



Evaluation of the Antimicrobial Effects of Nano-Essences of *Allium sativum* L., *Thymus vulgaris* L., *Mentha piperita* L., and *Chamaemelum nobile* L. on Common Causes of Otitis Externa

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Received: 28 June 2017
Accepted: 19 March 2018
ePublished: 17 April 2018

Abstract

Background: Inflammation or infection of the ear that refers to as otitis is a common ear disorder. The otitis externa infection involves outer ear and ear canal. *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* can be pointed out as the common causative agents of this infection. Due to the detrimental effects of chemical drugs on humans, utilizing the new formulation of herbal essential oils, such as nano-encapsulation is considered owing to the more efficient and reduction of adverse effects resulting from the direct application of essential oils. In this in vitro study, the antimicrobial effects of 4 nano-essences were compared to pure essential oils against four major microorganisms involved in otitis externa (namely, *S. pyogenes*, *S. aureus* and *P. aeruginosa*).

Methods: To evaluate the antibacterial effects of nano-essences, minimum inhibitory concentration (MIC) quantitative determination and minimum bactericidal concentration (MBC) were first carried out, and then qualitative disk diffusion tests were conducted.

Results: The MIC quantitative test results demonstrated that among the evaluated essential oils, garlic nano-essence had the most antimicrobial effect at the lowest concentration. Besides, the results showed that nano-essences had lower effect compared to pure essential oils. Diffusion disc test results revealed that nano-essences could not be released from paper discs diffusion in solid media.

Conclusions: Results suggested great antibacterial effects of nano-essences of garlic, thyme, peppermint, and chamomile on 3 strains of bacteria involved in otitis externa and it can be promised to produce new drugs, with lower side effects in eliminating these pathogens.

Keywords: Chamomile, Garlic, MIC, MBC, Nano-essence, Peppermint, Thyme



Background

Otitis externa or infection of outer ear, also called “swimmer’s ear” (1), is an inflammation or infection of the outer ear canal, which can be extended from the external ear to the eardrum. The incidence rate of the disease is 1% annually and it has a lifetime prevalence of 10%. On acute occasions, the infection invades the surrounding soft tissue and bone leading to malignant (necrotizing) otitis externa. Bacteria and fungi are common causative agents of this infection (1-3). *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* are the most common isolates (1-3). In recent years, according to studied chemical drug side-effects, people have been using medicinal plants to treat disease. The most advantages of medicinal plants are that they are cheap, accessible, and easy to use (4-7).

In various aromatherapy references, herbal essences such as garlic (*Allium sativum* L.), thyme (*Thymus vulgaris* L.), peppermint (*Mentha piperita* L.), and chamomile (*Chamaemelum nobile* L.) essential oils are established as antimicrobial agents (8,9). The garlic essence contains sulfurs, vinyl polysulfure, allyl, and allyl propyl; thyme essential oil mainly contains thymol (30%-70%) and carvacrol (15%-30%). Peppermint has tannins and chamomile major components are ethers of angelic acid, tiglic acid, free butyric acid, and azulene (8,9). Essence metabolism is faster than chemical medicine with least side effects. Due to some drawbacks such as volatility, low water solubility, and oxidation capability, it is necessary to develop new techniques with high antimicrobial activity, novel formulations, and modifications, which can be used on essence oils for improving their efficacy,

quality, and biological activity at the lowest concentration (10,12). Thus, it is widely used in recent years. In order to enhance the efficiency of herbal components, they can be used based on nanoscience. Several studies reported that herbal components have active ingredients that can be used for their antimicrobial effects (4-7,13). Nanogels or nanocapsules are the most common techniques for nanoformulations. Polymeric nanocapsules are produced by a simultaneous polymerization process in various sizes and shapes (10,11). Chitosan as a non-toxic, natural as well as biodegradable bio-polysaccharide, is one of the chitin products that can be applied as an antimicrobial agent. A powerful antimicrobial effect has been reported for chitosan on a wide range of microorganisms such as gram-positive and gram-negative bacteria as well as fungi (14,15). The antimicrobial activities of nanoencapsulated essential oils of garlic, thyme, peppermint, and chamomile are not fully evaluated yet. In this study, the antimicrobial properties of nanoencapsulated form of essential oils on common bacterial agents of otitis externa were explored.

