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# Evaluation of antimicrobial effects of copper–zinc oxide nanocomposites biosynthesized by pome pomegranate peel on some pathogenic bacteria

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**ABSTRACT**

Ever since humans started using antibiotics to treat bacterial diseases, resistance to treatment has been observed in bacteria. Studies have shown that by using metals we can take advantage of their antibacterial effects. The purpose of this research is to investigate the antibacterial activities of copper-zinc nanocomposite synthesized by green method (from pomegranate peel extract) on some pathogenic bacteria. Therfore, after green synthesis of copper-zinc oxide nanocomposite, the characteristics of nanocomposite were evaluated using UV-Vis spectrophotometry, X-ray diffraction analysis (XRD), FT-IR analysis, spectrum analysis (EDX) and scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Antibacterial activity of zinc-copper oxide nanocomposite against some pathogenic agents was investigated by well diffusion and blank disk methods in agar medium. The results of Well diffusion method were better compared to blank disc. It was demonstrated that the maximum inhibitory concentration on Staphylococcus aureus bacteria at concentrations of 100, 250, and 500 µg/ml showed; diameters of the non-growth halo of 17, 16 and 21 mm respectively. In contrast, the minimum inhibitory concentration on Enterococcus faecalis at concentrations of 100, 250, and 500 µg/ml, showed the diameters of the non-growth halo of 10, 12, and 14 mm respectively. The microbicidal properties of nanoparticles were investigated by two methods: the minimum growth inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). In the MIC method, the treatment concentration of 0.78 PPM stopped the growth of Escherichia coli and the treatment concentration of 1.56 PPM stopped the growth of other bacteria. MBC for Escherichia coli was 1.56 and for Pseudomonas aeruginosa, Shigella dysentery and Staphylococcus aureus was 3.12 PPM and had no effect on Enterococcus faecalis. These results showed that copper-zinc oxide nanocomposite synthesized from pomegranate peel inhibited the growth of the above-mentioned bacteria in all concentrations.

## Introduction

Due to the excessive use of antibiotics, antimicrobial resistance has emerged as one of the greatest threats to human health worldwide (Jan et al., 2019). This resistance is increasingly observed in various bacterial species. The trend of this resistance has accelerated in recent decades (Hawkey, 2008). Studies have shown that prior to the widespread use of antibiotics, the genes controlling drug resistance existed at very low levels in bacteria or were minimally expressed; however, with the increase in antibiotic usage, bacteria possessing genes such as the extended-spectrum beta-lactamase genes (CTX-M) have become more prevalent and abundant due to their selective advantage. Bacterial species such as Staphylococcus aureus, Enterococcus, Enterobacteriaceae, Pseudomonas, and Acinetobacter have become particularly resistant to antibiotic treatments, rendering the treatment of infections caused by these bacteria a complex challenge in medical science (Hawkey, 2008; Hawkey, 2009; Livermore, 2003). There are numerous reasons for this resistance, including the inappropriate use of antibiotics, such as the misuse of broad-spectrum antibiotics or the extensive use of antibiotics in livestock and food industries (Arnold, 2007), as well as in hygiene products and household cleaning agents (Aiello and Larson, 2003). These factors have led to the emergence of extensively drug-resistant bacterial strains that are resistant to a significant portion of antibiotic treatments. A strain is termed extensively drug-resistant if it is resistant to at least three classes of antibiotics. This situation has prompted the pursuit of novel approaches to combat bacteria (Jain and Dixit, 2008). Furthermore, with the advent of nanoscience and technology in the past decade, it has been demonstrated that reducing the size of materials from the micrometer scale to the nanometer scale results in a variety of properties concerning electrical conductivity, hardness, surface activity, and chemical reactivity. One of these diverse and significant properties is the antimicrobial effect of metallic nanoparticles such as silver, copper, titanium, and zinc. Metallic ions at high concentrations pose numerous health risks to humans; therefore, the use of these metals or their salts at high concentrations as a means to combat bacterial growth or as antibacterial agents is not a desirable option for the treatment or prevention of infections. However, advancements in technology have shown that utilizing these metals at the nanoscale and at concentrations significantly lower than harmful levels for humans can yield beneficial antibacterial effects (Blanc et al., 2005).

Recent investigations have demonstrated that pomegranate (a tree native to Iran) possesses significant amounts of polyphenols, which contribute to its essential antioxidant properties and exhibit antimicrobial effects (Yunfeng et al., 2006). Pomegranate, containing compounds such as pectin, ascorbic acid, tannins, anthocyanins, and flavonoids, has shown high antioxidant activity (Aviram et al., 2000). Among these, the extract of pomegranate peel specifically exhibits greater capacity to inhibit or prevent superoxide anions, hydroxyl radicals, and peroxides. Furthermore, it has been established that the ability to limit the oxidation of low-density lipoproteins by pomegranate peel extract is superior to that of other parts, such as the fruit pulp (Li et al., 2006). Research has indicated that pomegranate peel extract possesses antibacterial properties (Al-Zoreky, 2009; Danae and Lilian, 2009), antiviral effects (Martos et al., 2010), mutagenic inhibition (Lansky and Newman, 2007), and antioxidant activity (Ghasemian et al., 2006; Singh et al., 2002). The objective of the present study is to investigate the antibacterial activity of green-synthesized copper-zinc oxide nanocomposite (derived from pomegranate peel extract) against certain pathogenic bacteria, including Gram-negative bacteria such as Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (PTCC 1310), Shigella dysenteriae (PTCC 1188), and Gram-positive bacteria such as Enterococcus faecalis (PTCC 1778) and Staphylococcus aureus (PTCC 1431).

Nanotechnology

The simplest definition of nanotechnology is "technology at the nanoscale"; therefore, we cannot accurately define nanotechnology without first defining "nanoscale," which encompasses a range of 1 to 100 nanometers (Nasrollahzadeh et al., 2019). Various methods are employed for the preparation of metallic nanoparticles, which can be categorized into two main types: top-down and bottom-up approaches. The primary distinction between these two methods lies in the raw materials utilized for nanoparticle synthesis. Bulk materials serve as the raw material in top-down methods, and the creation of nanometric particles from them necessitates size reduction to the smallest possible scale, involving processes such as cutting, removing, grinding, crushing, shaving, melting, and milling. The size of the particles is reduced to nanoparticles through various physical, chemical, and mechanical processes, whereas in bottom-up methods, atoms or molecules are the raw materials, and manipulation of these atoms and molecules through self-assembly processes leads to the formation of nanostructures. This method can also be realized using biological approaches. Although top-down methods are relatively straightforward, they are not suitable for producing non-toxic particles and materials with very small sizes (Jamkhande et al., 2019; Semaltianos, 2010). Generally, nanoparticle synthesis methods can be divided into three categories: (1) physical methods, (2) chemical methods, and (3) biological synthesis methods (green synthesis), which can be further divided into microorganisms and plants (Dhand et al., 2015). Physical methods for nanoparticle synthesis include thermolysis, physical vapor deposition, pulsed laser methods, microwave-assisted synthesis, high-energy ball milling, laser ablation, ion implantation, sputter deposition, electric arc deposition, and others, each of which alters a specific physical parameter. For instance, temperature is modified in thermolysis, pressure in ball milling, pH in ion implantation, and radiation in laser ablation, among others. Desired particle size and shape can be achieved by optimizing and maintaining optimal parameters. However, the main disadvantages of physical methods include high equipment costs and time-consuming processes. Nevertheless, the advantage of these methods is that they can facilitate the uniform shape and size of nanoparticles (Thunugunta et al., 2015). Chemical methods utilize reactions such as phytochemical and electrochemical techniques, which require high energy, pressure, and temperature. The toxicity and relatively high cost of materials used in these methods limit their application (Santhoshkumar et al., 2019; Mali et al., 2020).

Green synthesis offers numerous advantages compared to chemical and physical methods: it is non-toxic, pollution-free, environmentally friendly, economical, and more sustainable. However, there are challenges related to the extraction of raw materials, reaction time, and the quality of final products. For instance, raw materials are not widely available (Guan et al., 2022; Dhand et al., 2015).

