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COMPARATIVE EVALUATION OF ANALYTICAL STANDARD COCONUT OIL AND VIRGIN COCONUT OIL WHEN INCORPORATED IN TISSUE CONDITIONER ON CANDIDA ALBICANS: AN IN VITRO STUDY

Dr. Preeti Satheesh Kumar*

Professor, Dept of Prosthodontics, The Oxford Dental College and Research Centre, Bangalore.

***Corresponding Author: Dr. Preeti Satheesh Kumar**
Professor, Dept of Prosthodontics, The Oxford Dental College and Research Centre, Bangalore.

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ABSTRACT

Aim: The emergence of antimicrobial resistance, coupled with the availability of fewer antifungal agents with fungicidal actions, prompted this present study to determine the effectiveness of coconut oil as an antifungal agent on these species. **Materials & methods**: Lyophilized culture of Candida albicans was inoculated in Tryptone Soya Broth (TSB) and was incubated overnight at 37°C. The analytical standard Cocos nucifera oil and commercially available virgin coconut oil were primarily screened for their antifungal activities by performing minimum inhibitory concentration (MIC) against C. albicans ATCC 24433. The lowest concentration of the oil needed to inhibit microbial growth compared to the positive control culture was considered as MIC. The results were observed after 48 hours of incubation at 37°C. MIC of analytical standard coconut oil against Candida albicans was observed to be 20%. MIC of commercially available coconut oil against Candida albicans was observed to be 100%, undiluted oil. Analysis of the the inhibition diameters (IDs) of eight concentrations (% v/v) of analytical standard Cocos nucifera oil in tissue conditioner (n = 20 each) revealed that the range of mean inhibitory diameter (MID) for different concentrations was between 5 and 16.55 mm at 48 hours. The ANOVA followed by post hoc test showed that even at the end of 7 days, 20% concentration was significantly [P value (0.000)] more effective than all other concentrations tested. **Conclusion:** Analytical grade coconut oil is active in killing C. albicans. More studies should be done to ascertain the mechanisms of actions of medium chain fatty acids on microorganism generally and their susceptibility pattern.

KEYWORDS: Tissue conditioner, coconut oil, Candida albicans, anti fungal, minimum inhibitory concentration.

INTRODUCTION

Tissue conditioners have been commonly used to enhance the recovery of denture-bearing tissues from trauma, damage or residual ridge resorption usually caused by ill-fitting dentures. However, these materials degenerate with time, are easily degradable and occasionally are susceptible to colonisation by microorganisms.[1] Candida-associated denture stomatitis has been found in 60-65% of the subjects carriers of prosthesis[2] with more diffused clinical manifestations, but considering also the subjects that do not manifest clinical signs of inflammation, the percentage increases to 75% of the population bearers of prosthesis. Candida albicans has been shown to be the principal Candida strain responsible for inflammatory pathology[3,4] isolated from 93% of patients with denture stomatitis.[5]

The treatment of Candida-associated denture stomatitis is complex because of its multifactorial etiology.[6] The therapeutic strategy adopted includes the use of topical and systemic antifungal drugs, the use of preservatives and disinfectants, the irradiation with microwaves and the scrupulous removal and control of the plaque present on the denture and on the oral mucosa.[2,7]

The most commonly used antifungal drugs are amphotericin B, nystatin, miconazole and fluconazole. Since the 1980s, the imidazoles have become increasingly more popular. Miconazole and ketoconazole have been found to be effective in in-vitro denture liners, but they are more expensive and the toxicity of ketoconazole is a problem.[8] Rapid relapse, resistance and cross resistance between the azoles have also been reported, particularly in association with immunosuppressed individuals.[8,9] The antifungal activity of nystatin in liners has been shown to decrease with time, and that the in vitro fungicidal activity is proportional to the concentration of nystatin administrated. The aim of antimycotic treatment is to reduce the acute candidal overgrowth to levels that can be controlled by the host's defences. It has been reported that nystatin does not cure denture stomatitis and

recolonization of the yeast occurs after cessation of drug therapy.[5]

