

Effects of combination of magnesium and zinc oxide nanoparticles and heat on *Escherichia coli* and *Staphylococcus aureus* bacteria in milk

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ABSTRACT

Objective: The objective of this study was to investigate the antibacterial activities of combination of MgO and ZnO nanoparticles in the presence of heat against *Escherichia coli* and *Staphylococcus aureus*.

Materials and Methods: Bacteria were grown on either agar or broth media followed by the addition of ZnO and MgO nanoparticles. Then the combined effect of ZnO and MgO nanoparticles was investigated. Furthermore, the media containing nanoparticles were treated with mild heat and their synergistic antibacterial activity was investigated against *E. coli* and *S. aureus* in milk.

Results: The data showed that the nanoparticles used in this study had no effect on the bacteria in the agar medium. However, the results showed that ZnO and MgO nanoparticles resulted in a significant decrease in the number of *E. coli* ($P < 0.000$) and *S. aureus* ($P = 0.05$) in the broth medium. The combination of nanoparticles and mild heat exhibited a significant decrease in the number of *E. coli* and *S. aureus* indicating the synergistic effects of nanoparticles and heat.

Conclusion: Using a combination of mild heat, ZnO and MgO nanoparticles, *E. coli* and *S. aureus* can be controlled successfully in the milk. Mild heating plus ZnO and MgO nanoparticles has a synergistic effect which would reduce the need for high temperature and also the concentrations of ZnO and MgO nanoparticles required for pathogen control in minimally processed milk during maintaining.

Keywords: *Escherichia coli*, Magnesium oxide, Nanoparticles, *Staphylococcus aureus*, Zinc oxide

INTRODUCTION

One of the significant problems nowadays is the increase of the prevalence of antibiotic resistance in various human and animal pathogens. In human and animal populations, antibiotics are used to treat and prevent infectious diseases. The main factor for the increase of the resistance of pathogenic bacteria is overuse of antibiotics and this has led to the emergence and spread of resistant pathogens and resistant genes in them [1].

Staphylococcus aureus and *Escherichia coli* are important pathogens in nosocomial infections. These

bacteria have been found on all surfaces of the hospital [2].

S. aureus is one of the most common and important pathogens in hospital infections that can cause infection anywhere in the body due to several enzymes, including coagulase, hyaluronidase, nuclease, lipase, leukocidin and hemolysin.

This bacterium also causes infections in the hospital and is the second cause of hospital infections after *Pseudomonas aeruginosa* and it has the ability to create serious infections such as septicemia, endocarditis and osteomyelitis in patients admitted to the hospital. It is also mentioned as the most common cause of mortality in hemodialysis patients [3-4].

E. coli is from the family enterobacteriaceae that has a role in infections such as sepsis, urinary tract infection,

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gastroenteritis, neonatal meningitis and intra-abdominal infections [5].

Antibiotic resistance is one of the problems for eliminating these bacteria. Access to materials with powerful antibacterial, antiviral and antifungal properties can be useful for disinfection and infection control in many cases. Due to the increasing spread of infectious diseases and food poisoning caused by different bacteria, including *E. coli* and *S. aureus*, as well as the resistance of microorganisms to different common antibiotics, researchers are considering the use of new antimicrobial agents against the bacteria. Researchers around the world are trying to find new pharmaceutical composition for ideal treatment of infections. Biological and physicochemical properties of inorganic nanomaterials have attracted the attention of many researchers.

Historically nanomaterials have been discussed on two categories which are metallic and nonmetallic nanoparticles [6].

Nanomaterials whose base is formed of metal ions have extensive cytotoxic activity against bacteria, fungi and viruses [7]. Sometimes, to increase the inhibition effects, combination of nanoparticles can be used.

The combined use of these materials can be considered as a good solution that, in addition to increasing antimicrobial properties, can overcome the limitations of each individual case the nanoparticles. The combination of nanoparticles with each other and their use against microorganisms has led to the development of different research areas.

One of the advantages of using a combination of nanoparticles is the extent of their performance, the use of lower concentrations of nanoparticles in combined state compared to single-nanoparticle state, reduced toxicity, prevention of emergence of resistance to each of the nanoparticles [8-3].

