Testing the antimicrobial activity of essential oils

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SUMMARY

The vapor phase of some essential oils proved to have antimicrobial activity. Utilization of the vapor phase of Eos is presently understood as one of the possible alternatives to synthetic food preservatives which could be used in the future. However, testing the vapor phase of EOs against microorganisms causing food-borne diseases (e.g. Salmonella enteritidis or Staphylococcus aureus) or food spoilage is relatively new. Consequently, due to the large number of known EOs, research on their antimicrobial activity is still largely in the phase of in vitro rather than in vivo testing. Moreover, no standard and reliable method for fast screening of a wide range of samples exists. Thus, the aim of this study is to show results concerning tests of the antimicrobial activity of EOs against S. enteritidis or S. aureus, which were conducted by two modifications of the disc volatilization method we developed. The lately developed method has the potential to become widely used for fast screening of EO antimicrobial activity in the vapor phase.

1. Introduction

Food safety is a big issue worldwide. Many steps have been made in securing healthy and safe food for consumers, e.g. the utilization of food preservatives, implementation of HACCP, which has to prevent food from contamination during its production or the implementation of an international system for monitoring the occurrence of dangerous products (RASFF). Despite all these safety measures, occurrence of food borne diseases is still relatively high. For example, 131,468 cases of salmonella, 3,157 cases caused by *Escherichia coli* and 1381 cases of listeriosis were reported in 2008 in EU. Moreover, 5332 food born-outbreaks causing 45,622 human cases, 6,230 hospitalizations and 32 deaths were reported European Union in 2008 (EFSA, 2009).

For this reason, new substances and methods for ensuring food safety and the prolongation of shelf-life are being intensively searched. Among the promising substances which could in the future play an important role in the food industry are essential oils.

Essential oils are natural aromatic oily liquids obtained from plant material (e.g. leaves, flowers, roots) and are present in many plant species. So far, over 3,000 EOs are known; however, only 10% of them are commercially used, mainly as flavors and fragrances and the cosmetics market (Burt et al., 2004). In the 1950s, scientific studies first confirmed the antimicrobial activity of some EOs (Cavallito et al., 1945) which had, in fact, unconsciously been used since antiquity, when different types of species were used for food preservation (Burt et al., 2004). Later studies discovered other qualities, such as antiviral, anti-inflammatory or anti-nociceptive activity, of which EOs dispose (Adorjan and Buchbauer, 2010). Nevertheless, not until recently were these properties in the focus of scientists and the food industry. The situation changed in the last decade, when consumer demand for healthy and green products, rather than products full of synthetic preservatives, started to increase.

Today, when EOs are understood as possible alternatives to synthetic food preservatives, a 'renaissance' in the research on antimicrobial properties of EOs can be observed. However, due to the large number of known EOs, research is still mostly in the phase of *in vitro* rather than *in vivo* testing.

Furthermore, no standard method of EO antimicrobial activity testing exists. The most common methods are based on direct-contact antimicrobial assays, such as different types of diffusion or dilution methods (Burt et al., 2004; Holley and Patel, 2005; Mann and Markham, 1998; Tripathi and Dubey, 2004). However, high hydrophobicity and volatility of the EOs causes many problems in direct-contact assays e.g., partitioning of the EOs in agar according to their affinity to water in diffusion assays. Another example is the possible influence of emulsifiers or solvents (Tween 80, DMSO), which are used in dilution direct-contact methods (Burt et al., 2004).

The less common and newer methods take advantage of the natural volatility of EOs, which could also have some potential for utilization in the food industry. EOs in the vapor phase have indeed proved to have an inhibiting effect on microorganisms (Delaquis et al., 1999; Innouye et al., 2001; Lopez et al., 2005). However, as mentioned above, no standard method for EO antimicrobial testing based on direct-contact in the vapor phase assays exists. Moreover, most of the methods are time and material consuming, consequently, not suitable for fast screening of large quantities of samples, which is necessary before beginning with *in vivo* tests. As one of the most suitable methods for fast screening of antibacterial activity of EOs in the vapor phase seems to be disc volatilization (or the microatmosphere) method and its further modification was done by us. Thus, the aim of this study is to show some our results to-date, which brought two modifications of the disc volatilization method (in detail described elsewhere (Lopez et al., 2005)) we developed.

