Green synthesis, characterization and biological activity of silver nanoparticles of Hypnum cupressiforme Hedw extract

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Mosses are one of the oldest land plants that constitute the second largest group of the plant kingdom, are found in almost every region, have known therapeutic effects, are rich in secondary metabolites, can withstand harsh climatic conditions and thirst for a long time. Volatile extracts of *Hypnum cupressiforme* Hedw. were analyzed by gas chromatography-mass spectrometry in this study, and the presence of 18 different phytocompounds was determined as secondary metabolites. Using these extracts, silver nanoparticles (AgNPs) were synthesized using a green synthesis method. The AgNPs that were created were characterized. The extracts and AgNPs were tested for antibacterial, antibiofilm, antioxidant, mutagenic, and DNA-cleavage activity. The tests revealed no evidence of mutagenicity. Antibiofilm and antibacterial activity of AgNPs against *P. vulgaris, P. aeruginosa*, and *E. faecalis* were demonstrated. Both oxidatively and hydrolytically, *H. cupressiforme* the extract was found to have higher antioxidant properties and high DNA cleavage activity.

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Keywords: Hypnum cupressiforme Hedw, Green synthesis, Biosynthesis, Silver nanoparticles, Biological activity

1. Introduction

The products obtained with new production methods provided by nanotechnology are being used in many fields, including the diagnosis and treatment of diseases, and it is seen that the use of products developed with nanotechnology is rapidly increasing in studies in disciplines such as physics, chemistry, biology, and engineering. Nanotechnology enables us to produce materials, devices, and systems with unique and superior functions by precisely and controlled placement of atoms and molecules at nanoscale [1]. The biological synthesis of nanoparticles is faster, simpler, cost-effective, and biocompatible since they do not contain toxic products, require less energy, and have fewer processing steps, making them advantageous. Thanks to these qualities, nanotechnology applications have become one of the most studied topics in biotechnology research in recent years.

Silver nanoparticles are distinguished from other biomedical nanomaterials by their distinct optical, electrical, physical, chemical, and biological properties, and they are thus used in the medical sector in a variety of fields such as pharmacology, biosensors, and biomedical applications as antiviral agents, antibacterial agents, and in the treatment of cancers such as breast cancer, leukemia, lung cancer, and skin cancer [2, 3, 4]. Silver nanoparticles have been reported to have antibacterial and anti-inflammatory effects that can shorten wound healing time [5].

Bryophytes, the first terrestrial plants, produce secondary metabolites in response to biotic and abiotic stresses such as interplant competition, microbial attack, insect and animal predation, climatic conditions such as drought and freezing, and UV protection [6]. Bryophytes are diverse and rich in secondary biologically active compounds due to their distinct morphology and physiology. Polysaccharides, terpenoids, lipids, amino acids, and phenylpropanoids are among the natural products found in bryophytes [7]. Mosses have been shown to have high antioxidant activity, which is higher than that of some highly structured plants [8, 9, 10, 11]. Furthermore, they

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can remain dormant for long periods before resuming photosynthesis when they meet water, which allows for low-cost production and storage. Yayintas and Demir (2021) investigated the potential biological effects of Marchantia polymorpha (Bryophyte) collected from Ida Mountain (Canakkale/Turkey) and discovered that M. polymorpha may be a potential source of antioxidants as well as having antiplasmid DNA effects [2].

H. cupressiforme is a cosmopolitan moss species in the Hypnum genus, Hypnaceae Schimp family, with a worldwide distribution. However, research on the secondary metabolites and therapeutic effects of this moss species is limited. H. *cupressiforme* has high antioxidant, antiproliferative [11, 13], antibacterial [14,15], and antifungal properties, and has the potential to be used in the treatment of neurodegenerative diseases [13]. In this study, the secondary metabolites contained in *H. cupressiforme* collected from the rocks of Ida Mountain (Ayazma, Canakkale) in spring season were determined and AgNPs were synthesized by green synthesis method. The biological activities, mutagenic effects, and DNA-cutting properties of both *H. cupressiforme* species and synthesized AgNPs were also investigated.

