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(RESEARCH ARTICLE)

Green synthesis and biochemical characterization of silver nanoparticles by using *Euphorbia umbellata* leaf extract and analysis of antimicrobial activity against plant pathogens

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Abstract

The science of nanotechnology and nanoparticles is the manipulation of matter on atomic and molecular weight, the nanotechnology and nanomaterial's refer to the particular technological goal which is precise manipulation of atom and molecules for used the fabrication process of microbial products and it is now concern to as molecular nanotechnology, it also known as the nanotechnology is science of designing, making and application of nano-structure and nanomaterials also used investigation of relationship various properties of materials with their nanometer dimensions. The exploitation of various plant materials for the biosynthesis of silver nano particles is considered a green technology. Because it does not involves any harmful chemicals. Nanotechnology field is one of the most attractive researches. The field of nanotechnology is applied to bio materials. Nanoparticles are generally considered as particles with a size up to 100 nm, that have completely new or improved properties as compared to the bulk material that they are collected based on particular characteristics such as size, distribution and morphology. Different groups of unorganized parts of the plants have been utilizing for the green synthesis of silver nanoparticles, in the present work the fresh leaves of euphorbia umbellate have been used for the synthesis of silver nanoparticles. Synthesis of AgNPs employing either microorganisms or plant extracts has emerged as an alternative approach. Silver nanoparticles is embedded with antibacterial properties because of its unique properties is considered in medical science, the main aim of work is green synthesis of silver nanoparticles using Euphorbia Umbellate leaf extract and its antibacterial activity, after the collection of sample, identification and extraction of Euphorbia Umbellate was performed the production of silver nanoparticles.

Keywords: Silver nanoparticles; Biochemical characterization; Antibacterial activity; TEM and SEM analysis; UV spectroscopy; XRD analysis

1. Introduction

Silver nitrate is commonly used in nano-system and employed in various biomedical and other research area, silver nanoparticles have excellent medical and non-medical properties and also its application when compared with the other metals of nanoparticles [1]. The main aim is green approach of nanoparticles synthesis possess reduced then no

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toxicity and number of plants extract has been concluded in such synthesis [2]. The plant extract contains number of secondary metabolites which plays a critical role during the green nanoparticles synthesis by acting as reducing agents [3]. Property of nanoparticles is utilized in the areas of biomedicine, solar energy conversion and catalysis and water treatment [4]. Among the various noble metals, silver is preferred as a nanoparticle because of its antibacterial catalytic properties and their nontoxicity towards human in comparison to other metals [5].

Several methods have been used for the preparation of silver nano-particles which can be physical, chemical or biological methods. Earlier methods used for the synthesis of silver nano-particles were toxic and hazardous chemicals were used for their synthesis [6]. Thus the use of eco-friendly processes, for the synthesis of silver nano-particles is known as "Green synthesis". Green synthesis is preferred over conventional synthesis because it is eco-friendly, cost-effective, single-step method that can be easily scaled up for large scale synthesis and does not require high pressure, temperature, energy and toxic chemicals [7].

Nano biotechnology provides a crucial technique for the development of a clean, nontoxic, and environment-friendly process for metal nanoparticles synthesis which has the ability to reduce metals by specific metabolic pathways [8]. Nanoparticles show specific characteristics as compared to large particles such as their morphology, size, and distribution. Chemical and physical methods for synthesis of nanoparticles are costly and releases toxic byproducts in nature [9]. Due to these problems, there is a requirement of an alternative for synthesis of nanoparticles [10]. It has also seen that silver nanoparticles synthesized from chemical methods show less antibacterial activity as compared to the nanoparticles synthesized from biological approach [11].

Various nanoparticles have been synthesized by using plant extracts which includes silver, gold, and copper oxide [12]. Use of plant extracts for nanoparticles synthesis is favorable over the other biological material as it removes the long process of maintenance of cell culture. Among various metal nanoparticles, silver nanoparticles obtain more attention due to its good conductivity, stability and antimicrobial activity [13]. The biological activity of silver nanoparticles depends on various factors such as size, shape, size, surface chemistry, distribution, particle composition, particle morphology, capping, agglomeration, etc [14].