Methods

Bacterial Strains

Streptococcus pyogenes PTCC (1447) CIP (5405), *Staphylococcus aureus* PTCC (1112) ATCC (6538) and *P. aeruginosa* PTCC (1430) ATCC (27853) were used in the present study. All microbial strains were purchased from Iranian Centre of Industrial and Medical Fungi and Bacteria Collection.

Culture Media

Brain Heart, Muller Hinton agar, Blood agar and Infusion broth were used purchasing from Merck Chemical Company.

Essential Oils

All essential oils including *Allium sativum* L., *Thymus Vulgaris*, *Mentha piperita* L., and *Chamaemelum nobile* were purchased from Barij Essence Co. Kashan, Iran.

Experimental Methods

In the present study, all standard strains were cultured on Muller Hinton and Blood Agar after being transferred to the laboratory. Culture media were placed in an incubator at 37°C for 24 hours. Then, minimum bactericidal concentration (MBC), minimum inhibitory concentration (MIC), and the disk diffusion assays were carried out using pre-prepared nano-encapsulated essential oils on Muller Hinton agar (13).

Preparing the Nano-Encapsulated Essence

To produce 0.5% chitosan solution, used for nanogel production, 0.5 g chitosan was solved in 1% acetic acid (pH = 3-3.5) and mixed with magnetic stirrer. In order to

homogenize, the solution was sonicated for 20 minutes. Carbodiimide myristic acid was added to the chitosan solution and sodium hydroxide (0.1 M) was used to adjust pH in the range of 4.5-6.5. The obtained viscous gel was centrifuged 3 times and absolute ethanol was added to the gel to remove any impurity. Eventually, in order to obtain 5000 ppm of nano-essence, about 25 µL essence was added to 5000 µL of nanogel, and then it was placed in an ultra-sonicate device for 5 minutes (13).

Morphological Assay of Nanocapsule with Electron Microscope

Transmission electron microscope (TEM) and scanning electron microscope (SEM) were used to study the surface and shape of nanocapsule walls. In order to prepare the samples, the obtained formulation was diluted with distilled water, and then was placed in ultrasonic device for 15 minutes at 40 W.

Microbial Suspension

Microbial suspension was prepared by transferring 4-5 colonies from (24 hours) the sterile serum culture, and then incubated at 37°C. After 2-5 minutes, the turbidity of suspensions was compared with 0.5 McFarland turbidity standard, and physiologic serum or fresh colony was applied to adjust the turbidity.

To determine the MIC, 100 µL of BHI broth was injected into each well in a standard 96-well plate. A serial dilution of each garlic, thyme, peppermint, and chamomile nano-essences was prepared in 96-well plate (100 µL each well), then, 100 µL of solution was transferred from the first well and then across all columns; finally, 100 µL was discarded from the last well. The maximum and minimum concentrations were observed in the first and last wells, respectively. Next, 100 µL of the microbial suspension was poured into each well. Microbial suspension well with no nano-essence was selected as positive and the highest concentration of nano-essence well with no microbial suspension was used as negative control. Microplates were placed in an incubator for 48 hours. This test was repeated for each bacterium and nano-essence twice in sterile conditions. Microplates were observed visually and measured at 540 nm by ELISA reader. The lowest concentration with inhibition of growth considered the MIC for the organism (13).

Determination of MIC

First 100 µL of BHI broth was added to each well of standard 96-well plate. A serial dilution of each garlic, thyme, peppermint, and chamomile nano-essences was prepared in 96-well plate (100 µL each well), then, 100 µL of solution was transferred from first well to the second and this 1:2 dilution inoculated across all columns. Eventually, 100 µL of the solution was removed from the last well. The highest and lowest concentrations

were observed in the first and the last wells, respectively. Then, 100 μL of the standardized microbial suspension was added to each well. Two separate wells were used as positive (microbial suspension without any nano-essence) and negative control (highest concentration of nano-essence without any microbial suspension). Microplates were incubated for 48 hours at 37°C (16-17). This test was performed twice for each bacterium and nano-essence under sterile conditions. Microplates were observed visually and measured at 540 nm by ELISA reader. The lowest concentration with growth inhibition was considered as the MIC of the organism (13,16,17).

