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Original Article

Antifungal activity of rosemary essential oil against *Candida spp.* isolates from HIV/AIDS patients with oral candidiasis

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Abstract

Background Oral candidiasis (OC) develops in most HIV/AIDS patients. Increasing resistance to antifungals leads to searches for alternative treatment for OC. This study evaluated the *in vitro* activity of rosemary (*Rosmarinus officinalis L.*) essential oil compared to fluconazole against *Candida spp*.

Methods The rosemary essential oil used in this study is a commercial product (Young Living Ltd.). This essential oil and fluconazole were tested by broth microdilution assay against 40 isolates of *Candida spp.* (20 *C. albicans* and 20 *C.* non-*albicans*) obtained from HIV/AIDS patients with OC.

Results Against *C. albicans*, MIC and MFC of rosemary essential oil were 6.25% and 25%, and fluconazole were 100% and >100%. Against *C.* non-*albicans*, MIC and MFC of rosemary essential oil were 12.5% and 25%, and fluconazole were 100% and >100%.

Conclusion Rosemary essential oil was a potential antifungal candidate showing higher antifungal activity compared to fluconazole. It was fungistatic against *C. albicans* but fungicidal against *C.* non-*albicans*, suggesting its potential role in the treatment of OC due to *C.* non-*albicans.* Further studies are needed to determine safety and toxicity of this essential oil prior to human use especially in immunocompromised patients.

Key words
Rosmarinus officinalis; Essential oil; Candida; Antifungal resistance; HIV.

Introduction

Oral candidiasis (OC) is infection of the oral mucosa by *Candida* yeasts. It occurs in up to 95% of patients with human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS).[1–3] It is more prevalent and can disseminate to gastrointestinal tract and other organs along with the progression of HIV infection, where the viral load is increasing and CD4+ T cell count is decreasing.[4,5]

Fluconazole is currently recommended as the first-line treatment for OC in HIV/AIDS patients. It inhibits conversion of lanosterol to ergosterol, a bioregulator which maintains the integrity of fungal cell membrane. However, increasing rate of fluconazole resistance has been reported in many studies due to widespread use of this medication.[6–8] Fluconazole resistance was also attributed to the emergence of *Candida (C.)* non-*albicans* as significant causes of OC in HIV/AIDS patients, commonly in those with lower CD4 count. Some *C.* non-*albicans* species

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possess intrinsic resistance against azole antifungals.[4,5,9] Drug interactions between fluconazole and antiretrovirals or other medications are also of important concern, which may limit its use to treat OC among HIV/AIDS patients.[10] Thus, alternative therapies for OC are being investigated.[6,7]

Rosemary (Rosmarinus officinalis L.), a common shrub for food additive, has been historically used to treat human diseases such as renal colic, asthma, and mental fatigue.[11] Several studies have also reported antifungal activity of rosemary essential oil. However, there were differences in the minimum inhibitory concentration of this essential oil between studies.[7,12,13] This may be partly attributed to variation in plant sources and extraction method resulting in variable concentration of the constituents of the rosemary essential oil.[14] In order to have more reproducible result, a commercially-available rosemary essential oil was chosen for this study. This study evaluated *in vitro* activity of rosemary essential oil compared to fluconazole against *Candida spp*. isolated from patients with HIV infection.

Methods

Strains isolation and identification and inoculum preparation Forty (20 *C. albicans* and 20 *C.* non-*albicans*) out of 149 isolates stored in cryotube from Department of Microbiology at the hospital at which this study was conducted were randomly sampled and used for this study. Species of *C.* non-*albicans* comprised of nine isolates of *C. krusei*, four isolates of *C. glabrata*, two isolates of *C. parapsilosis*, two isolates of *C. tropicalis*, two isolates of *C. dubliniensis*, and one isolate of *C. lipolytica*.

