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Comprehensive risk assessment of Ni-Cu ferrite nanoparticles and their action against dental caries and lung infections causing bacteria

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ABSTRACT

Isometric nickel copper ferrite nanoparticles with composition $Ni_{0.5}Cu_{0.5}Fe_2O_4$ synthesized using hydrothermal technique. Nanoparticles were characterized using powder X-ray diffraction, Fourier transform infrared spectroscopy, dynamic light scattering technique, scanning electron microscopy and energy dispersive spectroscopy. The nanoparticles obtained were of ~85 nm in size. Haemolysis of blood, microbial toxicology, and teratology on chick embryos were performed using various standard biological assays. The material was found to be non haematotoxic and non-teratogenic. The Bradford's assay showed that the level of total protein content to be insignificantly altered in the treated chick embryos. The material demonstrated an excellent antioxidant property of 75% when studied using DPPH free radical scavenging assay. The nanoparticles synthesized have shown substantial inhibition of pathogenic bacteria such as Streptococcus mutans, Bacillus substilis, Pseudomonas aeruginosa and Escherichia coli. Based on the current evidences and in comparison with the known ferrites, it was inferred that the biocompatibility and the ability of nickel copper ferrite nanoparticles to inhibit the growth of the selected pathogenic bacteria, opens the doors to a new arena for the usage of nanoparticles synthesized in clean room filters as antibacterial agents. They can also be considered for use as air filters for pathogens in nasal masks. Because of the inhibition it has caused against Streptococcus mutans, a significant contributor for tooth decay, it can be incorporated in tooth pastes, mouth washes as a potent antibacterial agent.

Keywords: Hydrothermal route, haemolysis, embryo toxicology, teratology, microbial toxicity, free radical scavenging, filters.

INTRODUCTION

Increased focus on cancer therapy and cancer eradication in the scientific field has led to the investigations on magnetic materials in the application of targeted drug delivery and hyperthermia [1]. Magnetite is one such substance which is widely used in biomedical applications [2,3]. Nickel copper ferrite particles are inverse spinel structure, with isometric symmetry, that are usually represented by the formula $[Fe^{3+}]tet[A^{2+}, Fe^{3+}]octO_4^{2-}$ (A= Ni, Cu,..) [4]. Nickel copper ferrites were previously used in microwave applications and electromagnetic devices. Copper substitution in nickel ferrite is done to modify the properties of the material which is used in making of many devices, but the material has been sparingly used for biomedical applications [5].

Among several synthetic routes available, hydrothermal technique is known to be one of the most effective methods for processing advanced materials [6]. The technique is defined as "any heterogeneous or homogeneous chemical reaction in the presence of a solvent (whether aqueous or non-aqueous) above the room temperature and at pressure greater than 1 atm in a closed system" [7]. The method facilitates the size and morphology control of the nanoparticles and it does not involve any post synthesis heat treatment, which may cause secondary agglomerations [8]. It is one of the most efficient techniques for processing nano-hybrid and nanocomposite materials [9].

Risk assessment is the major aspect that is to be considering in order to use the nanoparticles in biological systems. In this regard, chick embryos are mainly studied for understanding the embryonic development of higher mammals because of their anatomical similarities [10]. They are cost effective and ethical restrictions are limited to day 18 of the embryonic development and after hatching [11]. These embryos are employed in most of the industries to assess toxicology of several chemicals, drugs, materials, plant extracts, etc [12].

The present study aims at investigating the transcendency of nickel copper ferrite nanoparticles over the known biocompatible ferrites like magnetite, zinc ferrite, etc. Possibility of biomedical applications of nickel copper ferrite with composition $Ni_{0.5}Cu_{0.5}Fe_2O_4$ synthesized by hydrothermal route was investigated [13]. *Streptococcus mutans* is a gram positive bacteria that is said to be a major contributor for dental caries [14], *Pseudomonas aeruginosa* is a gram negative bacteria which is one of the most common air borne pathogen, that cause pneumonia, septic shock, gastrointestinal infection, urinary tract infections, skin infections [15], *Bacillus subtilis* is gram positive bacteria which is known to infect immunocompromised patients [16] and *Escherichia coli* gastroenteritis, urinary tract infections, and neonatal meningitis [17]. Antibacterial activity of the title material was tested against these bacteria. Toxicology of the material was assessed using chick embryos by assaying its teratogenicity [18] and also by analysing the total protein content of the embryos [19,20]. In addition, haematotoxicity [21] of the material was also tested, along with determination of the antioxidant nature by DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging assay [22] and microbial toxicity by antibacterial assay [23].

