BIOSYNTHESIS OF ZINC OXIDE NANOPARTICLES AND ITS CHARACTERIZATION FOR ANTIBACTERIAL AND ANTICANCER PROPERTIES

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ABSTRACT
Nanotechnology is a burning field for the researchers and more so for commercial applications. The nanoparticles finds its use extensively in medical chemistry, environmental remediation, atomic physics, energy and several other fields. Zinc oxide (ZnO) is a multifunctional material with its unique physico-chemical properties, and due to its low toxicity, makes it a material of interest for biomedical applications. Hence our aim was to extract ZnO nanoparticles from low cost materials like plants and determine its suitability for applications for antibacterial or anticancer properties. The biosynthesis of ZnO nanoparticles (ZnO NPs) was carried out from the aqueous solution of Azadirachta indica leaf extract using Zinc sulphate as reducing agent. The synthesized nanoparticles were characterized by UV-vis Spectrophotometer, FTIR, XRD, SEM and TEM. The nanoparticles were in a powder form of spherical shapes from 10-75 nm in size with average size ~25nm. These nanoparticles were used for evaluating the antibacterial activity against Gram-negative (Escherichia coli) which were more sensitive than Gram-positive (Streptococcus mutans) bacteria. We have also tested ZnO NPs on leukemic cancer cells (CML) using MTT assay, by
exposing the cancer cells to various concentrations of ZnO NPs solution; the results showed good activity- however this needs further confirmation. It is concluded that ZnO NPs synthesized from plant extract (biological method) could be standardized and proposed as an antibacterial compound further, we present here the complete protocol for the green synthesis of ZnO NPs which was validated using physicochemical, and analytical methods.

KEYWORDS: Nanotechnology, Nanoparticles (NPs), Biosynthesis, Zinc oxide, Characterization, Antibacterial properties.

INTRODUCTION
Rapid industrialization and urbanization has been affecting our environment leading to release of several toxic chemicals and gases, promoting the need to investigate the use of natural products in the synthesis processes of nanoparticles. Nanotechnology involves the research and technology development at the atomic molecule in the length scale of approximately 1 – 100 nanometer range, to provide a fundamental understanding of phenomena and materials at the nanoscale and to create and use structures, devices and systems that have novel properties and functions because of their small or intermediate size (NSET, 2000). Nanotechnology applications are highly suitable for biological molecules, because of their exclusive properties. The biological molecules undergo highly controlled assembly for making them suitable for the metal nanoparticles synthesis which was found to be reliable and eco-friendly (Bar et al., 2009). The novel and differentiating properties and functions are developed at a critical length scale of matter typically under 100 nm.

Nanotechnology research and development includes manipulation under control of the nanoscale structures and their integration into larger material components, systems and architectures. Within these larger scale assemblies, the control and construction of their structures and components remains at the nanometer scale (NSET, 2000). The synthesis of metal and semiconductor nanoparticles is a vast area of research due to its potential applications, implemented in the development of novel technologies. Nanotechnology is one of the upcoming areas of research in material science with the increasing variations in properties such as size, distribution and morphology of the Nanoparticles etc. This has led to development and use of novel applications of nanoparticles and nanomaterials in various fields.
Richard Feynman, Nobel Prize winner (1959) in physics gave a lecture to the American Physical Society called “There’s Plenty Room At The Bottom” The focus of his speech was about the principles of nanotechnology (Feynman, 1960). He was the first to predict the future of nanotechnology and to bring forward the idea to build “nano-scale” machines and have them build millions of factories. In 1974 Tokyo scientist Norio Taniguchi from Tokyo University, first defined nanotechnology and his statement was “Nanotechnology mainly consists of the processing of separation, consolidation and deformation of materials by one atom or one molecule.”