2. Experimental

2.1. Plant collection and description

Hypnum cupressiforme Hedw. was collected from rocks in Ayazma, Ida Mountain (Canakkale) in spring and identified by Prof. Dr. Ozlem Yayintas (Canakkale Onsekiz Mart University) (Fig.1). The plant was first washed with tap water, then washed again with deionized water, and dried in a cool environment with continuous air circulation in such a way that the parts above the soil remained in the shade. After drying, 10 g of the powdered sample was weighed and extracted in 100 mL deionized water at 60^0C. The extract obtained was allowed to reach room temperature and centrifuged at 8000 rpm for 45 minutes. The extract obtained after centrifugation was first filtered with Whatman Filter paper and then passed through a 0.45µm membrane filter.

Fig.1. Hypnum cupressiforme Hedw.

2.2. Silver nanoparticle preparation

For the synthesis of silver nanoparticles, a 1 mM solution of AgNO3 (silver nitrate) was used. The extracted (10 mL) was then mixed with a 90 mL solution of AgNO3 and stirred for 24 hours on a magnetic stirrer. A color shift was observed during this time. The resulting AgNPs were centrifuged at 10,000 rpm for 30 minutes, and the pellet was washed at 10,000 rpm with distilled water for 30 minutes. The washing process was repeated three times, and the silver nanoparticles were dried in an oven at 60^0C in a watch glass.

2.3. Characterization of biosynthesized silver-nanoparticles

Biosynthesized silver nanoparticle exposed to Ultraviolet-Visible (UV-Vis) Spectrometer (Spectro UV-Vis Dual Beam with 8 Auto Cell, Labomed, Inc) range of 250nm-600nm. Morphology, size, and element analysis of silver nanoparticles investigated by Transmission Electron Microscope (TEM) (Hitachi HT-7700) and Scanning Electron Microscope (SEM).

2.4. Antibacterial activity analysis

Analyses to determine the antibacterial effects of the extract and synthesized NPs were performed using agar disk diffusion and broth microdilution methods as recommended by the Clinical and Laboratory Standards Institute [16]. Antibacterial activity of NPs was tested against *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (NRRL B-14617), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Proteus vulgaris* (NRRL-B-123) bacteria.

2.5. Free radical scavenging activity

Free radical scavenging activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to Blois (1958) [17] method. 0.5 mL of 1 mM DPPH solution prepared daily in methanol was added to the samples and the samples were kept in the dark for 30 minutes at room temperature and then their absorbance was measured at 517 nm in a spectrophotometer against the blind. Standard (BHT) was used as positive control and solvent (methanol) was used as negative control. The study was performed in three replicates.

2.6. The interaction of NP with DNA

pBR322 plasmid DNA (in 90% super-coil form) was used in DNA cleavage studies. Tris-HCl buffer samples (10mM, pH: 7.4) were used to prepare plasmid pBR322 DNA. After 3 hours at 37^0C in the incubator, 6X loading dye is added, and 1 hour at 60V in 1% agarose gel, 1X TAE buffer (40 Tris-20 mM acetic acid, 1mM EDTA pH: 8.2) gel electrophoresis is performed. The bands were then photographed under UV light using a gel imaging system [18].

2.7. Mutagenic activity determination

The mutagenic activity will be determined using the Ames/Salmonella test. The frameshift is determined by the TA98 strain of Salmonella typhimurium, while the mutations that cause base-pair change are determined by the TA100 strain [19; 20]. The positive controls were 4-nitro-o-phenylenediamine (NPD) for strain TA98 and sodium azide (SA) for strain TA100; water was used as the negative control for both stains. To determine whether the NPs are mutagenic, the doses will be compared to a negative control. To speak of the mutagenic effect, the number of revertant colonies obtained after varying concentrations of the tested NPs should be twice that of the negative control [20].

