Green synthesis of cobalt oxide nanoparticles and the effect of annealing temperature on their physiochemical and biological properties

Sirajul Haq\(^{1,}\), Farwah Abbasi\(^{1}\), Manel Ben Ali\(^{2}\), Amor Hedfi\(^{3}\), Amine Mezni\(^{4}\), Wajid Rehman\(^{5}\), Muhammad Waseem\(^{6}\), Abdul Rehman Khan\(^{1}\) and Hamayun Shaheen\(^{1}\)

\(^{1}\) Department of Chemistry, University of Azad Jammu and Kashmir Muzaffarabad, 13100 Pakistan

\(^{2}\) Department of Biology, College of Sciences, Taif University, PO Box 11099, Taif 21944, Saudi Arabia

\(^{3}\) Department of Chemistry, College of Science, Taif University, PO Box 11099, Taif 21944, Saudi Arabia

\(^{4}\) Department of Chemistry, Hazara University Mansehra, 21300, Pakistan

\(^{5}\) Department of Chemistry, COMSATS University Islamabad (CUI), Islamabad, Pakistan

\(^{6}\) Department of Botany, University of Azad Jammu & Kashmir, Muzaffarabad, 13100, Pakistan

\(^{*}\) Author to whom any correspondence should be addressed.

E-mail: cii_raj@yahoo.com

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Abstract

The cobalt oxide nanoparticles (Co\(^{3}\)O\(^{4}\) NPs) were synthesized via the green method using \textit{Piper nigrum} (\textit{P. nigrum}) leaves extract and were calcined at different temperatures. The crystalline nature was studied through x-ray diffractometer (XRD). With increasing calcination, the amorphous phase transitioned to the crystalline phase. Diffuse reflectance spectroscopy (DRS) was used to observe UV-Visible light absorbance phenomenon, and Tauc’s plot was used to calculate the band gap energy, which was found to decrease with calcination. Scanning electron microscopy (SEM) was used to analyze the surface morphology, and Fourier transform infrared (FTIR) spectroscopy was used to classify the surface functional groups. The antibacterial efficacy of the Co\(^{3}\)O\(^{4}\) NPs was examined against Gram-positive bacteria (GPB) and Gram-negative bacteria (GNB), whereas its antioxidant potential was explored against ABTS free radicals. Antibacterial and antioxidant potentials of Co\(^{3}\)O\(^{4}\) NPs were found to decrease with increasing calcination.

1. Introduction

The extensive and improper use of antibiotics led to the development of multi-drug resistant bacteria, which are difficult to treat and is a serious health problem worldwide [1, 2]. To encounter MDR species and secure human health, researchers are consistently working on the development of new ways and materials to combat this issue [3, 4]. Upon the emerging of nanobiotechnology, a new hope is arisen due to the exposure of new and enhanced properties of the materials. Nanostructures got significant attention owing to their small size and increased surface to volume ratio, which result in improved biological, chemical, mechanical, physical and chemical properties as compared to the bulk material of the similar chemical composition [5].

The metal oxide nanoparticles (MO-NPs) have attract the attention of researchers in the recent years due to their small size, high surface area and are used in several environmental, industrial and medical applications [6–9]. Cobalt belong to transition metal with variable oxidation states and more secure state in an oxide form and due to the involvement of 3d orbital in Co\(^{3}\)O\(^{4}\) make it multi-electronic valences and rich incoordination due to which it possesses many states like low, high and intermediate spin [10]. The CoO and Co\(^{2}\)O\(^{3}\) are the most stable oxide of cobalt known as both of them are stable at room temperature [11, 12]. Co\(^{3}\)O\(^{4}\) is a p-type semiconductor with the direct band gap in the range of 1.48–2.19 eV and widely used as heterogeneous catalysts, battery cathodes, gas sensors and magnetic materials [10]. In Co\(^{3}\)O\(^{4}\), 3d energy level of Co\(^{3}\) becomes complicated due to crystal field splitting and have a variety of spintronic applications due to the possible states of
Several chemicals and physical methods were reported for the synthesis of Co$_3$O$_4$ NPs, however the very limited literature is available on the green synthesis of Co$_3$O$_4$ NPs and biological applications [14, 15]. The green process was used to synthesize Co$_3$O$_4$ NPs in this study because it is a more cost-effective, environmentally friendly, and simpler process [16]. In the recent past, several researcher have synthesized Co$_3$O$_4$ NPs using Calotropis procula latex, Punica granatum peel and Aspalathus linearis leaf extract [17, 18]. P. nigrum is a perennial climbing plant in the Piperaceae family that produces a fiercely aromatic spice. The P. nigrum is a common crop in Southeast Asia’s tropics and a vital component of overland trade between Asia and Europe. The active phytochemical like Piperine has antibacterial, antioxidant, antitumor and anti-inflammatory properties.

