As. J. Food Ag-Ind. **2010**, 3(06), 567-579

OPEN ACCESS

Asian Journal of Food and Agro-Industry
ISSN 1906-3040
Available online at www.ajofai.info

Research Article

Commercial virgin coconut oil: assessment of antimicrobial potential

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Abstract

Virgin coconut oil is defined as the oil obtained from fresh coconut meat, without undergoing any chemical processes. Virgin coconut oil is mainly sold on the basis of its antimicrobial activity which is contributed to by its lauric acid. This study aimed to investigate the antimicrobial activity of commercial virgin coconut oil. Agar well diffusion test and disc diffusion test were first done to test on both methanol extracted oil and pure oil samples against four types of bacteria. Both methods showed no antimicrobial activity. Broth dilution method was then done to determine the minimum inhibitory concentration of the oil samples against eight types of bacteria. Again, no antimicrobial activity was observed. In turn, gram negative bacteria (*Coccus*, *Bacillus*, *Streptobacillus* and *Coccobacillus*) were isolated from all the oil samples. The triglyceride in the oil samples have to be broken down to monoglyceride or free fatty acids by enzyme lipases in order to exert antimicrobial activity. Bacteria have the ability to produce non-specific enzymes to release free fatty acids and sn-1,3 lipase to produce sn-2 monoglyceride. In fact, the free lauric acids released by lipase only possess weak antimicrobial activity and the sn-2 monoglycerides formed may not exert any antimicrobial activity. Bacteria were isolated from the oil samples because raw and unprocessed coconut meat can support growth of bacteria and no sterilization is done to prevent microbial contamination.

Keywords: contamination, lauric acid, agar well diffusion test, agar disc diffusion, broth dilution, Malaysia.

Introduction

Tuberculosis, malaria and leprosy that are some of the examples of microbe-causing diseases affecting millions of people around the world. Studies have been done to find cures to microbe-causing diseases. Fleming was the first person who discovered the use of antibiotics in controlling microbe-causing diseases [1]. However, problems arose when bacteria became resistant to

antimicrobial agents as a result of chromosomal changes or the exchange of genetic material via plasmids [2]. Antibiotic resistant bacteria cause an increase in the rate of infectious diseases and mortality worldwide. As a result, scientists turn to other alternatives to look for a cure or prevention.

According to The Philippines National Standard, Virgin coconut oil (VCO) is defined as the "oil obtained from the fresh, mature kernel of the coconut by mechanical or natural means, with or without the use of heat, without undergoing chemical refining, bleaching or deodorizing (RBD) processes, and which does not lead to the alteration of the nature of the oil" [3]. It was described as being colourless with an acid, *cocojam*, *lantik*, nutty and rancid aroma [4]. VCO at room temperature is in liquid form. It will solidify and turn white below 22ºC [5].

In older times, virgin coconut oil (VCO) was widely used as a massage oil. It was applied onto women's abdomens to reduce stretch marks after delivery and to babies to prevent skin infection [6]. Recently, virgin coconut oil supplemented diet has been shown to improve the antioxidant level in rats [7]. One study also showed that virgin coconut oil supplemented diet exerts antithromnotic properties and prevents the formation of lipid peroxides in rats [8].

Virgin coconut oil (VCO) is found to contain a high amount of medium chain fatty acids such as lauric acid, caproic acid and caprylic acid. Studies found that medium chain fatty acids contain powerful antimicrobial properties. For example, lauric acid has been proven to inhibit the growth of *Chlamydia trachomatis, Helicobacter pylori*, *Neisseria gonorrhoeae* and *Candida albicans* effectively [9, 10, 11, 12]. This resulted in an increased interest in the research of antimicrobial properties of virgin coconut oil. The objective of this study was to investigate the antimicrobial activity of pure and methanol extracted VCO through agar disc diffusion assay, agar well diffusion and broth dilution assay.