Biological methods present significant advantages, as they can be conducted using various microorganisms, including bacteria, fungi, actinomycetes, and enzymes. Compared to physical and chemical methods, biological methods reduce processing time. Green synthesis pathways provide additional benefits such as easy accessibility, non-toxicity, cost-effectiveness, and ease of transport. The biosynthesis of nanoparticles has been achieved using plants, microbes, enzymes, proteins, starches, and amino acids. Green synthesis methods have been employed to produce both enzymatic and non-enzymatic copper nanoparticles through the interaction of copper salts with organic compounds (Santhoshkumar et al., 2019).

Moreover, it can be performed at room temperature and pressure. Green synthesis of nanoparticles can be regarded as an alternative for the synthesis of biocompatible nanoparticles, representing the latest feasible method for bridging materials science and biotechnology. Consequently, green synthesis of nanoparticles with controlled shape and size using genetic engineering methods, molecular simulation, plant extracts, and other biological techniques is expected to yield remarkable advancements in nanobiotechnology (Hoseinpour and Ghaemi, 2018).

Pomegranate is native to Iran and grows in all coastal areas of the Caspian Sea, thriving on sandy beaches and sometimes forming pure forests or mixed with other plants in lowland areas away from the coast. In steppe regions, it is also mixed with other trees, such as in the western and southwestern forests of Iran. Pomegranate in Iran has several cultivated varieties and breeds, with the weight of its large fruits sometimes reaching 500 grams, containing numerous large seeds with either sweet or sour flavors. The large and beautiful flowers of the pomegranate, known as the ornamental variety, never develop into fruit. One variety of this species is called Punica granatum var. pleniflora. Another variety, known as the dwarf or short pomegranate, is cultivated in pots and does not exceed half a meter in height (Punica granatum var. nanagracilisima). Wild pomegranates in northern Iran almost all have sour seeds, and if wild seedlings are planted in fertile soil and cared for, they yield larger fruits. In the northern coasts of Iran, fresh pomegranate seeds are used to make pomegranate juice as a condiment, and the dried seeds, known as "nardon" or pomegranate seeds, are used as a food seasoning (Ahmad Ghahraman, 1993).

In addition to plants, many fruits and vegetables contain chemicals that can help reduce various diseases. The skin of the fruit contains compounds that possess antimicrobial and antioxidant activities, which can prevent diseases caused by spoiled food. These compounds are generally secondary metabolites, particularly phenolic compounds, steroids, and alkaloids, which have numerous beneficial effects on human health. Pomegranate is a fruit group that is well-known for its medicinal properties (Sing et al., 2019).

The pomegranate fruit plays a beneficial role in human health. The presence of antioxidant compounds and bioactive phenolic contents in pomegranate fruits has been reported to be advantageous for human health. The skin of the pomegranate is rich in phytochemicals. Pomegranate peel is a common remedy for diarrhea. In the future, pomegranate peel may be developed into an anti-diarrheal medication (Qahir et al., 2021).

Pomegranate peel constitutes approximately 24 percent of the total weight of the fruit and is rich in carbohydrates and crude fibers. Additionally, pomegranate peel contains significant amounts of amino acids, including lysine, leucine, aromatic amino acids (phenylalanine and tyrosine), threonine, and valine. The primary minerals found in pomegranate peel are potassium, calcium, phosphorus, and sodium, while substantial amounts of iron, zinc, copper, and selenium have also been reported. Furthermore, pomegranate peel contains high levels of vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin C, and vitamin A (retinol) (Kaderides et al., 2021).

Pomegranate is a natural source of phenolic compounds that contain antioxidants such as tannins, polyphenols, and flavonoids. Other antioxidants present in pomegranate include tocopherols and anthocyanins. The fruit of the pomegranate consists of 85.4 percent water, 10.6 percent sugars, 1.4 percent pectin, and 0.2-1 percent polyphenols. Other substances found in very small amounts include fatty acids, amino acids, organic acids, indoleamines, sterols, triterpenoids, and alpha-tocopherol (Pirzadeh et al., 2021). The pomegranate fruit exhibits antimicrobial activity, acting as an antiparasitic agent for living organisms. The use of pomegranate peel for controlling various microbes has proven to be highly effective (Qahir et al., 2021).

The objective of the present study is to investigate the antibacterial activity of green-synthesized copper-zinc oxide nanocomposite (from pomegranate peel extract) against certain pathogenic bacteria, including Gram-negative bacteria such as Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (PTCC 1310), Shigella dysenteriae (PTCC 1188), and Gram-positive bacteria such as Enterococcus faecalis (PTCC 1778) and Staphylococcus aureus (PTCC 1431).

## -3Review of Previous Studies

Dahham et al. (2010) examined the antimicrobial activity of pomegranate peel extract, seed extract, juice, and whole fruit against several bacteria and fungi. The results indicated that, compared to other plant components, the peel extract exhibited the highest antimicrobial activity. Among the bacteria and fungi cultured, the most significant antibacterial activity was demonstrated against Staphylococcus aureus. Bhumi and Savithramma (2014) synthesized zinc oxide nanoparticles (ranging in size from 23 to 59 nanometers) using zinc acetate and leaf extract of Catharanthus roseus, evaluating the antimicrobial properties of the zinc oxide nanoparticles against Pseudomonas, Streptococcus, Escherichia coli, and Bacillus thuringiensis, with the highest antimicrobial effect observed against Pseudomonas and the least against Escherichia coli. Fuku et al. (2016) investigated the antimicrobial activity of green-synthesized zinc oxide nanoparticles from pomegranate peel, assessing their antimicrobial properties against certain pathogenic microorganisms, including Staphylococcus aureus, Proteus vulgaris, and Escherichia coli. The results indicated that the zinc oxide nanoparticles (ranging in size from 7 to 200 nanometers) exhibited potential activity against the tested bacteria at various concentrations. Ifeanyichukwu et al. (2020) evaluated the antimicrobial activity of zinc oxide nanoparticles using aqueous extracts of pomegranate leaves and flowers. The antimicrobial activity of the synthesized nanoparticles demonstrated that zinc oxide nanoparticles were effective against all selected pathogenic strains (Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis, Escherichia coli). Duffy et al. (2018) assessed the antibacterial activity of silver, zinc oxide, and copper oxide nanoparticles against Salmonella and Campylobacter isolated from poultry. The results indicated that silver nanoparticles had the most significant effect against Salmonella.

Awwad et al. (2020) synthesized nanoparticles using an aqueous extract of Ailanthus altissima fruit. The average size of the synthesized nanoparticles ranged from 5 to 18 nanometers. The synthesized zinc oxide nanoparticles exhibited antibacterial activity against the bacterial strains Escherichia coli and Staphylococcus aureus. The results indicated that zinc oxide nanoparticles possess high antibacterial activity and are considered a suitable alternative to toxic chemical and physical agents. Dhanasegaran et al. (2021) evaluated the antimicrobial effect of zinc oxide nanoparticles on Pseudomonas aeruginosa. The findings of this study demonstrated a significant and dose-dependent inhibition of the growth of Pseudomonas aeruginosa treated with 5 to 150 grams per milliliter of zinc oxide nanoparticles.

Rajeshkumar et al. (2019) synthesized copper nanoparticles using the rare medicinal plant Cissus arnottiana and assessed their antibacterial activity against both Gram-negative and Gram-positive bacteria. The synthesized copper nanoparticles exhibited superior antibacterial activity against the Gram-negative bacterium Escherichia coli. The observed antioxidant property was relatively comparable to the standard antioxidant agent ascorbic acid at a concentration of 40 micrograms per milliliter. The highest antibacterial activity was observed against the Gram-negative strains, particularly Escherichia coli, attributed to the thin peptidoglycan layer and the electrostatic interactions between the bacterial cell wall and the surfaces of the copper nanoparticles.

Wang et al. (2012) investigated the antibacterial effects of zinc oxide nanoparticles under laboratory conditions. E. coli K88 was selected as the indicator pathogenic bacterium. The antibacterial activity against E. coli K88 was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The results indicated that zinc oxide nanoparticles possess strong antibacterial activity against the bacterium E. coli K88. These findings suggest that zinc oxide nanoparticles could serve as a potent antibacterial agent for the treatment of bacterial-induced diseases. Xie et al. (2011) examined the antibacterial effect of zinc oxide nanoparticles on Campylobacter jejuni to inhibit and inactivate cellular growth. The results demonstrated that

Campylobacter jejuni is highly sensitive to treatment with zinc oxide nanoparticles. To address the molecular basis of the behavior of zinc oxide nanoparticles, a large set of genes involved in cellular stress response, motility, pathogenesis, and toxin production was selected for gene expression studies. These results indicated that the antibacterial mechanism of zinc oxide nanoparticles is likely due to disruption of the cell membrane and oxidative stress in Campylobacter.

