ORIGINAL ARTICLE
Volume 14 Issue 2 2019
DOI: 10.21315/aos2019.14.2.359
ARTICLE INFO
Submitted: 1102/2019
Accepted: 05/09/2019
Online: 30/12/2019

Fatty Acid Evaluation and Antimicrobial Activity of Virgin Coconut Oil and Activated Virgin Coconut Oil on *Streptococcus mutans*

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To cite this article: Haron UA, Mukhtar NI, Omar MN, Abllah Z (2019). Fatty acid evaluation and antimicrobial activity of virgin coconut oil and activated virgin coconut oil on *Streptococcus mutans*. *Arch Orofac Sci*, **14**(2): 87–98. https://doi.org/10.21315/aos2019.14.2.359

To link to this article: https://doi.org/10.21315/aos2019.14.2.359

ABSTRACT

For decades, coconut oil was reported to possess a broad spectrum of antimicrobial activity due to its abundant fatty acid's contents. *Streptococcus mutans* (*S. mutans*) has been strongly implicated as the main etiological factor in dental caries. Regardless of the ongoing medical advances, the therapeutic resources for dental caries remain ineffectual, and this has led to renewed interest in using virgin coconut oil (VCO) as a possible choice for dental caries control. In this study, the ability of VCO and activated virgin coconut oil (AVCO) combatting cariogenic *S. mutans* ATCC 25175 has been evaluated. Fatty acids contents were compared through gas chromatography-mass spectrum (GC-MS) analysis, and their antimicrobial activity was determined using disc diffusion and minimum inhibitory concentration (MIC) test. From the GC-MS analysis, AVCO (59%) was found to have a slightly higher medium-chain fatty acids (MCFA) as compared to VCO (54.1%), and the long-chain fatty acids (LCFA) contents in VCO (45.9%) was found to be higher than AVCO (41%). Interestingly, *S. mutans* ATCC 25175 was found to be susceptible towards AVCO (MIC: 6.24 mg/ml) and resistance towards VCO in vitro. The excellent antimicrobial activity of AVCO as a result from (i) the release of individuals fatty acids after activation of VCO by lipase digestion and (ii) the present of MCFA and LCFA that are significant in antimicrobial activity. Further study can be designed to specifically examine the activity of individuals fatty acids present in oils against *S.mutans* virulence genes/protein using molecular dynamic assessment.

Keywords: *Activated virgin coconut oil; antimicrobial activity; fatty acids; GC-MS analysis; virgin coconut oil.*

INTRODUCTION

Historically, coconut oil has been stapled in the diets of those who live in tropical regions (Kumar, 1997; Eyres *et al.*, 2016; Pham, 2016). The coconut tree (*Cocos nucifera*), is a member of Arecaceae family (palm tree) that can produce two kinds of oils which are coconut oil (copra oil) and virgin coconut oil (VCO) (DebMandal and Mandal, 2011). While coconut oil is a product from the extraction of heated copra, VCO is a product derived from coconut milk extracted from the freshly shredded coconut and not from copra by processes like fermentation, centrifugal separation and enzymatic action (Seneviratne *et al.*, 2009).

VCO has been commercialised for decades in medication fields as it possesses anti-microbial, antioxidant, anti-inflammatory, acute chemotherapy and anti-diabetic effects (Famurewa *et al.*, 2017a; 2017b; Kinsella *et al.*, 2017; Lima *et al.*, 2017; Nafar *et al.*, 2017; Sheela *et al.*, 2017; Varma *et al.*, 2019). The lauric acid content present in the VCO was reported to be enormously high where it is attached to a glycerol backbone to form a triglyceride (Ali *et al.*, 2014; Sheela *et al.*, 2016; Manivannan *et al.*, 2018). Previous study has shown that, of the free fatty acids present in coconut oil, lauric acid (C:12:0) is proven to be more active as antibacterial agent compared to caprylic acid (C8:0), capric acid (C10:0), and myristic acid (C14:0) (DebMandal and Mandal, 2011). The triglycerides present in VCO can be converted into free unsaturated fats; mainly lauric acid and monolaurin which act as anti-inflammatory, antibacterial and antiprotozoal components when present in the human gastrointestinal tract (Shilling *et al.*, 2013; Dayrit, 2015; Faciola and Broderick, 2014; Harris *et al.*, 2017; Varma *et al.*, 2019). It is notable that VCO possesses an excellent antimicrobial activity due to its abundant medium-chain fatty acid contents and for decades, the interest in studying VCO as a possible alternative for an antimicrobial agent has been on the incline.

