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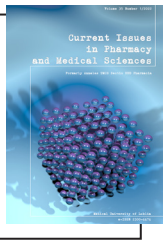

Comparison of disc-diffusion and disc-volatilization assays for determining the antimicrobial activity of *Thymus vulgaris* L. essential oil

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ABSTRACT

Essential oils (EOs), being mixtures of various natural compounds, constitute an attractive source of novel antimicrobial agents. Along with multiple studies concerning their rich biological activity, there are different methods allowing to determine both antimicrobial activity in direct contact, as well as through the activity of vapours. Thyme constitutes one of the most common aromatic herbs used for pharmaceutical, cosmetic and culinary purposes. The aim of the study was to compare the antibacterial activity of liquid and vapour phases of *Thymus vulgaris* L. essential oil (TEO) against most common pathogenic Gram-positive (*Staphylococcus aureus* ATCC 25913, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 10876) and Gram-negative (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853) microorganisms. To determine TEO antibacterial properties, two methods were used: disc-diffusion assay so as to test activity in direct contact; disc-volatilization assay so as to determine activity in gaseous contact.

Statistical analysis showed that Gram-positive bacteria were more sensitive to TEO vapours than Gram-negative (p=0.005), while in the case of disc-diffusion assay, no such differences were evident. Moreover, sensitivity towards TEO differed among Gram-positive bacteria in disc-diffusion (p=0.009) and disc-volatilization assay (p=0.001). Among Gram-negative rods, a statistically higher sensitivity regarding *E. coli* was noted when compared to *S. typhimurium* in both direct (p=0.011) and gaseous (p=0.006) TEO contact. No activity of TEO against *P. aeruginosa* was found.

As thyme essential oil exhibited significant antimicrobial activity against Gram-positive bacteria in gaseous contact, this justifies its use as an addition to inhalation agents to prevent or support the treatment of upper respiratory tract infections. Further studies should therefore apply other methods for determining the antibacterial activity of TEO vapours to assess the exact minimal concentration inhibiting bacterial growth.

INTRODUCTION

Essential oils (EOs) are a mixture of various compounds obtained from plants, mainly by steam distillation. These volatile and lipophilic liquid plant extracts are characterized by a wide range of desirable properties significant for simple aromatherapy, but also cosmetology, dentistry and medicine [1]. Due to their known antimicrobial activity, EOs have already found application in stomatology, in maintaining oral hygiene, as well as in the treatment of gingivitis and periodontal diseases [2]. The high content of volatile biologically active compounds makes them also useful in medicine for inhalation or for external use as warming agents in muscle and joint pain [3].

Thymus vulgaris L. essential oil (TEO) is a known powerful antiseptic EO, exhibiting both antifungal and antibacterial activity. It is extracted from *Thymus vulgaris* L.

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(a member of the *Lamiaceae* family). This small subshrub originating from the Mediterranean Sea area and is widely disseminated in Europe. It reaches 30-40 cm in height and has branched stems and elongated inflorescences with small purple flowers [4,5].

The chemical composition of TEO is well known and includes phenolic derivatives such as thymol, carvacrol, p-cymene, linalool, beta-caryophyllene, myrcene, p-entha-1,3-diene, 4-carvomenthenol, camphene, limonene, 1-octen-3-ol, geraniol or terpinolene. However, the antimicrobial properties of TEO are associated mainly with two phenolic compounds, i.e. thymol and carvacrol [6]. It is worth mentioning that the composition and concentration of compounds in TEO are variable and depend on cultivating region, environmental conditions, and plant chemotype [7]. The mechanisms of antibacterial action of the main TEO compounds – thymol and carvacrol – are not fully understood, except that their hydroxyl groups affect cytoplasmic membrane permeability, lipid ordering and bilayer stability [8]. The monoterpenoids represented by thymol and sesquiterpenoids represented mainly by β-caryophyllene are the two dominant groups of volatile compounds that determine the activity of TEO in gaseous contact [6]. TEO is well-known for its wide biological activity, including anticancer, antimicrobial, anti-inflammatory and antioxidant properties. It is often used in the case of upper and lower respiratory tract infections manifested by coughing, runny nose and sinus inflammation [9]. The inhalation facilitates expectoration, increases the secretion of mucus in the respiratory tract, and stimulates the movements of the ciliary epithelium. Consisting of a wide range of active compounds, it can be a component of various forms of therapeutic agents, e.g. syrups, tablets and drops [10].

