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Research Article

The Effect of Zinc Oxide, Copper, and Silver Nanoparticles Synthesized by the Green Method for Controlling Strawberry Gray Mold Fungus, *B. Cinerea* Pers

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Keywords: Nanoparticle; Strawberry; *Botrytis cinerea*; Post-harvest control; Biocontrol

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Abstract

Gray mold disease, caused by the fungus *Botrytis cinerea*, causes heavy losses in strawberries. The use of chemical fungicides due to the dangers for humans and the environment has caused attention to reduce their consumption and use biological methods. In this research, the effects of zinc oxide, copper, and silver nanoparticles have been synthesized from an aqueous extract of cloves, and the probiotic bacteria *Lactobacillus casei* by the green method was investigated on the gray mold disease of strawberries. The results showed that concentrations of 10% of zinc oxide nanoparticles synthesized from aqueous extract of cloves can completely control this pathogen on the culture medium and the fruit. Zinc and silver nanoparticles produced by *Lactobacillus casei* prevented 93.7% and 81% of fungal growth in the culture medium, respectively. Other treatments did not show a good inhibitory effect on the fungus. All treatments were able to prevent 100% to 50% of fungal growth after 96 hours on strawberries. The investigation of the storage characteristics showed the positive effect of the examined nanoparticles on reducing the rate of change of the physicochemical characteristics of the strawberry fruit tissue. Apparent decay was significantly reduced and samples treated with nanoparticles scored higher in sensory evaluation compared to control. Also, investigating the toxicity of nanoparticles in this experiment on the HepG2 cell line showed that Compared to the control, copper and zinc nanoparticles did not have significant toxicity on cells, but silver nanoparticles led to 25% cell death. This research provides promising results in the field of using nanoparticles for pre-harvest and post-harvest control of plant diseases.

Introduction

The strawberry (*Fragaria ananassa* Duc, family (Rosaceae), is an important and valuable plant. The fruit is one of the most consumed berries in the world due to its unique taste and nutritional and health benefits, However, strawberries are exposed to various plant pathogens, including viruses, bacteria, and fungi [1]. *B. cinerea* is one of the most common fungal pathogens of strawberries in the world, it is estimated that this disease can cause up to 25% yield reduction for untreated strawberries [2]. The fungus can affect flowers, fruits and attack the leaves. Infection may occur in the flower and persist until the fruits mature, and then develop after harvesting strawberry fruits during storage and cause fruit rot with pathogen sporulation [3] and there are many casualties. To date, the most efficient tactics for post-harvest control of the *B. cinerea* pathogen have been mainly the use of fungicides [4]. However, pathogen resistance to common fungicides is well known. Fungicide residue on fruits may also cause toxicity. In addition, the time of application of commercially available fungicides can reduce pollination and cause the Deformation of fruits. Because of these adverse effects, Integrated Pest Management (IPM) has been used as a safe strategy to minimize the risk of pesticides in agriculture worldwide. IPM is an ecosystem approach to protect the environment, human health and reduce the problems of chemical pesticides. Biological control is one of the IPM strategies. Biodegradable and non-toxic chemicals from plants with selective activity against pathogens can become safer disease control agents [5].

Plants are a natural source of bioactive compounds that have antifungal, aromatic, medicinal, etc. properties. Some essential oils have different anti-fungal properties, such as: disrupting cell membranes, cell walls, or mitochondria, reducing cell growth, inhibiting biological growth, etc. In addition, with different extraction methods, essential oils, and extracts can be obtained from plants [6]. Due to its valuable compounds and properties, clove bud extract is one of the available options in this field. Clove bud extract mainly consists of Eugenol and Eugenyl acetate derivatives (Olea, et al. 2019). Determined the antifungal activity of Eugenol and its derivatives against *Botrytis cinerea*. Higher efficiency of biological control agents can be obtained by changes in the structure of these compounds. The use of nanoscience to produce micro-sized particles (1 to 100 micrometers) and more penetrating ability can improve efficiency. On the other hand, the synthesis of nanoparticles using plant extracts and biological organisms compatible with the environment is a cost-effective method hence, it is considered an alternative technique to physical and chemical technologies of nanoparticle synthesis [7]. As an excellent candidate for the synthesis of nanoparticles, bacteria have several characteristics, including ease of cultivation, short production time, extracellular production of nanoparticles, and high stability [8]. *Lactobacillus casei* (*L. casei*) is a non-pathogenic and useful probiotic bacteria that can be found in several parts of the human body such as the mouth, intestine, and urinary tract. This species is able to reduce metal ions in a very fast process due to its long growth time and production of several enzymes [9]. In this study, zinc oxide, copper, and silver nanoparticles were produced by two mechanisms of green synthesis, the use of plant extract (water extract of clove bud) and the use of microorganisms (*Lactobacillus casei* bacteria). The antimicrobial effect of these nanoparticles against gray mold fungus was investigated by laboratory method and on strawberry fruit. After determining the best inhibitory dose, the effect of these compounds on storage characteristics (acidity (PH), Organic Acids (TS), Total Soluble Solids (TSS), Total Protein content (TP), taste index, and sensory characteristics of the Strawberries fruit were studied.

Materials and methods

Preparation of *Botrytis cinerea* fungus and strawberry fruit

The desired pathogen was obtained from the microorganism collection of Tehran University and used for investigation. Strawberry fruits of the Sabrina variety in the full ripening stage, which were usable for consumers, were obtained from the Karaj fruit and vegetable market. After selecting healthy fruits with almost similar shapes and sizes, they were stored without any packaging in disposable containers without a lid at 4 ºC.

Preparation of water extract of cloves and nanoparticles of zinc oxide (ZNPM), copper (CNPM), and silver (SNPM)

Clove buds were obtained from a herbal shop. After removing the waste material, it was crushed in a mortar. The extract was extracted by boiling method. This process is suitable for extracting effective substances that are soluble in water and thermally stable. The ratio of plant to water was constant and equal to 1:4. The process of extracting and boiling the water continued until the volume of the solvent reached a quarter of the initial volume. Then the concentrated extract was filtered. The obtained extract was stored in dark glasses at a temperature of 1 ^{o}C in the refrigerator until the next test.

10 milliliters of water extract from the clove plant was added to 90 milliliters of each 1 millimolar solution of silver nitrate, copper sulfate, and zinc sulfate separately. In order to reduce silver, copper, and zinc oxide ions, the solutions were kept at room temperature and in the dark for one day and night. The color change of extracts to dark brown indicated the production of nanoparticles [10].

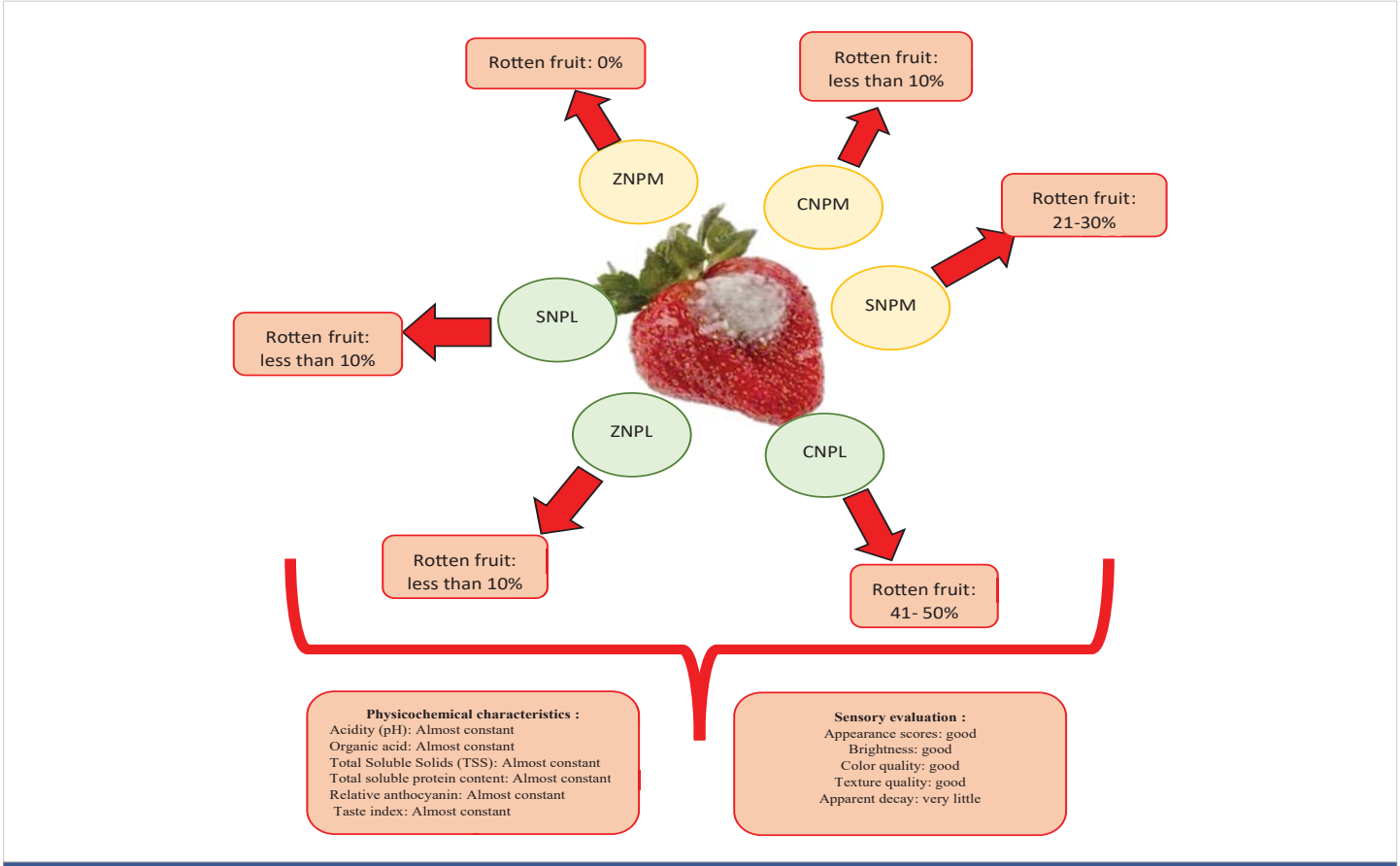
Graphical diagram: The effect of zinc oxide, copper, and silver nanoparticles synthesized by the green method for controlling strawberry gray mold fungus, *B. cinerea* Pers.

