

Modified Coconut Oils with Broad Antimicrobial Spectrum

Field of the Invention

This invention relates to modified oil having antimicrobial properties, in particular to modified coconut oil compositions which contain mixtures of medium chain fatty acids having 8 to 12 carbon atoms such as caprylic acid, capric acid, lauric acid and their corresponding monoglycerides. Said modified coconut oil is derived from catalytic activity of 1,3 positional specific lipase on coconut oil.

Background of the Invention

Medium chain free fatty acids and their corresponding monoglycerides have been found to have a broad spectrum of anti microbial activity against enveloped viruses and various bacteria in vitro (Kabara, 1978; Shibasaki and Kato, 1978; Welsh et al. 1979 Thormar et al., 1987; Isaacs et al. 1995), including human pathogens like herpes simplex virus (Thormar et al. 1987; Kristmundsdottir et al. 1999), *Nisseria gonorrhoeae* (Bergsson et al., 1999), *Candida albicans* (Bergsson et al, 2001), *Chlamydia trachomatis* (Bergsson et al., 1998), *Helicobacter pylori* (Bergsson et al, 2002) and *Staphylococcus aureus* (Kabara 1984). In addition, these compounds also known to have antimicrobial effect against food-borne pathogens like *Listeria monocytogenes* (Wang and Johnson, 1992), enterotoxigenic *Escherichia coli* (Petschow et al 1998) and *Clostridium botulinum* (Glass and Johnson, 2004). The mechanism by which these lipids kill bacteria is not known, but electron microscope studies indicate that they disrupt cell membrane permeability barrier (Bergsson et al, 1998; Thormar et al. 1987).

Saturated medium chain fatty acid are fatty acids that have 8 to 12 carbon atoms (C_6 to C_{12}). Among the medium chain fatty acids, lauric acid and its corresponding monoglyceride, monolaurin, has been investigated extensively as an antimicrobial agent for foods and cosmetics (Shibasaki and Kato, 1978; Kabara, 1984). Lauric acid is a disease fighting agent that it is present in breast

milk. The body converts lauric acid to a fatty acid derivative (monolaurin), which is the substance that protects infants from viral, bacterial or protozoal infections. Hierholzer and Kabara (1982) showed that monolaurin has virucidal effects on RNA and DNA viruses, which are surrounded by a lipid membrane.

5 The use of medium chain fatty acids and its corresponding monoester as anti microbial compound in several applications had been patented. i.e. monolaurin have been used in cleansing and conditioning hairs as well as the coat of animals (US Patent 5,378,731). This antimicrobial shampoo composition containing monolaurin which is safe for human and animal use. Monolaurin
10 can also widely employed in toiletries and household articles, which need antifungal properties (US Patent 5,569,461 and 5,658,584). In addition its can be combined with bacteriocin i.e. nisin for the treatment of bacterial infections of the genus *Helicobacter* that cause various gastrointestinal disease including gastritis and ulcer (US Patent 5,660,842 : US Patent 5,804,549). These fatty
15 acids and their derivative thereof were also claims as a dietary supplement that controlling or reducing human's weight. (US Patent 6,054,480). Additionally these compounds and their monoester can be used to reduce the microbial contamination of processed meat and is particularly related to a product and a process to disinfect poultry carcasses. Moreover its help to kill harmful
20 microbes on the under of a milk-producing animal (US Patent 6,699,907). They are also effective course of treatment for skin, mucous membrane and hair lesion (US Patent 5,208,257). The latest invention showed that with these fatty acids could be a therapeutic agents for the treatment of Alzheimer's disease and other diseases associated with reduced neuronal metabolism, including
25 Parkinson' disease, Huntington's disease and epilepsy (US Patent 6,835,750)