EXPERIMENTAL SECTION

2.1 Hydrothermal synthesis of nickel copper ferrite

Nickel copper ferrite nanoparticles of composition $Ni_{0.5}Cu_{0.5}Fe_2O_4$ were synthesized by hydrothermal route in General Purpose stainless steel (SS 316) autoclaves provided with Teflon liners. 1% aqueous solution of EDTA (Ethylene diamine tetraacetic acid, Rankem) was prepared in a beaker using ultrapure water (purity of up to 18.2 MΩ-cm, Elga Option Q7) and placed for continuous stirring using a magnetic stirrer. To that, 0.125 M Nickel Chloride Hexahydrate ($NiCl_2.6H_2O$, Sigma-Aldrich), 0.125M Copper (II) chloride dihydrate ($CuCl_2.2H_2O$, Sigma-Aldrich) and 0.5M Ferric Chloride Hexahydrate ($FeCl_3.6H_2O$, Sigma-Aldrich) were added in 1:1:2 ratio of Ni:Cu:Fe. Here, EDTA acts as hexadentate chelating agent and helps in the formation isometric nickel copper ferrite. To ensure proper mixture of the chemicals, stirring was done for at 1 hr [24]. To this mixture, 8 M NaOH (Sodium hydroxide, Rankem) aqueous solution was added, until the *pH* reached 12, which was measured using ELICO *pH* meter. Here NaOH acts as a reducing agent. As the *pH* increased, precipitation occurred. The precipitate thus formed was transferred into a Teflon liner. A measure volume of 20µL of 1% SDS was added into the liner, which acts as a surfactant. which was then placed in the General Purpose autoclave and kept inside the furnace (Thermotek, Mysore, India). Temperature of the furnace was adjusted to 180°C and the process was run for 4 hr. After the completion of the process, the synthesized nickel copper ferrite were collected from the liner and washed at least 8 to 10 times using double distilled water, with the help of a neodymium magnet (NdFeB magnet).

2.2 Characterization studies

Powder XRD analysis was done to confirm the phase purity of the synthesized nanoparticles using Rigaku Mini Flux II (Japan) instrument, with Cu as target material (1.5406 Å). The crystallite size was determined using Debye-Scherrer's formula,

$$D = \frac{0.9\,\lambda}{\beta \cos\theta}$$

Where, D is the average crystallite size, λ is the wavelength of the X-ray used, β is the angular line width of half maximum intensity, and θ is the Bragg's angle in degrees. Fourier transform infrared spectroscopy (FTIR) analysis was done to study the functional groups present in the desired material using Jasco FTIR 360 plus, Japan. Size of the desired nanoparticles was confirmed by using Dynamic light scattering (DLS) technique (Nanotracwave, Microtrac®, USA). Morphological and elemental composition was analysed using scanning electron microscope (SEM) and energy dispersive spectroscopy (EDS) respectively (Hitachi® S-3400N, Japan).

2.3 Evaluation of antioxidant activity using DPPH assay

Antioxidant property of the Nickel copper ferrite synthesized was assessed by its 1, 1-diphenyl 2-picrylhydrazyl (DPPH) free radical scavenging ability [25-27].

DPPH free radical scavenging was observed upon exposure to the desired nickel copper ferrites nanoparticles, by the colour change in the solution from violet to pale yellow. It was measured at 517 nm using UV-Vis

spectrophotometer (ELICO® SA 165, India). DPPH is easily photodegradable; hence this experiment was performed under less intense light.

Percentage of DPPH-free radical scavenging activity was calculated based on the absorbance readings using the following formula:

$$\frac{(A_c - A_s)}{A_c} \times 100$$

Where, (A_c) is the absorbance of the control and (A_s) is the absorbance of the sample.

2.4 Haemolysis assay

Haemolysis assay was performed in order to study the lysis of RBC with respect to the change in isotonicity of the solution in the presence of the title compound synthesized [28].

Blood stock solution was prepared using Dulbecco's phosphate buffer saline (DPBS) such that the mixture contains ~5×10^8 RBC/ml [29]. It was then exposed to different concentrations of the synthesized nanoparticles for 3 hrs. Then contents were later centrifuged and the absorbance of the supernatant was measured at 541 nm using UV-Vis spectrometer using 1× DPBS as reference.

Using the absorbance reading, percentage of haemolysis was calculated as bellow

$$\%\ haemolysis = \frac{(A_s - A_n)}{(A_p - A_n)} \times 100$$

Where, (A_s) is the absorbance of the sample, (A_n) is the absorbance of the negative control and (A_p) is the absorbance of the positive control.

2.5 Teratogenicity assay

Embryotoxicity assay was performed using 4-day-old fertilized hen eggs. These eggs were collected from National Hatcheries (Gundlupete, Karnataka, India).

About 18 eggs of approximately equal weight were collected from hatcheries and were divided into three sets of 6 each, namely set A, set B and set C. In each set, the eggs were labelled as B, N1, N2, N3, N4 and N5, where B denotes for blank eggs in which 20 μl of sterile ultrapure water was injected and N1 to N5 represent the eggs in which the serially diluted concentrations (6 mg/ml to 0.375 mg/ml) of the material synthesized were injected.

