Preparation of An Effective Antimicrobial Agent from Virgin Coconut Oil

Mi Mi Yee*

Abstract

Virgin coconut oil (VCO) was extracted from locally abundantly available coconut by cold press method. The physicochemical properties VCO were investigated. To synthesize potent antimicrobial agent (monoglycerides of MCFAs that rich in monolaurin), VCO was enzymatically hydrolyzed and MCFAs rich fraction was then separated. By using this fraction monoglycerides of MCFAs was prepared via enzymatic glycerolysis using RM IM immobilized lipase. The MICs of this glyceride were found to be <1.7 μg/mL against *S. aureus* and *P. morganii*.

Keywords: monolaurin, medium chain fatty acids, monoglyeride, antimicrobial agent

Introduction

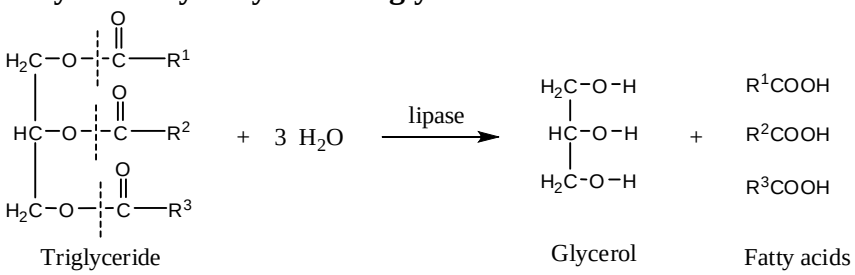
The main aim of present work is to explore cost effective antimicrobial agent (monolaurin) from locally abundantly available coconut oil. Coconut (*Cocos nucifera* Linn) is classified as a "functional food" as it provides many health benefits beyond its nutritional content (Enig, 2004). Coconut is highly nutritious and rich in fiber, vitamins, and minerals. It provides a nutritious source of meat, juice, milk, and oil. Among these, coconut oil is of special interest because it possesses healing properties far beyond that of any other dietary oil and is extensively used in traditional medicine among Asian and Pacific populations. Modern medical science is now confirming the use of coconut may provide a wide range of health benefits (Enig, 1998).

Coconut oil is one of the primary natural products produced from dry fruit (copra) of coconut. Different methods produce different types of coconut oil that their properties are little varied. Virgin coconut oil (VCO) seemed to be the purest form of coconut oil, water white in colour, contains natural vitamin E and with very low free fatty acid content and low peroxide value. It has a mild to intense fresh coconut aroma. VCO may be defined as the naturally processed, chemically-free and additive-free product from fresh coconut meat or its derivative (coconut milk and coconut milk residue) which has not undergone any further chemical processing after extraction. Desiccated coconut and coconut cream contained about 69 % of coconut oil. Full coconut milk is approximately 24 % of oil. Coconut oil has a melting point ranging from 23 to 26 °C. It does not become rancid (oxidation) easily. Coconut oil is a fat consisting of about 90% saturated fat. The oil contains predominantly medium chain triglycerides, with roughly 92% saturated fatty acids, 6% mono unsaturated fatty acids, and 2% polyunsaturated fatty acids. Approximately 50% of the fatty acids in coconut fat are lauric acid (C12:0) and 5-9% are capric acid (C10:0). Other medium-chain triglycerides such as caprylic acid (C8:0) and myristic acid (C14:0) are also present (Handayani *et al*, 2009).

Coconut oil was once mistakenly believed to be unhealthy because of its high saturated fat content, it is now known that the fat in coconut oil is a unique and different from most all other fats and possesses many health giving properties. It is now gaining long overdue recognition as a nutritious health food (Enig, 1998).

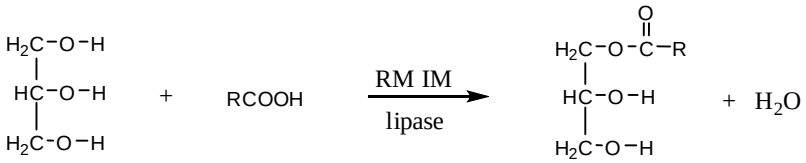
*Dr., Lecturer, Department of Chemistry, Dagon University

Enzymatic Hydrolysis of Triglycerides



Triglyceride Glycerol Fatty acids

Enzymatic Esterification of Glycerol



Glycerol Fatty acid Monoglyceride

Materials and Methods

General

Two lipase enzymes, viz., lipase (*Candida rugosa*) and immobilized lipase (*Rhizomucor miehei*, Lipozyme RM IM), were purchased from Sigma Co. Coconuts (*Cocos nucifera* Linn) were collected from Yankin Township, Yangon Division and used for the extraction of virgin coconut oil.

