

Antimicrobial Effect of Nanofluid Including Zinc Oxide (Zno) Nanoparticles and *Trachyspremum Copticum* Essential Oils on Food-Borne Pathogens

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Received: June 06, 2016; Accepted: June 23, 2016; Published: June 30, 2016

Abstract

This study was carried out to evaluate the *in vitro* antibacterial activity of nanofluid based on *Trachyspremum copticum* essential oils and Zinc oxide (ZnO) nanoparticles against different bacterial species. The essential oil was obtained by hydro-distillation and analyzed by gas chromatography-mass spectrometry (GC-MS). The agar disk diffusion and micro-dilution methods were used to study the antibacterial activity. GC-MS analysis of the essential oil revealed 16 compounds, representing 99.61% of the total oil in which Thymol (29.17%) and ρ -cymene (27.17%) were the main components. The diameters of inhibition zones varied in the ranges of 28-33, 18-37, 17-32 and 16-29 mm, respectively for positive control and treatments containing 1000, 500 and 300 ppm ZnO NPs. Minimum inhibitory concentration (MIC) of ZnO against *B. cereus*, *S. aureus*, *S. entrica* and *E. coli* was determined respectively 250, 250, 500 and 500 ppm. Minimum bactericidal concentration (MBC) of ZnO against the mentioned bacteria was respectively 250, 500, 1000 and 500 ppm. *B. cereus* and *E. coli* were respectively the most and least sensitive species against the nanofluid. ZnO NPs improved the antibacterial activity of *T. copticum* essential oil and show the potent application of the particles in different industries like food packaging, food and pharmaceutical systems.

Keywords: Nanofluid; *Trachyspremum copticum*; Antibacterial activity; Zinc oxide; Nanoparticles; GC-MS

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].

Trachyspermum copticum is an aromatic herb used for its medicinal values against fungi, bacteria. Micro-propagation can be used for large-scale multiplication of essential oil producing plants thus avoiding an overexploitation of natural resources [8]. *T. copticum* which belongs to the family Apiaceae includes 428 species widespread all around the world. Several studies investigated chemical composition of the essential oil of *T. copticum* and focused on their antimicrobial activities [9]. Recently, the essential oils of various species of the *T. copticum* have been screened for their traditional indigenous uses and investigated intensive as promise sources of antibacterial, antifungal, antioxidant and other natural products [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]. This technology is capable of providing miscellaneous novel applications that range from innovative fabric compounds, food processing, and agricultural production to sophisticated medicinal techniques [12]. In recent years, ZnO has received considerable attention because of its unique optical, piezoelectric, and magnetic properties [13]. 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 types of safe and cost-effective antibiotics in controlling the spread of resisted pathogens in food processing environment [14]. 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 [15].

The aim of this study was to assess the antimicrobial activities of nanofluid based on ZnO NPs and *T. copticum* essential oil against typical food borne pathogens.

Materials and Methods

Chemicals materials

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

Plant material and essential oil

T. copticum essential oil (Gol Ghatre Toos, Iran) was purchased and stored in a sterilized vial at 4°C until use.

Gas chromatography/mass spectroscopy

GC-MS analysis was carried out using Shimadzu-QP2010SE GC/MS operating at 70 eV ionization energy, equipped with a Restek-5 capillary column (phenyl methyl siloxane, 0.25 µm film thicknesses) with Helium as the carrier gas, flow rate 0.9 ml/min and a split ratio of 1:20. Retention indices were determined using retention times of n-alkanes that had been injected after the oil under the same chromatographic conditions. The retention indices for all components were determined according to the Van Den Dool method using n-alkanes as standards. The compounds were identified by comparison of retention indices (RRI, HP-5) with those reported in the literature and by comparison of their mass spectra with the Wiley and Mass Finder 3 libraries or with the published mass spectra. Data was obtained from qualitative and quantitative determination of the oil sample.