1.1. About the plants

Euphorbia umbellata is a rather succulent, ever green or small bushy tree that usually grows up to 5 meters tall, this plant is harvested from the wild for local medicinal use; it is sometimes cultivated as a greenhouse plant in temperate region or as a garden plant in Kenya. *E. umbellata* also known as *Synadenium grantil* is a base and in it habitat becoming up to 3.5 m in height with an equal spread. It is widely grown in the tropics for its giant fleshy leaves as an ornamental and hedge or cover plant and under glass in colder regions.

1.2. Cultivation and propagation

Euphorbia umbellata is very suited to growing on sunny terraces of warm areas need full sun to light shade with a very well-drained soil mix with sand or perlite with small gravel added to ensure good drainage, water them thoroughly and allow to dry before watering again, fertilize the plants only once during the year with the balanced fertilizer [15].

1.3. Medicinal uses

Although toxic and very caustic to the skin and mucous membranes, the latex has sometimes been used internally, particularly to deal with internal parasite, several drops of latex from warmed leaves are taken to latex to expel intestinal parasites and sometimes tapeworm, the leaf sap is also used to treat cardiac problems and excessive menstruation [16]. A few drops of the latex are applied topically to warts, the latex is also applied to source as a treatment for syphilis, and the latex is applied to order abscesses in order to mature them [17].

1.4. Physiology and phonology

This plant is very interesting texture of the stem and leaves is similar to that of some succulent, inside has latex, somewhat reminiscent of the gums, it is native to Africa, it scientific name is Synadenium *grantil* hook or Africa, in urban courtyard takes sizes of shrub or tree zones near zero degree has an outdated behavior, dropping leaves, although frost can dry, the leaves are very colorful, green, with small dark spots and takes a reddish color with sun and throughout its development [18].

It can spread quite easily from segment, a segment of about 10 cm with a blade at least and it is growing rapidly, it is a very attractive for growing in pots on a patio plants, sun requires little irrigation in cold weather and moderate watering in hot seasons [19].

1.5. Green synthesis

The nanotechnology and bioscience opens the possibility for wide variety of biological research topic and medical uses at molecular and cellular levels, the biosynthesis of nanoparticles has been proposed as cost- effectives and environmentally eco- friendly and alternative to chemical and physical methods [20]. The plant mediated green synthesis of nanoparticles is a green chemistry approach that connects nanotechnology with plants.

Utilization set of principle that reduces or eliminates the generation of hazardous substance in the design, manufacture and the application of chemical product. Time and energy consuming synthesis at high temperature and pressure, simple, inexpensive and low temperature synthesis methods is use of toxic reducing and stabilizing agents makes its harmful [21]. Green synthesis provides advancement over chemical and physical methods as it is cost effective which is considered as environmentally eco- friendly, easily scaled up for large scale synthesis, in this methods there is no need to use high pressure energy, temperature and toxic chemical [22].

1.6. Biochemical synthesis of Ag nanoparticles

Chemical approaches are the most popular methods for the preparation of nanoparticles. However, some chemical methods cannot avoid the use of toxic chemicals in the synthesis protocol. Since noble metal nanoparticles such as gold, silver and platinum nanoparticles are widely applied to human contacting areas, there is a growing need to develop environmentally friendly processes of nanoparticles synthesis that do not use toxic chemicals. Biological methods of nanoparticles synthesis using microorganism, enzyme and plant or plant extract have been suggested as possible ecofriendly alternatives to chemical and physical methods. Using plant for nanoparticles synthesis can be advantageous over other biological processes by eliminating the elaborate process of maintaining cell cultures. It can also be suitably scaled up for large-scale synthesis of nanoparticles [23]. It is well known that biological systems can provide a number of metal or metal [18].

Containing particle in the nanometer size range. The synthesis of magnetite nanoparticles by magneto tactic bacteria, siliceous materials by diatoms and gypsum and calcium carbonate layers by S-layer bacteria are some of the examples [24]. The synthesis and assembly of nanoparticles would benefit from the development of clean, nontoxic and environmentally acceptable ‘‘green chemistry’’ procedures and involving organisms ranging from bacteria to fungi and even plants. Hence, both unicellular and multicellular organisms are known to produce inorganic materials either intra-or extracellular [25].

