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Synthesis, characterization and antimicrobial activity of Cassia fistula mediated Cobalt doped Copper oxide nanoparticle against Salmonella typhi a step toward antibacterial nanomedicine

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ABSTRACT

The green synthesis route is becoming an emerging field of study in nanotechnology due to its biodegradability, eco-friendly nature, and non-hazardous nature. Among a variety of industrial metal nanoparticles (NPs), copper oxide nanoparticles (CuONPs) are the most attractive topic for researchers because of their effective surface area to volume ratio, chemical properties, and antimicrobial activity. The current study consists of the synthesis of Copper oxide nanoparticles (CuONPs) using Cassia fistula extract as a reducing and capping agent and studies its antibacterial activity. The formation of Cassia Fistula mediated CuONPs was identified by color change and was confirmed by FTIR and UV-visible spectrophotometry, revealing an absorbance peak at 235 nm. A shift of 45 nm was observed when the NPs were coated with PEG. Astonishingly, CuONPs showed a virtuous result on 485 ug/ml, showing only 3% of hemolysis; meanwhile, many different concentrations of NPs were used to check whether it would exceed the standard value. However, none of the diluted concentrations were above the standard value, making them biocompatible. The MIC test was performed, showing 250 ml and 350 ml of diluted CuONPs were prominent concentrations for the elimination of bacteria. ROS quantification and identification showed that the ROS produced followed the Type II mechanism in which the singlet oxygen transfers energy to triplet oxygen. We settled that CuONPs possess the ability to delimit the bacteria, specifically Salmonella typhi.

Keywords: *Copper oxide nanoparticles, nanomedicine, Cassia fistula, antibacterial*

1. INTRODUCTION

Nanomedicine is the most attractive topic for researchers in the field of pharmaceutical and material sciences. Nanomedicines replace conventional medicine due to highly targeted, rapid and efficient treatments. The concept of delivering certain medicines to a specific part of the living body completely changed after the basic development of nanotechnology. Nanomedicines are more efficient in many diseases like disease due to bacteria and fungi [1]. Green synthesis is also fast compared to other synthesis techniques. For example, silver NPs were prepared through green synthesis and chemical reduction methods, and it was found that the production of the green route is 20 times faster than that of the chemical route [2]. The reducing agents present in plants are commonly enzymes, proteins and phytochemicals like flavonoids and terpenoids etc. Besides the fact that microbes are used for the preparation of NPs, the plant extract is generally preferable because its waste product is environmentally friendly, the extract can be used for mass production, and it is easily available. Abundant research has been carried out in the preparation of NPs, which are used for the production of semiconductor and metallic NPs. The common methods are physical and chemical, but a relatively modest number of studies show that they are not eco-friendly and not cheaper than that of using plant extract.

From the beginning, plants were given a lot of importance in the medical field and were used to cure many diseases. Plants do have natural ingredients to reduce and give a composed valuable size to the NPs. Few plants have gained praise from the scientific community, and cassia fistula is one of them that has been used in many parts of the world and has had medical applications since the 19th century [3]. The organs of the cassia fistula have a wide variety of secondary metabolites in greater percentages and are ideal for the preparation of NPs. The most noticeable is phenolic compounds. Also, reports have confirmed the presence of Fistucacidin and an optically inactive leucoanthocyanidin in it [4]. Many reports and studies have shown that cassia fistula has pharmacological activities, including antibacterial, antifungal, and antitumor. In traditional medicines, Cassia fistula was used for the tumors of the throat, glands and liver [5]. In most states, it is used as a mosquito repellent, and different literature has proven the activity of Cassia fistula against mosquitoes. The larvicidal, insecticide, and ovicidal activity of Cassia fistula is known to many researchers and is recommended by WHO methods [6]. The antioxidant activity of Cassia fistula is in descending order from the stem to the pulp. The reason for the low amount of antioxidants in the pulp and the flower of the

Cassia fistula is the presence of chrysophanol and reducing sugars, which are prooxidants [7].