One of the nanoparticles, Zinc oxide (ZnO) is used on an industrial scale in many countries [9-10]. Nanoparticles of Magnesium oxide (MgO) is used in industry and medicine.

Studies show that magnesium oxide nanoparticles, alone or in a combination with other antibacterial groups, act as a potential effective antimicrobial factor to enhance food safety [11].

The objective of this study was to investigate the antibacterial activities of combination of MgO and ZnO

nanoparticles and its synergistic effect in combination with heat against foodborne pathogens including *E. coli* and *S. aureus*.

MATERIALS AND METHODS

Materials

Standard strains of *S. aureus* (PTCC: 1431) and *E. coli* (PTCC: 1394) used in this research was purchased in the form of lyophilized microorganisms from The Iranian Center for Collection of Industrial Microorganism. These strains of bacteria were cultured in tryptic soy agar medium (Merck, Germany) and were stored at 0-2 ° C for use in the subsequent steps. ZnO nanoparticles used in this study were purchased from TECONAN Company and had the purity of 99.98 percent. MgO nanoparticles with the purity of 99.98 percent were purchased from US Nano Company. Transmission electron microscope (TEM), visible-ultraviolet spectrometer and X-ray diffraction were used to evaluate and verify the chemical and physical properties and morphology of the nanoparticles purchased.

Preparation of MgO and ZnO nanoparticles mixture

Nanoparticles mixture was prepared using deionized water. In order to achieve a concentration of ZnO and MgO nanoparticles that is the effective against both bacteria, the effect of nanoparticles in the agar media was explored in two stages.

In the first stage, concentrations of 0.25, 0.50, and 0.75 mg/ml and combined nanoparticles concentrations of $0.50_{(ZnO)} + 0.50_{(MgO)}$, $0.25_{(ZnO)} + 0.75_{(MgO)}$, $0.25_{(MgO)} + 0.75_{(ZnO)}$ mg/ml were used.

In the second stage, with increasing the concentration, the effect of the nanoparticles with concentrations of 0.50, 1, 1.5 mg/ml and combined concentrations of $1_{(ZnO)} + 1_{(MgO)}$, $0.50_{(ZnO)} + 1.50_{(MgO)}$, $0.50_{(MgO)} + 1.5_{(ZnO)}$ mg/ml against the bacteria were measured [12].

Evaluation the effect of combined nanoparticles against *E. coli* and *S. aureus* in agar media.

Disk diffusion method was used to assess the effects of MgO and ZnO nanoparticles in agar medium. For this purpose, 20 µl of the suspension of nanoparticles at concentrations [$0.50_{(ZnO)}$, $1_{(ZnO)}$, $1.50_{(ZnO)}$, $0.50_{(MgO)}$, $1_{(MgO)}$, $1.50_{(MgO)}$, $1_{(ZnO)} + 1_{(MgO)}$, $0.50_{(ZnO)} + 1.50_{(MgO)}$, $0.50_{(MgO)} + 1.50_{(ZnO)}$] mg/ml were evaluated. Disks with a diameter of 1 mm were prepared using Whatman paper.

Using sterile forceps, disks were placed on plates containing 10^7 cfu/ml of each of bacterium. Then, 20 μ l of different concentrations of nanoparticles of ZnO and MgO were placed on plates.

Then the plates were incubated at 37 °C for 24 h and after that time, inhibition zone was measured using a caliper. Inhibition zone around the disk shows the antibacterial activity of nanoparticles.

The control group at this stage was consisted of raw disk and disks contained distilled water and tetracycline antibiotic discs [13].

Evaluation of the effect of combined nanoparticles in liquid medium

The tryptic soy broth (TSB) containing concentrations of 0.5, 1, 1.5 mg/ml and combined concentrations of 1_(ZnO) + 1_(MgO), 0.5_(ZnO) + 1.5_(MgO), 0.5_(MgO) + 1.5_(ZnO) mg/ml of nanoparticles were prepared. Samples were then inoculated with the 10^7 cfu/ml suspension of *S. aureus* (PTCC: 1431) and *E. coli* (PTCC: 1394). The samples were stored at room temperature on the shaker at around 100 rpm and examined every 2 h for 24 h. Manitol salt agar (MSA, Merck, Darmstadt, Germany) was used for the isolation and enumeration of *S. aureus*.