Materials and methods

Chemicals and reagents

Methanol and the media (nutrient broth and nutrient agar) used in the study were purchased from Merck, Germany. Other chemicals such as gram stain alcohol, gram stain iodine and gram stain crystal violet were purchased from Labchem, Malaysia.

Source of virgin coconut oil

Three samples of commercial virgin coconut oil were used in this study, organic virgin coconut oil, extra virgin coconut oil and cold-pressed virgin coconut oil. The organic virgin coconut and extra virgin coconut oil were purchased from an organic shop in Malaysia. Cold-pressed virgin coconut oil was purchased from a local pharmacy in Kuala Lumpur, Malaysia.

Extraction of virgin coconut oil by using methanol

The commercial organic virgin coconut oil and extra virgin coconut oil were extracted by using three different concentrations of methanol, 100%, 90% and 80% in 1:1 ratio. The extracted samples were concentrated in a rotary evaporator at 35ºC.

Experimental design

In this study, the antimicrobial activity of the virgin coconut oil was investigated by using three antimicrobial assays. In agar well diffusion test and agar disc diffusion assay, both pure and methanol extracted samples of two commercial virgin coconut oils namely, organic virgin coconut oil and extra virgin coconut oil were investigated. As no antimicrobial activity could be observed in both organic and extra virgin coconut oil, another sample of cold-pressed virgin coconut oil was added in the broth dilution test. In this test, three pure oil samples of commercial virgin coconut oil (organic virgin coconut oil, extra virgin coconut oil and cold-pressed virgin coconut oil) were used to determine their minimum inhibitory concentrations.

Antimicrobial assay

In agar well diffusion test and agar disc diffusion test, the antimicrobial assay was performed on four types of bacteria (*Staphylococcus aureus, Bacillus cereus, Escherichia coli,* and *Serratia marcescens)*. In broth dilution test, eight types of bacteria were tested which included *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Bacillus subtilitis, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa* and *Serratia marcescens.* All the bacteria were obtained from Microbiology Laboratory of UCSI University, Kuala Lumpur, Malaysia. In all the three antimicrobial assays, the bacteria were standardized to 10^6cfu/ml. Overnight cultures (200µL) of each organism were mixed into 20ml of sterile nutrient broth. In order to obtain a bacteria count of 10^6cfu/ml, all the inoculated nutrient broths were then incubated 3-5 hours [13]. The bacteria counts were confirmed by hematocytometer counting [14].

Agar well diffusion test

This assay was first done by inoculating 100µL of 24 hours broth culture of test bacteria with 20mL of nutrient agar in individual petri dishes [15, 16]. After that, 20µL of samples were pippetted into three 5mm wells to obtain triplicate results [16]. Another two wells were used as control. Methanol was used as the negative control and ampicilin was used as the positive control [14, 17]. All the plates were incubated at 37°C for 24 hours [16].

Disc diffusion test

Agar plates were first prepared by pouring 20mL of nutrient agar into each petri dish and air dried in the laminar flow. After that, 100µL of the cultures were pippetted onto the agar surface by using a glass spreader and air dried for 20 minutes [18]. Filter paper discs (6mm) were then impregnated with 10µL of samples and were then placed onto the surface of the inoculated agar plates [17, 19]. Similar to agar well diffusion test, methanol was used as the negative control and ampicilin as the positive control [14, 17]. All the plates were sealed, inverted and incubated for 24 hours at 37°C [19].

