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Activity of Nano-Formulated Antibacterial Pomegranate Peel Extract

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Abstract: Punica granatum (pomegranate) peel has been used for centuries by many civilisations as it contains antibacterial and health-promoting effects. Rich in polyphenols, the peel has garnered interest for its possible use as an adjunct treatment of cancer and infection, particularly in integrative approaches among patients with compromised immune systems. Materials and Methods: Pomegranate peel extracts were prepared using solvents with increasing polarity, emphasizing the ethyl acetate fraction (PPE-EA). A nano-formulated version (n-PPE-EA) was obtained using a broad-spectrum method for nano-encapsulation. Antimicrobial activity against Proteus mirabilis, Escherichia coli, Aeromonas hydrophila and Klebsiella pneumoniae was analyzed. By way of agar diffusion, Proteus penneri, Pseudomonas aeruginosa, Enterococcus faecalis, Kocuria kristinae, Staphylococcus aureus and Staphylococcus hominis. Results: Pomegranate (Punica granatum) peel has been traditionally used for centuries in several ancient civilisations as an antibacterial and health promoting agent. The peel, abundant in polyphenols, has attracted interest as a candidate for cancer and infection therapy, particularly within integrative approaches to caring for patients with compromised immune systems. Materials and methods: Pomegranate peel extracts (PPEs), successively extracted with increasing polarity solvents, were prepared and evaluated for activity of the ethyl acetate fraction (PPE-EA). A nano-formulated form (n-PPE-EA) was prepared using a well-established nano-encapsulation technique. Antimicrobial activity was assessed against Proteus mirabilis, Escherichia coli, Aeromonas hydrophila and Klebsiella pneumonia. Via agar diffusion: Proteus penneri, Pseudomonas aeruginosa, Enterococcus faecalis, Kocuria kristinae, Staphylococcus aureus and Staphylococcus hominis Conclusions: evaluate antibacterial potential of nanoformulated PPE compared to conventional extract against pathogenic bacteria. Explicitly state in a dedicated Objectives subsection.

Key Words: Pomegranate Peel Extract, Antibacterial Activity, Nano-Formulated Pomegranate Peel, Silver Nanoparticles, Proteus Penneri, Gram-Positive, Gram-Negative

INTRODUCTION

Antimicrobial resistance (AMR) is a growing threat to global health due to the ineffectiveness of traditional antimicrobials and increasing morbidity and mortality globally. Various mechanisms of resistance, such as enzymatic degradation of antibiotics, increased expression of efflux pumps, and biofilm formation, have evolved in multidrug-resistant (MDR) bacteria such as Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa, leading to a significant reduction in treatment efficacy [1,2]. As a result, the discovery of new antimicrobial agents with novel mechanisms of action from natural resources is becoming increasingly relevant. Recently, some plant-derived natural bioactive compounds have attracted wide attention due to their broad antibacterial spectrum, safety and accessibility. Pomegranates (Punica granatum L.) are among the most therapeutic medicinal plants in this group [3]. The peel accounts for almost 40% of the weight of the fruit and contains polyphenolic compounds like punicalagins, ellagic acid, gallic acid, and flavonoids that display potent antibacterial and antioxidant activities [4]. The mechanisms of antibacterial action of these phytochemicals include damage to bacterial membranes, inhibition of essential enzymes, interference with endogenous nucleic acid synthesis, and induction of oxidative stress in microbial cells [5,6].

The therapeutic use of traditional plant extracts has been limited by their poor solubility and volatility with low

bioavailability compromised their antibacterial power. Nanotechnology, by enhancing the transport, stability and biological activity of phytochemicals of plant origin, provides a potential solution for these issues [7]. Increasing the surface area, penetration, and controlled-release characteristics of nano-formulated extracts allows for better interaction with bacterial cells [8,9]. Nanoparticles can directly bind to bacterial cell walls and membranes, destabilising their architecture and morphology, which leads to cell death. Nanodelivery devices also improve antibacterial efficacy against resistant strains by safeguarding bioactive materials from biodegradation and allowing them to accumulate at the target site [10].