Previous works have shown that susceptibility to medium chain fatty acids varies considerably among species, certain microbes were sensitive to certain fatty acids and monoglycerides. For example, Bergsson et. al 1998 showed that lauric acid, capric acid and monocaprin caused a greater than 10,000-fold
30 reduction in the infectivity titer. When the fatty acids and monoglycerides were

further compared at low concentration and shorter exposure times, lauric acid was more active than capric acid and monocaprin, causing a greater than 100,000 fold inactivation of *C. trachomatis* at a concentration of 5 mM for 5 min. Compare with monocaprin, monolaurin and monocaprylin at 10 mM concentration had negligible effect on *C. trachomatis*. In other worked done by Bergsson et al. (2002) found that none of the medium chain fatty acids (C_8 , C_{10} and C_{12}) and its monoglycerides derivatives showed significant antibacterial activity against *Salmonella* spp and *E. Coli*. However, in this experiment they found out that monocaprin and monolaurin at 10 mM concentration for 30 min at 37°C proved to be the most active against *H. pylori*. Incorporation of monolaurin at 250 and 500 ppm into naturally contaminated cottage cheese was resulted in more than 90% inhibition of both *Pseudomonas* spp and coliform (Bautista et al., 1993). In the case of *C. albicans*, capric acid and lauric causes the fastest and most effective killing compare with its derivatives monoglycerides (Bergsson et al. 2001). However, myristic acid, palmitoleic acid, oleic acid and its derivates monoglycerides do not shown antimicrobial effect.

It seems that the ability of these medium chain fatty acids and their monoglycerides to act as antimicrobial agents are varied. Effectiveness of these compounds are always depending on their concentrations and the type of microbes involved. So far, test for antimicrobial activity of medium chain fatty acids and their corresponding monoglycerides were tested individually. Test was performed either using the lauric or capric or caprylic acid or their corresponding monoglycerides. The synergistic effect of both medium chain fatty acids and their respective monoglycerides in oil medium never being conducted and reported.

Summary of the Invention

It is therefore an object of the invention to provide for modified coconut oil derived form catalyzing coconut oil with 1,3 -specific lipase, said modified coconut oil contains effective amounts of medium-chain fatty-acids and their

corresponding monoglycerides, wherein said modified oil has antimicrobial properties. Said modified coconut oil having medium-chain fatty-acids comprises of caprylic acid (C₈), capric acid (C₁₀) and lauric acid (C₁₂).

Detailed Description of the Invention

5 In accordance with the object of the invention, efforts have been done in this work to get profiles of the modified coconut oil that contain medium chain fatty acids (special emphasis on C₈-C₁₂) and its respective monoglycerides which have antimicrobial activities towards bacteria, yeast, fungi and viruses. Coconut oil was selected because by nature it was rich source of medium chain fatty
10 acids. It contains 90% saturated fatty acids, and of these, 45-48% is lauric acid and 30-36% are other short- and medium chain fatty acids. Preliminary work showed that coconut oil in the present form don't have antimicrobial activities (contains 7% diacylglycerol and remaining is the triglycerides). Analysis by HPTLC showed that fatty acids and monoglycerides are detected in the oil
15 compositions. The invention also includes methods using 1,3 positional specific lipase to obtain not only oils that have antimicrobial properties but also effective and powerful antimicrobial agents.

According to the present invention 1,3 positional specific lipases is used to modify the coconut oil under specific reaction conditions to obtain profiles of
20 saturated medium chain fatty acids and their respective monoglycerides that have broad antimicrobial spectrum towards bacteria and yeast. The modified coconut oil mixtures preferably contain high amount free fatty acids (C₈- C₁₂) and their corresponding monoglycerides that can be obtained through partial hydrolysis or glycerolysis reaction. The enzyme used in the present invention is
25 an enzyme such as lipase, and preferably immobilized onto a suitable enzyme carrier. The said specific lipase possess 1,3 -position i.e. Lipozyme TL IM (*Rhizomucor miehei*). Reactions to obtain modify oils were as followed.

Reaction 1- Modified 1 and Modified 2

Enzymatic reaction was carried out using 2.5g 1,3- positional specific lipases with 250g coconut oil and 2.5 ml distilled water. The reaction was conducted at 45 °C at 250 r.p.m. Samples were withdrawn for analysis after 24 h reaction (Modified 1) and 120h reaction (Modified 2). Samples were then passed through funnel containing sodium sulfate powder to remove water form the sample. The reaction mixture was centrifuged to separate the oil phase. High performance thin layer chromatography technique and gas chromatography technique were carried out to determine the lipid classes and fatty acids compositions of the oil sample respectively. The modified oils were then analyzed for their antimicrobial activities of following Test 1: Minimal Microcidal Concentration (MMC, > 90%) and Test 2: Time-kill studies

Reaction 2- Modified 3

Twenty ml of coconut oil was added into a 125 ml flask containing 8 g glycerol and 160ul of distilled water. The reaction mixtures were incubated at 35°C, 300 r.p.m for a time until the incubation temperature reached 35°C. Lipozyme TL IM at 250 mg was then added into the reaction mixture and incubated for 24h at 35°C. Consequently, the oil sample was then subjected at 25°C for another 3days.

