Antimicrobial activity of essential oils against food-borne bacteria evaluated by two preliminary methods

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Abstract

The purpose of this research was to evaluate the in vitro antimicrobial activity of seven essential oils against two Gram-positive bacteria (Staphylococcus aureus ATCC 25923, Bacillus cereus ATCC 11778) and two Gram-negative bacteria (Escherichia coli ATCC 25922, Salmonella enteritidis ATCC 13076) using two preliminary methods: agar disc diffusion method and disc volatilization method.

Results showed that all seven essential oils presented antibacterial activity against all the test strains in direct contact method. On the other hand, only two EOs presented significant antibacterial effect through volatilization method against test bacteria. Oregano oil, clove bud oil and white thyme oil showed maximum activity against all the bacteria tested in direct contact method, having a greater inhibition diameter than the reference control (streptomycin 50 mg/ml).

The results support the high efficacy of oregano, white thyme and clove bud essential oils to control pathogenic microorganisms and their use in developing new systems to prevent bacterial growth, extend the shelf life and increase the safety of the processed food.

Key words: essential oils, food-borne pathogens, antibacterial activity, agar diffusion method, vapor phase activity

Introduction

Food safety is a known problem worldwide, affecting hundreds of millions of people that suffer from contaminated food. World Health Organization (WHO) defines this issue as “one of the most widespread health problems and a major cause of the reduction in economic productivity” [21].

Nowadays, consumers are continuously concerned with the growing number of illness caused by some pathogenic and spoilage microorganisms in food and also for the safety of foods containing synthetic preservatives. Thus, it shows a growing interest about the replacement of synthetic preservatives with natural, effective and nontoxic antimicrobial compounds. There is growing interest in using natural antimicrobial compounds, such as extracts and essential oils (EOs) of spices and herbs, for food conservation. Essential oils are volatile oily liquids obtained from different plant parts and widely used as food flavors [3]. They are variable mixtures of essential terpenoids, especially monoterpenes (C10) and sesquiterpenes (C15), although diterpenes (C20) may also be present, and of a variety a low molecular weight aliphatic hydrocarbons, acids, alcohol, aldehydes, phenolic compounds, acyclic esters, or lactones [19]. The composition, structure, as well as functional groups of the oils play an important role in determining their antimicrobial activity. It has been demonstrated that the essential oils exercises their antimicrobial activity by causing structural and functional damages to the bacterial cell membrane. It is also indicated that the optimum range of hydrophobicity is involved in the toxicity of the essential oils [11].
Essential oils from spices and herbs are the most promising natural antimicrobials, because they do not cause microbial resistance due to the diversity of mechanisms of action. They have a GRAS status given by the U.S. Food and Drug Administration [10], meaning that they are generally recognized as safe for human consumption without limitations on intake and commonly accepted by consumers.

The purpose of this research was to create directly comparable, qualitative data on the capacity of seven essential oils to prevent a diverse range of foodborne and spoilage microorganisms strains based on two preliminary test methods: agar disc diffusion method and disc volatilization method. These two methods were used to screen essential oils for antimicrobial activity, generating the preliminary, qualitative data only, and permitting the selection of the most active essential oils for further evaluation.

Materials and methods

ESSENTIAL OILS

Within this research seven high purity essential oils were used: cinnamon leaf oil (Cinnamomum zeylanicum, CAS No: 8015-91-16), garlic oil (Allium sativum, CAS No: 8000-78-0), onion oil (Allium cepa, CAS No: 8002-72-0), white thyme oil (Thymus vulgaris, CAS No: 8007-46-3), oregano oil (Thymus capitatus, CAS No: 8007-11-2), basil oil (Ocimum basilicum, CAS No: 8015-73-4) and clove bud oil (Eugenia caryophyllata, CAS No: 8000-34-8), obtained by steam distillation, purchased from Sigma Aldrich, Germany.

Essential oils quality parameters (appearance, color, purity, odor, density at -20°C and refraction index at -20°C) were described in an accompanying technical report. These oils were selected based on literature survey with documented antimicrobial activity.