These stored isolates came from oral rinse solutions from 40 HIV/AIDS patients with OC, which were cultured in Chromagar media, and then subsequently cultured in VITEK2 for species identification. All stored isolates were activated by culturing on Sabouraud Dextrose Agar within aerobic conditions and temperature of 37°C for 24 hours. Yeast suspensions in 5 mL 0.9% NaCl solution were prepared in sterile tubes. These suspensions were vortexed for 2 minutes. The turbidity was adjusted to 0.5 McFarland standard which approximately equal to a concentration of 10^6 CFU/mL.

Rosemary (Rosmarinus officinalis L.) essential oil Rosemary *(Rosmarinus officinalis L.)* essential oil used in this study was a commercial product by Young Living, Ltd (Utah, United States). This product contained rosemary leaf extract with concentration of 0.920 g/ml, and the component of this product according to gas chromatography–mass spectroscopy (GC–MS) analysis provided by the manufacturer was listed on **Table 1**.

Table 1 The chemical composition of rosemary essential oil.

No.	*Compounds*	*Retention Time*	*Relative Percentage*
1	1,8-Cineole	8.94	45.12
2	Camphor	11.06	12.78
3	α-Pinene	6.46	10.08
4	β-Caryophyllene	15.17	5.12
5	β-Pinene	7.56	4.91
6	Camphene	6.82	4.17
7	Borneol	11.41	3.50
8	γ-Terpinene	6.04	1.18
9	Terpinolene	9.99	1.31
10	Bornyl acetate	13.27	0.97
11	β –Mircerene	7.97	0.97
12	δ-Cadinene	14.18	0.82
13	β -Cadinene	16.44	0.82
14	α -Caryophyllene	15.60	0.68
1	α-Copapaene	15.55	0.59
16	α-Amorphene	15.87	0.57
17	Isoledene	16.15	0.46
18	Germacrene-D	16.24	0.29
19	Phellandrene	8.22	0.21
20	β-Guainen	15.21	0.14
21	α-Cubebene	14.18	0.11

This pale yellow-colored essential oil was stored in an amber-colored glass vial at room temperature. The undiluted rosemary essential oil was designated as 100% v/v concentration.

Fluconazole Fluconazole used in this study is in the forms of 2 mg/ml solution, which was designated as 100% v/v concentration.

Antifungal assay Serial microdilution using 96-well cell plates culture was performed according to the Clinical and Laboratory Standards Institute (CLSI) 2002, M27-A2.[15] A plate of 96-well microtiter was filled with 100µL of rosemary essential oil or fluconazole solution with ten-level concentration of 100% v/v; 50% v/v; 25% v/v; 12.5% v/v; 6.25% v/v; 3.125% v/v; 1.56% v/v; 0.78% v/v; 0.39% v/v; and 0.19% v/v using multilevel dilution in Saboraoud Dextrose Broth. A 100µL inoculum was subsequently added to each wells, but leaving the first row left as negative control. MIC was the lowest concentration at which the well began to clear without turbidity which indicates inhibition of *Candida* growth. Minimal fungicidal concentration (MFC) was evaluated by obtaining 10 µL aliquots from the wells with MIC, MIC x 2 and MIC x 4 concentrations and subculturing in Saboraoud Dextrose Agar plates incubated at 37°C for 48 hours. MFC was the lowest concentration without growth. The tested substance was defined to be fungistatic if the MFC/MIC ratio ≥ 4, and fungicidal if the MFC/MIC ratio < 4.[16]

Statistical analysis Statistical differences were analyzed by SPSS 17 version. Results from microdilution assay were analyzed by Chi-square test for normality of distribution and Mann-Whitney test for non normally distributed data. Statistical significance was defined at $p<0.05$.

Results

MIC against *Candida albicans* was at 6.25% concentration for rosemary essential oil and at 100% concentration for fluconazole. Significant difference ($p<0.05$) between fluconazole and rosemary essential oil were observed at concentration between 6.25%-50% **(Table 2)**.