Broth dilution test

In this assay, the antimicrobial activity was tested against five different concentrations of samples (100%, 50%, 25%, 12.5% and 6.25%), which were achieved through serial dilutions. Assay was done by mixing 500µL of nutrient broth with 500µL of oil samples at different concentrations. After that, all the tubes were added with 10µL of standardized bacteria culture and were incubated for 24 hours at 37°C. Control tubes were also prepared. Bacteria control was prepared by adding 500µL of nutrient broth with 10µL of 24 hours bacteria culture. Another control was prepared by adding 500µL of nutrient with 500µL of oil samples [20]. After 24 hours, the growths of the bacteria were observed by comparing the turbidity of all the tubes with the controls. Growths of bacteria were also confirmed through pour plate method. In this method, around 20mL of nutrient

agar were mixed with 25µL of samples and were incubated for 24 hours at 37ºC [20]. The growth of bacteria can be observed through the milky coloured bacteria colonies growing on the agar plate

Gram staining

Bacteria isolated from all three samples (organic virgin coconut oil, extra virgin coconut oil and cold-pressed virgin coconut oil) were tested with gram staining to identify the type of bacteria. The smear was first prepared by mixing a small drop of distilled water with a loopful of bacteria and was air-dried [21]. The smears of the colonies were first stained with crystal violet for 60 seconds and were washed away. After that, the smears were covered with iodine, a mordant for another 60 seconds [22]. Alcohol was then applied to decolorize the purple colour on the cells. Lastly, the smears were stained with safranin and washed away after 60 seconds [22]. All the smears were allowed to air-dry and were observed under microscope. Gram negative bacteria was stained as pink colour while gram positive bacteria was stained as purple colour.

Results and Discussion

From the results of the three antimicrobial assays, no antimicrobial activity can be observed from both methanol extracted oil samples and pure oil samples. In turn, gram negative bacteria were isolated from the oil samples.

Antimicrobial susceptibility test by using well diffusion method

As shown in Table 1 and Table 2, no inhibition zone can be observed in both pure oil and methanol extracted oil samples for all the tested bacteria. The only inhibition zone that can be observed was the antibiotic solution. *Serratia marcescens* is the most susceptible to the antibiotic solution. For *Bacillus cereus* and *Staphylococcus aureus,* both bacteria showed intermediate susceptibility to antibiotic solution whereas *Escherichia coli* were resistant to the antibiotic solution.

Table 1. Agar well diffusion test for methanol-extracted organic virgin coconut oil and extra virgin coconut oil.

Test bacteria	Methanol extracted organic V.C.O			Methanol extracted Extra V.C.O			Control	
	Conc. 100%	Conc. 90%	Conc. 80%	Conc. 100%	Conc. 90%	Conc. 80%		
	Mean	Mean	Mean	Mean	Mean	Mean	Antibiotic	Methanol
S. aureus	-	-	-	-	-	-	15.0mm	-
B. cereus	-	-	-	-	-	-	13.0mm	-
E.coli	-	-	-	-	-	-	10.0mm	-
S. marcescens	-	-	-	-	-	-	30.0mm	-

"-": No inhibition zone observed.

Table 2. Agar well diffusion test for pure oil samples (sample without extraction).

Test bacteria	Organic V.C.O	Extra V.C.O	Control	
	Mean	Mean	Antibiotic	Methanol
S. aureus	-	-	11.0mm	-
B. cereus	-	-	10.0mm	-
E.coli	-	-	9.0mm	-
S. marcescens	-	-	27.0mm	-

"-": No inhibition zone observed.

One of the most common problems in oil study is the poor solubility of the oil samples [23]. It was suspected that the virgin coconut oil has a complex chemical composition and the molecular size of the oil is too big to diffuse into the agar. There is also a possibility that the methanol used failed to extract lauric acid from the oil samples. As the lauric acid are attached to the glycerol backbones of the triglyceride (not present as free fatty acid in the oil samples), methanol might have difficulty in extracting the lauric acid from the oil samples.

Antimicrobial susceptibility test by using disc diffusion method

Table 3 and Table 4 show that in this assay no inhibition zone can be observed in both pure oil samples and methanol extracted samples. The only inhibition zone that can be observed was again the antibiotic solution. *Serratia marcescens* showed the highest susceptibility, while *Staphylococcus aureus* and *Bacillus cereus* showed intermediate susceptibility. *Escherichia coli* showed intermediate susceptibility, with 11mm zone of inhibition in methanol extracted samples, whereas in pure oil samples it is resistant to the antibiotic solution which only gives 10mm of inhibition zone.