Reaction 3- Modified 4

A mixture of 30.4g glycerol, 1.09 ml of water, 1g of Lipozyme TL IM and 100g of coconut oil was prepared. The reaction mixture was first incubated at 30°C for 6 h with constant stirring at 800 r.p.m. The mixture was then transferred to 5°C for up to 3 days before analyze.

Reaction 4- Modified 5

The reaction mixture was prepared according to Modified 4. The reaction was carried out at 30°C, 800 r.p.m. for 16 h. before analyze.

Reaction 5- Modified 6

Twenty ml oil from Modified 2 was added into a mixture containing 8 g glycerol, and 160µl sterile distilled water. The reaction mixture was pre incubated at 35°C , 300 r.p.m. The reaction was initiated by adding 250 mg Lipozyme TL IM into the mixture and reaction was carried for 24h. Consequently the mixture was then incubated at 25°C for 3 days

Test 1: Minimal Microcidal Concentration (MMC, > 90% kill)

All well were inoculated with 120µl broth (BHI, contain 0.1% Tween 80, for Gram-positive bacteria; TSB for Gram-negative bacteria; PDB for yeast). 120µl of antimicrobial agent was inoculated into first well. From first well, 120µl of the mixture was transferred into the second well and so on until the 12th well. Inoculum which was adjusted to 10^5 - 10^6 cfu/ml was inoculated into each well. The plates were incubated at 37°C (2 days) for bacteria and 32°C (3 days) for yeast. Results were expressed in terms of MMC₉₀ (minimal bactericidal concentration, >90% killing) as shown in Table 1.

Test 2: Time-kill studies

Inocula were developed by inoculation of a loopful cell in 50ml broth (BHI for Gram-positive bacteria; TSB for Gram-negative bacteria; PDB for yeast) in flask and shaken at optimum temperature overnight. This was used to inoculate BHI, TSB or PDB broth that contained 50% of filtered sterilized treated VCO. The initial inoculum was adjusted to 10^4 - 10^6 cfu/ml. At time interval, 1ml of reaction mixture was withdrawn and serial dilution was carried out in ringer solution. Viable colony was enumerated by plating dilutions on plate count agar (PCA). The plates were incubated at 37°C (2 days) for bacteria and 32°C (3 days) for yeast. A control experiment was done in the presence of 50% sterile distilled water.

Table 1: Minimum Microcidal Concentration (MMC₉₀) of modified virgin coconut oils against pathogenic microorganisms

	MBC, mg/ml	Strain					
		<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>C. albicans</i>	<i>E. coli</i>	<i>S. pyogenes</i>	<i>P. acne</i>
5	Untreated Virgin Coconut Oil	none	none	none	none	none	none
	Modified 1	156.25	156.25	156.25	none	156.25	none
	Modified 2	78.13	78.13	78.13	none	78.13	none
10	Modified 3	4.88	4.88	2.44	2500	4.88	none
	Modified 4	78.13	78.13	2.44	2500	78.13	none
	Modified 5	78.13	78.13	4.88	2500	78.13	none
	Modified 6	4.88	4.88	2.44	2500	4.88	none

Table 2: Inhibition of pathogenic microorganisms by modified virgin coconut oils evaluated by time-kill studies