Copper Oxide Nanoparticles (CuONPs) have many applications in the nano-world. They have a lot of diverse functions regarding environmental remediation, catalysis, and making personal care products [8]. Many studies have shown that CuONPs have a great prospect in medicine and cosmetics; however, the Copper oxide prepared through Physiochemical methods shows genotoxicity. The small size of their NPs can penetrate the lungs and kidney cell lines to cause oxidative stress in the cells and can damage the mitochondria and many other cytoplasmic contents of the cell [9]. The bulky size of the copper oxide particles might stick in the lining of the intestine, which can cause serious problems to one's body, and if the dose is taken over the amount suggested, it can cause apoptosis in the cells [10]. If prepared through the green route using any plant, just like the Cassia fistula, the toxicity level can decrease marginally further, and its aspect ratio can be drastically better when interacting with cells. The surface area and the capping agents will make the NPs more stable and specific to their site [11]. The mean detection range (MDR) bacteria is becoming a bigger problem, but using copper oxide, which shows a promising result over traditional drugs, might be a better solution. Studies have shown that Shigella sonnei and Salmonella typhimurium bacteria show resistance to the most recently available drugs, but copper oxide shows the elimination of these two bacteria [12]. Copper oxide, which is made through the green synthesis route, is more lucrative. These NPs show activity against microbes such as bacteria, bacterial parasites, fungi and many others. Better photodegrading ability shows the importance of the delivery of drugs and many treatments, for example, anticancer, antitumor, and antileishmanial. Doping a nanoparticle is a necessity nowadays because it can enhance many properties of NPs, such as their optical and magnetic abilities and antimicrobial activities [13]. Previous studies suggest that doping can amend the electronic configuration, customize the structure and surface area of the NPs, and examine the activity of Ag NPs with and without the doping agent [14].

Long before the Typhoid was given a name and the genus "Salmonella" was discovered. In 1880, Karl Leibermeister and his colleagues thought Bacillus was the agent responsible for typhoid, and they also thought that the epidemic of the disease was due to water contamination and unclean drinking water [15]. A doctor in Bristol, UK, William Bud, had an opinion in 1873 that typhoid could be due to the specific toxin present in the waste of patients. The disease spreads because of the water that is infected by the feces of patients [16]. Many scientists and researchers have investigated the possible culprits of the disease. In short, it was a doctor, Karl Joseph Eberth, in 1879 who revealed the pathogen (bacillus) in the abdominal and spleen lymph nodes. Karl published his discoveries in 1880, and in 1881, after that, his discoveries were supported and long-established by German and English biologists, one of whom was Robert Koch [17]. An American veterinarian and pathologist (director of the USDA research project, Daniel Elmer Salmon), the genus was named after him: "Salmonella." Despite the facts, many researchers and scientists have investigated and contributed to the finding of the pathogen [18]. However, the history of "typhoid" will always be remembered, and we should not forget the "Marry Typhoid." Many assumed names were given to her; she was Irish and born in 1869 [19]. Mary Mallon was the first carrier of Typhoid disease, and Many studies show that in 1906, only 11 people were present in the population who might have been infected by Typhoid fever. Different literatures suggest that at the time of 1906, typhoid fever was still fatal in 10% of cases and mainly was found in underprivileged people. Upon relocating her home to Manhattan, New York, reports suggest that over 3000 people were infected by Salmonella typhi, and perhaps Mary was considered the main reason for the outbreak. In 1911, no immunization was discovered against the disease; moreover, till 1948, no antibiotic treatment was available for the patients, due to which many people died [20]. However, being in newspaper and one of the major concerns was due to her stubbornness for not doing any operations regards that she was a carrier of the pathogen. In 1932 she moved out to North Brother Island where she remained until her death. The death of Marry Typhoid left many mysteries behinds, studies suggest what would have happened if she had recognized to do the operation beside all the facts Mary gain an awkward place in the history of medicine [21].

