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Original Article

Investigating the Antibacterial Activity of ZnO Nano-particles Suspension Containing Acetic Acid against Staphylococcus *aureus* in Mango Juice

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ABSTRACT

Background and Objectives: Zinc oxide (ZnO) nano-particles have been proven to have strong antibacterial activity against food borne pathogens. The practical applications of different concentrations (Control, 0, 1, 2, 4, 6 and 8 mM) of ZnO suspensions containing 1% acetic acid were investigated against the pathogenic bacteria like *Staphylococcus aureus.*

Materials and Methods: The projects of laboratory and field number 3 were repeated. The order of 1% acetic acid, and the 6 mM and 8 mM suspension concentrations of zinc oxide nanoparticles containing acetic acid was/were used as antibacterial juices. The experiments were repeated three rounds. The data were analyzed by ANOVA software, and the $P<0.05$ level of significance was identified.

Results: The results also exhibited that ZnO and acetic acid had inhibitory effect on the growth of all strains during the 24 h culture period in mango juice, as compared to the control experiment, which was further confirmed in the liquid culture.

Conclusions: This is the first report describing the antibacterial activity of ZnO NPs in mango juices that showed the potential of these nano-particles for use as an antibacterial agent in the food industry.

Keywords: Antibacterial, Acetic acid, Food, Nano-particles, Mango juice, Zinc oxide

Introduction

The consumption of unpasteurized fruit juices, defined as the product obtained by pressing or squeezing the fruits, has increased in recent years presumably due, in part, to their characteristics of freshness, high vitamin content, low calorie contribution, and an active promotion of a healthy diet. However, food-borne disease outbreaks caused by *E.coli 0157:H7* and different serovars of *salmonella* have been associated with unpasteurized fruit juices (1). This demonstrates that such products can serve as a vehicle for pathogenic microorganisms. In addition, incidence of survival growth of *Listeria innocua*, *Salmonella* serovars and *E.coli0157:H7* has been demonstrated in fruit juices and apple cider (2, 3, 4, 5). In response to the high number of outbreaks, caused by these pathogenic microorganisms following the consumption of fresh products, the regulatory organizations have recommended the use of good cleaning and sanitation practices (6), as well as the application of the food hazard analysis and ascetical control-point plural for the productions of juice (7). Likewise, the Food and Drug Administration (FDA) has established regulations for juice manufacturing, indicating that the treatments for commercial preparation of fresh juices should be capable of reducing pathogenic loads by a minimum of 5.0 log (8). The use of organic acids is considered as a good alternative in the fruit processing industry because of their low cost, as well as natural origin preservative, antioxidant, flavoring and acidifying properties. However, some important aspects such as the kind of juice, characterization of the spoilage with the help of pathogenic flora, and characteristics of the organic acid used for disinfection must be considered before selecting it as an antimicrobial agent for fruit juices.

In recent years, inorganic antimicrobial agents are being increasingly used for the control of microorganisms in various areas (9). The antibacterial activity of metal oxides including MgO and ZnO was first shown by a Japanese group (10). Reduction of materials to nano-size alters their activity and properties (11). Nano-particles have attracted much attention for their distinct characteristics that are unavailable in the conventional macroscopic materials (12). From the standpoint of nutrition and health, Magnesium (Mg), Zinc (Zn) and Calcium are essential to human health, as they are needed for more than 300 biochemical reactions in the body. ZnO powder has been used for a long time as an active ingredient for dermatological application in creams, lotions and ointments for its antibacterial

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properties (13). Also ZnO NPs, which are nontoxic and biocompatible, have been utilized as drug carriers and medical filling materials (14).

The objective of this experiment was to determine the effect of ZnO nano-particles and acetic acid on the survival of Staphylococcus *aureus* (inoculated onto the specific culture media), and estimating their antibacterial effect in mango juice.