The *Verticillium* sp. fungal biomass when exposed to aqueous AgNO3 solution resulted in the intracellular formation of silver nanoparticles, while *Fusarium oxysporum* biomass resulted in the extracellular silver nanoparticles [26]. The use of microorganisms such as bacteria, yeast, fungi and actinomycetes has been described for the formation of nanoparticles and their applications.

Nanotechnology which denotes the manipulation of matter with at least 1 dimension size from 1 to 100 nanometers. In other words we can say that the technology which deals with the synthesis and control of matter at molecular level [27]. Science is expanding in a broad spectrum from the past few decades and the introduction of nanotechnology has gain lights in the recent decades because of its wide applicability in the field of research for scientist for the treatment of diseases and its wide application in the field of electronics, microchips, chemicals and much more. The synthesis of these nanoparticles is also cost effective and ecofriendly thus not hampering the natural constraints [28].

Nanotechnology provides the ability to engineer the properties of materials by controlling their size, and this has driven research toward a multitude of potential uses for nanomaterials [29]. Because of the unique physicochemical characteristics of metal nanoparticles, including catalytic activity, optical properties, electronic properties, antibacterial properties, and magnetic properties, they are gaining the interest of scientist for their novel methods of synthesis [30].

2. Material and methods

- Plant material**:** Fresh *Euphorbia umbellata* Leaves.
- Chemicals**:** L-ascorbic acid, silver chloride solution, Hydrazine hydrate, NaOH solution, distilled water, ethanol.
- Glass wares**:** Chopping Knife, Conical Flask, Petri plates, Beaker, Culture Tube, Spreader, Gel Puncher.
- Instruments**:** Autoclaves, Centrifuge, Spectrophotometer, Hot Air Oven, Hot Plate, Transmission Electron Microscope, Weighing Machine.
- Cultures: Pure culture of gram positive and gram negative bacteria.

2.1. Collection of plant

Euphorbia umbellata was collected from Bangalore, dist- kolar, Karnataka, India and identified by Dr. Kirankumar B, Reva University was collected and preserved the sample in Department of microbiology, Bangalore City College, Bangalore, Karnataka.

2.2. Preparation of leaf extract

Leafs of *Euphorbia umbellata* plants were collected and washed in water to remove the dirt and sand. Then again it was washed with distilled water to remove the adhering particles and contaminates. 20 g of leaf sample was weighed and chopped in fine pieces excluding the leaf vein. The chopped leaf pieces was then again washed with distilled water to remove the contaminants and then it was transferred to 250 ml beaker and 100 ml of distilled water was added to it then it was boiled at 60 °C for 30 minutes. The beaker was covered so that to ensure no loss of the particles of the leaf. With the help of Whatmann filter paper the plant extract was collected in a borosil made sample collecting bottle and it was covered with paper such that to prevent it from light exposure so that photosynthesis may not occur.

2.3. Preparation of $AgNO_3$ Solution

Required molar $AgNO_3$ solution was prepared by accurate amount of silver nitrate was dissolved in required volume of water .generally for the preparation of silver nanoparticles we use 1 mM silver nitrate solution. The solution was stored at dark color bottle for prevents to auto oxidation. [0.0168 g $AgNo_3$ /100 ml D.W] (4).

2.4. Green synthesis of leaves silver nanoparticles

Generally 80 or 90 ml of $AgNO_3$ was added to 20 or 10 ml of leaf extract and follow some physical techniques like heat, stirring then the solution was incubated some time. The color change was observed, it is indicated by formation of silver nanoparticles, which was confirmed by uv-visible spectrophotometer. The formed silver nanoparticles were centrifuge separated and dried.

Figure 1 Plant extract and silver nitrate

Figure 2 Formation of nanoparticles

2.5. Biochemical characterization

2.5.1. Indole test

Peptone broth was prepared and sterilized at 121 °C for 15 min and inoculated with test organism, incubated the medium at 37 °C for 24 hours, Added 1 ml of Kovac reagent to tubes including control. Shook and observed the tubes for presence of rings.

