Mechanism of Antifungal Activity of Virgin Coconut Oil on Cell Membrane of Candida Albicans

Nor Izzah Mukhtar[1], Zurainie Abllah[2]*, Azrul Naim Mohamad[1],
Intan Azura Shahdan[3], Ummi Aqilah Haron[1]

1. Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia.
2. Department of Paediatric Dentistry and Dental Public Health, Kulliyyah of Dentistry, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia.
3. Department of Biomedical Sciences, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia.

Abstract

Oral candidiasis is typically caused by diploid yeast, Candida albicans through the colonization of the yeast which may cause oral tissue damage and tissue invasion. The limitation of antifungal drugs such as problem with resistance development has led researchers to investigate the potential use of natural product as new target for antifungal drug development. The aim of this study is to identify the components cytoplasmic release and the morphology of C. albicans in the presence of activated virgin coconut oil (AVCO) and the crude extract of virgin coconut oil (VCO). The fungal suspensions were treated with AVCO and VCO while nystatin and 1% Tween were used as a positive and a negative control respectively. Treatment with AVCO has caused disruption of the cell membrane of C. albicans which leads to the leakage of the cytoplasmic contents while treatment with VCO which did not show any changes on the cell membrane of C. albicans after 4 h of exposure. Our results suggest the potential use of AVCO as a novel antifungal agent to control oral candidiasis and it is likely to become an alternative for conventional drugs available in the market.

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Introduction

Oral candidiasis is a common opportunistic oral infection affecting oral mucosa caused by colonization of Candida species, which the prevalent being *Candida albicans*.[1,2] In healthy people, about 30% to 50% of *C. albicans* lives as normal oral microflora or commensal fungi.[3] The overgrowth of a hyphal form of *C. albicans* may be harmful in several conditions such as immunocompromised people due to mucosal or cutaneous barrier damage thus causes the development of lesions in the oral cavity or oral candidiasis.[4] Current treatments of *C. albicans* infections are still debatable due to several setbacks such as toxicity issues of antifungal drugs, the availability of effective drugs, multidrug resistant and high cost of antifungal agents.[5] Therefore, the development of effective antifungal treatment from natural remedies are necessary to reduce the current problems caused by conventional antifungal therapies.

***Corresponding author:**
Zurainie Abllah
Department of Paediatric Dentistry, Orthodontic and Dental Public Health, Kulliyyah of Dentistry, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia.
E-mail: drzura@iium.edu.my

Virgin coconut oil (VCO) is obtained from the kernel of the coconut fruit and processed at low temperature. VCO is composed of medium-chain fatty acids predominantly that is not deposited in adipose tissue and easily oxidized.[6,7] Medium-chain fatty acids (MCFA) of VCO has been reported to have antifungal, antiviral and antibacterial activities from previous studies as it is the action of lauric acid in the MCFA on bacteria, fungi and viruses.[8-10] The presence of higher level of MCFA (capric, caprylic and lauric acid) in enhancing the activity of antifungal/antimicrobial properties of hydrolyzed VCO.[11,12] Hydrolysis of VCO can be done by using biocatalyst, for example, lipase enzyme. Previous study reported that lipolysis of VCO inhibited the growth of *Clostridium difficile* significantly whereas nonlipolyzed VCO showed no growth inhibition of *C. difficile*.[13] A recent

study showed that AVCO has high sensitivity against *L. casei*, *C. albicans* and *S. mutans* and killed the organisms rapidly at 8-hour point of time.[14]

The challenge of the antifungal resistant of *C. albicans* on current treatments emphasizes the needs for alternative therapies that can reduce the cases of oral candidiasis especially in immunosuppressed patients. Thus, this study is carried out to identify the antifungal activity of AVCO which can act as a promising antifungal treatment in the future. In the present study, in vitro techniques have been employed with the purpose to investigate the leakage of the cytoplasmic components and ultrastructural changes of *C. albicans* cells after being treated with activated virgin coconut oil (AVCO) and the crude extract of VCO.