Activated virgin coconut oil (AVCO) was a name given to a partially hydrolysed virgin coconut oil that was derived from an enzymatic reaction between the VCO and 1, 3-positional specific lipase (Long, 2009; Koh and Long, 2014). By activating the coconut oil content, it was reported that the antimicrobial activity was more excellent as compared to the VCO, of which the AVCO has a broader spectrum of antimicrobial effects (in vitro and in vivo) against pathogenic microorganism ranging from Gram-positive bacteria, Gram-negative bacteria, mycoplasma, fungus and also some viruses (Long, 2009; Koh and Long, 2014; Koh *et al.*, 2016).

The excellent reports of coconut oils as potential antimicrobial agents have led to the interest of the present study to investigate its antimicrobial activity against *Streptococcus mutans* (*S. mutans*) ATCC 25175. *S. mutans* is well-known for its vast virulence factors that initiate dental caries. The most common virulence factors of *S. mutans* includes its acidogenicity and acid-tolerant properties, a multitude of genes expressions that involve in transporting and metabolising sucrose and formation of biofilm (Loesche, 1986; 1996). The interest in finding an alternative for classic regime against *S. mutans* such as chlorhexidine has gained interest among researchers over the years (Figueiredo *et al.*, 2010; Choi *et al.*, 2016; Pires *et al.*, 2018). This is owing to the fact that the usage of the antimicrobial agent may lead to specific side effects, while the use of natural and eco-friendly products is becoming increasingly popular due to its lesser toxic contents, thereby, possess lesser or no side effect (da Costa *et al.*, 2017).

Evidently, numerous studies have shown a promising use of coconut oil as an antimicrobial agent. Nevertheless, there is a lack of research evaluating the activity of VCO and AVCO towards cariogenic *S. mutans*. Hence, this leads to the interests of the present study to evaluate the fatty acids contents for both VCO and AVCO by gas chromatography-mass spectrum

(GC-MS) method and to compare the antimicrobial activity of the oils against *S. mutans* ATCC 25175 using disc diffusion test and minimum inhibition concentration (MIC) test.

MATERIALS AND METHODS

AVCO and VCO Preparations

VCO used in this study was extracted in our laboratory (Natural Product Laboratory, Kulliyyah of Science, International Islamic University Malaysia, Kuantan, Pahang, Malaysia). The coconut fruits were obtained from Taman Agrotechnology, Malaysian Agricultural Research and Development Institute (MARDI, Cherating, Pahang, Malaysia). The oil extraction process was performed according to the method described by Seneviratne *et al.* (2009). The meat of mature brown coconuts was blended with the blender until it is well shredded. The coconut milk was filtered using cheesecloth over a wide-mouth jar, and the process was repeated, and the coconut milk was gathered into a jar. The coconut milk was rested for more than 24 hours. As it sets, the coconut milk and oil were separated, and a layer of curd appeared at the top of the jar. The curd was scooped out, and the pure VCO is left in the jar and store in dark glass bottle at room temperature until needed to be used. In the present study, the AVCO was provided from the Malaysian Agricultural Research and Development Institute (MARDI, Selangor, Malaysia).