Eosin methylene blue (EMB, Merck, and Darmstadt, Germany) agar was employed for the isolation and identification of *E. coli* [9].

Combined effect of mild heat, MgO and ZnO nanoparticles in milk

Culture conditions

Each strain was cultured in a tryptic soy broth (TSB; Merck, Germany) at 37 °C for 24 h, harvested by centrifugation at 4000 g for 20 min at 4 °C and washed three times with buffered peptone water. The final pellet was resuspended in buffered peptone water, corresponding to approximately 10^7 - 10^8 cfu/ml and mixed cocktails were prepared by blending the equal volumes of each test strain.

Sample treatment

The milk samples containing 0.5_(ZnO), 1.5_(MgO), 1.5_(MgO) + 0.5_(ZnO) mg/ml nanoparticles were prepared. Samples were then inoculated with the prepared mixed culture cocktails (10^6 - 10^7 cfu/ml of each strain). Heat treatments were performed at 25 °C, 50 °C and 60 °C in a water bath, with the temperature monitored by inserting a thermometer into a bottle of milk. During the heat

treatment, the sample bottles were vigorously agitated to facilitate uniform distribution of the inoculums. Once the temperature of the sample reached the target treatment temperature of 50 and 60 °C the glass bottles were removed and immediately cooled in crushed ice. The samples were stored at room temperature and examined after 3, 8, 24 and 48 h, respectively, to allow for the recovery and then enumeration of injured cells [13]. *Bacterial enumeration* Aliquots (1ml) of the treated milk samples were dispersed in 9 ml of 0.2% (w/v) sterile peptone water and then serially diluted (10^{-1} - 10^{-5}) in 0.1% sterile peptone water. Manitol salt agar (MSA, Merck, Darmstadt, Germany) was used for the isolation and enumeration of *S. aureus*.

Eosin methylene blue (EMB, Merck, and Darmstadt, Germany) agar was employed for the isolation and identification of *E. coli*. Microscopic examination of the isolates was performed by staining smears according to the Gram method. Identification and characterization of bacterial isolates up to the species level were implemented using various biochemical and sugar fermentation tests [9-14].

Statistical analysis

Antimicrobial experiments were conducted in triplicate. Data points were expressed as the mean \pm standard deviation. Data were analyzed using analysis of variance from SPSS version 16 software. Duncan's multiple range tests were used to determine the significant difference of mean values. Unless stated otherwise, significance was expressed at 5% level.

RESULTS AND DISCUSSION

Physicochemical properties of MgO nanoparticles

Fig 1 shows physicochemical properties of MgO nanoparticles. Fig 1(a) shows X-ray diffraction pattern for magnesium oxide nanoparticles. As shown in Fig 1, diffraction peaks at 2 θ values have been absorbed. A sharp increase in the peak of XRD shows that particles have a crystalline nature. Fig 1(b) shows the characteristics of the ultraviolet visible spectrum of MgO nanoparticles. As can be seen in the Fig, absorption peak in the 200-700 nm area proves the existence of magnesium oxide nanoparticles. Fig 1(c) shows Transmission Electron Microscopy (TEM) image of the MgO nanoparticles.

According to the results of the TEM studies, the diameter of the MgO nanoparticles is in the range of 20 nm.

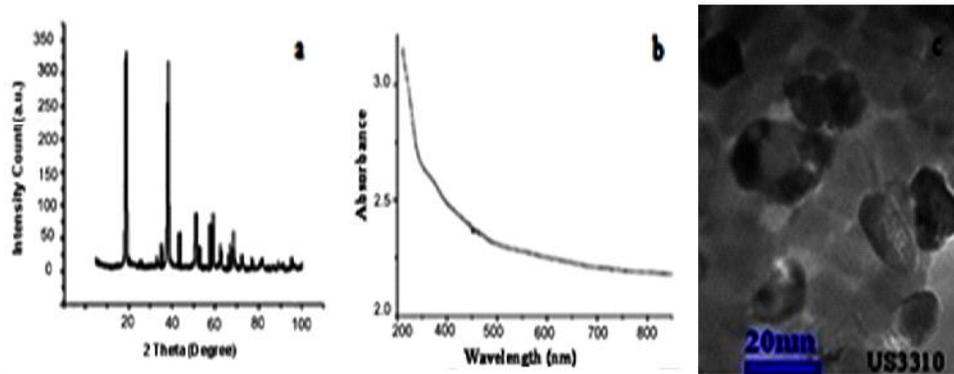


Fig. 1. The physicochemical properties of MgO nanoparticles. 1(a), X-ray diffraction pattern. 1(b) the visible spectrum - ultraviolet. 1(c) TEM image