This work describes the synthesis of Co$_3$O$_4$ NPs utilizing P. nigrum leaves extract as a reducing and capping agent, both of which have not been studied previously. The Co$_3$O$_4$ NPs were calcined at 100, 300, 600, and 900 °C. The physicochemical characteristics were investigated using XRD, SEM, DRS, and FTIR spectroscopies. To investigate its biological potential, all of the prepared Co$_3$O$_4$ NPs were tested against (GPB), (GNB), and ABTS free radicals.

2. Material and methods

2.1. Chemicals used
Sigma-Aldrich provided the laboratory grade materials, which included cobalt sulphate, ethanol, and sodium hydroxide, which were used without further purification. The Department of Botany taxonomically described the P. nigrum leaves obtained from the Chehlla campus. The working solutions were made with deionized water, and the glassware was cleaned in a 15 percent nitric acid solution before being rinsed with deionized water.

2.2. Preparation of leaf extract
P. nigrum healthy leaves were washed in running tap water followed by deionized water and were incised into small pieces with sterile knife. About 30 g of these small pieces of leaves was weighed and taken in a beaker and 1000 ml of deionized water added to it. Then heated for 3 h at 60 °C. By this time aqueous part turns yellow. The extract was filtered by Whatman No. 1 filter paper. This filtrate was made up to 1000 ml in a standard measuring flask. The extract was stored for further use.

2.3. Synthesis of Co$_3$O$_4$ NPs
For the formulated cobalt oxide NPs, 20 ml of leaf extract of P. nigrum and 80 ml of cobalt sulphate were taken in a flask and mixed. Then 0.1 M solution of NaOH added drop by drop to maintain pH at 10. The colour turns green to pink. Then the flask was kept at the hot plate with a magnetic stirrer for stirring and heating at 60 °C for 30 min. The Co$_3$O$_4$ NPs in solution were collected and washed with distilled water thrice. The Co$_3$O$_4$ NPs were dried in oven at 100 °C and calcined at 300, 600 and 900 °C in muffle furnace. The obtained powder of Co$_3$O$_4$ NPs was stored in airtight bottle for further analysis.

2.4. Characterization
The change in crystallographic characteristics with calcination was investigated using the Panalytical X-pert Pro at 2-theta (20°–80°). The Debye–Scherrer equation was used to estimate crystallite sizes based on the width at half maxima. The particle morphology was studied using a Japanese SEM model 5910. The band gap energy was calculated using the transmittance edge of the DRS spectrum operating in the 400–1000 nm region. An FTIR analysis was performed in the region of 4000–400 cm$^{-1}$ with a Nicolet 6700 FTIR analyzer to detect the functional groups contained in the samples (USA).

2.5. Antibacterial activity
Agar diffusion well methodology was used to check the antibacterial activity of prepared and calcined Co$_3$O$_4$ NPs against bacterial species of our interest. In growing bacterial culture the selected bacterial species were uniformly spread on the agar medium in sterilized petri dishes, and kept for some time. Using sterilized micropipette tip, five wells of 6 mm were created in the petri dish. The suspensions containing sample dissolve in distilled water and calcined at different temperatures A (Co$_3$O$_4$ NPs calcined at 100 °C) B (Co$_3$O$_4$ NPs calcined at 300 °C) C (Co$_3$O$_4$ NPs calcined at 600 °C) and D (Co$_3$O$_4$ NPs calcined at 900 °C), about 30 microliter of each suspension poured into the prepared wells and placed in incubators for 24 h. Pseudomonas aeruginosa (P. aeruginosa), Escharchia coli (E. coli), Klebsiella pneumonia (K. pneumonia) and Staphylococcus aureus (S. aureus) bacterial species were used, all these bacterial species were taken from microbial biotechnology laboratory, department of biotechnology in AJK University.
2.6. Antioxidant activity

The stock solutions were prepared by dissolving 5 mg of each calcined Co$_3$O$_4$ NPs in 5 ml of deionized water. For preparing 7 mM ABTS solution, 360 mg ABTS was dissolve in 100 ml of deionized water. 2.5 mM solution of potassium persulphate was synthesized by taking 3.4 mg of it in 10 ml of water and it is mixed with ABTS.