Alexandre et al. (2019) conducted a study to investigate the effects of high pressure (300 and 600 megapascals) and enzymatic extraction (pectinase and cellulase) on the phenolic compound profiles, antioxidant capacity, and antimicrobial activity of pomegranate by-products. The antimicrobial activity was assessed against eight different strains of pathogenic bacteria and five beneficial bacteria, including Lactobacillus and Bifidobacterium. All pomegranate peel extracts exhibited selective antimicrobial activity against all pathogenic bacteria without affecting the beneficial bacteria. The results indicated that the antioxidant activity and the content of phenolic compounds were strongly correlated with antimicrobial activity .

According to the study conducted by Atmaca et al. (1998) regarding the antibacterial properties of zinc oxide nanoparticles against Staphylococcus aureus and Staphylococcus epidermidis, it was concluded that zinc oxide nanoparticles possess significant antibacterial properties and can inhibit the growth of these microorganisms .

Faiz et al. (2011) investigated the antibacterial effect of zinc nanoparticles against certain intestinal pathogenic bacteria (Escherichia coli, Shigella, Salmonella). Their results indicated that the growth of all examined bacteria was inhibited.

## -4Research Methodology :

Plant extracts contain several hydroxyl groups and water-soluble heterocyclic compounds, which contribute to the reduction and stabilization of nanoparticles. In this study, the presence of reducing agents such as phenols, flavonoids, and vitamin C in pomegranate peel extract was evaluated. Due to the polar components present in the structure of flavonoids, phenolic compounds, and vitamin C, they can dissolve in water, which is a polar solvent, and thus be present in the prepared aqueous extract. The total amounts of phenolic compounds, flavonoids, and ascorbic acid in the plant extract are summarized in Table 1. In the examination of the plant extract, the concentration of total phenols was found to be higher than that of ascorbic acid and flavonoids. In this research, a nanocomposite was synthesized using pomegranate peel extract. The pomegranate peel extract, due to its high concentration of phenolic compounds and ascorbic acid, has a significant potential for the reduction of nanoparticles and nanocomposites, and it can play an effective role in their biosynthesis

Table (1) - The amount of reducing compounds in the aqueous extract of pomegranate peel

| **Concentration in extract (mg/ml)** | Restorative substance |
|---|---|
| 479±2.8 | Phenol total |
| 0.32±51 | |
| 48.3 ± 6.5 | Flavonoid |

The biosynthesis of copper-zinc oxide nanocomposite was conducted according to the method established by Yedurkar and colleagues. In this study, the effects of the volume of copper and zinc salts, temperature, and time under optimal conditions were investigated. Various volumes ranging from 10 to 30 milliliters of copper and zinc salts were added, and the absorption of the copper-zinc oxide nanocomposite was examined using pomegranate peel extract. The highest absorption was observed with the addition of 20 milliliters of copper and zinc salts to a solution containing 5 milliliters of extract and 50 milliliters of distilled water. Consequently, the optimal volume for the biosynthesized nanocomposite was determined to be 20 milliliters of copper and zinc salts .

To determine the optimal conditions, different volumes ranging from 10 to 30 milliliters of copper and zinc salts at concentrations from 0.0001 to 0.1 molar were added, and the absorption of the biosynthesized copper-zinc oxide nanocomposite from pomegranate peel extract was examined. Thus, the optimal volume for the biosynthesized nanocomposite was again established as 20 milliliters of copper and zinc salts. The results of this investigation are illustrated in Figure 1 .

The effect of the reaction environment temperature on the synthesis of copper-zinc oxide nanocomposite from pomegranate peel extract was also examined, as shown in Figure 4-1. To optimize the temperature in the synthesis of copper-zinc oxide nanocomposite, a temperature range of 30°C to 60°C was investigated. The absorption increased from 30°C to 50°C, with the highest absorption observed at 50°C. Therefore, 50°C was selected as the optimal temperature for the synthesis of copper-zinc oxide nanocomposite .

To optimize the time in the biosynthesis of the nanocomposite, a time range of 5 to 20 minutes was examined. The results of the investigation into the effect of time on the synthesis of copper and zinc nanoparticles using pomegranate extract are presented in Figure 4-1. These results indicate that from the beginning of the reaction until 10 minutes, the absorption gradually increases. After 10 minutes, the absorption decreases. A duration of 10 minutes was selected as the optimal time for the biosynthesis of the nanocomposite using the extra

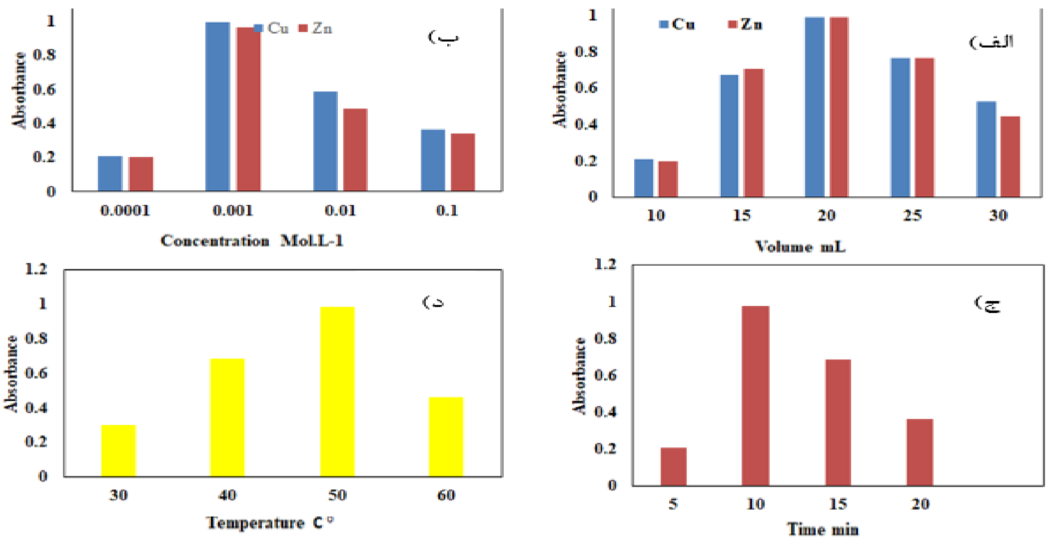


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Figure (1). Image related to the optimization of the effect (a) volume of copper and zinc salt at a concentration of 0.001 molar, (b) concentration of copper and zinc salt, (c) investigation of reaction time on the biosynthesis of nanoparticles, and (d) investigation of water bath temperature in degrees Celsius.

By adding copper and zinc oxide compounds to the pomegranate peel extract, we observed a color change in the extract, indicating the initiation of the formation process of the desired nanocomposite. To confirm the biosynthesis of the copper-zinc oxide nanocomposite, UV-Vis spectrophotometry was employed to assess the color change. To verify the presence of the desired nanocomposite in the samples and their stability, the absorption spectra were recorded after the color change in the range of 300-700 nanometers using a spectrophotometer. The optical absorption spectrum of the biosynthesized nanocomposite from the plant extract is illustrated in Figure (2). In the spectra obtained from the nanocomposite, the highest absorption was observed at a wavelength of 398 nanometers.

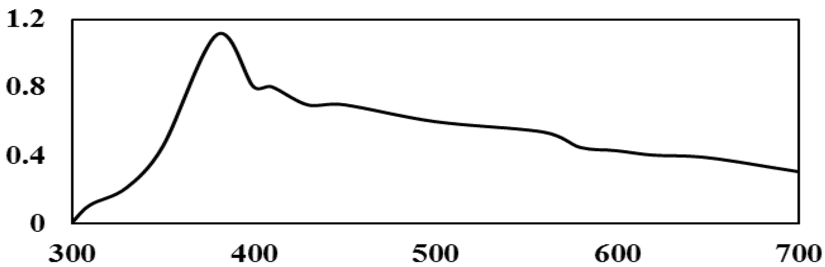


Figure (2). Spectrophotometric spectrum of the biosynthesized nanocomposite from pomegranate peel

EDX analysis was employed to investigate the constituent elements and purity of the copper and zinc nanocomposite. The biosynthesized nanocomposite exhibits an absorption peak of less than Kev 5, which is indicative of nanoparticles and metallic nanocomposites. The results obtained from EDX are illustrated in Figure (3). The analysis of the sample reveals the presence of oxygen, zinc, and copper elements. The high percentage of zinc and copper observed in this spectrum confirms the high purity of the synthesized nanoparticle.

