International Journal of Phytopharmacology

Research Article

www.onlineijp.com

e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

MINIMUM INHIBITORY CONCENTRATION OF SAPONIFIED VIRGIN COCONUT OIL AGAINST *STREPTOCOCCUS MUTANS*

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ABSTRACT

The increase of drug resistant pathogens produces challenges to the successful treatment of microbial diseases, including tooth decay. *S.mutans*, the major etiological agent of dental caries are resistant to many antibiotics commonly used to treat infections. Natural products could offer an effective alternative to antibiotics and represent a promising approach to prevention and therapeutic strategies for various oral infections. Virgin Coconut oil(VCO), is the oil resulting from the fresh and mature kernel of the coconut has captured the attention of public recently. VCO is predominantly made up of lauric acid and studies have proven that lauric acid possess antimicrobial activity against *S.mutans*. The anticariogenic effect of the saponified VCO which contains the salts of all the fatty acids such as Lauric acid, Caprylic acid, Capric acid, Myristic acid, Palmitic acid, Stearic acid, Oleic acid and Linoleic acid has not been studied. Hence this study is aimed to evaluate the antimicrobial activity of fatty acid salts of Virgin coconut oil and determination of minimum inhibition concentration of the saponification product of Virgin Coconut oil against *Steptococcusmutans.*Saponification of Virgin Coconut oil was done to obtain the potassium salts of all fatty acids in VCO. Minimum inhibitory concentration was determined using broth microdilution method. Fatty acid salts of VCO exhibited antibacterial effect even at low concentrations.Fatty acid salts of VCO can be used for different formulations of oral care agents against *S.mutans*.

Key words: Dental caries, *S.mutans*, Virgin coconut oil, Antimicrobial, Broth microdilution, Oral care agents.

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INTRODUCTION

Dental caries is the most common and diffuse oral infectious disease, wide spread over the world in every segment of the population (Marsh PD, 2003). The major etiological players are thought to be the two α-haemolytic 'mutans group' streptococci; *Steptococcus mutans* and *S.Sorbinus*, although several other types of bacteria (notably lactobacilliandactinomyces) may also be involved (Hardie JM and Whiley RA, 1999). *S.mutans* is a facultative anaerobic cocci which is generally found in the human oral cavity. This bacteria has the ability to bind to tooth surfaces and produce lactic acid in the presence of fermentable sugars which contributes to corrosion of enamel (Bowden GH, 1990). Targeting *Streptococcus mutans* is the most important measure for prevention of dental caries. Numerous new antimicrobial agents have been introduced in the last decade resulting in the evolution of drug resistance by microorganisms (Sakoulas G and Moellering RC, 2008; Kohanteb J *et al.*, 2007). The increase of drug resistant pathogens produces challenges to the successful treatment of microbial diseases, including tooth decay. Natural products which are safe, non-toxic, and cost effective are gaining popularity these days because many of them have pharmacological

Access this article online		
DOI: http://onlineijp.com/ DOI: http://dx.doi.org/10.21276/ijp.2018.9.3.2		Quick Response code
Received:14.02.18	**Revised**:22.02.18	**Accepted**:01.03.18

properties, such as antimicrobial, anti-inflammatory and cytostatic effects. Herbal extracts are used in dentistry for treatment of various dental disorders. Natural phytochemicals could offer an effective alternative to antibiotics and represent a promising approach to prevention and therapeutic strategies for various oral infections. *S.mutans* are resistant to many antibiotics commonly used to treat infections and are thus prime candidates for targeted investigations of natural products which are suspected to have antimicrobial properties.

In the present study, the effect of saponified Virgin coconut oil on *S.mutans* was evaluated.Virgin Coconutoil (VCO)is defined as the oil resulting from the fresh and mature kernel of the coconut (*Cocosnucifera* L.) through mechanical and natural means, either with the use of heator not, provided that it does not lead to alteration or transformation of the oil (Asian and Pacific Coconut Community(APCC) 2003). VCO has many advantages, which include the health benefits from the retained vitamins and antioxidants, the antimicrobial and antiviral activity from the lauric acid components and through its easy digestibility from the medium chain fatty acids (MCFA). VCO is predominantly made up of lauricacid. The total lauricacidall concurred with the APCC (2003) standard for VCO, Codex (2001) standard for coconut oil (45.10 – 53.20%) (Marina AM and Che MI, 2009; Dayrit F M *et al.*, 2007).