		Microbes	Modified Virgin Coconut Oil	Number of viable bacteria (log10 cfu/ml) at time interval (hours)						
				0	2	4	6	8	24	48
5			Modified 1	5.69	5	3.6	3.11	2.62	0	0
		<i>S. aureus</i>	Modified 2	6.32	0	0	0	0	0	0
			Modified 3	6.27	0	0	0	0	0	0
			Modified 4	6.04	4.25	0	0	0	0	0
10			Modified 5	6.08	3.96	0	0	0	0	0
			Modified 6	5.87	0	0	0	0	0	0
			Modified 1	5.71	5.02	3.62	3.15	2.66	0	0
		<i>L. monocytogenes</i>	Modified 2	6.28	0	0	0	0	0	0
			Modified 3	6.15	0	0	0	0	0	0
15			Modified 4	6.06	4.25	0	0	0	0	0
			Modified 5	6.08	3.76	0	0	0	0	0
			Modified 6	5.34	0	0	0	0	0	0
			Modified 1	4.59	4.61	4.54	4.44	4.29	2.81	2.71
			Modified 2	6.48	5.56	5.48	5.65	5.38	2.16	2
20		<i>C. albicans</i>	Modified 3	5.76	4.98	3.21	3.45	3.11	0	0
			Modified 4	5.98	5.65	5.15	4.18	3.46	0	0
			Modified 5	6.02	5.68	5.43	4.75	3.86	0	0
			Modified 6	5.98	4.13	3.06	2.99	2.14	0	0
			Modified 1	ND	ND	ND	ND	ND	ND	ND
25		<i>E. coli</i>	Modified 2	6.32	6.48	7.52	8.34	9.16	10.27	11.16
			Modified 3	6.21	6.54	5.82	5.96	4.32	2.58	2.69
			Modified 4	6.01	6.26	5.64	5.28	5.04	2.96	2.71
			Modified 5	6.02	6.15	6.01	5.78	5.44	2.84	2.56
			Modified 6	6.14	5.28	5.35	6.02	4.67	2.64	2.63
30			Modified 1	5.48	5.21	4.23	3.08	2.12	0	0
			Modified 2	6.1	0	0	0	0	0	0
		<i>S. pyogenes</i>	Modified 3	5.7	0	0	0	0	0	0
			Modified 4	6.01	4.68	0	0	0	0	0
			Modified 5	6.04	4.74	0	0	0	0	0
35			Modified 6	5.49	0	0	0	0	0	0
		<i>V. cholerae</i>	Modified 2	6.34	0	0	0	0	0	0

Table 3: Inhibition of pathogenic microorganisms by modified palm kernel oil evaluated by kill studies

5	Microbe	Number of viable bacteria (log cfu/ml) at time interval (hours)						
		0	2	4	6	8	24	48
	<i>S. aureus</i>	7.28	7.24	7.18	7.18	5.31	5.85	0
	<i>E. coli</i>	6.93	7.30	10.76	12.10	13.56	10.22	8.56
	<i>C. albicans</i>	5.71	6.03	5.92	5.95	5.87	5.98	5.74

10 The following profiles of modified virgin coconut oils and palm kernel oil have been tested for their antimicrobial activities and found to be active against a substantial group of microorganisms

MODIFIED 1- Profile 1

	Lipid Classes	Percentages (% area by HPTLC)	Medium chain length	Concentration (mg/g)
15	Fatty acids	10.58	Caprylic acid (C ₈)	10.44
			Capric acid (C ₁₀)	7.27
			Lauric acid (C ₁₂)	54.61
20	Monoglycerides	1.32	Monocaprylin	0.67
			Monocaprin	0.42
			Monolaurin	20.96
	Diglycerides	22.81		
	Triglycerides	65.29		

MODIFIED 2- Profile 2

	Lipid classes	Percentages (% area by HPTLC)	Medium chain length	Concentration (mg/g)
5	Fatty acids	14.13	Caprylic acid (C ₈)	13.95
			Capric acid (C ₁₀)	9.89
			Lauric acid (C ₁₂)	70.54
10	Monoglycerides	1.51	Monocaprylin	1.30
			Monocaprin	0.50
			Monolaurin	27.00
	Diglycerides	26.88		
	Triglycerides	57.48		

MODIFIED 3-Profile 3

	Lipid Classes	Percentages (% area by HPTLC)	Medium chain length	Concentration (mg/g)
15	Fatty acids	23.40	Caprylic acid (C ₈)	23.10
			Capric acid (C ₁₀)	16.08
			Lauric acid (C ₁₂)	116.81
20	Monoglycerides	14.28	Monocaprylin	16.04
			Monocaprin	10.35
			Monolaurin	75.54
	Diglycerides	37.24		
	Triglycerides	25.08		

