RESEARCH ARTICLE

Investigation of Antimicrobial and Genotoxic Effects of Fe_2O_3, NiO and CoO NPs Synthesized by Green Synthesis

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ARTICLE INFO

Article History:
Received 2020-02-22
Accepted 2020-03-15
Published 2020-08-01

Keywords:
α-Fe_2O_3
NiO and CoO NPs
Green synthesis
Antimicrobial activity
Genotoxic effect
Pathogen microorganisms

ABSTRACT

It is seen that metal nanoparticles are used in many areas due to their antimicrobial effects. For this reason, our study focused on the production of α-Fe_2O_3, NiO and CoO NPs of golden nanoparticles, which are easily obtained with the use of Erzincan grape extract, safe to use, environmentally friendly and cost-effective. Metal ions synthesized by the green synthesis method were characterized using the Scanning Electron Microscope (SEM) analysis. From the SEM diagrams of the synthesized nanoparticles, it was determined that the nanoparticles were approximately 5 to 65 nm in size. Both antimicrobial, genotoxicity and cytotoxicity effects were investigated to determine the rates at which nanoparticles can be used as biosafe. Synthesized α-Fe_2O_3, NiO and CoO NPs showed excellent antibacterial properties on pathogen bacteria against human. In addition, it was determined that α-Fe_2O_3, NiO and CoO M-NPs showed genotoxic properties in parallel with increasing concentrations. This study, as far as we know, is the first report on microbial α-Fe_2O_3, NiO and CoO NPs and their biological properties synthesized by this statistical approach.

How to cite this article
Nadaroglu H., Alaylı A., Çeker S., Ogutcu H., Agar G. Investigation of Antimicrobial and Genotoxic Effects of Fe_2O_3, NiO and CoO NPs Synthesized by Green Synthesis. J. Nanoanalysis., 2020; 7(3): 216-224. DOI: 10.22034/jna.006.

INTRODUCTION

Nanotechnology is a multidisciplinary science that incorporates the ever-developing dynamics and is the design, synthesis and application of materials and devices in different fields such as medicine, biology, physics, chemistry and engineering [1,2]. The use of nanoparticles of different sizes and shapes in fields such as drug transport and imaging in medicine is a widely studied research subject today. In addition, the widespread use of nanoparticles is increasing day by day due to their electrical chemical and physical properties. Therefore, developed countries allocate high budgets for this kind of research every year [1-4].

Metal-Nanoparticles (M-NPs), which are frequently encountered among nanoparticles, are nanoparticles that have found a quite common use. In general, although different strategies are followed in the synthesis of nanoparticles, synthesis can be carried out by methods that are collected under 3 groups, physically, chemically and biologically [5]. There are physical, chemical and biological methods for the synthesis of M-NPs. Among these methods, biological methods are a new

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approach that can handle physical and chemical methods conditions [6-8]. Nano-technology in which biochemical processes are applied is called nanobiotechnology and plants, algae, fungi and bacteria are used for this purpose in the synthesis of nanoparticles [8-16]. In this way, nanoparticle synthesis is also called green nano synthesis because it offers environmentally friendly, biocompatible and inexpensive alternatives. Nanoparticles synthesized in this way are used in many fields such as dressings, cell labeling, photo-imaging, sensors, drug delivery, gene delivery, photothermal therapy [7]. In addition, as a result of researches, they explained the biomedical and pharmaceutical potential of nanoparticles and revealed that they were more effective than other therapeutic drugs. MNPs' large surface area, high cellular penetration ability and important catalytic activity increase their toxic effects, even though they are among the reasons for their use in this field. [17].

Nickel NPs have gained great importance because they have biological activities, effective photocatalysts, [10, 11], and are cytotoxic to microorganisms [11]. Nickel (II) compounds function as antibacterial [12], antifungal [13], antioxidant [14, 15] and antiproliferative agents [16]. It is assumed that nickel nanoparticles penetrate the bacterial cell wall and damage sulfur and phosphorus-containing compounds such as proteins and DNA [17]. As a result, metal nanoparticles inhibit cellular metabolism, causing the death of the microorganism [18,19].