Preparation of nanoparticles of zinc oxide (ZNPL), copper (CNPL), and silver (SNPL) using *Lactobacillus casei* bacteria

The biosynthesis of nanoparticles was performed according to Markus et al.'s method with minor modifications. Briefly, *L. casei* was grown in MRS broth and incubated at 37 ºC and 150 rpm for 24 h. Cells were separated by centrifugation (5000 rpm) for 15 minutes and washed three times with sterile distilled water. Then, 20 ml of 1 mM solutions of silver nitrate, copper sulfate, and zinc sulfate were separately added to the falcons containing the cell biomass, and the biomass was suspended in the solutions. The falcons were kept in the dark overnight at room temperature [11].

MIC and MBC determination

The study of the antimicrobial efficacy of nanoparticles was done by evaluating the visible growth of *Lactobacillus Casei* bacteria in a nutrient broth culture medium. Serial dilutions of nanoparticles (zinc oxide, copper, and silver) in concentrations ranging from 2 to 100 percent with adjusted bacterial concentration (10^{8} CFU/ml, 0.5 McFarland's standard) were used to determine MIC in nutrient broth. The positive control contained nutrient broth medium plus bacteria and the negative control contained medium plus nanoparticles without bacteria. Samples were incubated for 24 h at 37 ^{0}C. The MIC is the lowest concentration of antimicrobial agent that completely inhibits the growth of the organism in tubes as detected by the unaided eye. The visual turbidity of the tubes was recorded, before and after incubation to confirm the MIC value. After the MIC determination of the



Graphical diagram: The effect of zinc oxide, copper, and silver nanoparticles synthesized by the green method for controlling strawberry gray mold fungus, *B. cinerea* Pers.

nanoparticles, aliquots of 50 µL from all the tubes which showed no bacterial growth were cultured on Nutrient Agar (NA) plates and incubated for 24 h at 37 ºC. When 99.9% of the bacterial population is killed at the lowest concentration of an antimicrobial agent, it is termed an MBC endpoint. This was done by observing pre and postincubated NA plates for the presence or absence of bacteria.

Investigating the formation of nanoparticles by FESEM microscope

The size and morphological characteristics of nanoparticles were determined using FESEM according to the standard protocol [12].

MTT assay

To determine the toxicity effects of nanoparticles synthesized in this research, a cell viability study was conducted using the conventional MTT reduction assay. Briefly, HepG2 cells were seeded in 96-well plates at the density of 5,000 cells/well in the presence of 100 µL cell culture medium (RPMI supplemented with 10% FBS and 1% penicillin–streptomycin solution). Cells were incubated for 24 hours in an incubator containing 5% CO_2 at 37 °C. After 24 hours of seeding, the medium inside the wells was replaced with fresh medium along with different concentrations of nanoparticles synthesized (1, 5, and 10 %) and incubated for 10 days at 37 °C. To detect cell viability, the old medium was replaced with 100 µL of fresh medium, then 10 µL of MTT solution (5 mg/mL in DMSO) was added to each well, and the plates were incubated further for another 4 hours. The MTT solution was then discarded, and 100 µL of DMSO was added to each well followed by incubation for 40 minutes in the dark. The solution was then pipetted and its absorbance was recorded at 540 nm using a microplate reader (Heales MB580 Microplate Reader - MB- Model).

The antifungal effect of nanoparticles on *B. cinerea* fungus by *in vitro* method

The antifungal effect of compounds was investigated in laboratory conditions on *B. cinerea* by mixing with culture medium [13]. To the prepared solutions, some toluene 80 solution (0.05%) was added to make an emulsion. Each concentration of 20, 40, 60, 80, and 100 milliliters of nanoparticle solution was added separately to one liter of cooling culture medium, and after mixing, it was divided into Petri dishes Iprodione-carbendazim fungicide was prepared at a ratio of 1:1000 and added to the cooling sterile culture medium. From the four-day-old young cultures of *B. cinerea*, 5x5 mm fungus discs were taken and placed in the center of the Petri dishes containing the culture medium. The inoculated Petri dishes were kept at 25 ºC in the Incubator fungus growth was measured daily in the sample and control Petri dishes

until the surface of the control medium was completely occupied by the fungus. The inhibition percentage of fungal growth in different treatments was calculated by Abbott's formula (Formula 1) [14] Formula 1:

$$IG = [(C-T)/C] \times 100$$

IG = The inhibition percentage of mycelial growth of the pathogenic fungus

C = The growth diameter of the pathogenic mycelium in the control

T = Growth diameter of pathogenic mycelium in each treatment

The control treatment included a PDA culture medium without extract and fungicide, containing Tween 80 solution (0.05%).

The antifungal effect of nanoparticles on *B. cinerea* fungus on fruits

For surface disinfection, the fruits were immersed in 2% sodium hypochlorite and then washed twice with sterile water, and finally immersed in 70% ethanol for 20 seconds. After drying, the fruits were immersed in the Nanoparticle solutions with concentrations of 2, 4, 6, 8, and 10% and poisoned separately. Then, a hole with a diameter of 1.5 mm and a depth of 3 mm was created in each fruit with a toothpick. 20 microliters of pathogenic fungus suspension with a concentration of 10^6 CFU/ml were injected into the wound. The fruits were packed in disposable containers and kept for 96 hours at 20 ºC and normal relative humidity. Control strawberries were inoculated with sterile distilled water.

The amount of fruit decay was evaluated according to Asghari Marjanlou, et al.'s method [15] and graded as follows: 0 = healthy fruit, 1 = less than 10% rotten fruit, 2 = 11-20%, 3 = 21-30%, 4 = 40-31%, 5 = 5-41%, 6 = 51-65, 7 = 65-80, 8 = more than 80% of the fruits are rotten.

Measuring the storage characteristics of strawberry fruit

After determining the best inhibitory concentration of nanoparticles, strawberry fruits were immersed in the nanoparticle solution and dried at room temperature. Then they were packed in closed disposable containers and stored at 4 ºC and 70% relative humidity for 10 days. The experiment was carried out in the form of a completely random design and Sampling was done every 3 days.

Total Soluble Solids (TSS): The fruits were completely homogenized in a mixer in each iteration. The measurement of total Soluble Solids was done by using a handheld refractometer, ATAGO model, by dropping a few drops of fruit extract on the device and reading it at an ambient temperature of 20 ºC.

Acidity (pH): pH of fruits was measured with a digital pH meter PM12E model after calibrating the device.

Amount of organic acid: The amount of organic acids that can be measured using fruit extract in each repetition was carried out according to the A.O.A.C standard titration method. 50 cc of the extract was diluted with 50 cc of distilled water and titrated with 0.1 normal sodium hydroxide using a magnetic stirrer, the titration process was carried out until the pH of the endpoint was 8 ± 0.2. The amount of organic acid in terms of the percentage of citric acid (the dominant acid in strawberries) was obtained from the following formula:

$$z = \frac{v \times N \times \mu Eqvt}{y} \times 100$$

Z: Acid percentage in the sample in terms of citric acid

V: milliliters of sodium hydroxide solution for titration

N: normal consumption sodium hydroxide solution equal to 0.1 µEqvt: milliequivalent of acid, which is equal to 0.64 for citric acid.

Y: milliliters of sample volume or its weight in grams

Total soluble protein content: this feature was measured by the method of Bradford [16]. For this purpose, 0.5 grams of fresh fruit tissue was mixed with 6.25 ml of extraction buffer and placed in a refrigerator at 4 degrees Celsius for 24 hours. Then, the sample with the extraction buffer was centrifuged for 20 minutes at a speed of 6000 rpm. 0.1 ml of the supernatant phase was removed and 5 ml of Bjord's reagent was added to it. The obtained mixture was shaken for a few seconds and the light absorbance of the samples was read at a wavelength of 595 nm using a spectrophotometer EV-2800 DS model. By comparing the absorption curve of bovine serum albumin as a standard, the concentration of soluble protein was calculated.