2.5.2. Methyl red test

Prepared MR-VP broth in two flasks, inoculate the broth with the test organism and incubated for 24 hours at 37 °C, after 24 hours of incubation transferred 5 ml of broth into two test tubes. To the each broth culture added 5 drops MR indicator the tubes and shake them. Examine the colors of the each culture.

2.5.3. Voges – proskauer test

Prepared MR-VP broth in two flask, inoculate the broth with the test organism and incubated foe 24 hours, prepared BARRITT regent A and B. after 24 hours of incubation 0.5 ml of reagent A and 0.2 ml of reagent B was added to the broth and observe for color change.

2.5.4. Citrate utilization test

Prepared citrate agar slant and inoculated each of the test organism into appropriately labeled tubes by means of a loop, the slant was left un-inoculated that serve as control, incubated for 24 hours at 37 °C. After 24 hours all agar slant were examined for the presence of growth and coloration of the medium.

2.5.5. Catalase test

Transferred small quantity of culture from the plates on glass slide, add 1 drop of 3% H_2O_2 observe bubbles formation.

2.5.6. Oxidase test

Taken oxidase disc in clean microscopic slide, pasted the culture on the oxidase disc and observed for color changes.

2.5.7. Nitrate test

Prepared nitrate broth and inoculated each of the test organisms into its appropriately labeled tubes means of a loop. The last slant was left un inoculated that serve as control, incubated all culture for 24 hours at 37 °C , after 24 hours add one dropper full of sulfanilic acid and one dropper full of α naphthylamine to each broth. Broth were examined for the change in coloration of the medium, a color change to red indicates a positive nitrate reduction test.

2.5.8. Starch test

Prepared starch agar and inoculated each of the test organism into its appropriately labeled tubes by means of a loop, the last plates was left un inoculated that serve as control, incubated all culture for 24 hours at 37 °C. after 24 hours all agar slants were examined for the presence of growth and zone formation on the medium, add iodine solution to see the zone formed more vividly.

2.5.9. Gelatin test

Prepared gelatin slant and inoculated each of the test organism into its appropriately labeled tubes by means of a loop. The slant was left un inoculated that serve as control, incubated all culture at the bacterium optimal growth temperature for up to 1 week and checked every day for gelatin liquefaction. Gelatin normally liquefies at 280c and above, so to confirm that liquefaction was due to gelatinase activity. The tubes are immersed in an ice bath for 15 to 30 min, Afterwards tubes are tilted to observe if gelatin has been hydrolyzed, hydrolyzed gelatin will results in liquid medium even after exposure to cold temperature (ICEBATH), and while the UN inoculated control medium will remain solid.

2.6. Characterization of silver nanoparticles

2.6.1. Transmission electron microscope

Transmission electron microscope is microscopy technique in which a beam of electron is transmitted through a specimen to form an image, the specimen is ultrathin section which is less than 100 nm thickness, the image is formed form the interaction of the electrons with the samples is the beam transmitted through the specimen. Then the image is magnified and also focused an imaging device which is a fluorescent screen, a layer of photographic film.

Transmission Electron Microscopy (TEM) uses an electron beam to interact with a sample to form an image on a photographic plate or specialist camera. The sample must therefore be able to withstand the electron beam and also the high vacuum chamber that the sample is put into. The sample preparation can be difficult as a thin sample on a support grid must be prepared. High-Resolution TEM looks at the interference of the electron beam by the sample rather than the absorbance of the beam as with ordinary TEM. This gives a higher resolution which is beneficial when studying nanoscale samples. However it does require understanding of the sample to allow interpretation of the results, as the phase-contrast resulting information can be difficult to interpret. This can therefore restrict the use of HRTEM. Environmental TEM allows TEM to be carried out in-situ by using the relevant gaseous atmosphere as opposed to the vacuum used for TEM.

2.6.2. Analysis

TEM analysis give the information about the morphology of the silver nano particles .generally silver nano particles are spherical or crystal structures. Tem also give average mean size of silver nano particles. TEM measurements were performed on JEOL model JEM 2100 instrument operated at an accelerating voltage at 120 kV.