Iron NPs are used in large quantities, especially in the field of health and the environment. It has attracted great interest in iron nanoparticles in the search for a simple, inexpensive and effective method to eliminate toxic paint-induced impurities [20,21]. While magnetic iron nanoparticles are used in medicine, transportation systems, cancer therapies and imaging systems in the health sector, zero valent iron nanoparticles have been used for a long time in the environmental field. For more than 20 years, iron has been used for the treatment of waters. With an average size range of 50-300 nm and highly reactive, nZVI is still used for environmental improvement [22,23].

Cobalt-based materials (for example, cobalt metal, salts, hard metals, oxides and alloys) are widely used in various industrial and medical applications. In recent years, the production and use of various nanoparticles have increased in society. Nano-sized, cobalt particles exhibit structural, electrical, magnetic and catalytic properties that depend on interesting sizes in size. Cobalt nanoparticles showed high chemical reactivity from large surface areas, making them suitable for catalysis. Cobalt-based NPs are now used as pigments, catalysts, sensors, magnetic contrast agents and in energy storage devices [24,25].

Genotoxicity is a problem associated with the use of M-NPs, especially it plays an important role in the initiation and progression of abnormalities in the DNA structure. Genotoxicity can be defined as destructive genetic changes that may be caused by gene toxins in nanoparticles, including gene mutations, structural chromosomal abnormalities, and recombination [17-19]. The substances that make changes in the morphological structure that replicates the gene sequence are called genotoxins. Genotoxins can cause cancer, mutation and birth defects [18].

Genotoxicity of drugs should be investigated after the production phase [19]. Metal nanoparticles have a strong antimicrobial effect due to the unique properties they have gained. In order to benefit from these powerful antimicrobial properties, it is also necessary to adjust the dosage of use correctly, so the study of the genotoxicity of the nanoparticles has become very important. Genotoxicity has become very important in researching genotoxicity due to its characteristics such as the intake of cells and increased activity in the organism of these clinically synthesized nanoparticles [26].

In our study, we systematically reviewed laboratory studies evaluating the genotoxicity of MNPs using *in vitro*. It has been observed that α-Fe_2O_3, NiO and CoO M-NPs can be synthesized using Erzincan grape extract in various shapes and sizes. Genotoxicity studies with M-NPs are extremely important to identify potential damages before studies on living things. Over the past decade, numerous articles have reported green synthesis methods for MNPs that have benefited from biological ways as an environmentally friendly approach. However, laboratory studies on the genotoxicity of biologically synthesized MNPs are still scarce. Therefore, it is quite difficult to compare and extract the results from genotoxicity data. A better understanding of the genotoxicity mechanisms of α-Fe_2O_3, NiO and CoO M-NPs will enable us to prevent potential damage. In this way, the safe use concentration of metal nanoparticles, whose antimicrobial properties will be used, will be determined.