2. Materials and methods

2.1. Bacterial strains

The tests were performed on 2 bacteria: Gram-positive *Staphylococcus aureus* and Gram-negative *Salmonella enteritidis*. In case of *S. aureus* following strains were included: Methicilin-suspectible (MSSA) MSSA ATCC 2592, ATCC 25913 and Methicilin-resistant (MRSA) MRSA 43300. In case of *S. enteritidis* ATCC 13076 strain was used. For the tests, Mueller-Hinton agar was purchased from Oxoid (Brno, CZ).

2.2. Antimicrobial assays

2.2.1. Disc dilution method A

In vitro antibacterial activity of 27 EOs in the vapor phase was evaluated by modified disc volatilization method (Lopez et al., 2005) at eight different concentrations (0.0042–0.53 $\mu l/cm^3$). In brief, 15 ml of warm medium was poured into 90 mm plastic Petri dish and 5 ml into its cover, and after solidification the medium in the dish part was inoculated with 20 µl of Tris buffer saline containing 10^6 CFU/ml of the microorganism under study. Medium in Petri dish cover served as a sealing and prevented adsorption of EOs onto the plastic material of Petri dish cover. Then, eight doses of EOs in a two fold dilution manner (32, 16, 8, 4, 2, 1, 0.5, 0.25 µl) were added to 6 mm sterile blank paper disks (Oxoid) and placed on the medium in the cover of each Petri dish. The two lowest doses of EOs were diluted in diethyl ether 1:10, because pipetting of these small volumes was inaccurate, and the discs were left to dry for 1 min. The Petri dishes were incubated at 37 ˚C for 18–24 h. After incubation, the minimum inhibitory concentrations (MICs) were recorded. The MIC (expressed as microlitres of EOs per volume unit of atmosphere above the organism growing on the agar surface) was defined as the lowest concentration which made the inhibition zone clearly visible. Blank discs with and without diethyl ether served as negative control. All tests were carried out in triplicate. This is the first method we used for the evaluation of EO activity.

2.2.2. Disk dilution method B

The tests were then performed in 90 mm PDs divided into four sections. Into each section, 5 ml of warm agar were poured, as well as into the lid. After solidification, three compartments were inoculated by spreading 20 µl of the prepared suspension with different microorganism, fourth was left uninoculated as a contamination control. EOs were diluted in ethyl acetate in a two-fold dilution manner to give final volume of 250 µl. This solution was evenly distributed on a 85 mm round sterile filter paper by micropipette, and the paper was left to dry for 1 minute to evaporate ethyl acetate. Finally, the filter paper was laid into the PD on walls dividing the compartments, so the distance between paper and agar surface was approximately 2 mm, and the PD was hermetically closed with its lid containing solidified agar. The Petri dishes were incubated at 37°C for 18-24 hours. After incubation, the minimum inhibitory concentrations (MICs) were recorded. The MIC (expressed as microlitres of EOs per volume unit of atmosphere above the organism growing on the agar surface) was defined as the lowest concentration, which absolutely inhibit visible growth of the microorganism. Blank filter papers with and without ethyl acetate served as negative control. All tests were carried out in triplicate and were performed in class II microbiological safety cabinet (Faster, Italy).

2.3. Essential oils

In total, the antimicrobial activity of 108 essential oils was tested. One third of the essential oils were obtained from plants cultivated at the experimental field of the Czech University of Life Sciences, Prague. Essential oils were gained by Clevenger hydrodistillation method described in detail elsewhere (Nedorostova, 2009).

The rest of the essential oils were purchased from Essential Oil University (Charlestown, IN, USA). EOs were kept in -18°C until use.

3. Results and discussion

At the beginning of our research, we used disk dilution method A. Through this method, antimicrobial activity of 27 EOs against *S. enteritidis* and *S. aureus* was tested. Thirteen of the EOs were effective at least to one of the strain. The results are shown in *Table 1*.

Table 1

MIC (µl/cm^3) of esential oils in vapor phase obtained by two methods against foodborne bacteria

Essential oils	method A		method B	
	SA	SE	SA	SE
Allium sativum	0.0083	0.26	0.25	-
Armoracia rusticana	0.0083	0.0083	0.03125	0.03125
Caryopteris x clandonensis	0.53	-	nt	nt
Hyssopus oficinalis	0.53	-	nt	nt
Mentha x villosa	0.53	-	nt	nt
Nepeta x faassenii	0.53	-	nt	nt
Ocimum basilicum var. Grant verte	0.53	-	nt	nt
Origanum majorana	0.53	-	nt	nt
Origanum vulgare	0.017	0.13	0.0625	0.0625
Satureja montana	0.033	0.26	nt	nt
Thymus pulegioides	0.033	0.26	nt	nt
Thymus serpyllum	0.033	-	0.250	0.250
Thymus vulgaris	0.017	0.033	0.125	0.125