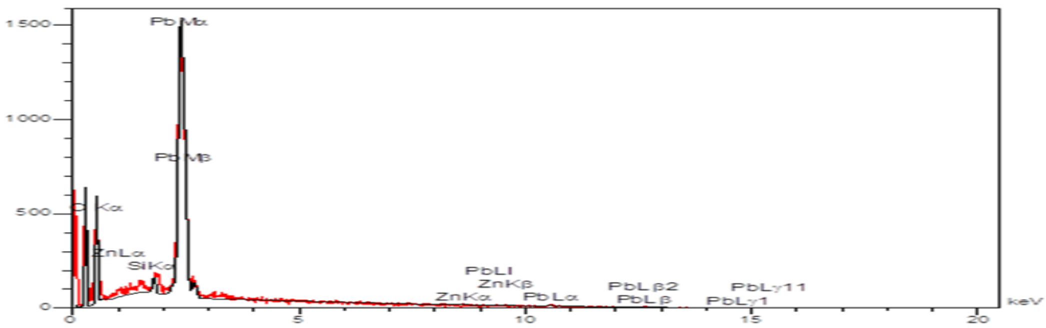

**Figure (3). EDX spectrum of nanoparticles**

To investigate the morphology and surface of the copper-zinc oxide nanocomposite, scanning electron microscopy was employed. The results of the scanning electron microscopy analysis of the biosynthesized copper-zinc oxide nanoparticles using pomegranate peel extract are illustrated in Figure 4. In this study, the copper and zinc oxide nanoparticles are approximately spherical in shape, with a size of about 50 nanometers.

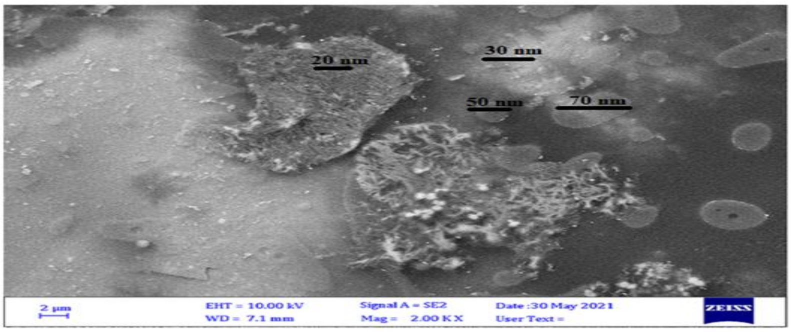


**Figure (4). SEM image of the biosynthesized nanocomposite from pomegranate peel.**

The determination of the size of nanoparticles and nanocomposites through FESEM is not precise, as the resolution of FESEM is lower than that of TEM. Consequently, TEM analysis is employed to ascertain the average particle size. Considering the images obtained in Figure 5 of this study, the shape of the nanoparticles was approximately spherical, with a size of less than 30 nanometers.

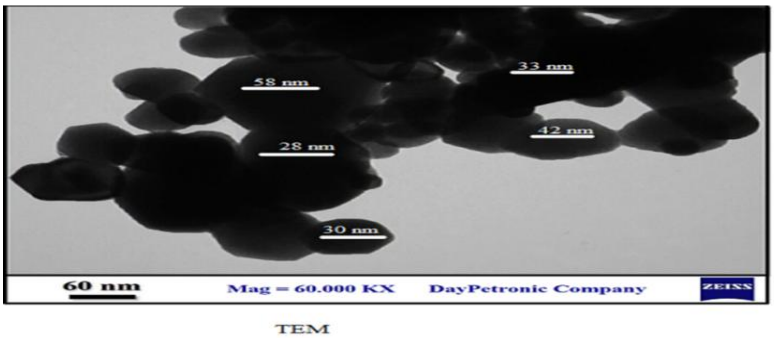


**Figure (5). TEM image of biosynthesized nanocomposite from pomegranate peel**

To investigate the crystalline structure of the biosynthesized nanocomposite derived from the plant, X-ray diffraction (XRD) was employed. XRD is a valuable tool for confirming the formation of the nanocomposite, determining the crystal structure, and calculating the size of the crystalline particles in a liquid environment. The XRD pattern of the biosynthesized nanocomposite extract is illustrated in Figure 6 .

The X-ray diffraction peaks correspond to the Miller indices 111, 200, 220, 311, and 222, which provide evidence for the formation of the copper-zinc oxide nanocomposite (Alswat et al., 2017; Sajjad et al., 2018; Gurgur et al., 2020). The Miller indices in crystallography are used to specify the planes in a crystal lattice. A family of crystal planes is identified by three integers. The degree of displacement of a plane relative to each axis is denoted by a number, with a plane parallel to an axis indicated by zero. The size, width, and angle of the peaks contain data regarding the sample. X-ray diffraction measurements for experimental samples were utilized to determine the size and structure of the biosynthesized nanocomposite derived from the plant. The results obtained corroborate the microscopic findings of the nanocomposite in question.

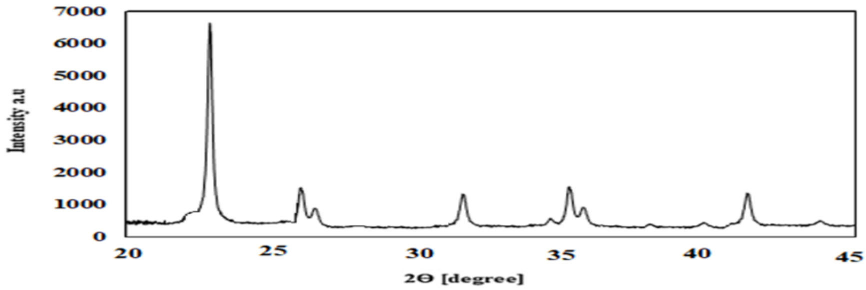


**Figure (6). XRD pattern of the biosynthesized nanocomposite from pomegranate peel.**

To investigate potential biomolecules in the biosynthesized nanocomposite derived from plants and to examine the surface characteristics of the produced nanoparticles, FT-IR analysis was employed. The results of the FT-IR analysis of the plant-derived biosynthesized nanocomposite are presented in Figures (7) and (8). The distinct peak at 3454 corresponds to the hydroxyl functional group in alcohols and phenolic groups. The peak recorded at 2945 pertains to the C-H stretching bond in alkanes. Additionally, the peak observed at 1610 is associated with the stretching vibration in the C=O bond. The presence of these peaks in the spectrum of the nanoparticles synthesized with plant extract substantiates the notion that these nanoparticles have achieved adequate stability following synthesis through surface modification with the active compounds of the plant.

