The MIC means the lowest concentration that inhibits at least 80% of microbial growth — initially identified by the visual method. The data for microdilution methods were analyzed using the Mann-Whitney test. The statistical significance was determined at $p \leq 0.05$. This research has been reviewed and approved by the Ethics Committee at Dr. Soetomo General Academic Hospital (1614/KEPK/XI/2019).

RESULT

Microdilution assay showed efficient Essential Oils activity against all tested *Candida* species. This suggests that by using the microdilution method, the MIC of nystatin cannot be determined because even with 100% nystatin concentration, the result still seemed turbid. MIC produced statistically rosemary essential oil through the chi-square test on *Candida albicans* species showed a significant difference in the results of microdilution at a concentration of 100% to 6.25% between nystatin and rosemary essential oil ($p < 0.05$). At a concentration of 3.125%, there was no significant difference ($p > 0.05$) and at a concentration of 1.56% to 0.19%, it could not be analyzed because the results were turbid.

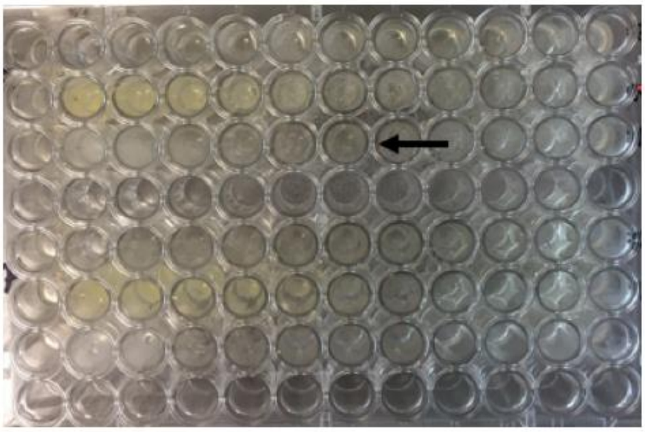

Figure 1. A clear zone of rosemary essential oil and nystatin against *Candida albicans*. The black arrow points to the clear zone boundary produced by rosemary essential oil at a concentration of 3.25%.

Table 1. The Minimum Inhibitory Concentration values of rosemary essential oil and nystatin against *Candida albicans*

Concentration (%)	Fungal growth (clear zone)	Nystatin (n)	Rosemary essential oil (n)	p-value
100	(+)	20 (100%)	0 (0%)	< 0.001
	(-)	0 (0%)	20 (100%)	
50	(+)	20 (100%)	0 (0%)	< 0.001
	(-)	0 (0%)	20 (100%)	
25	(+)	20 (100%)	0 (0%)	< 0.001
	(-)	0 (0%)	20 (100%)	
12.5	(+)	20 (100%)	4 (20%)	< 0.001
	(-)	0 (0%)	16 (80%)	
6.25	(+)	20 (100%)	10 (50%)	0.001
	(-)	0 (0%)	10 (50%)	
3.125	(+)	20 (100%)	19 (95%)	1.000
	(-)	0 (0%)	1 (5%)	
1.56	(+)	20 (100%)	20 (0%)	-
0.78	(+)	20 (100%)	20 (0%)	-
0.38	(+)	20 (100%)	20 (0%)	-
0.19	(+)	20 (100%)	20 (0%)	-

Note: (+) = growth; (-) no growth

This suggests that by using the microdilution method, the MIC of nystatin cannot be determined because even with 100% nystatin concentration, the result still seemed turbid. MIC produced statistically rosemary essential oil through the chi-square test on *Candida albicans* species showed a significant difference in the results of microdilution at a concentration of 100% to 6.25% between nystatin and rosemary essential oil ($p < 0.05$). At a concentration of 3.125%, there was no significant difference ($p > 0.05$) and at a concentration of 1.56% to 0.19%, it could not be analyzed because the results were turbid.