Relative content of anthocyanins: Pietrini and Massacci method was used to measure anthocyanin in fruit extract. First, 1 milliliter of fruit extract was placed in a boiling water bath with 3 milliliters of the extraction mixture (N-propanol 1 normal, hydrochloric acid 32%, and distilled water in a ratio of 18:1:18) for 5 minutes. And it cooled down in 3 hours. Then, separation was done with the help of a centrifuge, and the amount of light absorption of the samples was read at wavelengths of 535 and 650 nm by a spectrophotometer model EV-2800 DS. The extraction mixture was considered Blank. The relative content of anthocyanin was calculated and reported using the formula "Aanth = $A._{535}$–2.2 ($A._{650}$) [17].

Fruit Taste Index: The TSS/TA ratio was used to express the fruit taste index. This ratio affects the edible quality of the fruit [18].

Apparent decay: Fungal decay was evaluated by visual inspection of signs of growth of mushroom mycelium and also by microscopic observation of the growth of fungal mycelium

causing decay on fruit tissue. A numerical scale was considered to express the amount of fruit decay: 5 = no decay, 4 = less than 5%, 3 = 6-10%, 2 = 11% - 15%, and 1 = 16% - 20% decay [19].

Sensory evaluation of the product

In order to check the sensory and quality characteristics of strawberry samples during the storage time, the taste test and 5-point hedonic method were used. First, explanations were given to the evaluators about the color, brightness, texture, appearance, and presence or absence of mold and rot of the product and grading based on Very good = 5, good = 4, neither good nor bad = 3, bad = 2 and very bad = 1 done. The number of evaluators was considered to be 10 and in 3 repetitions.

Statistical analysis

This experiment was conducted in the form of a completely randomized design. Data were analyzed using SAS 9.1.3 statistical software (2001). To compare the means, Duncan's multiple range test was used at the 5% probability level. All experiments were performed in three replicates.

Results and discussion

SEM analysis was used for structural and morphological analysis and to confirm the synthesis of nanoparticles. Figure 1 shows SEM images. According to the images obtained from the SEM electron microscope, all the solutions of synthesized nanoparticles showed a decrease in the size of the particles compared to the control. Zn nanoparticles synthesized from clove aqueous extract clearly form almost sponge-like and flower-like structures, and nanoparticles obtained from *L. casei* bacteria show a spherical structure. The size of the nanoparticles was irregular and for the nanoparticles obtained from the aqueous extract of clove and lactobacillus, they were 115.05 - 74.74 nm and 111.8 - 35.08 nm, respectively.

In the images obtained from copper nanoparticles, it can be directly observed that the nanoparticles have a spherical morphology. The size of nanoparticles obtained from the water extract of clove and Lactobacillus were 41.84 - 51.55 nm and 44.77 - 68.73 nm, respectively.

The results also showed that the silver nanoparticles synthesized by clove aqueous extract have a good and homogeneous dispersion and a size of 67.59 - 70.06, while the nanoparticles produced by *L. casei* bacteria show almost spongy and spherical structures with a size of 73.967.04. Researchers found that controlling the size of nanoparticles is very important to achieve the best bactericidal response, and nanoparticles with smaller sizes show the highest

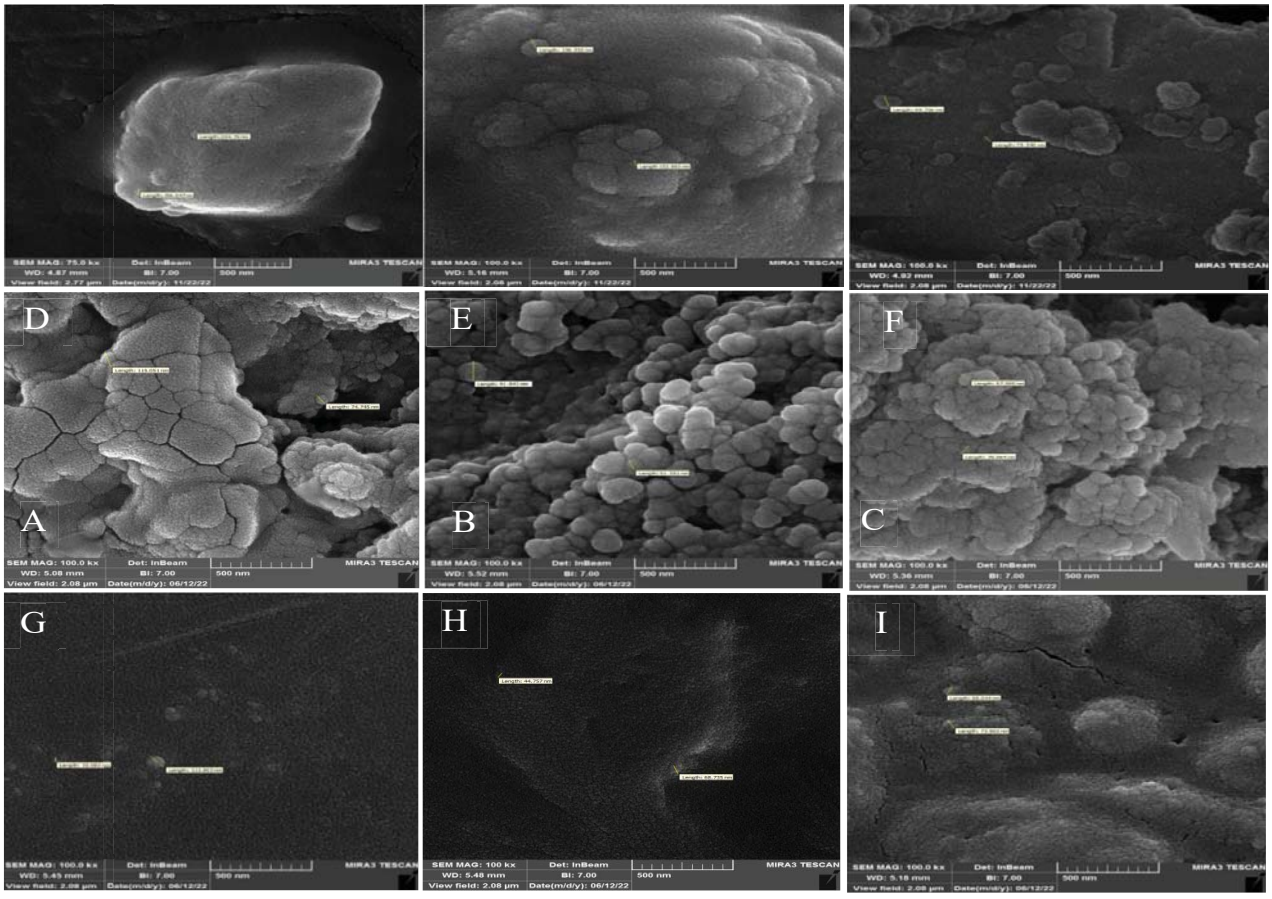


Figure 1: SEM images of nanoparticles A, B, and C respectively 1 millimolar solution of zinc sulfate, copper sulfate, and silver nitrate. D, E, and F respectively zinc Oxide, copper, and silver nanoparticles synthesized by clove aqueous extract, G, H, and I respectively zinc oxide, copper, and silver nanoparticles synthesized by *L. casei bacteria*.

antibacterial activity [20]. In this study, green synthesis of nanoparticles was done by two methods: 1- using clove plant extract and 2- synthesis with the help of *L. casei* bacteria. The aim was to compare the Control function of nanoparticles synthesized from these two methods on *B. cinerea* fungus. According to the investigation carried out in this study, the size and morphology of the copper and silver nanoparticles obtained from the aqueous extract of cloves and *lactobacillus casei* bacteria are almost similar. But in the case of zinc oxide nanoparticles, we saw a difference in the size and morphology of the nanoparticles obtained from these two methods. Zinc oxide nanoparticles synthesized by bacteria were smaller in size compared to nanoparticles obtained from clove extract. (35.08 *vs.* 74.74).

MIC and MBC study

After 24 h of incubation in aerobic conditions at 37 ^{0}C, no turbidity was seen in the concentration of 4% and 6% copper nanoparticles synthesized from aqueous extract of clove and *Lactobacillus casei* bacteria, respectively. Also, no turbidity was observed in the case of zinc oxide nanoparticles obtained from clove extract at a concentration of 30% and nanoparticles synthesized by bacteria at a concentration of 60%. Silver nanoparticles synthesized from both methods in this article did not show inhibition of bacterial growth. Suspension from tubes without turbidity was inoculated in nutrient agar plates and incubated for 24 hours. Bacterial growth was observed at all concentrations, Therefore, it was determined that nanoparticles do not have any bactericidal properties for the beneficial bacteria *Lactobacillus casei.* Studies demonstrated that nanoparticles have antibacterial properties against many bacteria [21-23]. This variation might be due to the methodology used to prepare nanoparticles and the size of the nanoparticles used. Particles with very small sizes can be effective in lower concentrations. In this study, the size of nanoparticles synthesized by the green methods was relatively large compared to the samples of nanoparticles synthesized by industrial methods, and they had an inhibitory effect on the growth of bacteria, but they did not have any lethal effect on bacteria.