**Figure 1.** Proposed reaction mechanism for green synthesis of Co$_3$O$_4$ NPs.

**Figure 2.** Pattern of XRD of Co$_3$O$_4$ NPs calcined at 100, 300, 600 and 900 °C temperature.
solution and kept in dark overnight. By mixing both solutions we get working solution which was kept for 24 h in dark overnight, giving time that both solutions can react with each other and generate ABTS free radical. The wavelength of spectrophotometer was adjusted at 405 nm, then absorbance of ABTS $^\cdot$ solution was examined, whose value should be less than 2. For activity 1 ml of ABTS $^\cdot$ solution, 100–400 μl of Co$_3$O$_4$ NPs solution and 1600–1900 μl deionized water was added to check percentage activity. The equation (1) was used to compute the % radical scavenging activity, where $A_o$ is the control absorbance and $A_i$ is the test absorbance. During the investigation, a standard of ascorbic acid with the same concentrations was employed.

$$\text{%RSA} = \left( \frac{A_o + A_i}{A_o} \right) \times 100$$  \hspace{1cm} (1)

3. Results and discussion

The Co$_3$O$_4$ NPs were calcined at 100, 300, 600, and 900 degrees Celsius in an environmentally friendly process utilizing P. nigrum leaves extract. XRD, SEM, DRS, and FTIR methods were used to investigate the crystal structure, surface morphology, optical characteristics, and surface functional moieties of produced Co$_3$O$_4$ NPs. Although the precise mechanism of the green synthetic route is unknown, phytochemicals are thought to act as both reducing and capping agents. Ion reduction, nucleation, clustering, growth, and oxidation of metallic oxide nanoparticles are all involved in their formation [19]. The nanoparticles are formed by the most major phytochemicals contained in P. nigrum, and each of these processes is regulated by the structure of the reducing agent, its concentration, the concentration of precursor salts, temperature, and pH [20]. The hydroxyl group in phytochemicals reduces metallic ions coated with phytochemicals. However, it was previously assumed that the hydroxyl group present in the catechol moiety of a flavonoid had a low dissociation energy when compared to other flavonoid and phytochemical hydroxyl classes based on DFT studies [21]. When metal ions that have been
coated with phytochemicals are exposed to air, they become metal oxides [22]. In the presence of polyols, a proposed chemical process for the creation of Co$_3$O$_4$ NPs is shown in figure 1.

3.1. XRD analysis
The XRD diffractograms of the produced Co$_3$O$_4$ NPs are shown in figure 2 and show the typical diffraction peaks. There is no diffraction peak in the XRD spectrum of as-synthesized Co$_3$O$_4$ NPs calcined at 100 °C. The XRD spectrum display in figure 2(b) represent the Co$_3$O$_4$ NPs calcined at 300 °C also lack any diffraction band. The spectrum is noisier than the obtained for the sample calcined at 100 °C, which is due to the transition state. The particles form rearranging themselves under the influence of heat. Thus the absence of diffraction bands in the spectrum of both samples calcined at 100 and 300 °C have amorphous nature. The XRD spectrum of Co$_3$O$_4$ NPs calcined at 600 °C shown in figure 2(c) shows diffraction peaks at 2 with associated hkl values of 37.09 with matching (110) hkl plane, which was determined to be identical to peak described in the reference card 01-074-1657, confirming the synthesis of Co$_3$O$_4$ NPs with cubic geometry. The crystallite had a size of 29.10 nm and 0.376 percent crystal lattice. The XRD pattern of Co$_3$O$_4$ NPs calcined at 900 °C shown in figure 2(d) shows a diffraction peak at 2 theta positions 31.34, 37.00, and 59.64 with corresponding hkl values (220), (311) and (511). The peaks and hkl values corresponded to those reported in JCPDS card 01–074–1657, indicating that Co$_3$O$_4$ NPs with cubic geometry were synthesized. The Debye–Scherer equation yielded a crystallite size of 42.05 nm with 0.257 percent imperfection. The XRD spectra demonstrated that with initial raise in calcination accompanied by the increase of noise in the spectrum, which due to the restructuring and rearrangement crystallites to form bigger crystal. However further increase in calcination results in the transformation of amorphous phase into crystalline phase accompanied by the increase in crystal size and ultimate decrease in the lattice strain.