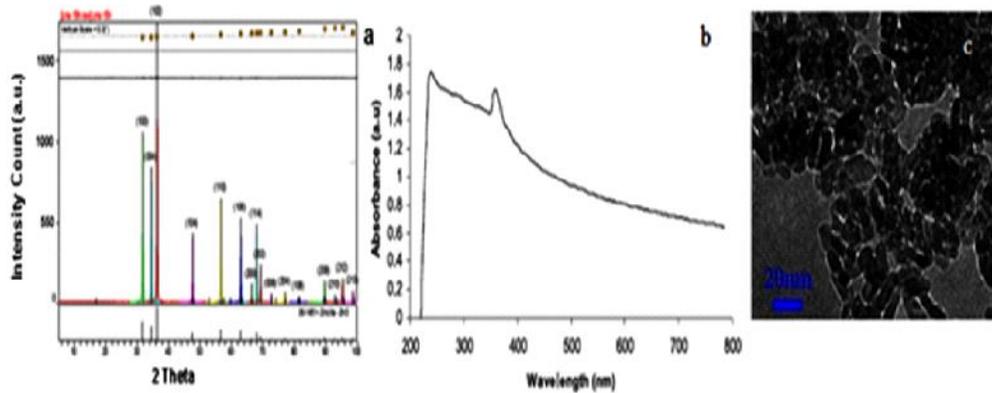


Fig. 2. The physicochemical properties of ZnO nanoparticles. 1(a), X-ray diffraction pattern, 1(b) the ultraviolet visible spectrum, 1(c) TEM image

Physicochemical properties of ZnO nanoparticles

Fig 2 shows physicochemical properties of zinc oxide nanoparticles. Fig 2(a) shows X-ray diffraction pattern of ZnO nanoparticles. Fig 2(b) shows the characteristics of the ultraviolet visible spectrum of ZnO nanoparticles. As can be seen in Fig 1, absorption peak in the 200-800 nm area proves the existence of ZnO nanoparticles. The peak lack of sharpness indicates the production of nanoparticles in different sizes and verifies the results of the ultraviolet visible spectrum and electron microscopy data. Fig 2(c) shows TEM image of the ZnO nanoparticles. According to the results of the TEM studies, the diameter of the ZnO nanoparticles is in the range of 20-25 nm.

Antimicrobial assays

Antibacterial properties of concentrations of $0.5_{(ZnO)}$, $1_{(ZnO)}$, $1.5_{(ZnO)}$, $0.5_{(MgO)}$, $1_{(MgO)}$, $1.5_{(MgO)}$, $1_{(ZnO)} + 1_{(MgO)}$, $0.5_{(ZnO)} + 1.5_{(MgO)}$, $0.5_{(MgO)} + 1.5_{(ZnO)}$ mg/ml of MgO and ZnO nanoparticles were evaluated using inhibition zone measurement, against *S. aureus* (PTCC: 1431) and *E. coli* (PTCC: 1394). Data showed that no inhibition zone was found in the presence of MgO and ZnO nanoparticles. In fact, these nanoparticles have no effect against *E. coli* and *S. aureus* in agar media culture. Fig 3 shows the effect of combination of nanoparticles of MgO and ZnO on the growth of *S. aureus* (PTCC: 1431) and *E. coli* (PTCC: 1394) in TSB medium at 37 °C within 24 h.