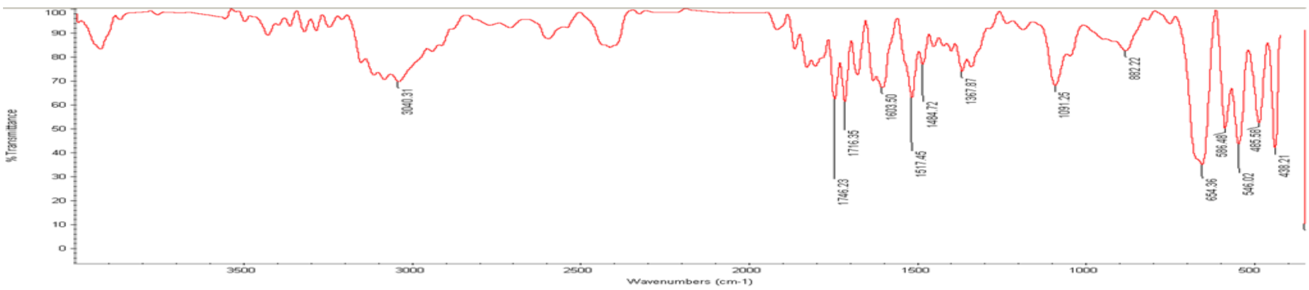


**Figure (7). FT-IR spectrum of pomegranate peel extract**

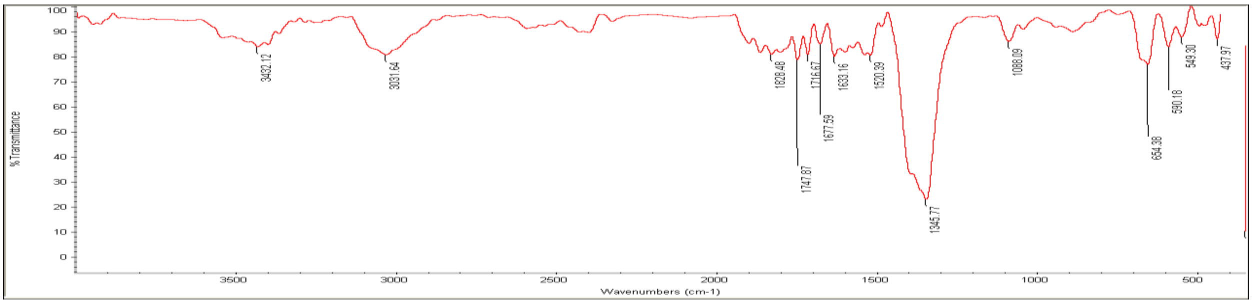

**Figure (8). FT-IR spectrum of the biosynthesized nanocomposite from pomegranate peel extract.**

The antibacterial effects of the copper-zinc oxide nanocomposite synthesized with pomegranate peel extract against certain pathogenic bacteria (Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteriae, Enterococcus faecalis, and Staphylococcus aureus) were investigated using the agar well diffusion method .

Figure (9) illustrates the diameter of the inhibition zones of some pathogenic bacteria created by various concentrations of the colloidal solution of the copper-zinc oxide nanocomposite synthesized from pomegranate peel (100, 250, and 500 micrograms per milliliter), the pure pomegranate peel extract, and tetracycline, as determined by the well diffusion method .

Part A pertains to the bacterium Escherichia coli, which exhibited sensitivity at concentrations of 100, 250, and 500 micrograms per milliliter, as well as to tetracycline and the pomegranate peel extract .

Part B relates to the bacterium Staphylococcus aureus, which is sensitive to the applied treatment concentrations, tetracycline, and the pomegranate peel extract .

Part C concerns the bacterium Shigella dysenteriae, which demonstrated sensitivity to the various treatments applied .

Part D refers to the bacterium Enterococcus faecalis, which was sensitive to the concentrations of 100, 250, and 500 micrograms per milliliter but resistant to the pomegranate peel extract and tetracycline .

Part E pertains to the bacterium Pseudomonas aeruginosa, which responded to the applied treatment concentrations, the pomegranate peel extract, and tetracycline, indicating sensitivity.

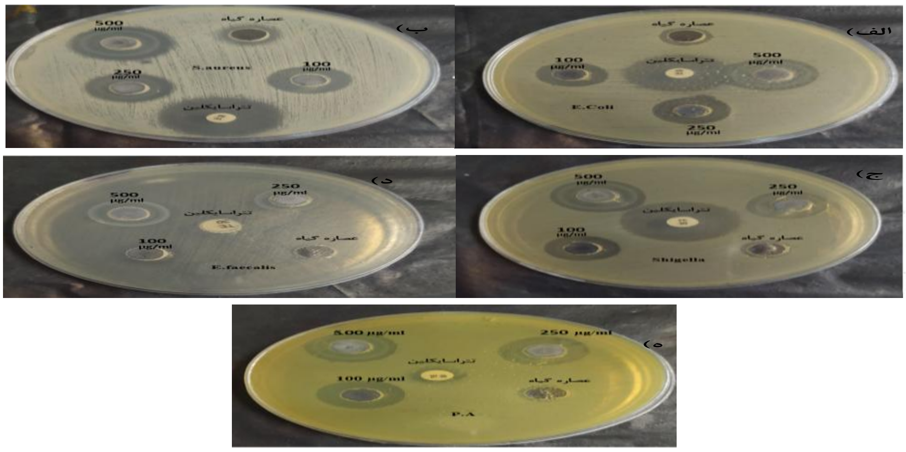

Figure (9) illustrates the diameter of the inhibition zone resulting from the application of antibacterial treatments against the following bacteria: a) Escherichia coli, b) Staphylococcus aureus, c) Shigella dysenteriae, d) Enterococcus faecalis, and e) Pseudomonas aeruginosa using the well diffusion method.

The results of the antibacterial activity test of the copper-zinc oxide nanocomposite synthesized with pomegranate peel extract, pure pomegranate peel extract, and tetracycline, as determined by the well diffusion method in agar, are also presented in terms of the diameter of the inhibition zone in Table .(2)

Table (2). Average diameter of the inhibition zone of the copper-zinc oxide nanocomposite synthesized with aqueous pomegranate peel extract, pure pomegranate peel extract, and tetracycline against certain pathogenic bacteria using the well diffusion method (measured in millimeters).

| Watch (Tetracycline) | Pomegranate peel extract | thickness (500 μg/ml) | thickness (250 μg/ml) | thickness (100 μg/ml) | Sample |
|---|---|---|---|---|---|
| 24 | 9 | 18 | 16 | 15 | Escherichia coli |
| 6 | 5 | 16 | 15 | 15 | Pseudomonas aeruginosa |
| 24 | 9 | 19 | 15 | 14 | Shigella dysenteriae |
| resistant | resistant | 14 | 12 | 10 | Enterococcus faecalis |
| 25 | 11 | 21 | 17 | 16 | Staphylococcus aureus |

Based on the results obtained from Table (2), it was observed that the synthesized copper-zinc oxide nanocomposite using pomegranate peel extract inhibited all the examined bacteria. Furthermore, the copper-zinc oxide nanocomposite synthesized with the plant extract exhibited a stronger antibacterial effect against Pseudomonas aeruginosa and Enterococcus faecalis compared to tetracycline .

The antibacterial effects of the copper-zinc oxide nanocomposite synthesized with pomegranate peel extract against the aforementioned pathogenic bacteria were evaluated using the disk diffusion method on agar. Figure (10) illustrates the diameter of the inhibition zones for the pathogenic bacteria under the influence of various concentrations of the colloidal solution of the copper-zinc oxide nanocomposite synthesized from pomegranate peel (100, 250, and 500 micrograms per milliliter), the pure pomegranate peel extract, and tetracycline, as assessed by the disk diffusion method .

Part (a) pertains to Escherichia coli, which was sensitive to the concentrations of 100, 250, and 500 micrograms per milliliter and tetracycline, but resistant to the pomegranate peel extract. Part (b) relates to Staphylococcus aureus, which was sensitive to the applied concentrations and tetracycline, yet resistant to the pomegranate peel extract. Part (c) concerns Shigella dysenteriae, which was sensitive to all antibacterial treatments but resistant to the pomegranate peel extract. Part (d) refers to Enterococcus faecalis, which was resistant to all applied treatments. Finally, part (e) pertains to Pseudomonas aeruginosa, which was sensitive to the concentrations of 250 and 500 micrograms per milliliter and tetracycline, but resistant to the concentration of 100 micrograms per milliliter and the pure extract.

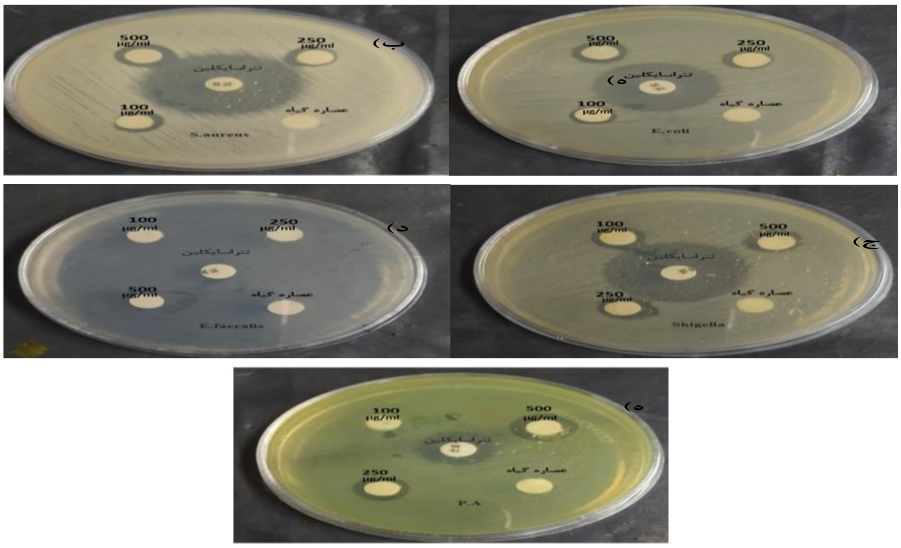


Figure (10). The image illustrates the diameter of the inhibition zone resulting from the application of antibacterial treatments against the bacteria a) Escherichia coli, b) Staphylococcus aureus, c) Shigella dysenteriae, d) Enterococcus faecalis, and e) Pseudomonas aeruginosa using the disk diffusion method .