Nanotechnology research deals with design, synthesis, and manipulation of nanoparticle structures of the order of approximately 1-100 nm in one dimension. There has been remarkable growth in this upcoming technology which has led to novel fundamental and applied frontiers, including the synthesis of nanoscale materials and investigation of their interesting physicochemical and optoelectronic properties. Nanotechnology is swiftly gaining importance in a numerous areas such as health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, reorography, single electron transistors, light emitters, nonlinear optical devices and photoelectrochemical applications (Korbekandi and Iravani, 2012). Nanomaterials are seen as solution to many technological and environmental challenges in the field of solar energy conversion, catalysis, medicine and water treatment. Nanomedicine is rapidly emerging as a field with a wide variety of nanotechnology applications (Freitas, 2005; Wagner et al., 2006). In the biomedicine field, nanoparticles are being investigated for use as antimicrobial (Taylor et al., 2005; Seil et al., 2012) and anticancer agents (Magrez et al., 2005). Although a few studies have reported the potential toxicity to normal human cells associated with nanoparticle use (Gupta and Gupta, 2005; Long et al., 2006), largely the immense applications of nanoparticles suggest the need to continue research into their biomedical applications, in conjunction with extensive research into their toxicity. In the context of global efforts to reduce hazardous waste, the continuously increasing demand of nanomaterials must be accompanied by green synthesis methods.

Nanotechnology is essentially changing material synthesis and device fabrication methods. A “bottom-up approach” is applied in transforming nanoscale building blocks into functional assemblies and construction of multifunctional devices. The unique features of nano sized
materials in terms of their varied optoelectronic, magnetic and mechanical properties have propelled research into their synthesis (Ingole et al., 2010).

**Zinc oxide nanoparticles (ZnO NPs)**

Zinc oxide is an inorganic compound, which is a white powder, insoluble in water. The structural arrangement and configuration of ZnO nanoparticles has been investigated by the use of new atomistic potentials. Mechanical properties like internal stress and adhesion characteristics are required to maintain patterning accuracy and durability for various applications of nanoparticles.

**Applications of zinc oxide nanoparticles**

Zinc oxide particles are used for many applications such as various products as it acts as an invisible obstacle which scatters UV radiation away from the skin relatively than permitting its destructive energy to be absorbed, making them useful in sunscreens, paints, varnishes, plastics and cosmetics, especially for broad UV-A and UV-B blocking (Stypuła et al., 2011; Lu et al., 2015). A similar kind of active ingredient is used in the calamine lotion and diaper cream. ZnO is nontoxic and the antimicrobial effects of Zinc oxide and zinc oxide nanoparticles have also been demonstrated and confirmed by various researches which show evidence of antibacterial activity which increases with decreasing the particle size (Söderberg et al., 1990; Yamamoto, 2001; Gordon et al., 2011). Further, Zinc nanoparticles are used as preservative for various materials and products such as plastics, ceramics, glass, pigments, foods, etc. Zinc nanoparticles have also been explored for use as quantum dots for use in photoconduction devices (Kim et al., 2003). ZnO nanoparticles are also used in industries involved in environmental remediation, synthetic textile manufacture and food packaging (Rajamanickam et al., 2012). Zinc oxide, an II–VI semiconductor, is particularly important because of its unique optical or electronic properties and promising applications in various fields such as photonic catalysis, light emitting diodes, field emission, gas sensors and solar cells. Hence, our aim was to synthesize ZnO Nanoparticles from cheap sources such as plant materials and to test for some of their antibacterial and anti-cancer properties.

**MATERIALS AND METHODS**

**Methods for nanoparticles synthesis**

Green synthesis provides advantage over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals (Ingle et al., 2008). So, in
the search of cheaper pathways for nanoparticles synthesis, we used plant extracts (phytochemicals). With their antioxidant or reducing properties they are usually responsible for the reduction of metal compounds into their respective nanoparticles.

**Preparation of ZnO nanoparticles from Plant Extract**

*Azadirachta indica* leaves

Fresh leaves of *Azadirachta indica* (classification presented in Fig. 1), were collected from O.U. campus- Hyderabad, and washed several times with water to remove the dust particles and then sun dried to remove the residual moisture and grinded to form powder. Then plant extract was prepared by adding 20 gms of powder to 300 ml of deionized water and boiled up to $80^\circ$C for 30min with the stirring. Zn Sulphate was added to precipitate the Zn NPs. Then the solution was centrifuged for 15 min at 7000 rpm. The supernatant was separated and filtered with whatman filter paper. The extract was stored at -20°C for further use. UV-Visible Spectroscopy was used to detect the compound in the extract.