Materials and methods

Preparation of the VCO extract

The fresh coconut fruit was bought from Taman Pertanian Malaysia Agriculture Research and Development Institute (MARDI) and the solid endosperm of coconut was de-husked and grated was made into a viscous slurry. The slurry was squeezed through a cheese cloth to get coconut milk. The coconut milk was left overnight or 24 hours in room temperature. Three layers were formed from the coconut milk. As the layers of oil and water became separated, the upper oil layer was discarded and VCO was seen on the second layer. Next, the oil was scooped out slowly with a spatula and centrifuged to remove the coconut residue. The separatory funnel was used to isolate the remains of coconut residue to obtain the crude extract of VCO.[15] Meanwhile, AVCO product was obtained from the collaborator, Dr. Kamariah Long, produced by MARDI (UPM, Selangor, Malaysia).

AVCO and VCO Stock Preparations

An initial stocks solution of AVCO and VCO were prepared by dissolving 6.24 mg of oil with 1% Tween 80 and were stored at 4 ℃ maximum at 12 h prior to the test. The concentration of 6.24 mg/ml was used for both AVCO and the crude extract of VCO since this concentration is the minimum fungicidal concentration (MFC) value of AVCO to inhibit the growth of *C. albicans*.[14] The stock of AVCO and VCO were used for the release of cytoplasmic assay and transmission electron microscopy (TEM) analysis.

Growth and culture conditions of *C. albicans*

C. albicans (Robin) Berkhout (ATCC MYA 4901) was bought from American Type Culture Collection (ATCC), (Manassus, VA, USA) which was used in this study. *C. albicans* was sub cultured on yeast extract peptone dextrose (YPD) agar (Invitrogen), USA plates for 24 hours at 36 ℃. After incubation, a single colony of *C. albicans* was transferred to 10 ml of YPD broth culture media at 36 ℃ for 12 hours. The optical density of the fungal suspension was standardized to 1×10^7 CFU/ml [~0.38 optical density (OD)].[16]

Release of cytoplasmic materials at 260 nm and 280 nm assay

The leakage of cytoplasmic contents was measured using a 96-well plate by a microplate reader (TECAN INFINITE 200 PRO, Mannedorf, Switzerland). *C. albicans* with an inoculum concentration of 1×10^7 CFU/ml was centrifuged at 10,000 × g for 15 min and was washed three times with PBS buffer at pH 7.4. The supernatant was discarded and the pellet was suspended in 10 ml of nystatin as a positive control and 1% Tween 80 as a negative control at 6.24 mg/ml respectively. AVCO and the crude extract of VCO at 6.24 mg/ml was added to the pellet of *C. albicans* cells respectively and incubated for different time periods (1, 2, 3 and 4 h) at 37 ℃. After incubation, the suspension cell was centrifuged at 10,000 × g for 15 min, and the absorbance reading at 260 nm for DNA and 280 nm for protein from the supernatant was recorded. The experiment was done in triplicates.[17]

Proteins contained in the supernatant

The total level of soluble protein contained in the fungal suspension treated with AVCO, VCO and controls after incubation at a different time point (1, 2, 3 and 4 h) was measured by Bradford protein assay (Bio-Rad) using bovine serum albumin (BSA) as the standard. Spectrophotometric measurements were performed at 595 nm using 96-well plate by a microplate reader (TECAN INFINITE 200 PRO). The supernatants of the untreated and treated cell were added with the 1x Bradford dye reagent in the 96- wells. All samples were incubated for 5 min and the readings of optical density for each sample were taken. The experiments were carried out in triplicates.[18]

Transmission electron microscopy (TEM)