Preparation of Fatty Acids Methyl Esters (FAME)

Methyl-esterification of both samples (VCO and AVCO) used in the analysis was performed by BF3-MeOH method, according to Ainie *et al.* (2005) with some modifications. A 20 mg of sample oils were added to 6 ml 0.5 mol/L NaOH-methanol solutions, and the mixture was heated at 100°C for 10 minutes using the reflux condenser. After cooling, 7 mL of 14% BF3-MeOH reagent was added, and the vessel was covered and heated at 100°C for 5 minutes. After cooling the vessel to the room temperature, 2 mL of hexane and approximately 10 mL of saturated NaCl solution were added, followed by a thorough shaking. The resulting hexane layer (2 mL) was then used as a sample solution for gas chromatography (GC).

Gas Chromatography-mass Spectrum (GC-MS) Analysis

GC-MS CLARUS® 680 (PerkinElmer, UK) equipped with a splitless injector was used to study the analysis of FAME for VCO and AVCO in the present study. Separations were achieved using a fused silica Elite-5MS capillary column (30m × 0.25mm ID, 0.50μm film thickness) and helium gas was used as the carrier gas at flow rates of 1.99 mL/min and a split ratio of 1:10. The temperature of 250°C was set up for the injector and another temperature was programmed at 140°C for a hold of 10 minutes and increased to 250°C at a rate of 7°C/min and hold at the final temperature for 10 minutes for the oven setup. The operation of GC-MS was controlled using Turbo Mass software. At the range width m/z 40-500, interface temperature 255°C, ion source temperature 210°C, solvent cut time 3 minutes, the MS spectra were finally obtained. The start time was set at 2.5 minutes, and the end time was set at 40 minutes. By comparing their retention time and equivalent chain length with respect to standard FAME obtained from Sigma Chemicals, the FAME peaks were identified. All mass spectra were also compared with the Wiley Registry of Mass Spectral Data, 11th Edition (2016), and the automated report was generated from the library. The percentage yield detected by GC-MS were calculated using the following formula of area normalising:

$$\text{Yield (\%)} = \frac{\text{Peak area of one peak}}{\text{sum of all peak}} - (100)$$

Bacterial Strain and Culture Conditions

The *S. mutans* ATCC 25175, were obtained from the American type culture collection (ATCC), USA. *S.mutans* ATCC 25175 were cultured in brain heart infusion (BHI) broth (Oxoid™, MD, USA) and on BHI agar. The bacteria were maintained in anaerobic condition and incubated at 37°C for 24 hours prior to the test.

Disc Diffusion Test

The efficacy of VCO and AVCO against the *S. mutans* ATCC 25175 was determined by disc diffusion method. Both VCO and AVCO stock was diluted with 1% Tween 80 at the following concentration of >5,000 and 20 mg/ml respectively. The selection of the concentration of VCO and AVCO was made based on the previous screening test. A volume of 20 µL of each concentration was, respectively, infused into the paper disc with 6 mm diameter (Oxoid, Badhoevedorp, Netherlands), and then placed onto Mueller-Hinton agar (MHA) plates (Oxoid™, MD, USA), which were previously inoculated on the surface agar with 200 µL of CFU/mL suspension of *S. mutans* ATCC 25175. A (1%) Tween 80 was used as a negative control and a standard reference antibiotic, chlorhexidine (20 mg/disc) was used as a positive control. The plate was then incubated at 37°C for 24 hours, and the test was conducted in triplicates. The inhibitory zone was measured in millimetres (mm) and the mean expressed as the results of three determinations.

