

In-Vitro antibacterial activity of glass ionomer cements containing silver nanoparticles synthesized from leaf extract of *Mentha piperita*

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Abstract

PURPOSE: The aim of this study was to evaluate the antibacterial activity of glass ionomer cement incorporated with silver nanoparticles (AgNPs) synthesized using mint leaf extract (*Mentha piperita*, *M. piperita*) on some oral cavity bacteria.

MATERIALS AND METHODS: In the present study, *M. piperita* leaf extract was used for the synthesis of AgNPs. A total of 60 glass ionomer cement (GIC) disk-shaped specimens were prepared and divided into two groups: conventional GIC (C-GIC), and glass ionomer cement with 2 wt% AgNPs (GIC-AGNPs). The antibacterial activity of the GIC specimens in comparison with Ampicillin disk (10 µg/ml) was investigated against *Streptococcus mutans*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Streptococcus aureus* by measuring the diameter of growth inhibition zones.

RESULTS: C-GIC specimens failed to show any antibacterial effect against the studied bacteria. However, the GIC-AgNPs had relatively significant antibacterial effects on *S. mutans*, *L. acidophilus*, *L. casei* and *S. aureus*. The highest antibacterial effect of GIC-AgNPs specimens was reported against *L. acidophilus* (P <0.001). GIC-AgNPs had no antibacterial effect on *E. faecalis*.

CONCLUSION: Glass ionomer cement incorporated with AgNPs synthesized using *M. piperita* showed a promising antibacterial effect against oral cariogenic pathogens.

KEYWORDS: Antibacterial activity; Glass ionomer cement; *Mentha piperita*; Silver nanoparticles

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Introduction

The number of people who seek orthodontic treatment is increasing. Based on the 2012 report of the American Association of

Orthodontists (AAO), the number of adults who underwent orthodontic treatment increased about 39% compared to the previous report in 1996 [1]. The impact of orthodontic

treatment on oral health is not evident because of the lack of high-quality published evidence [2]. Some researchers believe, orthodontic treatment is a strong risk factor for

dental caries [3, 4]. The reason for that is the accumulation of dental plaque around fixed appliances, subsequent enamel demineralization, and the occurrence of dental caries [5- 7]. Orthodontic treatment has the potential to change the composition of dental plaque. It has been revealed that a large number of *Pseudomonas* species exist in normal oral microflora of orthodontic patients [8]. It has been said that orthodontic treatment is a risk factor for dental caries especially in younger patients and responsible for 45.8% of newly formed caries lesions [9]. Despite the above-mentioned facts, some studies support the improved ability of orthodontic patients to maintain oral hygiene [10] and show that in the presence of good oral hygiene, orthodontic treatment would not be a risk factor for dental caries [11]. Therefore, there is little agreement on the relationship between orthodontic treatment and dental caries and while debate continues in this regard, it seems logical to apply methods and materials which lessen the caries activity to the fewest possible extent. Coating of materials with antimicrobial nanoparticles is one of the proposed methods to reduce caries activity during orthodontic treatment. Incorporation of nano Cinnamon powder in orthodontic resin [12], wires coated with AgNPs [13], nanoparticles of silver and nitrogen-doped TiO₂ applied on orthodontic brackets [14], incorporation of arginine to

commercial Orthodontic light-cured resin cements [15], and nanosilver mouthwash [16] are examples of promising modifications for improvement of orthodontic treatment. Attempts also have been made to modify orthodontic cement such as glass ionomer with nanoparticles to enhance the antibacterial properties of the cement. Glass Ionomer Cement with TiO₂ nanoparticles showed a significant antibacterial effect on *S. mutans* [17]. The incorporation of N-acetylcysteine (NAC) into nanosilver-containing resin-modified glass ionomer cement revealed favorable antibacterial capability and biocompatibility [18]. The rechargeable nano-CaF₂ orthodontic cement showed promising results in inhibiting white spot lesions in orthodontics [19].