The results of the antibacterial activity test of the copper-zinc oxide nanocomposite synthesized with pomegranate peel, the pure extract of pomegranate peel, and tetracycline against certain pathogenic bacteria are presented in Table (3) as the diameter of the inhibition zone .

Table (3). Average diameter of the inhibition zone in the copper-zinc oxide nanocomposite synthesized with pomegranate peel extract, pure pomegranate peel extract, and tetracycline against certain pathogenic bacteria using the disk diffusion method (measured in millimeters).

| **Observe (tetracycline)** | **Pomegranate peel extract** | **viscosity )g/mlμ (500** | **viscosity )g/mlμ (250** | **viscosity )g/mlμ (100** | **Sample** |
|---|---|---|---|---|---|
| 21 | resistant | 7 | 7 | 6 | Escherichia coli |
| 13 | resistant | 10 | 8 | resistant | Pseudomonas aeruginosa |
| 24 | resistant | 10 | 8 | 7 | Shigella dysenteriae |
| resistant | resistant | resistant | resistant | resistant | Enterococcus faecalis |
| 28 | resistant | 10 | 8 | 7 | Staphylococcus aureus |

Based on the results obtained from Table (3), it was observed that all bacteria, except for Enterococcus faecalis, which was resistant to the various treatments applied, and Pseudomonas aeruginosa, which was resistant to a concentration of 100 micrograms per milliliter, were sensitive to the synthesized copper-zinc oxide nanocomposite derived from pomegranate peel. Furthermore, it was noted that the antibacterial effect of the copper-zinc oxide nanocomposite synthesized from pomegranate peel was significantly greater than that of the plant extract on the bacteria of interest, with all examined bacteria being completely resistant to the pure extract. It can be concluded that in the disk antibacterial method, the extract did not inhibit bacterial growth. The minimum inhibitory concentration (MIC) of the copper-zinc oxide nanocomposite synthesized with the plant extract against the targeted pathogenic bacteria was investigated. The minimum concentration at which no bacteria were observed was considered as the MIC, and the turbidity observed thereafter indicated that the antibiotic was ineffective, allowing bacterial growth.

Figures (11, 12, 13, 14, and 15) illustrate the minimum inhibitory concentration of the copper-zinc oxide nanocomposite synthesized with the plant extract from pomegranate peel against Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteriae, Enterococcus faecalis, and Staphylococcus aureus, respectively. The presence of turbidity, which indicates bacterial growth or lack thereof, was recorded, and according to the definition, the most diluted well that exhibited no turbidity was considered equivalent to the MIC. The results indicated that a treatment concentration of 0.78 micrograms per milliliter (PPM) halted the growth of Escherichia coli, while a treatment concentration of 1.56 micrograms per milliliter (PPM) inhibited the growth of Pseudomonas aeruginosa, Shigella dysenteriae, Enterococcus faecalis, and Staphylococcus aureus.

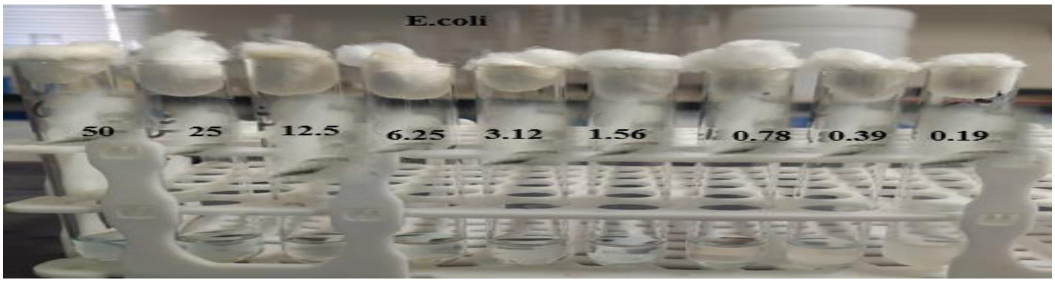


Figure (11) illustrates the minimum inhibitory concentration of the nanocomposite synthesized with .pomegranate peel extract against the bacterium Escherichia coli

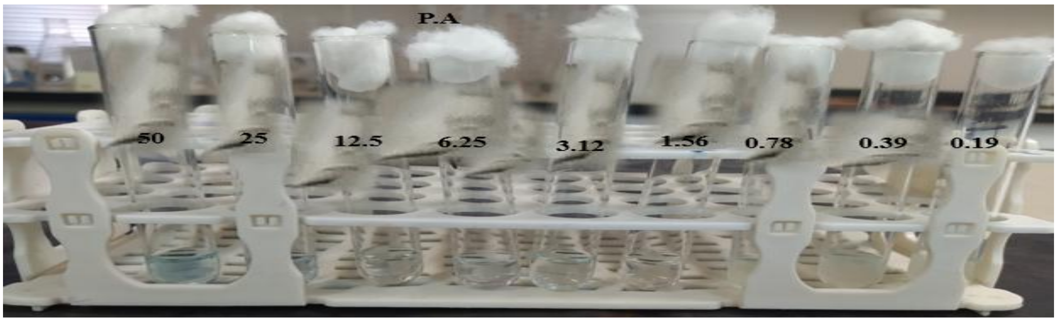


Figure (12). Image of the minimum inhibitory concentration of the nanocomposite synthesized with pomegranate peel extract against Pseudomonas aeruginosa.

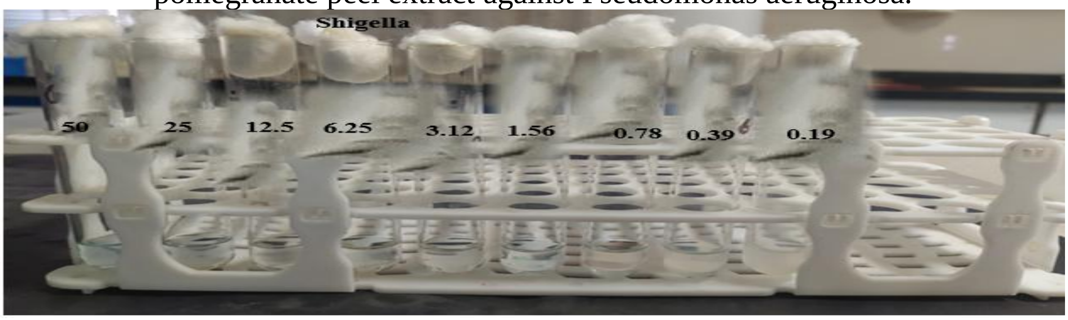

Figure (13). Image of the minimum inhibitory concentration of the nanocomposite synthesized with pomegranate peel extract against Shigella dysenteriae.

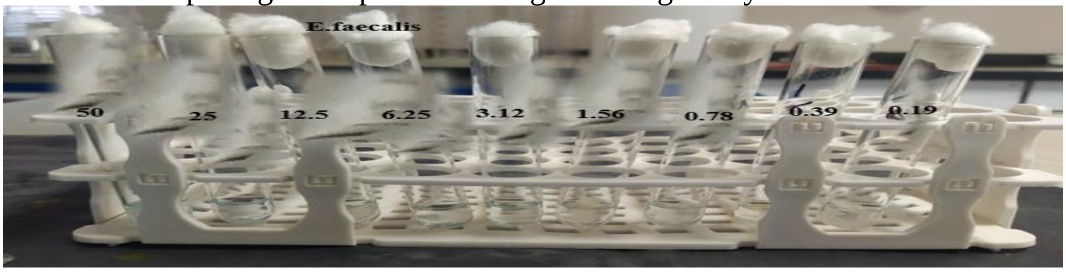


Figure (14). Image of the minimum inhibitory concentration of the nanocomposite synthesized with pomegranate peel extract against the bacterium Enterococcus faecalis.