In the current study, CuONPs were prepared by green synthesis using Cassia fistula as a reducing agent. The production of nanoparticles was confirmed by UV visible spectroscopy and Fourier Transform Infrared Spectroscopy (FTIR), and their activity against Salmonella typhi was checked and discussed. It is found that CuONP formed by green synthesis can be used as an antimicrobial against Salmonella Typhi.

2. METHODS AND MATERIALS

The collected leaf sample from the ring road Peshawar was washed with distilled water (DiW), and the midrib of the fresh leaf was removed and cut into small different sizes. 20 gm leaf put in 200 ml distilled water and heated for about 1 hour at 37 °C in a water bath. Prepared 50 ml of DiW solution with 0.513 g/ml of Copper oxide and 0.109 g/ml of dopant Cobalt nitrate separately. Then, both solutions were mixed and got 100 ml solution with a 5 pH value. To obtain the optimal PH value, leaf extract was added at a leisurely pace to the solution till the solution turned into a dark green color. After that, the solution was taken in 50 ml of 2 falcon tubes and centrifuged for 10 mins at 9000 rpm. Then, the supernatant was discarded, and finally, prepared NPs (NPs) were dried in an incubator for 1 day at 60°C. The prepared CuOCoNPs were coated with a peg using the sonication technique. The concentration of the NPs was set at 1mg/ml, and sonicated NPs were used for the photothermal activity. 4 ml of DI water was mixed with 1 ml of NPs and the solution in sunlight for about 1 minute to increase the temperature, then measured by PH meter. We repeated the same procedure for 11 minutes and took readings. The same process was followed in the absence of light to check the variations in temperature. Cultured *Salmonella Typhi* was taken from a master petri plate in a Finnpipette tip for antibacterial activity, and then tips were hurled into a 50 ml

falcon tube. The falcon tubes were stored in a Shaker Incubator (unimax 1010 Heidolph, Germany) for 24 hours (For rigorous growth). The media preparation was performed in the Class II Biological Safety Cabinet.

For hemolysis in this methodology 2cc blood was taken for hemolysis. This fresh blood was attenuated with 12ml of PBS (phosphate buffer saline) solution. The test was performed in light and then in the dark. Triton X-100(0.1%) was taken as a positive control, and pure blood was a negative control. The attenuated blood was fetched into 1.5 ml of Eppendorf tubes in different concentrations as 485uL, 242.5uL, 121.5uL, 60.2uL, and 30.3uL. Nanoparticles in different concentrations were used as 515µg/ml, 757.5 µg/ml, 878.5µ g/ml, 939.8 µg/ml, 969.7µg/ml. As for the light set of tests, the Eppendorf tubes were exposed to sunlight for 8 minutes, and as for the dark set of tests, they were in an Eppendorf tube without sunlight, then all the Eppendorf tubes were stored in Incubator (Panasonic) for 3 hours at 37 °C temperature. After that, all tubes were centrifuged for 15 as minutes at 1500 rpm, the supernatant was taken with great care, and OD was checked in a Spectrophotometer. The wavelength was set to 576nm. For the graph, see figure (5). The data were collected through the following formula.

Hemolysis percentage = OD at 576 nm in the nanoparticles / OD at 575nm in 0.1% Triton X-100×100.