Inoculation was done on the day 4 as mentioned earlier, with the help of a sterile needle and a Borosil micropipette. After injection, the opening was sealed using candle wax. The eggs were then placed in the incubator at 37 °C (Lead® instruments (P) Ltd) until the 12th day.

After the completion of the incubation period, eggs were dissected on 12th day to check the inhibition in the growth of the embryo and teratogenicity [30-32]. Heart and liver from the embryos were collected for biochemical assay.

2.6 Bradford protein assay

On 12th day of administration of the synthesized nanoparticles, embryos were removed and washed in phosphate buffer saline and dissected to isolation of heart and liver. Heart and liver were removed carefully and were homogenised properly using 1× DPBS in agate mortar in a ratio of 1:5 (w/v). The homogenised mixture was then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected and a total protein assay was performed using Bradford's standard assay protocol [33-35].

After incubation, the contents in the vials were read at 595 nm using UV-Vis spectrometer for absorbance readings. For positive control, the prepared BSA stock solution was used instead of nanomaterial and for negative control, 800 µl of ultrapure water and 200 µl of the Bradford reagent were used. The results obtained were tabulated and calculation was done as follows:

$$\frac{(A_s - A_n)}{(A_p - A_n)} \times 100$$

Where, (A_s) is the absorbance of the sample, (A_n) is the absorbance of the negative control and (A_p) is the absorbance of the positive control.

2.7 Antibacterial assay

Antibacterial property of the nickel copper ferrite synthesized was assessed against *Escherichia coli* (gram-negative facultative anaerobe, MTCC 1698), *Pseudomonas aeroginosa* (gram-negative aerobe, MTCC 4673), *Bacillus subtilis* (gram-positive bacilli, MTCC *121), *Streptococcus mutans* (gram-positive facultative anaerobe, MTCC 497), procured from MTCC (Microbial Type Culture Collection and Gene Bank, Chandigarh, India).

The details of the media used are given in Table 1. Experiments were performed in 96-well microplates by adding respective nutrient media and different concentrations of nanoparticles synthesized into the wells [36-38].

Table. 1 Types of media used for different bacteria employed in the current assay

Name of the Bacteria	Medium used	Manufacturer
Escherichia coli	Luria Broth	HIMEDIA
Pseudomonas aeroginosa	Luria Broth	HIMEDIA
Bacillus subtilis	Luria Broth	HIMEDIA
Streptococcus mutans	Brain Heart Infusion Broth	HIMEDIA

The plates were then labelled and placed in incubator shaker for a 24 hr of incubation. After the incubation period, plates were scanned using microplate reader at 600 nm. The absorbance readings were noted and calculations were done using the following formula to obtain the growth percentage of the bacteria.

$$P_g = \frac{S - S_b}{C_p - C_n} \times 100$$

Where, P_g is the percentage growth, S is the absorbance of the sample with inoculum, S_b is the absorbance of the sample blank, C_p is the absorbance of the positive control and C_n, the absorbance of the negative control.

Percentage inhibition (P_i) can be calculated from the formula:

$$P_i = 100 - P_g$$

RESULTS

3.1 Characterization results

Synthesized nickel copper ferrite material was dried at 100 °C for 24 hr. The dried material was then scraped and ground gently using agate mortar before taking it for characterization.

3.1.1 Powder XRD analysis

Graph shows that the major peaks (111), (220), (311), (222), (301), (422), (511) and (440) correspond to cubic inverse spinel structures of nickel ferrites as they match with JCPDS card no. 74-2081. The major peaks also correspond to the typical cubic ferrite structures according to JCPDS card No. 03- 875. Further, lattice parameters were calculated and it was confirmed that a = b = c and α = β = γ = 90°, proving that the material synthesized belongs to isometric symmetry. The average crystallite size of the title material synthesized was calculated using Debye-Scherrer's formula and was found to be 32nm. The powder XRD pattern obtained is shown in Fig. 1.

3.1.2 FTIR analysis

FTIR spectrum of the title compound is shown in Fig. 2. The absorption band at ~400cm^{-1} corresponds to the stretching vibrations of Fe^{+3}–O^{-2} and M^{+2}-O^{-2} bonds in octahedral sites. Whereas the letter 'M' corresponds to metal ions, which in this case are Cu^{+2} and Ni^{+2}. The absorption band at ~580 cm^{-1} corresponds to the stretching vibrations of Fe^{+3}–O^{-2} in tetrahedral sites. C=C bonds and C-O bending vibrations were noted at ~1600cm^{-1} and ~1150cm^{-1} locations. A broad band at ~3400cm^{-1} denoted the presence of O-H bonds mostly adsorbed from atmospheric moisture by KBr during pellet making.