Silver nanoparticles (AgNPs) possess a great and long-lasting antibacterial activity [20] and are subjected to less bacterial resistance than antibiotics [21]. Dental materials which have been modified with nanosized silver particles (NAg) show effective antibacterial properties [18]. There are various techniques for synthesizing AgNPs, including laser ablation, microwave-polyol, sonochemical, sonoelectrochemical, microwave dielectric heating, solvothermal, and electrochemical which most of them are expensive and produce toxic chemicals [20]. In recent years there has been an increasing tendency and interest

toward the green synthesis of AgNPs as a clean and ecofriendly synthesis technique [20]. This technique which is based on the usage of bio-organisms, plant leaves, and fruit extracts, is cost-effective and fast and lessens the risk of toxicity [20, 22]. Green synthesis of Ag nanoparticles using plants is highly preferable since plants are more resistant to metal toxicity and offer a truly green alternative for AgNPs synthesis [23]. AgNPs synthesized with *Mangifera indica* leaves [20], and gum arabic [24] showed promising results against oral pathogens. *M. piperita* leaf extracts have been shown to have antimicrobial properties against oral pathogens [25]. Biosynthesis of AgNPs using mint leaf extract (*M. piperita*) revealed antibacterial activity against pathogenic bacteria such as *Escherichia coli* and *S. aureus* [26]. *M. piperita* is a natural support for AgNPs that showed antifungal activity against *Candida albicans* [27]. To the best of our knowledge, there was no report on green synthesis of AgNPs using *M. piperita* leaf extract for enhancement of antibacterial properties of glass ionomer cement, therefore, this study aimed to evaluate the antibacterial activity of glass ionomer cement incorporated with AgNPs synthesized using mint leaf extract (*M. piperita*) on *S. mutans*, *L. acidophilus*, *L. casei*, *E. faecalis* and *S. aureus* bacteria.

Material and Methods

Materials

Silver nitrate (AgNO₃) (99%) and Mueller-Hinton agar (MHA) were purchased from Merck Company, Germany. Conventional Glass Ionomer Cement (GIC) (powder/liquid form) supplied by Fuji Pharma, Japan. *M. piperita* leaves were obtained from a local provider (Birjand, South Khorasan province, Iran). The 10 µg/ml Ampicillin disk was purchased from MAST Company, UK. Five standard strains of bacteria (ATCC bacterial strains) were purchased from Pasteur Institute (Tehran, Iran).

Preparation of Plant Extract

In the present study, *M. piperita* leaf extract was used for the synthesis of AgNPs based on its ease of availability, cost-effectiveness, and medicinal properties. The fresh and healthy leaves of *M. piperita* were washed with distilled water and air-dried at room temperature. *M. piperita* leaf aqueous extract was prepared (50 g of finely powdered leaves in 500 ml double-distilled water) at 60 °C for 10 minutes. The extract was cooled down and filtered with Whatman filter papers. Finally, the filtered extract was centrifuged at 8000 rpm for 20 minutes, and the supernatant was collected and stored at 4 °C for further use.

Biosynthesis and Characterization of Silver Nanoparticles (AgNPs)

Biosynthesis of AgNPs was carried out by adding 10 ml of *M. piperita* leaf extract to 90 ml of 1mM silver nitrate

(AgNO₃) solution with continuous stirring at room temperature for 48 hours. The reduction of silver nitrate to AgNPs was confirmed by the color change of the prepared colorless solution to brown. The fully reduced solution was centrifuged at 8000 rpm for 20 minutes. The supernatant liquid was discarded and the pellet containing AgNPs was washed 2-3 times with deionized water to remove silver ions and the leaf extract residue. The resultant pellet was dried in a desiccator for 72 hours and stored in dark condition for further characterizations.

The synthesized AgNPs were characterized by UV-visible spectroscopy. The particle size of the samples was determined by the Zetasizer (Nano-ZS, Malvern, UK). Furthermore, the Fourier-transform infrared (FTIR) spectra of the nanoparticles were obtained on a Perkin-Elmer spectrometer (USA) in the absorption mode in the range of 4000-450 cm⁻¹. To determine the shape and structure, the nanoparticles were subjected to transmission electron microscopy (TEM) at 120 kV (LEO 912AB Zeiss electron microscope-Germany).

