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Investigation of antibacterial activity of ZnO nanoparticles suspension containing citric acid against *Salmonella typhimurium* in mango and carrot juice

Yadullah Edalatpanah*, Freshteh Rahedan, Manejeh Rostami, Hadi Rezaei, Khadeigeh Sanaeiyan, Parisa HosseiniAlvand

Department of Biology, University of Applied Sciences Dehdasht, Dehdasht, Iran

*correspondence should be addressed to Yadullah Edalatpanah, Department of Biology, University of Applied Sciences Dehdasht, Dehdasht, Iran; Tell: +989175175068; Fax: +98; Email: edalatpanah1367@gmail.com.

ABSTRACT

Nanoparticles (NPs) are widely recognized because of their usage in biological applications including nano-medicine and food safety. The objective of this experiment was to determine the effect of ZnO NPs and citric acid on the survival of *Salmonella typhimurium* inoculated on to mango juice and carrot juice. Antibacterial activities of different concentrations (0, 1, 2, 4, 6, 8, 10 mM) of zinc oxide (ZnO) suspension containing 1% citric acid were tested against the *Salmonella typhimurium* inoculated on to culture media by spot on the lawn method and culture turbidity as a qualitative measure of the cell growth. Results manifested more inhibitory effect of ZnO in liquid medium than the solid culture against *Salmonella typhimurium*. These data suggested that the antibacterial activity of ZnO was concentration dependent which was previously confirmed by the agar diffusion test. ZnO NPs were more effective in initial reduction of *Salmonella typhimurium*. Results demonstrated that ZnO NPs suspensions containing citric acid had synergic effect against studied bacteria in media. Citric acid 1% dilution in concentrations of 10^2,10^4,10^6 and 10^8 in mango and carrot juice, which show the highest antimicrobial effect in concenteration 6 mM and 8 mM dilution of the $10^6$and 10^8 . The effect of zinc nano oxide synergic and citric acid 1% has stronger antimicrobial effect in the face with net citric acid 1%.

Key words: Salmonella typhimuriumin, Citric acid, Zinc oxide Nanoparticles, Pathogens

1. INTRODUCTION

Lactic and citric acids have been reported to be less effective in decontamination of some pathogenic microorganisms such as some *Salmonella serovars* and *E. coli*, which have developed some resistance to acidic conditions (1). It is therefore imperative that new decontaminants sought against such pathogenic micro-organisms. ZnO is one of the five zinc compounds that are currently listed and generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (21CFR182.8991). Zinc salt has been used for the treatment of zinc deficiency (2). Besides, ZnO powder has been used for decades as an active ingredient for dermatological applications in creams, lotions and ointments on account of its antibacterial properties (3-7). In addition ZnO NPs, which are nontoxic and biocompatible have been utilized as drug carriers and medical filling materials. Biological sensors based on nano-materials have been designed to be used in the diagnosis of molecular

and genetic diseases. Nano-materials have been used extensively in the food industry they can used to produce juices without microbial organisms. Such nano-materials that are widely used, we can mention that zinc oxide (ZnO) nanoparticles show strong antibacterial properties against gram-positive and gram–negative bacteria. These nanoparticles are used as drug carriers and impact on the activities of *Aspergillus niger* and *Salmonella typhimurium* (8-10). ZnO nanoparticles make damages in to the bacteria lipid and cell membrane proteins. In addition, as a matter of antibacterial frequency against gram–positive and gram–negative bacteria is considered. In another study done by Nicole Jones Etal on zinc oxide nanoparticles antibacterial properties, determined that this nanoparticle, stops the growth of *staphylococcus aureus* (11). The objective of this experiment was to determine the effect of ZnO NPs and citric acid on the survival of *Salmonella typhimurium* inoculated on to mango juice and carrot juice.

2. MATERIALS AND METHODS

2.1. Bacterial strains, media and materials

The following bacterial strains were used in this study: Salmonella typhimurium PTCC1371. These mentioned bacteria were obtained from the culture collection of the I.R. Department. Moreover, Stock cultures were maintained at -80 °C. The stains were propagated on Tryptic Soy Agar (TSA; Merck, Darmstadt, Germany) at 37 °C and maintained at 0–2 °C before use. Zinc oxide nanoparticles were purchased from TECONAN, Spain (particle diameters: 20–25 nm). The purity of ZnO NPs was more than 99.98%.