3.2. SEM analysis
SEM micrographs with high magnification were used to determine the morphology and grain size of all calcined Co$_3$O$_4$ NPs. The highly magnified micrograph (figure 3(a)) clearly shows that the small particles joined together forming a bunch like structure, where clear boundaries among the particles were seen. Upon increasing the calcination up to 300 °C, visible boundaries between the grains are disappeared and the bunch like structure leading to the formation of a bit denser structures. The clusters formation occurred when the calcination increase up to 600 °C and the size of the cavities seen between the particles get increases. Further increase in calcination (up to 900 °C) reveals the cracking of these clusters into small flat particles whereas the size of the cavities between the particles squeezed. The SEM micrographs show the growth of grains leading to the formation of flakes, when the calcination increased from 600 to 900 °C. For Co$_3$O$_4$ NPs calcined at 100, 300, 600, and 900 °C, the particle sizes estimated from SEM micrographs using ImageJ software are 21.68, 38.35, 51.85, and 77.48 nm.
3.3. DRS analysis

Figures 4(a)–(d) shows the DRS spectra of Co$_3$O$_4$ NPs calcined at various temperatures, revealing that the highest absorption occurred in the visible range. The wavelength of the transmittance edge was established by matching the steep rising section of the UV-vis curve with the X-axis of the DRS spectrum, according to prior results [23]. Table 1 demonstrates a distinct drop in transmittance owing to light absorption, which causes electrons to move from the valance band to the conduction band in excited state. Table 1 lists the band gap energies for the Co$_3$O$_4$ NPs analogue computed using a Tauc plot. The band energies were lessen with raising temperature of the sample. When compared to as-synthesized Co$_3$O$_4$ NPs calcined at lower temperatures, the Co$_3$O$_4$ NPs calcined at 900°C has a band gap of 1.65 eV, while the Co$_3$O$_4$ NPs calcined at lower temperatures has a band gap of 1.42 eV. The decrease is caused by a rise in crystallite size, surface roughness, phase structure and electron energy.

3.4. FTIR analysis

The figures 5(a)–(d) shows the FTIR spectra of Co$_3$O$_4$ NPs calcined at temperatures of 100, 300, 600, and 900°C temperature. The stretching vibration of the hydroxyl group was seen in the FTIR spectra of Co$_3$O$_4$ NPs analogue in the region of 3565–3398 cm$^{-1}$, whilst the bending vibration of the O-H vibration was seen in the band about 1647 cm$^{-1}$. The reduction in the strength of the aforementioned bands as the calcination of the sample increased suggested that the water content in the sample evaporated at high temperatures [23]. Table 1 lists the band gap energies for the Co$_3$O$_4$ NPs analogue computed using a Tauc plot. The band energies were lessen with raising temperature of the sample. When compared to as-synthesized Co$_3$O$_4$ NPs calcined at lower temperatures, the Co$_3$O$_4$ NPs calcined at 900°C has a band gap of 1.65 eV, while the Co$_3$O$_4$ NPs calcined at lower temperatures has a band gap of 1.42 eV. The decrease is caused by a rise in crystallite size, surface roughness, phase structure and electron energy.

![Figure 5. FTIR spectra of Co$_3$O$_4$ NPs calcined at 100, 300, 600 and 900°C temperature.](image)

| Calcination temp (°C) | Transmittance edge (nm) | Band gap energy (eV) | Particle size (nm) |
|-----------------------|-------------------------|---------------------|--------------------|
| 100                   | 626.61                  | 1.65                | 21.68              |
| 300                   | 654.09                  | 1.45                | 38.35              |
| 600                   | 659.18                  | 1.43                | 51.85              |
| 900                   | 661.65                  | 1.42                | 77.48              |

Figure 5. FTIR spectra of Co$_3$O$_4$ NPs calcined at 100, 300, 600 and 900°C temperature.
The rearranging of the crystals in the lattice, the intensity of the band found in the FTIR spectra decreases as the calcination rises.