Extraction of Virgin Coconut Oil (VCO) by Cold Pressed Method

Mature coconuts were dehusked the shell with a sharp knife or a scraper. The shredded meat was pressed at room temperature to get coconut milk and filtered through a cheese cloth to remove the impurities. The coconut milk was separated into four layer parts by centrifugal method. Virgin coconut oil at the top, cream layer at the second, skim milk in third and shredded meat (solid) at the bottom were collected. Water like colour of virgin coconut oil was separated from top layer by decanting.

Determination of Physicochemical Properties of Virgin Coconut Oil

The physical properties such as moisture content, pH, iodine value, saponification value, acid value, peroxide value, viscosity, colour density, and specific gravity of VCO were determined according to AOCS method.

Analysis of Fatty Acid Compositions

The quantitative composition of fatty acids (as methyl esters) was determined by GC-MS at the Universities' Research Center.

Isolation of Medium Chain Fatty Acids from Virgin Coconut Oil

Virgin coconut oil (100 g) was taken in a 250 mL stoppered Erlenmeyer flask and water (60 % wt. of neutral glycerides) containing lipase powder (*Candida rugosa*) was added. The mixture was magnetically stirred at 35 ± 2 °C for 11 h. The degree of hydrolysis was monitored via determining the amount of free fatty acid liberated by titration method during hydrolysis. After reaction completed, the oil layer and water layer containing enzyme and glycerol were separated by centrifugation. The oil layer contained mixture of fatty acids which was subjected to steam distillation. The steam carried volatile fatty acids and was collected as the distillate. This distillate was extracted with pet-ether (40-60 °C) using a separating funnel. PE extract was dried over anhydrous sodium sulphate, filtered and evaporated to get medium chain fatty acid containing fraction. This fraction (**F-I)** was weighed and the yield was recorded. It was then kept in air-tight bottle. The residual fatty acids from the steam distillation were fractionally distilled. The distillate that collected at (100-140 °C) under 4 mm Hg pressure mainly contained medium chain fatty acids (MCFAs). The distillate as fractions (**F-II)** and residual fractions **(R)** were then weighed and the yields were calculated on the basis of VCO.

Enzymatic Esterification of MCFAs with Glycerol

MCFAs (**F-II**) (lauric acid as major constituent) and glycerol (1 : 1.2, mole ratio) were placed into a round bottomed flask. RM IM lipase (10% wt. fatty acids) was added and reaction was carried out at 60 ± 2 °C for 4 h under stirring. The stopper of flask was kept open to eliminate the water during esterification reaction. The progress of reaction was monitored by TLC analysis that was performed on 0.25 mm precoated silica gel (60 F_{254}, Merck) using chloroform : acetone : acetic acid (96 : 4 : 1 v/v). Visualization was done by spraying with 20% sulphuric acid followed by heating at 180 °C for 25 minutes.

Preparation of Monolaurin via Enzymatic Esterification of Lauric Acid with Glycerol

Lauric acid and glycerol (1 : 1.2, mole ratio) were placed into a round bottomed flask. RM IM lipase (10% wt. fatty acids) was added and reaction was carried out at 60 ± 2 °C for 4 h under stirring. The reaction mixture was then cooled to room temperature and poured into distilled water. Then, it was partitioned with equal volume of chloroform for three times. The organic layer was dried over anhydrous sodium sulphate and filtered through filter paper. Evaporation of chloroform under reduced pressure provided colourless plates.

Separation of monolaurin from this mixture was carried out by silica gel column chromatography using chloroform : acetone : acetic acid (96 : 4 : 1 v/v). Quantitative amount of monolaurin was obtained. Characterization of monolaurin was carried out by determination of melting point and measurements of spectra such as FT-IR, NMR and GC-MS.

Screening of Antibacterial Activity

Antibacterial activity was investigated by the agar disc diffusion technique. Screening was done by the use of impregnated paper disc (6 mm). These discs were sterilized by autoclaving and followed by heating at 60 °C for 1 hour. It was then impregnated with concentrated sample (20 μg/disc) and allowed to dry at 42 °C in an oven. The bacterial suspension from trypticase soy broth was streaked evenly onto the surface of the trypticase soy agar plates with sterile cotton swab. After the inoculums had allowed drying for minutes, the

dried discs impregnated with test sample were placed on the agar with a flamed forceps and gently pressed down to ensure proper contact. Two control discs impregnated with solvent only and with clinical drug (tetracycline) were also included. After inoculation, the plates were incubated immediately or within 30 minutes. After overnight incubation at 37°C, the diameters of inhibition zone including 6 mm discs were measured by dial calipers.