For ROS quantification,1,3 Diphenylisobenzofuran (DBPF) is used as a scavenger for the singlet oxygen. For this specific purpose, the reactivity of cobalt doped copper oxide was checked through 1,3 -Diphenylisobenzofuran assay. The assay was done in two sets, dark and light. The solution was prepared by deliquesce 5.4 mg of 1,3-DBFP in 50 ml of ethanol to make a 0.2 mM solution. The prepared solution was in a falcon tube, which had aluminum foil warped around it to protect it from exposure. Then, 1 ml of 1,3 DBPF was added to the cuvette, and 1 ml of DiW was mixed with it. The cuvette was fetched into the Spectrophotometer, and it was set up to 410 nm. The cuvette was also blacked out from both sides. The absorbance was carefully measured, and during the measurement, the cuvette was exposed to sunlight for 30 seconds every time, and the absorbance length was from 400-800 nm. Due to the photochemical alteration of 1,3-DBFP, the measured value decreased over time. Methylene blue was used as a standard. The same procedure was done for dark, except the exposure to sunlight for 30 seconds was not done. ROS plays an important role in defense and in a number of pathological and physiological processes in the human body. In the current study, CuOCoNPs follow a type II reaction. In this case, the reaction energy transfers from the 3O2 excited to generate singlet oxygen (1O2).

For the scavenging of singlet oxygen, sodium azide is used, and for ROS identification, different solutions were mixed with NPs. Sodium azide solution was mixed with NPs, and as the solution was prepared, 1000 uL of sonicated CuOCoNPs, 1000 uL of Sodium azide, and 1000 ul of DBFP (dye) were taken in a quartz cuvette, which was blocked from two sides, and its absorbance was taken at 410 nm in spectrophotometry. After every 30 seconds, the quartz cuvette was exposed to sunlight, and the absorbance was checked 10 times.

Mannitol was used for the hunting of hydroxyl radicals. A mixed solution of mannitol along with 1000 ul of DBFP, 1000 ul of mannitol, and 1000 ul of CuOCoNPs was prepared in a cuvette (blacked from two sides) for a total of 5 minutes. The absorbance was checked at 410 nm, and the cuvette was exposed to sunlight.

For the scavenging of hydroxyl radical and hydrogen peroxide (H2O2), DMSO was used. 1000 ul of DMSO, 1000 ul of DBFP and 1000 ul of NPs were mixed in a cuvette to check the absorbance, and the spectrophotometry was set to 410 nm. The blacked-out cuvette was exposed to sunlight. MIC is a test performed to check the growth of bacteria on the lowest concentration of antibacterial agent under specific conditions. A fresh *Salmonella typhi* culture was taken in tip and fetched into 50 ml falcon tubes. The falcon tube had 35 ml LB media and was grown overnight in a Shaker Incubator. After that, the MacFarland solution was prepared to standardize the CFU (colony-forming unit). The freshly prepared bacterial solution was compared with the MacFarland solution in order to check the turbidity. Then, the first priority was to clean the Class II Biological Safety Cabinet with 70% ethanol, and a fresh 96 well plate was opened in the mentioned cabinet. The antibiotic solution was diluted in different concentrations and was taken in a 15 ml falcon tube. The different concentrations of diluted antibiotics were prepared in a calculated manner, and 5 ml of DiW was taken in a falcon tube. The 16 ul DiW was removed again, and after that, 16 ul ciprofloxacin was added, and 5 ml of media was added. This procedure was repeated, but only the concertation of Ciprofloxacin was decreased (14 ul, 12 ul, 10 ul, 8 ul, 4 ul, 2 ul, 1 ul). After the preparation of the dilution, the experiment was performed in 96 well plate first columns were taken as a positive control, which had 192 ul of ciprofloxacin from 16 ul diluted falcon tube and 8 ul of cultured media. So, the same amount was taken from all the diluted falcon tubes (14 ul, 12 ul, 10 ul, 8 ul, 4 ul, 2 ul, 1ul) in the same concentration. The second column was taken as a negative control. In the 4 wells, there was only cultured media, and the remaining 4 wells had sterile media. The 3rd and 4th columns had coated (PEG) NPs and cultured media in different concentrations. The coated (PEG) NPs were already diluted in falcon tubes in 950 ul, 750 ul, 450 ul, 350 ul, 250 ul, 150 ul, 50 ul, and 25 ul. In the well, 142 ul of NPs were taken from the 950ul falcon tube, and then 8ul cultured media was added to the well, and 50 ul sterile media was added, so a total of 200ul well was filled. The concertation was taken from the different falcon tubes (750 ul, 450 ul, 350 ul, 250 ul, 150 ul, 50 ul, 25 ul), but the unit value was the same (142 ul NPs, 8 ul cultured media and 50 ul sterile media) when poured into the wells. The 96 well plate was stored in an Incubator for 24 hours. After 24 hours, the result was checked in Spectrophotometry on 600 nm using a microplate reader, and in the first 4 wells, the *Salmonella typhi* was completely eliminated. The bacteria were taken from 950 ul, 450 ul, 350 ul, 250 ul, and in the last 4 wells, the bacteria were not killed.