Materials and Methods

Bacterial strains, media and materials

The following bacterial strains were used in this study: S. *aureus*. They were obtained from the Culture Collection Department (Tehran, Iran). Moreover, stock cultures were stored in a -80^{0}C freezer. The strains were propagated on Tryptic Soy Agar (TSA; Merck, Darm Stadt, Germany) at 37^{0}C, and maintained at 0-2^{0}C before use. ZnO NPs were purchased from TECONANO, Spain (particle diameters: 20-25nm; purity: 99.98%).

Determination of the antibacterial activity of acetic acid: In order to test the antibacterial activity of acetic acid (Merck, Germany), spot on the lawn method was employed. Antibacterial activity of acetic acid was tested by spotting 20μL of acetic acid solution (1, 1.5, 2% w/v) onto the soft agar lawn (0.6%), seeded with 10^7 cells/ml of S. *aureus*, respectively. Each concentration of acetic acid was placed on the surface–inoculated TSA agars, and incubated at 37 ^{0}C for 24 hours. Inhibition zone around the specimens was used to indicate the antibacterial activity of each acetic acid concentration (16).

Antibacterial activity of ZnO NP suspension containing acetic acid: To test the antibacterial activity of different concentrations of ZnO NP suspensions containing 1% acetic acid, spot on the lawn method was employed (as stated in the above section). ZnO nano-particles were re-suspended in sterile 1% acetic acid in the form of a uniform suspension. Antibacterial activity of ZnO NP suspension containing acetic acid was tested by spotting in different ZnO concentrations (Control,1, 2, 4, 6 and 8mM) onto the soft agar lawn (0.6%), seeded with 10^7cells/ml of S. *aureus*. Each concentration of ZnO NP suspension, containing acetic acid, was placed onto the surface – inoculated at 37 ^{0}C for 24h. Inhibition zones around the specimens were used to indicate the antibacterial activity of each suspension (16).

Detection of inhibitory activity: For detecting the combined antibacterial and pathogenic growth inhibitory activity of ZnO NPs and acetic acid, the liquid Tryptic Soy Broth culture (TSB; Merck, Germany) containing 1,2,4,6 and 8 mM NP suspensions with added acetic acid 1% was inoculated with 10^7 cells/ml of S. *aureus*, respectively. The bottles were shaken at the speed of 50 rpm at 37^{0}C. Afterwards, the optical density (OD) of the cultures was certainly monitored every 1-2 h for up to 10-12h at 600nm with a final reading taken at 24h.The cultured strains without any NP or acetic acid formulation and under the same growth conditions were used as a control. To avoid potential optical interference during the optical measurements of the growing cultures caused by the light's scattering properties of the NPs, the same liquid medium without microorganism but containing the identical concentration of NPs and 1% acetic acid was cultured under the similar conditions as blank controls.

Fruits and juices preparation: Mango fruits were purchased from a supermarket of Yazd (Iran) for extracting their juices. Each fruit was washed, peeled, cut into pieces, and blended using a Centrifuges blender (Model BP 4512, Vitoria, Spain). The obtained fruit juices were then centrifuged at 12500rpm for 15 min at 4^{0}C in an Avanti ^{TM}J-25 centrifuged (Iso Lab, Germany). Each supernatant of the juice was filtered, bottled and autoclaved for 15 min in an autoclave (Iran Tolid, Tehran, Iran) at 121^{0}C, to obtain sterile fruit juices.

PH determination: The pH of mango juice was determined using a microprocessor pH meter (Hanna Instrument pH210: Vernon Hills, USA). The pH was recorded to be about 6.8± 0.4.

Addition of acetic acid and ZnO nano-particles to fruit juices: 6 and 8 mM ZnO NP suspensions containing 1% acetic acid were separately prepared. The pH of the mango juices when acetic acid and ZnO NP were added reached to 5.3±0.2, 6.25 ±0.2, and 5.35±0.6, respectively. The above stated solutions were used as antimicrobial agents for the mango juice samples. Two experimental groups of mango juices were separately added with 6 and 8 mM NP suspensions containing 1% acetic acid, and then inoculated with 10^7cells/ml concentration of S. *aureus*, respectively. The bottles were incubated in an orbital shaker at the speed of 50rpm at 30^{0}C for 24h. Following inoculation, the number of bacterial cells was determined every 4hours for up to 24 hours by agar count plate method. All experiments were carried out twice.