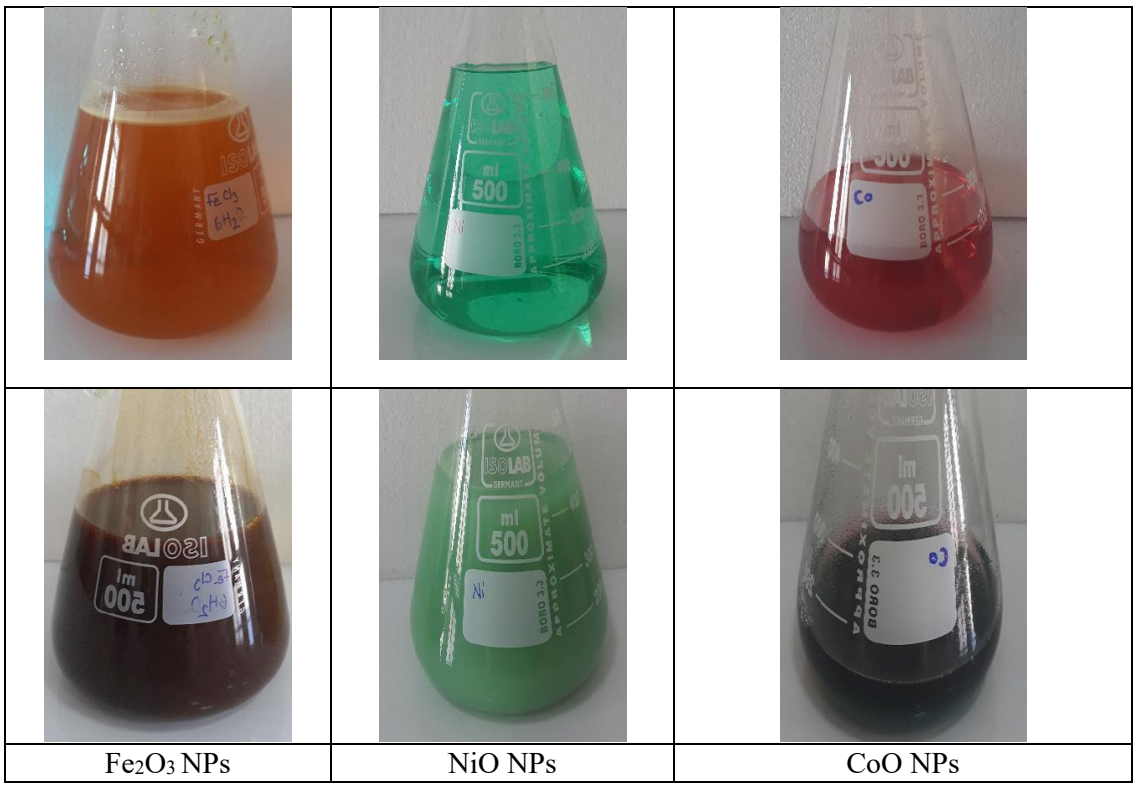

Fig. 1. Post-green synthesis color change results of Fe2O3, NiO and CoO Nanoparticles

EXPERIMENTAL

Green Synthesis of α-Fe_2O_3, NiO and CoO M-NP

Synthesis of magnetic α-Fe_2O_3, NiO ve CoO NPs has been achieved by using 1 mM $FeCl_2$-$FeCl_3$, $Ni(NO_3)_2$, $Co(NO_3)_3$ solution and Erzincan Cimin grape (*Vitis vinifera cimin*) using green synthesis method as Nadaroglu group [27, 28].

Characterization of α-Fe_2O_3, NiO and CoO M-NPs

The resulting magnetic α-Fe_2O_3, NiO and CoO M-NPs were identified and characterized using SEM (Scanning Electron Microscope) after the washing and drying steps.

The morphology of the obtained M-NPs was performed with Zeiss brand Sigma 300 model Scanning Electron Microscope (SEM).

Antimicrobial Effect

Test Microorganisms

The pathogenic bacterial cultures; Gram (+) bacteria (*Staphylococcus aureus* ATCC25923, *Staphylococcus epidermis* ATCC12228, *Micrococcus luteus* ATCC9341, *Bacillus cereus* RSKK-863), Gram (-) (*Escherichia coli* ATCC1280, *Salmonella typhi* H NCTC901.8394, *Klebsiella pneumonia* ATCC 27853, *Proteus vulgaris* RSKK 96026, *Pseudomonas aeroginosa* sp. *Brucella abortus* RSKK 03026) and yeast (*Candida albicans* Y-1200-NIH) were used.

Detection of Antimicrobial Activity

The synthesized α-Fe_2O_3, NiO and CoO MNPs were examined for their antimicrobial activity by the well-diffusion method against Gram-negative bacteria, Gram-positive bacteria and one yeast [29, 30, 31].