The increased resistance to antifungal agents is the main clinical complication that is associated with biofilm formation. Antimicrobial resistance is a common phenomenon in cells recovered from biofilms. Increased resistance of C. albicans biofilms grown on denture acrylic to fluconazole, amphotericin B, nystatin and chlorhexidine has already been demonstrated.[10] Furthermore, it has been found that C. albicans cells resuspended from a biofilm typically maintain some degree of resistance to antimicrobials compared to planktonic cells, even after the biofilm has been disrupted.[5,7]

Treatment methods should therefore be directed towards reducing initial fungal attachment and subsequent biofilm development on denture acrylic to reduce the incidence and severity of this condition. Chemically used antimycotic drugs have side effects regarding toxicity, development of fungal resistance, lack of fungicidal efficacy, drug-drug interactions, high cost. Therefore, there is a need to isolate new antifungal agents, mainly from plant extracts, with the goal of discovering new chemical structures without the above disadvantages.

Systemic administration of drugs may not be that effective against candidal infection because the organism usually limits its activity to the oral mucosa. Also, the success of topical application of drugs in the oral cavity may be compromised by the copious flow of saliva as well as by the lack of patient compliance. Therefore, antifungal agents can be incorporated in tissue conditioners to simultaneously treat injured peri-prosthetic tissues and infection by Candida.[11]

Certain herbal medicines known as phytotherapeutic agents have also been shown to have antibacterial, antifungal, and antiviral activity.[11] The major advantages of natural medicinal plant extracts as antimicrobial agents include enhanced safety and stability without any side effects, which are lacking in conventional antifungal agents. The antimicrobial activity shown by plant oils is mainly due to a number of phenolic and terpenoid compounds, which have antibacterial or antifungal activity.[5,7,11,12,13] Several studies using plant essential oils have been carried out. Essential oils like Tea tree oil, Lemon grass oil, Citronella oil, and some of their constituents have been tested against the in vitro growth of C. albicans and found to be effective against candida.[14]

Coconut oil (Cocos nucifera) is one such phytotherapetic agent that is useful for the development of medicines against various diseases because of its antimicrobial and antioxidant property.[15] Due to its wide applications, there have been many researches and investigations focusing on a variety of its applications, among which is its antimicrobial activity first shown by Prof. John Kabara in the 1970s. His research has shown/established that coconut oil has broad spectrum antimicrobial activities including antibacterial, antiviral and anti fungal.[16,17,18,19]

No reported studies in the literature to ascertain the antimicrobial effect of Cocos nucifera (Coconut oil) in treating denture stomatitis have been undertaken. This study therefore aims to determine the effectiveness of coconut oil as an antifungal agent and to study the effect of Cocos nucifera incorporated into tissue conditioner and evaluate its efficacy against C. albicans. There are varieties of coconut oil available in the market, so, to analyze if both share the anti fungal property, this study also has evaluated the efficacy of analytical grade coconut oil (Sigma Aldrich) and commercially available coconut oil.

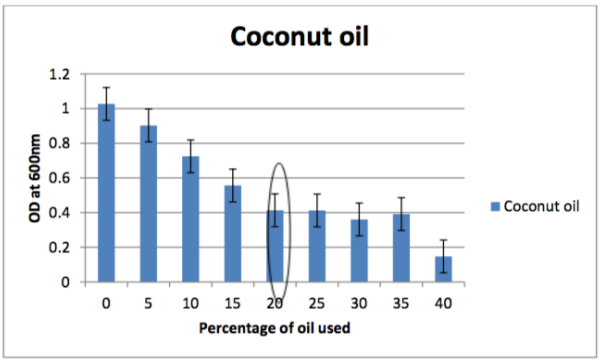
MATERIALS AND METHODS

A lyophilized culture of Candida albicans was inoculated in Tryptone Soya Broth (TSB) and was incubated overnight at 37°C. The turbidity of the culture was adjusted to 0.5 McFarland standards at 600nm. The analytical standard Cocos nucifera oil and commercially available virgin coconut oil were primarily screened for their antifungal activities by performing minimum inhibitory concentration (MIC) against C. albicans ATCC 24433. The lowest concentration of the oil needed to inhibit microbial growth compared to the positive control culture was considered as MIC. For the analytical standard Cocos nucifera oil, in a 96-well plate, 150µl of media was added to which 1.5 µl of culture and 7.5 µl of different concentrations of the oil were added. (Table 1)

Table 1: Micro broth dilution test.