### 3.5. Antibacterial activity

The agar well diffusion methodology was used to examine the antibacterial property of Co$_3$O$_4$ NPs against (GNB) and (GPB). As a result of activity inhibitions zone were created which were calculated in millimeters (mm) as shown in figure 6 and table 2 [26]. The activity of Co$_3$O$_4$ NPs calcined at 100 °C demonstrate more activity to other samples calcined at higher temperature because antibacterial activity strongly depends upon the

![Figure 6. Antibacterial activity of Co$_3$O$_4$ NPs (A = E. coli, B = K. pneumoniae, C = P. aeruginosa, D = S. aureus); each well were loaded with 30 microliter of the prepared suspension of the calcined Co$_3$O$_4$ NPs.](image)

| Bacterial strains          | Zone of inhibition in millimeter (mm) |
|----------------------------|--------------------------------------|
|                            | 100 °C  | 300 °C  | 600 °C  | 900 °C  |
| Gram negative bacteria     |         |         |         |         |
| E. coli                    | 13.5    | 9       | 5.3     | 4       |
| P. aeruginosa              | 8       | 7.2     | 6.2     | 5.5     |
| K. pneumonia               | 7.5     | 5.4     | 3.5     | 2.8     |
| Gram positive bacteria     |         |         |         |         |
| S. aureus                  | 8       | 5.5     | 4.5     | 2.1     |
morphology and structure of NPs which are effected by calcination. By increasing temperature particle size and surface area of NPs increased, which reduces their chance to form interaction with bacteria. At lower temperature like 100 °C size of particles is small and specific surface area to volume ratio increases which is capable of forming more interactions with bacterial species. The binding of small size Co₃O₄ NPs on the surface of bacteria react much rapidly with cell and disturb its important life activities [2, 27].

The Co₃O₄ NPs showed greater activity against GNB instead of GPB. This is attributed to differences in the chemical structure of these two types of bacterial species' cell walls. When compared to GNB, the GPB contains a dense coating of peptidoglycan in the cell wall, which provides more strength and makes the entry of Co₃O₄ NPs on the surface of bacteria react much rapidly with cell and disturb its important life activities [11, 28]. The activity of Co₃O₄ NPs was found elevated towards GNB as contrast to GPB due to the presence of phospholipid and lipopolysaccharide, which convey strong negative charge to bacteria surface, which interact with Co₃O₄ NPs and disturbed bacterial cell [29, 30]. Whereas the surface negative charge of GPB lower than the GNB so Co-cation form weaker interaction with the Gram positive bacteria surface [31, 32].

The antibacterial action of metal oxide nanoparticles can be explained in a variety of ways. In aqueous solutions of Co₃O₄ NPs, reactive species such as cobalt cation, super oxide radical anions, and hydroxyl radicals develop. Cobalt ions released by cobalt oxides interact with the thiol group of key bacterial enzymes, causing inactivation and cell death, or radicals present in aqueous Co₃O₄ NPs interact with the negatively charged bacterial cell surface, interfering with vital life functions like breathing and cell reproduction. As absorbed oxygen reacts with the surface of Co₃O₄ NPs, an electron is excited, which reacts with the oxygen ion to form oxygen ion, which then reacts with the H₂O molecule to form hydrogen peroxide. As a result, H₂O₂ enters the bacterial cell, disrupting cytoplasmic functions and rendering the microorganisms lethal [23, 33].

