



Antimicrobial effect of nanofluid including Zinc oxide (ZnO) nanoparticles and *Mentha pulegium* essential oil

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ABSTRACT

This study was carried out to evaluate the in vitro antibacterial activity of nanofluid based on *Mentha pulegium* essential oil and Zinc oxide (ZnO) nanoparticles (NPs) against different bacterial species. The essential oil was obtained by hydro-distillation using Clevenger and then ZnO NPs were added at the rates 0, 300, 500 and 1000 ppm to prepare nanofluids. The agar disk diffusion and micro-dilution methods were used to study the antibacterial activity. Minimum inhibitory concentration (MIC) against *B. cereus*, *S. aureus*, *S. entrica* and *E. coli* was determined respectively 7.8, 3.9, 15.6 and 62.5 ppm for ZnO NPs. Minimum bactericidal concentration (MBC) against the mentioned bacteria was respectively 15.6, 7.8, 31.2 and 125 ppm for ZnO NPs. *S. aureus* and *E. coli* were respectively the most and the least sensitive species. ZnO nanoparticles improved the antibacterial activity of *M. pulegium* essential oil which shows the potent application of the particles in different industries like food packaging, food and pharmaceutical systems.

1. INTRODUCTION

Many food products are perishable by nature and require protection from spoilage during their preparation, storage and distribution to give them desired shelf-life [1]. Food conservation is based on an intermittent search for foods that have high nutritional quality and microbial stability, and it involves controlling the growth/survival of spoiling and pathogen foodborne microorganisms. The improvement of the shelf-life of foods has an important economic impact by reducing losses attributed to spoilage and allowing the products to reach distant and new markets [2]. Nowadays, the excessive use of synthetic antimicrobial compounds in food manufacture as additive agents is well known, many of which are suspected for their residual toxicity [3]. Because of increasing pressure of consumers and legal authorities, the food industry has tended to reduce the use of chemical preservatives in their products to either completely nil or to adopt more natural alternatives for the maintenance or extension of product shelf life [4]. Several essential oils (EOs) [4] and plant extracts [5] offer potential applications in food preservation which reduce the addition of chemical preservatives

as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organic properties^[3]. Several compounds found in plants, which have long been used as natural agents for food preservation, are generally well accepted. Amongst these naturally occurring compounds, essential oils and extracts of various species of edible and medicinal plants, herbs and spices are considered by the food industry because of their antimicrobial potential.

The aptitude of essential oils to inhibit the growth of certain microorganisms is of paramount importance, particularly, when it is expressed against food-borne pathogens [6] and the antimicrobial activities of plant oils and extracts have formed the foundation of many applications such as in raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [7]. *Mentha* is a distinctive genus of the Dead-nettle family (Lamiaceae, Labiatae) which is instantly recognized by its distinctive minty smell. *Mentha pulegium* has a European-southern temperate distribution, with its absolute northern limit in the British Isles. It is widespread in Europe, with the range extending throughout the Mediterranean, Macaronesia, Asia and North Africa, with the southern range limit reached in Madeira. *M. pulegium* is naturalized in north and South America and is considered an invasive alien species in Australia [8]. The antibacterial effect of *M. pulegium* essential oil has been studied on limited strains [9].

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The antimicrobial efficacy of *Mentha* essential oil has been found to vary from moderate to significant often correlating with the composition of the oil. Besides, the antimicrobial and biofilm formation preventive properties of *M. pulegium* essential oil against *Streptococcus* mutants and *Streptococcus pyogenes* in vitro and in vivo have also been assessed [10]. In another hand, nanotechnology has attracted global attention because nanoparticles (NPs) have properties unique from their bulk equivalents. A common feature of NPs is their antimicrobial activity [11-12]. This technology is capable of providing miscellaneous novel applications that range from innovative fabric compounds, food processing, and agricultural production to sophisticated medicinal techniques [13]. In recent years, ZnO has received considerable attention because of its unique optical, piezoelectric, and magnetic properties [14]. In addition, ZnO NPs has the potential to impact many aspects of food and agricultural system because of its antimicrobial efficacy especially with the growing need to find alternative methods for formulating new type of safe and cost-effective antibiotics in controlling the spread of resisted pathogens in food processing environment [15]. Nano-ZnO has been reported to have extremely good safety profile and no toxicity observed when taken at different nano sizes of the Zinc particles [16-17]. The aim of this study was to assess the antimicrobial activities of nanofluid based on ZnO NPs and *M. pulegium* essential oil against typical food borne pathogens.

2. MATERIALS AND METHODS

2.1. Chemicals materials

Gentamicin (Padtan teb, Iran), methanol, dimethyl sulfoxide (DMSO) and Mueller Hinton Agar (MHA) (Merck, Germany), Mueller Hinton broth (MHB) (Liofilchem, Italy), were purchased.

2.2. Plant material and essential oil

Aerial parts of *M. pulegium* were collected from Khorasan razavi province (Iran) in spring 2015. A voucher specimen for this plant was deposited at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Mashad University, Mashad, Iran. The plants were dried in a dark place at room temperature. Dried leaves were powdered using an electric device and stored in refrigerator (4°C) until use. The essential oil was prepared from 200g leaves of plant by hydro-distillation in 3h using Clevenger [18]. The essential oil was dried over anhydrous sodium sulfate, filtered and stored in refrigerator [19].