Statistical analysis: The experiment was repeated two times. Data points were expressed as mean±SD. The data were analyzed based on the analysis of variance (ANOVA), with the help of SAS software. Ducant's multiple range test (DMRT) was used to determine the significant difference between the mean values. Significance was expressed at 5% level.

Results

Antibacterial activity of acetic acid: Antibacterial properties of 0.5, 1 and 2% acetic acid against *Staphylococcus aureus* were measured according to the inhibition zone method. Table 1 shows the results of inhibition zones taken at different concentrations of acetic acid. According to the obtained results, the inhibition zones increased instantly at once in relation with the percent content of acetic acid for all strains.

Antibacterial activity of ZnO nano-particles suspension containing acetic acid: Antibacterial properties of the suspension of various concentrations (1, 2, 4, 6 and 8mM) of ZnO NP containing 1% of acetic acid against *S. aureus* were measured according to the inhibition zone method. The results showed that the inhibition zones increased instantly in relation with the molar content of ZnO NPs for all strains (Table 1).

Table 1: Inhibition zone diameter of acetic acid and ZnO nano-particle suspensions containing acetic acid

Concentration of acid acetic and ZnO nano-particles	Inhibition zone *(mm)*
0.5 % Acid	-
1% Acid	Gr
1% Acid + 1mM ZnO	Gr
1% Acid + 2mM ZnO	Gr
1% Acid + 4mM ZnO	Gr
1% Acid + 6mM ZnO	8± 0.2
1% Acid + 8mM ZnO	9± 0.2
2% Acid	9± 0.1
2% Acid + 1mM ZnO	9± 0.1
2% Acid + 2mM ZnO	9± 0.2
2% Acid + 4mM ZnO	10± 0.2
2% Acid + 6mM ZnO	10.5±0.1
2% Acid + 8mM ZnO	11± 0.2

Detection of inhibitory activity: The growth curves of *S. aureus* are shown in Fig. 1. It is evident that different concentration of ZnO NPs had affected these strains' growth rate. Increase in concentration of ZnO NPs significantly decreased the growth rate of S. aureus comparing to the control. Marginal reduction in the specific growth rate was observed at 8 mM concentration ($P<0.05$). Thus 6 and 8 mM of ZnO were selected for further antimicrobial studies to be carried out on mango juices.

Antibacterial effect of ZnO nano-particles suspension containing acetic acid in mango juice: Two concentrations of ZnO (6 and 8 mM) were used in the antimicrobial treatments in the mango juice samples. Table 2 shows the cell counts of S. aureus in the mango juices treated with different concentrations of ZnO (6 and 8 mM) suspensions with added acetic acid (1%) during a culture period of 24h at 30^0C. Treating the mango juices with 6 and 8 mM ZnO suspensions containing1% acetic acid caused a striking reduction in S. aureus counts during the 24 h of culture at 30^0 C (Fig. 2), which were recorded to be ranging between 7 and 5.79 log CFU/ml, respectively ($P < 0.05$). S. aureus count in the mango juices treated with 6 and 8 mM ZnO suspensions was 5.91 and 5.79 log CFU/ml after 24 h of incubation, respectively. Whilst, cell count in the mango juice treated with water (control) was 10.34 log CFU/ml.