### 3.6. Antioxidant activity

The fundamental principle of antioxidant activity is the potential of molecule exhibiting antioxidant activity for long lasting ABTS reduction. ABTS is chemical compound utilized to monitor the reaction rates of precise enzymes. It is mostly used as substrate with hydrogen peroxide for a peroxidase enzyme or alone with blue multicopper oxidase enzyme. The free radicals are constantly produced in human body and get stable by extracting an electron/hydrogen led to the degradation of the target molecules including DNA, lipids and proteins led to cancer, heart and neurodegenerative disorders [34]. The ethanolic solution of ABTS shows maximum absorption at 405 nm and the decrease in the absorbance maxima suggest the stabilization of ABTS free radicals. The P. nigrum mediated Co₃O₄ NPs calcined at different temperatures were screened against ABTS free radicals in dose-dependent manner as shown in table 3. The ABTS free scavenging activity was found to increase with increasing administered dose of Co₃O₄ NPs, which might be due to the larger number of the nanoparticles that can encounter the ABTS free radicals [35]. However, a clear decrease was seen in the antioxidant activity of Co₃O₄ NPs calcined at higher temperature. This gradual decreased is may be due to the increase in particles size of the Co₂O₃ NPs particles with ultimate decrease in the active surface available for the

| Sample name | Concentration (µg µL⁻¹) | %RSA | IC₅₀ (µg mL⁻¹) |
|-------------|-------------------------|------|----------------|
| A           | 100                     | 87.91| 79.8           |
|             | 200                     | 91.59|                |
|             | 300                     | 92.24|                |
|             | 400                     | 93.22|                |
|             | 100                     | 76.4 |                |
| B           | 200                     | 86.20| 105.18         |
|             | 300                     | 88.73|                |
|             | 400                     | 90.5 |                |
|             | 100                     | 77.06|                |
| C           | 200                     | 83.51| 106.7          |
|             | 300                     | 89.71|                |
|             | 400                     | 90.2 |                |
|             | 100                     | 63.4 |                |
| D           | 200                     | 78.44| 225.15         |
|             | 300                     | 85.91|                |
|             | 400                     | 88.57|                |

A = Co₃O₄ NPs calcined at 100 °C, B = Co₃O₄ NPs calcined at 300 °C, C = Co₃O₄ NPs calcined at 600 °C and D = Co₃O₄ NPs calcined at 900 °C.
stabilization of ABTS free radicals. The decrease in the antioxidant activity are may also be due to the condensation of cobalt hydroxides to cobalt oxide with increasing calcination, which slowdown the hydrogen donation process to stabilized ABTS free radicals [36, 37].

Under the current experimental circumstances, the IC50 values represent the concentrations of Co2O3 NPs required to scavenge 50% of the ABTS+ radical cations. Table 3 lists the IC50 (g/mL) values for all synthesized compounds. Co3O4 NPs calcined at 100 °C had the highest antioxidant potential of any calcined sample, with an IC50 of 79.8 g/mL. The antioxidant activity, on the other hand, decreases as the calcination rises. This is because of the increase in particle size of Co2O3 NPs calcined at 100 °C, which provides a larger active surface to scavenge the ABTS+ radical cations. As noted in XRD and SEM research, that with increasing calcination, particles size rises. This indicates that when the calcination temperature rises, the number of Co3O4 NPs in the DMSO solution decreases. Because of a decrease in the amount of Co3O4 NPs in solution, which is responsible for the stabilization of ABTS+ radicals, the activity dropped as the calcination increased [38]. When compared to previous studies, the Co2O3 NPs described in this study had stronger antioxidant activity.

4. Conclusion

The green methodology was used to synthesize Co3O4 NPs using P. nigrum leaves extract. For the production of nanoparticles, this process is easy, ecologically benign, the most efficient and less expensive. The structure of Co3O4 NPs was verified by XRD, and the sample calcined at 100 °C was amorphous. The amorphous phase evolves into a crystalline phase as the calcination rises. The XRD also confirms the cubic geometry of synthesized Co3O4 NPs. Because of the increase in particle size, the band gap energy decreases with temperature. The antibacterial activity of produced Co3O4 NPs was tested against Gram negative and Gram positive bacteria, and the activity against Gram negative bacteria was shown to be quite strong. The antioxidant activity was performed then reduced the toxic effect of ABTS free radicals and the antioxidant activity of Co3O4 NPs calcined at 100 °C was significantly high than those calcined at high temperature, which might be due to the increase in particle size.

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Data availability statement

The data generated and/or analysed during the current study are not publicly available for legal/ethical reasons but are available from the corresponding author on reasonable request.

ORCID iDs

Sirajul Haq @ https://orcid.org/0000-0002-9424-2531
Amor Hedfi @ https://orcid.org/0000-0002-3476-4178
Muhammad Waseem @ https://orcid.org/0000-0002-6118-8057

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