![Image of Azadirachta indica leaves](image)

**Fig. 1: Image shows the leaves of Azadirachta indica (Neem).**

**Characterization of the Nanoparticles**

(i) **UV-visible spectroscopy**: was used for determining the nanoparticle preparation. The progress of the reaction between metal ions and the leaf extracts were monitored by UV-visible spectra of ZnO nanoparticles in aqueous solution with 30 min reaction time.

(ii) **Fourier Transform Infrared (FTIR) Spectroscopy**

Using Spectrum one – Perkin Elmer (USA) spectroscopy, we could determine the functional groups present in the synthesized Nano powders. For FTIR measurements, the ZnO nanoparticles solution was centrifuged at 10,000 rpm for 30 min. The pellet was washed three
times with 20 ml of de-ionized water to get rid of the free proteins/ enzymes that are not capping the Zinc Oxide nanoparticles. The samples were dried and grinded with KBr pellets and analyzed on a Spectrum One, Perkin Elmer, USA model in the diffuse reflectance mode operating at a resolution of 400-4000 cm\(^{-1}\).

(iii) **X-Ray Diffraction**

The XRD method was especially suitable for evaluating crystallite size. The methodology of XRD-based evaluation of crystallite size was done to evaluate the crystalline size of the NPs. A thin film of the ZnO nanoparticles was made by dipping a glass plate in the solution and carried out the X-ray studies. The diffraction pattern was recorded by Co–K\(\alpha_1\) radiation with a wavelength of 1.78 Å. The scanning was done in the region of 200 to 900 for 20 at 0.020/min and the time constant was 2 s. The crystalline nature of ZnO nanoparticles was confirmed from the X-ray diffraction analysis.

(iv) **Scanning Electron Microscopy (SEM)**

We used SEM to detect the sample shape and size on the nano-materials prepared as above. In this work, morphology of the ZnO was studied using scanning electron microscope (ZEISS- EVO 18). The powder was mounted on a metallic stub and an ultrathin coating of was deposited by low vacuum sputter coating. This is done to prevent the accumulation of static electric fields at the specimen due to the electron irradiation during imaging and to improve contrast. The SEM can produce high resolution images of the sample surface.

(v) **Transmission Electron Microscopy (TEM)**

Which is the actual size of the nano-particles prepared could be measured using TEM. Transmission electron microscopy is the premier tool for understanding the internal microstructure of materials at the nanometer level. This allowed us to obtain real-space images of materials with resolutions of the order of a few tenths to a few nanometers.

**Determining the Biological activity of the ZnO nanoparticles**

To determine the biological activity of the ZnO nanoparticles we carried out two types of assays – first to determine its antimicrobial activity and second to determine its anticancer activity.
Determination of antimicrobial activity
The test organisms *E. coli* and *S. mutans* were procured from MTCC, Chandigarh, India, and cultured in our laboratory. The agar well diffusion method was employed to determine the antimicrobial activities of the ZnO Nanoparticles. Well-assay was found to be a simple, cheap and reproducible practical method. The second test was the minimum inhibitory concentration (MIC) assay, which detects lowest inhibition concentration for tested bacteria and all experiments were done in triplicates.

Bacterial Culture Preparation
In this method the culture media was prepared in 150 ml Mueller-Hinton agar, which was poured in 4 Petri dishes. After solidification we plated the culture on the agar by rod spreader using 50 mm H₂O + 50 mm of bacterial culture. Then the plates were left for the inoculated cultures to grow and form a layer on the media.

**For MIC method:** 3 wells were made in each plate and different amounts of the nanoparticles solution were added with 10mM, 25mM and 50mM concentrations. The plates were incubated at 37°C, for 24 hours. The diameters of inhibition zones were measured in millimeters.

**For Disc Method:** A solution of each sample to be tested was prepared by diluting to 10-1, 10-2 and 10-3. Then 1 ml of 10⁸ cells/ml was spread on a solid agar medium in the petri dishes (Nutrient agar). Filter paper discs (4 mm in diameter) were soaked in 5 μl of the Nano-solution (test solution) and placed on the inoculated plates and allowed to dry for 7 to 10 min, then incubated at 37°C for 24 h. The diameters of the inhibition zones were measured in millimeters.