2.6.3. Scanning electron microscope

Scanning electron microscope has been indispensable tool in science and research area its invented in the year of 1962 and has been significantly associated with biology and the fields of research and innovation, Scanning Electron Microscopy also uses a high energy electron beam but the beam is scanned over the surface and the back scattering of the electrons is looked at. The sample must again be under a vacuum and foe SEM it must be electrically conductive at the surface. This can be achieved by sputter coating a non-conductive sample. This requirement can be restrictive and again this technique can be time consuming and expensive. Environmental SEM is available where samples can be looked at again in a low pressure gas environment as opposed to a vacuum. Scanning Transmission Electron Microscopy combines the ideas of looking at the surface of the sample and into the sample with an electron beam.

SEM scans samples that are electrically conductive for a non- conductive materials a special layer or coating of carbon, gold, platinum is applied on the sample to make it conductive and for this sputtering coating machine is used to serve this purpose, in SEM conventional imaging the specimen must be conductive electrically at least at the surface and it is made sure that they are electrically grounded to prevents buildup of electrostatic charge superficially, apart from cleaning and mounting the specimen on stub, metals require no specific preparation for SEM.

2.6.4. Analysis

SEM analysis shows uniformly distributed silver nano particles on the surfaces of the cells. The suspended silver nano particles in sterile distilled water were used for scan electron microscope analysis by fabricating a drop of suspension onto a clean electric stubs and allowing water to completely evaporate. SEM analysis gives size of silver nanoparticles. Majority cases a large size silver nanoparticles was observed due to agglomeration of smaller ones.

2.6.5. UV- visible spectroscopy

In UV visible spectroscopy the sample is irradiated with the broad spectrum of the UV visible radiation, if a particular electronic transition matches the energy of a certain band of UV visible will be absorbed, the remaining UV visible light passes through the sample and is observed, from this residual radiation a spectrum is obtained with gaps at these discrete energies this called an absorption spectrum. Ultra violet and visible absorption spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface, absorption measurement can be at a single wavelength or oven an extended spectral range.

Every times a molecule has bonds, the atoms in bonds their atomic orbital which can be occupied by electrons of different energy levels, grounds state molecular orbital can be excited to anti- bonding molecular orbital. These electrons when imparted with energy in the forms of light radiation get excited from the highest occupied molecular orbital to the lowest unoccupied molecular orbital and the resulting species is known as the excited state or the anti-bonding state.

2.7. Plant pathogen

Infectious plant disease are caused by living biotic agent or pathogen, these plant pathogen can be spread from an infected plant to a healthy plants, microorganism that cause plant disease include nematode, fungi, bacteria and some kind of mycoplasmas. Photosynthesis is an essential function of plants and any pathogen that interferes with it will cause disease that may appear as chlorosis and necrosis of the leaves and stem, pathogen can affect translocation of water and nutrient through the vascular system of the host plant. All viruses that spread within their host tissue can be transmitted by grafting branches or buds from diseased plants on healthy plants, most disease causing viruses are carried and transmitted naturally by insects and mites which are called vectors of the virus.

2.8. Citrus canker

Citrus canker is a disease affecting citrus species caused by the bacterium *Xanthomonas axonopodis*; infections cause lesions on the leave, stem and fruits of citrus tree including lime, orange and grape fruits. While not harmful to human canker significantly affects the vitality of the citrus tree that causing leaves and fruits to drop prematurely that fruits infected with canker is safe to eat but too unsightly to be sold. The disease which is believed to have originated in Southeast Asia, is extremely persistent when it becomes established in an area, citrus groves have been destroyed in attempts to eradicate the disease.

3. Results

3.1. UV spectroscopy

The spectral study of synthesized silver Nanoparticles was done using UV visible spectrophotometer in range of 200-800 nm.The production of copper nanoparticles due to the reduction of copper ions by the addition of leaf extract to the copper chloride solution. The band observed in the spectrum confirmed copper nanoparticles.