3. RESULTS AND DISCUSSION

3.1. UV-Visible Spectrophotometry

The NPs were sonicated for 30 minutes before the UV-spectroscopy was done. After that, NPs were tip-downed through the FINN pipette. The SPR values with a maximum wavelength were found to be 235 nm for bare cobalt-doped copper oxide nanoparticles, and 280 for polyethylene glycol (PEG) coated CuOCoNPs. A shift of 45 nm was observed that indicated the coating of PEG, as shown in Figure 1.

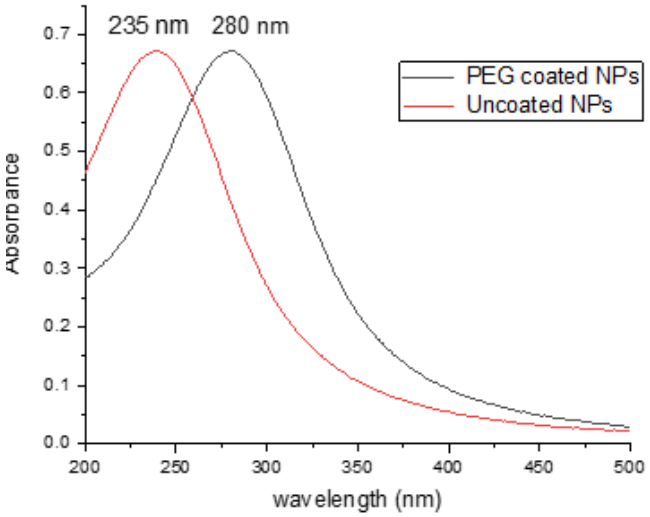


Fig. 1. UV visible spectroscopy of the coated and uncoated NPs.

3.2. Fourier Transform Infrared Spectroscopy (FTIR)

The liquid and non-doped samples of CuOCoNPs were dried in an incubator for 24 hours at 50 °C. In the FTIR study, different functional groups are revealed, as depicted in Figure 2. Data collected from both coated and non-coated shows the formation of CuOCoNPs. The abortion band at 1642-1666 occurred due to stretching of the C=C bond and Alkene compound absorbed at 1640 – 1690. The C=N bond stretching an Amine group 1635-1132 a strong N-O a Nitro compound, a peak is absorbed which is due to *Cassia fistula* extract biomolecules, a 1385-1380 stretching of C-H an alkene bonding, 1335-1370 S=O stretching a Sulfonamide 1275-1200 a C-O stretching an alkyl group, 1030-1070 a S=O stretching a sulfoxide group, 730-665 C=C bonding an Alkene group, 642-520 strong CI stretching a Halo compound. The results of PEG-coated and non-coated were compared, and there was not much difference. Fig. 2. showing the Pattern of FTIR.

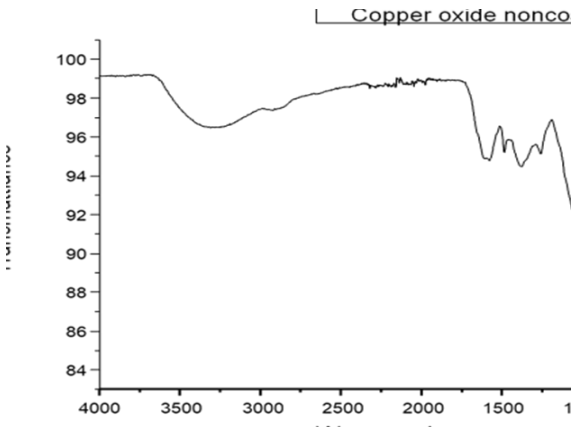


Fig. 2. FTIR for Copper oxide noncoated.