Several studies showed that nanoscale pomegranate peel extract had a much higher antibacterial effect compared to traditional extracts. [11]. This enhanced activity is ascribed to improved penetration of effective particulates into bacterial cells, more stable active chemical species and a sustained release of antimicrobial agents. [12, 13]. Hence, this study aimed to evaluate the antimicrobial potential of nanoformulated pomegranate peel extract in comparison to conventional forms of extract and determine its efficacy against pathogenic bacteria [14].

METHODS

In order to assess and contrast the antibacterial activity of traditional and nano-formulated pomegranate (Punica granatum L.) peel extract against specific pathogenic bacteria, this experimental study was carried out.

Collection and Preparation of Plant Material

In the Diyala Governorate of Iraq, fresh pomegranate fruits were purchased from neighbourhood markets. After being separated, the peels were cleaned with distilled water and allowed to air dry for ten to fourteen days at room temperature. Before being extracted, the dried peels were kept in airtight containers after being finely ground with an electric grinder.

Preparation of Pomegranate Peel Extract (PPE)

After organically produced pomegranates (Punica granatum L.) were cleaned with double-distilled water (DW) and left to air dry for 62 hours at 44±2 °C, fruit skins were manually extracted. The dried peels were mechanically ground into a powder (100 g, around 60 mesh size), then extracted using 1 L of 70% diluted ethanol, agitated at 110×g for 65 hours at room temperature (RT; 25±2 °C), and filtered to remove any leftover plant debris. The P. granatum peel extract (PPE) was vacuum-dried at 41 °C and then redissolved in DW to achieve a 10% concentration [12]. As illustrated in Figure 1.

Biosynthesis of PPE- Silver nanoparticles (AgNPs)

In DW, a 10 mM aqueous solution of silver nitrate ($AgNO_3$) has been prepared. After that, 10 ml of PPE (1%, w/v) and a solution of silver nitrate ($AgNO_3$) (10 mM) were mixed and agitated at 610 × g for 55 minutes at room temperature. The fluid's yellow hue demonstrated the PPE biosynthesis of AgNPs. Centrifugation at 11.600 ×g for 37 minutes precipitated the PPE/AgNPs matrix from the solution. Parts of the PPE/AgNPs matrix were then washed twice with ethanol and three times with DW to produce plain AgNPs. Centrifugation was carried out following each wash. The PPE/AgNPs and ordinary AgNPs were then freeze-dried [13].

Characterisation of AgNPs

AgNPs were scanned within the 400–4000 cm-1 wavelength range. atomic force microscopy (AFM), a scanning electron microscope (SEM), and a Hitachi S-3400N TEM with a 500 nm resolution were used to capture the images. The qualitative and quantitative states of elements that might be involved in the creation of nanoparticles can be determined using it. These tests were carried out in the Chemistry Department labs of the University of Baghdad's College of Sciences, which serviced [14].

Determination of Minimum Inhibition Concentration (MIC)

As previously reported [15], the minimum inhibitory concentrations (MICs) of CS-AgNPs against indicator strains were calculated using the microdilution method. In the appropriate medium, each indicator strain was cultivated for an entire night. We dissolved and diluted the NPs using the appropriate culture medium selected depending on indicator microorganisms. The minimum inhibitory concentration (MIC) of NPs against various pathogenic organisms was found using the broth microdilution method.

A (B) (C)

Figure 1: Pomegranate Peel Extract (Ppe) Preparation A) Pomegranate Peel B) Dried Peel C) Peel Extract

NPs were created in three different concentrations (1000, 500, and 250 µg/mL). To evaluate NPs, bacterial species were grown on Mueller-Hinton Agar. The agar-well diffusion assay was then employed.

Well diffusion method

The effectiveness of the synthetic AgNPs against clinical isolates was investigated using the agar well diffusion method. Mueller-Hinton agar (MHA) plates were inoculated with a pure bacterial suspension from an overnight culture on nutritive agar medium at 37°C using sterile cotton-tipped swabs in order to create confluent bacterial growth. Five similarly spaced wells were created on the agar surface using a sterile borer. Eighty microlitres of the tested NPs suspension in MHB (2000 µg/ml) were added to each well. The remaining wells were subsequently inoculated with either ceftriaxone (50 µg/ml) as a positive control or copper acetate or MHB as a negative control (NC).