Viable cells of *C. albicans* (MYA 4901) were harvested from 10 ml of fungal culture. The cell number was adjusted to OD_{600} 0.38 ~1×10^{7} cell/ml.[16] The fungal suspension was centrifuged at low speed to obtain the pellet of candida cells. The pellet was washed with PBS buffer (1x, pH 7.4). Different aliquots of cell suspensions were treated with AVCO and VCO at a concentration of 6.24 mg/ml respectively and incubated at the room temperature. The fungal suspension treated with 6.24 mg/ml of nystatin was used as a positive control, while fungal suspension treated with 1% Tween 80 was served as a negative control. *Candida albicans* cells were treated with AVCO, VCO, nystatin and 1% Tween 80 were centrifuged at 10,000 × g for 15 minutes. The supernatant of the samples was discarded and the pellet was resuspended in 5 ml of *McDowell-Trump* fixative prepared in I ml of 0.1 M phosphate buffer (pH 7.2) for at least 2 hours. The resuspended sample was centrifuged, and the supernatant was discarded. Next, the pellet was resuspended in 0.1 M phosphate buffer. The sample was then rewashed again, centrifuged, and the supernatant was discarded while the pellet was resuspended in 1 ml of 0.1 M phosphate buffer. For post-fixation, the pellet was then centrifuged, and the pellet was resuspended in 1% osmium tetraoxide (OsO_4) prepared in the 0.1 M phosphate buffer for 2 hours. Next, the pellet was centrifuged, the supernatant was discarded, and the pellet was resuspended in 1 ml of distilled water to discard the OsO_4. The sample was centrifuged again, and the pellet was resuspended in 1 ml of distilled water, and the supernatant was removed. The resuspended sample was centrifuged, the supernatant was discarded, and the pellet of fixed cells was placed in a tube and was further incubated at 45℃ about 30 minutes in a water bath. The YPD agar was dissolved in boiling distilled water to prepare a two percent of the solution of agar. The solution was poured into a test tube, while it is still molten and placed in the water bath at 45℃. At this temperature, the agar remains liquid. After the temperature of both the agar and the pellet have equilibrated to 45 ℃, a small drop of the agar was transferred to the tube containing the pellet of cells.

The pellet was then stirred up to break into small blocks and was suspended in the YPD agar. The YPD agar with the suspended pellet blocks was poured on to a glass microscope slide and left to sit for 2 min. The agar containing the cells was cut into small cubes, about 1 mm^3 with a razor-sharp blade and was placed in a vial containing 50% ethanol. These cubes were processed in the same way as pieces of a cohesive pellet or tissue with 50% ethanol for 15 min, 75% ethanol for 15 min, 95% ethanol for 15 min twice, 100% ethanol for 30 minutes twice and 100% acetone for 10 minutes twice for dehydration process. The mixture of acetone was used to infiltrate the Spurr's resin mix in a rotator for 30 minutes. Then the Spurr's mix resin was infiltrated overnight in the rotator. The new change of mix resin again was infiltrated for another 5 hours in the rotator. The mix was embedded and cured at 60℃ for 12-48 hours.[19] Ultra-thin sections of 90 nm of *C. albicans* treated with AVCO, VCO, nystatin and 1% Tween 80 were cut with glass knives and were stained with UAR (Uranyl Acetate Replacement) stain for 20 min at room temperature and washed thrice with distilled water. All samples were subjected to the observation of cellular morphology of *C. albicans* under transmission electron microscopy (LIBRA 120-ZEISS, Singapore).[20]

Statistical analysis

One-way ANOVA and Post-hoc Tukey analysis were performed for statistical analysis. The *p*-value < 0.01 was considered statistically significant.

Results

The mechanism of action of AVCO was studied through the quantification of leakage of cytoplasmic material (protein and DNA) of *C. albicans* after treatment. The concentration of AVCO used in this study is 6.24 mg/ml which is the (MFC) of AVCO against *C. albicans.*[14] Maintenance of membrane potential of *C. albicans* is vital to show its virulence on the host niche. From Figure 1, it could be seen that the amount of protein and DNA leakage of *C. albicans* treated with AVCO is significantly increased at 1, 2 and 3 h incubation period. Treatment with AVCO has higher leakage rate than treatment with VCO where it demonstrated the enhanced membrane permeability of *C. albicans* to the activated VCO.