Minimum Inhibitory Concentration (MIC) of AVCO and VCO

A broth microdilution method proposed by Wiwattanarattanabut *et al.* (2017) was applied with some modifications to determine the MIC value required for VCO and incorporated into the 96-wells containing Mueller Hinton (MH) broth medium and is serially diluted by double technique to achieve concentrations ranging from 49.92–0.39 mg/ml. As for VCO, a much higher concentration was used for the MIC test because the high concentration of VCO used in the disc diffusion test was not able to exhibit inhibition of *S. mutans* ATCC 25175. A 100 µl of 10,000mg/ml VCO was dissolved in 1% Tween 80 and incorporated into the 96-wells containing MH broth medium and a serially diluted by double technique to achieve concentrations ranging from 7692.3–60.09 mg/ml. *S. mutans* ATCC 25175 was grown overnight and the bacterium cells suspensions, at OD510 was diluted 10-fold and 100µl of the suspensions were dispensed into each well. The plate was incubated at 37°C overnight with setup of appropriate cell control, chlorhexidine control, Tween 80 control, and media control. The test was performed in triplicates. The plate was assessed visually for turbidity, which signifies cell growth, and the optical density was measured at the wavelengths of 510 nm. The MIC was determined to be the lowest concentration of VCO, AVCO and chlorhexidine which produced an optical density of ≤25% than that of the cell control, or the lowest concentration of VCO, AVCO and chlorhexidine with no visible cell growth (as seen by absent of turbidity or clear broth medium).

RESULTS AND DISCUSSION

In the present study, gas-chromatography coupled with mass spectrometry (GC-MS) was used to identify and measure the composition of fatty acids present in VCO and AVCO (Table 1, Fig. 1, Fig. 2). Gas chromatography (GC) and infrared (IR) spectroscopy are the two commonly used methods for the determination of trans-fatty acids. It is noted that IR spectroscopy is much straightforward; however, the methodology is not considered accurate below 5%, and it's prone to the subject of interferences (Moigradean *et al.*, 2013). In contrast, GC analysis of fatty acid methyl ester (FAME) has been noted to be more precise for fatty acids analysis (Cropper and Heywood, 1953).

Table 1 Fatty acid compositions of VCO and AVCO obtained by the GC-MS method

Fatty acids	Molecular formula	Saturation	RT (min)	VCO (%)	RT (min)	AVCO (%)	Identification
MCFA							
Caproic	C6H12O2	Saturated	6.50	0.20	6.01	0.30	RT, MS
Caprylic	C8H16O2	Saturated	11.80	4.90	11.73	6.40	RT, MS
Capric	C10H20O2	Saturated	17.17	6.00	17.18	6.30	RT, MS
Lauric	C12H24O2	Saturated	22.17	43.00	22.43	46.00	RT, MS
LCFA							
Myristic	C14H28O2	Saturated	26.57	24.00	26.71	20.70	RT, MS
Palmitic	C16H32O2	Saturated	30.60	13.00	30.67	12.00	RT, MS
Stearic	C17H40O2	Saturated	-	-	33.86	1.20	RT, MS
Oleic	C18H34O2	Monounsaturated	33.95	8.50	34.33	7.10	RT, MS
Arachidic	C20H40O2	Monounsaturated	35.21	0.40	-	-	RT, MS

Note: VCO – virgin coconut oil; AVCO – activated virgin coconut oil; MCFA – medium-chain fatty acid; LCFA - long chain fatty acid; RT – retention time; MS – mass spectrum.

Table 2 Biological activity for the fatty acid compositions present in VCO and AVCO

Fatty acids	Molecular formula	Biological activity	Reference
MCFA			
Caproic	C6H12O2	Flavouring agent	National Centre for Biotechnology Information (NCBI), 2018a
Caprylic	C8H16O2	Antimicrobial	NCBI, 2018b
Capric	C10H20O2	Antimicrobial	NCBI, 2018c
Lauric	C12H24O2	Antimicrobial	NCBI, 2018d
LCFA			
Myristic	C14H28O2	Antimicrobial	Huang *et al.*, 2011
Palmitic	C16H32O2	Antimicrobial	Huang *et al.*, 2011
Stearic	C17H40O2	Antimicrobial	Karimi *et al.*, 2015
Oleic	C18H34O2	Antimicrobial	Karimi *et al.*, 2015
Arachidic	C20H40O2	Plant metabolite	NCBI, 2018e

