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Role of Direct Bioautographic Method for Detection of Antistaphylococcal Activity of Essential Oils

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The aim of the present study was the chemical characterization of some traditionally used and therapeutically relevant essential oils (thyme, eucalyptus, cinnamon bark, clove, and tea tree) and the optimized microbiological investigation of the effect of these oils on clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA). The chemical composition of the oils was analyzed by TLC, and controlled by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). The antibacterial effect was investigated using a TLC-bioautographic method. Antibacterial activity of thyme, clove and cinnamon oils, as well as their main components (thymol, carvacrol, eugenol, and cinnamic aldehyde) was observed against all the bacterial strains used in this study. The essential oils of eucalyptus and tea tree showed weak activity in the bioautographic system. On the whole, the antibacterial activity of the essential oils could be related to their most abundant components, but the effect of the minor components should also be taken into consideration. Direct bioautography is more cost-effective and better in comparison with traditional microbiological laboratory methods (e.g. disc-diffusion, agar-plate technique).

Keywords: thin layer chromatography, direct bioautography, essential oils, antibacterial activity, methicillin-resistant *Staphylococcus aureus* (MRSA).

Methicillin-resistant *Staphylococcus aureus* (MRSA), a bacterium responsible for several difficult-to-treat infections in humans, is also responsible for nosocomial infections. This bacterium has developed resistance to β-lactam antibiotics, which include the penicillins (for example, methicillin, dicloxacillin, nafcillin, oxacillin), and cephalosporins. In diagnostic microbiology, MRSA strains can be identified with a rapid latex agglutination test that detects the PBP2a protein encoded by the *mecA* gene, which is a penicillin-binding protein and responsible for the antibiotic resistance [1].

MRSA is especially troublesome in hospitals, where patients with open wounds and weakened immune systems are at greater risk of infection than the general public [2-4]. Other risk factors include the use of inappropriate antibiotics, underlying diseases, central venous catheterization, endotracheal intubation, enteral feeding, quality of nursing staff work, and compliance with hand disinfection procedures [5].

Today, treatment of MRSA infection is complicated, because only a few antibiotics (for example, vancomycin, teicoplanin, linezolid, tigecycline) are effective against this bacterium. Moreover, several newly discovered strains of MRSA show antibiotic resistance even to vancomycin and teicoplanin [6]. This fact has led to research on new antimicrobial agents from medicinal plants.

Essential oils are used in a variety of fields, such as pharmacology, medical microbiology, phytopathology and food preservation. The number of studies has recently increased focusing on these substances, as well as their application as new potential antibiotic agents against plant and human microorganisms [7-10]. There are some *in vitro* studies related to antistaphyllococcal activity of essential oils and plant extracts [11-13]. These experiments are important because, unfortunately, antibiotics and chemical sprays applied for prevention and treatment in agriculture and human medicine can cause selective pressure leading to the spread of resistant mutants.

Previous studies on the antimicrobial activity of essential oils *in vitro* described a wide range of assays with different parameters (agar recipes, incubation times, solvents, microorganisms) [14-15], so the results from the assays are very different, and sometimes their reliability is questionable. Essential oils are volatile, complex and viscous substances that are insoluble in water so the common screening methods (disc diffusion, agar absorption, agar and broth dilution) are inadequate for their antimicrobial testing. Therefore, there is a need for an optimized and reproducible assay for assessing the antibacterial effect of these oils.

Direct bioautography combined with thin layer chromatographic (TLC) separation is a rapid and sensitive screening method for the detection of antimicrobial compounds. Test microorganism cultures are capable of growing directly on the TLC plate, so each step of the assay is performed on the sorbent. Similar to the common antimicrobial screening methods, TLC-bioautography must be carried out under controlled conditions since the experimental conditions (for example, solvent, sample application, resolution of compounds, type of test microorganism, incubation time) may influence the result [16].

The advantages of direct bioautography are that it is suitable for evaluating complex plant extracts and facilitates rapid, economic and easy evaluation. The use of bioautography to detect antimicrobial compounds effective against plant and human pathogenic bacteria has been reported in the literature [17-20]. *In vitro* antimicrobial activity of eucalyptus, clove, thyme, cinnamon and tea tree essential oils has been shown in several studies [21-24].

The present study aimed at the chemical characterization of the essential oils of thyme (*Thymus vulgaris* L.), clove [*Syzygium aromaticum* (L.) Merr. et Perry], eucalyptus (*Eucalyptus globulus* Labill.), tea tree [*Melaleuca alternifolia* (Maiden et Betche) Cheel] and cinnamon bark (*Cinnamomum ceylanicum* Nees.) by TLC and the detection of antibacterial activity of the essential oils and their main components against some strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) using a direct bioautographic method. These essential oils are important from therapeutic as well as economic aspects. The composition of the oils was checked using gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). To the best of our knowledge, the present study is the first to investigate the biological activity of the above essential oils against different *Staphylococcus* strains using direct bioautographic assay.

With the gas chromatographic analysis we wanted to check only the main components of the essential oils investigated. Eucalyptol (84.2%) was the main component of the essential oil of eucalyptus, eugenol (83.7%) of clove oil, and *trans*-cinnamic aldehyde (73.2%), thymol (49.9%) and terpinen-4-ol (45.8%) of cinnamon bark, thyme and tea tree oils, respectively. Among the other constituents, *p*-cymene, β-caryophyllene, limonene, carvacrol, eugenol and γ-terpinene were also characteristic of the oils, but smaller amounts were present.