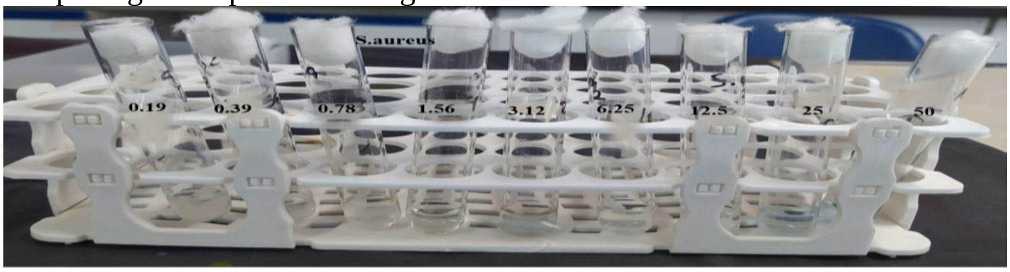


Figure (15) illustrates the minimum inhibitory concentration of the nanocomposite synthesized with pomegranate peel extract against Staphylococcus aureus.

The results of the minimum inhibitory concentration of the nanocomposite synthesized with aqueous pomegranate peel extract against certain pathogenic bacteria are also presented in Table .(4)

Table (4) displays the minimum inhibitory concentration of the nanocomposite synthesized with aqueous pomegranate peel extract against various pathogenic bacteria.

| Staphylococcus aureus | Shigella dysentery | Pseudomonas aeruginosa | Enterococcus faecalis | Escherichia coli | sample |
|---|---|---|---|---|---|
| 1.56 | 1.56 | 1.56 | 1.56 | 0.78 | **minimum inhibitory concentration** |

The minimum inhibitory concentration of the copper-zinc oxide nanocomposite synthesized with pomegranate peel plant extract against pathogenic bacteria was investigated. The minimum concentration at which no bacterial growth occurred was considered the minimum inhibitory concentration. Figure (16) illustrates the minimum inhibitory concentration of the copper-zinc oxide nanocomposite synthesized with pomegranate peel plant extract against certain pathogenic bacteria .

Part A pertains to Escherichia coli, where a treatment concentration of 1.56 micrograms per milliliter (PPM) effectively halted bacterial growth. Part B relates to Pseudomonas aeruginosa, where a treatment concentration of 3.12 micrograms per milliliter (PPM) resulted in the cessation of bacterial growth. Part C concerns Shigella dysenteriae, which also exhibited a treatment concentration of 3.12 micrograms per milliliter (PPM) that stopped bacterial growth. Part D addresses Enterococcus faecalis, which demonstrated resistance to the applied treatment concentrations. Finally, Part E pertains to Staphylococcus aureus, where a treatment concentration of 3.12 micrograms per milliliter (PPM) successfully inhibited bacterial growth

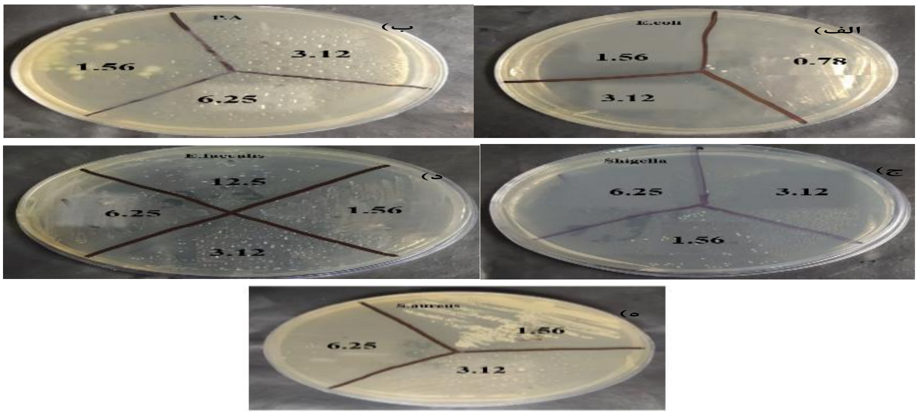


Figure (16). Image related to the minimum inhibitory concentration resulting from the nanocomposite synthesized with pomegranate peel extract against bacteria: a) Escherichia coli, b) Pseudomonas aeruginosa, c) Shigella, d) Enterococcus faecalis, e) Staphylococcus aureus .

The results of the minimum inhibitory concentration of the nanocomposite synthesized with aqueous extract of pomegranate peel against certain pathogenic bacteria are presented in Table 5.

Table (5) Minimum Lethal Concentration of Nanocomposite Synthesized with Aqueous Extract of Pomegranate Peel Against Pathogenic Bacteria

| *Staphylococcus aureus* | *Shigella dysentery* | *Pseudomonas aeruginosa* | *Enterococcus faecalis* | *Escherichia coli* | **sample** |
|---|---|---|---|---|---|
| 3.12 | 3.12 | 3.12 | _ | 1.56 | **minimum lethal concentration** |

## Research and Conclusion :

The utilization of nanoparticles as antimicrobial agents can overcome bacterial resistance mechanisms, as the microbicidal nature of nanoparticles operates through direct contact with the bacterial cell wall, without the necessity for intracellular penetration. Consequently, the likelihood of developing antibacterial resistance when employing nanoparticles is comparatively lower than with antibiotics. The antibacterial activity of nanoparticles varies among different types of nanoparticles (Rudramurthy et al., 2016). Metallic nanoparticles such as silver, copper, and zinc are extensively used in controlling infectious diseases caused by bacterial resistance. These metallic nanoparticles exhibit relatively lower toxicity and demonstrate stable treatment efficacy, effectively preventing bacterial resistance (Ashfagh et al., 2016).

This study investigated the green synthesis of copper-zinc oxide nanocomposites using pomegranate peel extract and examined their antibacterial effects. Following the addition of the extract to the copper and zinc salt solution, the color of the nanoparticles transitioned from light blue to greenish-brown, indicating the synthesis of copper and zinc nanoparticles. The observed color change to greenish-brown in the plant extract serves as a clear indication of the formation of copper-zinc oxide nanocomposites during the reaction. The color difference in the two states is attributed to the phytochemicals present in the plant extract (Ashtaputrey et al., 2017).

The optimal conditions for synthesizing copper-zinc oxide nanoparticles revealed that temperature and time significantly influenced the process, with a temperature range of 30-50 °C identified as the best for nanoparticle synthesis, leading to increased absorbance. Maximum absorbance was observed at 50

°C, which was selected as the optimal temperature. As the temperature exceeded this optimal value, absorbance decreased. Additionally, from the beginning of the reaction until the tenth minute, absorbance gradually increased, after which it declined, indicating the reduction of copper and zinc nanoparticles. The reduction of copper and zinc nanoparticles is attributed to the instability of the formed nanocomposite.

The green synthesis method involved the reduction of copper and zinc ions using pomegranate peel extract. In this process, the free electrons present in the copper and zinc nanoparticles are excited by visible light absorption, elevating them to a higher energy level. However, since the excited state of the electron is unstable, it returns to the ground state, emitting a photon. The data obtained from UV-Vis spectroscopy of the nanoparticles indicated the presence of an absorption peak for the biosynthesized copper-zinc oxide nanocomposite at a wavelength of approximately 398 nanometers, signifying the correct formation of the nanocomposite (Norouzi et al., 2021).

TEM analysis confirmed the desirable size of the nanoparticles. Furthermore, observations made using these techniques revealed that the particles possess a spherical shape and appropriate morphology. In this study, XRD analysis was employed to ascertain the size of the crystals and to confirm the existence and synthesis of the copper-zinc nanocomposite using pomegranate peel extract. According to the XRD pattern, the copper-zinc oxide nanoparticles exhibited diffraction peaks corresponding to Miller indices 111, 200, 220, 311, and 222.

To investigate the potential biomolecules involved in the formation and coating of the synthesized nanocomposites, FT-IR analysis was conducted. The FT-IR analysis indicated the presence of functional groups that likely play a role in the synthesis of copper-zinc oxide nanoparticles. These groups are instrumental in the reduction of copper and zinc ions to the copper-zinc nanocomposite. Additionally, these groups play a crucial role in the stability and prevention of aggregation and agglomeration of the plant-based nanocomposite. The presence of these peaks in the spectrum of the nanoparticles synthesized with plant extract substantiates the notion that these nanoparticles achieved adequate stability through surface modification with the active compounds from the plant (Hosseini-Sarvari et al., 2014 and Norouzi et al., 2021).