**MTT ASSAY**
Cell viability was quantified using the metabolic dye MTT, as previously described (Giordano et al. 2006; 2008). Briefly, cells were treated with ZnO NPs for 24 hours at 37°C. At the end of exposure, the medium was removed and cells were incubated with 500 μl/well of Locke's buffer solution containing 2 mg/ml MTT for 30 minutes. MTT was then removed and the reaction product was dissolved in 0.25 ml DMSO/well. Absorbance was read at 570 nm, and the results expressed as the percentage of viable cells relative to the DMSO exposed controls. Untreated controls and blanks were incubated in the same plates and under the same
conditions. Percentage of anticancer activity (Cell viability) = Control- Treated/control X 100. All experiments were done in triplicates.

Statistical Analysis
Results are expressed as the mean±SD of at least three independent experiments.

RESULTS AND DISCUSSION
Bio Synthesis of Zinc Oxide Nanoparticles
The ZnO nanoparticles were biosynthesized by following the co-precipitation method. The precursor solution of solution (0.2mM) of zinc acetate Zn(O₂C₃H₇)₂ was prepared in 250 mL conical flasks and to this 30 ml of Neem leaf extract was added for reduction into zinc+ ions. The composite mixture was then kept on turntable of the microwave oven for complete bio-reduction at a power of 300W for 4 min. intermittently to prevent an increase of pressure. In the meantime, the colour change of the mixture from faint light to deep blue dark was monitored periodically (time and colour change were recorded along with periodic sampling and scanning by UV-visible spectrophotometry) for maximum 30 min. The reactions were carried out in darkness. Suitable controls were maintained all through the conduction of experiments. Complete reduction of Zn (O₂C₃H₇)₂ to Zn²⁺ ions was confirmed by the change in colour from colourless to colloidal brown. After irradiation, the dilute colloidal solution was cooled to room temperature and kept aside for 24 h for complete bio reduction and saturation denoted by UV-visible spectrophotometric scanning. Then, the colloidal mixture was sealed and stored properly for future use. The formation of ZnO NPs was furthermore confirmed by spectrophotometric analysis.

The qualitative analysis of zinc oxide nanoparticles was carried out based on the visual observation of color formation. Appearance of white colored precipitate was observed as the end product. The natural products, namely flavanones, terpenoids and reducing sugars are the main constituents of the Neem leaf broth that acts as stabilizing agents. It is observed that the aldehyde groups are responsible for reduction of zinc oxide to zinc oxide nanoparticles. In addition, these molecules also act to stabilize the nanoparticles. Thus, it is seen that the Neem leaf extract acts as reducing and stabilizing agents for the formation of zinc oxide nanoparticles.
UV-Visible Absorption Studies

Reduction of Zn ions into ZnO nanoparticles during exposure to plant extracts was observed as a result of the color change. The color change was due to the Surface Plasmon Resonance phenomenon. The metal nanoparticles have free electrons, which give the SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave (Fig. 2).

![UV-Visible Absorption Spectra of ZnO Nanoparticles reduced with Zinc acetate for Azadirachta indica leaf extract](image)

**Fig. 2:** UV-Vis absorption Spectra of ZnO Nanoparticles reduced with Zinc acetate for *Azadirachta indica* leaf extract

X- Ray Diffraction Studies

X-ray diffraction is a non-destructive and analytical method for identification and quantitative analysis of various crystalline forms of ZnO, also known as phases of the compound present in the samples. Diffraction occurs when the waves collide with a regular structure in which the repeating distance is approximately same as the wavelength of the wave. It happens that X-rays have wavelengths in the order of a few angstroms. This means that the X-rays can be easily diffracted from materials which, are crystalline and have repeating and regular atomic structures. When the required parameters met, the X-rays that get scattered from a crystalline solid can interfere constructively, thus producing a diffracted beam of light. In 1912, W. L. Bragg derived a relationship among several factors: (i) the inter atomic spacing which is known as d-spacing and is measured in angstroms. (ii) The angle of diffraction which is known as the theta angle and is measured in degrees and (iii) the wavelength of the incident X-rays, denoted by the lambda and, in this case, is equal to 1.54 angstroms (Bragg, 1913).
Fig. 3 shows the XRD pattern with the diffraction peaks at 31.89°, 34.38°, 36.64° and 39.36° corresponding to the (100), (002) and (101) reflection planes of hexagonal wurtzite structure of ZnO nanoparticles.