3.3. ROS Quantification

For the quantification of ROS, DBFP was used as a dye for the identification of Nascent oxygen and hydroxyl groups. DBFP showed very sensitive behavior in light and air and rapidly lost color because of ROS species. The color-nesses may be depending on the reaction and the rate of reaction.

3.4. Photothermal Activity

DiW was taken as a control and, for 5 minutes, was exposed to sunlight. The same procedure was applied to CuOCoNPs, and no significant increase in the temperature was observed. This showed the copper oxide cobalt doped was not photosensitizer; the data is shown below in Figure 3.

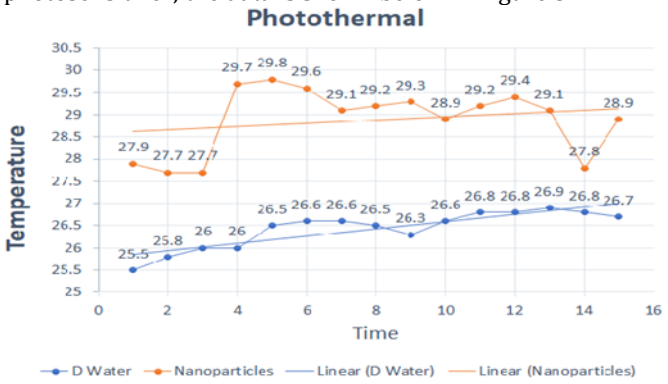
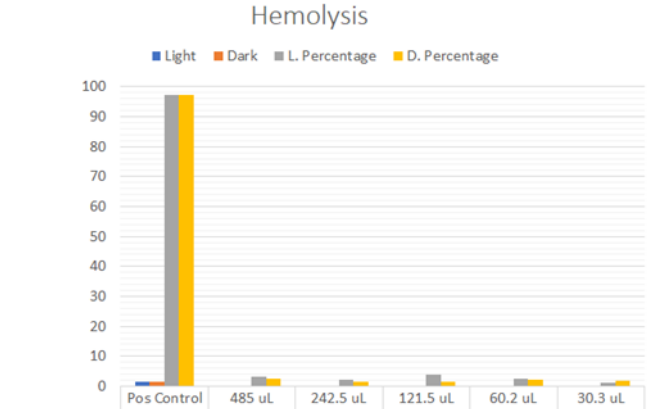


Fig. 3. The data for the photothermal activity.

3.5. Hemolysis

Different concentration of CuOCoNPs was treated with RBC (red blood cells). Spectrophotometry showed none of the concentrations of copper oxide cobalt were toxic. The highest concentration used was 485 ug/ml, which showed 3% of hemolysis, and surprisingly, 121 ug/ml showed 3.8 % of hemolysis, which is not alarming and is below the toxic level (5%). The rest of the percentages are given in Figure 4. Triton X-100 was used as a positive control in light and dark. The difference absorbed was major and revealed that CuOCoNPs have biocompatibility.



	Pos Control	485 uL	242.5 uL	121.5 uL	60.2 uL	30.3 uL
Light	1.5411	0.0476	0.0338	0.0588	0.0377	0.0182
Dark	1.4975	0.0368	0.0211	0.0232	0.0318	0.028
L. Percentage	97.17085199	3.088702875	2.193238596	3.815456492	2.446304588	1.180974629
D. Percentage	97.17085199	2.457429048	1.409015025	1.549248748	2.123539232	1.869782972

Fig. 4. Showing the data table and graph for the percentage of Hemolysis.