Determination of Minimum Inhibitory Concentration

MICs of the active samples were determined by agar plate method. A tube serial dilution technique was applied with little modification (Cruickshank, 1986). The samples to be tested were prepared at different concentration levels, viz. 27.54, 13.77, 6.89, 3.44 and 1.714 μg mL^{-1}. The equal volume of various concentration of test solution was individually place in serial labeled well. After drying the plates, 20 μL of the bacterial suspensions was transferred by using a micropipette and allowed to dry the suspension. The agar plates were incubated at 37 ˚C for 18-24 h. The least concentration of sample showing no growth of bacteria was termed as minimum inhibitory concentration (MIC).

Results and Discussion

Extraction and Properties of Virgin Coconut Oil

Virgin coconut oil was extracted from freshly harvested mature coconut by cold press method. It is a unique production process; from harvesting of coconut final up to VCO, is within 24 hours and this important factor ensures optimal retention of the coconuts natural flavor, natural vitamin E and micronutrient. No heat was used in this process and no fermentation occurred during the process. High process temperature and bacterial contamination of the coconut meat before oil extraction cause the yellow colour of the coconut oil. Therefore, for the coconut oil to be categorized as virgin, its color should be water white. The yield % of VCO in this work was found to be 21 %.

Physicochemical properties of VCO were found to be: moisture content (0.03%), pH (5.3), iodine value (14.21), saponifaction value (345.02), acid value (0.031 mg KOH/g), viscosity (45 cp), colour density (-1.14), peroxide value (0.0014 meq/kg), and specific gravity (0.8730). Elemental analysis was carried out via EDXRF method that indicated the presence of calcium, iron, copper and zinc in VCO.

Fatty acid profile of VCO was investigated by GC-MS. Identification of GC peak was made by comparison of their mass spectra on both columns with those stored in NIST 02 and Wiley 275 Libraries or with mass spectra from literature. Component relative concentrations were calculated based on GC peak areas without using correction factors. The retention time, relative concentration (% area) and identification of each GC peaks are summarized in Table **1**. As represented in the table, VCO was found to be rich in medium chain fatty acids (MCFAs) that got some special importance, as they provide a quick source of energy for infants and stressed adults. MCFAs have several distinguished characteristics, such as high oxidative stability, low melting point and viscosity, and high solubility in water. Not all the coconut oils have the same in quality, and do not all provide the same health benefits.

Table 1. Fatty Acid Profile of Virgin Coconut Oil and MCFAs

Fatty Acids	Relative Concn. (%)	
	VCO	MCFA
Caproic acid (C 6:0)	3.75	-
Caprylic (C 8:0)	20.83	2.27
Capric acid (C 10:0)	18.32	23.64
Lauric acid (C 12:0)	33.75	66.45
Myristic acid (C14:0)	23.19	4.27

Table 2. The Yield of FFA Fractions isolated from Enzymatic Hydrolyzed Coconut Oil

Isolated Fatty Acid Fractions	Yield (%)
F-I (rich in SCFAs)	4.2
F-II (rich in MCFAs)	54.6
R (LCFAs and non-volatile matter)	41.2

SCFAs = short chain fatty acids, MCFAs = medium chain fatty acids, LCFAs = long chain fatty acids

Separation of Medium Chain Fatty Acids and Preparation of 1-Glycerol Ester

Enzymatic hydrolysis of VCO was made so as to obtain MCFA fraction (rich in lauric acid) which was further used as a starting material for the synthesis of monoglycerides (rich in monolaurin). Hydrolysis of VCO provided free fatty acids and glycerol. 98.1% of FFA released from VCO after 11 h reaction.