The oils were dissolved in DMSO (Dimethyl sulfoxide, >= 99.0%, CAS No: 67-68-5, Sigma Aldrich, Germany) 1:2 (v/v) to give stock solutions after which they were mixed for total solubility at 180 rpm for 10 minutes. For the bioassay, the stock solutions of essential oils were sterilized by filtration using sterile membrane filters (Millex – GP, pore size 0.22 µm). Until subsequent use, stock solutions of essential oils were stored in a refrigerator at +4°C.

TEST ORGANISMS

In this research were used pure references strains of food-borne microorganisms involved in food toxinfections. The selected test organisms used to evaluate the antimicrobial activity of the essential oils were as follows: Gram positive (Bacillus cereus ATCC 11778, Staphylococcus aureus ATCC 25923) and Gram negative (Salmonella enteritidis ATCC 13076, Escherichia coli ATCC 25922) all purchased from MicroBioLogics, SUA. The source of bacteria strains was ATCC, American Type Culture Collection.

All the bacterial and fungal strains used in this research were a part of the collection of reference strains of Microbiology – ELISA Laboratory from National Institute of Research & Development for Food Bioresources – IBA, Bucharest.

PREPARATION OF TEST ORGANISMS

The cultures of test organisms were maintained in agar slants at +4°C (Plate Count Agar – PCA; Biokar Diagnostics, France) and used as stock cultures.

Bacterial inoculums were obtained from reference stock culture inoculated in TSB medium, which was incubated at 37°C for 18-24 hours. From the fresh grown cultures decimal dilutions were made in sterile PS, up to the concentration of 10⁶ CFU/mL, used for testing essential oils.

IN VITRO ANTIMICROBIAL ACTIVITY TESTING

Because it appears that no standardized test has been developed for evaluating the antimicrobial activity of possible preservatives against food-related microorganisms, the CLSI
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(Clinical and Laboratory Standards Institute) method for antimicrobial susceptibility testing has been modified for testing essential oils. We used two preliminary methods: agar disc diffusion method and disc volatilization method (vapor phase activity) for detecting the most efficient essential oils against test organisms.

**AGAR DISC DIFFUSION METHOD**

The agar diffusion method is the most widespread technique of antimicrobial activity assessment. This method is normally used as a preliminary check and to select between efficient essential oils [15, 2, 13]. The appropriate solidified medium (Plate Count Agar - PCA) was inoculated with 100 µl of bacterial inoculum (10^6 CFU/mL) and spread over the plates using a sterile rod display in order to get a uniform microbial growth on both control and test plates. After inoculum absorption by agar, sterile filter discs (Whatman no 1, England, 6 mm diameter) were impregnated with 10 µl of stock solutions of essential oils and placed on the agar surface using forceps dipped in ethanol and flamed.

Growth cultures containing essential oils were accompanied by DMSO solution as a negative control. Positive control cultures with streptomycin solution (50 mg/mL) were used to assess the susceptibility of tested strains and to compare with them the essential oils efficiency.

All Petri dishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the essential oils. The dishes were left for 30 min at room temperature to allow the diffusion of oil, and then were incubated at 37°C for 24h. After the incubation period, the mean diameter of inhibition halo where test microorganism did not grow (clearly visible inhibition zone) was measured in millimeters, for each disc and evaluated for susceptibility or resistance.

**DISC VOLATILIZATION METHOD** [15]

This method evaluates the activity of essential oils vapors on the same strains, technically near to disc diffusion method. It is used for defining the activity of essential oils, which are to be employed as atmospheric preservatives.

Solidified medium was inoculated with 100 µl of bacterial inoculums. Then, 10 µl of each stock solution of essential oil were added to 6 mm diameter sterile blank filter discs and placed in the center of the cover of the Petri dish in which was previously cast a thin layer of medium to avoid adsorption of essential oils onto the plastic material of the cover. The dishes were then sealed using sterile laboratory parafilm to avoid eventual evaporation of the essential oils, followed by incubation at 37°C for 24h. Blanks were prepared by adding 10 µl of DMSO solution to the filter discs. The effectiveness of the essential oils was calculated by measuring the diameter (in mm) of the zone of microorganism growth inhibition above the disc.