Composition	Coding for Cocos nucifera oil
0% oil + 150 µl Media+ 1.5µl Culture	C-Positive Control
5% oil + 150 µl Media+ 1.5µl Culture	C-1
10% oil + 150 µl Media+ 1.5µl Culture	C-2
15% oil + 150 µl Media+ 1.5µl Culture	C-3
20% oil + 150 µl Media+ 1.5µl Culture	C-4
25% oil + 150 µl Media+ 1.5µl Culture	C-5
30% oil + 150 µl Media+ 1.5µl Culture	C-6
35% oil + 150 µl Media+ 1.5µl Culture	C-7
40% oil + 150 µl Media+ 1.5µl Culture	C-8
40% oil + 150 µl Media + 0µl Culture	C-Negative Control

The results were observed after 48 hours of incubation at 37°C. MIC of analytical standard coconut oil against Candida albicans was observed to be 20%. (Graph 1)



Graph 1: MIC of Analytical Standard Cocos nucifera (Coconut oil).

Similarly, Minimum Inhibitory Concentration of commercially available virgin coconut oil was calculated by Micro broth dilution Method using different test concentrations (100, 50, 25, 12.5, 6.25, 3.125, and 1.56%) in 96 well plates in triplicate. The results were observed after 48 hours of incubation at 37°C. MIC of commercially available coconut oil against Candida albicans was observed to be 100% undiluted oil.

Based on the preliminary screening results for MIC, tissue conditioner samples were prepared to evaluate the zone of inhibition.

Group 1 specimen preparation: This served as the control group. 20 specimens of Visco gel conditioner were prepared according to manufacturer's recommended powder-liquid ratio. Any incomplete specimens or that containing voids were discarded and new specimens were made to replace them.

Group 2 specimen preparation: Different concentrations of analytical standard Cocos nucifera oil (Sigma Adrich) were measured in a micropipette and mixed with the conditioning liquid of the tissue conditioner. Each subgroup of a particular concentration contained 20 specimens each. Cocos nucifera oil and the tissue conditioner liquid were homogenized in a sterile glass beaker for 30 seconds. This liquid was mixed with the tissue conditioner powder immediately and coated.

The study was conducted strictly aseptically using standard barrier technique donning sterile latex gloves, face mask, and cap. All laboratory procedures, including plating of agar plates, were carried out in a laminar airflow cabinet hood.

Preparation of Tissue Conditioner Samples

Different volumes of the oil were added to the monomer of self cure tissue conditioner separately in a measuring beaker with demarcations to achieve the respective concentrations in v/v.

5 parts oil with 95 parts monomer, 10 parts oil with 90 parts monomer, 15 parts oil with 85 parts monomer, 20 parts oil with 80 parts monomer, 25 parts oil with 75 parts monomer, 30 parts oil with 70 parts monomer, 35 parts oil with 65 parts monomer, 40 parts oil with 60 parts monomer were mixed to get concentrations of 5, 10, 15, 20, 25, 30, 35 and 40 % v/v respectively. Liquid monomer without the oil (Control) and with different concentrations of the oil was then mixed with the powder in the ratio suggested by the manufacturer to formulate the autopolymerized tissue conditioner samples. Tissue conditioner samples each with a thickness of 3mm were then applied to one side of the heat cured denture base resin.

Zone of Inhibition (ZOI)

Diluted C. albicans solution (0.5 ml) was dropped on each sterile Tryptone Soya agar plate and a lawn culture was made. Three wells (7-mm deep, 5 mm in diameter) were created in each agar plate for all concentrations of materials to be tested. A sterile wooden stick was used to carry the antifungal and tissue conditioner mixtures into the punch holes in the inoculated petri-plates. Plates were incubated at 37◦C for 7 days. Significant antifungal effect was looked for in all the plates with the objective of determining the minimum most effective concentration. Mean inhibition diameter (MID) for each test punch hole was measured in millimeters across the punch hole at 48 hours and on day 7 using a metal graduated ruler. (Figure 1)