MTT assay

The results of long-term toxicity (10 days) showed that copper and zinc oxide nanoparticles synthesized with an aqueous extract of cloves and *L. casei* bacteria had almost no significant toxic effects on HepG2 cells compared to the control, However, silver nanoparticles at a concentration of 10% decreased cell viability by 25%(Figure 2a, 2b). The toxicity effect of nanoparticles is strictly dose-dependent, and HepG2 is the most sensitive cell line, providing a convenient and sensitive tool for the rapid screening of nanomaterial samples with potentially genotoxic and cytotoxic effects. The liver is of particular importance to toxicological research; therefore, using in vitro hepatic systems for nanoparticle toxicity studies is of great interest [24].

The effect of nanoparticles in inhibiting the growth of *B. cinerea* in PDA culture medium

The inhibitory effect of zinc, copper, and silver oxide nanoparticles synthesized from aqueous extract of cloves and *L. casei* bacteria, as well as the effect of iprodione-carbendazim fungicide in PDA culture medium on *B. cinerea,* are shown in Table 1. As can be seen, the concentration of 10% nanoparticles showed the best inhibition of fungal growth compared to other concentrations.

At this concentration, the surface of the culture medium in the control Petri dish was completely occupied by the fungus after 72 hours. The results showed that the concentration of 100 ml/l of zinc oxide nanoparticles synthesized from the aqueous extract of cloves can completely prevent the growth of *B. cinerea* fungus during the study period (96 hours), which was similar to iprodione-carbendazim poison. Copper and silver nanoparticles obtained from this extract prevented the

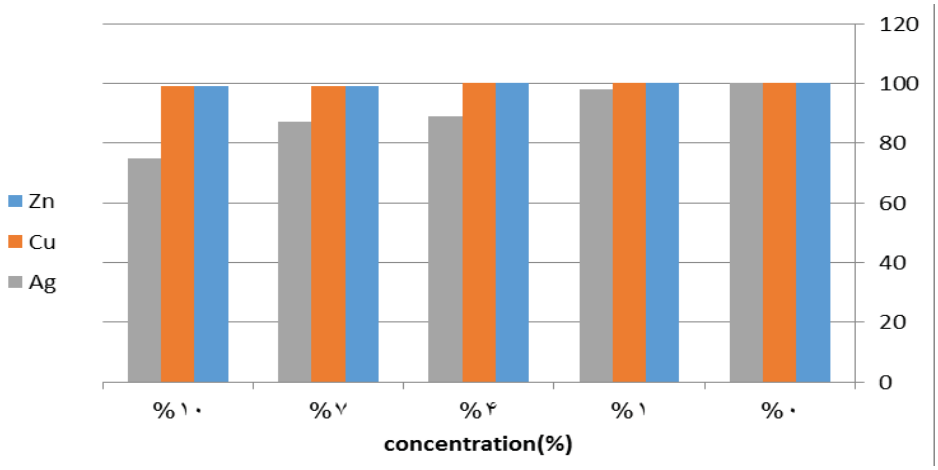


Figure 2a: Long-term exposure cytotoxicity, as determined by the MTT assay after 10 days. The data are means ± SD calculated for at least three replicates of each experimental point. All the results are compared to the negative controls (100% viability).

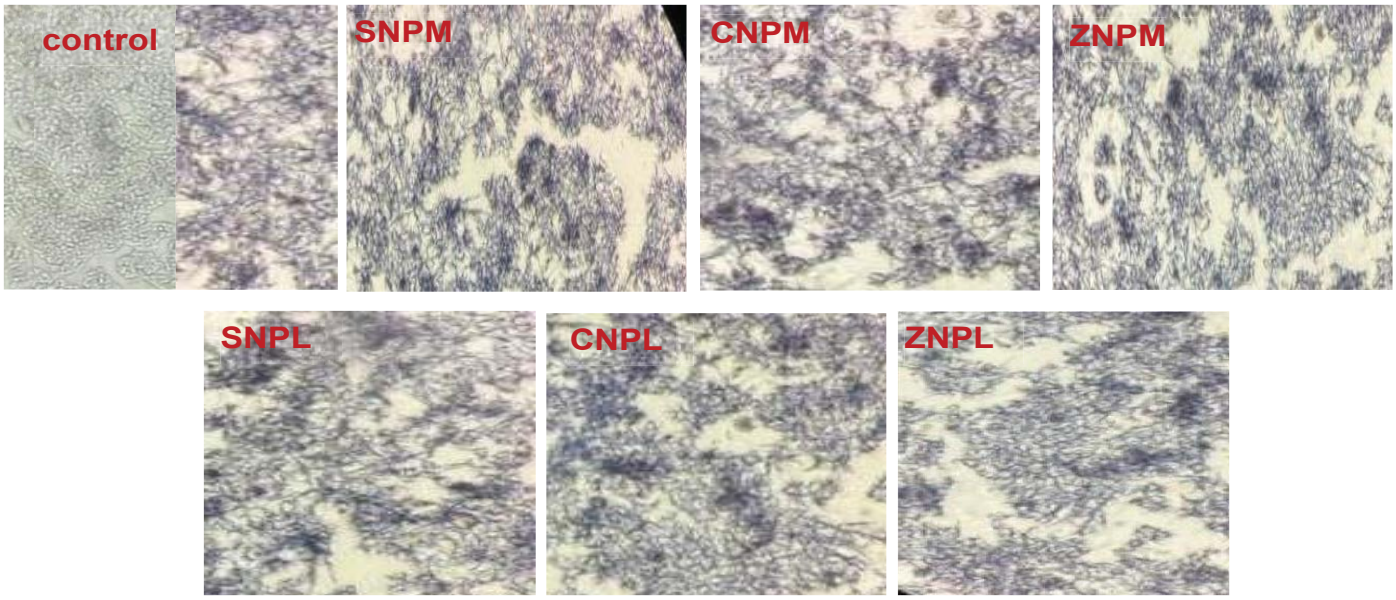


Figure 2b: Long-term exposure cytotoxicity, as determined by the MTT assay after 10 days.

growth of fungus in the first 24 hours, but their inhibitory effect decreased in the following hours so that after 24 hours, the fungal growth in the Petri dishes of these two treatments was similar to the control (Figure 3, Table 1).

In the medium containing 100 ml/liter of zinc oxide and silver nanoparticles obtained from *Lactobacillus casei* bacteria, 93.7 and 81% inhibition of fungal growth was observed, respectively, while copper nanoparticles obtained from bacteria led to 40% inhibition of fungal growth (Table 1, Figure 3). A significant decrease in the radial growth and colony formation of gray mold in the PDA culture medium containing nanoparticles, especially zinc oxide nanoparticles, can be attributed to the inhibitory effects of carbon nanomaterials such as MWCNTs, SWCNTs, GO, and rGO, which can suppress spore germination by blocking the water channels of spores. Our findings are supported by Gonelimali and colleagues [25]. They reported that the extract extracted from cloves has a significant inhibitory effect against harmful microorganisms and can be used as a food preservative. Research shows that both clove oil and eugenol have significant inhibitory effects on several types of food source microorganisms. The inhibitory mechanism of nanoparticles is related to reducing migration and adhesion and inhibiting biofilm synthesis of various pathogenic agents, binding to the membrane of microorganisms, electrostatic interactions, and disrupting the cell wall and intracellular processes such as DNA, RNA, and protein synthesis [26,27]. Also, oxidative stress has been

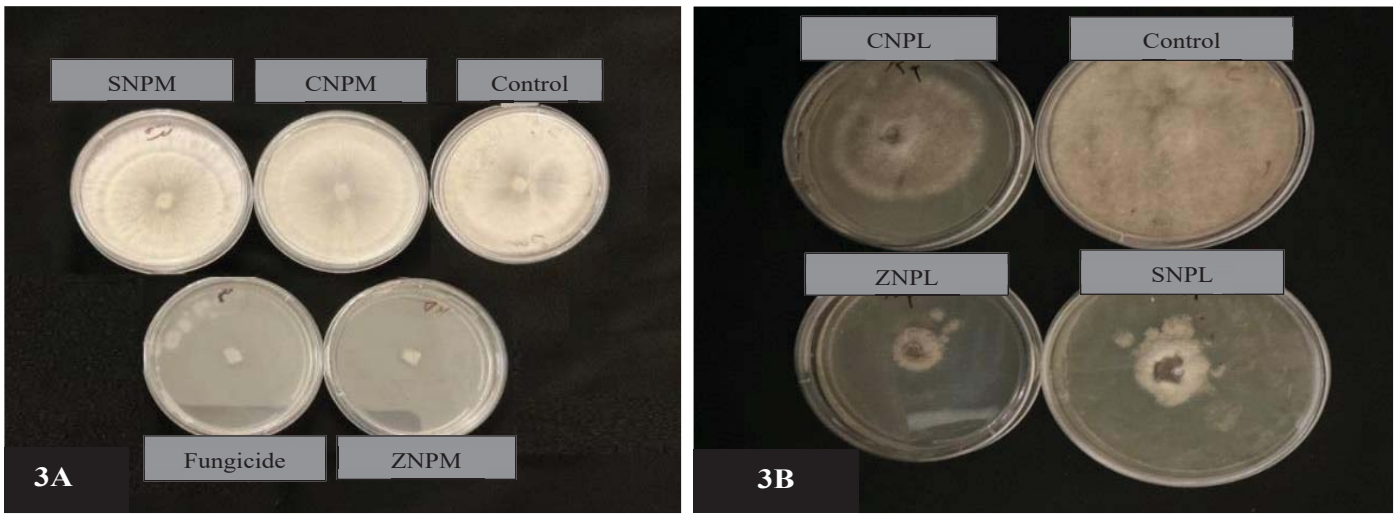


Figure 3: Investigating the antifungal effect of 10% solution of copper, zinc oxide, and silver nanoparticles and iprodione-carbendazim fungicide on *B. cinerea* fungus in PDA culture medium after 96 hours, A2: the antifungal effect of 10% solution of copper, zinc oxide, and silver nanoparticles produced from clove aqueous extract and iprodionecarbendazim fungicide. B2: Antifungal effect of 10% solution of copper, zinc oxide, and silver nanoparticles produced by *L. casei bacteria*.