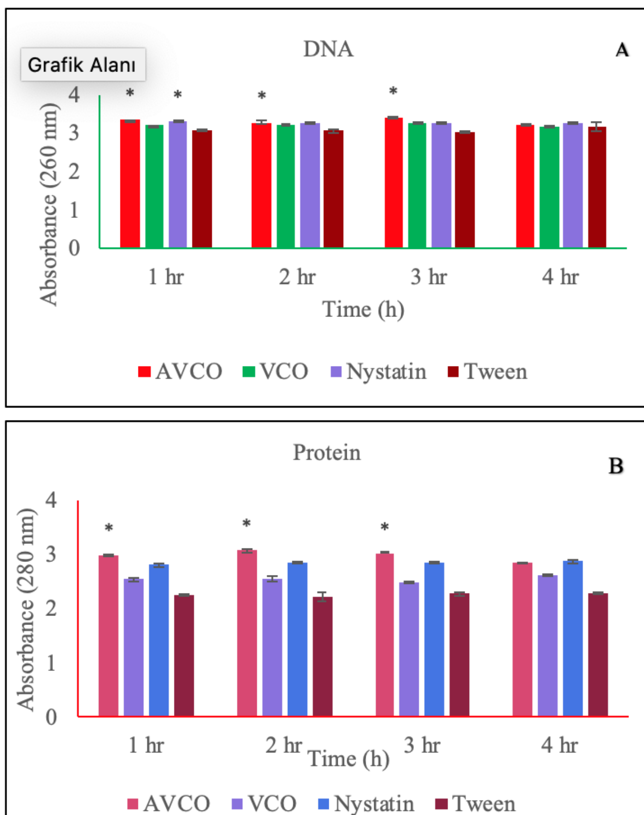

Figure 1. *C. albicans* cells treated with VCO, AVCO and nystatin. Supernatant of the treated cells shows the presence of a) protein at 260 nm and b) DNA at 280 nm due at different time points (1, 2, 3 and 4 h) at room temperature. Values represent mean ± SD of triplicate of experiments. One-way ANOVA and Tukey's test were performed (*$p < 0.01$).

Discussion

Protein content measurements by Bradford assay showed a higher total protein level of *C. albicans* cells when treated with AVCO in contrast to the crude extract of VCO which did not show any significant result of protein leakage from *C. albicans* as shown in Figure 2. The data of cytoplasmic material release and total protein content in this study suggested that AVCO damages the physical network of *C. albicans* cell membrane. The leakage of cytoplasmic materials (DNA and protein) is due to the disturbance of the cell membrane. This alteration could have affected the the membrane permeability indicating the mechanism of AVCO on the solubilization of the cell membrane of *C. albicans* by disrupting the cell membrane permeability barrier with its MCFA.[21,22]

Sudden decrease of protein content at 4-hour incubation in AVCO showed that *C. albicans* has released the proteolytic enzyme in which the enzyme degraded the protein released after cell membrane damage.[23]

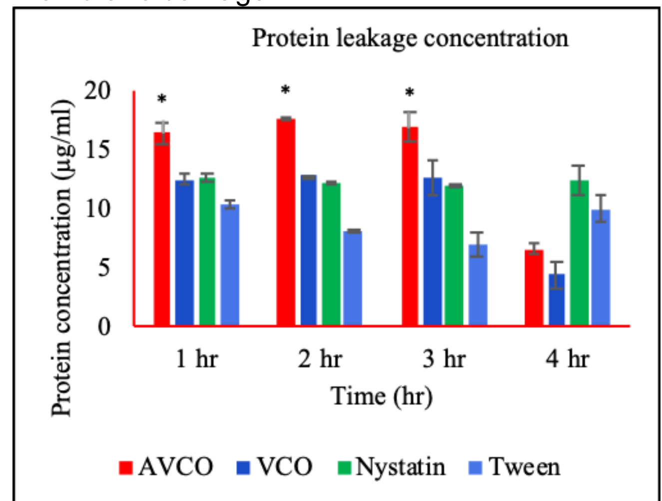

Figure 2. Bradford assay for protein leakage analysis of *C. albicans* cells treated with Tween 80, nystatin, VCO and AVCO. Values represent mean ± SD of triplicate experiments. One-way ANOVA and Tukey's test were performed (*$p < 0.01$).