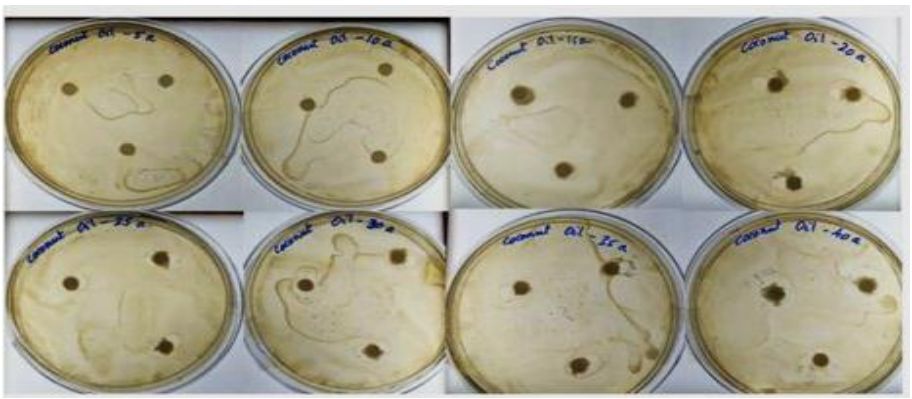


Fig 1: Zone of inhibition.

Statistical Analysis

Antifungal activity was evaluated using MID as the parameter at 48 hours and on day 7 while carrying out the monitoring every day. The minimum most effective concentration of the oil was assessed using one way Analysis of variance (ANOVA) and post hoc test. A 5% significance level was used for all tests.

RESULTS

Analysis of the inhibition diameters (IDs) of eight concentrations (% v/v) of Cocos nucifera oil (Analytical standard coconut oil) in tissue conditioner (n = 20 each) revealed that the range of mean inhibitory diameter (MID) for different concentrations was between 5 and 16.55 mm at 48 hours. 40% analytical standard Cocos nucifera /coconut oil-tissue conditioner samples showed the highest MID (16.55 mm). (Table 2)

Table 2: Analysis of the inhibition diameters (Zones of inhibition) of eight concentrations (% v/v) at 48 hrs.

Concentration (in %)	Sample size	Mean (mm)	Std. Deviation	F value	P Value
5	20	5.0000	.00000	1460.0	0.000
10	20	5.2000	.41039		
15	20	7.2000	.61559		
20	20	11.0000	.64889		
25	20	12.3000	.47016		
30	20	13.7500	.44426		
35	20	14.4000	.50262		
40	20	16.5500	.68633		

However, the percentage increase in the mean inhibitory diameter from 15% to 20% concentration was the highest (152.78%). The ANOVA followed by post hoc test showed that at 48 hours, 20% concentration was significantly [P value (0.000)] more effective than all other concentrations tested. (Table 3)

Table 3: Analysis of the inhibition diameters (Zones of inhibition) of eight concentrations (% v/v) at 48 hrs.

Concentration	5%	10%	15%	20%	25%	30%	35%	40%
Mean (mm)	5.00	5.20	7.20	11.00	12.30	13.75	14.40	16.55
Percentage of increase	100.0	104.0	138.46	152.78	111.82	111.79	104.73	114.93

It was also noted that except for the pair of 5% and 10% concentration, the MID of all other pairs of concentration levels was significantly different. (Table 4)

Table 4: Analysis of the inhibition diameters (Zones of inhibition) of eight concentrations (% v/v) at 48 hrs.

Concentration (%)	Mean Difference						
	5	10	15	20	25	30	35
10	0.200						
15	2.200*	2.000*					
20	6.000*	5.800*	3.800*				
25	7.300*	7.100*	5.100*	1.300*			
30	8.700*	8.550*	6.550*	2.750*	1.450*		
35	9.400*	9.200*	7.200*	3.400*	2.100*	0.650*	
40	11.550*	11.350*	9.350*	5.550*	4.250*	2.800*	2.150*

At the end of 7 days, it was noted that only for the 10% concentration, the MID decreased to 5 mm due to the regrowth of the fungus. For all other concentrations, even though there was a decrease in the MID of respective concentrations, it was maintained within the same range suggesting that the antifungal activity was observed even at the end of 7 days. The range of mean inhibitory diameter (MID) for different concentrations was between 5 and 16.2 mm at the end of 7 days. 40% Cocos nucifera /coconut oil showed the highest MID (16.2 mm). (Table 5).