**The antibacterial effect of copper-zinc oxide nanocomposite against pathogenic bacteria** was investigated using the well diffusion and disk diffusion methods on agar. The diameter of the inhibition zone was measured by varying concentrations of the colloidal solution of the copper-zinc oxide nanocomposite synthesized from pomegranate peel extract against the bacteria. A larger diameter of the inhibition zone indicates a greater antibacterial effect. Metal nanoparticles possess significant antibacterial properties. The results obtained from determining the antibacterial properties of the copper-zinc oxide nanocomposite indicated a direct correlation between the concentration of nanoparticles and the percentage of bacterial elimination. As the concentration of the copper-zinc oxide nanocomposite synthesized from pomegranate peel extract increased, the inhibition zone diameter against pathogenic bacteria also increased. This implies that an increase in the concentration of the copper-zinc oxide nanocomposite leads to an enhancement in its antibacterial effect.

In comparing the inhibition zones of certain pathogenic bacteria (Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteriae, Enterococcus faecalis, and Staphylococcus aureus) using the well diffusion method, it was shown that the copper-zinc oxide nanocomposite synthesized with plant extract at various concentrations had the most significant effect on Staphylococcus aureus, achieving maximum inhibitory concentrations at 100, 250, and 500 micrograms per milliliter with inhibition zone diameters of 17, 16, and 21 millimeters, respectively, compared to the pomegranate peel extract, which produced an inhibition zone of 11 millimeters. Conversely, the least effect was observed on Enterococcus faecalis, with minimum inhibitory concentrations at 100, 250, and 500 micrograms per milliliter yielding inhibition zone diameters of 10, 12, and 14 millimeters, respectively, demonstrating resistance compared to the pomegranate peel extract and tetracycline (control), which is a significant finding of this study. Additionally, the bacteria Escherichia coli, Shigella dysenteriae, and Pseudomonas aeruginosa exhibited nearly similar results; specifically, Escherichia coli, Pseudomonas aeruginosa, and Shigella dysenteriae showed inhibition zone diameters of 15, 15, and 14 millimeters, respectively, at a concentration of 100 micrograms per milliliter, and 16, 15, and 15 millimeters at 250 micrograms per milliliter, and 18, 16, and 19 millimeters at 500 micrograms per milliliter, compared to the pure extract which yielded 9, 5, and 9 millimeters.

In comparing the inhibition zones on the examined bacteria using the disk diffusion method, it was shown that the copper-zinc oxide nanocomposite synthesized with pomegranate peel extract at various concentrations produced similar results across all bacteria (except for Enterococcus faecalis, which was resistant to all applied treatments), generating comparable inhibition zone diameters. The results also indicated that Pseudomonas aeruginosa was resistant at a concentration of 100 micrograms per milliliter. The copper-zinc oxide nanocomposite synthesized with plant extract at various concentrations, along with pomegranate peel extract and tetracycline (control), exhibited the most significant effect on Shigella dysenteriae and Staphylococcus aureus, achieving maximum inhibitory concentrations at 100, 250, and 500 micrograms per milliliter with inhibition zone diameters of 7, 8, and 10 millimeters, respectively. In contrast, the applied treatments had the least effect on Enterococcus faecalis, which remained resistant to all treatments. Furthermore, Escherichia coli produced inhibition zone diameters of 6, 7, and 7 millimeters at concentrations of 100, 250, and 500 micrograms per milliliter, respectively, while Pseudomonas aeruginosa was resistant at 100 micrograms per milliliter but produced inhibition zone diameters of 8 and 10 millimeters at concentrations of 250 and 500 micrograms per milliliter, respectively. Additionally, all examined bacteria in this study were resistant to the pure pomegranate peel extract, indicating a stronger antibacterial effect of the nanocomposite synthesized from plant extract compared to the pure extract.

Yousefi et al. (2013) investigated the antibacterial effects of copper nanoparticles on Escherichia coli, Klebsiella, Staphylococcus aureus, and Enterococcus faecalis using the disk diffusion method. The results obtained in this study indicated that the strains of Escherichia coli and Staphylococcus aureus exhibited greater sensitivity to copper nanoparticles, which is consistent with the present findings.

In the study conducted by Hosseinkhani et al. (2011), it was demonstrated that the use of zinc nanoparticles can inhibit the growth of pathogenic bacteria such as Shigella, which aligns with the findings of this study.

A comparison between the well and disk methods on agar revealed that various concentrations of copper-zinc oxide nanocomposite were slightly more diffusible in the well method than in the disk diffusion method on agar. It is quite evident that the diffusion of copper-zinc oxide nanocomposite in suspension in the well method on agar is greater; this is because, in the disk method on agar, each side of the disk was coated with 10 microliters of solution and was allowed to dry for 24 hours under sterile conditions. However, in the well method on agar, 100 microliters of the solution were poured into each well. Additionally, in the disk method on agar, some of the colloidal solution of nanoparticles is absorbed by the various layers of the disk, and after drying, a small amount of this solution also evaporates. Considering the results from both the well and disk methods, which were derived from the extract and compared with the nanocomposite, it was determined that the effect of the extract was not significant compared to the effect of the copper-zinc oxide nanocomposite. Therefore, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were only conducted on the copper-zinc oxide nanocomposite. The antibacterial tests for MIC and MBC are effective methods for assessing the antibacterial effects of compounds and formulations and are among the most important and widely used analyses in microbiology. The MIC refers to the concentration of an antibacterial agent that can inhibit bacterial growth in a laboratory environment, while the MBC refers to the minimum concentration of the drug that kills the bacteria.

The lower these values are in the antibiogram analysis, the stronger the antibacterial potency of the therapeutic compound. This test serves as a complement to the determination of the inhibition zone. However, it is generally a more suitable method for insoluble particles, such as nanoparticles. The significance of determining MIC and MBC has increased considerably today due to the rise of antibiotic resistance in bacteria. To investigate the antibacterial effect of the synthesized nanoparticles, a tube dilution method (macrodilution) was employed. The lowest inhibitory concentration (MIC) and bactericidal concentration (MBC) were found to be: the minimum inhibitory concentration in Escherichia coli was 0.78 and in other bacteria was 1.56 micrograms per milliliter, while the minimum bactericidal concentration in Escherichia coli was 1.56 and in Shigella dysenteriae, Staphylococcus aureus, and Pseudomonas aeruginosa was 3.12 micrograms per milliliter, with Enterococcus faecalis being resistant to all concentrations. The results of the minimum inhibitory concentration and minimum bactericidal concentration confirmed the sensitivity of the examined bacteria to the applied treatments.

The findings of this research indicated that the antibacterial effect of the copper-zinc oxide nanocomposite synthesized with plant extract is greater than that of the pure plant extract. Furthermore, these results demonstrated that the copper-zinc oxide nanocomposite synthesized from pomegranate plant extract possesses good antibacterial properties and was able to inhibit the growth of nearly all targeted pathogenic bacteria, which is attributed to the antibacterial effects of copper and zinc nanoparticles. Based on the findings of this study, the antibacterial effect of these materials is enhanced with the pomegranate plant extract, which may be due to the presence of phenolic compounds in this plant. Additionally, the results confirmed that varying amounts of salt, as well as temperature and reaction time, significantly influence the production of nanoparticles. An important point to note is that the plant extract alone, without the need for any other substances, is capable of producing nanoparticles. Copper and zinc nanoparticles, due to their advantages such as small size, biocompatibility, low consumption, and long lifespan, as well as the side effects of antibiotics, can serve as a good alternative to antibiotics and may receive greater attention in the treatment of bacterial infections. Therefore, it is expected that the good antibacterial properties of the biosynthesized nanoparticles in this study are due to their relatively small size and appropriate shape.

Recommendations
- Investigate the stabilizing factors of copper-zinc oxide nanocomposite
- Prepare and optimize copper and zinc nanoparticles for market release
- Examine the clinical effects of the resulting copper-zinc oxide nanocomposite on wounds and burn injuries
- Investigate the cytotoxic effects of the synthesized copper-zinc oxide nanocomposite on human and animal cells

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