Preparation of Zinc oxide NPs

ZnO NPs were prepared by Sol-Gel method. To prepare ZnO NPs, 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 1 h) to the above solution under high speed stirring (300 rpm). The beaker was sealed at this condition for 1 h. Then the solution was heated at 220°C for 5 h and ZnO NPs was prepared [16].

Preparation of nanofluid including *T. copticum* essential oil and ZnO NPs

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

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 Entrica* (PTCC 1709), *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 MIC determinations [17]. Finally, suspensions were adjusted to 0.5 McFarland standard turbidity. Bacterial suspensions were standardized to concentrations of 1.5×10^8 CFU ml⁻¹ by adjusting the optical density to 0.1 at 600 nm by Shimadzu UV-120-01 spectrophotometer [18].

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 MBC [19].

Disk-diffusion method

The antibacterial activity test was carried out on nanofluid including ZnO NPs and *T. copticum* essential oil using disk diffusion method against the mentioned microorganisms [20]. 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). 15 μ L of the nanofluids samples containing 300, 500 and 1000 ppm NPs 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 [21]. DMSO was used as a negative control, while gentamicin was used as the positive one [22]. 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 [23].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (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, 100 μ l of the nanofluid was added in a well containing 95 μ l of MHB and 5 μ l of inoculum (1.5×10^6 CFU ml⁻¹). 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, 10 μ l broth was taken from each well and inoculated in MHB for 24 h at 30°C or 37°C. MBC is defined as the lowest concentration the nanofluid at which inoculated microorganism was completely killed (99.99%) [24].

Statistical analyses

Treatments contained for nanofluid including ZnO NPs and *T. copticum* essential oils at 10 concentrations in triplicate. Data were analysed using Excel 2010 and results reported as $X \pm SD$.

Results

Chemical composition of *T. copticum* essential oil

The chemical composition of *T. copticum* essential oil is summarized in TABLE 1. According to the obtained data, 16 compounds were identified, representing 99.61% of the total oil. Thymol (29.17%) and ρ -cymene (27.17%) were the main components, followed by γ -terpinene (19.27%), phenol (8.84%), benzenemethanol (5.27%), carvacrol (4.40%), β -pinene (1.27%) and ρ -cymene α -ol (1.04%).

TABLE.1. Chemical composition of essential oil from *T. copticum* analyzed by GC-MS.

S.No	Compound ^a	RI ^b	Rt ^c	%
1	Cyclohexane	677	9	0.3
2	α Pinene	933	9.3	0.5
3	β -Pinene	980	11.4	1.3
4	β -Myrcene	991	12.2	0.4
5	ρ Cymene	1026	14.3	27.2
6	γ -Terpinene	1059	16.1	19.3
7	Bornanone	1135	20.8	0.5
8	Benzene	642	22.1	0.4
9	Cuminic aldehyde	1239	25.3	0.4
10	Thymol	1290	28.1	29.2
11	Phenol	1149	28.2	8.8
12	ρ -Cymen- γ -ol	*	28.2	1
13	Carvacrol	1244	28.4	4.4
14	Benzenemethanol	1186	33.3	5.3
15	Croweacin	*	37.2	0.7
16	Caryophyllene oxide	1573	39.5	0.4

Results of disk-diffusion test

The diameters of inhibition zones varied in the ranges of 28-33, 18-37, 17-32 and 16-29 mm, respectively for positive control and treatments containing 1000, 500 and 300 ppm ZnO NPs. Among the bacterial species, as summarized in TABLE 2, *B. cereus* was the most sensitive (37 mm) and *S. enterica* had the lowest sensitivity (16 mm).

TABLE. 2. Inhibition zone in diameter (mm) for nanofluid including ZnO NPs and *T. copticum* essential oil.