Table 1: Percentage inhibition of *B. cinerea* fungus growth in PDA culture medium using different concentrations of nanoparticles of copper, zinc oxide, and silver synthesized from clove aqueous extract, *L. Casei* bacteria, and iprodione-carbendazim fungicide after 96 hours.

Treatment \ Concentration (%)	2	4	6	8	10
CNPM	0	0	0	0	0
SNPM	0	0	0	0	0
ZNPL	37	45	68	91	93.7
CNPL	0	0	10	18	25
SNPL	15	36	64	78	81
fungicide	100	100	100	100	100

proposed as a common cause of cell death caused by many types of nanoparticles. It has been shown that nanoparticles induce lipid peroxidation and oxidative stress that lead to DNA damage and apoptosis. It is generally believed that nanoparticles cause oxidative stress due to their small size [28]. In this study, one of the causes of cytotoxicity caused by exposure to nanoparticles can be considered oxidative stress. The synthesis of nanoparticles by the green synthesis method leads to the production of reactive oxygen species more effectively than other methods. For example, the dissolution of ZnONPs leads to the production of Zn^{2+}, which reacts with hydrogen ions to produce H_2O_2 molecules, which can penetrate the cell membrane and acts as a killer for microorganisms [20]. Several studies have shown that CuNPs can be used as an antifungal agent. They can inhibit several animal and plant pathogenic fungi. They have been used as an antifungal agent against fungal strains such as *Fusarium oxysporum* Schltdl., *Alternaria solani* (Ellis & G. Martin) L.R. Jones, *Aspergillus niger* Tiegh., *Penicillium citrinum*, *Curvularia lunata* (Wakker) Boedijn, *Phoma destructive* Plur [29]. In a study by Suba, et al. [30], the antimicrobial properties of zinc oxide nanoparticles were investigated using the agar well diffusion method. The results showed that zinc oxide nanoparticles have strong antimicrobial properties against Gram-positive (*Clostridium difficile*, *Clostridium perfringens*) and Gram-negative (*E. coli*, *Salmonella typhi*) bacteria and fungi (*Candida albicans*, *Aspergillus flavus*). The findings, therefore, show that the biosynthesized zinc oxide nanoparticles have excellent antimicrobial activity compared to the available synthetic commercial antibiotics [30]. The antifungal activity of silver nanoparticles has also been investigated in several studies and confirmed that these nanoparticles have antifungal activity against fungi such as *F. oxysporum* [31] *R. solani* [32], *Bipolaris sorokiniana* and the species of sclerotium and Colletotrichum [33].

The effect of different concentrations of nanoparticles on *B. cinerea* fungus on fruit

The results showed that the best concentration to inhibit the growth of *B. cinerea* fungus on strawberry fruit is 10% (Table 2). At the end of 96 hours, all treatments effectively reduced the growth of the fungus compared to the control (Figures 4 and 5). Among the applied treatments, zinc oxide nanoparticles synthesized with clove aqueous extract

Table 2: The percentage of decay of fruits treated with different concentrations of nanoparticles obtained from aqueous extract of cloves and *L. casei* bacteria in 10 days.

Treatment \ Concentration (%)	2	4	6	8	10
ZNPM	5	3	2	1	0
CNPM	6	5	3	3	2
SNPM	6	5	4	4	3
ZNPL	5	5	3	2	1
CNPL	6	6	6	5	5
SNPL	5	5	3	2	1

(0 = healthy fruit, 1 = less than 10% rotten fruit, 2 = 20-11%, 3 = 21- 30%, 4 = 31-40%, 5 = 41-50 %, 6 = 51-65%, 7 = 65-80%, 8 = more than 80%).

completely prevented the growth of gray mold even after 10 days, which was similar to the fungicidal effect of iperdione-carbendazim. At the end of the experiment, the rate of inhibiting the growth of copper and silver nanoparticles synthesized with clove aqueous extract was calculated as 80% and 75%, respectively (Table 2 and Figure 4). The rate of inhibition of fungal growth by zinc oxide, silver, and copper nanoparticles synthesized by bacteria was 97, 97 and 58%, respectively (Figure 4 and Table 2). Studies have stated that nanoparticles may cause the cytoplasmic content to leak by changing the structure of the pathogen's cell membrane and eventually lead to the death of the pathogen's cells. Several studies reported that the binding of zinc oxide nanoparticles to cells may stimulate lipids and membrane proteins that alter cell membrane permeability [34]. On the other hand, nanoparticles may affect the function of the cell and increase the contents of nucleic acid and carbohydrates in the cell, this increase may be due to the self-protection mechanism against nanoparticles [35].

The deformed structures of the hyphal cells may also be due to excessive accumulation of nucleic acid and carbohydrates. The antifungal mechanism of nanoparticles can also lead to the accumulation of ROS, lipid peroxidation, ergosterol content, and changes in the integrity of the pathogen's membrane. The increase in ROS level eventually disrupts proteins, lipids, and nucleic acids and can cause the death of fungi by apoptosis caused by oxidative stress [36]. In support of the present study, several studies have shown that nanoparticles, physical agents [37], synthetic fungicides [38], and fungicides Biological agents [39] can induce the death of fungi through apoptosis caused by oxidative stress and ROS increase. Mitra, et al. [40] reported that silver nanoparticles inhibit fungal growth and aflatoxin production in *Aspergillus parasiticus* and showed that silver nanoparticles inhibit aflatoxin production by reducing the expression of aflatoxin biosynthesis genes in an amount lower than the lethal dose [40].

Investigating the characteristics of strawberry fruit storage

The results of the variance analysis of the effect of the applied treatments on the physicochemical properties of

