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APPLICATION OF DIRECT FLUORESCENCE-BASED LIVE/DEAD STAINING FOR ASSESSMENT OF ANTIFUNGAL ACTIVITY OF COCONUT OIL AGAINST *Candida albicans*

(Aplikasi Pewarnaan Hidup/Mati Berasaskan Pendarfluor Langsung untuk Penilaian Aktiviti Antikulat Minyak Kelapa terhadap *Candida albicans*)

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Abstract

Candida albicans is one of the common causes for oral candidiasis worldwide. The proliferation of antifungal-resistant *C. albicans* has become a major concern. This study was carried out to evaluate activated virgin coconut oil (AVCO) and crude extract of virgin coconut oil (VCO) as new antifungal agents for treatment of oral candidiasis. *C. albicans* viability was determined using LIVE/DEAD bacterial viability kit. *C. albicans* cells were grown in yeast peptone dextrose (YPD) broth overnight. The fungus was treated with AVCO and VCO at the concentration of minimum fungicidal concentration (MFC) of 6.24 mg/mL and incubated for three different time points (1, 2, and 3 h). To evaluate the viability of *C. albicans*, SYTO 9 and propidium iodide (PI) staining were used and the cells were observed using fluorescence microscopy. *C. albicans* treated with AVCO showed more dead cells compared to cells treated with VCO. The data indicate that exposure of *C. albicans* to AVCO was the most inhibitory to its growth ($p < 0.01$).

Keywords: LIVE/DEAD staining, `antifungal activity, *Candida albicans*, virgin coconut oil

Abstrak

Candida albicans ialah salah satu penyebab kepada kandidiasis mulut di seluruh dunia. Malahan, pembiakan *C. albicans* rintang antikulat semakin mendapat perhatian. Kajian ini dijalankan untuk menilai kesan-kesan minyak kelapa dara teraktif (AVCO) dan ekstrak minyak kelapa dara (VCO) dalam pencarian agen antikulat yang baharu untuk rawatan kandidiasis mulut. Kebolehhidupan sel *C. albicans* ditentukan dengan penggunaan kit daya kebolehhidupan bakteria HIDUP/MATI. *C. albicans* dibiakkan semalaman di dalam kultur cecair yis pepton dekstrosa (YDP). Kulat tersebut dirawat dengan minyak kelapa teraktif dan ekstrak minyak kelapa dara pada kepekatan fungisidal minimum (MFC) sebanyak 6.24 mg/mL dan dieramkan pada tiga masa yang berbeza (1, 2, dan 3 jam). Untuk menilai kebolehhidupan *C. albicans*, pewarnaan SYTO 9 dan propidium iodida digunakan dan sel dilihat dengan menggunakan mikroskop pendarfluor. *C. albicans* yang telah dirawat dengan minyak kelapa dara teraktif menunjukkan lebih banyak sel yang mati berbanding sel yang dirawat dengan ekstrak minyak kelapa dara. Data menunjukkan minyak kelapa dara teraktif adalah paling berkesan untuk menghalang pembiakan *C. albicans* ($p < 0.01$).

Kata kunci: pewarnaan HIDUP/MATI, aktiviti rintang kulat, *C. albicans*, minyak kelapa dara

Introduction

Candida albicans is an opportunistic fungal species in the oral cavity where it can become pathogenic under several circumstances like weakened immune system or damaged mucosal barriers, leading to mucous membrane infections [1–7]. Occasionally, candida infection may cause superficial infections such as oral candidiasis which can disseminate to other organs, causing systemic infections that can be fatal [8, 9]. Generally, oral candidiasis occurs in immunocompromised people such as HIV-positive patients, transplant recipients, patients with lymphoma, drug abusers, and pregnant women [10–11].

Despite having numerous types of antifungal agents to treat oral candidiasis, current antifungal treatments are ineffective due to increased antifungal resistance, which contributes to fungal growth causing oral candidiasis [12, 13]. *C. albicans* has been shown to develop resistance against amphotericin B and fluconazole [14–16]. The low success rate in treating candidiasis also is due to the biofilm growth not only on host tissues, but also on dental devices and prosthesis [16, 17]. Therefore, an alternative antifungal therapy is necessary to combat candidiasis. The major constituents of activated virgin coconut oil (AVCO) are medium-chain fatty acids (C_6 to C_{14}), which are widely known for their antifungal and antibacterial activities.