Microorganism	300 ppm NPs	500 ppm NPs	1000 ppm NPs	Positive control (Gentamicin)	Negative control (DMSO)
<i>B. cereus</i>	29 \pm 2.9	32 \pm 0.9	37 \pm 2.8	33 \pm 3.1	0
<i>S. aureus</i>	17 \pm 1.4	19 \pm 2.6	23 \pm 1.6	31 \pm 1.9	0
<i>S. enterica</i>	16 \pm 1.2	17 \pm 0.8	18 \pm 0.9	28 \pm 1.8	0
<i>E. coli</i>	19 \pm 3.1	20 \pm 3.3	22 \pm 2.7	30 \pm 2.1	0

All values were expressed as mean \pm SD of duplicate determination.

Results of MIC and MBC

The MIC and MBC values of the nanofluids are summarized in TABLE 3, which shows that all treatments were able to prevent the growth of all the four studied microorganisms, including gram-positive and negative bacteria.

TABLE. 3. MIC and MBC for nanofluid including ZnO NPs and *T. copticum* essential oils (ppm).

Microorganism	<i>T. copticum</i> NF (ppm)	
	MIC	MBC
<i>B. cereus</i>	208.3 ± 71.2	416.7 ± 144.3
<i>S. aureus</i>	250 ± 0	666.7 ± 288.7
<i>S. enterica</i>	416.7 ± 144.3	1000 ± 0
<i>E. coli</i>	416.7 ± 144.3	833.4 ± 288.7

All values were expressed as mean ± SD of triplicate determination.

Discussion

Application of nanotechnology in food industry has different aspects and using nano particles is one of the best examples. ZnO, silver and gold nano particles are widely studied and used in food systems and specially packaging. These nano particles might be used directly on a carrier such as essential oils which are widely used for their antioxidant and antibacterial activities. There are many papers on antimicrobial activity of different varieties of *T. copticum*. Usha et al. showed that ethanolic extract of *T. copticum* had antibacterial activity against *Pseudomonas spp.* [25]. Murthy et al. indicated that *T. copticum* extract was effective on *B. cereus* with 48 mm zone [26]. Hazzit et al. showed antimicrobial activity of the essential oil against *C. albicans* and *Helicobacter pylori* [27]. Kaure et al. indicated to antibacterial activity of *T. copticum* essential oil against *Enterococcus faecalis* and *S. aureus* [28]. Malekinejad et al. indicated that *T. copticum* essential oil had MIC value of 1.25 mg/ml against bacterial species [29].

Also different studies have been done on ZnO antimicrobial activities. Mirhosseini et al. [30] reported the antibacterial activity of ZnO against *L. monocytogenes*, *E. coli*, *S. aureus* and *B. cereus* in apple juice. This study suggested that the application of ZnO NPs as antibacterial agent in food systems and medicine may be effective to inhibit certain pathogens. Also the same result, carried out to reduce *E. coli* and *S. aureus* in milk samples [30].

Tam et al. investigated antibacterial activity of ZnO NPs prepared by a hydrothermal method against *E. coli* and *B. atrophaeus* [31]. In another study, Jehad et al. used nano-ZnO as antimicrobial agent in food systems [32]. Salianni et al. showed that ZnO had antibacterial activity against *E. coli* and *S. aureus* and the inhibitory effect increased with increasing the concentration and the antibacterial activity was influenced by temperature and pH [33].

In the present study, bacterial species including gram (+) and (-) 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. 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 [34].

Nanoparticles have larger surface area available for interactions, which enhances bactericidal effect than the large sized particles, hence, they impart cytotoxicity to the microorganisms [35]. 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 [36], leaving the bacterial cells incapable of properly regulating transport through the plasma membrane, resulting into cell death [37].

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 [38]. 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 [39].

Conclusions

This is the first study to provide data on the effect of nanofluid including ZnO NPs and *T. copticum* essential oil against pathogenic bacteria. Results showed synergistic effect between ZnO NPs and *T. copticum* essential oil. Findings suggest the possibility of using the mentioned nanofluid as natural antimicrobials in food industry to control food-borne pathogens and to be used as a natural preservative in food systems against the well-known causal agents of foodborne diseases and food spoilages.

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