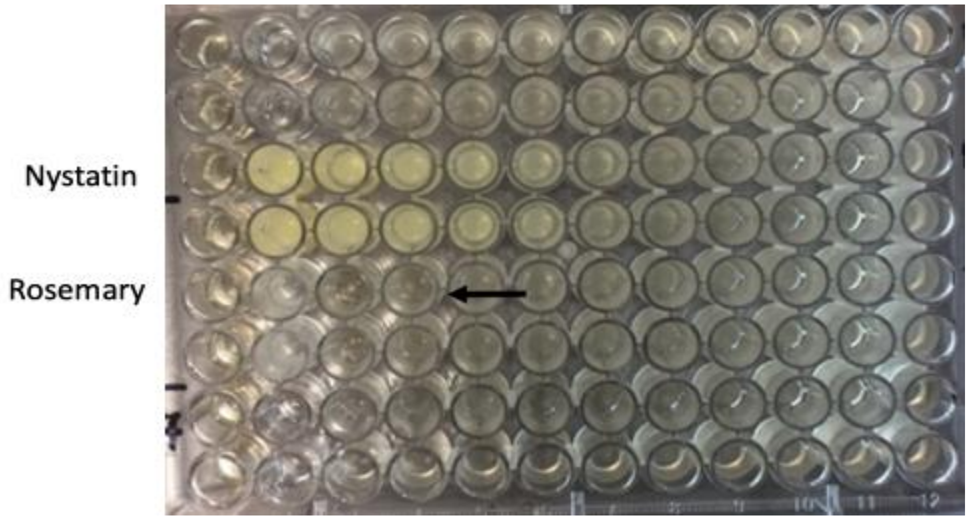

Figure 2. A clear zone of rosemary essential oil and nystatin against *Candida non-albicans*. The black arrow points to the clear zone boundary produced by rosemary essential oil at a 25% concentration.

Table 2. The Minimum Inhibitory Concentration values of rosemary essential oil and nystatin against *Candida non-albicans*

concentration (%)	Fungal growth (clear zone)	Nystatin (n)	Rosemary essential oil (n)	p-value
100	(+)	20 (100%)	0 (0%)	< 0.001
	(-)	0 (0%)	20 (100%)	
50	(+)	20 (100%)	0 (0%)	< 0.001
	(-)	0 (0%)	20 (100%)	
25	(+)	20 (100%)	2 (10%)	< 0.001
	(-)	0 (0%)	18 (90%)	
12.5	(+)	20 (100%)	8 (40%)	< 0.001
	(-)	0 (0%)	12 (60%)	
6.25	(+)	20 (100%)	16 (80%)	0.106
	(-)	0 (0%)	4 (20%)	
3.125	(+)	20 (100%)	19 (95%)	1.000
	(-)	0 (0%)	1 (5%)	
1.56	(+)	20 (100%)	20 (0%)	-
0.78	(+)	20 (100%)	20 (0%)	-
0.38	(+)	20 (100%)	20 (0%)	-
0.19	(+)	20 (100%)	20 (0%)	-

Note: (+) = growth; (-) no growth

The Chi-square test results on *Candida non-albicans* species showed significant differences in the microdilution concentrations of 100% to 12.5% ($p < 0.050$). There was no significant difference ($p > 0.005$) at 6.25% and 3.125% concentrations, and at 1.56% to 0.19% concentrations, the results could not be analyzed as fungal growth was observed. By using the microdilution method, the MIC of nystatin on *Candida non-albicans* could not be determined because even at 100% concentration, turbidity was present.

The MFC of rosemary essential oil was 25%. This means that at a 25% concentration, there was no growth of the *Candida albicans* and *Candida non-albicans*. The MFC value of nystatin was higher than 100% concentration. The Shapiro-Wilk test result showed that the data were not normally distributed ($p < 0.005$). Therefore, we used the Mann Whitney test to analyze the difference in MFC between *Candida albicans* and *Candida non-albicans*. Mann Whitney test results showed no significant difference between the MFC *Candida albicans* and *Candida non-albicans* ($p > 0.005$).

Table 3. Minimum Fungisidal Concentration value of rosemary essential oil

Species	n	*Means* (%)	p-value
Candida albicans	20	25	0.775
Candida non-albicans	20	25	

DISCUSSION

This study examined the antifungal activity of rosemary essential oil and nystatin against *Candida albicans* and *Candida non-albicans* in vitro to obtain the MIC using the liquid microdilution method. The liquid dilution method has several advantages, including a greater homogeneity between the media, test materials, suspensions and fungi as they interact better. Also, the method needs fewer media and test materials.[5]

Rosemary essential oil through the diffusion method before formed a zone of inhibition, but not as strong as the positive control of nystatin. This microdilution method contained a series of dilution of rosemary essential oil and nystatin in microplates containing media and *Candida albicans* and *Candida non-albicans*. The observations were made visually by assessing the presence of sediment or turbidity after the microplates were incubated for 18–24 hours at 37^0 C.

The initial concentration of rosemary essential oil is 100% then in the microplate series dilution of columns 12 to 3 is carried out so that the lowest concentration is in column 12 at a concentration of 0.19 % and the highest concentration in column 3 is 100%. At 100% and 50% concentrations of rosemary essential oil, all *Candida albicans* isolates showed no turbidity, meaning that the fungal growth could be inhibited. The lowest concentration of rosemary essential oil to inhibit the growth of *Candida albicans* was 3.125%, where 1 out of 20 isolates showed inhibited growth.