2.2. Determination of the antibacterial activity of the citric acid

In order to test the antibacterial activity of citric acid (Merck), spot on the lawn method was employed. Antibacterial activity of citric acid was tested by spotting 20µL of the citric acid solution (0, 0.5, 1, 2 % V/V) on to soft agar lawn (0.6%) seeded with 107 cell/mL Salmonella typhimurium, respectively. Each concentration of citric acid was placed on surface-inoculated TSA agars and incubated at 37 °C for 24 h. Inhibition zone around specimens was used to indicate antibacterial activity of each citric acid concentration (12).

2.3. Antibacterial activity of ZnO NPs suspension containing citric acid

Spot on the lawn method

To test the antibacterial activity of different concentrations of ZnO NPs suspensions containing 1and 2% citric acid, spot on the lawn method was employed. ZnO nanoparticles were resuspended in sterile citric acid (1, 2%) and formed a uniform suspension. Antibacterial activity of ZnO NPs suspension containing citric acid was tested by spotting ZnO NPs suspensions(1, 2, 4, 6, 8, 10 mM)containing 20 µL of the citric acid (1, 2%) on to soft agar lawn (0.6%) seeded with 107 cell/mL Salmonella typhimurium, respectively. Each concentration of ZnO NPs suspension containing citric acid was placed on surface-inoculated TSA agar and incubated at 37 °C for 24 h. Inhibition zones around specimens were used to indicate antibacterial activity of each suspension (12).

2.4. Detection of inhibitory activity

The Tryptic Soy Broth culture (TSB culture; Merck) which contained (0, 1, 2, 4, 6, 8, 10 mM) of ZnO nanoparticle suspensions along with 1% citric acid inoculated with 107 cell/mL of Salmonella typhimurium, respectively. The bottles were shaken at 50 rpm at 37 °C. Afterwards, the OD (600 nm) of the cultures was serially monitored every hour or every 2 h up to 10–12 h, with a final reading at 24 h (11). Free culture medium of nanoparticles and citric acid under the same growth conditions was used as a control. To avoid potential optical interference during optical measurements of the growing cultures caused by the lights catering properties of the NPs, the same liquid medium without micro-organisms but containing the identical concentration of NPs and 1% citric acid was cultured under the similar conditions as blank controls.

2.5. Statistical analysis

Antimicrobial experiments were conducted in triplicate. Data points were expressed as the mean ± SD. Data were analyzed based on analysis of variance (ANOVA) from SAS software. DUNCAN s multiple range tests were used to determine the significant difference between mean values if stated. Otherwise, significance was expressed at 5% level.

2.6. The effect zinc nano oxide synergic (Zno) and citric acid 1% on the growth rate Salmonella typhimurium in carrot juice

The amount of 160cc of carrot juice is late by means of centrifuges apparatus and measure. Its pH is equal to 6.8. The carrot juices can be sterilized at 121^0 by an autoclave apparatus. After the sterilization of 40cc of the carrot juice we can use it as a control without adding any substance as a sample in two containers to pour, for every each sample containing a volume of 20cc for Salmonella typhimurium we weight the amount of 1.2gr of citric acid and dissolve in the 120cc of the carrot juice .The amount 40cc of the carrot juice that we add to 1% acid in two containers we pour every each container containing a volume of 20cc for Salmonella typhimurium ; For the Acetock solution, we add an amount of the 0.2 gr citric acid 1% with 0.08gr zinc nano oxide dissolved in 20 cc sterile distillated water and according to the calculations for concentrations of 6mM and 8Mm from acetock solution we carry out the dilution up to 24 hours and we can count the number of formed colonies by counter colony (12).

2.6.1. Acetock solution 6mM of citric acid 1%

$50v_1 = (6)20 \rightarrow v_1 = 2.4cc \rightarrow 2.4cc\ acetock\ solution + 17.6cc\ citric\ acid$

According to the above calculations for each of the Salmonella typhimurium amount of 2.4cc acetock solution 6mM of citric acid 1% with 17.6cc carrot juice that we have already added to it citric acid 1% pour in two containers containing the volume 20cc.