Preparation of Glass Ionomer Cements (GICs)

In this study, a total of 60 GIC (Fuji II LC, GC Corporation, Tokyo, Japan) disk-shaped specimens were prepared using a silicone mold (7 mm in diameter and 2 mm in height). GIC powder and liquid were mixed with a

plastic spatula for the 30 seconds according to the manufacturer's instructions and placed in the molds. The AgNPs powder was weighed and mixed with GIC powder in a percentage of 2% (w/w) immediately prior to manipulation. Specimens of cements were divided into two groups: conventional GIC without any addition (C-GIC), and cement with 2% (w/w) AgNPs (GIC-AgNPs).

Noteworthy, the material and specimens were prepared under laminar flow conditions. Finally, the prepared GIC specimens were sterilized for 30 min using ultraviolet (UV) radiation on each side to prevent contamination during the antibacterial activity tests.

The prepared specimens (*n* = 90) were classified into 3 main groups (30 specimens each) including C-GIC, GIC-AgNPs, and Ampicillin groups.

Antibacterial Activity Assessment

The antibacterial activity of the GIC specimens along with Ampicillin disk (10 µg/ml) was investigated by the Kirby-Bauer disk-diffusion method and according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [45]. Experimented bacteria were *S. mutans* (ATCC 35668), *E. faecalis* (ATCC 29212), *L. acidophilus* (ATCC 4356), *L. casei* (ATCC 39392), and *S. aureus* (ATCC 25923). The disks were placed on MHA inoculated with 0.5 McFarland of the studied bacteria at a distance of 15-20 mm. After incubation (24-48

hours) at the temperature of 37°C in 5% CO₂ (aerobic conditions for *S. aureus*), antibacterial activity was assessed by measuring the diameter of growth inhibition zones. The results represented the average of the three replicates.

Statistical Analysis

Statistical analysis was performed using SPSS version 21 (SPSS, Chicago, IL). Mean values and standard deviations for diameter of inhibition zone were reported. Antimicrobial activity of Glass Ionomer Cement (GIC) and GIC- AgNPs specimens, and Ampicillin disks against various standard bacterial strains were analyzed using Independent-samples t-test. (Since no inhibition zone was observed for GIC specimens, Independent-samples t-test was used for comparison of the antibacterial effect of the two other disks (GIC- AgNPs, and Ampicillin) against each bacterial strain.). The effect size (Hedge's g) and the results of statistical analyses are presented in Table 2. The significance level for all analysis was set at P <0.05.

Results

Characterization of AgNPs

In this study, the biosynthesis of *M. piperita* AgNPs was confirmed by the color change of the initial colorless solution to brown. Furthermore, the UV-Vis spectral analysis showed the peak at 420 nm, which attributed to the Surface Plasmon Resonance (SPR)

of AgNPs (Figure 1). The size distribution of the AgNPs was

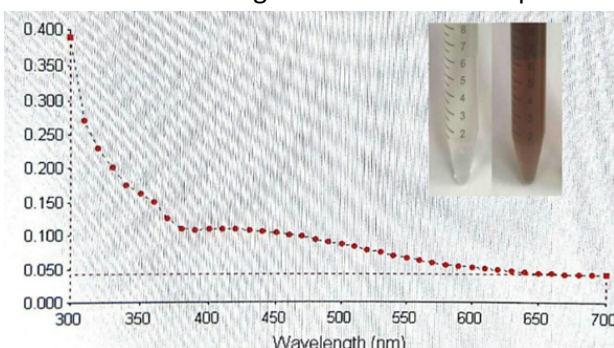


Figure 1. UV-Vis spectral analysis of silver nanoparticles (AgNPs) synthesized by *M. piperita* leaf aqueous extract.

measured by DLS and their average size was found to be 71.67±0.13 nm (Figure 2). As per TEM analysis, AgNPs

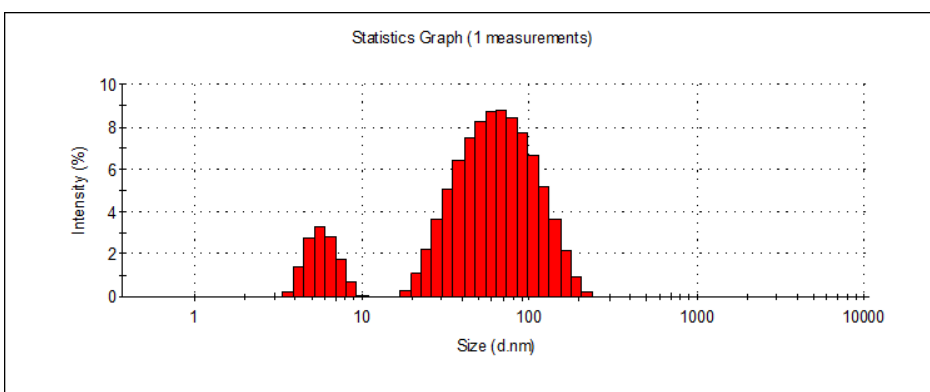


Figure 2. Particle size of *M. piperita* AgNPs.