Table 2 Minimum inhibitory concentration (MIC) determination of fluconazole and rosemary essential oil against Candida albicans.

Concentration (v/v)	*Turbidity*	*Treatment*		*p-value*
		Fluconazole	*Rosemary essential oil*	
100%	(+)	0 (0%)	0 (0%)	1.000
	(-)	20 (100%)	20 (100%)	
50%	(+)	19 (95%)	0 (0%)	<0.001
	(-)	1 (5%)	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 (100%)	1.000
	(-)	0 (0%)	0 (0%)	
0.78%	(+)	20 (100%)	20 (100%)	1.000
	(-)	0 (0%)	0 (0%)	
0.38%	(+)	20 (100%)	20 (100%)	1.000
	(-)	0 (0%)	0 (0%)	
0.19%	(+)	20 (100%)	20 (100%)	1.000
	(-)	0 (0%)	0 (0%)	

Table 3 Minimum inhibitory concentration (MIC) determination of fluconazole and rosemary essential oil against Candida non-albicans.

Concentration (v/v)	*Turbidity*	*Treatment*		*p-value*
		Fluconazole	*Rosemary essential oil*	
100%	(+)	0 (0%)	0 (0%)	1.000
	(-)	20 (100%)	20 (100%)	
50%	(+)	11 (55%)	0 (0%)	<0.001
	(-)	9 (45%)	20 (100%)	
25%	(+)	14 (70%)	1 (10%)	<0.001
	(-)	6 (30%)	19 (90%)	
12.5%	(+)	17 (85%)	8 (40%)	<0.001
	(-)	3 (15%)	12 (60%)	
6.25%	(+)	19 (95%)	16 (80%)	0.342
	(-)	1 (5%)	4 (20%)	
3.125%	(+)	19 (95%)	19 (95%)	1.000
	(-)	1 (5%)	1 (5%)	
1.56%	(+)	19 (95%)	20 (100%)	1.000
	(-)	1 (5%)	0 (0%)	
0.78%	(+)	19 (95%)	20 (100%)	1.000
	(-)	1 (5%)	0 (0%)	
0.38%	(+)	19 (95%)	20 (100%)	1.000
	(-)	1 (5%)	0 (0%)	
0.19%	(+)	20 (100%)	20 (100%)	1.000
	(-)	0 (0%)	0 (0%)	

Table 4 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of fluconazole and rosemary essential oil against *Candida spp.*

Species	*Fluconazole*			*Rosemary essential oil*		
	MIC	*MFC*	*MFC/MIC ratio*	*MIC*	*MFC*	*MFC/MIC ratio*
C. albicans	100%	>100%	C/E	6.25%	25%	4
C. non-*albicans*	100%	>100%	C/E	12.5%	25%	2

C/E = cannot be evaluated.

MIC against *Candida* non-*albicans* was at 12.5% concentration for rosemary essential oil and at 100% concentration for fluconazole. Significant difference ($p<0.05$) between fluconazole and rosemary essential oil were observed at concentration between 12.5%-50% **(Table 3)**.

MFC analysis showed that 15 of 20 (75%) plates showed growth of *C. albicans* at 100% concentration of fluconazole, indicating that the MFC of fluconazole was above 100% and fluconazole was fungistatic against *C. albicans*. On the other hand, the MFC of rosemary essential oil against *C. albicans* was 25%. The MFC/MIC ratio of rosemary essential oil against *C. albicans* was 4, indicating that rosemary essential oil was fungistatic against *C. albicans* **(Table 4)**.

MFC analysis showed that 19 of 20 (95%) plates showed growth of *C.* non-*albicans* at 100% concentration of fluconazole, indicating that the MFC of fluconazole was above 100% and fluconazole was fungistatic against *C. albicans*. On the other hand, the MFC of rosemary essential oil against *C.* non-*albicans* was 25%. The MFC/MIC ratio of rosemary essential oil against *C. albicans* was 2, indicating that rosemary essential oil was fungicidal against *C.* non-*albicans* **(Table 4)**.