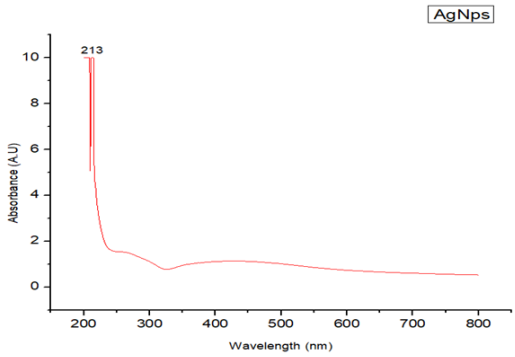

Figure 3 Synthesized AgNps with *Euphorbia umbellata* leaf plant extract shows UV ranges between 200-300 nm and preliminary confirmed by UV results

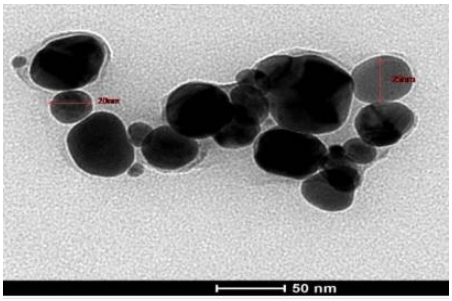

Figure 4 TEM analysis

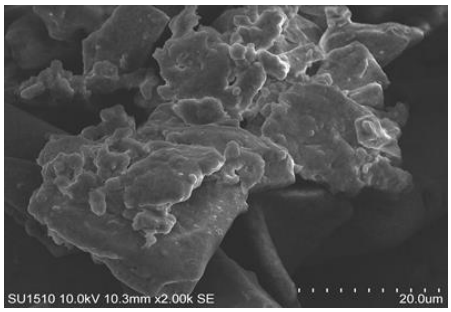

Figure 5 SEM analysis

3.2. Biochemical characterizations

Table 1 Biochemical Analysis

Test	Results
Indole	+
Motility	-
Gram staining	-
Methyl red	+
Citrase	+
Gelatin	-
Oxidase	-
Starch	+
Triple sugar	+
Gram staining	-

3.3. Antimicrobial activity against plant pathogen (*Xanthomonas citrus*)

Figure 6 Antimicrobial tests against plant pathogens

3.4. Disc diffusion test silver nanoparticles of *Euphorbia umbellate*

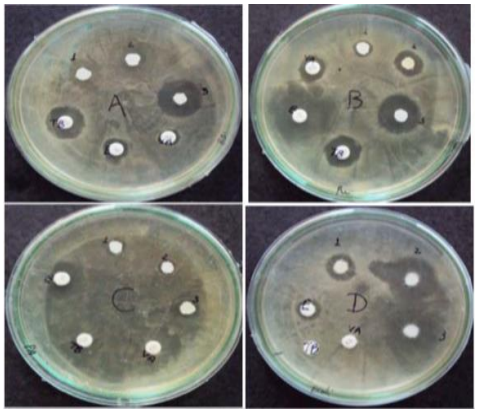

Figure 7 Disc diffusion methods of antimicrobial activity

Table 2 zone of inhibition

Bioactive agent	**Zones of inhibitions**		
	E. coli	***Bacillus* spp.**	***Pseudomonas* sp.**
Silver nanoparticles	2.5	3.2	3.1
Erythromycin	Nil	Nil	0.6
Ampicillin	3.4	4.2	3.3
Vancomycin	0.7	Nil	0.9
Kanamycin	2.3	3.1	Nil
Penicillin	2.4	2.1	3.1

4. Conclusion

Given the wide ranging applications of AgNPs in recent years, many researchers have focused on the development of modified or novel synthetic strategies for AgNPs as opposed to the use of conventional methods which are strongly associated with toxic environmental footprints. This study reports on the antimicrobial activities of AgNPs prepared from leaf extract samples of the medicinal tree species. Microscopic analysis (SEM) provided an inconclusive indication that AgNPs were spherically shaped. In addition, the particles appeared to be highly agglomerated, possibly owing to the physical dehydration exerted during the SEM sample preparation procedure. In contrast, TEM analysis of aqueous silver nanoparticle samples provided unequivocal evidence that the prepared AgNPs were spherical in shape. Furthermore, the particles were observed to be stable and dispersed, even within aggregates. In recent years, however, studies have suggested that the bioactivity of AgNPs occur in a size-dependent manner with smaller particles exerting better bioactivities than larger ones Size class distribution studies of the AgNPs prepared In this study indicate that the particles had narrow size distribution ranges and no significant differences were noted for the nanoparticle preparations in terms of their size. The particles produced here are relatively.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest**.**

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