**Results and discussion**

**DIRECT CONTACT VERSUS VAPOUR PHASE METHOD**

Antimicrobial activity of selected essential oils, both by direct contact and vapor phase, against four bacterial species was qualitatively assessed by the presence or absence of the inhibition zone. Each assay in these experiments was repeated three times and the results (mm of zone of inhibition) were expressed as average values (± standard deviation). Bacteria susceptibility to the essential oils, as determined by the agar diffusion method, showed that oils with the highest inhibitory effects produced inhibition zones of 20-46 mm diameter. The antibacterial activity of the selected essential oils is summarized in Table 1.
Table 1. Antibacterial activity of tested essential oils

<table>
<thead>
<tr>
<th>EOs</th>
<th>Direct contact mean diameter of the inhibition zone diameter in mm</th>
<th>Vapor phase</th>
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<tbody>
<tr>
<td></td>
<td>Gram positive</td>
<td>Gram negative</td>
</tr>
<tr>
<td></td>
<td>B. cereus</td>
<td>S. aureus</td>
</tr>
<tr>
<td>White thyme</td>
<td>35.5 ± 0.31</td>
<td>37.7 ± 0.31</td>
</tr>
<tr>
<td>Clove bud</td>
<td>28 ± 0.07</td>
<td>22 ± 0.21</td>
</tr>
<tr>
<td>Oregano</td>
<td>45.2 ± 0.67</td>
<td>43.5 ± 0.42</td>
</tr>
<tr>
<td>Cinnamon leaf</td>
<td>20 ± 0.03</td>
<td>21.2 ± 0.03</td>
</tr>
<tr>
<td>Onion</td>
<td>38.2 ± 1.09</td>
<td>-</td>
</tr>
<tr>
<td>Garlic</td>
<td>25 ± 0.21</td>
<td>-</td>
</tr>
<tr>
<td>Basil</td>
<td>10 ± 0.35</td>
<td>12 ± 0.95</td>
</tr>
</tbody>
</table>

* The diameter of the filter paper disc (6 mm) is included. - No inhibition (< 6 mm diameter)

The results revealed that all the selected essential oils showed antibacterial activity with a higher activity in direct contact method and only two essential oils presented significant antibacterial effect through volatilization method against test bacteria.

Among the essential oils, oregano, clove bud and white thyme oil presented a higher antibacterial activity in direct contact method especially against E. coli and B. cereus, with inhibition zones of 42 mm, 31.2 mm and 39.3 mm and 45.2 mm, 28 mm and 35.5 for B. cereus, having a greater inhibition diameter than the control sample (streptomycin 50 mg/mL). Such an activity could be strictly related to their chemical composition: in fact, carvacrol, thymol and eugenol found in these oils, are able to disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP, deteriorate the cell wall and inhibit production of amylase and proteases [3].

For the reference control results are shown in Table 2. DMSO solvent as negative control presented no antimicrobial activity against the tested strains in any method used proving that is a suitable solvent for testing oils.

Table 2. Diameter of inhibition zones of streptomycin for the test bacteria by direct contact method

<table>
<thead>
<tr>
<th>Reference control</th>
<th>Mean inhibition zone diameter (mm) after 24 h of incubation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Gram positive</td>
</tr>
<tr>
<td></td>
<td>B. cereus</td>
</tr>
<tr>
<td>Streptomycin 50 mg/ml</td>
<td>28 ± 0.1</td>
</tr>
</tbody>
</table>

* The diameter of the filter paper disc (6 mm) is included.