Table 5: Analysis of the inhibition diameters (Zones of inhibition) of eight concentrations (% v/v) at 7th day.

Concentration (in %)	Sample size	Mean (mm)	Std. Deviation	F value	P Value
5	20	5.0000	.00000	1675.11	0.000
10	20	5.0000	.00000		
15	20	6.5000	.51299		
20	20	9.7000	.65695		
25	20	11.8000	.41039		
30	20	13.3000	.47016		
35	20	13.1500	.36635		
40	20	16.2000	.69585		

However, the percentage increase in the mean inhibitory diameter from 15% to 20% concentration was the highest (149.23%). The ANOVA followed by post hoc test showed that even at the end of 7 days, 20% concentration was significantly [P value (0.000)] more effective than all other concentrations tested. (Table 6) Except for the pairs of 5% and 10% concentrations and 30% and 35% concentrations, the MID of all other pairs of concentration levels was significantly different. (Table 7)

Table 6: Analysis of the inhibition diameters (Zones of inhibition) of eight concentrations (% v/v) at 7th day.

Concentration	5%	10%	15%	20%	25%	30%	35%	40%
Mean (mm)	5	5	6.5	9.7	11.8	13.3	13.15	16.2
Percentage of increase	100.0	100.0	130.00	149.23	121.65	112.71	98.87	123.19

* - significant at 5% level

Table 7: Analysis of the inhibition diameters (Zones of inhibition) of eight concentrations (% v/v) at 7th day.

Concentration (%)	Mean Difference						
	5	10	15	20	25	30	35
10	0.000						
15	1.500*	1.500*					
20	4.700*	4.700*	3.200*				
25	6.800*	6.800*	5.300*	2.100*			
30	8.300*	8.300*	6.800*	3.600*	1.500*		
35	8.150*	8.150*	6.650*	3.450*	1.350*	-0.150	
40	11.200*	11.200*	9.700*	6.500*	4.400*	2.900*	3.050*

It was also noted that the control group did not exhibit any significant antifungal activity against C. albicans as compared to the different concentrations of the Cocos nucifera /Analytical standard coconut oil with Visco-gel tissue conditioner.

DISCUSSION

Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing. In dilution tests, microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) or in broth (broth dilution) containing dilutions of the antimicrobial agent.[19,20] The lowest concentration of an antimicrobial agent (in mg/L) that, under defined in vitro conditions, prevents the appearance of visible growth of a microorganism within a defined period of time, is known as the MIC. The MIC is a guide for the clinician to the susceptibility of the organism to the antimicrobial agent and aids treatment decisions.[19,20,21,22]

A variety of laboratory methods can be used to evaluate or screen the in vitro antimicrobial activity of an extract or a pure compound. The most known are the disk-diffusion and broth or agar dilution methods.[23] Hence Broth Dilution methods[21,22,23] were used to determine the MIC and Agar disk diffusion method was used to record ZOI. Disk-diffusion assay offers many advantages over other methods: simplicity, low cost, the ability to test enormous numbers of microorganisms and antimicrobial agents, and the ease to interpret results provided.[23]

The antimicrobial agent diffused from the disc into the medium and the growth of the test organism was inhibited at a distance from the disc that is related (among other factors) to the susceptibility of the organisms. Strains susceptible to the antimicrobial were inhibited at a distance from the disc whereas resistant strains have smaller zones of inhibition or grow up to edge of the disc. Following incubation, the agar plate was examined for zones of inhibition (areas of no growth) surrounding the discs. Zone of inhibition indicates antimicrobial activity against the organisms. Absence of zone of inhibition indicates that the antimicrobial was ineffective against the test organisms or the organisms are resistant to the antimicrobial.[24] It was found that inhibition against Candida albicans was exhibited when 20% Cocos nucifera/ Analytical standard

coconut oil was present in the media. Hence the MIC determined for the analytical grade coconut oil was 20 % v/v. This was less than that when Ogbolu,et al conducted a study on C. albicans and found coconut oil with a minimum inhibitory concentration (MIC) of 25% concentration. This could be because of the difference in the composition of the coconut oil. The MIC of the commerically available coconut oil was 100% undiluted oil and it did not exhibit a Zone of inhibition.