Over the years, virgin coconut oil (VCO) has been stated to have a broad spectrum of antimicrobial/antifungal activity against Gram-positive bacteria, e.g. *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Listeria monocytogenes*; Gram-negative bacteria, e.g. *Vibrio cholerae* and *Escherichia coli*; and fungi, e.g. *Candida krusei*, *Pityrosporum ovale*, and *Candida albicans* [18]. Recent reports have shown that AVCO has greater antimicrobial activity against mycobacterium and some viruses [19, 20]. The major constituents of activated virgin coconut oil are medium-chain fatty acids (C_6 to C_{14}), which are widely known for their antifungal and bactericidal activities [21–22]. Considering the potential antifungal effect of AVCO, this study investigated the viability of *C. albicans* against AVCO with the aid of live/dead viability kit.

Materials and Methods

Preparation of VCO and source of AVCO

Coconuts were bought from Taman Pertanian Agroteknologi MARDI, Cherating, Kuantan. The endosperm of the coconut was made into a viscous slurry and was squeezed through a cheesecloth until all the creamy milk was obtained. The creamy coconut milk was left at room temperature for at least 48 h. The coconut milk formed three layers after fermentation. The second layer was the VCO. It was gently scooped out, centrifuged to remove coconut residue, and kept at room temperature in a dark bottle. AVCO (100% purity) was bought from MARDI (UPM, Selangor, Malaysia).

Growth and maintenance of *Candida albicans*

Candida albicans (strain MYA 4901) was obtained from American Type Culture Collection (ATCC), subculture from freezer stocks at 4 °C onto yeast extract peptone dextrose (YPD) agar plates and incubated at 36 °C overnight to generate *C. albicans* yeast used for the experiments.

Culture conditions and preparation of fungal suspensions

Preparations for fungal suspensions were done according to the LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Invitrogen, Carlsbad, CA, USA) manual that has been previously used for *C. albicans* by Vasconceles et al. [7]. Briefly, 30 mL culture of *C. albicans* was grown from a single colony in nutrient YPD broth and incubated at 36 ℃ for 12 hours. Reading of optical density of the broth culture was taken using a spectrophotometer at 600 nm by a microplate reader and was adjusted to 1×10^7 cell/mL (OD_{600} ~0.38) [13]. The culture then was concentrated by centrifugation at 10,000 × *g* for 15 minutes. The supernatant was removed and the pellet was suspended in 2 mL of 0.85% NaCl. Each mL of this suspension was added to tubes containing 10 mL of 0.85% NaCl, 10 mL of 70% ethanol, 10 mL of AVCO, and 10 mL of VCO. All samples were incubated at room temperature for 1 hour and were pelleted by centrifugation at 10,000 × *g* for 15 minutes. After centrifugation, the supernatant was discarded and all pellets were resuspended in 10 mL of 0.85% NaCl. The optical density of a 200 µL aliquot of the fungal suspensions was determined in 96-well plates at 600 nm.

Staining fungi in suspension with SYTO 9 and propidium iodide (PI)

Cell viability was assessed using SYTO 9 and propidium iodide (PI) included in the LIVE/DEAD BacLight Bacterial Viability Kit, as per manufacturer's directions. Equal volumes of SYTO 9 and PI in a microfuge tube were combined and mixed thoroughly. Three microlitres of the dye was added for each mL of the suspension. The suspension was mixed thoroughly and incubated at room temperature in the dark for 15 minutes. Then, 15 µL of the stained suspension was trapped between a slide and an 18-mm^2 coverslip. The suspension was observed under a fluorescence microscope equipped with filter sets FITC (excitation wavelength: 467–498 nm, emission wavelength: 513–556 nm) and TRITC (excitation wavelength: 540–580 nm, emission filter: 600–660 nm). The viability of yeast cells was visualized by using fluorescence microscopy (Nikon Eclipse T*i*, Japan) at a resolution of 1280 × 960. Cells with intact membranes appeared green, whereas cells with compromised membranes appeared red.

Calculations for live and dead cells

To determine the number of cells, five sides of the slide covered with cover slid were chosen. The stained cells were counted manually for live and dead cells separately using ImageJ software (https://imagej.nih.gov/ij/). The following formula was used to calculate the percentage of dead cells.

$$\frac{Total\ number\ of\ dead\ cells}{Total\ number\ of\ cells} \times 100\% \quad (1)$$

Statistical analysis

Data were analysed statistically by one-way ANOVA and Tukey's test to evaluate the mean difference with standard deviation (mean ± standard deviation) between the control and the test groups and the level of significance was compared at $p < 0.01$. All experimental results were triplicated.