Note: MCFA – medium-chain fatty acid; LCFA - long chain fatty acid

From the GC-MS analysis of fatty acids for VCO and AVCO (Table 1), it was found that, the fatty acids contents present in the VCO's sample are; the medium-chain fatty acids (MCFA [caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0)] and long-chain fatty acids (LCFA) [myristic acid (C14:0), palmitic acid (C16:0), arachidic acid (C20:0) and oleic acid (C18:1)]. Meanwhile, the fatty acids contents that were detected in the AVCO's sample are; the MCFAs [caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0)] and LCFAs [myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1) and stearic acid (C17:0)]. The biological activity of the fatty acids was summarised in Table 2, where it is interesting to note that, most of the fatty acids present in both VCO and AVCO has been reported to possess antimicrobial activity towards a broad spectrum of microorganism. From the GC-MS analysis, we observed that the LCFAs contents

present in both VCO and AVCO have some notable differences. The amount of LCFAs (myristic acid, palmitic acids, and oleic acid) in VCO were slightly higher in concentration as compared to those present in the AVCO. It is also notable that, stearic acids which reported to have an antimicrobial activity was present in AVCO but not in VCO and arachidic acids which are a common minor compound in peanut butter that acts as plant metabolite was present in VCO and not in AVCO.

The current study was in agreement with the previous finding where the MCFAs acids were determined as the major fatty acid's compositions of VCO and lauric acid is the most abundant of the other medium-chain fatty acids (DebMandal and Mandal, 2011). Activation of VCO resulted in a high number of MCFAs, suggesting that the activation by enzymatic reaction does not possess any significant changes to the 'backbone' of the oil. From the present finding, it is revealed that the total MCFAs of VCO was 54.1%, which is lower than AVCO, that has 59.0% of total MCFAs. It can be seen from the present study that the enzymatic reaction had improved the oil to produce a high number of MCFAs which is significantly beneficial as MCFAs was reported to be the active compound involves for antimicrobial (Huang *et al.*, 2011). As stated previously, AVCO is the product of the enzymatic reaction between the VCO and 1, 3-positional specific lipase, a previous study has shown that the use of the 1, 3-positional specificity of lipases is crucial in obtaining a high yield of products for biodiesel production, suggesting an astounding purpose of the enzyme reaction in the improvement of the lipid productions (Li *et al.*, 2015).

Another finding that is worth mentioning is that, after activation with enzymatic reaction, we have determined that the lauric acid content in AVCO (46%) is much higher than lauric acid present in the VCO (43%).

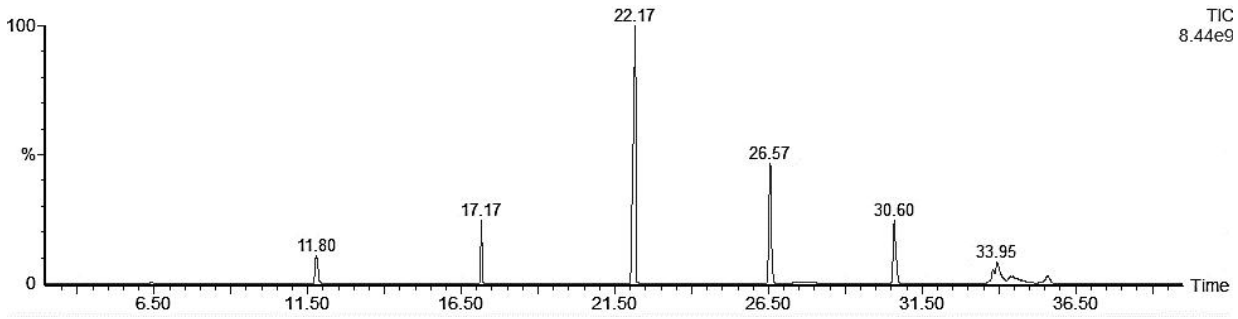

Fig. 1 VCO's gas chromatography.