![XRD pattern](image)

Fig. 3: XRD pattern of as synthesized ZnO nanoparticles from *Azadirachta indica* leaf extracts.

By applying Scherrer formula, the size of the crystallite ZnO samples was found to be same as the value measured by particle size analyzer. Here it was seen that the diffract grams were almost same but there were some undesired picks in between, which was most probably because of the impurities present in the samples.

**Fourier Transform Infrared Spectroscopy (FTIR) Studies**

The peak at 459 cm$^{-1}$ corresponds to the standard peak of ZnO due to Zn–O stretching frequency of Zn–O bonds confirms the presence of M–O vibrational bands (Sharma et al., 2010; Rajiv et al., 2013). The intense broad line at 3420 cm$^{-1}$ is characteristic of the hydroxyl functional group in alcohols and phenolic compounds. The band 1577 cm$^{-1}$ corresponds to amide-I and 1416 cm$^{-1}$ is C–C stretching vibrations. 1032 cm$^{-1}$ and 1050 cm$^{-1}$ can be attributed to C–N stretching vibrations of aliphatic, aromatic amides and aliphatic amines, alcohol and phenolic groups and stretching vibrations of secondary amine (Baskar et al., 2013). The bands at 784, 677, 664 and 617 cm$^{-1}$ corresponds to C–H stretching of alkanes, C–H (aromatics), CQC–H (alkynes) and –OH stretching of intra molecular H-bond, COO and C–C stretching of alkanes Figure-4.
Fig. 4: FTIR spectrum recorded by making KBr disc as synthesized ZnO nanoparticles prepared from the extract.

Scanning Electron Microscopy (SEM) Analysis

Fig. 5: (a, b) SEM images of ZnO nanoparticles with various magnifications (c) EDS spectra.

Transmission Electron Microscopy (TEM)

TEM technique was employed to visualize the size and shape of ZnO nanoparticles. The 200 kV Ultra High Resolution Transmission Electron Microscope (JEOL-2010) has been used. TEM grids were prepared by placing a drop of the particle solution on a carbon-coated copper grid and drying under lamp. Fig. 6a shows the bright-field TEM micrograph of the synthesized ZnO nanoparticles with different magnifications. It is observed that most of the ZnO nanoparticles were spherical in shape. At all magnifications quite a few agglomerated ZnO nanoparticles were observed in some places, there by indicating possible sedimentation at a later time or the stabilizing agent was not properly selected. It is evident that there is variation in particle sizes and the average size estimated was 25 nm and the particles size ranged from 5 nm to 50 nm and Fig.7(e) corresponds to high resolution lattice image form one such particle.
Fig. 6: (a,b,c,d,e) different magnifications of TEM microscopy of ZnONPs.

Fig. 6 (a-d) a typical bright field TEM image of bio reduced ZnO nanoparticles and (e) High resolution diffraction pattern shown below

Evaluation of Antimicrobial properties of ZnO nanoparticles

Antimicrobial activity by Well-diffusion method (Zone inhibition)

The need for novel antibiotics comes due to the high incidence of resistance to bacterial infections by existing antibiotics. In this study we determined the antimicrobial activity of the synthesized zinc oxide nanoparticles against both Gram-negative (E.coli) and Gram-positive (S.mutans) bacteria by using two different methods viz MIC and well diffusion method.