The antimicrobial properties of TEO have been relatively well investigated through the use of standard microbiological methods, however, little is known about the activity of volatile compounds alone. Therefore, the aim of our study was to investigate the antibacterial activity of volatile compounds of TEO with the use of disc-volatilization assay so as to screen vapour-phase activity, and to compare the results to the standard disc-diffusion method that is applied to provide data on antimicrobial activity in direct contact.

MATERIALS AND METHODS

Culture conditions and bacterial suspensions

Antibacterial activity of *Thymus vulgaris* L. essential oil (TEO; Etja, Poland) was tested against six reference microbial strains of the most common human pathogens from the American Type Culture Collection (ATCC). These included: gram-positive bacteria - *Staphylococcus aureus* ATCC 25913, *Enterococcus faecalis* ATCC 29212 and *Bacillus cereus* ATCC 10876; gram-negative bacteria – *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028 and *Pseudomonas aeruginosa* ATCC 27853. All reference bacterial strains were cultured on Mueller-Hinton Agar (MHA; Biorad, USA) in aerobic conditions at 35±2°C. Bacterial inoculums used in both disc-diffusion and disc-volatilization assays described in detail below were prepared from overnight cultures on MHA in sterile sodium chloride solution (0.9%; POCH, Poland) to obtain 0.5 mFc density equal to 1.5×10^8 colony forming units (CFU)/mL. The bacterial suspensions were then diluted 100-times in 0.9% NaCl to obtain final cell concentration equal to approximately 1.5×10^6 CFU/mL.

Disc-diffusion assay

For each tested microorganism, 100 µL of bacterial suspension (1.5×10^6 CFU/mL) was plated on MHA (4 mm of thickness) with the use of a sterile microbiological spreader (Medlab, Poland). Next, a sterile blank paper disc of a diameter of 6 mm (Becton Dickinson, USA) was placed in the middle of the MHA (diameter 85 mm) patch and loaded with 10 µL of TEO. The plates were subsequently thoroughly sealed with parafilm to avoid the evaporation of EO and incubated for 48 h at 35±2°C. A growth inhibition zone was defined as a circular zone of no bacterial growth around the paper disc. The inhibition zone was measured after 24 h and 48 h of incubation. Growth control for all tested bacterial strains was performed via sterile paper disc in triplicate.

Disc-volatilization assay

In the disc-volatilization assay, MHA was first poured into a Petri dish bottom plate to obtain a thin medium layer. Afterwards, 100 µL of bacterial suspension ($1.5 \times /10^6$ CFU/mL) was plated on the MHA with the use of a sterile spreader. Next, a sterile blank paper disc was placed in the middle of the petri dish lid and loaded with 10 µL of TEO. The bottom plate was then placed on top of the lid so as to allow vapour contact but not direct contact, and the plates were subsequently thoroughly sealed with parafilm and incubated for 48 h at 35±2°C. A growth inhibition zone was defined as a circular zone of no bacterial growth above the paper disc. The inhibition zone was measured after 24 h and 48 h of incubation. Growth control for all tested bacterial strains was performed via sterile paper disc in triplicate.

Statistical analysis

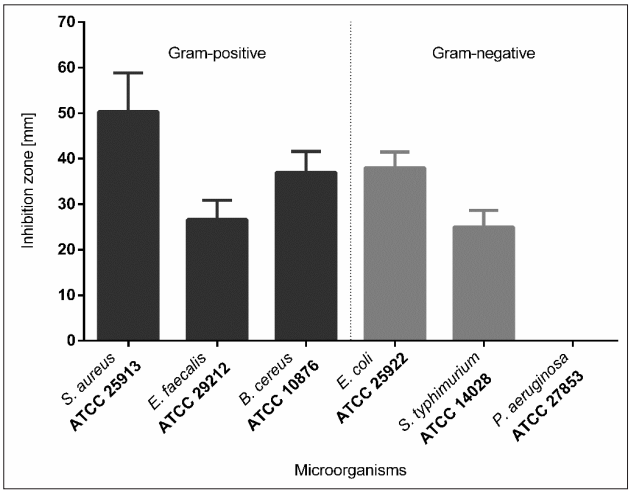
Statistical analysis of the results was conducted by t-Student test and One-way analysis of variance (ANOVA) with the post-hoc Fisher's NIR test (statistical significance being set at $p \leq 0.05$). The assumption of normality was verified by the Shapiro-Wilk test, while the homogeneity of the variance was confirmed by the Levene's test (STATISTICA 13.0 software; StatSoft, Krakow, Poland).