There was a significant difference between nystatin and rosemary essential oil at 100% to 6.25% concentrations ($p < 0.05$). However, at 3.125% concentration, there was no significant difference as $p > 0.05$. This shows that rosemary essential oil at 6.25% concentration inhibited the growth of the fungus, *Candida albicans.*

This study found that nystatin did not have a clear zone at 100% concentration as fungal growth was still present. The results of this study differ from several studies. Mariz et al. in 2012 compared the antifungal activity between rosemary essential oil and nystatin and reported that the nystatin had MIC as evidenced by the presence of clear zones.[7]

Turbidity produced by nystatin is influenced by active substances that are concentrated and turbid. This made a direct visual observation on the level of clarity not viable. We used analytic nystatin antifungals or serial dilution of nystatin for initial concentrations to determine the MIC value of nystatin and not be affected by the concentration of the nystatin active substance. Nenoff and colleagues investigated the sensitivity of nystatin using the microdilution method in vitro. They used pure nystatin by serial dilution that concentrated, and turbid nystatin became clear.[10]

Overall, the MIC of rosemary essential oil was lower than nystatin. A lower MIC value suggests a higher antifungal activity. The rosemary essential oil had a low MIC, ranging from 50%–6.25%, compared to nystatin. In this microdilution method, no clear zone observed in all isolated treated with nystatin.

The study also evaluated the MIC of rosemary essential oil and nystatin on *Candida non-albicans* species. We found no turbidity in all *Candida albicans* isolates at 100% and 50% concentrations of rosemary essential oil. This means that the fungal growth was inhibited. The lowest concentration of rosemary essential oil to inhibit the growth of *Candida non-albicans* was 3.125%, where 1 out of 20 isolates of *Candida non-albicans* showed inhibited growth. The specific *Candida non-albicans* species was *Candida dubliniensis* species.

There was a significant difference between nystatin and rosemary essential oil at 100% to 12.5% concentrations ($p < 0.05$). However, at 3.125% concentration, there was no significant difference as $p > 0.05$. This suggests that at 6.25% concentration, rosemary essential oil can inhibit the growth of *Candida non-albicans*. However, it was not statistically significant as $p > 0.05$. This was in line with a study conducted by Gauch et al. In 2014, they compared the MIC of rosemary essential oil and nystatin and reported that the MIC produced by rosemary essential oil was quite small by 8%.[11]

In this study, we did not only use the diffusion method in the microdilution. The antifungal activity can indeed be seen by measuring the diameter of inhibitory zones, yet the strength of antifungal activity is rather determined by the MIC. The MIC shows the antifungal ability that can inhibit microbial growth in its minimum concentration, whereas an assessment based on inhibition zones only illustrates the inhibition power of an antifungal agent without specifying the minimum concentration.[12]

Antifungal activity testing using the microdilution method can also measure antimicrobial or antifungal activities that have slow inhibition.[4] This study was similar to one conducted by Agarwal and colleagues in 2010 on the MIC of various of essential oils. The study reported that rosemary essential oil had little effectiveness with MIC of more than 3%. The study also reported small inhibitory zones from using the diffusion method. However, higher activity was observed when the microdilution was implemented as evidenced in lower MIC.[13]

The next research is to determine the minimum fungicidal concentration by taking a colony of 3 concentrations of MIC rosemary essential oil and plant it to agar media. The MFC means the concentration needed for *Candida albicans* and *Candida non-albicans* to fully absent from the agar media[14]. The MFC was determined by taking 10 microliters from the hole that showed clarity, the MIC of rosemary essential oil and nystatin then examined on the agar media. Three concentrations that indicate a clear zone were taken. The inspection was conducted after the 24-hour incubation period. MFC was then determined by the absence of the growth in the agar media.

We found that the MIC of rosemary essential oils on *Candida albicans* was 100% to 6.25%. Statistically, at a concentration of 25%, no growth of *Candida albicans* and *Candida non-albicans* was observed, even after being retested. Khorram et al. reported that the MFC of rosemary essential oil for nystatin was very small, which was 1%. On the contrary, the MFC of rosemary essential oil was still quite large, at 25% concentration. The MFC of nystatin was obtained at more than 100% concentrations because the MIC of 100% nystatin still had turbidity. This means that nystatin at higher than 100% concentrations and microdilution method are needed to form a clear zone.[15]

This study concluded that medicinal plants are believed to be an important source with potential therapeutic effects. Hence, the present study has shown the antimycotic activity of rosemary essential oil on *Candida species*.

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