Treatment of the bacteria with concentrations of $0.5_{(ZnO)}$, $1_{(ZnO)}$, $1.5_{(ZnO)}$, $0.5_{(MgO)}$, $1_{(MgO)}$, $1.5_{(MgO)}$, $1_{(ZnO)} + 1_{(MgO)}$, $0.5_{(ZnO)} + 1.5_{(MgO)}$, $0.5_{(MgO)} + 1.5_{(ZnO)}$ mg/ml resulted in significant reduction of the growth of bacteria compared to the control group. Among the 9 evaluated concentrations, the concentration of $0.5_{(ZnO)} + 1.5_{(MgO)}$ mg/ml shows the highest level of impact against *E. coli*. Also, 0.5 concentration of ZnO had the lowest level of impact on *E. coli*. The results showed that the reduction in the number of bacteria cells compared with the control group is significant $p < 0.000$. These results showed that combination of MgO and ZnO nanoparticles had a significant effect on *S. aureus* in a way that these nanoparticles showed a significant decrease compared to the control group (p value $d^* 0.05$). The results show that among the studied concentration, the concentration of $0.5_{(ZnO)} + 1.5_{(MgO)}$ mg/ml resulted in a significant decrease in the number of bacterial cells compared to the control group. The data also showed that *S. aureus* was more sensitive than *E. coli* to the nanoparticles in liquid medium. Also the results showed that the MgO nanoparticles have stronger effect on *E. coli* and *S. aureus* compared to ZnO nanoparticles in TSB medium. The results showed that the antibacterial activity of nanoparticles depends on the amount consumed. Therefore, concentrations o) $1.5_{(MgO)} + 0.5_{(ZnO)}$ mg/ml was used for studying the antibacterial effect of nanoparticles in milk.

The use of the combination of MgO and ZnO nanoparticles and mild heat

Three concentrations of MgO, ZnO nanoparticles including $0.5_{(ZnO)}$, $1.5_{(MgO)}$, $1.5_{(MgO)} + 0.5_{(ZnO)}$ and heat were used as antibacterial treatments in milk. Fig 4 shows the effect of the combination of MgO and ZnO nanoparticles against *E. coli* in milk for 48 h at ambient temperature, 50 °C and 60 °C. The results showed a reduction in bacterial counts in the milk in the presence of nanoparticles, compared to the control group. The results indicated that among the concentrations studied, the concentration of $0.5_{(ZnO)} + 1.5_{(MgO)}$ mg/ml resulted in the highest reduction in the number of cells. In fact, after inoculation with bacterial suspension of $7 \log$ cfu/ml, at 48 h, control group showed increase to $10 \log$ cfu/ml. However, the treatment group with the concentration of $0.5_{(ZnO)} + 1.5_{(MgO)}$ mg/ml the number of cells were reduced to $6 \log$ cfu/ml in ambient temperature and to $4 \log$ cfu/ml and $3 \log$ cfu/mL at temperatures of 50 °C and 60 °C respectively, compared to the control group. The results show that the nanoparticles used in this study have a stronger activity on *S. aureus* than

E. coli. Our data indicate a decrease in the number of *S. aureus* cells (p value < 0.000) in the presence of the nanoparticles. Among the concentrations, the highest level of effect belonged to the concentration of $0.5_{(ZnO)} + 1.5_{(MgO)}$ mg/ml (Fig 5). The results showed that the nanoparticles tested against *S. aureus* is more sensitive. The results show that with increasing the temperature, growth of the bacteria decreased in the presence of nanoparticles and among three temperature conditions studied in this research, 60 °C has the greatest level of effect. The present study explored the effect of the combination of ZnO and MgO nanoparticles on Gram-positive bacteria such as *S. aureus* and Gram-negative such as *E. coli* in both agar and liquid media as well as milk. The results showed that these nanoparticles had no effect on *E. coli* and *S. aureus* in agar medium. Among 9 concentrations studied in this experiment, the concentration of $0.5_{(ZnO)} + 1.5_{(MgO)}$ mg/ml showed highest level of effect against *S. aureus* and *E. coli* after 24 h in broth medium. The data also showed that *S. aureus* was more sensitive than *E. coli* to nanoparticles in liquid medium. The results showed that the antibacterial activity of nanoparticles depends on the amount consumed. Also, the results showed that MgO and ZnO nanoparticles have synergic effect. Recently, the use of inorganic nanomaterials for the control of pathogens in food is widely increased [15-16]. The use of ZnO nanoparticles to suppress *S. aureus* and *E. coli* in milk has been proven [17]. Recently, the use of ZnO nanoparticles against pathogens that are transmitted through food, including *E. coli O157: H7* and *Enterotoxigenic*, has increased [14-18]. It has been found that the use of ZnO nanoparticles on the fabric results in 87 percent reduction in bacterial cells. Also the use of nanoparticles in polyester fabrics resulted in 80.7 percent reduction in fungal cells [19]. The results of the present study showed that the ZnO and MgO nanoparticles are good candidates for protection of agricultural products and foodstuffs. According to reports, the minimum dose of zinc in adults diets is 40 mg daily [20] while 0.4 mg of zinc is used in each 100 ml of milk. In addition to the nanoparticles concentration, temperature can also have an important role in controlling milk pathogens. The data showed that combination of ZnO and MgO nanoparticles at 60 °C had the highest level of effect on the studied bacteria. In fact, it can be said that high temperatures result in the increase of the fluidity of cell membranes of microorganisms and facilitate nanoparticles entrance into bacterial cells [21]. In addition, leakage of