RESULTS

The assessed antimicrobial activity of TEO against six reference microorganisms via disc-diffusion and disc-volatilization assay is summarized in Table 1, Figure 1 and Figure 2. All reference bacterial strains for which the antibacterial activity of TEO was observed had a greater growth inhibition zone in the disc-diffusion assay as compared with the disc-volatilization assay. Comparison of sensitivity of tested microorganisms in gaseous contact revealed a significant difference: Gram-positive bacteria were more sensitive to TEO than Gram-negative ($p=0.005$). In contrast, no such differences were observed in the case of disc-diffusion assay

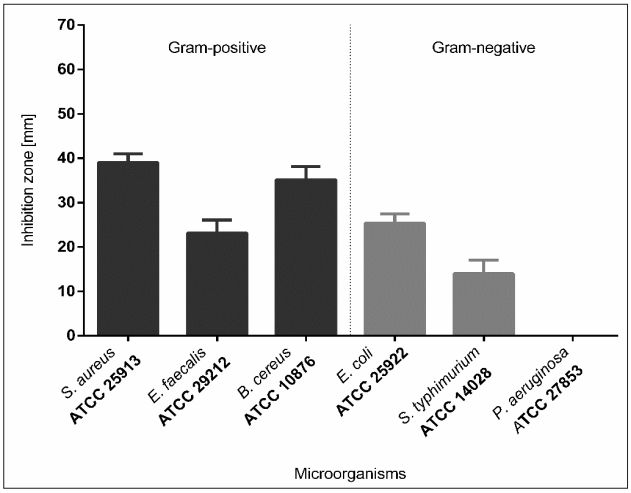
Table 1. Growth inhibition zone diameters (mm) for *Thymus vulgaris* L. essential oil against six reference microorganisms as obtained via disc-diffusion and disc-volatilization after 24 hours of incubation

	Microorganism	Disc-diffusion assay	Disc-volatilization assay
		mean ± SD (mm)	
Gram-positive	*Staphylococcus aureus* ATCC 25913	50.3±8.5	39.0±2.0
	Enterococcus faecalis ATCC 29212	26.7±4.2	23.2±2.9
	Bacillus cereus ATCC 10876	37.0±4.6	35.2±3.0
Gram-negative	*Escherichia coli* ATCC 25922	38.0±3.5	25.3±2.1
	Salmonella typhimurium ATCC 14028	25.0±3.6	14.0±3.0
	Pseudomonas aeruginosa ATCC 27853	0	0



Growth inhibition zone diameters are given as mean ± SD (mm)

Figure 1. Antimicrobial activity of *Thymus vulgaris* L. essential oil against six reference microorganisms in disc-diffusion assay after 24 hours



Growth inhibition zone diameters are given as mean ± SD (mm)

Figure 2. Antimicrobial activity of *Thymus vulgaris* L. essential oil against six reference microorganisms in disc-volatilization assay after 24 hours

(p=0.614). Additionally, statistical analysis showed that TEO exhibited higher antibacterial activity against Gram-negative bacteria in direct rather than gaseous contact, namely for *S. typhimurium* (p=0.016) and *E. coli* (p=0.006).

Tested EO exhibited a statistically different activity among Gram-positive bacteria. The ANOVA analysis demonstrated significant differences in the activity of TEO in direct contact between tested Gram-positive bacteria (p=0.009), while post-hoc comparisons showed that *S. aureus* was significantly more sensitive to TEO than *E. facealis* (p=0.003) and *B. cereus* (p=0.036).

Similar observations have been made in disc-volatilization assay. The ANOVA test showed that TEO was characterized by different antibacterial activity against Gram-positive microorganisms (p=0.001). Further analysis with post-hoc comparisons showed significant differences in susceptibility between *S. aureus* and *E. faecalis* (p<0.001) and between *B. cereus* and *E. faecalis* (p=0.002), which proved that *E. faecalis* was less sensitive to TEO vapours than the other two Gram-positive reference strains.

Among Gram-negative bacteria included in this study, statistical analysis demonstrated that E. coli was more sensitive to TEO than *S. typhimurium* - both in disc-diffusion (p=0.011) and in disc-volatilization assay (p=0.006). We observed a lack of antibacterial activity of TEO against the tested strain of *P. aeruginosa*.

Despite that the inhibition zones were measured after 24 h and 48 h of incubation, no differences in their size were observed. However, for *S. typhimurium*, two types of inhibition zone were observed after incubation: the first was characterized by the total lack of bacterial growth (reported as the growth inhibition zone in this study), and the second was characterized by visibly weaker growth.