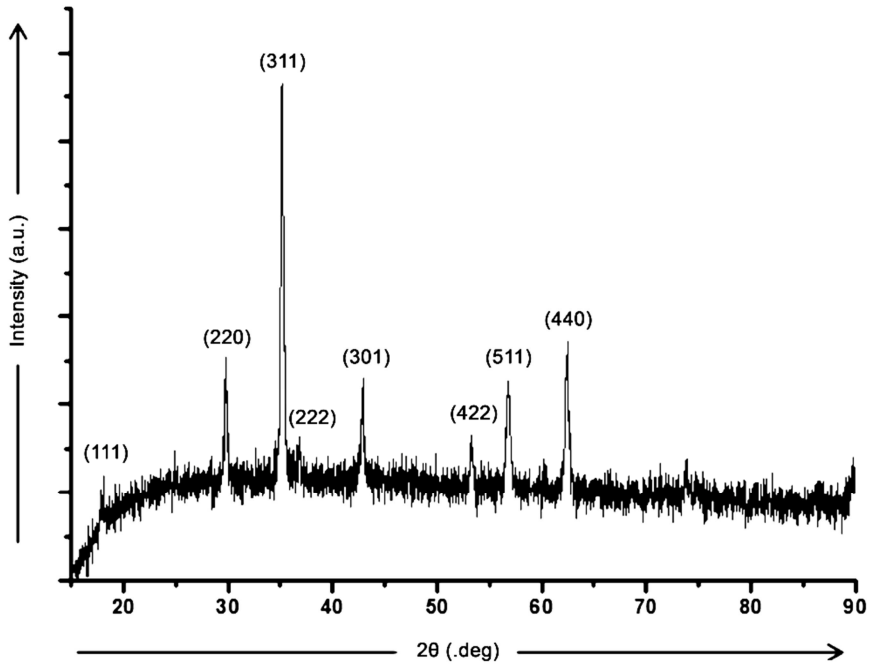


Fig. 1. Powder XRD analysis showing isometric symmetry of the nanoparticles

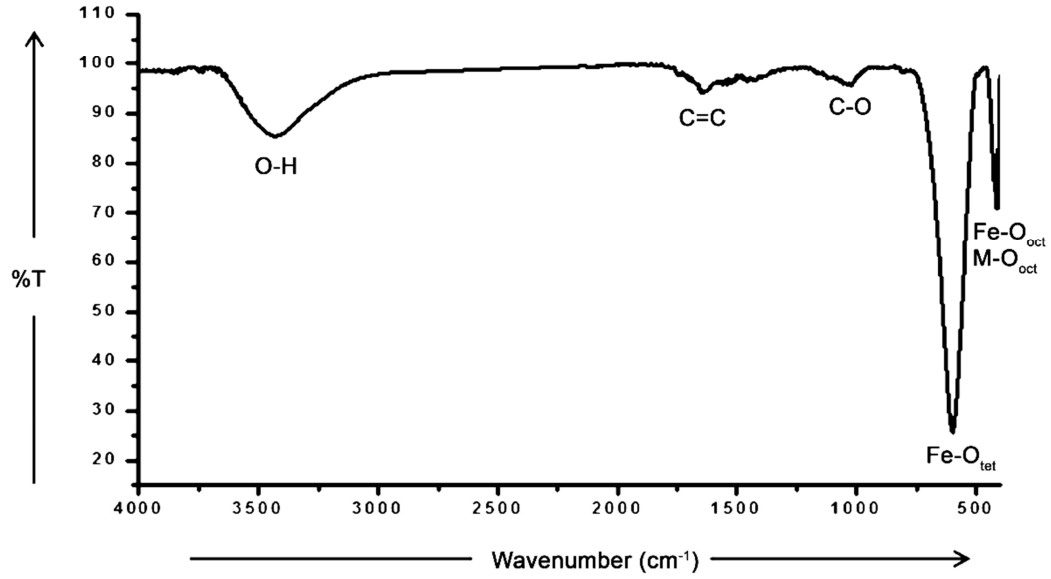


Fig. 2. FTIR analysis of the material prepared

3.1.3 SEM analysis

Scanning electron microscopy was carried out to understand the morphology of the synthesized material. The results show that the cube-like nickel copper ferrite nanocrystals were obtained by the hydrothermal synthesis. Uniform morphology of nanoparticles was also observed as seen in the images in Fig. 3.