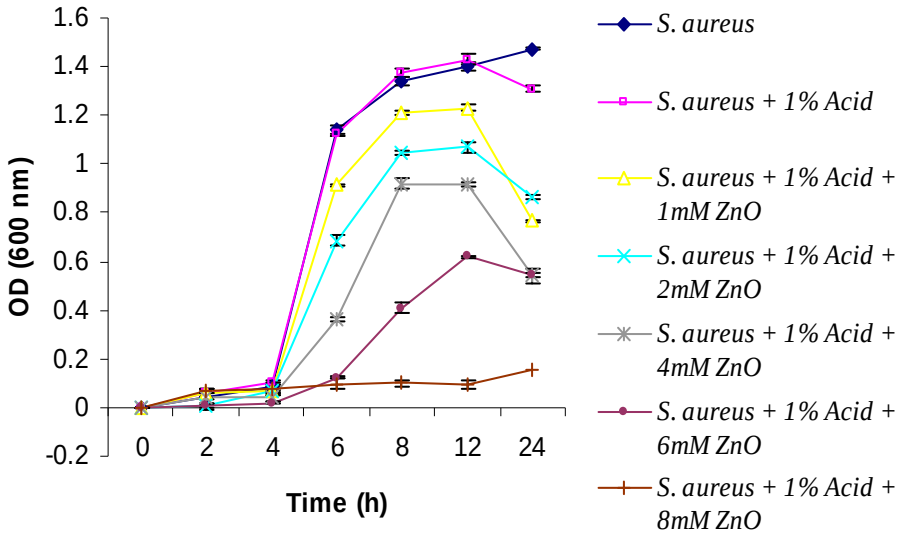

Fig. 1. Effect of ZnO suspensions (Control, 0 , 1, 2, 4, 6 and 8 mM) containing 1% acetic acid on the growth of S. *aureu*.

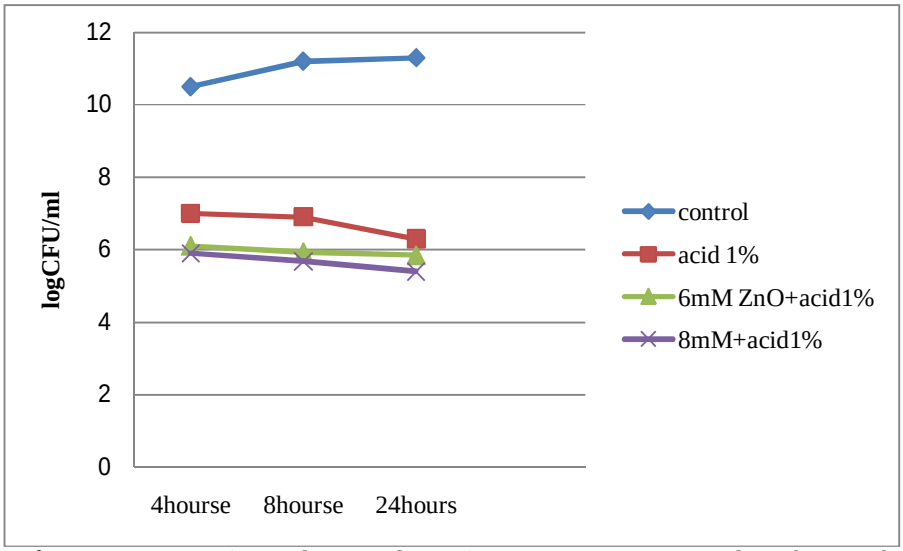

Fig. 2. Effect of ZnO suspensions (Control, 0, 6 and 8 mM) containing 1% acetic acid on the growth of S.*aureus*.

Discussion

To apply hurdle technology in food processing, sensory quality must also be considered when determining the appropriate microbial intervention strategies (17). However, some hurdles influence the sensory qualities of products such as color, flavor and texture. In this study, we investigated of the effect of ZnO nano-particles on the antibacterial activity of acetic acid. The ZnO NP suspension containing 1% acetic acid was chosen for further studies in TSB media and in mango juice. Treatments encompassing the antibacterial effect of ZnO NP suspension containing acetic acid were surprisingly significant in reducing the cell count and survival of S.aureus. Our results further showed that ZnO and acetic acid had better inhibitory effect against all experimental strains, when cultured in the liquid medium, rather than in the solid culture. These data suggest that the antibacterial activity of ZnO was concentration-dependent, which was further confirmed by the agar diffusion test. Thus, 6 mM and 8 mM concentrations of ZnO were selected for further studies in mango juice; they showed a significant growth inhibition in the bacterial strains, when cultured in TSB (Fig. 1).