Results and Discussion

The results on cell quantification of *C. albicans* treated with AVCO and VCO for three different periods of exposure (1, 2, and 3 hours) by fluorescence microscopy are shown in Table 1. The average value of the three exposure times of the viable cells of the negative control (saline) and crude extract of VCO was similar with no significant statistical difference between them ($p < 0.01$). In contrast, the result assays showed that AVCO efficiently reduced cell viability like the positive control ($p < 0.01$) after 2 hours incubation period. No significant changes between three different incubation periods were seen after treating *C. albicans* with AVCO or VCO (Table 1).

This research utilised the minimal fungicidal concentration of AVCO and VCO to assess the viability of *C. albicans*. Generally, antifungal agents target various part of fungal cells such as cell walls, nuclei, membranes, and microtubules. An increase in plasma membrane permeability is associated with the disturbance of intracellular electrochemical gradient. The viability test for fungal cells by using the combination of SYTO 9 and PI is suitable for the present study because they stain tissues regardless of cell morphology [6]. The change in membrane integrity of *C. albicans* was evaluated using PI where the dye penetrates and fluoresces the damaged membrane of the cell.

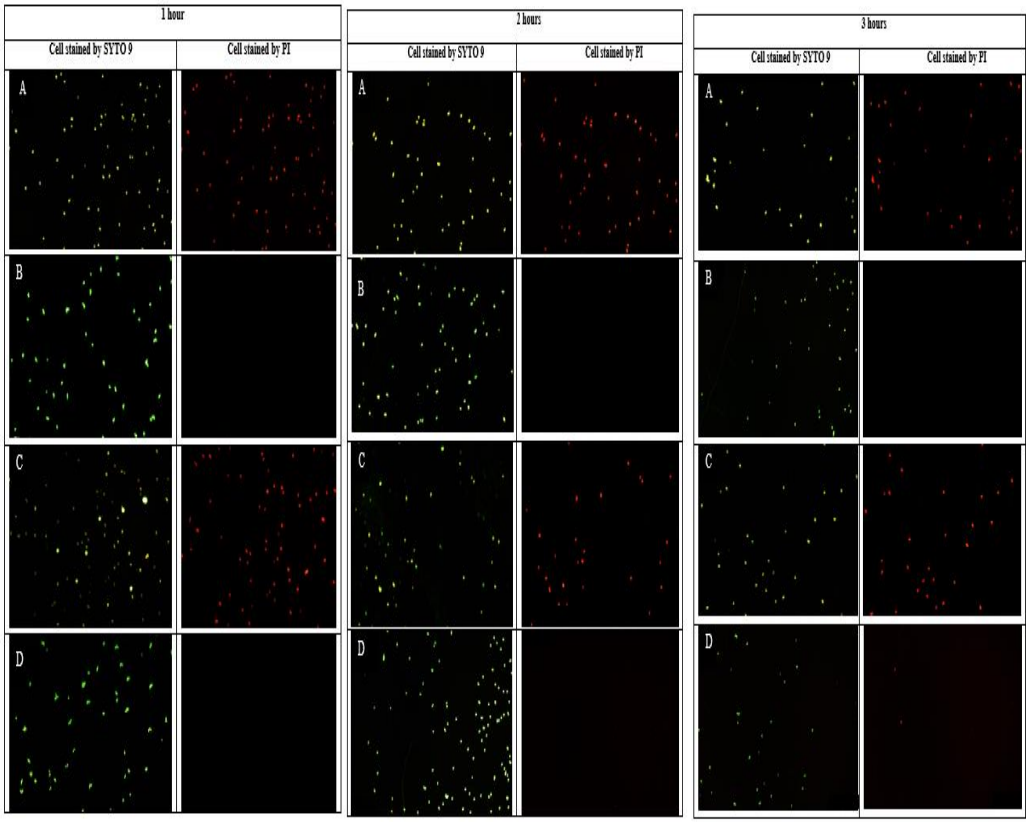
Our finding is consistent with a recent report that showed AVCO having a significant antifungal activity with an MFC value of 6.24 mg/mL and a *p*-value of less than 0.01 compared to the crude extract of VCO, which did not show any major activity against *C. albicans* [24]. The mechanism of action of AVCO is related to the solubilisation of the cell membrane that causes the cells to be lysed, which is indicated by the permeation of PI on the cell membrane after exposure to AVCO. SYTO 9 penetrates intact membranes of viable cells and sometimes stains non-viable cells and appears green/yellow. Meanwhile, PI only stains dead cells and appears red.