To enable the NPs suspension to pre-diffuse into the MHA, all plates were chilled for half an hour. The following step was a 24-hour aerobic incubation period at 37°C. The diameter of the clear zone that developed around NP-containing wells was measured in triplicate during the experiment and is shown as mean±standard deviation (mean±SD).

$$\text{Activity Index (AI)} = \frac{\text{IZD caused by the PE}}{\text{IZD caused by the standard antimicrobial}} \quad (1)$$

$$\text{Percent InhI)} = \text{AI} \times 100 \quad (2)$$

All isolation of gram-positive and negative bacteria obtained from the lab at Baghdad University, Iraq.

Bacterial Isolates

Gram-negative (Aeromonas hydrophila, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Proteus penneri, Pseudomonas aeruginosa) and Gram-positive (Enterococcus faecalis, Kocuria kristinae, Staphylococcus aureus, Staphylococcus hominis) bacterial species were tested for antibacterial activity. These isolates were discovered using conventional microbiological techniques after being extracted from clinical samples.

Preparation of Bacterial Suspension

Bacterial suspensions were prepared in sterile saline and adjusted to a 0.5 McFarland standard, equivalent to approximately 1.5×10^8 CFU/mL.

Antibacterial Activity Test

The agar well diffusion method was used to assess the antibacterial activity. A sterile swab was used to inoculate Mueller-Hinton agar plates with bacterial solution. After creating 6 mm-diameter wells in the agar, 100 µL of the conventional extract, nano-formulated extract, positive control (antibiotic), and negative control (distilled water) were introduced. For a whole day, the plates were incubated at 37°C. Millimetres were used to measure the inhibitory zone's diameter. Every test was run three times.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined using the broth microdilution method. Serial dilutions of the extracts were prepared, and bacterial growth was evaluated after 24 hours of incubation.

Statistical Analysis

Data were analysed using SPSS software (version 26). Results were expressed as Mean±Standard Deviation. Differences between groups were analysed using Student's t-test and One-way ANOVA. A P-value ≤ 0.05 was considered statistically significant. Include effect size; ensure clarity of units; confirm normality assumptions for t-test/ANOVA.

RESULTS AND DISCUSSION

AgNPs Synthesis

Color change from pale yellow to dark brown in 5–20 minutes after addition of PPE to the $AgNO_3$ solution confirmed the reduction of Ag^+ ions (metallic silver nanoparticles, Ag^0) in the reaction mixture. As time progressed, the brown color deepened, further confirming the gradual development of nanoparticles (Figure 2).

Characterisation AgNPS

Atomic force microscopy (AFM): Data were analysed using atomic force microscopy (AFM) to study the size distribution and surface morphology of AgNPs. A droplet of suspended nanoparticles was placed on a glass slide and air dried. Tapping mode imaging was used. The obtained images confirmed the nanoscale size and surface properties of the generated nanoparticles (Figure 3).

Characterisation of AgNPs Using Transmission Electron Microscopy (TEM)

The size, form, and morphology of the produced silver nanoparticles (AgNPs) were assessed using transmission