intracellular contents of *E. coli* and *S. aureus* at high temperatures resulted in cell death [18]. Data showed that compared to *E. coli*, *S. aureus* is more sensitive to both nanoparticles. Differences in the sensitivity of the bacteria studied in this experiment can be attributed to the physiology of the bacteria. The Gram-negative bacteria have one outer wall more than Gram-positive bacteria [22, 24]. For increasing the inhibitory effect of antimicrobial compounds such as nanoparticles, in some cases, the combined use of these materials can be considered as a good solution in a way that in addition to increasing antimicrobial effect, can overcome the limitations of each individual nanoparticles [3]. The combination of two or more nanoparticles to determine the antagonistic or synergistic behavior has been the subject of many studies. For example, in one study, Reddy et al. used a combination of ZnO nanoparticles and other metal oxide nanoparticles against pathogenic bacteria [23]. Ren et al used a combination of ZnO nanoparticles

with other metal oxides against strains of methicillin resistant *S. aureus* (MRSA) and some Gram negative bacteria pathogens. They also revealed that the combination of these nanoparticles results in synergism effect. Although the exact mechanism of nanomaterials on microorganisms has not been established, several studies suggest that nanomaterials release ions that react with thiol group (-SH) in protein on the surface of the bacterial cells and thus reduce adhesion and biofilm formation [25]. Metal ion homeostasis is very important for living bacteria. These metal ions have a role in the regulation of metabolic functions such as coenzyme, the co-factors, catalysts and DNA-binding proteins. However, increase in metal ions can be considered as toxic [26]. In addition, the production of hydrogen peroxide ions in the presence of zinc oxide nanoparticles can also be considered as the key mechanism against pathogens [16].

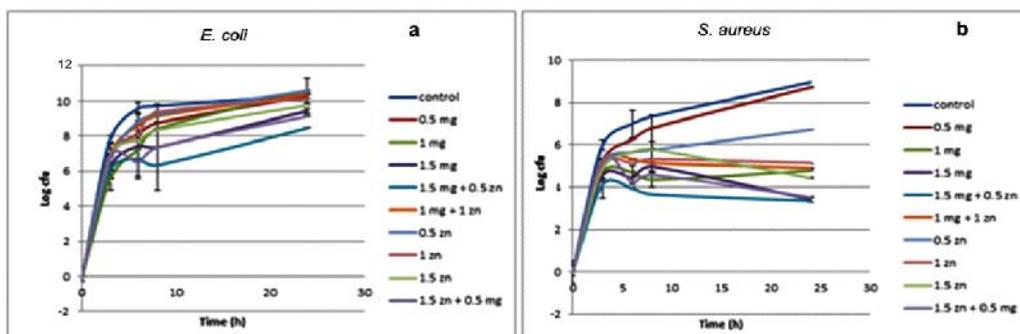


Fig. 3. The effect of the combination of MgO and ZnO nanoparticles on *E. coli* and *S. aureus* in liquid medium, 3(a) *E. coli*, 3(b) *S. aureus*