3. Result and discussions

3.1. Volatile compounds *H. cupressiforme* Hedw

The main bioactive chemicals present in the *H. cupressiforme* extract were identified using gas chromatography-mass spectrometry (GC-MS). As secondary metabolites, the presence of 18 different phytocompounds, which are thought to contribute to the therapeutic abilities of this plant species. According to the results of this test, the most abundant compound in the extracts of *H. cupressiforme* was (3E,5E,7E)-6-Methyl-8-(2,6,6-trimethyl-1-cyclohexenyl)-3,5,7-octatrien-2-(3E,5E,7E). Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester (14.16%), and Neophytadiene (10.27%) were the most abundant phytocompounds in the extract's secondary metabolites. These three compounds accounted for roughly 60% of the extract. The remaining 40% is made up of 15 different compounds with total extract concentrations of 5% or less. Sujana et al. (2012) found (3E,5E,7E)-6-Methyl-8-(2,6,6-trimethyl-1-cyclohexenyl)-3,5,7-octatrien-2- phenolic acid in Passiflora incarnata L. plant compounds and emphasized that this

bicin has antitumor activity. Sharma et al. (2015) [21] discovered Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-methyl ester compound in the extract of *Brassica juncea* L. species, and Xian et al. (2018) [22] emphasized in their study that this plant has anti-inflammatory effect. Vieira et al. (2018) [23] comprehensively examined the effects of limonene on health. They emphasized that many studies are revealing the anti-inflammatory, antioxidant, antinociceptive, anticancer, antidiabetic, antiviral, and gastroprotective effects of limonene published in the scientific literature between 2008 and 2017.

3.2. Synthesis and characterization of silver nanoparticles

When a colorless AgNO3 solution (B) was added to the dark green *H. cupressiforme* extract (A), the extract turned yellow brown (C). The color change in the extract indicates the presence of silver nanoparticles (Fig2.).

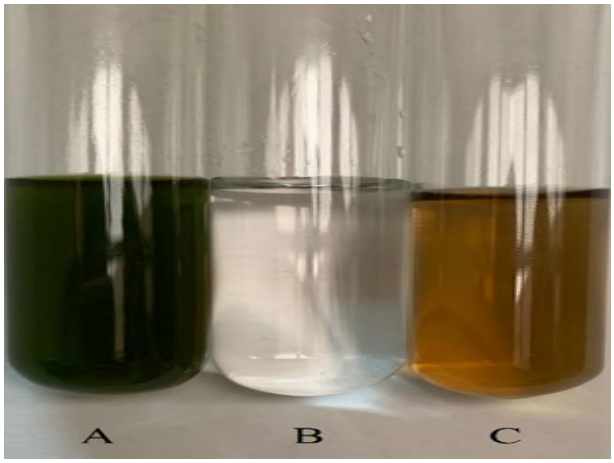
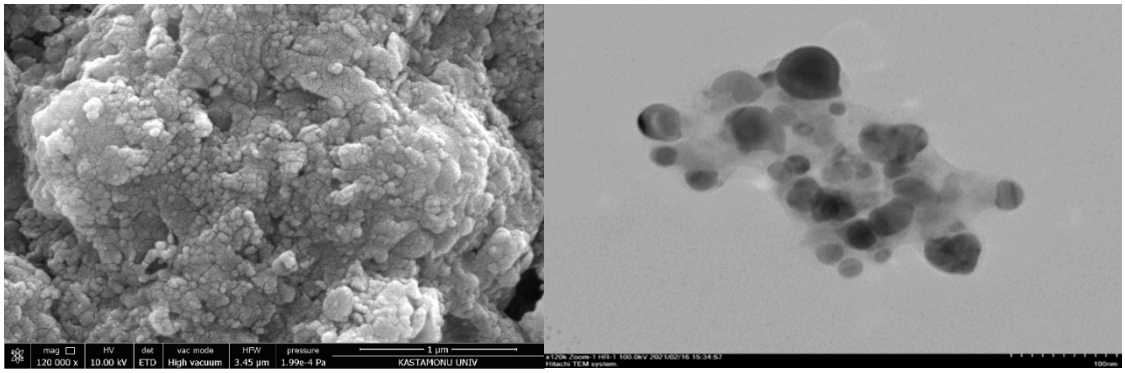


Fig. 2. AgNP synthesis.



(a) *(b)*

Fig. 3. SEM (A) and TEM (B) image of AgNPs synthesized from H. cupressiforme.