Table 3. Agar disc diffusion test for methanol-extracted organic virgin coconut oil and extra virgin coconut oil.

Test bacteria	Methanol extracted organic V.C.O			Methanol extracted Extra V.C.O			Control	
	Conc. 100%	Conc. 90%	Conc. 80%	Conc. 100%	Conc. 90%	Conc. 80%		
	Mean	Mean	Mean	Mean	Mean	Mean	Antibiotic	Methanol
S. aureus	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	14.0mm	-
B. cereus	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	13.0mm	-
E.coli	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	11.0mm	-
S. marcescens	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	6.0±0.00	30.0mm	-

Table 4. Agar disc diffusion test for pure oil samples (sample without extraction).

Test bacteria	Organic V.C.O	Extra V.C.O	Control	
	Mean	Mean	Antibiotic	Methanol
S. aureus	6.0±0.00	6.0±0.00	11.0mm	-
B. cereus	6.0±0.00	6.0±0.00	12.0mm	-
E.coli	6.0±0.00	6.0±0.00	10.0mm	-
S. marcescens	6.0±0.00	6.0±0.00	28.0mm	-

It was again suspected that the oil samples have difficulty in dissolving onto the surface of the agar from the discs due to its complex chemical structure and the low solubility of the oil samples [23]. Another suspected reason is the low lipase activity in the agar as this will only be activated in an oil-water interface [24]. The triglycerides in the oil samples have to be broken down by lipase to lauric acid or monolaurin in order to express their antimicrobial activity [25]. Thus, with a low lipases activity on the agar plate, the amount of lauric acid formed will not be enough to exert any antimicrobial activity. Considering the results of the agar well diffusion and disc diffusion tests, it is also possible that the oil samples do not posses any antimicrobial activity.

Broth dilution assay

No antimicrobial activity can be observed for all the samples in all the tested bacteria as shown in Table 5. The growth of bacteria can be observed as all the tubes became turbid and bacteria growth was confirmed through pour plate method for all the tubes. Contamination was a common problem in the broth dilution test, especially when pour-plate was done. Bacteria colonies were observed on the agar plate of sample control (virgin coconut oil only), the agar became turbid and bacteria colonies were observed on the agar plates. The colonies observed on the agar were then isolated for gram staining.

Table 5. Minimum inhibitory concentrations of organic virgin coconut oil, extra virgin coconut oil and cold-pressed virgin coconut oil.

Test bacteria	Concentration (%)															Sample control	Bacteria control
	Organic V.C.O					Extra V.C.O					Cold-pressed V.C.O						
	100	50	25	12.5	6.25	100	50	25	12.5	6.25	100	50	25	12.5	6.25		
Gram positive																	
S. aureus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
S. epidermidis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
B. cereus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
B. subtilis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Gram negative																+	+
E. coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
S.typhi	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
P. aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
S. marcescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		

"+": Growth of bacteria on agar plate.

Gram staining

From the results of the gram staining, four types of bacteria were found in the oil samples with different morphologies as summarized in Table 6. All the bacteria were stained pink colour, indicating that they are gram negative bacteria.

Table 6. Summary of bacteria found in V.C.O.

Sample	Type of bacteria	Shape
Cold-pressed V.C.O	Gram negative	Coccus, Streptobacilli
Extra V.C.O	Gram negative	Bacillus
Organic V.C.O	Gram negative	Coccobacillus, Streptobacillus

Generally, lipids that can be found in virgin coconut oil are in the form of triglyceride. Both triglyceride and diglyceride are not active compounds, they do not possess antimicrobial activity [25, 26]. The antimicrobial activity of medium chain triglycerides is reported to be the reaction of the free fatty acids and its derivatives known as monoglyceride [12, 27]. Monoglyceride is defined as a glyceride molecule with only one fatty acid attached to either the sn-1 or sn-2 position of the glycerol. The monoglyceride that is discovered to be most effective in inhibiting microorganisms is the monolaurin which is the monoester of the lauric acid.