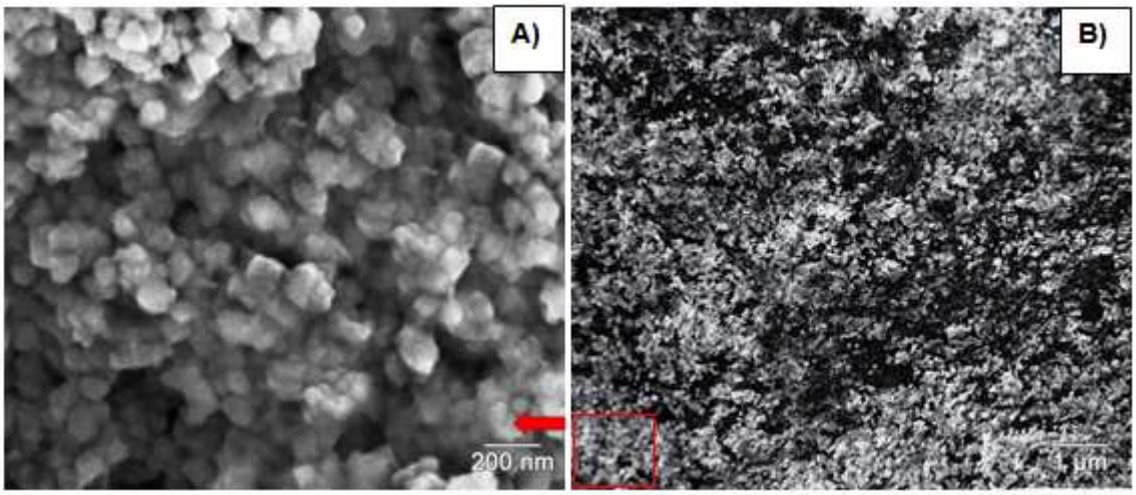


Fig. 3. (A,B). SEM analysis of the synthesized Ni-Cu Ferrites showing cubic nanocrystals

3.1.4 EDS analysis

The EDS analysis in the Fig. 4 is indicating that the material synthesized exhibits the presence of nickel, copper, iron and oxygen conforming that the synthesized material is nickel copper ferrite.

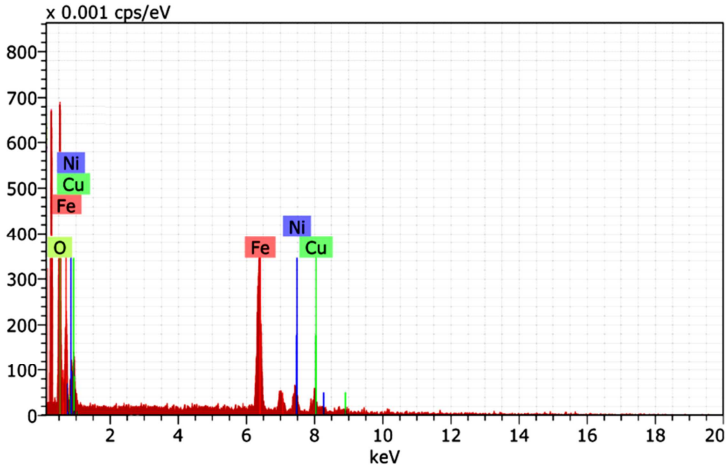
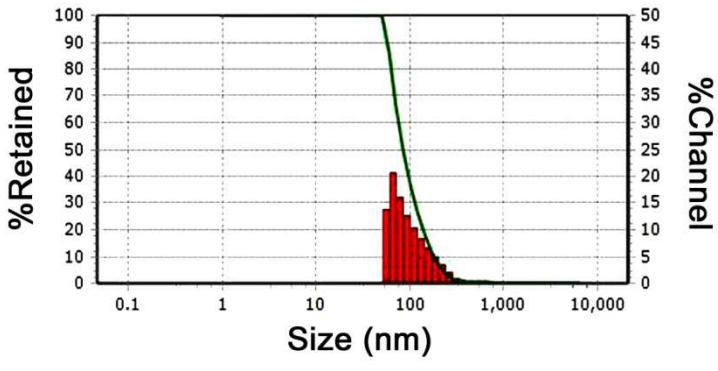


Fig. 4. EDS elemental analysis showing the presence of Ni, Cu, Fe, O

3.1.5 DLS analysis

Dynamic light scattering analysis was done using the nanoparticles size analyser and the results are shown in Fig. 5. The analysis shows an average particle size of 84.7 nm and 95 percentile of the particles fall into the range of 55.55 nm.



Percentiles	
%Tile	Size(nm)
10.00	184.5
20.00	140.5
30.00	115.0
40.00	98.01
50.00	85.74
60.00	76.57
70.00	69.75
80.00	64.27
90.00	58.73
95.00	55.55

Peaks Summary		
Dia(nm)	Vol%	Width
85.7	100	92.6

Fig. 5. Particle size distribution of Ni-Cu nanoparticles

3.2 DPPH free radical scavenging assay

DPPH-free radical scavenging activity of the samples was calculated by taking absorbance reading at 517 nm using UV-Vis Spectrophotometer and is tabulated. The results obtained showed a good free radical scavenging activity of 72% at a concentration of 6 mg/ml nickel copper ferrite material as evident from Fig. 6. It was also observed that there was only a slight reduction in the activity with the reduction in the concentration of the material by half (i.e., 3 mg/ml). At 0.375 mg/ml, the nanoparticles showed 53% of DPPH-scavenging activity.