of *M. piperita* appeared to be quasi-spherical in shape with a size range of less than 100 nm (Figure 3).

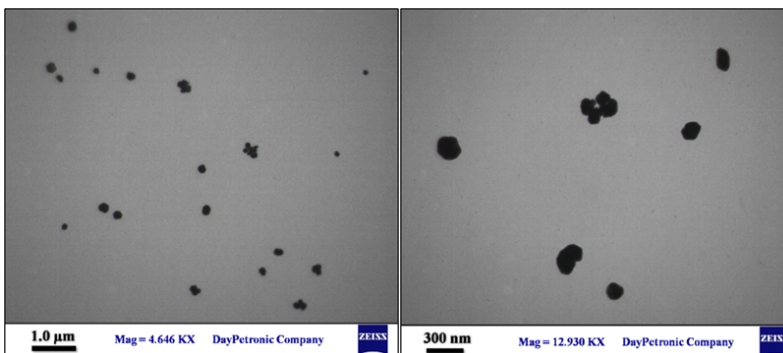


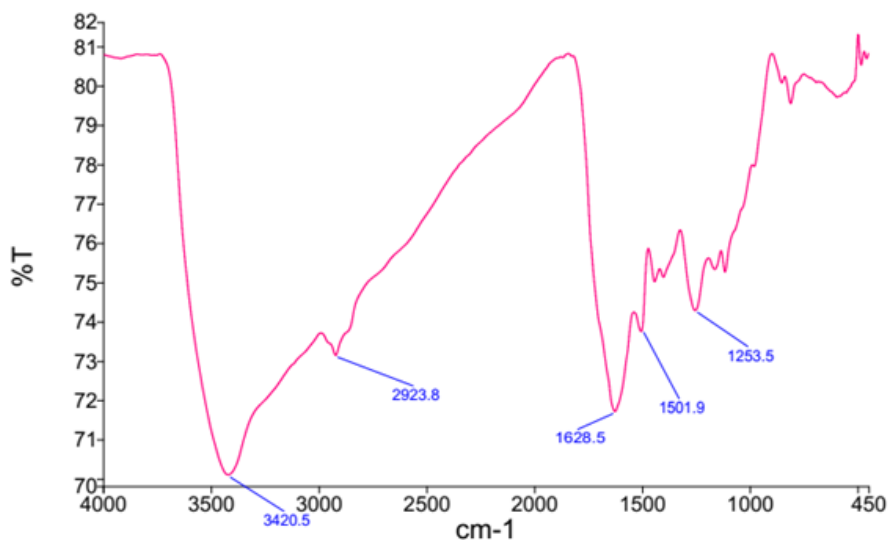
Figure 3. TEM images and respective size distribution *M. piperita* AgNPs.

Fourier transform infrared (FTIR) spectral measurements were carried out to identify the possible biomolecules in *M. piperita* leaf aqueous extract as reducing and capping agents of AgNPs (Figure 4). In the FTIR spectrum of *M. piperita* AgNPs, the 3420 cm⁻¹ peak represents the N-H bending vibration of the amine groups. Also, two peaks were observed at 2924 cm⁻¹ and 1628 cm⁻¹ which are related to C-H groups and carbonyl groups from polyphenols,

respectively. The results indicate the presence of plant biomolecules on AgNPs that these compounds can play an important role in stabilizing

nanoparticles and increasing their antibacterial properties.

specimens in comparison with Ampicillin was investigated for



(C-GIC) specimens did not have any antibacterial effects against the studied bacteria. However, the GIC-AgNPs had antibacterial effects on *S. mutans*, *L. acidophilus*, *L. casei* and *S. aureus*. The highest antibacterial effect of GIC-AgNPs specimens with an average inhibition zone diameter of 11.67 ± 0.29 mm was reported against *L. acidophilus* (Table 2). All studied bacteria were sensitive to Ampicillin with a minimum inhibition zone diameter of >26 mm ($P < 0.001$). Overall, the results of this study showed that the antibacterial effects of GIC-AgNPs against *S. mutans*, *L. acidophilus*, *L. casei* and *S. aureus* were relatively significant compared to conventional GIC, whereas significantly lower compared to Ampicillin (Table 2).