For antibacterial activity (viability) test, the bacteria (10⁷ to 10⁸ CFU/mL) were incubated with different concentrations of ZnO NPs for 4 h, and then 20μL of a serial 6 to 10-fold dilution of each bacterial suspension in sterile deionized water was spread onto LB plates and allowed to grow for 24 at 37ºC. Colonies were counted and the cell mortality (% of the control) was expressed as the percentage. All treatments were individually repeated at least
three times. The diameter of inhibition zones around each well is represented in table 1. From the results it was observed that the gram positive bacteria were more sensitive when compared to Gram negative bacteria. The mechanism of the antibacterial activity of ZnO nanoparticles is still not well understood. Some researchers have proposed in their study that the generation of hydrogen peroxide is the main factor of the antibacterial activity, while they also indicate that the binding of the particles on the bacterial surface due to the electrostatic forces could be another factor (Sirelkhatim et al., 2015).

Table 1: Zone inhibition values of ZnO nanoparticles with *E.coli* and *S.mutans* bacteria

<table>
<thead>
<tr>
<th>S.No</th>
<th>ZnO nanoparticles Concentration(mM)</th>
<th><em>E.coli</em> Zone of Inhibition (mm)</th>
<th><em>S.mutans</em> Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10µg/ml ZnO NPs</td>
<td>1.0 mm</td>
<td>3.2 mm</td>
</tr>
<tr>
<td>2</td>
<td>25 µg/ml ZnO NPs</td>
<td>2.2 mm</td>
<td>5.6 mm</td>
</tr>
<tr>
<td>3</td>
<td>50 µg/ml ZnO NPs</td>
<td>3.5 mm</td>
<td>7.2 mm</td>
</tr>
</tbody>
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![Image](image1.jpg)

*E.coli* and *S.mutans*

Fig. 7: Image shows the zone of inhibition of *E.coli* and *S.mutans*.

Minimum inhibitory concentration (MIC)

![Image](image2.jpg)

Fig. 8: Graph indicates the MIC of *E. coli* and *S. mutans*. 

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MTT Assay
ZnO nanoparticles and their potential toxicity to cancer cells was investigated in vitro by exposing leukemic cells to various concentrations of ZnO NPs. (Fig. 9) The results were positive – but this is a preliminary report, which needs to be tested on several samples.

However, literature shows that nanotechnology has revolutionized selective cancer targeting. The design and modification of properties of nanoparticles such as size, shape, chemical and physical properties, and so forth, aids their application in targeting the desired cells. Further, the targeting of the neoplastic cells can be either active or passive (Sutradhar and Amin, 2014).

The mechanism of cytotoxicity and DNA damage by ZnO NPs could be attributed in large part to the presence of ionic zinc.

![Graph](image)

**Fig.10:** Graph shows the % cell viability of cancer cells at different concentrations of ZnO NPs.

**CONCLUSIONS**
The conclusion drawn from the present investigations are as follows: ZnO nanoparticles were synthesized successfully by Biological methods (from *Azadirachta indica* leaf extract). The UV-Vis spectroscopic study shows the Plasmon resonance property, confirmed the reduction of metal ion and formation of nanoparticle with plasma resonance peak at 279 nm. The SEM analysis confirms the formation of ZnO nanoparticles with spherical size of diameter ranging 64.6 nm to 1.27μm. The XRD study confirms the structure of ZnO nanoparticles and the formation of narrow peak with the Bragg’s angle of 2θ=34.83° suggests the crystalline nature
and wurtzite structure of ZnO nanoparticles. FTIR measurements were carried out to identify the possible biomolecules responsible for capping and efficient stabilization of the metal nanoparticles synthesized by the Neem leaf extract. Antibacterial potential of ZnO nanoparticles as a function of nanoparticles concentration was tested against bacteria like *E. coli* and *S. mutans*. The test was performed by Well diffusion method. From the study, ZnO nanoparticles were observed to have strong antimicrobial potential. It is observed that the gram positive bacteria were more sensitive when compared to Gram negative bacteria. The minimal inhibitory concentration assay (MIC) of ZnO NPs showed positive towards both the Gram positive (*S. mutans*) and Gram negative (*E. coli*) bacteria. ZnO nanoparticles were active against cancer cells in MTT assay- but this is a preliminary result and this may need further confirmation. However, future development of ZnO NPs-based as therapeutic agents will require more work to optimize physicochemical properties and to understand the medicinal properties. The detailed physicochemical analysis and dosage preparations of ZnO NPs, presented in our work may provide some basic information for future studies.

**Conflict of Interest:** None

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