Fig. 6. Free radical scavenging ability of the synthesized material

3.3 Haemolysis assay

Percentage of haemolysis activity of the samples was calculated by taking absorbance reading at 541 nm using UV-Vis Spectrophotometer. The results obtained showed only 1% of haemolytic activity for 0.375mg/ml concentration of the nickel copper ferrite nanoparticles synthesized as evident from Fig. 7. It was also observed that there was only slight increase in the haemolysis activity with increase in the concentration of the material. At 6mg/ml concentration, the material showed only 4% of haemolysis. From this observation, we can infer that the synthesized nickel copper ferrite nanomaterials are haematologically compatible.

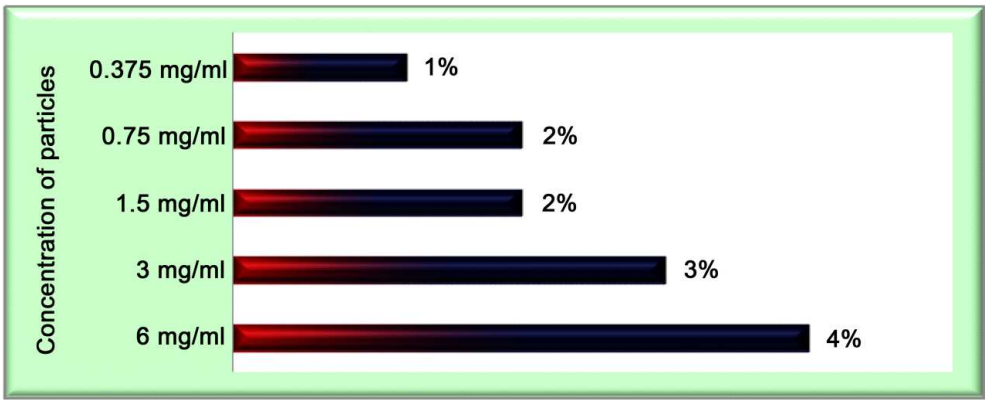


Fig. 7. Haemolysis assay

3.4 Embryotoxicity assay

3.4.1 Teratogenicity assay

Embryos were observed carefully for morphological abnormalities. The wing formation in test group was checked and compared with that of blank. Likewise, the limbs and beak formation were also observed. As such there was no strong evidence of the growth inhibition and teratogenicity in the embryos. There were no signs of hepatomegaly and cardiomegaly and the breast plate was found to be properly formed. There were no deformations in beak, limbs and wings found in the embryos at all the concentrations of the material used (i.e., from 6mg/ml to 0.375mg/ml), as evident from Fig. 8. This shows that the injected nickel copper ferrite nanoparticles did not show any teratogenicity in the embryos.

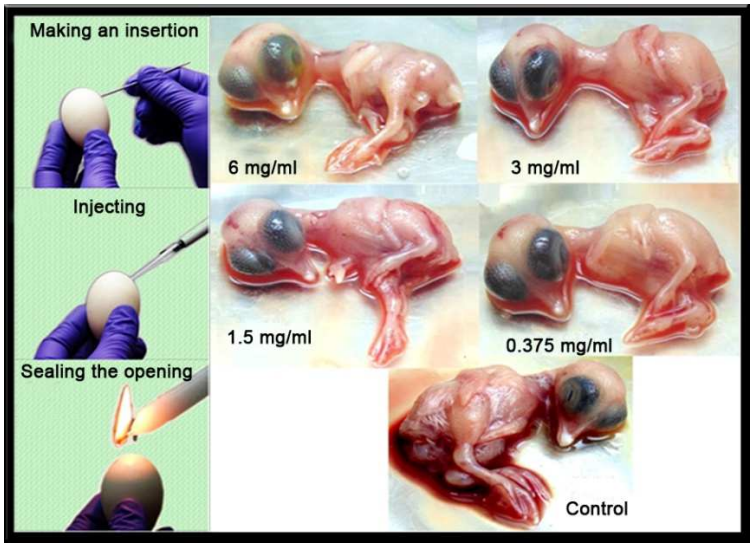


Fig. 8. Teratogenecity assay at different concentrations of Ni-Cu ferrites

3.4.2 Total protein assay

The homogenates of the heart and liver were collected, after observing the teratogenicity of the embryos on day 12 and total protein assay was performed using Bradford's reagent and Bovine serum albumin as standards. Absorbance readings were taken at 595 nm and percentage of protein content was calculated. The results obtained are represented in Fig. 9. The protein content in the liver slightly reduced in the presence of nickel copper ferrite material, when compared to that of blank. There was not much variation in the total protein content as compared to that of blank, in the presence of 1.5 mg/ml and 0.75 mg/ml concentrations of nickel copper ferrite material. Whereas in the presence of 3 mg/ml and 6 mg/ml concentrations, a noticeable decrease in the total protein content to 40% was observed. Similar results were observed in the case of the total protein content of the heart as well. From the results obtained, we infer that the desired nickel copper ferrite nanoparticles caused a minimal amount of protein degradation within the biological system.