MODIFIED 4- Profile 4

	Lipid Classes	Percentages (% by HPTLC)	Medium length	chain	Concentration (mg/g)
5	Fatty acids	13.44	Caprylic	acid	13.35
			(C ₈)		9.34
			Capric acid (C ₁₀)		68.61
			Lauric acid (C ₁₂)		
10	Monoglycerides	8.04	Monocaprylin		8.00
			Monocaprin		5.40
			Monolaurin		41.10
	Diglycerides	31.36			
	Triglycerides	47.16			

MODIFIED 5- Profile 5

15	Lipid Classes	Percentages (% area by HPTLC)	Medium chain length	Concentration (mg/g)
20	Fatty acids	9.40	Caprylic acid (C ₈)	9.40
			Capric acid (C ₁₀)	6.56
			Lauric acid (C ₁₂)	71.77
	Monoglycerides	12.10	Monocaprylin	12.25
25			Monocaprin	8.05
			Monolaurin	71.77
	Diglycerides	39.98		
	Triglycerides	38.47		

MODIFIED 6- Profile 6

	Lipid Classes	Percentages (% area by HPTLC)	Medium length chain	Concentration (mg/g)
5	Fatty acids	25.01	Caprylic acid (C ₈)	24.63
			Capric acid (C ₁₀)	17.81
			Lauric acid (C ₁₂)	133.70
10	Monoglycerides	11.45	Monocaprylin	11.82
			Monocaprin	8.29
			Monolaurin	57.16
	Diglycerides	35.98		
	Triglycerides	27.56		

Modified Palm Kernel Oil

	Lipid Classes	Percentages (% area by HPTLC)	Medium length chain	Concentration (mg/g)
15	Fatty acids	12.87	Caprylic acid (C ₈)	16.10
			Capric acid (C ₁₀)	13.06
			Lauric acid (C ₁₂)	58.19
20	Monoglycerides	1.31	Monocaprylin	1.64
			Monocaprin	2.03
			Monolaurin	19.71
	Diglycerides	21.66		
	Triglycerides	64.16		

25 According to the invention, methods to obtain antimicrobial compositions containing i.e. medium chain fatty acids and their corresponding monoester through partial hydrolysis and glycerolysis of coconut oil are provided. Modified oil 1 and Modified oil 2 were obtained from hydrolysis reaction of virgin coconut oil at 24 h and 120h, respectively. Test for antimicrobial

activities as MMC₉₀ (Table 1) and time-kill studies (Table 2) showed that the Modified 2 have more powerful antimicrobial activities compare to Modified 1. This is probably because of high amount of fatty acids (14%) present in Modified 2 (Profile 2). The amount of saturated medium chain fatty acids especially caprylic, capric and lauric (mg/g) oil were increased (Profile 2). However, it was noticed that modified oil compositions from profile 1 and profile 2 can't stopped the growth of *E. coli* and *P. acne*. Obviously, from the time-kill studies *C. albicans* growth was not 100% inhibited even after 48h incubation (Table 2). Modified 1 and Modified 2 were effective toward gram positive bacteria where 100% inhibitions were noted after 8 h and 2 h incubation, respectively. Preliminary results indicated that the amount fatty acids i.e. C₈, C₁₀ and C₁₂ might play important role in inactivation of the growth of gram positive bacteria. Modified 3, 4, 5 and 6 were prepared differently from Modified 1 and 2. They were obtained through glycerolysis reactions. In all reactions glycerol and Lipozyme TL IM were added.

In comparison, Modified 3, 4, 5 and 6 were more effective in killing *C. albicans* than Modified 1 and 2. Hundred percent inhibitions were noted after 8 hours incubation. Minimal Microbial Concentration towards *C. albicans* was obtained at 2.44 mg/ml whereas Modified 5 need higher concentration e.g. 4.88 mg/ml. Among the modified oil samples, Modified 3 and Modified 6 contained powerful antimicrobial activities. In addition it was noted that MMC₉₀ for gram positive bacteria like *S. aureus*, *L. monocytogenes*, *S. pyogenes* of Modified 3, 4, 5 and 6 were lower than the Modified 1 and 2. The most interesting thing is that these modified coconut oils have antimicrobial activity towards the gram negative, *E. coli* although the MMC₉₀ required is still higher compare to the gram positive bacteria. Modified 3 and 6 were also proved to be more potent toward gram positive bacteria compare to Modified 4 and 5. Detail analysis of the lipid classes of the Modified 3, 4, 5 and 6 showed that high amount of fatty acids and monoglycerides content play important role in broaden the antimicrobial spectrum of these modified oils. About 8% to 14% monoglycerides were required to control and reduce the growth of *E. coli* and