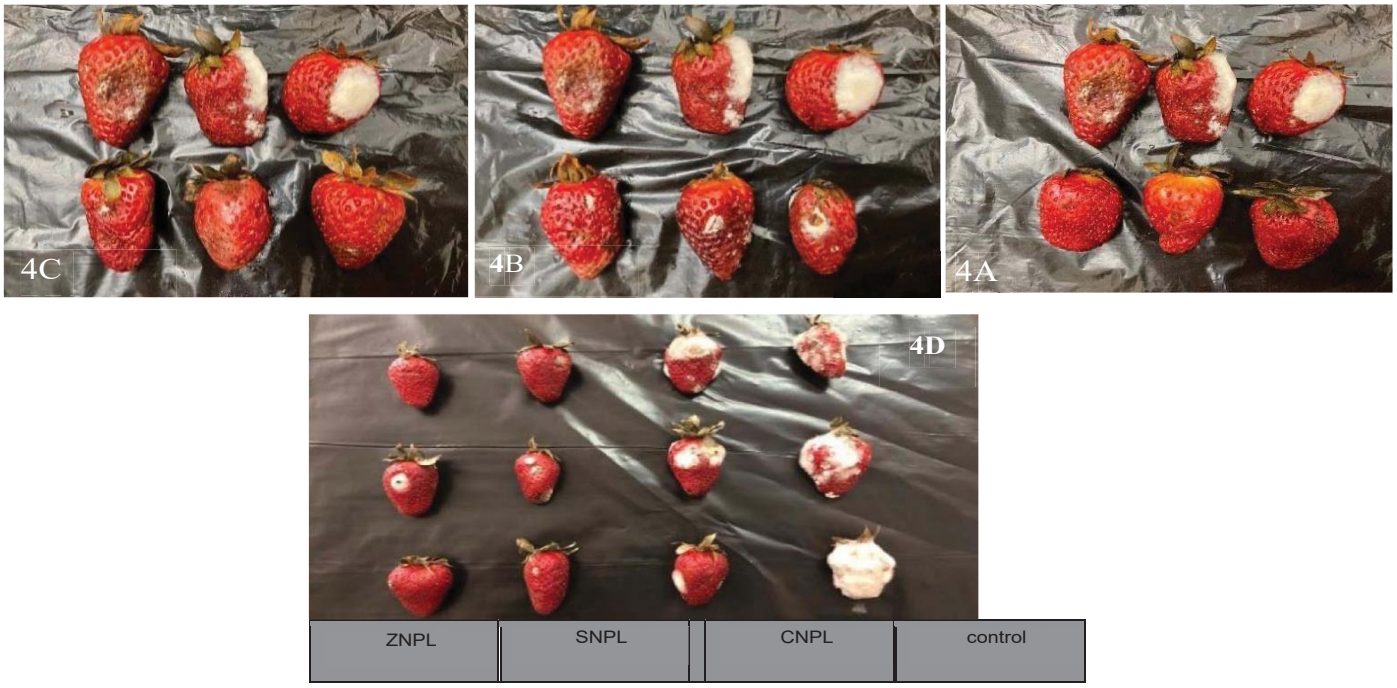


Figure 4: Investigating the antifungal effect of a 10% solution of copper, zinc oxide, and silver nanoparticles produced compared to the control treatment on *B. cinerea* fungus after 96 hours, 3C, 3B, 3A, respectively, the effect of zinc oxide, copper, and silver nanoparticles synthesized Using clove aqueous extract in inhibiting the growth of gray mold compared to the control, 3D effect of 10% concentration of zinc, copper and silver oxide nanoparticles synthesized using l.casei in inhibiting the growth of gray mold compared to the control.

strawberry fruit after 10 days of storage at 4 ºC are shown in Table 3. As can be seen, the applied treatments had a significant effect on all the examined indicators except total soluble protein.

pH changes

As can be seen in Table 4, the amount of total acidity and the degree of acidity of the fruit in the treatments have a significant difference at the level of 1% compared to the control. The data showed that in the control and all the treatments, except for the fruits treated with zinc oxide nanoparticles, there was an increase in acidity during 10 days, and the highest pH was observed on the 10th day (Figure 4A). There was no significant difference between fungicide treatment with zinc oxide nanoparticles obtained from aqueous extract of cloves and *Lactobacillus casei* bacteria and copper and silver nanoparticles synthesized by bacteria. In the treatment of zinc oxide, a 111% decrease in acidity was observed compared to the control. The sharp increase in pH in the control samples can be attributed to the reduction of organic acids and the increase of sugars. The fruits of the control treatment have higher cellular respiration than the fruits of the other treatments, which leads to the decomposition of organic acids that are used as substrates for the enzymatic activities of respiration in fruits treated with nanoparticles, due to low oxygen permeability and lower respiration rate, oxidation of organic acids and increase in acidity is prevented [41]. Willis (1998) believes that the pH changes of the fruit extract during ripening are mostly due to the leakage of organic acids from the vacuoles to the cell cytoplasm, which is confirmed by the comparison of pH changes and organic acids. It should be mentioned that due to the over-ripening of the fruit, the pH of the extract increases and turns from acid to alkaline [42]. This was clearly seen in the fruits of the control treatment and the severity of the changes was reduced by applying the coating. Therefore, fruit treatment with nanoparticles has preserved the quality of strawberry fruit by preventing pH changes.

Changes in the amount of organic acid

According to the results obtained from the data analysis, the effect of using nanoparticles during the storage period on the amount of total organic acids during storage at 4 ^{0}C is significant at the probability level of 1%. (Table 4). By looking at Figure 5B, we can see that with the passage of time, the amount of organic acids has decreased in all samples, But the amount of this reduction in the applied treatments was lower than the control. So that at the end of 10 days of storage, the amount of total organic acids in strawberries treated with poison, zinc oxide, copper, and silver nanoparticles obtained from aqueous extract of cloves and strawberries treated with zinc oxide, copper, and silver nanoparticles synthesized by *L. casei* Bacteria were 1.2, 1.45, 1.08, 1.18, 1.45, 1.21, 1.23 times the control, respectively. Therefore, it can be concluded that the treatments have played an important role in maintaining the amount of organic acids such as citric acid and malic acid, which shows the decrease in the speed of fruit metabolic processes and the decrease in the rate of conversion of fruit organic acids into sugars [43]. These results are consistent with the

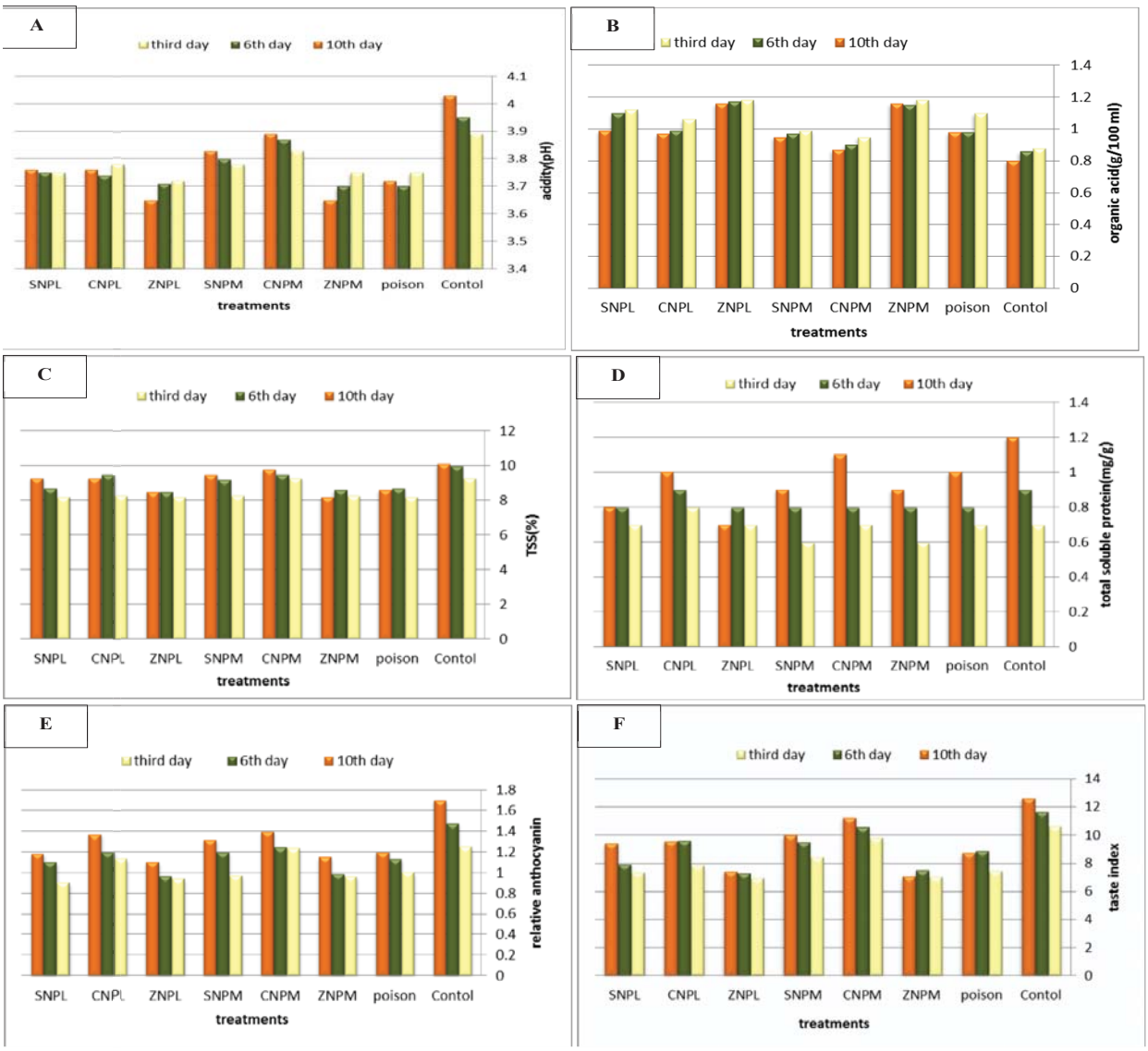


Figure 5: Changes in physicochemical characteristics of "Sabrina" strawberry fruit treated with iperdione-carbendazim, zinc oxide, copper, and silver nanoparticles synthesized from aqueous extract of cloves and *L. casei bacteria* during 10 days of storage, (A) acidity (pH), (B) organic acid, (C) Total Soluble Solids (TSS), (D) total soluble protein content, (E) relative anthocyanin, (f) taste index.

Table 3: Variance analysis of physicochemical characteristics of strawberry fruit after 10 days of storage period.