2.6.2.Acetock solution 8mM of citric acid 1%

$50v_1 = (8)20 \rightarrow v_1 = 3.2cc \rightarrow 3.2cc\ acetock$ solution $+ 16.8cc\ citric\ acid$

according to above calculations for Salmonella typhimurium , amount of the 3.2cc acetock solution 8mM of citric acid 1% with 16.8cc carrot juice that we have already added to citric acid 1% poured in two containers containing the volume of 20cc. To all of the containers containing a solution, amount of 500λ we pour by using a sampler of Salmonella typhimurium, and put it at 20^Oc in an incubator refrigerator.

2.7. The effect zinc nano oxidesynergic (Zno) and citric acid 1% on the growth rate Salmonella typhimurium in mango juice

The amount of 160cc of the mango juice isolate by centrifuges apparatus and measure, it's pH that is equal to 6.4. The mango juice can be sterilized at 121^Oc using an autoclave apparatus. After sterilization, we can use the mount of 400cc of the mango juice as a control, without adding any substance as a sample in two containers, that each of them containing a volume of 20cc for Salmonella typhimurium. We weight the amount of 1.2gr of citric acid and dissolve it in the 120 cc of the mango juice. The amount of 40cc of the mango juice that we are adding it in to acid 1%, as acid 1% in two containers ,that each of them containing a volume of 20cc for Salmonella typhimurium. From acetock solution, we dissolve the amount of the 0.2gr citric acid 1% with 0.08gr zinc nano oxide, in 20 cc sterile disulted water and according to the calculations for concentration of 6mM and 8mM, from acetock solution. we carry out the dilution up to 24 hours and we can count the number of formed colonies by counter colony (12).

2.7.1. Acetock solution 6mM of citric acid1%:

50v_1=(6)20→v_1=2.4cc→2.4cc acetock solution+17.6cc citric acid

According to the above calculations for each of Salmonella typhimurium . Amount of the 2.4cc acetock solution 6mM of citric acid 1% with 17.6cc mango juice that we have already added to citric acid 1%, poured in two containers containing the volume 20cc.

2.7.2. Acetock solution 8mM of citric acid 1%:

50v_1=(8)20→v_1=3.2cc→3.2ccacetock solution+16.8cc citric acid

According to above calculations for Salmonella typhimurium, an amount of 3.2cc acetock solution 6mM of citric acid 1% with 16.8 cc mango juice that we have already added to citric acid 1%, poured in two containers containing of 20cc volume. To all of containers a solution, amount of 500λ, we pour by using a sampler of Salmonella typhimurium,and to put it at 20^0c in an incubator refrigerator.

2.8. Dilution

For both bacteria, dilution in mango juice and carrot juice, carried out at intervals of 48 and 24h at temperature of 20^Oc, a dilution of $10^{\wedge}2$,$10^{\wedge}4$,$10^{\wedge}6$,$10^{\wedge}8$, after dilution, the amount of 100 λ, pick up from each dilution. On solution of the test tubes by using a sampler and pour in agar culture plates and by using slice bar of slice culture, dispread at side of flame and to put in an incubator at temperature of 37^Oc for 24 hours, until they form a colony.

3. RESULTS AND DISCUSSION

3.1. Antibacterial activity of citric acid

Antibacterial properties of 0.5, 1and 2% citric acid were measured according to the inhibition zone method against Salmonella typhimurium. Table 1 shows the results of inhibition zones for different concentrations of citric acid. Results showed that 0.5% citric acid had no inhibition zone on none of the strains. Furthermore, it was concluded that 1% citric acid had no inhibition zone on none of the strains, but it had Growth Reduction on all of them. In addition, results proved that 2% citric acid had 8, 9, 10 and 11 mm inhibition zones against Salmonella typhimurium, respectively. As it is depicted in Table 1, the inhibition zones increased at once in relation with the percent content of citric acid.

3.2. Antibacterial activity of ZnONPs suspension containing citric acid

Antibacterial properties of various concentrations (1, 2, 4, 6, 8, 10 mM) of ZnO NPs suspensions containing (1, 2%) citric acid were measured according to the inhibition zone method against Salmonella typhimurium. Table 1 shows the inhibition zones for diverse concentrations of citric acid (0.5, 1, 2%) and various concentrations of ZnO NPs suspensions containing (1, 2%) citric acid. According to the obtained results, the inhibition zones increased instantly in relation with the molar content of ZnO NPs (Table 1).