The essential oil with the widest spectrum of activity was found to be oregano oil followed by white thyme oil > clove bud oil > cinnamon oil > garlic oil > onion oil > basil oil, in that order.
White thyme essential oil presented a higher activity against gram-negative bacteria like *S. enteritidis* and *E. coli*, both in direct contact and vapor phase method, while oregano essential oil was most efficient against gram-positive bacteria (*B. cereus* and *S. aureus*). Clove bud essential oil demonstrated antibacterial activity against all the food-associated bacteria with zone of growth inhibition ranging from 22 mm to 31.2 mm, but only in direct contact method. The maximum zone of inhibition showed by this oil was showed against *E. coli* (31.2 mm) followed by *B. cereus* (28 mm), *S. enteritidis* (25.2 mm) and *S. aureus* (22 mm).

Activity was also shown by cinnamon leaf oil and basil oil against the same bacterial strains and with the same amount (10 µl/paper disc), with inhibition zones fewer than 30 mm as those presented by white thyme and oregano essential oils. Cinnamon leaf oil and basil oil expressed a moderate action against tested strains in direct contact method while in vapor phase method only cinnamon leaf oil showed antibacterial activity against *B. cereus* with a growth inhibition diameter of 12.3 mm. Cinnamon leaf oil presented a greater antibacterial activity against gram-positive bacteria than basil oil. Basil oil was more active against gram-negative bacteria *E. coli* (diameter of inhibition of 24.5 mm).

In direct contact method, onion essential oil inhibited weakly the development of gram-negative bacteria; both bacteria tested expressed the same sensitivity against onion oil. On the tested gram-positive bacteria, *B. cereus* was strongly inhibited by onion and garlic essential oils, with a diameter of the inhibition zone of 38.2 mm and 25 mm, respectively. The vapor phase of onion and garlic essential oils presented no inhibition against the test strains, except garlic essential oil that was active against *B. cereus* with a growth diameter inhibition larger than that in direct contact.

The zone of inhibition resulting from the exposure to selected essential oil vapors varied maybe because of the presence of different volatile chemical components. Only oregano and white thyme essential oils vapors exhibited antibacterial activity against all the test strains, activity higher than that present in direct contact method. Zone of inhibition due to vapors generated by 10µl white thyme essential oil was higher (i.e. 48.3 mm against *S. enteritidis*) than oregano oil (43 mm also against *S. enteritidis*). The antimicrobial activity of the essential oils in vapor phase is closely associated with their chemical composition. In the direct contact assays for liquid phase, the activity depends upon the diffusibility and solubility of the essential oil compounds into the agar while the antimicrobial activity of the vapor assay depends upon the volatility of each compound [11].

**Figure 1.** Antibacterial activity (zones of inhibition) of white thyme essential oils in direct contact method (1) and volatilization method (2).
Conclusion

The investigations on antimicrobial activity of seven essential oils against foodborne bacteria confirmed the potential of plant volatile oils to be used in food conservation as alternative to chemical preservatives.

The results suggested that the evaluation techniques described could be used as a preliminary, qualitative step, which can determine the sensitivity of many microorganisms to essential oils and select the oils with the best antimicrobial activity, in order to use them for further evaluations. Disc volatilization method proved to be a useful method for simple screening of antimicrobial activity of vapor phase of essential oils.

In our study, selected essential oils exhibit inhibitory effects against the selected bacterial strains, both in liquid form and volatile vapors. The result of the direct contact method showed that white thyme, oregano and clove bud oil were the most active essential oils against foodborne bacteria. Gram-positive bacteria, B. cereus and S. aureus, as a mean sensitivity against all essential oils tested, were less sensitive than the Gram-negative bacteria E. coli and S. enteritidis, but the difference in susceptibility were not that evident. By disc volatilization method only two essential oils represented by white thyme and oregano oil were found highly effective.

The importance of these preliminary results is that pathogenic bacteria can be controlled using plant essential oils. Further investigations include quantitative tests in order to determine the concentration of essential oils (minimum inhibitory concentration) needed to exhibit antimicrobial activity against food related microorganisms in order to use them as natural antimicrobial agents to extend the shelf life and increase the safety of the processed food.

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