Average of squares							
Indicator / Treatment	Degrees of freedom	Taste index	relative anthocyanins	total soluble protein (mg/g)	Total Soluble Solids (TSS)	organic acid (g/100 ml)	acidity (pH)
Treatment	7	0.075*	7.22**	0.016^{ns}	0.84**	0.039**	0.024**
Error	16	0.03	0.33	0.03	0.12	0.02	0.018
Total	23	0.105	7.55	0.046	0.96	0.059	0.042

**Significance at the 0.01 level, *significance at the 0.05 level, ns is not significant

Table 4: Comparison of the average effect of treatments on the physicochemical characteristics of strawberry fruit after 10 days of storage conditions

Indicator / Treatment	TSS/TS	Anthocyanins	TP	TSS	TS	pH
Control	11.6^{a}	1.47^{a}	0.93^{a}	9.8^{a}	0.84^{a}	3.95^{a}
poison	8.36^{b}	1.11^{b}	0.83^{b}	8.5^{b}	1.02^{b}	3.72^{b}
ZNPM	7.18^{c}	1.03^{c}	0.76^{c}	8.36^{c}	1.16^{c}	3.7^{b}
CNPM	10.56^{d}	1.29^{d}	0.86^{b}	9.5^{d}	0.9^{d}	3.86^{c}
SNPM	9.28^{e}	1.16^{b}	0.76^{c}	9^{e}	0.97^{b}	3.83^{c}
ZNPL	7.2^{c}	1^{c}	0.73^{c}	8.43^{c}	1.17^{c}	3.69^{b}
CNPL	9^{f}	1.23^{d}	0.9^{a}	9.03^{e}	1^{b}	3.76^{b}
SNPL	8.2^{g}	1.06^{bc}	0.76^{c}	8.73^{f}	1.07^{b}	3.75^{b}

In each column, the numbers that have at least one letter in common do not have a significant difference at the 5% level.

results of Hmmam, et al. [44]. They stated that the coating of mango fruit with CMC and silver nanoparticles based on guar gum prevents the reduction of organic acids during mango storage. Also, organic acids may be used to improve the carbon skeleton and synthesis of phenols such as anthocyanin and non-anthocyanin phenols [44]. The respiration of strawberry fruit during the storage period reduces organic acids. This reduction may be due to the increase in malic dehydrogenase enzyme activity and pyruvate decarboxylation reactions during the respiration phase. Acids can be considered a source of fruit energy [45]. In a study by Ali, et al. [46], the effect of silver nanoparticle coatings based on carboxymethyl cellulose and guar gum on the stability of the storage characteristics of Mani Lokot (*Eriobotrya japonica* Lindl.) during one month of storage at two different temperatures (4 and 8 °C) were investigated. The results showed that the coated fruits showed a lower decrease in weight, total soluble solids (TSS), reducing sugars, and nonreducing sugars compared to the control at both temperatures. In addition, coated samples had higher concentrations of ascorbic acid, total phenolic contents (TPCs), antioxidants, and organic acids. The general results showed that the coating based on silver nanoparticles can be used commercially to increase the physicochemical and nutritional properties of loquat during storage [46].

Changes in total soluble solids (TSS)

According to the data of Figure 5C, it can be seen that in the control and the used treatments, the amount of total soluble solids increased until the sixth day of storage, but in the poison treatments, zinc nanoparticles, copper, and silver nanoparticles obtained from *Lactobacillus casei* after that, a decreasing trend was observed in soluble solids. So the highest and lowest amount of TSS is related to the third and tenth days. The increase in the concentration of total soluble solids in strawberries during the storage period can be related to the decrease in fruit weight. Of course, fruit spoilage and respiration also cause an increase in soluble solids by breaking down polysaccharides and turning them into simpler compounds. Any factor that prevents or reduces the breakdown of cell walls, will prevent the abnormal changes of dissolved solids. In addition, the reduction of organic acids and their conversion into simple sugars to provide the energy needed for respiration is one of the reasons for the increase in the total amount of soluble solids in strawberry samples. Therefore, covering the strawberry fruit reduces the rate of aging due to the reduction of respiration rate and prevents the excessive increase of soluble solids. These results are consistent with the results of Mahajan and Sharma (2000) [47].

Total soluble protein content

With the increase in storage time, an increase in the content of total soluble proteins was observed in all samples, while this increase was not significant between the treatments and the control at the 5% probability level. According to Figure 5D, this increase in silver and zinc oxide nanoparticle treatments was lower than in other treatments. The highest increase in total protein during the storage period was observed on the 10th day, which was the highest in the control with 1.2 mg/g and the lowest in the zinc nanoparticles synthesized by *L. casei* bacteria with 0.7 mg/g. There is evidence of an increase in the abundance of some proteins that are involved in the metabolism of sugars, ethylene production, oxidative stress, defense responses, etc., so maybe part of the increase in protein content can be attributed to the metabolic processes of strawberry fruit during storage. According to the obtained results, it can be stated that the coating of fruits with nanoparticle compounds, especially zinc and silver nanoparticles, by reducing respiration and other metabolic activities, prevents the excessive increase of total soluble proteins compared to the control.

Relative anthocyanin changes

The effect of nanoparticle treatment on the amount of anthocyanin during storage is shown in Table 4. According to the results obtained from the data analysis, the effect of using treatments during the storage period on the amount of anthocyanin is significant at the probability level of 1%. As can be seen in Figure 5E, the relative anthocyanin index value increased during the storage period, and this increase was more severe in the control and less in other treatments, especially zinc oxide nanoparticles. Based on these results, it can be concluded that the use of nanoparticle treatment has a positive effect on preventing the accumulation of relative anthocyanin during fruit ripening [48]. In strawberry fruit, anthocyanins are the main flavonoids of the fruit that accumulate during ripening and play an important role in distinguishing the color of quality strawberry fruits. Therefore, it can be stated that there is some kind of relationship between fruit ripening and anthocyanin increase. Nanoparticles, especially zinc nanoparticles, indirectly affect the marketability of fruits by preventing the rapid changes of anthocyanins and over-ripening of fruits. On the other hand, the greater increase of anthocyanin in the control and copper nanoparticles treatment may be due to microbial contamination, because one of the biochemical changes that occur in plants when faced with stress is the accumulation of reactive oxygen species. Plants with an antioxidant system that includes enzymes (superoxide dismutase, catalase, etc.) and polyamine compounds, this system keeps the level of reactive oxygen species balance in the cell and protects the plant against environmental stress. Since polyamines can play a role in cell survival and homeostasis, plasma membrane permeability, preventing chlorophyll decomposition and stimulating the biosynthesis of special proteins and Compounds [49]. it can be concluded that nanoparticles by causing oxidative stress lead to an increase in antioxidant system activity and an increase in anthocyanin production and total phenol content in fruit.

Taste index

The ratio of total soluble solids to titratable acid is an important indicator that is usually used to determine the taste and flavor characteristics of fruits, it depends on the maturity of the fruit. Therefore, factors that increase the total soluble solids can increase it, and factors that increase the amount of titratable acid can decrease it. It was observed that this ratio was minimum in the treatment of zinc oxide nanoparticles and maximum in the control treatment (Figure 5F). These changes can be attributed to the preservation of organic acids. In the control treatment, large changes in the taste index indicate a large change in the taste of the fruit and in fact a departure from the initial desirable taste of the fruit at the beginning of the study. If the strawberry fruit is stored in inappropriate conditions, glycolytic enzymes and alcoholic fermentation enzymes such as Alcohol Dehydrogenase (ADH) and Pyruvate Decarboxylase (PDC) are activated to provide the necessary energy for cellular respiration [50]. Therefore, with these enzymatic changes, the taste of strawberries changes. According to the results obtained from this research, it can be stated that the treatment of nanoparticles by creating a suitable coating reduces respiration and inhibits the activity of the mentioned enzymes, therefore, it is expected to prevent severe changes in the taste of the fruit.

Sensory evaluation results (The appearance quality, brightness, color, and texture quality, and the appearance decay)

As can be seen in Table 5, All the treatments used, including different nanoparticles and the fungicide iperdione-carbendazim, were significantly different from the control samples in terms of appearance quality, brightness, and color and in terms of the mentioned sensory characteristics, they scored higher than the control samples. According to the results obtained, control samples and samples treated with poison, copper nanoparticles obtained from aqueous extract of cloves and *L. casei* bacteria, and silver nanoparticles synthesized with aqueous extract of cloves had a downward trend in the score of sensory factors with increasing storage time. While the samples were treated with nanoparticles synthesized from two methods of using an aqueous extract of cloves and *L.casei* bacteria, these parameters increased with increasing storage time. The treatments had a significant difference at the probability level of 5% in apparent decay with the control treatment (Table 6, Figure 6).

Fruits and vegetables with the passage of storage time due to respiration and an increase in pH, their pigments are decomposed, as a result of which their color and appearance quality decreases. In the treated samples, due to the reduction in respiration rate, evaporation, and transpiration, the breakdown of pigments is less and the external quality of the samples is significantly maintained, Therefore, a significant difference was observed in the evaluation of sensory parameters between the treatments and the control sample. One of the consequences of preventing respiration in strawberry fruit is maintaining the amount of organic acids in the fruit and reducing the pH in strawberry fruit during storage which leads to the prevention of the destruction of strawberry fruit pigments and as a result, the level of color quality and brightness is maintained [51]. The decrease in the texture quality score of strawberry samples occurs as a result of changes in the structure of the cell wall, including the reduction of hemicellulose, galactose, and the dissolution and depolymerization of pectin during storage. Covering the fruits reduces the loss of water in the fruit and maintains the weight of the fruit, and this maintenance of the weight of the fruit helps to maintain the firmness of the tissue [52]. On the other hand, nanoparticles and Iperdione-Carbendazim fungicide inhibit and reduce the activities of fungal pathogens and lead to the reduction of the activity of cell wall degrading

Table 5: Analysis of variance of sensory evaluation of strawberry fruits during 10 days of storage period.