The results of the present microbiologic in vitro study suggest that 20 % v/v analytical grade coconut oil (Sigma Aldrich) when mixed with Visco-gel showed substantial antifungal activity, resulting in complete inhibition of C. albicans growth after 24 hours ($p < 0.001$). This phenomenon could be due to maximum effect for the concentration of the drug and/or an inhibitory effect at higher drug concentrations. Thus, 20% coconut oil in Visco-gel was the minimum most effective concentration and was considered for further comparison. An important finding of the study was the continued, unabated antifungal effect even at the end of 7 days. 20% concentration was significantly [P value (0.000)] more effective than all other concentrations tested implying that its antifungal effect remains active within its tissue conditioner polymeric structure for at least 7 days.

Most tissue conditioners have maximum effect between 24 and 72 hours. Graham et al reported that tissue conditioners continue to flow for 7 days and suggested that they are clinically effective throughout this period. The study time parameters of 48 hours and day 7 were therefore decided upon for the study.[11,25]

Visco-gel, which is a commonly used tissue conditioner, was used in the study. It is an elastomer based on polymethymethacrylate with a plasticizer. Tissue conditioners are short-term soft liners made from amorphous polymers, formed in situ from a mixture of a polymer powder and a liquid plasticizer.[11,24] Kanathila et al,[26] Thomas et al.[27] observed that Viscogel alone was completely inert and, therefore, would not be beneficial without antifungal agents in the treatment of denture stomatitis. In a study conducted by Shino et al[16] coconut oil has shown antifungal activity that is comparable to that of ketoconazole.

Coconut oil[24] is a potent nondrug or natural yeast fighter, which contains three medium chain fatty acids, all of which have antibacterial and antifungal effect against lipid coated bacteria such as staphylococcus species and fungi such as Candida spp. Medium-chain free fatty acids have been found to have a broad spectrum of microbicidal activity though the mechanisms by which the lipids kill bacteria is not known, but electron microscope studies indicate that they disrupt cell membranes.[11,14,24,28]

The three most valuable medium chain fatty acids in coconut fat are lauric acid (C12:0), capric acid (C10:0), and caprylic acid (C8:0). Lauric acid may reach a proportion of upto 50% in coconut oil and is the Medium chain fatty acid with the highest potential effect on harmful microorganisms.[29]

Variations in composition, plant, and genetic disparity among bacteria and fungi of the same or different species have been found to be responsible for the few inconsistencies in the antibacterial and antifungal properties of plant extract.[18]

Virgin Coconut oil obtained by cold pressing on the other hand does not show a zone of inhibition against candida albicans. This is in accordance with the study conducted by Nguyen et al[30] who concluded that virgin coconut oil and hydrolyzed virgin coconut oil did not show any antibacterial property. This was attributed to the absence of pure Medium chain fatty acid used in the previous studies, which were used in the form of pure chemical.[30] They concluded that hydrolyzed virgin coconut oil used did not show antibacterial ability even though it might contain monoglyceride, which could only be present in a small amount, not enough to inhibit bacteria. The findings of this study was also in accordance with that found by Tangwatcharin et al[31] who also concluded that virgin coconut oil did not possess antimicrobial potential and was not active against S. aureus.

Anzaku[24] conducted a study on the antimicrobial activity of coconut oil and its derivative (Lauric Acid) and concluded that organisms showed resistance to coconut oil at the various dilution concentrations and lauric acid exhibited appreciably high antimicrobial activity in some clinical isolates than others and the zones of inhibition varied based on their dilution concentration, declining as the concentration decreased. They recommended use of lauric acid in treating some of the emerging and re-emerging diseases as well as improving health status.

Since, the present study was performed under controlled laboratory conditions therefore, in-vivo studies are suggested for more precise results. It is also apparent that it is the medium chain fatty acids, especially lauric acid that has the antimicrobial potential. Further studies on quantifying the amount of fatty acids will allow for using coconut oil in routine anti fungal therapy.

CONCLUSION

It can thus be said, through this study, that analytical grade coconut oil (Sigma Aldrich) is active in killing C. albicans. More studies should be done to ascertain the mechanisms of actions of medium chain fatty acids on microorganism generally and their susceptibility pattern.

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