Fig. 2 shows the log-reduction of all strains inoculated onto the mango juices treated with different concentrations (0, 6 and 8 mM) of ZnO suspensions containing 1% acetic acid. ZnO NP suspensions were more capable of reducing the initial counts of all strains. The results exhibited that ZnO and acetic acid had inhibitory effects on the growth of all strains in the mango juices during the culture period of 24 h, as compared to the control, which was further confirmed in the media culture. Similar antibacterial effects on reducing the survival of pathogens in other acidified food samples have been observed by combining the mild heating with acidic addition (18). Anellis, Lubas and Rayman (1954) reported that low pH increased the effectiveness of heating on inactivating Salmonella spp in liquid whole egg (20). The study of Raybaudi-Massilia, Mosqueda-Melgar, and Martin-Belloso (2009) revealed the antimicrobial activity of malic acid against *L. monocytogenes*, *Salmonella enteritidis* and *E. coli O157:H7* in apple, pear and melon juices (5). However, Bell, Marshall, and Anderson (1986) observed the effect of acetic and formic acid (1:1), and noted 65% reduction in the average cell count in Salmonella, Yersinia, Pseudomonas and Staphylococcus *faecalis*, while *E. coli* was found to be the most resistant one (20). Kim, Kim, and Song (2009) reported the antimicrobial activities of combination of fumaric acid and aqueous chlorine dioxide or UV-C irradiation against *E. coli O157:H7*, *Salmonella enterica* serovar, Typhimuriumand L. monocytogenes inoculated on alfalfa and clover sprouts (21). Shin, Lee, Dougherty, Rasco, and Kang (2006) demonstrated that using a combination of mild heat and acetic acid treatment can successfully control *E. coli O157:H7*, *L. monocytogenes* and *Salmonella typhimurium* in pickled asparagus, and effective treatments can be selected to reduce the possible adverse effects on color, which occurs during the product storage (22). The bactericidal effect of organic acids such as acetic acid is due to the reduction of pH below the optimal range for microbial growth and metabolic inhibition by un-dissociated acid molecules, which penetrate into the bacterial cell membrane. The accumulation of un-dissociated weak acid in the cell cytoplasm, eventually, leads to the acidification of the microbial cytoplasm (23). The results obtained for ZnO NP suspensions have shown their significant antibacterial activity against *S. aureus*. ZnO is one of the five zinc compounds that are currently listed as *generally recognized as safe* (GRAS) by the U.S. Food and Drug Administration (US-FDA) (21CFR182.8991). Zinc salt

has been used for the treatment of zinc deficiency (24). Currently, there are very few reports related to the application of NPs in food safety. For example, ZnO quantum dots were used as the antimicrobial agents in liquid egg white samples. Depending on their concentration, ZnO quantum dots could significantly inhibit or reduce *L. monocytogenes* and *S. enteritidis* in liquid egg white (24). Similar inhibitory effects of ZnO NPs on reducing *S. aureus* and *E. coli* in milk samples have been observed (15). ZnO NPs are believed to destruct lipids and proteins of the bacterial cell membrane, resulting in a leakage of intracellular contents, and ultimately, leading to the death of bacterial cells (25). In addition, generation of hydrogen peroxide and Zn2þ ions has been suggested to be key antibacterial mechanisms of ZnO NPs (13). Also the recommended maximum dietary allowance for Zn was 40 mg per day for adults (24), which is equivalent to 100 mL of milk daily intake, if 0.4 mg of ZnO per mL of food is used (15). This is important disadvantage of these nano-particles for use as an antibacterial agent in the food industry. Therefore, further research is necessary to investigate the efficacy of ZnO at lower concentrations to inactivate the pathogens existing in foods.

Conclusion

ZnO NP suspension containing acetic acid can provide an approximately 5-log reduction in S. *aureus* in mango juices, with low survival of injured cells during the room temperature storage. The process information presented here can help in determining the necessary food processing conditions that assure food safety while producing a product of higher sensory quality at lower energy costs.

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