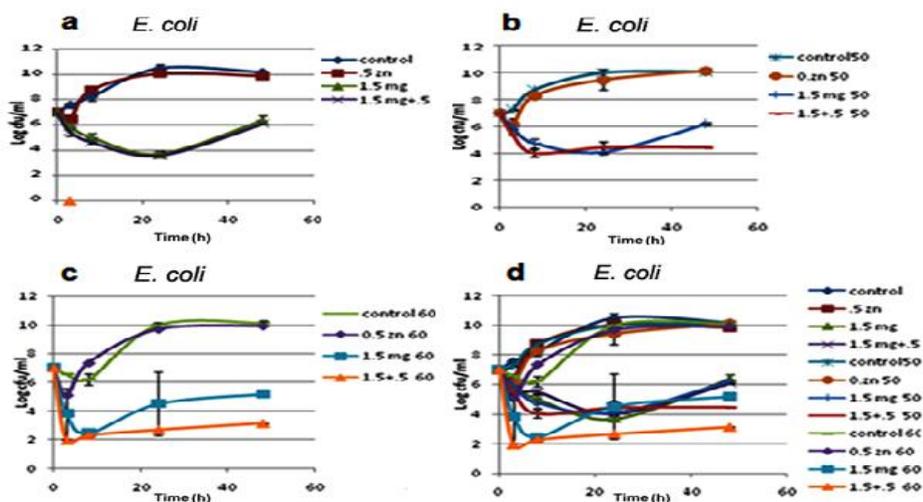


Fig. 4. The effect of the combination of MgO and ZnO nanoparticles against *E. coli* in milk. a (room temperature), b (50 °C), c (60 °C)

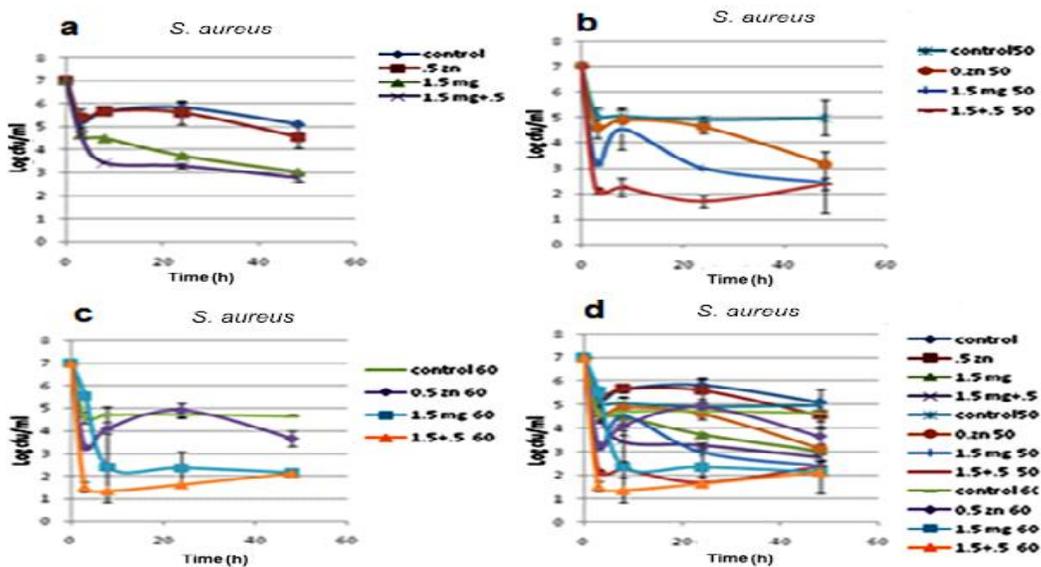


Fig. 5. The effect of the combination of MgO and ZnO nanoparticles and mild heat against *S.aureus* in milk. a (room temperature), b (50 °C), c (60 °C)

CONCLUSION

The results showed that the combination of ZnO and MgO nanoparticles has a favorable effect against *S. aureus* and *E.coli* in liquid media and milk and it has a synergistic behavior. A pharmaceutical composition with a new formulation with magnesium oxide and zinc oxide nanoparticles can function effectively to treat infections in the future. Also creating a new compound containing these nanoparticles can have a protective role in food safety. This is the first report describing the antibacterial activity of the combination of ZnO and MgO nanoparticles in milk and it showed the aforementioned combination to be a potential for use as an antibacterial agent in the food systems.

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