SA - *Staphylococcus aureus*; SE - *Salmonella enteritidis*, nt – not tested

The most effective inhibitors of *S. aureus* (MIC 0.0083 µl/cm^3) were EOs from *Allium sativum* and *Armoracia rusticana*, followed by *Origanum vulgare* and *Thymus vulgaris* (MIC 0.018 µl/cm^3). Only six EOs were effective against *S. enteritidis*. Again the lowest MIC (0.0083 µl/cm^3) showed *Armoracia rusticana* followed by *Origanum vulgare* (MIC 0.013 µl/cm^3). Similar MICs were obtained for *A. rusticana* when tested against *S. enteritidis, L. monocytogenes, E. coli* and *S. aureus* (Ward et al., 1998), However, the method is not fully comparable.

Mentha x villosa, *Nepeta x faassenii* and *Caryopteris. x clandonensis* showed antimicrobial activity in the vapor phase (Tab.1); this fact has never been reported before.

Due the high susceptibility of *S. aureus* to EOs, second part of the test was focused on antimicrobial activity of 7 most effective EOs against strains MRSA and MSSA strains of *Staphylococcus aureus*. Selected EOs together with their MIC measured for each strain are shown in *Table 2*. Similarly to our previous research, the most effective again all strains was *A rusticana* (MIC 0.0083 – 0.017 µl/cm^3) followed by *Origanum syriacum* (0.0083 – 0.13 µl/cm^3). The least effective was *Thymus serpyllum* (0.033 – 0.530 µl/cm^3). Several studies dealt with EOs activity in vapor phase against MRSA (Edwared-Jones et al., 2004; Roller et al., 2009); however, none of them tested EOs from plants used in our research.

Table 2

MIC (µl/cm^3)of essential oils in vapor phase against *S. aureus* strains

Essential oils	MSSA		MRSA
	ATCC 25923	ATCC 25913	ATCC 43300
Armoracia rusticana	0.0083	0.0083	0.017
Allium sativum	0.0083	0.017	0.26
Origanum syriacum	0.0083	0.0083	0.13
Satureja hortensis	0.017	0.017	0.13
Satureja montana	0.017	0.017	0.13
Thymus serpyllum	0.033	0.033	0.26
Thymus vulgaris	0.033	0.033	0.26

Despite the promising results we obtained from these experiments, the method was still very time and material consuming. Moreover, due to the different volatility of EO components, we came to the conclusion that the resulting inhibition zone would not be the only function of its activity, but it could also strongly depend on the speed of the evaporation of its active constituents when applied to a small area, such as paper disc. For these reasons, we decided to make this method more effective and objective. As a result, we started to apply Disk dilution method B (described in section 2.2.2 of the text). This method eliminates possible problem with different volatility of EOs components. Furthermore, possibility of testing antimicrobial activity of EOs again three microorganisms placed in one Petri dish significantly saves the time, material and consequently, the cost of all tests. For example, so far we tested 81 essential oils in six dilutions against 6 microorganisms in triplicate. Tests required usage of approx. 3,000 Petri dishes, if we had done it by the older method, we would have needed approx. 9,000 Petri dishes.

If we compare the MICs of some EOs measured by method B with MICs of the same EOs tested by the pervious method (Tab. 1) and other researchers (Inouye et al., 2001; Lopez et al., 2005; Maruzzella & Sicurella, 1960), it can be seen that the values of MIC are higher. Nevertheless, we believe the method brings more objective and reliable results for practical use. This is because of the well known fact that, in food model samples, the concentrations needed for microorganism inhibition are higher than that obtained by *in vitro* methods (Burt et al., 2004). This could be partially caused by the fact that, in practice, the MIC has to be achieved in all points of the treated space to ensure adequate effect.

4. Conclusions

Due to the modification of the disk diffusion method, we are able to assay the MIC of wide range of EOs. Thus identify most suitable and also new EOs which will be used in further test concerning research in vivo. Thus, in further research, we will test antimicrobial activity of the most promising EOs (selected by our screening) against several pathogens causing food borne diseases, moreover, against microorganisms causing food spoilage of fresh fruit and vegetable and other ready to eat products.

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