Figure 2: AgNPs Synthesis after Addition of PPE

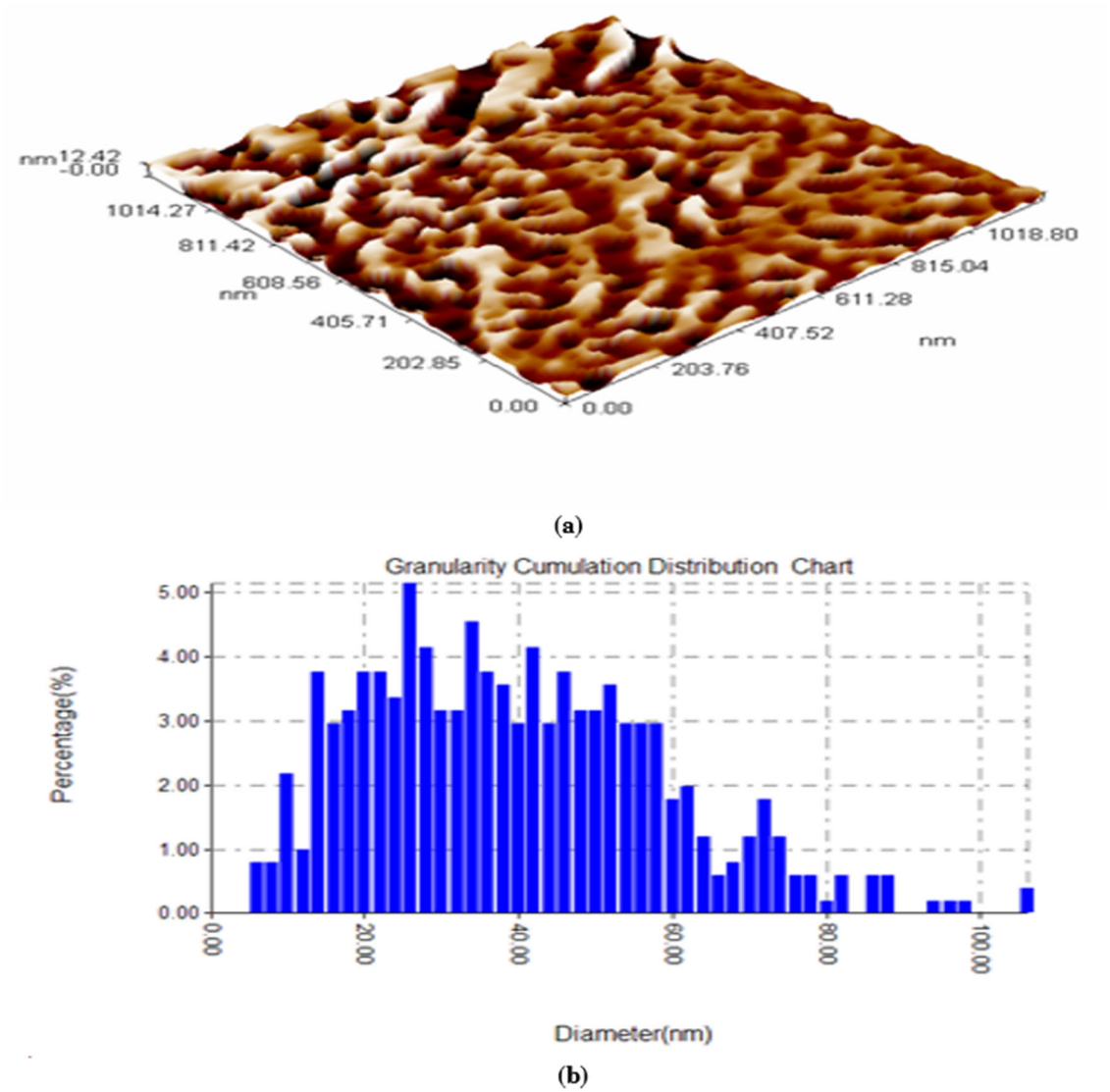

Figure 3: The Biosynthesised AgNPs under AFM a) 2D and 3D Images b) Histogram

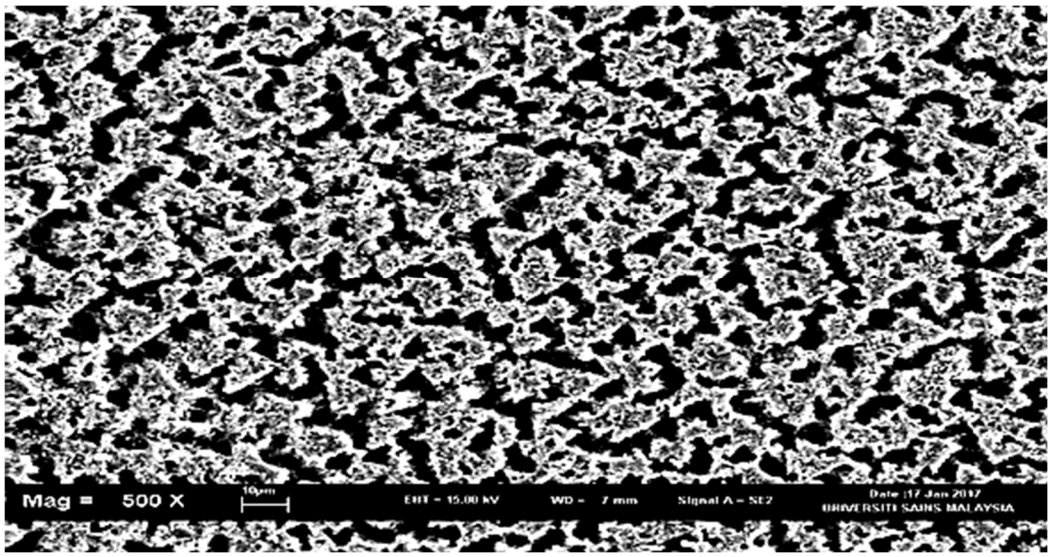

Figure 4: Characterisation of TEM Image

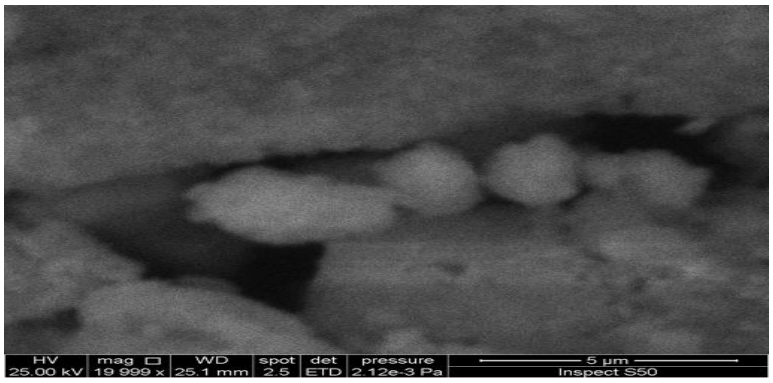

Figure 5: Characterisation of SEM Image

Table 1: Mean±SD Zone of Inhibition in mm Treated with AgNPs against Bacteria spp at Different Concentrations between 1000, 500, 250, µg/mL.: Standard Deviation, (n = 3)

AgNPs				
250	500	1000		
µg/ml			Bacteria spp	
6.3±0.8	8.3±0.8	10.3±0.8	Aeromonas hydrophila	Gram negative
10.3±0.6	10.7±0.7	13.8±2.6	Escherichia coli	
7.3±0.8	10.5±1. 8	16.0±0.2	Klebsiella pneumoniae	
.5 0±89.	11.5±0.9	17.5±0.9	Proteus mirabilis	
11.3±1.2	11.8±1.4	14.8±0.4	Enterococcus faecalis	Gram positive
±10.368.	16.7±0.6	23.8±0.3	Kocuria kristinae	
8.3±0.8	9.0±0.9	17.3±0.6	Staphylococcus aureus	
11.3±0.6	12.2±0.3	13.3±0.6	Staphylococcus hominis	

electron microscopy (TEM). A drop of the nanoparticle suspension was put on a copper grid coated with carbon and allowed to cure at room temperature in a sterile environment. A transmission electron microscope was used for TEM analysis. running at a 200 kV accelerating voltage. To assess the size distribution and particle shape, the photos were captured at various magnifications. Using image analysis software, the diameter of randomly chosen nanoparticles was measured in order to determine the average particle size. The effective production of silver nanoparticles was confirmed by the TEM images, which showed that the AgNPs were primarily spherical in shape with nanoscale dimensions (Figure 4).

Figure 5 is a scanning electron microscopy (SEM) image that displays the size distribution and surface morphology of the synthesised silver nanoparticles (AgNPs). The nanoparticles were mostly spherical in shape, distributed rather uniformly, and slightly aggregated, according to the micrograph. The particle size fell within the nanoscale range, indicating that AgNPs were successfully synthesised.

DISCUSSION

These present results demonstrate that AgNPs are broad-spectrum in their antibacterial effect, with a marked dose-dependent increase in efficacy. These diameters of the inhibition zones grew in accordance with the used concentration. This pattern aligns with the results of Liu *et al.* with the exception of an Iraqi study involving plant-derived AgNPs, where a similar linear relationship was observed between concentration and antibacterial effect. In the global study of Ahmed *et al.* These results also agree with that pointed out the dose-dependent activity as one of the most distinctive properties of silver nanoparticles owing to their greater surface interaction with bacterial cells. [15,16].