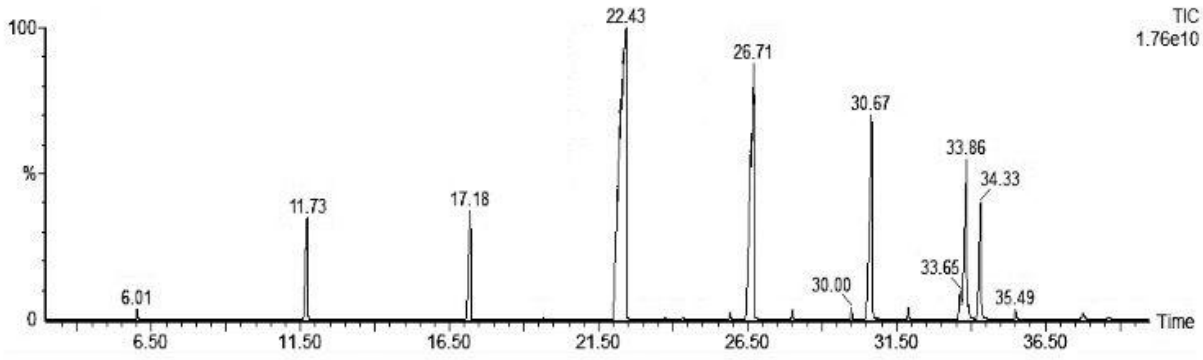

Fig. 2 Activated VCO's gas chromatography.

Table 3 Microdilution method MIC (mg/ml) and disc diffusion (mm) values results of VCO and AVCO against *S. mutans* ATCC 25175

Microbial strain	VCO		AVCO		Chlorhexidine		*p*-value
	ZI (>200mg disc mm)	MIC (mg/ml)	ZI (20mg/ disc mm)	MIC (mg/ml)	ZI (20mg disc mm)	MIC (mg/ml)	
Streptococcus mutans ATCC 25175	ND	>5000.00	15.00 ± 0.30	6.24	8.00 ± 0.20	12.48	<0.001

Note: VCO – virgin coconut oil; AVCO – activated virgin coconut oil; ND – not determined; MIC – minimum inhibitory concentration; ZI – zone of inhibition. The outcomes were carried out in triplicates and data were expressed as means ± standard error (SEM) and one-way ANOVA test was used to determine the significant difference between each tested group (VCO and AVCO) with the control group (chlorhexidine) which the level of statistical significance was set at *p*-value (<0.05).

A similar finding after hydrolysing VCO with lipolytic *Geotrichum candidum* was observed by Khoramnia *et al.* (2013), suggesting that the enzymatic reaction had improved the medium-chain fatty acids contents in VCO and its antimicrobial activity. This finding is essential as, lauric acid has been reported to be the main compound from the MCFAs contributed to the excellent antimicrobial activity (Nakatsuji *et al.*, 2009; Yang *et al.*, 2009; Huang *et al.*, 2014; Salleh *et al.*, 2014; Chang *et al.*, 2015; Ma *et al.*, 2016; Nitbani *et al.*, 2016; Sheela *et al.*, 2017).

From the fatty acid's analysis, it is noted that there were slight differences in the fatty acids profile of VCO when compared to AVCO. We further investigate the antimicrobial activity of VCO and AVCO via disc diffusion test and minimum inhibitory concentration (MIC) test (Table 3, Fig. 3). To the best of our knowledge, this is the first attempt to evaluate the antimicrobial activity of VCO and AVCO on *S. mutans* ATCC 25175.