Determining MBC

In order to determine the MBC, MIC concentration and the concentration before and after MIC were cultured on Blood agar and Muller Hinton agar (50 μL from each well was added to culture medium). The culture media were incubated at 37°C, and then were assayed. In order to form and grow colonies, the concentration in which the 99.9% of bacteria were killed, evaluated as the MBC (13,16,17).

Disc Diffusion Test

After inoculation of microbial suspension (adjusted to 0.5 McFarland standard) to the Muller Hinton agar medium with swab, 9 mm discs dripped with 10 μL nano-essence were placed on agar surface in a regular order. Plates were incubated for 48 hours at 37°C. The *S. pneumoniae* and *S. pyogenes* were cultured on blood agar. Some of the commonly antibiotic discs were applied as positive controls (13).

Results

Evaluating the Surface Morphology of Nanocapsules With Electron Microscope

Figure 1 shows the SEM surface of nanocapsules in which the essence is trapped and released slowly. The image of

TEM (Figure 2) illustrates the core and wall structure of the nanocapsules. The exterior surfaces of nanocapsules are smooth and uniform.

Determining the Size of Nanocapsules

In order to determine the size of particles in liquid, dynamic light scattering was used. The distribution of the size of nanoencapsulated essences is shown in Figure 3.

Fourier Transform Infrared Spectroscopy of Nano-Essences Components

The Fourier transform infrared (FTIR) spectroscopy is used in biological evaluation as a powerful tool. Figures 4-6 depict the infrared spectroscopy of nanogel major components. The peaks resulted from mathematical equations on FTIR spectrum can be used to obtain information quickly and easily in relation to dose-response curve as well as pharmacological and toxicological information (16).

The Antimicrobial Activity of Nano-Essences

Results of MICs tests were as an indicator of high antimicrobial effects of nanoencapsulated essences of garlic and thyme on bacterial strains when compared to those of peppermint and chamomile (Tables 1 and 2). Measurements were carried out in duplicates and revealed similar results. In disc diffusion test, no inhibition zone

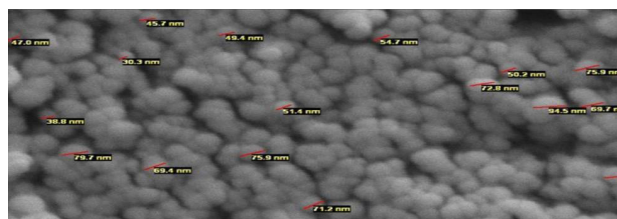


Figure 1. SEM of Chitosan Nanocapsules Containing Essence (SEM, Tescan, Vegall).

Table 1. The Results of MICs Determination of Nanoencapsulated Essential Oils of Garlic (*Allium sativum* L.), Thyme (*Thymus vulgaris*), Peppermint (*Mentha piperita* L.), and Chamomile (*Chamaemelum nobile*) on All Tested Microorganisms ($\mu\text{g/mL}$)

Microrganism Nano-Essence	<i>Staphylococcus aureus</i>	<i>Streptococcus pyogenes</i>	<i>Pseudomonas aeruginosa</i>
<i>Allium sativum</i> L.	0.390	0.195	1.562
<i>Thymus vulgaris</i>	0.781	0.781	1.562
<i>Mentha piperita</i> L.	6.25	6.25	6.25
<i>Chamaemelum nobile</i>	3.125	0.195	3.125

Table 2. The Results of MBCs Determination of Nanoencapsulated Essential Oils of Garlic (*Allium sativum* L.), Thyme (*Thymus vulgaris*), Peppermint (*Mentha piperita* L.), and Chamomile (*Chamaemelum nobile*) on All Tested Microorganisms ($\mu\text{g/mL}$)