The α-Fe_2O_3, NiO and CoO M-NPs were kept dry at room temperature and dissolved (0.25 µg/mL) in DMSO. DMSO was used as solvent for compounds and also for control. DMSO was found to have no antimicrobial activity against any of the tested organisms. 1% (v/v) of 24 hours' broth culture (pathogenic bacteria and yeast) containing 10^6 CFU/mL were placed in sterile plates. Mueller-Hinton Agar (MHA) (15 mL) kept at 45°C was then poured into the petri plate and allowed to solidity. After, 6 mm diameter wells were carefully drilled using a sterile cork borer and filled with fully synthesized compounds. Finally, plates were incubated for 24 h at 37 °C on the incubator. At the

end of incubation period, the mean value obtained for the two wells were used to calculate the zone of growth inhibition of each sample. The pathogenic bacteria cultures and yeast were tested for resistance to five standart antibiotics (Ampicillin, Nystatin, Kanamycin, Sulphamethoxazol, Amoxycillin) produced by Oxoid Lt., Basingstoke, UK [32-34].

Mutagenicity Assay

In vitro Micronucleus and Sister Chromatid Exchange Tests

The anti- genotoxic properties of the Fe / Ni / Co nanoparticles were studied in human lymphocyte cells by micronucleus (MN) and sister chromatid exchanges (SCE) assays.

The MN test was carried out by Fenech [35] as described in our previous study [36]. After completion of treatments, cells were centrifuged for 10 min at 1200 rpm and the pellet was then resuspended in hypotonic solution (0.05 M potassium chloride (KCl)) and incubated at 37 °C for 7 min. After the incubation period the cell suspension was centrifuged again for 10 min at 1200 rpm and fixed with freshly mixed methanol:acetic acid 3:1. The fixation procedure was repeated until the cells were completely cleaned and the tube was centrifuged. The prepared preparations were allowed to dry in room conditions for 3 days. Then, the slides were stained with 5% giemsa dye solution for 10 min and excess giemsa dye was removed with distilled water. The slides were air-dried and only bi-nucleated cells were scored for MN analysis. For each experimental group, approximately 1000 bi-nucleated cells were analyzed for the presence of MN [37].

The SCE test, tests the exchange of DNA replication products between the gene loci on homologous chromosomes and may show microscopically identifiable chromosomal damage. [38]. specifically, this test can be used to identify mutagen compounds that form DNA inserts or interact with DNA replication. [38]. mutagenic and carcinogenic effects of various agents in experimental studies, especially in the chromosomes as an indicator to investigate the structural changes in the structural changes [38]. Therefore, it is used as a suitable method to determine the mutagenic and carcinogenic effects of chemicals. The SCE, which is a fast, reliable, sensitive and simple test, was performed as described in our previous study [39].

RESULTS AND DISCUSSION

SEM, EDX Characterization of α-Fe_2O_3, NiO and CoO MNPs Structure

The surface morphology of α-Fe_2O_3, NiO and CoO MNPs NPs synthesized by green synthesis method was performed with Zeiss brand Sigma 300 model Scanning Electron Microscope (SEM). SEM device; It works on the basis of collecting and examining the interactions formed by dropping electrons from the tungsten tip onto the sample to be examined. In this way, the surface topography of the sample examined is informed about its structure.

α-Fe_2O_3, NiO and CoO MNPs SEM micrographs was shown in Fig. 2A-C. By looking at Fig. (2A), it was determined that α-Fe_2O_3 was nanosized and the synthesized NPs were in the range of 8-65 nm. It is seen that iron nanoparticles are properly shaped as plates and spherically. SEM analysis results confirmed the formation of iron oxide nanoparticles [42].