Discussion

Fluconazole is the recommended first line

antifungal for mucocutaneous candidiasis. However, resistance to fluconazole has been increasingly observed, either primary or intrinsic resistance of certain *C.* non-*albicans* species, or extrinsic resistance attributed to widespread use of this antifungal.[17,18] Thus, substances with antifungal properties were investigated to become alternative to fluconazole, including rosemary essential oil.

This study used microdilution assay to determine MIC and MFC value of fluconazole and rosemary essential oil. Microdilution assay is more suitable for rosemary essential oil compared to diffusion assay. Due to the volatility of rosemary essential oil, active ingredients will evaporate before diffusing into the paper disc resulting in the reduction of observed efficacy.[19] This assay was also reported to be more sensitive in determination of MIC and MFC of natural products.[20] Microdilution assay also has other advantages, namely less amount of samples required, qualitative and quantitative nature of the results, and homogeneity of the media which allowed better interaction between the yeast and test substances. However, microdilution assay is more complex and requires precise concentration of the test substances.[21]

The MIC of fluconazole against both *Candida albicans* and non-*albicans* were 100%. On the other hand, the MIC of rosemary essential oil against *Candida albicans* and non-*albicans* were 6.25% and 12.5% respectively in this study. *In vitro* studies on antifungal activity of rosemary essential oil against *Candida spp.* showed varying results. Study by Gauch *et al*. reported that the MIC of rosemary essential oil was 0.5% against *Candida albicans* and 1-2% against *Candida non-albicans*. The use of Tween 80 as surfactant to emulsify rosemary essential oil may account for the different MIC value compared to this study. Surfactant allowed hydrophobic active ingredients such as camphor and α-pinene to become soluble in the culture medium, which may increase antifungal activity of rosemary essential oil by two- to four-fold.[12]

This study showed that fluconazole has higher MIC than rosemary essential oil. Higher MIC corresponded to lower antifungal activity.[22] Fluconazole was also fungistatic against both *C. albicans* and *C.* non-*albicans*, as reported in other study.[19] Rosemary essential oil was fungistatic against *C. albicans*, but was fungicidal against all species of *C.* non-*albicans*. Greater inhibition of biofilm production by rosemary essential oil against *C.* non-*albicans* compared to *C. albicans* may be responsible for this finding.[23] Greater cell walls hidrophobicity of some *C.* non-*albicans* compared to *C. albicans* corresponding to higher susceptibilities to hydrophobic essential oil, may also explain this finding.[24]

Rosemary essential oil contained terpenes such as 1,8-cineole, α-pinene and camphor, which were considered responsible for its antifungal properties.[25–27] These terpenes were also constituents of the commercially-available rosemary essential oil used in this study, as shown in **Table 1**. These lipid soluble terpenes interacted with lipid-containing cell membrane, resulting in increased membrane permeability, electrolyte imbalance and cell death.[25] Chemotypes contained in rosemary essential oil especially cineole also inhibited the formation of biofilms of *Candida spp.*[28] However, the whole composition of rosemary essential oil should be taken into consideration, because synergistic interactions of the components, including the minority constituents, may be important for antifungal properties of this essential oil.[25,28]

Conclusion

In conclusion, this study highlighted rosemary

essential oil as a potential antifungal candidate. Rosemary essential oil showed higher antifungal activity compared to fluconazole, and was fungistatic against *C. albicans* but fungicidal against *C.* non-*albicans*, suggesting its potential role in the treatment of OC by some intrinsically fluconazole-resistant *C.* non-*albicans*. Further studies are needed to determine the active components of the essential oil and their mechanism of action against Candida spp. and to analyze the safety and toxicity of rosemary essential oil prior to human use especially in immunocompromised patients as medicine.

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