Average of squares						
Indicator / Treatment	Degrees of freedom	Appearance	Brightness level	Color quality	texture quality	Apparent decay
Treatment	7	1.42**	1.43**	1.39**	0.94*	0.94*
error	16	0.16	0.17	0.16	0.14	0.14
Total	23	1.75	1.77	1.76	1.32	0.84

**significant at the 0.01 level, *significant at the 0.05 level, ns not significant

Table 6: Comparison of the average effect of treatments on the physicochemical characteristics of strawberry fruit after 10 days of storage conditions.

Indicator / Treatment	Appearance	Brightness level	Color quality	texture quality	Apparent decay
Control	2.6^{a}	2.56^{a}	2.7^{a}	3^{a}	4^{a}
poison	3.93^{b}	3.6^{b}	3.93^{b}	4.1^{b}	5^{b}
ZNPM	4.7^{c}	4.6^{c}	4.73^{c}	4.63^{c}	5^{b}
CNPM	3.56^{d}	3.76^{d}	4.23^{d}	4.13^{b}	4.66^{c}
SNPM	4.13^{e}	4.2^{e}	4.56^{e}	4.43^{d}	5^{b}
ZNPL	4.86^{f}	4.8^{f}	4.76^{c}	4.73^{e}	5^{b}
CNPL	3.9^{b}	4^{g}	4.36^{f}	4.06^{b}	5^{b}
SNPL	4.3^{g}	4.23^{e}	4.63^{g}	4.66^{c}	5^{b}

In each column, the numbers that have at least one letter in common do not have a significant difference at the 5% level.

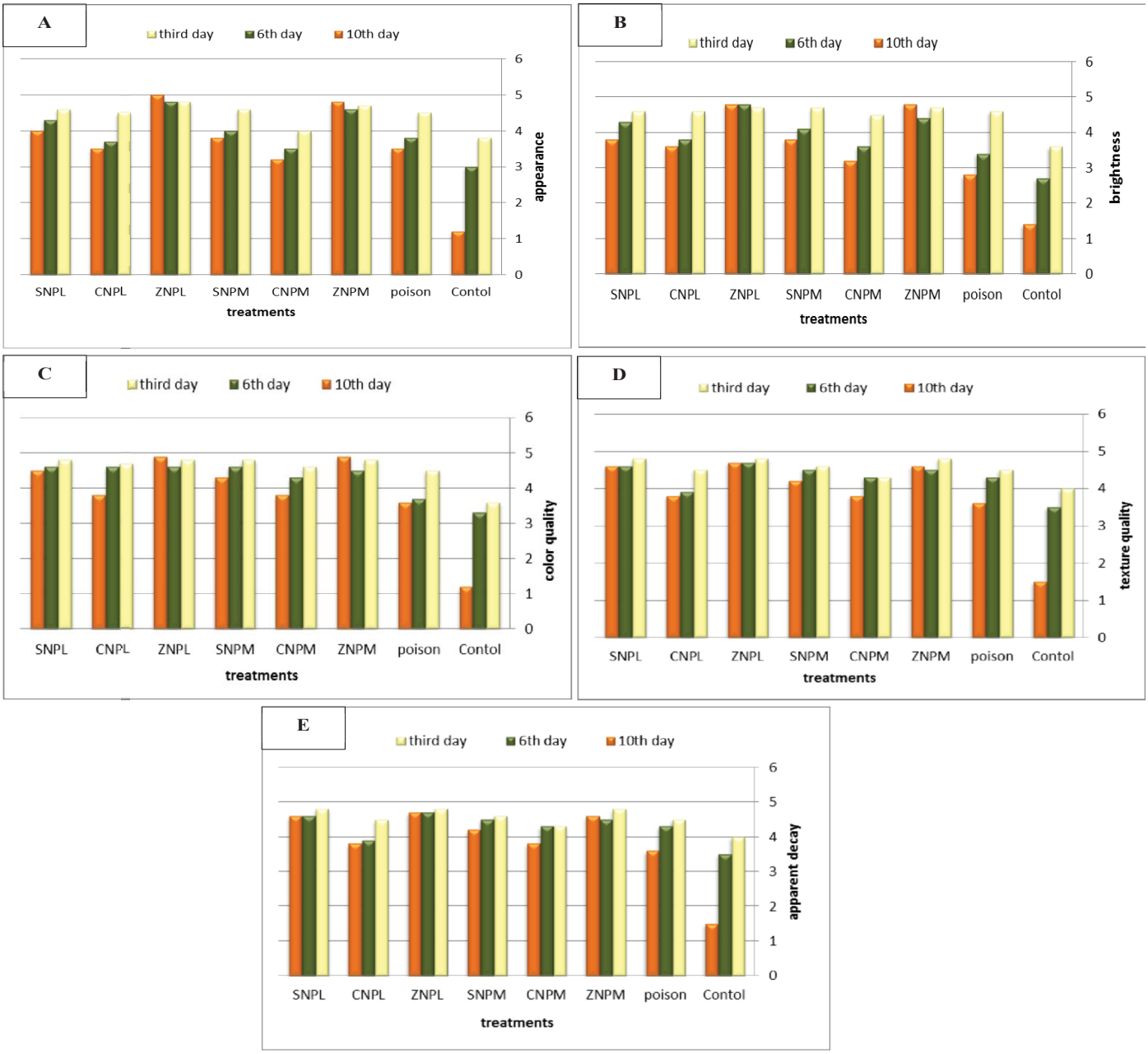


Figure 6: Changes in the Sensory evaluation of "Sabrina" strawberry fruit treated with iperdione-carbendazim poison, zinc oxide, copper, and silver nanoparticles synthesized from aqueous extract of cloves and L.casei bacteria during storage in 10 days, very good = 5, good = 4, neither good nor bad = 3, bad = 2 and very bad = 1, (A) appearance scores, (B) brightness, (C) color quality, (D) texture quality, (E) apparent decay.

enzymes such as polygalacturonase, pectin methylesterase and xylanase which are produced by these microorganisms. Therefore, in the evaluation of tissue quality parameters, the samples treated with nanoparticles and fungicides scored higher than the control. With the increase in storage time, the gradual destruction of the cell wall leads to the reduction of the defense barriers against the pathogen, and subsequently the microbial contamination in the fruits increases, which 10% - 6% contamination of the control treatment confirms this well. López-Vargas, et al. [53] stated that spraying copper nanoparticles on tomatoes increases the content of biologically active compounds and preserves the quality of the fruit [53]. It has been stated that edible coatings reinforced with nanoparticles can be effective in improving the quality of color, and strength, increasing antimicrobial properties, controlling enzyme activity, and reducing the weight of fruits. The incorporation of silver nanoparticles in sodium alginate prevents the growth of microbial diseases in pears. Because sodium alginate coatings combined with silver nanoparticles maintain their antibacterial activity against gram-positive and gram-negative bacteria. Joshy, et al. (2020) applied xanthan gum reinforced with zinc oxide nanoparticles to apple and tomato fruits. This coating protected the fruits from rotting and water loss. ZnO-starch coatings reduced the growth of anthracnose fungus, delayed texture changes, and preserved mango shelf life at low storage temperatures [54].

Conclusion

According to numerous reports, the use of nanotechnology to increase the shelf life of fruits is one of the most effective techniques, because these compounds are renewable, safe for humans, and environmentally friendly. In this research, it was shown that the treatment of fruits with nanoparticles of copper, zinc oxide, and silver has a very important role in maintaining the quality characteristics of Sabrina strawberry fruits compared to the control. They can effectively slow down the softening and other changes that lead to the loss of product quality after harvesting. The obtained results show that the treatment of zinc nanoparticles is very effective in maintaining the amount of soluble solids, organic acids, anthocyanin, and taste index and preventing the increase of acidity and total protein, and these factors play an important role in maintaining the quality of fruits after harvesting. The increase in antimicrobial activity can be attributed to the increase in the contact surface of nanoparticles. On the other hand, investigating the long-term toxicity of zinc and copper oxide nanoparticles at a concentration of 10% did not have any significant lethal effect on liver cells. And Zinc oxide nanoparticles also caused only 25% of cell death in these conditions. In general, according to the results obtained from this research, it can be stated that the treatment of nanoparticles, especially zinc oxide nanoparticles, has a positive effect on maintaining the physicochemical and sensory characteristics of strawberry fruit and they even improve the nutritional value of fruits; Because according to the analysis of MIC and MBC, it was found that these nanoparticles have no lethal effect on the probiotic bacteria *Lactobacillus casei.* Therefore, the solution of nanoparticles synthesized from lactobacillus bacteria contains the bacteria itself, which can help to strengthen the microbial flora of the body by entering the intestine. As a result, by conducting further research in the field of nanoparticles including chronic toxicity studies, biologically synthesized nanoparticles can be used as an effective method to control post-harvest diseases and maintain storage characteristics of strawberry fruit.

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