Lauric acid is the major fatty acid found in coconut and it can be found in all the three positions of the glycerol backbone as shown in Table 7. Lauric acid was found to be most abundant in the sn-2 position [28]. In human and animal bodies, monolaurin can be formed from the breakdown of triglyceride through enzyme activity [29].

Table 7. Positional distribution of fatty acid in coconut.

Sn position	Fatty acid %							
	8:0	**10:0**	**12:0**	**14:0**	**16:0**	**18:0**	**18:1**	**18:2**
1	4	4	39	29	16	3	4	-
2	2	5	78	8	1	1	3	2
3	31	13	38	8	1	1	3	2

[Adapted from Sikorski *et al.* 2002]

Studies have shown that bacteria will produce extracellular enzyme lipases that are able to break down triglyceride [28]. There are two types of bacterial lipases discovered by scientists, namely non-specific lipases and 1, 3-specific lipases [30, 31].

In the case of bacteria with 1, 3-specific lipases, their main target will be the sn-1 and sn-3 esters bonds [31]. The end products produced by this enzyme will be two free lauric acids and sn-2 monolaurins which are similar to human body lipid catabolism. In previous studies done on the antimicrobial activity of monoglyceride, researchers used the chemically synthesized monoglyceride (synthetic monolaurin) in which the lauric acid is attached to the sn-1 position while the monolaurin produced by the bacteria is attached to the sn-2 monolaurin [29, 32]. This means that the proven antimicrobial activity is due to the sn-1 monolaurin which is a different isomer from the monolaurin formed by the bacteria. These two isomers of monolaurin reflect different biological metabolism in the human body. In human body metabolism, the synthetic sn- 1 monolaurin is absorbed as free fatty acids and they are not reesterified into inactive triglyceride while the sn-2 monolaurin will be reesterified and formed into chylomicron [33]. Since there is no study done on the utilization of monoglyceride by bacteria, it is suspected that either the sn-2 monolaurin reacts differently in bacterial cells, or they may not have any reaction towards sn-2 monolaurin. Hence, direct usage of virgin coconut oil to inhibit the bacteria growth may not be as effective as the use of synthetic sn-1 monolaurin.

The non specific lipases will act on all the three ester bonds in the triglyceride backbone [31]. The end products of this lipase will be three free fatty acids and one glycerol molecule. In the case of virgin coconut oil, all the three lauric acids that are attached to the glycerol backbone will be released as free fatty acids, lauric acids. Both non-specific and 1, 3- specific microbial lipases will release lauric acid from the triglyceride backbone. However, lauric acid only possesses weak antimicrobial activity [9, 12]. Studies show that lauric acid is not able to inhibit the growth of bacteria at a lower concentration but it will only block the production of exoezymes, virulence factor and toxin formation by the bacteria [34].

Monolaurin was found to be more effective in inhibiting the growth of gram positive bacteria. This is mainly due to the structural difference in the cell wall of the gram positive and gram negative bacteria. However in this project, both tested gram positive and gram negative bacteria were not inhibited. In gram positive bacteria, the cell wall consists of a thick layer of peptidoglycan while the cell wall of the gram negative bacteria consists of a thin layer of peptidoglycan with an outer membrane consisting of lipopolysaccarides, lipoprotein and phospholipids [28]. The outer membrane of the gram negative bacteria plays an important role in protecting the bacteria from being attacked by monolaurin. Due to the O side chain of the lipopolysaccarides, the gram negative bacteria have a hydrophilic surface [12]. This causes hydrophobic molecules such as monolaurin to have difficulty

entering the bilayer. This means that lauric acid and monolaurin do not have the chance to react and destroy the membrane of the bacteria. Thus, the growth of gram negative bacteria were not inhibited.