Generally, lipids found in virgin coconut oil are in the form of triglycerides. Triglycerides do not possess antimicrobial activity (Preuss HG *et al.*, 2005; Petschow BW *et al.*, 1996). The antimicrobial activity of medium chain triglycerides is due to the combined effect of free fatty acids such as lauric acid and its monoglyceride, monolaurin (Nevin KG and Rajamohan T, 2006; Hughes PI, 2013; Chiaw MS, 2010). Hence, direct usage of Virgin Coconut oil to inhibit the bacterial growth may not be as effective as the use of monolaurin. Virgin Coconut oil is completely non-toxic to humans, and is referred to as the "drugstore in a bottle". Virgin Coconut oil is growing in popularity as functional food oil and the public awareness of it is increasing. Coconut imbibing a tremendous potential deserves a special attention of the scientific fraternity to emerge as a milestone for medical science of this millennium due to its various medicinal uses (Mandal M and Mandal S, 2011). So far in literature, studies have not been conducted to test the anticariogenic effect of the saponified VCO which contains the salts of all the fatty acids such as Lauric acid, Caprylic acid, Capric acid, Myristic acid, Palmitic acid, Stearic acid, Oleic acid and Linoleic acid. Hence this study is aimed at determination of minimum inhibition concentration of the saponification product of Virgin Coconut oil against *Steptococcus mutans.*

MATERIAL AND METHODS

Saponification of Virgin Coconut oil

Commercially available cold pressed Virgin Coconut oil- KLF Nirmal(10 grams oil) was taken in a round bottom flask to which 25 ml of 0.5 N methanolic potassium hydroxide was added. The mixture was placed in a water cool condenser and then placed on a heating mantle. The mixture was boiled by heating the mantle at 80ºC for one hour (Jansen S *et al.*, 2014) (Figure 1).

Mixture of fatty acids in Virgin coconutoil

The potassium salts of all fatty acids in Virgin Coconut oilsuch as Lauric acid, Caprylic acid, Capric acid, Myristic acid, Palmitic acid, Stearic acid, Oleic acid and Linoleic acid were obtained. (Figure 2)

MIC Determination –Broth Microdilution method

Pure strain of *Streptococcus mutans* (MTCC 890) was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. The bacterium (lyophilized culture) was in a vial and revival of pure strain was done by suspending in Brain Heart infusion broth.The strains were cultured on MitisSalivarius Bacitracin (MSB) agar plates. An overnight culture of bacterial inoculum in Brain heart infusion broth was standardized to 0.5 McFarland standard ($1.5*10^8$ CFU/ml) using a nephlometer.

The product obtained by saponification of Virgin coconut oil (mixture of potassium salts of fatty acids) were serially diluted to get the final concentrations of 1%, 0.5%, 0.25%, 0.12%, 0.06%, 0.03%, 0.01%, 0.008%, and 0.004%. 100 µl of test and control were added to 100 µl of bacterial inoculum and premixed since premixing helps in equal distribution ofthe components properly without any precipitation.200 µl of the premixed bacterial culture with test and control was added into each of the wells in 96 well micro titreplate. Chlorhexidine was taken as the positive control, broth with bacterial inoculum as blank control and plain broth as negative control. The micro titre plate was incubated anaerobically at 37^0C for 24 hours in an incubator shaker at 50 rpm/min. After 24 hours, microplate reader (Parkin-Elmer multiplate reader) was used to calculate the final optical density of different solutions in each of the wells. The degree of reduction in bacterial turbidity in the test and control wells were obtained by measuring the optical density at 600 nm. The Minimum inhibition was calculated according to the formula

$$\text{MIC in \%} = \frac{\text{Net OD of the culture-Net OD of the drug}}{\text{Net OD of the culture}} \times 100$$

RESULTS

The different concentrations of a mixture of fatty acid salts of VCO tested were 1%, 0.5%, 0.25%, 0.12%, 0.06%, 0.03%, 0.01%, 0.008%, and 0.004% and the mean OD values were 0.05, 0.027, 0.012, 0.028, 0.029, 0.102, 0.213, 0.302and 0.318 respectively(Table 1).