For Gram-negative bacteria, the most affected strain was Proteus penneri, and the least affected was Aeromonas hydrophila. Moderate activity was noted against Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa. These results were similar to an Iraqi study by Ahmed, Z. M., and Ahmed, M. E. exemplifying that the sensitivity of Gram-negative bacteria to AgNPs depends on the outer membrane structure [17]. Internationally, ALkhazraji *et al.* report that differences in outer membrane structure and the presence of transport proteins are crucial for silver nanoparticle sensitivity in Gram-negative bacteria [18].

For Gram-positive bacteria, Staphylococcus aureus and Kocuria kristinae had higher sensitivity than Enterococcus faecalis and Staphylococcus hominis. These results are in line with the Iraqi study of Hassan *et al.* which demonstrated that S. aureus is one of the most responsive species to AgNPs. In a large international study by Franci *et al.* This was supported by Shabir *et al.* who found that the thickness of the peptidoglycan layer and structure of the cell wall altered nanoparticle permeability, which ultimately influenced antibacterial properties. [19,20].

Silver nanoparticles exert an antibacterial action through various mechanisms, such as damage to the bacterial cell membrane permeability, penetration into the bacterial cell, interaction with biological entities, and production of reactive oxygen species (ROS), which leads to oxidative stress that ultimately causes cell death [21]. An Iraq-based

study by Abdul Majeed *et al.* mentioned the mechanism of action by AgNPs, in which oxidative stress was important, which inhibits bacterial growth. In contrast, the work of Thamer *et al.* already demonstrated that ROS generation is one of the major mechanisms involved in the bactericidal toxicity of nanoparticles [22].

These bacteria develop sophisticated resistance mechanisms like efflux pump systems and decreased membrane permeability, explaining the relatively lower susceptibility of some species like Aeromonas hydrophila and Pseudomonas aeruginosa) [23]. This assumption is supported by an Iraqi study which showed that P. aeruginosa demonstrates relative resistance because of its physiological properties [24]. Ahmed *et al.* described efflux pump systems as one of the most important robust mechanisms responsible for bacterial resistance to antimicrobial agents, including nanoparticles, on a global scale [25].

In general, the present study findings match with the majority of Iraqi and international studies that confirm AgNPs are effective and may represent potential antimicrobial agents, particularly at a time when antibiotics are becoming less useful. An Iraqi study Morones *et al.* recently revealed that AgNPs could be an effective therapeutic alternative, while Lara *et al.* they possess considerable potential as broad-spectrum antibacterial agents in medical applications. [26,27].

CONCLUSION

The findings in this study show that biosynthesised silver nanoparticles (AgNPs) are active against both Gram-positive and Gram-negative bacterial strains, with an increase in concentration associated with increased effectiveness of bacterial inhibition. In species like Proteus penneri and Staphylococcus aureus, high susceptibility was reported due to the presence of susceptible cellular structures while Aeromonas hydrophila and Staphylococcus hominis displayed lower susceptibility mechanisms owing to different cellular structures or resistance mechanisms. AgNPs exhibit antibacterial activity via multiple pathways including disruption of membrane permeability and oxidative stress. Analysing the face of antibiotic resistance, AgNPs could become an alternative or complementary antimicrobial agent that aims to further investigation towards biosafety and optimal application concentration.

Future Recommendations

- Determine optimal nanoparticle dosage
- Biosafety and in vivo studies
- Synergistic combination with antibiotics
- Expand microbial spectrum
- Enhance nanoparticle properties (size, surface modification)

Ethics Declarations

Ethical Considerations: No specific ethical approval or permissions were required for this study. Plant material was obtained from commercial markets in Diyala Governorate, Iraq. No formal ethical approval was required; bacterial isolates were from the university lab. Experiments were conducted in the laboratory available in Ba'aqubah teaching hospital; therefore, no ethical approval or informed consent was required in accordance with institutional and international guidelines

Competing Interests

The authors declare no competing interests.

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