Microrganism Nano-Essence	<i>Staphylococcus aureus</i>	<i>Streptococcus pyogenes</i>	<i>Pseudomonas aeruginosa</i>
<i>Allium sativum</i> L.	12.5	25	-
<i>Thymus vulgaris</i>	-	-	0.195
<i>Mentha piperita</i> L.	12.5	3.125	12.5
<i>Chamaemelum nobile</i>	25	25	25

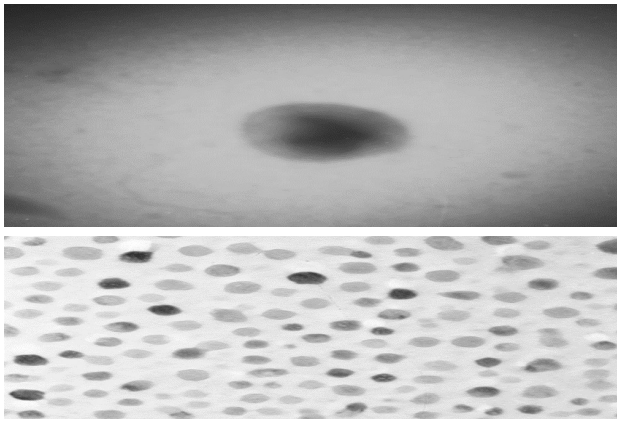


Figure 2. TEM of Chitosan Nanocapsules Containing Essence. (TEM, Philips, EM 208).

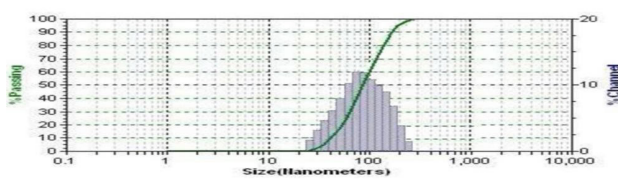


Figure 3. Dynamic Light Scattering Diagram of Distribution of the Size of Nanoencapsulated Essences.

was observed. The tests were repeated and evaluated for multiple times. Apparently, the essences were not released from nanocapsules on paper discs diffused on agarose.

Discussion

The common causative bacteria and fungi for otitis external include *P. aeruginosa* (swimmer's ear), *S. aureus* and group A streptococci (1-3). For treatment of these infections in severe cases, antibiotic drops such as ciprofloxacin or polymyxin eardrops are applied using oral routes. In addition to antibiotic treatments, pH of ear environment should be lowered to limit the microbial growth. To achieve this goal, acetic acid 1%-2% or boric acid in alcohol solution was exploited. Today antibiotic-based therapy of these infections is facing many problems that limits its application. Therefore, herbal resources have been taken a huge condensation due to their lower adverse effects and high efficiency. Previous literature has studied the antimicrobial activities of herbal essences in

Iran and other countries (4-9). Due to some drawbacks of herbal essences such as volatility, low solubility in water, and oxidation capability, developing new technologies is necessary to increase the antimicrobial activity of essences at the lowest concentration before utilizing them in the control and treatment of diseases. Harnessing nanoscience for formulation and manipulation of essences is one the most common techniques that can be used for increasing the quality and effect and also to prolong biological activities at minimum concentration (10,12). Few studies have been conducted on nanoencapsulated essences so far. Review of studies conducted in Iran and other regions have demonstrated the antimicrobial activities of herbal essential oils (4-9). The previous study concluded that nasturtium nano-encapsulated extract could inhibit reproduction of cancer cells in similar concentration and interval compared to non-capsulated extract (18). Our results were in line with their finding and the antimicrobial effects in nano-encapsulated essential were more than pure essential oils. In addition, Negahban et al studied the effect of Artemisia and Thyme nano-encapsulated essential oils on a plant pest in which their results represented an increase in efficacy and environmental sustainability and improvement of controlled release of these materials using nanotechnology (11). In the another study, MICs of thyme and eucalyptus essential oils antibacterial test were conducted against *S. aureus*. The results showed that thyme essence had the lowest MIC in comparison with other essential oils. The results of this study also confirmed that thyme essence had no bacteriostatic effect on *S. aureus*. However, nanoencapsulated essential oil had the MIC value of 0.781 µg/mL (19). Our results also demonstrated that thyme essential oil did not have any inhibitory effect on *S. aureus*. Hashemi et al studied the antibacterial activities of thyme against *P. aeruginosa*. It was revealed that thyme essential oil had a significant antimicrobial effect on *P. aeruginosa*. Moreover, this study showed that among all evaluated essential oils, thyme had the strongest effect on *P. aeruginosa* (20). In accordance with these results, our study demonstrated that peppermint essential oil (6.25 µg/mL) had a significant inhibitory effect on *S. aureus*. Likewise, Mahboobi and Feizabadi studied the antimicrobial effects of Thyme, Savory, and Eucalyptus on some bacterial species. Their results demonstrated higher