Table 1. The effect of supplemental zinc oxide nanoparticles and citric acid on bacterial growth Salmonella typhimuriumin shows

Concentration of Acid citric and ZnO nanoparticle	*Salmonella typhimurium mm*
0.5 % Acid	-
1% Acid	Gr
1% Acid + 1mM ZnO	Gr
1% Acid + 2mM ZnO	Gr
1% Acid + 4mM ZnO	8±0.1
1% Acid + 6mM ZnO	7± 0.2
1% Acid + 8mM ZnO	8± 0.2
1% Acid + 10mM ZnO	10± 0.1
2% Acid	9± 0.1
2% Acid + 1mM ZnO	10± 0.2
2% Acid + 2mM ZnO	9± 0.2
2% Acid + 4mM ZnO	11± 0.3
2% Acid + 6mM ZnO	10.5±0.1
2% Acid + 8mM ZnO	10.5± 0.2
2% Acid + 10mM ZnO	11±0.3

3.3. Detection of inhibitory activity

ZnO suspensions with diverse concentrations (1, 2, 4, 6, 8 mM) containing 1% citric acid were used as the antimicrobial treatments in TSB broth media. Figure 1 demonstrates the effect of ZnO treatments with various concentrations containing 1% citric acid on the growth of Salmonella typhimurium in TSB brought at 37°C. Results exhibited that these treatments had significant inhibitory effects on the growth of Salmonella typhimuriumduring with 24h incubation, comparing to the control, among the six diverse concentrations of ZnO, the 6 and 8 mM suspensions of ZnO were the most effective on Salmonella typhimurium. Results manifested that 6mM suspension of ZnO caused 55.65% growth reduction in Salmonella typhimuriumafter with 12 h in TSB. Additionally, results disclosed that 0 and 1 mM suspensions of ZnO had no inhibition effect against none of the strains during 12 h in TSB. Moreover, results showed that 2 and 4 mM suspensions of ZnO had inhibitory effects against Salmonella typhimurium TSB broth. The Results exhibited that 2mM suspension of ZnO leads to 23.58% growth reduction in Salmonella typhimurium after 12hours, respectively. In addition, results showed that 4mM suspension of ZnO caused 34.43% growth reduction in Salmonella typhimurium after 12h, respectively. Results showed more inhibitory effect of ZnO in liquid medium than the solid culture against Salmonella typhimurium. These data suggested that the antibacterial activity of ZnO was concentration dependent, which was previously confirmed by the agar diffusion test (Figure 1).

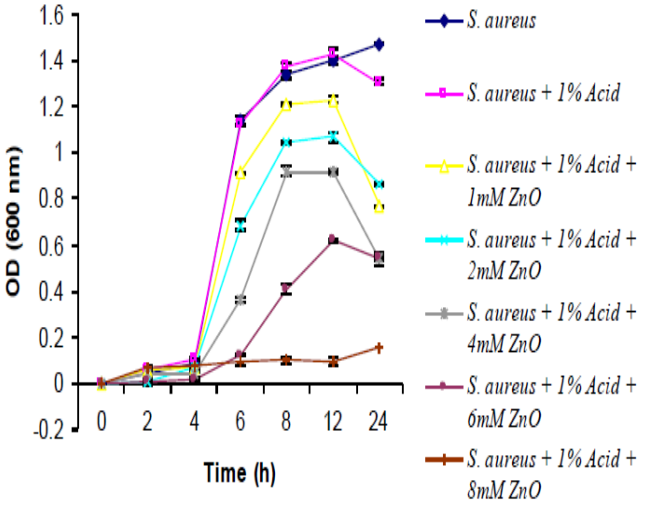

Figure 1. Effect (0, 1, 2, 4, 6, 8 mM) ZnO + 1% citric acid on growth Salmonella typhimurium in Tryptic Soy Broth at 37 °C

For Salmonella typhimurium bacteria a dilution in mango juice and carrot juice carried out at intervals of 4,8 and 24h at a temperature of 20^0c in a dilution of 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}; after the dilution of an amount of 100 λ which is picked up

from each dilution of the solution of the test tubes by using a sampler and poured in agar culture plates by using slice bar of slice culture which is dispread at a side of the flame and also put in an incubator at a temperature of 37^0c for 24 hours until they form a colony. The next day we count the number of formed colonies by countercolony apparatus according to tables 3 to 4, that with time lapse specified. The growth of bactria at concentration 6mM and 8mM at the dilution of $10^{\wedge 6}$ and $10^{\wedge 8}$ to zero the number of formed colonies at concentration of 1% citric acid showed high number of formed colonies that determined with the comparison of effect of pure citric acid and zinc nano oxide and citric acid. Effect of zinc nano oxide synergic and acid has a stronger antibacterial effect (Tables 2, 3).