Further analysis of cytoplasmic leakage can be confirmed by the TEM study, as shown in Figure 3. The TEM shows the cellular morphology of *C. albicans* cell after treatment with Tween 80 (negative control), nystatin (positive control), AVCO and the crude extract of VCO. After VCO treatment, most *C. albicans* cells showed no significant different from the cells treated with 1% Tween 80 in shape, or size, as illustrated in Figure 3. These digital measurement data (TEM) analysis give more evidence on the difference of the reduction of cytoplasmic density (Figure 3). Necrosis of cytoplasmic content were also observed in *C. albicans* after treated with AVCO at 3 hours. The necrosis effect is due to the transmembrane leakage of nucleic acids and other cytoplasmic materials through the pores formed on the cell membrane.[24] This finding is important as it has proven the excellent activity of AVCO as potential antifungal agent combatting *C. albicans* infections.

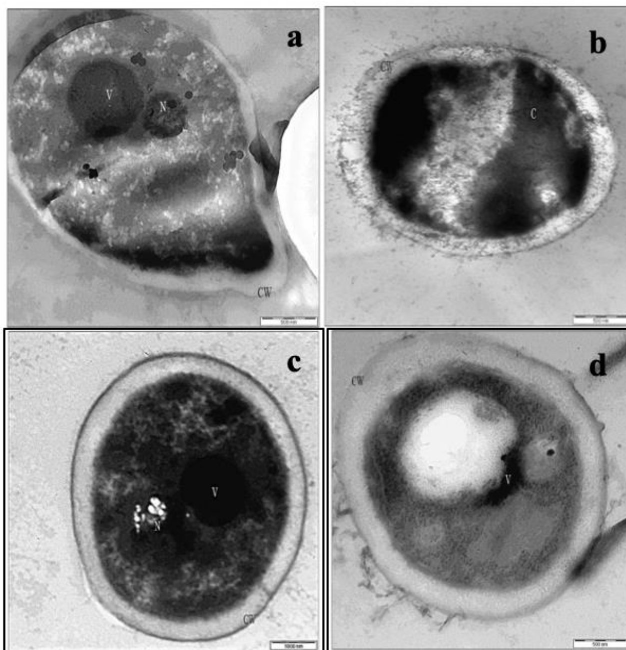

Figure 3. TEM images of *C. albicans* treated with different treatment after 3 h incubation. CW: cell wall; V: vacuole; N: nucleus. a) Cell treated with Tween 80 (negative control) showed normal vacuole, structured nucleus and regular outlined cell wall. b) Cell treated with nystatin (positive control) showed necrosis of cell membrane and ruptured cell wall. c) Cell treated with VCO exhibited normal vacuole and structured nucleus and enveloped regular cell wall. d) Cell treated with AVCO exhibited necrosis of cytoplasmic contents, abnormal shape of vacuole and shrunken cell membrane. Magnification:16.000X.

Conclusions

In conclusion, this study has suggested that treatment with AVCO can damage *C. albicans* cells through pores in the cell wall which lead to the death of cells or apoptosis due to membrane leakage of cytoplasmic content. Further investigations on the mechanism of action of AVCO on *C. albicans* are necessary to explain the penetration of AVCO on the cell membrane of *C. albicans* through the formation of pores and causes disorganization to the cell wall by interacting with the intracellular material.

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Declaration of Interest

The authors report no conflict of interest.

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