The first five concentrations of a mixture of fatty acid salts in VCO (i.e. 1%, 0.5%, 0.25%, 0.125% and 0.0625%) showed almost more than 90% inhibition of *S.mutans*. The lowest concentration of the product to show a sharp decline in the absorbance was 0.313 mg/ml (0.0313%) which showed an inhibition of 68.69% of bacterial growth and an absorbance value of 600nm. The mean percentage inhibition for 0.2% CHX (Chlorhexidine) was 94.28.This shows that higher concentrations of a mixture of fatty acid salts in VCO are comparable to the effect of 0.2% Chlorhexidine. Lower concentrations of the product such as 0.0156%, 0.0078% and 0.0039% showed 33.76,6.43,1.49 percentage reduction respectively.

MIC was determined by analysing the 50% percentage inhibition of bacterial growth using Prism software (Figure 4).

MIC of a mixture of fatty acid salts in VCO was estimated to be 0.02215% .i.e. 0.221mg/ml. At this concentration, a visible decrease in the bacterial turbidity was first observed indicating a 50% inhibition of bacterial growth. Hence, 0.02% (0.22 mg/ml)was taken as the MIC of saponified VCO.

Table 1. Mean Optical Density (OD) Values of different concentrations of a mixture of fatty acid salts in VCO against *S.mutans*

Bacterial broth (Positive control)	0.2% CHX (Drug positive control)	Test concentrations of a mixture of fatty acid salts in VCO								
		0.0039%	0.0078%	0.0156%	0.0313%	0.0625%	0.125%	0.25%	0.5%	1%
0.315	0.018	0.318	0.302	0.213	0.102	0.029	0.028	0.012	0.027	0.05

CHX-ChlorhexidineThe mean percentage inhibition of bacterial growth by each concentrations of a mixture of fatty acid salts in VCO and 0.2% Chlorhexidine was calculated(Table 2 and fig 3)

Table 2. Mean percentage inhibition of *S.mutans* growth by each concentration of a mixture of fatty acid salts inVCO and 0.2% Chlorhexidine

0.2%CHX	0.0039%	0.0078%	0.0156%	0.0313%	0.0625%	0.125%	0.25%	0.5%	1%
94.28	1.49	6.43	33.76	68.69	90.57	91.17	96.07	92.05	83.59

CHX-Chlorhexidine

Fig 1. Saponification of Virgin Coconut oil

Fig 2. Saponification Product

Fig 3. Dose dependent percentage inhibition of S.mutans growth by varying concentrations of a mixture of fatty acid salts in VCO with standard error

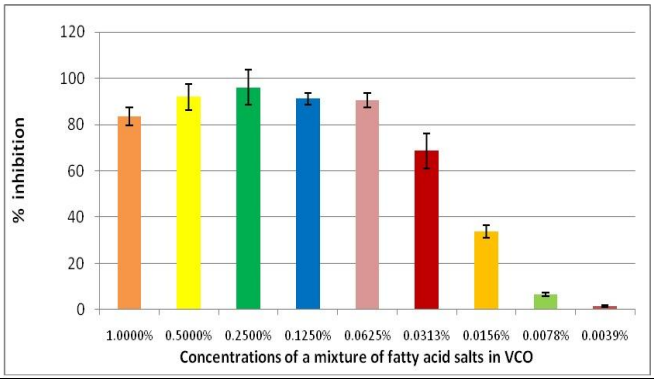
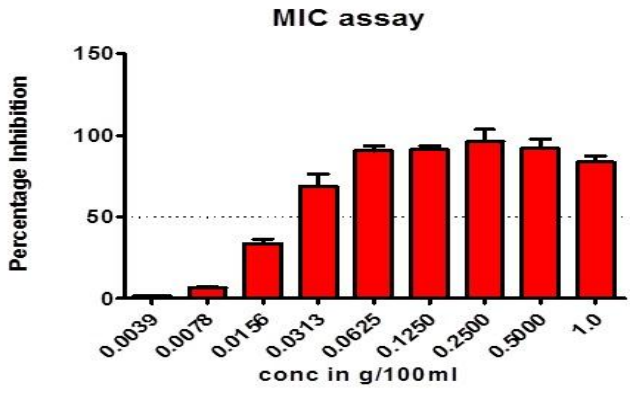