3.5 Antibacterial assay

Nickel copper ferrite nanoparticles showed 82% of inhibition in the growth of *P. aeruginosa*, 80.42% inhibition of *E. coli*, 77.32% inhibition of S. mutans and 76.30% inhibition in the growth of *B. subtilis*, as shown in Fig. 10.

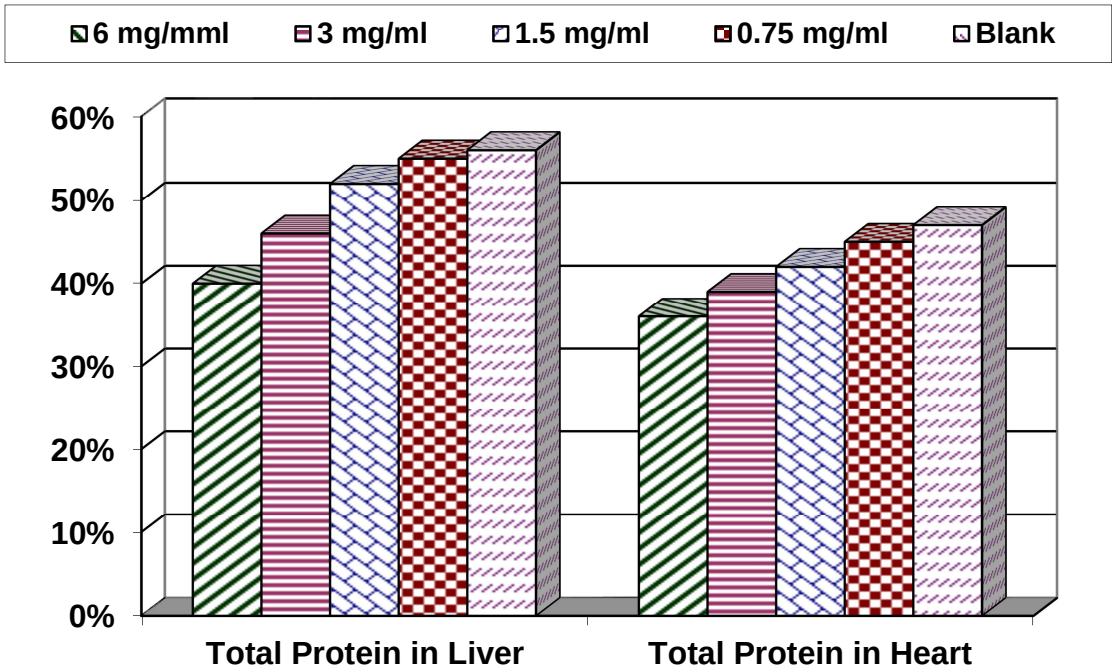


Fig. 9. Total protein assay

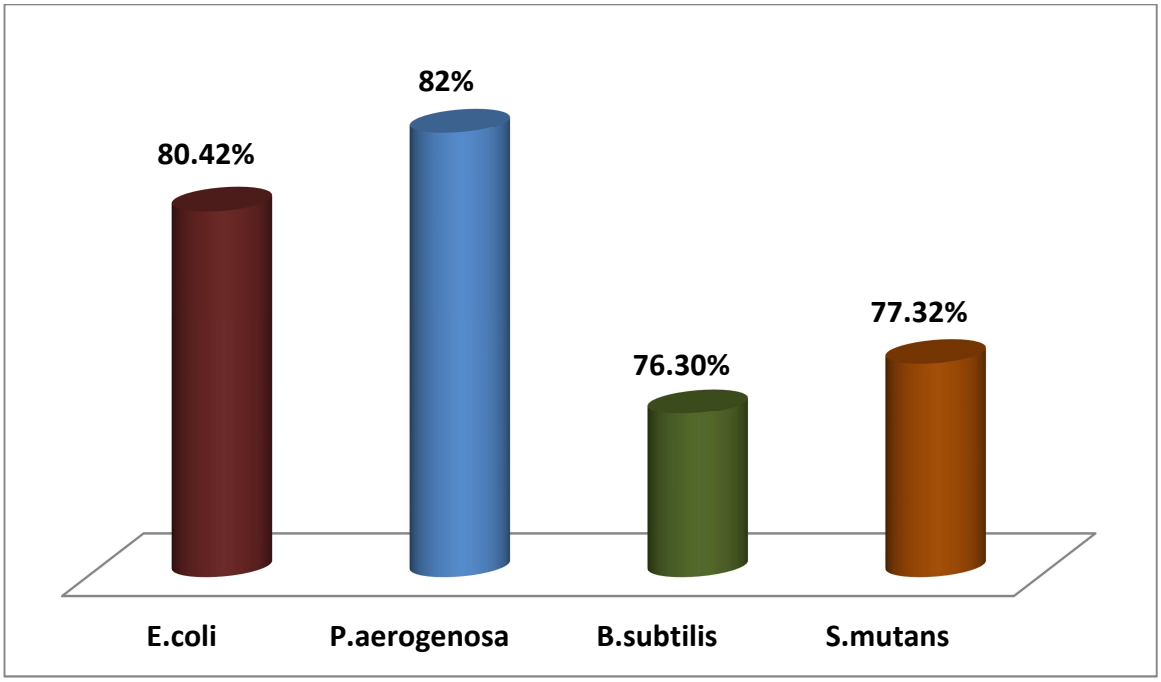


Fig. 10. Activity of the title nanocrystals against pathogenic bacteria

DISCUSSION

Cubic inverse spinel structured nickel copper ferrite nanoparticles were successfully fabricated under hydrothermal conditions. The method followed was a facile, one step hydrothermal route, which was cost effective and was employed in achieving the nanoparticles of desired size and shape. The synthesized nanoparticles were characterized using powder XRD, FTIR, DLS, SEM and EDS. The characterization results showed that the synthesized material is of cubic symmetry with an average crystallite size of 32nm and contains Ni, Cu, Fe and O as its components, confirming that the synthesized material was nickel copper ferrite. DLS results show an average particle size of ~85 nm. The material was tested against red blood corpuscles and against 4-day-old chick embryos to understand its haemolytic activity and teratogenicity. It was confirmed that the synthesized nickel copper ferrite nanoparticles did not show any toxicity towards blood and showed no teratogenic effects. Foreign objects that enter the body can cause several types of adversities which include protein degradation. Hence, the embryos were subjected to the synthesized material at its different concentrations to understand the changes in the level of total protein content in the heart and liver tissues. The material did not cause any significant degradation in the total protein content of the heart and liver of the chick embryos. In addition to that, the nanoparticles showed good antioxidant property and also caused a successful inhibition in the growth of *Escherichia coli, Streptococcus mutans, Bacillus subtilis,* and *Pseudomonas aeroginosa* bacteria.

Ferrites of heavy metals like nickel, cobalt, copper, and zinc are known to possess properties that are currently employed in magnetic storage devices, corrosion resistant coatings, biomedical devices, etc [39]. Fewer researches have been done in employing these ferrites for *in vivo* applications owing to the toxic nature of heavy metals. There are interesting studies on the antibacterial property of these heavy metals, which is a