DISCUSSION

We tested the antibacterial activity of TEO against the most common human bacterial pathogens that are responsible for a wide range of infections. It is worth mentioning that two ways of placing the disc soaked with TEO were used, i.e. in the first case it was placed directly on the medium on which the bacteria were growing, while the disc-volatilization assay relied on placing the disc on the Petri dish lid, so it had no direct contact with the medium and only the volatile compounds inhibited bacterial growth.

Results from our study show that Gram-positive bacteria are more sensitive to the TEO than Gram-negative bacteria in gaseous contact, while Gram-negative bacteria are more sensitive in disc-diffusion than disc-volatilization assay. This weaker antimicrobial activity against Gram-negative bacteria can be explained by the presence of hydrophilic lipopolysaccharides in the outer membrane, which prevents the hydrophobic EO from penetrating the bacterial cell membrane, whereas the higher activity of TEO against *E. coli* can be explained by the hydrophobic nature of its outer membrane consisting of shorter oligosaccharide chains [11]. Despite that many authors report a significant antimicrobial activity of TEO against *P. aeruginosa*, including even multidrug-resistant strains, we observed the lack of activity both in direct and gaseous contact. This was probably caused by the low content of the critical component of TEO responsible for the activity against this bacterium – thymol. It was observed that thymol affects cell membrane, intercalates DNA and inhibits biofilm formation in *P. aeruginosa* [12]. However,

the *Thumus vulgaris* L. genus can be represented by plants of various chemotypes, e.g. thymol chemotype containing up to 65% of thymol or carvacrol chemotype, where the content of thymol can be very low – 1 to 5% [13]. Despite that the antimicrobial activity of TEO in our studies was higher in direct than gaseous contact for every microorganism tested, for B. cereus the difference was very low. It has been shown that carvacrol, one of the main compounds of TEO, is able not only to inhibit *B. cereus* growth, but also decrease diarrheal toxin production, which creates a possibility to use TEO as an additive in food production [14].

The data presented in this study demonstrated statistically significant antimicrobial activity of TEO in vapour contact against Gram-positive than Gram-negative bacteria. Significant biological activity in gaseous contact and high volatility at room temperature is a particularly important physicochemical characteristic allowing for application of TEO in inhalation therapy, even in vulnerable patients. Ghahremani-Chabok et al. investigated the influence of inhalation therapy with the use of TEO in mechanically ventilated patients and demonstrated that it has not only limited the concentration of airway secretions by facilitating the discharge of mucous membranes, but also improved gas exchange by increasing the oxygen saturation [15].

Despite recent advances in testing biological activity of natural compounds, the evaluation of *in vitro* antimicrobial activity of EO in vapour phase remains technically challenging. Currently, different methods are being used, starting from screening methods relying on the diffusion of EO into the medium, through typically quantitative volatilization methods enabling to determine the minimum inhibitory dose (MID) of EO, ending on advanced assays, like macro- or microbroth volatilization assays allowing to determine EO activity simultaneously in direct and vapour contact [16-18].

It is noteworthy that in diffusion methods, different reservoirs of EO can be used, i.e. paper discs or filters, metal or glass cylinders placed on the medium surface or holes in the medium. The type of reservoir depends strongly on the characteristic of investigated substance – its polarity, solubility and volatility – and therefore the choice of the EO reservoir can be limited [16]. The most advanced methods for determining the vapour phase activity of EO rely on the microbroth volatilization assay allowing determination of the MIC (minimum inhibitory concentration) and MID values at once, that additionally can be developed into the broth volatilization checkerboard assay enabling assessment of the interactions between tested EO by calculating FIC (fractional inhibitory concentration) [19,20]. Unfortunately, because of the small volumes of liquid tested, as well as the rapid volatilization of EO constituents, the aforementioned methods require the use of additional sealing of the microtiter plate, therefore their standardization aiming at obtaining repeatable results may be difficult.

CONCLUSIONS

The global spread of bacteria resistant to commonly applied antibiotics creates the urge to discover and develop novel antimicrobials. Among many natural products attractive for drug development, thyme EO may represent an innovative source of biologically active compounds. Because of its antimicrobial activity, it has the potential to find an application in supporting the treatment of bacterial infections. This study has some limitations. Although the results proved that TEO exhibits antibacterial activity both in liquid and vapour phase, further research is needed to study the correlation between the content of volatile compounds and the antimicrobial activity.

AUTHORS' DECLARATION

Authors declare that this manuscript has not been published elsewhere and it is not under consideration by another journal.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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ETHICAL STANDARDS

Not applicable.

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