Figure 4. FTIR spectrum of AgNPs synthesized by *M. piperita* leaf aqueous extract.

Antibacterial Activity

In the present study, the antibacterial activity of the GIC and GIC-AgNPs

various standard bacteria strains (Table 1). The results indicated that Conventional Glass Ionomer Cement

Table 1: Antimicrobial activity of Glass Ionomer Cement (GIC), GIC- AgNPs, and Ampicillin specimens against various standard bacteria strains.

Bacteria Strains	Zone of Inhibition (mm)		
	C-GIC	GIC-AgNPs	Ampicillin (10 µg/ml)
<i>Streptococcus mutans</i> (ATCC 35668)	NI	10.17±1.04	51.33±1.04
<i>Lactobacillus acidophilus</i> (ATCC 4356)	NI	11.67±0.29	28.88±1.07
<i>Lactobacillus casei</i> (ATCC 39392)	NI	8.16±0.29	26.83±0.67
<i>Enterococcus faecalis</i> (ATCC 29212)	NI	NI	32.17±0.76
<i>Staphylococcus aureus</i> (ATCC 25923)	NI	8.67±0.76	32.83±0.29

C-GIC: Conventional Glass Ionomer Cement, GIC-AgNPs: GIC with 2 wt% AgNPs, NI: No inhibition zone

Table 2: Comparison of the inhibition zone diameter between GIC-AgNPs, and Ampicillin disks against various standard bacterial strains.

		<i>Streptococcus mutans</i> (ATCC 35668)	<i>Lactobacillus acidophilus</i> (ATCC 4356)	<i>Lactobacillus casei</i> (ATCC 39392)	<i>Enterococcus faecalis</i> (ATCC 29212)	<i>Staphylococcus aureus</i> (ATCC 25923)
GIC-AgNPs		10.17±1.04	11.67±0.29	8.16±0.29	NI	8.67±0.76
Ampicillin (10 µg/ml)		51.33±1.04	28.88±1.07	26.83±0.67	32.17±0.76	32.83±0.29
P value *		P <0.001	P <0.001	P <0.001	P <0.001	P <0.001
Effect size (Hedge's g) CI= 95%		-38.79	-21.51	-35.44	-	-41.16
95% CI values	Lower limit	-48.23	-26.78	-44.08	-	-51.19
	Upper limit	-30.39	-16.83	-27.77	-	-32.26

*Indicates the results of Independent-samples t-test for comparison of the antibacterial effect of the two disks against each bacterial strain.

Discussion

As previously mentioned, GIC is said to have antibacterial activity against cariogenic pathogen, though, its ability to arrest caries process is a matter of debate [28]. However, we failed to find any antibacterial activity of C-GIC in this study, which is corroborated with findings of some previous researches [29- 32]. Several attempts have been made to enhance the antibacterial activity of glass ionomer cement such as addition of Chlorhexidine

Gluconate and antibiotic mixtures to a Glass Ionomer Cement [28, 33]. However, these additives had different and often negative effects on physical and bonding properties of glass ionomer [28, 33]. Nano silver particles have shown great suppressive effect on oral pathogens [34]. They have inhibitory effect on adhesion and growth of cariogenic bacteria and impede the demineralization of enamel and dentin [34]. Nano silver particles are used in low concentrations and

therefore they don't cause changes in mechanical and color properties of dental materials or bacterial resistance [18, 34]. AgNPs, Nano silver fluoride, resin with AgNPs, and glass ionomer cement with AgNPs have been widely used and evaluated for dental applications [34]. Several researches have been done on the antibacterial activity of GIC incorporated with nano silver particles. Incorporating AgNPs into a resin-modified glass ionomer (resin containing 80 ppm of nanosilver)