Table 1. Number of dead cells and percentage distribution of dead cells after staining

Sample	Exposure Time (h)	Non-Viable Cells (%)
AVCO	1	91.00 ± 8.06^{a}
	2	92.42 ± 0.57^{a}
	3	97.68 ± 0.62^{a}
VCO	1	0.46 ± 0.12^{b}
	2	1.67 ± 1.48^{b}
	3	1.90 ± 0.99^{b}
PC	1	100.00 ± 0.00^{a}
	2	99.59 ± 0.42^{a}
	3	99.70 ± 0.53^{a}
NC	1	0.00 ± 0.00^{b}
	2	1.49 ± 0.30^{b}
	3	0.00 ± 0.00^{b}

Note. Mean values ± SD are shown. Positive control was ethanol (PC), negative control was saline (NC), VCO is virgin coconut oil, AVCO is activated virgin coconut oil. Statistical significance of data was evaluated using one-way ANOVA. The level of statistical significance was set at $p < 0.01$. Mean ± SD values in the same exposure time (h) group with no superscript in common differ significantly ($p < 0.01$). The abbreviations next to the mean ± SD values indicate from which groups the relevant group differs significantly (a = $p < 0.01$), (b = $p > 0.01$).

The fungicidal activity of AVCO was comparable to the positive control (70% ethanol) at 2 and 3 hours of incubation period (Figure 1). AVCO had higher antifungal activity against *C. albicans* compared to VCO (Table 1 and Figure 1). LIVE/DEAD staining executed on fungal suspensions from control, VCO, or AVCO treatments exhibited a change in membrane permeability as measured by PI. AVCO inhibited the growth of *C. albicans* and caused all fungal cells to transform immediately from green (live) to red (dead). The uniqueness of AVCO is due to the presence of significant amount of medium chain fatty acids (MCFA) through enzymatic hydrolysis process which can act as antifungal agent. Lauric acid is the major constituent of MCFA of AVCO [21–24]. Our findings suggest that AVCO exerts its potent fungicidal activity by inhibiting cell wall integrity of *C. albicans*. Meanwhile, fungal growth was not inhibited by the crude extract of VCO. AVCO significantly disrupted the cell membrane with its MCFA.



Note. Scale bars represent 100 µm. Viable and non-viable fungi were stained with SYTO 9 and appeared green. Non-viable fungi were stained with propidium iodide and appeared red.

Figure 1. *C. albicans* were exposed to (A) ethanol, (B) normal saline, (C) AVCO, and (D) VCO for 1, 2, and 3 hours incubation

Conclusion

AVCO has a great potential to be consumed as a new antifungal treatment against oral candidiasis. Such measure may serve to decrease the colonisation of the fungus in patients and reduce the cases of hospital-acquired oral candidiasis. A limited analysis has been shown in this *in vitro* study. Thus, further investigation of AVCO and its compositions should be undertaken to study their impact on *C. albicans*.

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References

1. Chanda, W., Joseph, T. P., Wang, W., Padhiar, A. A. and Zhong, M. (2017). The potential management of oral candidiasis using anti-biofilm therapies. *Medical Hypotheses*, 106: 15 - 18.
2. Gow, N. A., and Hube, B. (2012). Importance of the *Candida albicans* cell wall during commensalism and infection. *Current Opinion in Microbiology*, 15 (4): 406 - 412.
3. Katiraee, F., Khalaj, V., Khosravi, A., and Hajiabdolbaghi, M. (2014). Sequences type analysis of *candida albicans* isolates from iranian human immunodeficiency virus infected patients with oral candidiasis. *Acta Medica Iranica*, 52(3): 187 - 191.
4. Millsop, J.W. and Fazel, N. (2016). Oral candidiasis. *Clinics in Dermatology*, 34(4): 487 - 494.