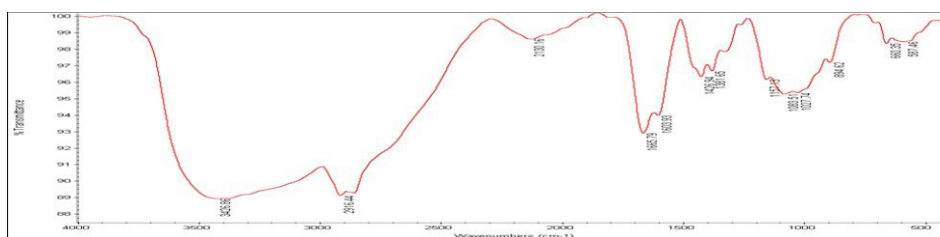


Figure 4. Infrared Spectroscopy of Chitosan Bio Polysaccharide Constituents.

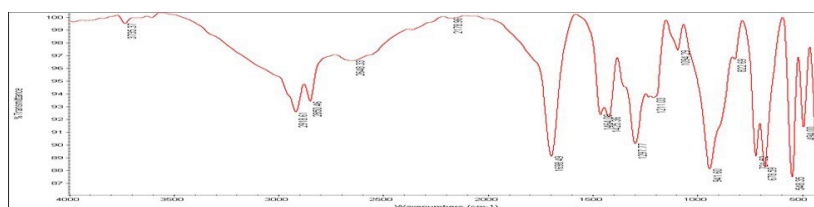


Figure 5. Infrared Spectroscopy of Myristic Acid.

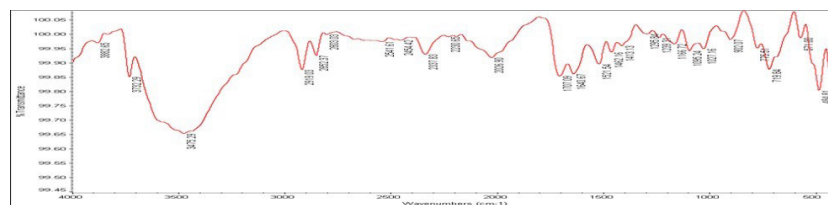


Figure 6. Infrared Spectroscopy of Chitosan and Myristic Acid After Bonding.

effects of Thyme and Savory compared to Eucalyptus (21). In this study, the results showed the higher effects of thyme essential oil on *P. aeruginosa*, *S. aureus*, and *S. pyogenes*, respectively. Furthermore, the MIC micro plates were checked and read using a standard ELISA reader. According to the results, there was no significant difference between the 2 studied methods ($P > 0.05$). Additionally, similar results were found in the evaluation of 42 and 72 hours incubation ($P > 0.05$) indicating that the active constituents of essences released at the initiating hours and increasing the incubation time did not have any significant effect on antimicrobial activity of essences.

Conclusions

The result showed that the antibacterial effects of nano-essences of garlic, thyme, peppermint, and chamomile on the 3 strains of bacteria involved in otitis externa and it can be promised to produce new drugs, with lower side effects in eliminating these pathogens.

Conflict of Interests

Authors do not have any conflict of interest.

Acknowledgements

The authors would like to thank Tehran University of Medical Sciences for providing all of the support for this study.

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