2.3. Preparation of ZnO NPs

ZnO NPs were prepared by Sol-Gel method. For this purpose, in a typical experiment, a 0.2M of Zinc tetrachloride (ZnCl₄) stirring with methanol for 2 hours at room temperature (white solution, pH=5) and 1M aqueous solution of sodium hydroxide (NaOH) were prepared in distilled water. Then, the white solution was added drop wise (slowly for 1h) to the above solution under high speed stirring (300 rpm). The beaker was

sealed at this condition for 1 h. Then the solution heated at 220°C for 5 h and ZnO NPs prepared [20].

2.4. Preparation of nanofluid

Specified amounts of ZnO NPs were added to *M. pulegium* of essential oil and DMSO (ratio 3:1) to achieve the final suspension which was sonicated for 10 min at 25°C. The final concentration of ZnO NPs was 0, 300, 500 and 1000 ppm.

2.5. Organisms and inoculation conditions

Authentic pure cultures of bacteria were obtained from Persian Type Culture Collection (PTCC). They included gram positive bacteria; *Bacillus cereus* (PTCC 1015), *Staphylococcus aureus* (PTCC 1431) and gram-negative bacteria; *Salmonella enterica* (PTCC 1709) and *Escherichia coli* (PTCC 1399). They were maintained on agar slant at 4°C and sub cultured on a fresh appropriate agar plates 24 h prior to any antimicrobial test. MHA was used for the activation of bacteria and the MHB was used for the Minimum Inhibitory Concentration (MIC) determinations [21]. Finally, suspensions were adjusted to 0.5 McFarland standard turbidity. Bacterial suspensions were standardized to concentrations of 1.5×10^8 CFU/ml by adjusting the optical density to 0.1 at 600 nm by Shimadzu UV-120-01 spectrophotometer [22].

2.6. Antimicrobial assay

The mentioned nanofluid was tested for antimicrobial activity using agar disc diffusion technique to determine the diameter of growth inhibition zones while broth micro-dilution method was used to determine the MIC and Minimum Bactericidal Concentration (MBC) [23].

2.7. Disk-diffusion method

The antibacterial activity test was carried out on nanofluid including ZnO NPs and *M. pulegium* essential oil using disk diffusion method against the mentioned microorganisms [24]. Sterile filter paper disks (6 mm diameters) were placed on plates containing a suitable medium (MHA) seeded with the test organisms (1.5×10^8). 15µL of the nanofluid samples were poured onto the disks. These plates were kept at 4°C for 15 min to allow maximum diffusion. A number of events take place simultaneously, which includes absorption of water from the agar medium by dried disks and dissolving the material which is under test. The test material diffuses from the disks to the surrounding medium according to the physical law that controls the diffusion of molecules through agar gel [25]. DMSO was used as a negative control, while gentamicin was used as the positive one [26]. Plates were then inverted and incubated at 37°C for 24 h for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the disks and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter [27].

2.8. MIC and MBC Tests

The antibacterial activity of nanofluids was tested using the micro-dilution antibacterial assay for MIC and MBC determination. MIC was determined by the broth micro-dilution method in a 96-wells micro-plate. All tests were performed in MHB. The nanofluids were serially diluted to give concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.81, 3.9, 1.95 ppm. Then, 100µl of the nanofluid was added in a well containing 95µl of MHB and 5µl of inoculum (1.5×10^6 CFU/ml). The micro plate was incubated at 37°C for 24 h. Dilution of the nanofluid corresponding to respective test organism showing no visible growth was considered as MIC. To determine MBC, 10µl broth was taken from each well and inoculated in MHB for 24 hrs at 30 or 37°C. MBC is defined as the lowest concentration the nanofluid at which inoculated microorganism was completely killed (99.99%) [28].

2.9. Statistical analyses

Treatments contained for nanofluid including ZnO NPs and *M. pulegium* essential oil at 10 concentrations in triplicate. Analysis of variance was performed using Statistical Analysis System (SAS; IBM) version 9.1. Statistical significance was judged at the 0.01 level.

3. RESULTS AND DISCUSSION

3.1 Disk-diffusion test

The diameters of inhibition zones varied in the ranges of 28-40, 18-37, 15-32 and 12-30 mm, respectively for positive control and treatments containing 1000, 500 and 300 ppm ZnO NPs. Among the bacterial species, as summarized in Table 1, *B. cereus* was the most sensitive (37 mm) and *S. enterica* had the lowest sensitivity (18 mm).

Table 1: Inhibition zone in diameter (mm) for nanofluid including ZnO NPs and *M. pulegium* essential oil

Microorganism	300 ppm NPs	500 ppm NPs	1000 ppm NPs	Positive control (Gentamicin)	Negative control (DMSO)
<i>B. cereus</i>	28	30	33	36	0
<i>S. aureus</i>	26	27	32	35	0
<i>S. enterica</i>	18	19	21	23	0
<i>E. coli</i>	20	21	24	25	0

3.2 MIC and MBC tests

The MIC and MBC values of the nanofluids are summarized in Table 2, which shows that all treatments were able to prevent the growth of all the four studied microorganisms, including gram-positive and negative bacteria. Results show that ZnO NPs improved the antibacterial activity of *M. pulegium* essential oil.