The surface morphological properties of the synthesized NiO NPs are given in Fig. 2B examined by scanning electron microscope. In Fig. 2, SEM image of NiO nanoparticles obtained by magnifying 5000 times is given. The results show that mono-dispersant and highly crystalline NiO nanoparticles are obtained. The appearances of some NiO NPs are mostly global. We can observe that the particles are highly agglomerated and mainly the luster of nanoparticles. This image can be attributed to the tendency of NiO nanoparticles to agglomerate due to high surface energy and ultra-thin nanoparticles due to high surface tension. It was determined from the SEM image that the NiO NPs obtained were 8-60 nm in size. The small particle size leads to a large surface area, which increases the catalytic activity of nanoparticles. Thus, we can conclude that the NiO particles prepared are in the nano meter range [43].

The SEM image of the produced CoO nanoparticles is shown in Fig. 2C. CoO nanoparticles have a solid shape, which indicates that they have a good crystal structure. CoO nanoparticles have a global configuration. Size distributions of CoO nanoparticles were analyzed using the Nano Measurer software and the average CoO NPs size was 8-55 nm. The obtained CoO NPs are compatible with iterator and obtained with high efficiency [44].

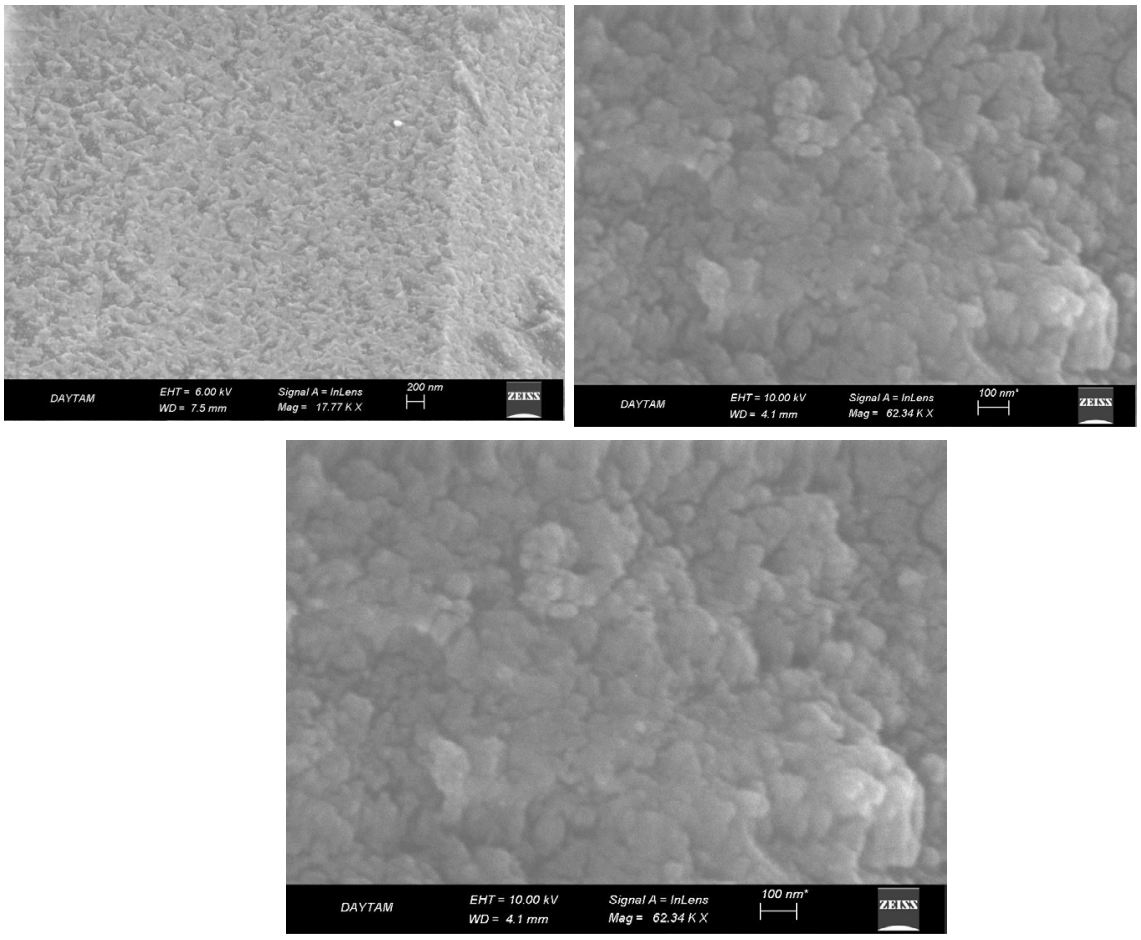

Fig. 2. SEM image of Fe_2O_3, NiO and CoO MNPs obtained by green synthesis method