Interestingly, *S. mutans* ATCC 25175 was found to be resistance towards VCO and highly susceptible to AVCO in vitro. Both disc diffusion and MIC test, have shown that the highest concentration of VCO fails to inhibit the growth of *S. mutans* ATCC 25175. In contrast, AVCO at the concentration of 20 mg/ml exhibit the inhibition zone of 15 ± 0.3 mm which is higher than the control antibiotic chlorhexidine (20mg/ml) which is 8 ± 0.2 mm and its significantly inhibited the growth

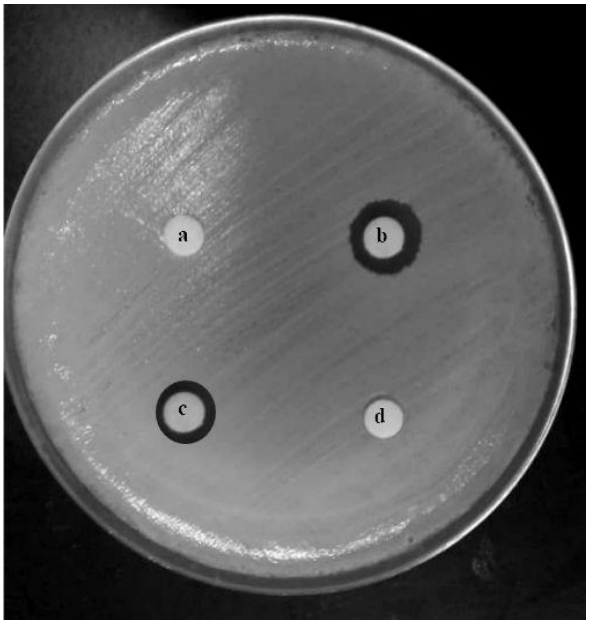

Fig. 3 Disc diffusion test to determine the effects of coconut oils on the growth of *S. mutans* ATCC 25175. Antimicrobial activity of VCO and AVCO was carried out according to the disc diffusion method by measuring the inhibitory zone size in mm.
Note: a: 1% Tween (negative control), b: AVCO 20 mg/ml, c: chlorhexidine 20 mg/ml, (positive control) d: >5,000 mg/ml VCO.

of *S. mutans* ATCC 25175 in vitro at levels as low as 6.24 mg/ml. We used chlorhexidine as a positive control in the study, because it is still commercially used as one of the treatment options for *S. mutans* (Eden, 2008) and interestingly, the MIC value for AVCO is found to be lower than the MIC value for chlorhexidine from previous study (Uzer Celik *et al.*, 2016). The excellent

antimicrobial activity of AVCO can be due to the digestion with lipase causing all the individual fatty acids to be released, therefore, allowing it to inhibit the growth of *S. mutans*. This finding was in agreement with the study done by Shilling *et al.* (2013) where they postulated that VCO, when consumed, would be digested by lipases of the digestive tract, releasing the individual fatty acids and ultimately inhibiting the growth of microorganisms in vivo. The fact that the test was performed in vitro, also can suggest that the lack of enzyme lipase present in *S. mutans* ATCC25175. Therefore, treatment with VCO in vitro does not show any antimicrobial activity despite having good fatty acids profiles. Hence, by having the digestion of the oil, it allows the individuals' fatty acids to be released and inhibits the growth of *S. mutans* ATCC 2157 in vitro. This finding can suggest the potential use of AVCO as one of the oral health aid options in the near future.

CONCLUSION

The present study has revealed the differences between the fatty acids content in VCO and AVCO as well as their antimicrobial activity towards *S. mutans* ATCC 25175. To the best of our knowledge, this is the first attempt to study the AVCO antimicrobial activity towards *S. mutans* ATCC 25175 in vitro. Ultimately, from our findings, we can postulate that the excellent antimicrobial activity of AVCO as a result from (i) the release of individuals fatty acids after activation of virgin coconut oil by lipase digestion and (ii) the present of MCFA and LCFA that are significant in antimicrobial activity. Further study can be designed to specifically examine the activity of individuals fatty acids present in oils against *S.mutans* virulence genes/protein using molecular dynamic assessment.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Kamariah Long from the MARDI, Serdang for her contribution to our study, to the International Islamic University Malaysia for the support throughout the study and to the Ministry of Higher Education for the funding of our study. This study was supported by the Fundamental Research Grant Scheme (FRGS 16-020-0519).

CONFLICT OF INTEREST

All of the authors report there is no conflict of interest relevant to this article.

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