5. Perlroth, J., Choi, B. and Spellberg, B. (2007). Nosocomial fungal infections: Epidemiology, diagnosis, and treatment. *Medical Mycology*, 45(4): 321 – 346.
6. Silva, S., Henriques, M., Hayes, A., Oliveira, R., Azeredo, J., and Williams, D. W. (2011). *Candida glabrata* and *Candida albicans* co-infection of an *in vitro* oral epithelium. *Journal of Oral Pathology & Medicine*, 40(5): 421 - 427.
7. Vasconceles, L. C., Sampaio, F. C., dos Reis Albuquerque, A. J. and Souza Vasconcelos, L. C. (2014). Cell viability of *candida albicans* against the antifungal activity of thymol. *Brazilian Dental Journal*, 25 (4): 227 - 281.
8. Kong, E. F., Kucarikova, S., Dijck, P. V., Peters, B.M., Shirtliff, M. E. and Jabra-Rizk, M. A. (2015). Clinical implications of oral candidiasis: host tissue damage and disseminated bacterial disease. *Infection and Immunity*, 83(2): 604 - 613.
9. Pfaller, M. A. and Diakema, D. J. (2007). Epidemiology of invasive candidiasis: A persistent public health problem. *Clinical Microbiology Reviews*, 20(1): 133 - 163.
10. Fidel, P. L. (2011). *Candida*-host interactions in HIV disease: Implications for oropharyngeal candidiasis. *Advances in Dental Research*, 23(1): 45 - 49.
11. Lyu, X., Zhao, C., Yan, Z., and Hua, H. (2016). Efficacy of nystatin for the treatment of oral candidiasis: A systematic review and meta-analysis. *Drug Design, Development and Therapy*, 10: 1161 - 1171.
12. Dhanasekaran, D., Vinothini, K., Latha, S., Thajuddin, N. and Paneerselyam, A. (2014). Human dental biofilm: screening, characterization, *in vitro* biofilm formation and antifungal resistance of *Candida spp. The Saudi Journal for Dental Research*, 5(1): 55 - 70.
13. Mosci, P., Gabrielli, E., Luciano, E., Perito, S., Cassone, A., Pericolini, E., and Vecchiarelli, A. (2014). Involvement of IL-17A in preventing the development of deep-seated candidiasis from oropharyngeal infection. *Microbes and Infection*, 16(8): 678 - 689.
14. He, M., Du, M., Fan, M. and Bian, Z. (2007). *In vitro* activity of eugenol against *Candida albicans* biofilms. *Mycopathologia*, 163: 137 - 143.
15. Ilyas, M. and Sharma, A. (2017). Cutaneous fungal infections in solid organ transplant recipients. *Transplantation Reviews*, 31(3): 158 - 165.
16. Jin, Y., Zhang, T., Samaranayake, Y. H., Fang, H. H. P., Yip, H. K., and Samaranayake, L. P. (2005). The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in *Candida albicans* biofilms. *Mycopathologia*, 159(3): 353 - 360.
17. Rasteiro, V. M. C., Costa, A. C. B. P., Araujo, C. F., Barros, P. P., Rossoni, R. D., Anbinder, A. L., Jorge, A. O. C. and Junquiera, J. C. (2014). Essential oil of *Melaleuca alternifolia* for the treatment of oral candidiasis induced in an immunosuppressed mouse model. *BMC Complementary and Alternative Medicine*, 14: 489 - 499.
18. Schelenz, S., Abdallah, S., Gray, G., Stubbings, H., Gow, I., Baker, P. and Hunter P. R. (2011). Epidemiology of oral yeast colonization and infection in patients with haematological malignancies, head neck and solid tumors. *Journal of Oral Pathology Medicine*, 40: 83 - 89.
19. Thompson III, G. R., Patel, P. K., Kirkpatrick, W. R., Westbrook, S. D., Berg, D., Erlandsen, J., Redding, S. W. and Patterson, T. F. (2010). Oropharyngeal Candidiasis in the Era of Antiretroviral Therapy. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, 109 (4): 488 - 495.
20. Williams D. W., Kuriyama T., Silva, S., Malic, S. and Lewis, M. A. O. (2011). *Candida* biofilms and Oral candidosis: Treatment and prevention. *Periodontology 2000*, 55: 250-265.
21. Lima, E. B. C., Sousa, C. N. S., Meneses, L. N., Ximenes, N. C., Júnior, S., Vasconcelos, G. S., Lima, N. B., Patocinio, M. C., Macedo., D. and Vasconcelos, S. M. M. (2015). *Cocos nucifera* (*L.*) (*Arecaceae*): A phytochemical and pharmacological review. *Brazilian Journal of Medical and Biological Research*, 48(11): 953 - 964.
22. Long K. Modified coconut oils with broad antimicrobial spectrum. Malaysian Granted Patent MY140578-A. 2009. https://patents.google.com/patent/WO2007067028A1. [Access online 26 November 2017].
23. Soo-Peng, K., Harun, D., Mat-Amin, M. and Long, K. (2016). Enhanced virgin coconut oil (EVCO) as natural postmilking teat germicide to control environmental mastitis pathogens. *International Journal of Biotechnology for Wellness Industries*, 5: 128 - 134.
24. Haron, U. A., Abllah, Z. and Nasir, N. A. M. M. (2018). The comparative antimicrobial effect of activated virgin coconut oil (AVCO) and virgin coconut oil (VCO) against dental caries-related pathogens. *Advances in Health Science Research*, 8: 312 - 317.