Antimicrobial Activity

Inhibition activity of α-Fe_2O_3, NiO and CoO MNPs on disease-causing pathogens was investigated.

As a result of the investigation of the inhibitory effects of α-Fe_2O_3 NPs on pathogenic microorganisms; It was determined that S. epidermis showed the same inhibition activity as AMP10 (26 mm) higher than two standard antibiotics (SXT25 AND K30 (25 mm)). Again, *E. coli* showed higher inhibition activity than two standard antibiotics (AMP10 (10 mm) and AMC30 (14 mm)). Moreover, it was determined that α-Fe_2O_3 NPs showed the same inhibition activity in *P. aeroginosa* as the standard K30 (20 mm) antibiotic. When α-Fe_2O_3 NPs activity in yeast is examined; it was determined that *C. albicans* had higher activity than standard antifungal (Table 1).

In this context, NiO NPs showed more inhibition activity (27 mm) in *P. aeroginosa* than all antibiotics (Table 1). Pseudomonas genus bacteria are common in nature and lead to opportunistic infections and hospital infections. Among these, *P. aeruginosa* is in the first place among the hospital infection agents and it can develop resistance against various antibiotics and cause high mortality and morbidity depending on the infection [45,46]. *P. aeruginosa* is held responsible for 10-25% of nosocomial infections [47,48]. Since *P. aeruginosa* can usually show multiple antibiotic resistance, it also causes problems in treatments. In addition, Green Nail Syndrome, Pseudomonal Pyoderma and Blastomycosis-Like Pyoderma, Otit Externa and Malignant Otitte Externa, Pseudomonal Folliculitis, Pseudomonas Hot Foot Syndrome, Ectima Gangrenosum are the most common clinical forms. *P. aeruoginosa* septicemia, on the other hand, occurs in especially weak and

Table 1. Antimicrobial activities of Fe_2O_3, NiO, CoO, NP (diameter of zone of inhibition (mm)).

	Bakteri adı	Fe	Co	Ni	Control	AMP* 10*	SXT 25	AMC 30	K 30	NYS 100
Gram (+)	*S.epidermis*	26	26	16	-	26	25	27	25	-
	S.aureus	13	26	16	-	30	24	30	25	-
	M.luteus	20	27	21	-	22	21	25	23	-
	B.cereus	15	30	17	-	23	25	20	28	-
Gram (-)	*S.typhi H*	-	28	13	-	11	17	19	20	-
	Br. abortus	25	25	30	-	-	-	-	-	-
	E.coli	17	33	25	-	10	18	14	25	-
	K.pneumonia	15	31	25	-	21	20	21	23	-
	P.vulgaris	16	32	21	-	17	19	20	21	-
	P aeroginosa	20	35	27	-	22	21	23	20	-
Maya	*C. albicans*	30	30	34	-	-	-	-	-	20

*Standard reagents (diameter of zone inhibition (mm). SXT25, Sulfamethoxazole 25 µg; AMP10, Ampicillin 10 µg; NYS100, Nystatin 100 µg; K30, Kanamycin 30 µg; AMC30, Amoxycillin 30 µg. N: not tried.
Fe : Iron (II) oxid NPs ; Ni : Nickel (II) oxid NPs ; Co : Cobalt (II) oxid NPs

immunosuppressed patients and has a mortality rate of 10-20%. Likewise, *K. pneumonia* also showed a higher inhibition effect (25 mm) than all antibiotics. It also showed a very high inhibitory activity (30mm) in *B. abortus* (Table 1). *Br. abortus* has gram-negative bacterium that causes premature abortion of cattle fetus [49] (Halling *et al.*, 2005), furthermore, it is a very serious, debilitating and sometimes chronic human pathogen that can affect various organs [50,51].