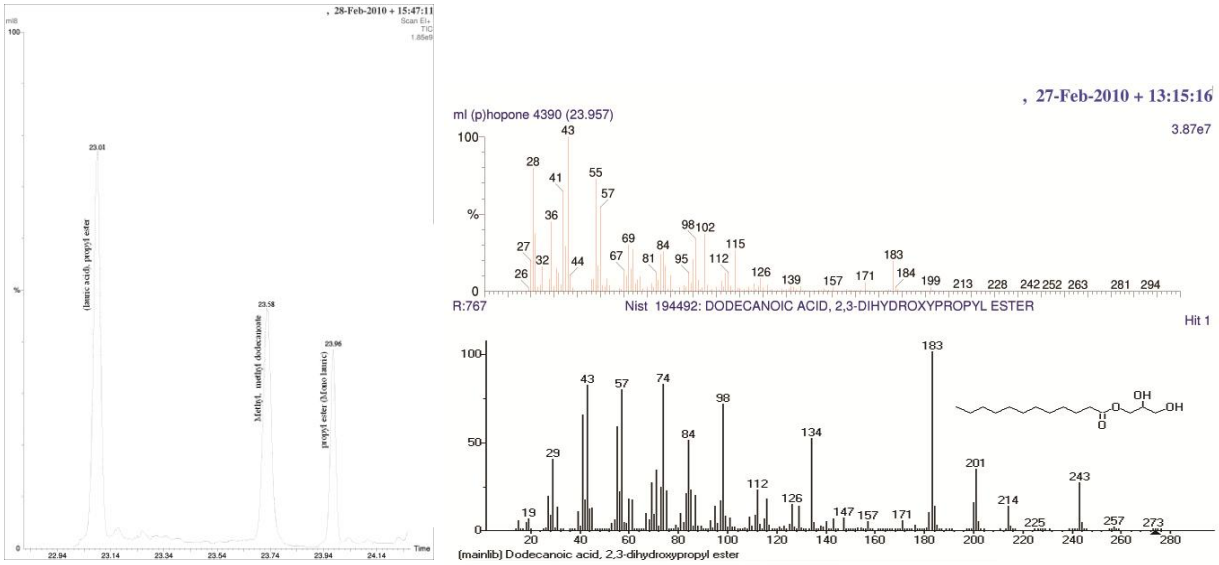
The hydrolyzed oil after removal of glycerol layer was subjected to steam distillation. Steam volatile fraction (F-I) was found to be rich in SCFAs, C 8:0 and C 10:0. Remaining oil was then fractional distilled under reduced pressure to provide fraction II (**F-II**) that was rich in MCFAs especially lauric acid (Table 1). Residual oil (R) consisted mainly of LCFAs. The yields of all isolated fractions were calculated on the wt. basis of VCO and reported in Table 2.

F-II was then used as a starting material to synthesize 1-monoglyceride of MCFA via glycerolysis using RM IM lipase. Glycerolysis was carried out at 60 ± 2 °C for 4 h. Prolong reaction time caused to form diglycerides and triglycerides. The progress of reaction was monitored by TLC. The product obtained from the reaction was found to be rich in monolaurin although small amount other glycerides and fatty acids were contaminated. Figure 1 represents the gas chromatogram of monoglycerides prepared from F-II indicating monolaurin as a major constituent.

Synthesis of Monolaurin

Monolaurin was also synthesized from lauric acid and glycerol so as to thoroughly understand not only the characteristics of glycerolysis but also the properties of monolaurin. In addition, monolaurin isolated via this experiment was used as a standard for further investigation. The progress of reaction was monitored by TLC analysis. Before the start of reaction, only lauric acid on TLC was observed (R_f=0.57). After 2 h, a new spot later identified as monolaurin appeared at R_f = 0.30. No spot corresponding to di- and tri-laurin was observed at this time. When reaction was allowed to proceed for 4 h, a new spot correspond to dilaruin was observed (R_f = 0.84). In addition, trilaurin (R_f = 0.93) formed when reaction was allowed to proceed for 6 h. No difference in spot pattern on TLC was observed between 6 h and 8 h. Both TLC revealed the presence of lauric acid, mono-, di- and tri-laurin in the reaction mixture, in fact, enhance in intensity of spots corresponded to di- and tri-laurin was observed at 8 h. Lauric acid (the starting material) remain present until the reaction was allowed to furnish 8 h. Therefore, reaction mixture was subjected to silica gel column chromatography so as to isolate the major compound.