The presence and morphological properties of AgNPs were determined by SEM characterization analysis. SEM analysis results showed the successful synthesis and presence of silver nanoparticles because of the green synthesis process. SEM analysis revealed that the majority of the synthesized AgNPs were approximately similar in size and spherical in structure (Fig. 3 A). TEM analysis revealed that the synthesized AgNPs ranged in size from 15 nm to 60 nm and were mostly spherical in shape (Fig. 3 B). The highest absorbance value of the synthesized AgNPs was measured at 450 nm (Fig. 4). When compared with previous studies, it was observed that the findings agreed with the literature [24, 25, 26, 27]

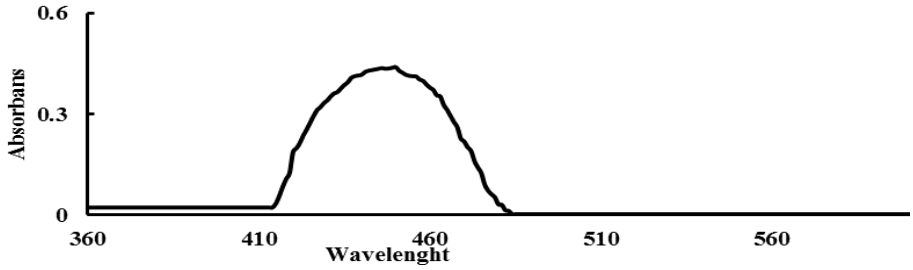


Fig. 4. UV-Vis spectra of the H. cupressiforme AgNP extract.

3.3. Determination of antimicrobial activity

The broth microdilution method was used to determine the MICs of nanoparticles and extracts. According to the findings (Table 1), *H. cupressiforme* extract had no antimicrobial effect on the tested bacterial strains, whereas HcAgNPs inhibited *P. vulgaris*, *E. faecalis*, and *P. aeruginosa* bacteria.

Table 1. Extract and AgNP MIC values.

Microorganisms	Extract	HcAgNP	Ampicillin
	MİK	MİK	MİK
*P. vulgaris (*NRRL-B-123)	>500	3.91	0.98
*P. aeruginosa (*ATCC 27853)	>500	15.63	3.90
E. coli (ATCC 25922)	>500	31.25	31.25
E. faecalis (NRRL B-14617)	>500	3.91	0.24
*S. aureus (*ATCC 25923)	>500	62.50	0.24
*B. subtilis (*ATCC 6633)	>500	62.50	0.98

The extract and AgNPs were evaluated for antibiofilm activity, and the extract showed no antibiofilm activity in both broth microdilution and agar disk diffusion tests (Table 2), whereas HcAgNP showed biofilm removal effect against *P. vulgaris, P. aeruginosa*, and *E. faecalis* bacteria.

Table 2. Agar well diffusion method.

Microorganisms	Zone of inhibition in millimeter (mm)		
	Extract	HcAgNP	Ampicillin
*P. vulgaris (*NRRL-B-123)	6	14	11
*P. aeruginosa (*ATCC 27853)	7	12	11
E. coli (ATCC 25922)	8	11	13
E. faecalis (NRRL B-14617)	6	14	11
*S. aureus (*ATCC 25923)	-	-	-
*B. subtilis (*ATCC 6633)	8	11	12

3.4. Antioxidant activity

The antioxidant capacities of the extract and HcAgNP synthesis were determined by the Blois method using DPPH. BHT was used as a positive control in the study, and antioxidant activity for each sample was calculated by measuring absorbance values at five different concentrations (25, 50, 100, 200, 400 µg/mL). DPPH was used to determine the antioxidant capacities of the extract and the synthesis of HcAgNP. Figure 5 shows that the extract and HcAgNP had similar free radical scavenging activity at low concentrations, but the extract's free radical scavenging activity increased with concentration. Free radicals cause cellular damage by causing oxidative stress when they proliferate more than necessary in the environment.