NiO NP showed a higher (13 mm) inhibition effect than only one standard antibiotic (AMP 10-11 mm) in *S. typhi* H (Table1). *Salmonella serovars* cause many different clinical symptoms, ranging from asymptomatic infection to severe typhoid-like syndromes in infants or in some high-sensitivity animals [52-54]. NiO NP had a higher (25 mm) inhibition effect than other antibiotics in *E. coli*, except for a standard antibiotic (K30). Similarly, in the compound *P. vulgaris*, the same (21 mm) as K30 antibiotic showed higher inhibition effect than other standard antibiotics (17 mm, 19 mm and 20 mm respectively) (Table 1). *P. vulgaris* is with ease isolated from patients in long-term care facilities and hospitals, and from patients with underlying diseases or patients with weak immune systems. Patients with repeated infection, those with constructional abnormalities in the urinary tract, those with urethral instrumentation and those who acquired infections in the hospital have a rose density of infection caused by Proteus spp. and other microorganisms. Finally, NiO NPs showed a higher (30 mm) inhibitory activity than standard anticandidale (NYS100) in *C. albicans* used in yeast study (Table 1). Systemic fungal infections, including C. albicans, have emerged as significant causes of death and disease in immunocompromised patients (organ or ligament transplantation, adjuvants, cancer chemotherapy) [55-57].

As a result; The pathogen, which is the causative agent of the synthesized NiO NPs, has a similar or higher inhibition effect similar to standard antibiotics and anticandida in Gram (+) and Gram (-) bacteria and yeast. In addition, it was determined that the synthesized compounds showed more inhibition activity in gram-negative bacteria (Table 1).

40-60 nm boyutunda sentezlenmiş olan NiO NPs'in Gram pozitif ve gram negative bakterilere karşı etkinli test edilmiştir. Elde edilen bulgularda her iki gruba

When the antimicrobial effect of CoO NPs is examined; The *S. epidermis* showed the same (26 mm) as the SXT25 antibiotic and higher inhibition activity (26 mm) than the AMC30. In *S. aureus*, it showed higher inhibition activity than two standard antibiotics (SXT25 and K30; 24 mm-25 mm respectively). *S. aureus* is an adaptive pathogen, versatile in nature and varies with the severity of the infection affects skin, soft tissue, respiratory system, bone joints and endovascular tissues. Again, CoO NPs showed higher inhibition activity than all of the standard antibiotics in Gram positive pathogen *M. luteus* (27 mm) and *B. cereus* (30 mm) (Table 1). *B. cereus* is known as opportunist pathogens and is associated with food-borne illness [58].

When the research results are examined; it

Table 2. The frequencies of SCE and MN in human lymphocytes treated with different concentrations of Fe_2O_3, NiO and CoO nanoparticules