Monolaurin: M.p. 58-60 °C; FT-IR (KBr): 3302 (υ_{O-H}), 2824, 2864 (υ_{C-H}) 1736 ($\upsilon_{C=O}$); 1466, 1396 (δ_{C-H}), 1180, 1111, 1049 (υ_{C-O}) 725 (ρ_{CH2}); ^{1}H NMR ($CDCl_3$, 600 MHz): 4.15 (*m*, 2H, O=C-O-C**H**$_2$), 3.9 (*m*, 1H, C**H**OH), 3.65, 3.56 (*m*, *m*, 1H, 1H, C**H**$_2$OH), 3.20 (*br*, 1H, CHO**H**), 2.85 (*br*, 1H, CH$_2$O**H**), 2.32 (*t*, 2H, O=CC**H**$_2$), 1.60 (*m*, 2H, O=CCH$_2$C**H**$_2$), 1.26 (*m*, 16H, 8 CH$_2$), 0.85 (*t*, 3H, CH$_3$); ^{13}C NMR ($CDCl_3$, 150 MHz): 174.18 (O=**C**<), 70.23 (**C**HOH), 65.08 (**C**H$_2$O-), 63.36 (**C**H$_2$OH), 34.18 (O=C-**C**H$_2$), 31.92 (**C**H$_2$CH$_2$CH$_3$), 29.62 (2CH$_2$), 29.48 (CH$_2$), 29.35 (CH$_2$), 29.28 (CH$_2$), 24.94 (CH$_2$), 22.71 (-**C**H$_2$CH$_3$), 14.15 (**C**H$_3$).



(a) **(b)**

Figure 1. (a) GC spectrum of monoglyceride prepared from F-II showing monolaurin (at R_t = 23.96 min) as a main constituent. (b) mass spectrum of peak at R_t = 23.95 min (scan 4390) in GC

Antimicrobial Activity of MCFAs and Glycerides derived from VCO

Ten strains of microorganisms used for preliminary test. Two Gram positive bacteria, *S. aureus* and *B. cereus*; seven Gram negative bacteria, *E. coli* (ATCC), *P. morgani*, *K. aeruginosa*, *P. aeruginosa*, *S. flexneri*, *S. flexneri* and *V. cholerae* 0139; and one fungus, *C. albicans*. According to the results presented in Table 3, *S. aureus*, *P. morganii*, and *S. flexneri* were found to be sensitive to test samples. All samples except VCO strongly inhibited *S. aureus* (Gram positive) with inhibition zone diameter ranging 15-20 mm. These samples also effective against Gram negative bacteria, *P. morganii* and *S. flexneri* with 13-21 mm inhibition zone diameter. MIC values of active fractions (Table 4) reveals that MCFAs and its monoglycerides have strongly anti-bacterial activity against Gram negative bacteria (*P. morganii* and *S. flexneri*) and Gram positive bacteria (*S. aureus*).

Table 3. Antimicrobial Activity of MCFA and Monoglycerides derived from VCO against Different Strains of Bacteria (disc diameter 6 mm)

No.	Bacterial Strain	Diameter of Inhibition Zone (mm)				
		VCO	**F-II**	**MG**	**$ML_{(m)}$**	**$ML_{(st)}$**
1.	*Staphylococcus aureus*	-	17	20	15	15
2.	*Bacillus cereus*	-	-	-	-	-
3.	*Escherichia coli* (ATCC)	-	-	-	-	-
4.	*Proteus morganic*	-	17	15	13	13
5.	*Klebsiella aeruginosa*	-	-	-	-	-
6.	*Pseudomonas aeruginosa*	-	-	-	-	-
7.	*Candida albicans*	-	-	-	-	-
8.	*Salmonella flexneri*	-	-	-	-	-
9.	*Shigella flexneri*	-	20	-	-	-
10.	*Vibrio cholerae* 0139	-	-	-	-	-

Table 4. MIC of MCFA and Monoglycerides derived from VCO

Bacterial Strain	MIC (μg/mL)			
	F-II	MG	$ML_{(m)}$	$ML_{(s)}$
Shigella flexneri	<1.714	-	-	-
Staphylococcus aureus	<1.714	<1.714	<1.714	<1.714
Proteus morganii	<1.714	<1.714	<1.714	<1.714

F-II Distillate collected at 100-140 °C and under 4 mm Hg pressure after removal of F-I
MG Monoglycerides obtained via enzymatic glycerolysis of F-II
ML(m) Product mixture obtained via enzymatic glycerolysis of lauric acid
ML(s) Monolaurin obtained via enzymatic glycerolysis of lauric acid followed by purification

Conclusion

Antibiotic resistance has become a worldwide problem for treating infections caused by numerous organisms. Antimicrobial super power of monoglycerides prepared from MCFAs (mainly consists of monolaurin) that derived from coconut oil was observed in this work. Therefore, this material can be considered as an effective alternative antimicrobial agent.

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