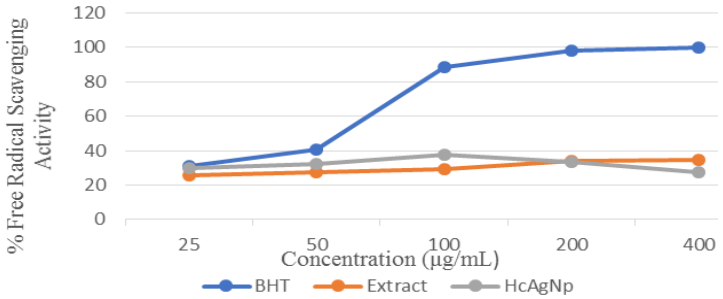


Fig. 5. Free radical scavenging activity.

3.5. DNA cleavage activity

The synthesized silver nanoparticles were analyzed by agarose gel electrophoresis using pBR322 plasmid DNA to determine their hydrolytic and oxidative shear properties. At all concentrations tested, AgNPs cut DNA both hydrolytically and oxidatively. In every concentration level, HcAgNPs converted DNA into a cyclic form, but no linear form was observed. Furthermore, the extract demonstrated high DNA-cutting activity both oxidatively and hydrolytically at all concentrations. The extract showed a linear form image as well as a cyclic form image in electrophoresis as the concentration increased oxidatively and hydrolytically at all concentrations, indicating that it has higher DNA-cleavage activity than HcAgNPs (Fig.6).

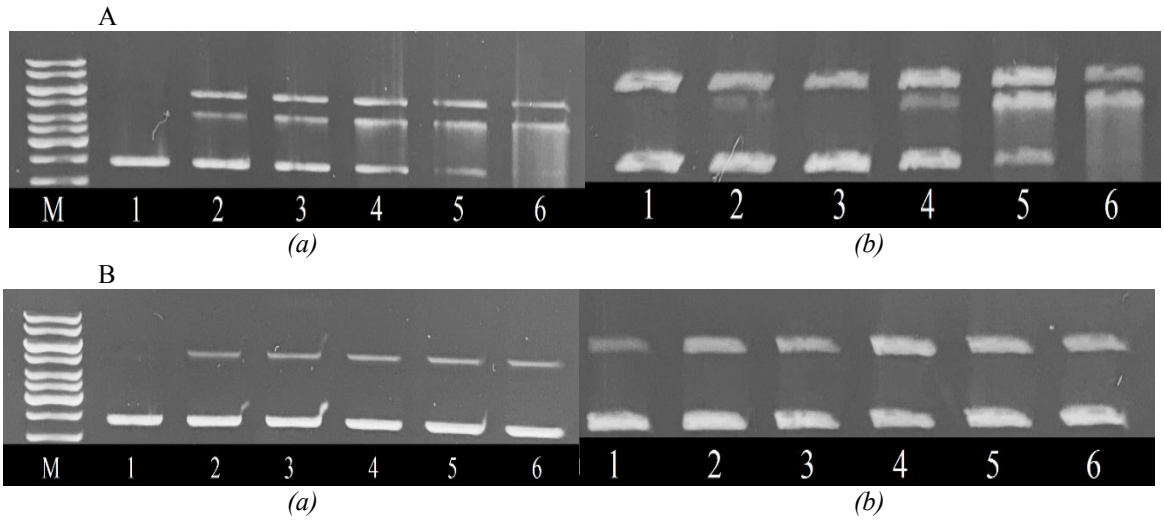


Fig. 6. Determination of DNA cleavage activity of H. cupressiforme water extract (A) and AgNP (B).

a. Hydrolytic, M. Marker, 1. DNA control, 2. DNA + 1.25 µg/mL, 3. DNA + 2.5 µg/mL, 4. DNA + 5 µg/mL, 5. DNA + 10 µg/mL, 6. DNA + 20 µg/mL.

b. Oxidative, 1. DNA + H2O2, 2. DNA + 1.25 µg/mL + H2O2, 3. DNA + 2.5 µg/mL + H2O2, 4. DNA + 5 µg/mL + H2O2, 5. DNA + 10 µg/mL + H2O2, 6. DNA + 20 µg/mL + H2O2.

3.6. Determination of mutagenic activity

Two different strains of *S. typhimurium*, TA98 and TA100, were used in the study and the mutagenic activity of the extract and HcAgNPs were determined by Ames/Salmonella test. As a result of the analysis, it was determined that the extracts and HcAgNPs did not show mutagenic effect (Table 3).