curious topic in this era of bacterial drug resistance against many of the standard antibiotics [40]. Magnetite has been extensively researched for its biological activity though it inherently lacks the antimicrobial activity only by the virtue of its biocompatibility [41]. Although magnetite is biocompatible to humans and animals, it is not known as an antibacterial substance. Some studies showed that magnetite can be a concentration dependent antibacterial material [42]. Based on the results obtained, we infer that the nickel copper ferrite nanoparticles synthesized are more advanced than magnetite and other known ferrites of heavy metals like nickel, cobalt, copper and zinc as nickel copper ferrite nanoparticles are not only biocompatible but also possess excellent antibacterial and antioxidant nature. This makes nickel copper ferrite as an ideal material for application in biological systems.

CONCLUSION

Eloquent hydrothermal technique was employed in order to synthesize biocompatible, cubic symmetric nickel ferrite nanoparticles. The nanoparticles synthesized were characterized using powder XRD, FTIR, DLS, SEM and EDS. Toxicological studies revealed that the synthesized material is not haematotoxic as it did not cause any disruption of

red blood corpuscles upon exposure. Embryotoxicity of nickel copper ferrite material was tested on chick embryos. The results obtained showed no evidences of teratogenicity in the embryos. Total protein content of the hearts and livers from these embryos was measured to confirm the effect of the nanoparticles on the embryos and it was evident from its results that there was only a slight variation in the amount of proteins upon exposure to title material. Apart from being completely biocompatible, the synthesized material also possesses excellent antioxidant property of 72% DPPH free radical scavenging activity. Nickel copper ferrite synthesized successfully inhibited the growth of *Escherichia coli, Streptococcus mutans, Bacillus subtilis,* and *Pseudomonas aeroginosa* bacteria. These bacteria are said to cause tooth decay, food infections, and several pulmonary infections. Based on the evaluated results and in comparison with the properties of other known ferrites, the present authors conclude that the nickel copper ferrite nanoparticles synthesized are ideal for biomedical applications as they are biocompatible and also antibacterial in nature, in addition to their excellent antioxidant nature.

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