Test Items	Concentrations	SCE/Cell ± S.E.	MN numbers ± S.E.
Control		6.04 ± 0.12^{a}	1.73 ± 0.09^{a}
NaN_3	5 µM	9.24 ± 0.08^{d}	3.30 ± 0.02^{d}
Fe	5 µg/mL	6.12 ± 0.04^{a}	1.74 ± 0.04^{a}
Fe	10 µg/mL	6.48 ± 0.03^{a}	1.77 ± 0.07^{a}
Fe	20 µg/ mL	6.58 ± 0.44^{a}	2.24 ± 0.06^{a}
Fe	40 µg/mL	6.92 ± 0.10^{a}	2.01 ± 0.15^{a}
Fe	80 µg/mL	7.04 ± 0.02^{ab}	2.43 ± 0.06^{b}
Fe	120 µg/mL	7.95 ± 0.06^{b}	2.65 ± 0.09^{b}
Ni	2.5 µg/mL	6.19 ± 0.11^{a}	1.90 ± 0.02^{a}
Ni	5 µg/mL	6.60 ± 0.03^{a}	2.40 ± 0.10^{ab}
Ni	10 µg/mL	7.09 ± 0.07^{ab}	3.12 ± 0.04^{cd}
Ni	20 µg/mL	7.46 ± 0.12^{b}	3.50 ± 0.05^{d}
Ni	40 µg/mL	7.78 ± 0.06^{b}	3.72 ± 0.03^{d}
Ni	80 µg/mL	8.03 ± 0.10^{bc}	4.10 ± 0.01^{d}
Co	0.05 µg/mL	6.50 ± 0.03^{a}	2.17 ± 0.02^{a}
Co	1 µg/ mL	7.14 ± 0.08^{ab}	2.75 ± 0.14^{b}
Co	2 µg/mL	7.94 ± 0.03^{b}	3.36 ± 0.11^{d}
Co	4 µg/mL	8.97 ± 0.18^{c}	3.62 ± 0.07^{c}
Co	8 µg/mL	9.92 ± 0.05^{d}	4.18 ± 0.04^{d}
Co	16 µg/mL	10.34 ± 0.14^{d}	4.40 ± 0.16^{d}

Sodium azide (NaN_3) was used as positive controls for human lymphocytes.
Fe : Iron (II) oxid NPs ; Ni : Nickel (II) oxid NPs ; Co : Cobalt (II) oxid NPs
^{a,b,c,d} Statistically significant differences in the same column are indicated by the different superscripts ($\alpha = 0.05$).

has been determined that CoO NP has a greater inhibition effect in Gram (-) pathogenic bacteria. In this context; It has been determined that CoO NP has a higher inhibition effect in *S. thyphi* (28 mm) than standard antibiotics. *Salmonella serovars* cause many different clinical symptoms, from asymptomatic infection to severe typhoid-like syndromes in infants or highly sensitive animals [59] Likewise, CoO NP has been shown to have a very high inhibition activity in *B. abortus*. It also showed a higher rate of inhibition activity (33 mm, 31 mm, 32 mm) in *E. coli* and *K. pneumonia* and *P. vulgaris*.

In addition, in *P. aeroginosa*, very high inhibitory activity (35 mm) was determined than all of the standard antiotics. It has been determined that CoO NPs are also highly effective on yeasts, especially in *C. albican* (30 mm) by observing higher inhibition activity than standard antifungal (Table 1).

In their study conducted by Dogra et al, using the agar well diffusion method to investigate the effectiveness of Co-based NPs against S. aureus bacteria, which are known to be human pathogens, they detected zones of 5.1-2.1 mm. It can be seen that the CoO NPs that we synthesize have more effect on bacteria from this study of the literature [60].

There are findings about the potential mechanisms of action by which metal oxide NPs try to explain the bactericidal effect. Some of these are explained by the mechanism of reactive oxygen species (ROS), their electrostatic interaction, accumulation, the mechanism by which NPs are transmitted and contacted, inducing various effects on the outside and bacteria [61,62].

Mutagenicity Assay

Genotoxic Potentials of α-Fe_2O_3, NiO and CoO nanoparticles

The genotoxic potentials of the α-Fe_2O_3, NiO and CoO nanoparticles are presented in Table 2. NaN_3 used as positive control was found to cause DNA damage compared to the control group. According to the findings; it was determined that α-Fe_2O_3, NiO and CoO M-NPs showed genotoxic properties in parallel with increasing concentrations and the results obtained from MN and SCE test systems supported each other. On the other hand, 5, 10, 20 and 40 µg/mL concentrations of α-Fe_2O_3, 5.5 µg/mL concentration of NiO and 1 µg/mL concentration of CoO were not genotoxic compared to the control group; it was determined that the increase in the results is not statistically significant ($p < 0.05$) [63-66].

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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