Table 3. Ames/salmonella test results.

Treatment	Concentration	Number of His+ Revertant Colony/ Plate	
	(µg/mL)	TA98	TA100
		Mean±SD	Mean±SD
Positive Control	NPD	491.67±5.51	
	SA		1264.67±33.50
Extract	6,25	26.33±2.52	101.00±6.56
	25	43.00±2.65	87.00±7.00
	100	46.67±4.04	59.00±5.29
	400	42.33±3.79	73.33±11.93
AgNP	6,25	44.67±3.06	93.67±8.39
	25	43.33±10.69	66.00±2.65
	100	52.33±11.24	72.33±7.51
	400	87.33±11.59	81.67±6.51
Negative Control	dH_2O	22.33±1.53	71.00±19.05
Spontaneous Control		24.67±4.51	90.00±14.53

NPD: 4-nitro-o-phenylenediamine, SA: sodium azide (SA)

Because of the toxic effects and high cost of physical and chemical methods for synthesizing nanoparticles, biological methods based on plants, bacteria, and fungi have emerged agents are used in the medical sector due to their antiviral, antitumor and antibacterial effects [2, 3, 4]. The most important use of nanotechnology in medicine is in "cancer diagnosis and treatment", often referred to as "theranostic or theragnostic" applications. Nanoparticles can overcome biological barriers and facilitate diagnosis, treatment, and monitoring of disease and treatment response [28].

Mosses, which can grow in almost any climate, are found in every region of the world and are high in secondary metabolites, they are known to have antioxidant, antibacterial, antifungal, and antitumor properties [8, 9, 10, 11, 29, 30]. Antibacterial and antioxidant properties have been demonstrated for biologically synthesized AgNPs from various moss species such as *Dicranum majus* Turner [27], *Fissidens minutus* [31], and *Bryum medianum* [32]. The antibacterial and

antioxidant activities of the HcAgNPs that we obtained were found to be consistent with previous research.

On the other hand, there are very limited number of studies on the secondary metabolites and therapeutic effects of *H. cupressiforme* Hedw. In previous studies, it was reported that *H. cupressiforme* ethanol [33, 14] and methanol [10, 15] extracts showed antibacterial effect on various bacteria, but the extract prepared with deionized water [11] was not effective on bacteria. The antioxidant effects of *H. cupressiforme* Hedw. species were studied by Lunic et al. (2022) [13], Yayintas and Demir (2019) [34], and Yayintas et al. (2019) [11]. The findings of this study corroborate previous research.

When the original superspiral form (Form I) of plasmid DNA is opened by damage, a circular form (Form II) is formed, and a linear form (Form III) can also be found with the formation of more breaks. In agarase gel electrophoresis, Yayintas et al (2021) detected Form I images of AgNPs synthesized with *Dicranum majus* Turner species and reported that AgNPs have DNA shearing activity [21]. Although these findings are consistent with our findings, Form III image was also detected in the extract in our study using agarase gel electrophoresis, and it was determined that *H. cupressiforme* Hedw. extract has high DNA cutting activity. However, neither the moss species nor the synthesized AgNPs were found to be mutagenic.

4. Conclusion

In this study, a novel and environmentally friendly *Hypnum cupressiforme* Hedw. AgNP was successfully green synthesized, characterized, and determined by its biological activity, which included antibacterial, antimutagenic, and antioxidant properties. The results obtained show that we have achieved the goal we aimed before starting the study and the values accepted in the literature.

In our study, AgNPs obtained by green synthesis method using *H. cupressiforme* aqueous extract were found to be effective on *P. vulgaris, P. aeruginosa*, and *E. faecalis* bacteria and had the ability to inhibit biofilm structures caused by these bacteria. The fact that they do not show mutagenic effect shows that they can be used as drugs. When the results of antibacterial, antibiofilm, and mutagenic activity tests of AgNPs, to which bacteria cannot develop resistance, are considered together, AgNPs have a high potential for use as a drug for the elimination of pathogenic microorganisms, as a disinfectant in the cleaning of medical devices and prostheses and will not cause a mutagenic effect as a result of these processes. In this regard, the findings of our study are encouraging.

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