showed significant antimicrobial activity against *S. sanguinis* and *S. mutans* in direct contact test [30]. Incorporation of nano silver base inorganic antibacterial powder into the glass ionomer cement decreased the demineralization rate around brackets without compromising bond strength [35]. In another study, Incorporation of AgNPs with GIC limited the *S. aureus* biofilm formation with insignificant effect on mechanical properties [36]. In a study conducted on a dental plaque microcosm biofilm model, resin-modified glass ionomer cement (RMGIC) containing nanoparticles of silver (NAg) reduced biofilm formation, plaque buildup, and white spot lesions formation around brackets without any adverse effect on the shear bond strength of brackets [37]. Incorporation of silver nanoparticle to orthodontic glass ionomer cements in the rat caries disease model enhanced its bactericidal effect on *S. mutans* [38]. The antibacterial effect of GIC-AgNPs against *S. mutans*, *L. acidophilus*, *L. casei* and *S. aureus* in this study is in line with the findings of a great deal of the previous work in this field [30, 36- 38]. We observed that GIC- AgNPs did not have any antibacterial effect against *E. faecalis* which is a Gram-positive bacterium usually found in persistent endodontic infections [39]. In a previous study, addition of up to 5 wt% nanosilver to zinc oxide sealer could not improve its antibacterial effect against *E. faecalis* [40].

However, we didn't find any study which had evaluated the antibacterial effect of nanosilver glass ionomer cement against *E. faecalis*.

In the studies conducted on the role of silver nanomaterials for caries prevention, the most commonly used bacteria were *S. mutans* which are the main cause of dental caries and various lactobacilli which are associated with progression of the caries lesion [34]. However, in oral cavity we are facing with biofilm rather than cariogenic monospecies strains, which enhance the resistance of microorganisms to antimicrobial agents [34]. Therefore, in order to simulate the complexity and heterogeneity of the biological biofilm, it is preferable to use oral microcosm cultured from various bacteria and the extracellular matrix removed from the oral environment [34]. However, an animal study or clinical trial would provide the environment of choice to evaluate antibacterial activity of nano silver materials [34].

Besides the antibacterial effects of nano silver materials, there are concerns regarding the silver nano particles cytotoxicity [41]. Free silver ions released from nanoparticles can penetrate cells and increase the production of reactive oxygen species and deleterious effects [42]. However, the cytotoxicity of AgNPs for human oral cells is lower than other silver compounds [34]. Besides the possible toxic effect of nano silver particles themselves, the techniques

used to produce these particles can lead to production of toxic chemicals [20]. The green synthesis of Ag nanoparticles can lessen the risk of toxicity and is a green alternative for AgNPs synthesis [20- 23]. We used the *Mentha piperita* leaf extracts for synthesis of Ag nanoparticles which to our best knowledge have not been used in dental applications so far. We found antibacterial activity of *Mentha piperita* leaf extracts synthesized GIC-AgNPs against the studied bacteria, which was in line with previous studies which used *Mentha piperita* leaf extracts against oral pathogens [25], *Escherichia coli* and *S. aureus* [26], and *Candida albicans* [27].

Although we found antibacterial activity of *Mentha piperita* leaf extracts synthesized GIC- AgNPs against bacteria strains, the stability of this effect should be further evaluated, since silver nanomaterials can be easily oxidized and aggregated [43]. In addition, further evaluation of different concentrations of AgNPs to find the optimized concentration which beside the antibacterial activity maintain the mechanical properties of the dental material seems necessary.

Conclusion

The findings of this study showed that glass ionomer cement incorporated with AgNPs synthesized using mint leaf extract (*M. piperita*) showed a promising antibacterial effect on *S. mutans*, *L. acidophilus*, *L. casei* and *S. aureus*.

Author Contributions

Author 1 contributed to conception, design, data interpretation

Author 4 and 5 contributed to conception, design, data interpretation, drafted and critically revised the manuscript.

Author 2 and 3 contributed to data acquisition.

Conflict of interests

Authors wish to declare there is no conflict of interest related to this study.

Ethical approval

The study protocol was approved by ethics committee of Birjand University of Medical Sciences (Ir.bums.REC.1397.65).

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