100% inhibition of *C. albicans*. Whereas high amount of fatty acids in Modified 3 and 6 make the modified oils more potent towards gram positive bacteria (Profile 3 and Profile 6). The MMC₉₀ for all gram positive bacteria for Modified 3 and 6 were at 4.88 mg/ml which are sixteen times lower than that of
5 Modified 4 and 5 . Fatty acids together with monoglycerides have a synergistic effect that inhibited 100% growth of *C. albicans* .

Palm kernel oil, which fatty acid composition similar to coconut oil, can also be modified using 1,3 specific lipase. The modified palm kernel oil was also found to have antimicrobial property towards *S. aureus*. Total inhibition of *S.*
10 *aureus* was noted after 24 h exposure. On the other hand it was noted that the amount of medium chain fatty acid and it corresponding fatty acids in the modified palm kernel oil didn't significant to stop the growth or kill the *E. coli* and *C. albicans*

Modified oils compositions in the present invention were found to exhibit good
15 shelf stability against oxidation and can be safely used to combat bacteria , yeast and viruses that affect human, as food preservatives, products for personal hygiene and prevention of skin infection.

As will be apparent to those skilled in the art in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of
20 this invention without departing from the scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the following claims.

CLAIMS

1. Treated virgin coconut oil comprises 24.63 mg/g caprylic acid (C₈), 17.8 mg/g capric acid (C₁₀), 133.70 mg/g lauric acid (C₁₂) and 11.8 mg/g monocaprylin, 8.29 mg/g monocaprin and 57.16 mg/g monolaurin wherein said treated virgin coconut oil completely inhibits the growth of gram-positive bacteria such as *S. aureus*, *L. monocytogenes* and *S. pyogenes* within 2 hours incubation time.
2. Treated virgin coconut oil according to Claim 1 wherein said treated virgin coconut oil reduces about 40% of the growth of gram-negative bacteria such as *E. coli* within 24 hours incubation time.
3. Treated virgin coconut oil according to Claim 1 wherein said treated virgin coconut oil completely inhibits the growth of yeast such as *C. albicans* within 24 hours incubation time.
4. Treated virgin coconut oil comprises 23.10 mg/g caprylic acid (C₈), 16.8 mg/g capric acid (C₁₀), 116.81 mg/g lauric acid (C₁₂) and 16.04 mg/g monocaprylin, 10.35 mg/g monocaprin and 75.54 mg/g monolaurin wherein said treated virgin coconut oil completely inhibits the growth of gram-positive bacteria such as *S. aureus*, *L. monocytogenes* and *S. pyogenes* within 2 hours incubation time.
5. Treated virgin coconut oil according to Claim 4 wherein said treated virgin coconut oil reduces about 40% of the growth of gram-negative bacteria such as *E. coli* within 24 hours incubation time.
6. Treated virgin coconut oil according to Claim 4 wherein said treated virgin coconut oil completely inhibits the growth of yeast such as *C. albicans* within 24 hours incubation time.

7. A pharmaceutical composition for treatment or prevention of skin diseases, comprising the treated virgin coconut oil according to Claim 1 or Claim 4.
8. A food preservative composition comprising the treated virgin coconut oil according to Claim 1 or Claim 4.
9. A personal hygiene product formulated with or incorporating as part of the formulation of said personal hygiene product the treated virgin coconut oil according to Claim 1 or Claim 4.
10. An antibacterial agent composition comprising the treated virgin coconut oil according to Claim 1 or Claim 4 wherein the dosage form is a capsule.
11. A pharmaceutical composition for preventing disease in animal caused by bacteria and yeast, comprising the treated virgin coconut oil according to Claim 1 or Claim 4 as a natural antibiotic agent.