Fig 4. MIC Assay



DISCUSSION

Pure strain of *S.mutans* (MTCC 890) was the test organism for the antimicrobial assay due to its cariogenic properties. It produces three types of glycosyltransferase (GTFB, GTFC and GTFD) which polymerize the glycosyl moiety from glucose and starch carbohydrates into α1, 3- and α1, 6-linked glucans. Binding of glucans to glucan binding proteins initiates bacterial adherence to tooth surfaces and leads to biofilm formation. This eventually leads to plaque formation, and upon exposure to sugar, there will be acid production which subsequently causes decalcification and cavitation of the enamel surface. Hypothetically, the reduction of *Streptococcus mutans* in the oral cavity should reduce the risk of caries occurrence (Tanzer JM *et al.*, 2001).

Virgin coconut oil is obtained by cold press processing of the kernel from the fruit of the coconut tree. The major fatty acid in virgin coconut oil is lauric acid (C12, 54.61%).The other fatty acids present in VCO are Lauric acid, Caprylic acid, Capric acid, Myristic acid, Palmitic acid, Stearic acid, Oleic acid and Linoleic acid. Previous studies have proven that VCO as such do not possess antimicrobial activity, whereas the medium chain fatty acid, lauric acid which is predominant in VCO possesses certain antibacterial and antiviral activities (Enig MG, 1996; Enig MG, 1998). The minimum inhibition concentration of lauric acid against *S.mutans* is 0.16-20M. Gram positive bacteria such as Streptococci are particularly susceptible to the antibacterial activities of fatty acids in VCO in comparison to Gram negative bacteria (Bergsson G et al., 2001).

An in vitro study conducted by Masuda et al has proved that the fatty acid salts such as potassium laurate (C12K), potassium linoleate (C18:2K), and potassium linolenate (C18:3K) have high antibacterial activity against *S. mutans*, and possess great potential as antibacterial agents. The antibacterial efficacy of fatty acid salt mixtures against *S.mutans* has also been tested and found that the addition of one fatty acid to another fatty acid does not affect its antimicrobial action (Pavia DL, 2004). Although the mechanisms by which fatty acids exert their antimicrobial activity is not fully understood, the disruption of the bacterial plasma membrane of lipid bilayer wherein the permeability of the bacterial cell is compromised has been suggested (Huang CB *et al.*, 2010; Kabara JJ *et al.*, 1972). Ababouch et al stated that the saturated fatty acid, lauric acid contain the optimal properties of water solubility and lipophilicity. Therefore, potassium laurate can easily adsorb onto the surface of cells and effectively act against *S. Mutans* (Ababouch L *et al.*, 1992). However, studies have not been done with a formulation using fatty acid salts in VCO as a chemotherapeutic agent.

The minimal inhibitory concentration (MIC) was determined by a broth microdilution method (CLSI, 2002).Broth dilution is one of the most sensitive and reliable method for the determination of MIC of oily extracts (Panos C, 2001). Solvent was not used in this MIC assay as the saponified VCO contains free fatty acid salts which were soluble in water without forming precipitate. The MIC was recorded as the lowest concentration that limited the turbidity of the broth to<0.05 at absorbance of 600 nm.The 0.2% Chlorhexidine showed 94.28% inhibition of bacterial growth in this study. These results indicate that a mixture of fatty acid salts possess antibacterial action against *S.mutans*. Hence it possess great potential as an oral care agent (mouthwash or other plaque controlling agents). Improving the oral cavity flora by removing gum disease bacteria and opportunistic organisms is of more significance than inhibiting all bacteria.

CONCLUSION

The results of this study demonstrated that fatty acid salts of VCO such as Lauric acid, Caprylic acid, Capric acid, Myristic acid, Palmitic acid, Stearic acid, Oleic acid and Linoleic acid have antimicrobial activity against *S.mutans*even at very low concentrations.These findings support the consideration of a strategy to use combinations of fatty acids as a potential therapeutic adjunct.So further research has to be carried out to deliver this product as oral health care agents against dental caries.

ACKNOWLEDGEMENT AND FUNDING

Nil

CONFLICTS OF INTEREST

There are no conflicts of interest

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