That all the oil samples in this study did not show any antimicrobial activity could be due to the low quality of the coconut sources. Once the coconut is harvested, lipolysis of oil will begin through microbial enzymatic process or through the natural occurring enzyme lipase in the oilseeds. The main product of this process is the free fatty acids that only possess weak antimicrobial activity. Thus, it is important to keep the amount of free fatty acids of the oil as low as possible. According to the Philippine National Standard, the amount of free fatty acid in the oil should be less than 0.2% [3]. The low amount of free fatty acids in the oil can be achieved by using a high quality coconut source to extract the oil. The harvested coconut should not be stored for a long time as lipolysis of oil to release free fatty acids will start once the coconut is harvested.

Factors supporting the growth of bacteria in virgin coconut oil

In 1960s, studies found that raw and unprocessed coconut meat and coconut milk can support the growth of *Salmonellae* and other enteric bacteria [35]. Researchers found that the enteric bacteria can be killed under pasteurization at 80°C for 8 to 10 mins. In most of the processed coconut products, including the RBD coconut oil, they do undergo heating process. The heating temperature used was high enough to kill the bacteria [36]. Hence, the presence of enteric bacteria in raw unprocessed coconut was not a concern.

In the extraction of virgin coconut oil, no sterilization process is done to prevent microbial contamination. The issue of the coconut meat ability to support the growth of enteric bacteria should become a concern regarding the safety of the end-product. According to the APCC standard, the total plate count in virgin coconut oil should be less than 10cfu/mL [37]. However, there is still no preservation method that can be used to prevent the division of bacteria in the oil. The enteric bacteria may use the fat as a source of energy and reproduce [28]. There is a concern that the number of bacteria will increase with time. Further study should be done on the total plate count of virgin coconut oil with storage time.

As claimed by the manufacturers, the lauric acids in coconut oil give the effect of antimicrobial activity. In fact, it is the monoglyceride of lauric acid, the monolaurin which kill the microorganisms as it is more biologically active than lauric acid. In coconut fat, the level of monolaurin formed is no greater than 3% [38]. In other words, the biological active compound of lauric acid is not strong enough to kill the enteric bacteria in coconut oil. The strong effect of antimicrobial activity is due to the combination effect of lauric acid and its monoglyceride, monolaurin [7, 17].

Conclusion

The antimicrobial activity of commercial virgin coconut oils were investigated through three methods, agar well diffusion test, agar disc diffusion test and broth dilution test. However, all three methods did not show any antimicrobial activity. In turn, bacteria were isolated from the oil samples. In the previous studies, scientists used synthetic monolaurin which is in the form of sn-1 monolaurin for antimicrobial testing. It is suspected that the sn-2 monolaurin formed by sn-1, 3 lipase producing bacteria does not exert any antimicrobial activity [32]. Apart from that, the non-specific lipase produced by bacteria will release all the three fatty acids attached to the glycerol backbone. Lauric acids released by both bacteria lipases only possess weak antimicrobial activity and will not be able

to inhibit the growth of bacteria at low concentration [9, 12]. Another possible factor that contributes to this result is the low quality of the coconut source. Once the coconut is harvested, lipolysis will begin through microbial or natural occurring enzymes to produce free lauric acid which possess weak antimicrobial activity.

Four different morphologies of gram negative bacteria were isolated from the oil samples. They are coccus, streptobacilli, bacillus and coccobacillus. Bacteria can be isolated from the samples because raw and unprocessed coconut can support the growth of bacteria and there is no sterilization process done during the whole process of the extraction [35]. Furthermore, the rate of conversion of lauric acid to monolaurin is less than 3% in coconut which is not enough to inhibit the growth of bacteria [38].

Acknowledgements

The authors wish to thank UCSI University for the financial and laboratory support.

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