There are many papers published on antimicrobial activity of different *Mentha* species. Our findings show that there is a good agreement with the findings of Cantore *et al.* [29] who reported that gram-positive bacteria are more sensitive to plant essential oils than gram negative ones. There is evidence in the

literature that essential oils of some Lamiaceae plants possess a moderate to good antibacterial activities [30-31]. The tested essential oils by Hazzit *et al.* showed antimicrobial activity against the microorganisms used, in particular against two important pathogens, *C. albicans* and *H.pylori* [30].

Table 2: MIC and MBC for nanofluid including ZnO NPs and *M. pulegium* essential oil (ppm).

Microorganism	<i>M. pulegium</i> nanofluid	
	MIC	MBC
<i>B. cereus</i>	7.81	15.625
<i>S. aureus</i>	3.9	7.81
<i>S. enterica</i>	15.625	31.25
<i>E. coli</i>	62.5	125

Mahboubi and Haghi (2008) screened the antimicrobial activity of essential oil from flowering aerial parts of Iranian *M. pulegium* L. against different microorganisms. They reported significant activity against gram positive bacteria with MIC values in the range of 0.25-4 ml/ml whereas the least susceptible were gram negative bacteria, especially *E. coli*. The essential oil of *M. pulegium* was earlier found to display good to excellent antimicrobial activities (MIC=1-8 ml/ml) against *E. coli*, *S. aureus* and *C. albicans* [9]. In contrast to the present study, Mirhosseini *et al.* reported the antibacterial activity of ZnO was tested against *L. monocytogenes*, *E. coli*, *S. aureus* and *B. cereus* in apple juice during storage at 25 and 4°C. This study suggested that the application of ZnO NPs as antibacterial agent in food systems and medicine may be effective at inhibiting certain pathogens. Also the same result, carried out to reduce *E. coli* and *S. aureus* in milk samples [32].

Tam *et al.* investigated antibacterial activity of ZnO nano-rods prepared by a hydrothermal method against a gram-negative bacterium *E. coli* and a gram-positive bacterium *B. atrophaeus* [33]. In another study, Jehad *et al.* used nano-ZnO as antimicrobial agent in food systems [34]. Analysis of the results by Saliani *et al.* demonstrated that exposure media of ZnO NPs and cultural factors play a role in their cytotoxic effects. The results showed that ZnO nanofluid had antibacterial activity against *E. coli* and *S. aureus* and the inhibitory effect increased with increasing the nanofluid concentration and the antibacterial activity was influenced by temperature and pH [35].

In the present study, bacterial species including gram (+) and gram (-) bacteria exhibited different degrees of sensitivity to the nano-fluid and essential oil which may be due to the differences in the chemical composition and structure of cell wall of both types of microorganisms [36]. The higher resistance of gram-negative bacteria to external agents has been earlier reported, and it is attributed to the presence of lipopolysaccharides in their outer membranes, which make them inherently resistant to antibiotics, detergent and hydrophilic dyes. The reason for higher sensitivity of the gram-positive bacteria than negative bacteria could be ascribed to the presence of an outer peptidoglycan layer which is an ineffective permeability barrier [37]. The hydrophilic cell wall structure of gram-negative bacteria, constituted

essentially by a lipo-polysaccharide, blocks the penetration of hydrophobic components of oils and for this reason, gram-positive bacteria are found to be more sensitive to the essential oils effects [38]. Nanoparticles have larger surface area available for interactions, which enhances bactericidal effect than the large sized particles; hence, they impart cytotoxicity to the microorganisms [39]. Studies suggest that when bacteria were treated with zinc oxide nanoparticles, changes took place in its membrane morphology that produced a significant increase in its permeability affecting proper transport through the plasma membrane, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane, resulting into cell death [40].

It is observed that zinc oxide nanoparticles have penetrated inside the bacteria and have caused damage by interacting with phosphorus and sulfur containing compounds such as DNA. Zinc oxide tends to have a high affinity to react with such compounds. Generally it is believed that nano-materials release ions, which react with the thiol groups (-SH) of the proteins present on the bacterial cell surface. Such proteins protrude through the bacterial cell membrane, allowing the transport of nutrients through the cell wall. Nano-materials inactivate the proteins, decreasing the membrane permeability and eventually causing the cellular death [41].

4. CONCLUSIONS

This is the first study to provide data on the nanofluid including ZnO NPs and *M. pulegium* essential oil. Our findings showed synergistic effect between the nanoparticles and essential oil. As ZnO is a nutritive additive with edible application, so using the nanofluid as a natural antimicrobial is possible for controlling food-borne pathogens in food systems, packaging and also for pharmaceutical application, however it is suggested to study the possible mechanism of binding the NPs with essential oil which improves the antimicrobial activity.

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