Tables 2. Shows the number of the formed colonies of Salmonella typhimurium at intervals 4,8 and 24 hours in mango juice

Type	4h	8h	24h
Acid citric1% 10^2	∞	∞	∞
Acid citric 1% 10^4	∞	∞	179±13
Acid citric 1% 10^6	176±10	56±12	51±6
Acid citric 1% 10^8	64±7	39±9	32±5
Acid citric1%+ 6mM10^2	∞	∞	∞
Acid citric1%+ 6mM10^4	∞	∞	65±13
Acid citric1%+ 6mM10^6	25±5	32±6	19±6
Acid citric1%+ 6mM10^8	14±3	17±2	5±1
Acid citric1%+ 8mM10^2	∞	∞	106±6
Acid citric1%+ 8mM10^4	∞	35±13	64±9
Acid citric1%+ 8mM10^6	17±3	14±4	4±2
Acid citric1%+ 8mM10^8	4±1	2	0

Tables 3. Shows the number of the formed colonies of Salmonella typhimuriumat intervals 4,8 and 24 hours in carrot juice

Type	4h	8h	24h
Acid citric1% 10^2	∞	∞	∞
Acid citric 1% 10^4	∞	∞	122±12
Acid citric 1% 10^6	142±11	121±14	97±8
Acid citric 1% 10^8	72±12	47±6	37±3
Acid citric1%+ 6mM10^2	∞	∞	∞
Acid citric1%+ 6mM10^4	∞	∞	59±10
Acid citric1%+ 6mM10^6	41±8	36±7	12±4
Acid citric1%+ 6mM10^8	25±4	31±5	2
Acid citric1%+ 8mM10^2	∞	∞	125±13
Acid citric1%+ 8mM10^4	∞	113±14	98±5
Acid citric1%+ 8mM10^6	23±6	18±5	4±1
Acid citric1%+ 8mM10^8	14±2	2	0

To apply hurdle technology in food processing, sensory quality must be also considered when determining the appropriate microbial intervention strategies. However, some hurdles influence sensory qualities of products, such as color, flavor and texture. In this study, treatments encompassing ZnO NPs suspensions containing citric acid were synergistic and effective on reducing the levels of Salmonella typhimurium. Quartey-Papafio and Carpenter who reported 55% of cultures became non-viable when treated with 1% citric and propionic acids in combination (13). However, Bell et al, observed the effect of citric and formic acid (1:1) for 10s and noted the reduction in average number of bacteria by 65% for Salmonella, Yersinia, Pseudomonas and S. Faecalis while E. coli was found to be the most resistant one (14). The bactericidal effect of organic acids such as citric acid is due to the reduction in pH below the growth range and metabolic inhibition by the disasociated molecules, which penetrate into the bacterial cell membrane. The accumulation of the disasociated weak acid in the cell cytoplasm eventually leads to the acidification of the cytoplasm of the microorganism (15). Obtained results for ZnO NPs have shown more antibacterial activity against Salmonella typhimurium. ZnO NPs are believed to destruct lipids and proteins of the bacterial cell membrane, resulting in a leakage of intracellular contents and ultimately the death of bacterial cells. In addition, generation of hydrogen peroxide and Zn^{2+} ions suggested it as being key for antibacterial mechanisms of ZnO NPs.

4. CONCLUSION

Results manifested that ZnO NPs suspensions containing citric acid had synergic effect against studied bacteria in media, that show highest antimicrobial effect in concentrations 6 mM and 8 mM of dilution of the $10^{\wedge 6}$ and $10^{\wedge 8}$. The effect zinc nano oxide synergic and citric acid %1 in the face with net citric acid%1, has strong antimicrobial effect.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration between all authors.

CONFLICT OF INTEREST

Authors have declared that no conflict interests exist.

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