3.6. MIC (Minimum Inhibitory Concentration)

Mic is the lowest concentration on which the growth of the bacteria is stopped by an antibacterial agent. The 96 well

plate method was applied to get the measurement CuOCoNPs, and the bacteria used was *Salmonella typhi*. Different concentrations of NPs were used, and the highest diluted concentration was 950 ul. *Salmonella typhi* was killed at different concentrations. The Mic value recorded was 950 ul. The decrease in the concentration of copper oxide cobalt coped NPs till 250 ul showed the elimination of bacteria. However, below this point, significant elimination was recorded.

4. DISCUSSION

Salmonella typhi is evolving and becoming resistant to traditional drugs. *Salmonella enterica serovar Typhi* is a rod-shaped, gram-negative bacteria and is a common etiological agent of Typhoid Fever, vomiting, and diarrhea. It is a lethal human disease. The statistics show that every year, there are 16 million cases recorded, causing 600,000 demise rates. Contaminated water and foods are responsible for the disease. When a living Salmonella typhi enters the body and goes to the intestine, the bacteria release toxins and attach them to the walls of the intestine, which then spreads to the bloodstream. However, it can evolve and spread to the spleen, tissues and liver.

CuOCoNPs, which are synthesized through the green route using cassia fistula (plant), are a better solution for eradicating the bacteria. CuONPs are gaining a lot of attention because of their smaller size and highly specific surface area, which are due to their physiochemical properties. The high surface area of CuONPs was confirmed through UV spectrophotometry, which revealed a peak at 280 nm for the elimination of bacteria. ROS are the first line of defense and are generated in two types, I and II. CuONPs follow the type II mechanism, in which the triplet oxygen transforms its energy, and singlet oxygen is produced, which is very reactive and has a high reaction rate. If they do not find anything to react to, they will immediately react with even the cells themselves.

In the current study, ROS was identified through the PDT, a two-step process in which a photosensitizer (chemical or dug) is carried to the site and a specific wavelength of light is required to activate the release of compound or drug, which in turn release the reactive oxygen species (singlet oxygen) which are lethal to kill the nearby cells to a specific unit of area. Type II produces singlet oxygen, which gains a lot of attention in the nanoworld. The electron configuration of (singlet oxygen) can be credited to it; the valence shell has two electrons, and they move antiparallel to each other. Copper oxide is a photosensitizer for PDT. It uses a type II mechanism. Gram positive bacteria will be killed immediately after the exposure to singlet oxygen. Meanwhile, gram negative bacteria are killed by moving singlet oxygen. The reaction rate of Gram positive is much faster than, the Gram negative. it is due to the protection offer to the Gram negative by the Lipopolysaccharide (LPS) coat. The LPS coat enables Gram – (negative) bacteria to protect the cytoplasmic material, but the singlet oxygens manage to find a barrier through the LPS and get its job done.

Copper oxide's primary antibacterial mechanism of action is the generation of ROS, which kills the bacteria and (ROS) residues in its surroundings of the cell wall. The ROS causes oxidative stress, which penetrates the wall of bacteria, gets internalized and damages the internal content.

5. CONCLUSION

Salmonella typhi has shown resistance to many traditionally available antibiotics, and copper oxide cobalt 15 % doped is a better alternative to eliminating the deadly *salmonella typhi.* They are synthesized through a green route using *cassia fistula,* and they are less toxic, biocompatible and eco-friendly. The mode of the mechanism of copper oxide can be considered very important in which ROS is produced, and the end product is singlet oxygen, which helps abolish the cell wall of the bacteria (*salmonella typhi*).

CuONPs have antimicrobial properties against known bacteria and can be used as a source of ROS. Many conventional antibiotics are not helpful as they were passed decades ago, but copper oxide can be used for different treatments of different diseases. CuONPs, along with some research, can be used in injection and drop forms to cure the disease. Leishmaniasis is a major world concern, and CuOCoNPs show promising results, and the antileishmanial activity can be checked.

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