Bioautography of the five essential oils and their components was performed by parallel analysis on TLC plates. In the eucalyptus oil, the main component was eucalyptol (R_f = 0.49). In the oil of thyme, thymol and carvacrol could be identified as a single red zone at R_f = 0.54. According to gas chromatographic analysis, *trans*-cinnamic aldehyde (73.2%) was the main component in the cinnamon oil and the amount of eugenol was only 2.7%. However, the two compounds could not be separated by TLC when developed with toluene – ethyl acetate (93: 7), due to their similar R_f values. Cinnamic aldehyde was only identified by reference to [25], while eugenol was determined as a brown zone at R_f = 0.49, on the basis of co-chromatography with eugenol.

Since the quantitative difference between thymol (49.9%) and carvacrol (1.1%), as well as cinnamic aldehyde (73.2%) and eugenol (2.7%) was so large in thyme and cinnamon oils, respectively, it was not worth trying to separate them by TLC. Instead, we applied standards of these compounds (thymol, carvacrol, eugenol) to the plate for comparison. In the oil of clove, the main component was eugenol (brown zone, R_f = 0.49) and a violet zone of β-caryophyllene could be detected at R_f = 0.82 by reference to [25]. In the oil of tea tree, we could not exactly identify the components without standards. Two blue zones were detected at R_f = 0.24 and R_f = 0.37. According to the Hungarian Pharmacopoeia, these compounds may be identified as monoterpene alcohols (terpinen-4-ol and terpineol) [26]. To confirm the main components of tea tree oil by TLC we need more standards for future examinations.

The antibacterial activity of the five volatile oils and their main components was detected by direct bioautography. Absolute ethanol was used as control, which had no antibacterial activity in the bioautographic system. Initially, TLC separation of the essential oils was not attempted. The antibacterial activity was expressed as the diameter (mm) of inhibition zones (Table 1). The essential oils of thyme, clove and cinnamon bark showed the 'strongest' activity against all strains of bacteria, and MRSA 13 was the most sensitive against these oils. Eucalyptus and tea tree oils had weak antibacterial activity, which was dependent on the concentration of the oil tested.

The antistaphylococcal activity of the essential oil components was determined after TLC separation. The minor components of the five essential oils had no apparent activity at the concentration tested. With oil of thyme, thymol-carvacrol and the standard thymol were

Table 1: Antibacterial activity (expressed as the diameter [mm] of inhibition zones) of the essential oils of thyme, tea tree, clove, eucalyptus and cinnamon bark detected by direct bioautography.

Bacterial strains	thyme oil	tea tree oil	clove oil	eucalyptus oil	cinnamon bark oil
methicillin-resistant *Staphylococcus aureus* (MRSA 6)	8-14-18	3-4-4	7-10-15	0-6-8	5-8-10
methicillin-resistant *Staphylococcus aureus* (MRSA 13)	9-16-22	0-3-4	8-12-15	0-0-7	12-16-21
methicillin-resistant *Staphylococcus aureus* (MRSA 14)	5-11-14	0-2-4	5-8-12	0-4-6	6-11-17
methicillin-resistant *Staphylococcus aureus* (MRSA 15)	7-13-16	3-6-6	6-9-11	0-0-5	9-15-19
methicillin-susceptible *Staphylococcus aureus* (MSSA 70)	8-11-14	4-5-5	6-10-13	0-0-7	4-8-10
methicillin-susceptible *Staphylococcus aureus* (MSSA 86)	13-17-22	2-4-5	8-11-13	0-6-8	7-12-16
methicillin-susceptible *Staphylococcus aureus* (MSSA 10)	6-11-13	3-5-5	5-8-12	0-4-6	8-10-14
methicillin-susceptible *Staphylococcus aureus* (MSSA 107)	7-16-19	2-5-6	6-10-14	0-0-5	9-16-19

The values are averages of three parallel measurements. First number: inhibition zone of 1 μL oil; second number: inhibition zone of 5 μL oil; third number: inhibition zone of 10 μL oil

tested, but only thymol had antibacterial activity against all the bacterial strains. With oil of cinnamon, *trans*-cinnamic aldehyde-eugenol, and in oil of clove, eugenol and the standard eugenol inhibited the growth of all test bacteria, respectively. In tea tree oil the monoterpene alcohols, α-terpineol (R_f = 0.24) and terpinen-4-ol (R_f = 0.37) showed antibacterial effects only against MRSA 15. In the TLC-bioautographic system, the essential oils of eucalyptus and tea tree showed weak activity, and of the standards, eucalyptol had no antibacterial activity against the strains tested in this study. It should be noted that the solvents (toluene and ethyl acetate) applied in TLC separation as mobile phase components had no inhibiting effect in the direct bioautographic system and thus they did not influence detection of biological activity.

On the whole, the antibacterial activity of the essential oils seemed to be associated with their most abundant components, but the effect of the minor compounds should also be taken into consideration. In our experiments, only the main components of the essential oils had an antibacterial effect in the bioautographic system.

Our study showed that the essential oils of thyme, cinnamon bark and clove have antimicrobial activity against the clinical isolates of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* used in this study, and, therefore, they might be useful in controlling MRSA infections. Further research is required to assess the practical value of the therapeutic application of these oils. Antimicrobial screening methods used in essential oil research show a great variety [27]. The lack of standardized assays makes direct comparison of results between studies impossible. Therefore there is a need for elaborating standardized, reproducible and reliable methods.

We succeeded in optimizing the direct bioautographic method for studying antibacterial activity of some essential oils. Methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* (MRSA and MSSA) were used as test microorganisms. This assay is more cost-effective and better in comparison with traditional microbiological laboratory methods and is also suitable for analysis of complex, viscous, lipophilic extracts, such as essential oils. Interest in the idea of using volatile oils against pathogenic microbes keeps growing, because their side-effects are not significant and often the main effect intended can be achieved. The individual components of the essential oils clearly had antibacterial properties [28-29], although the mechanism is poorly understood.

There can be no doubt that direct bioautography combined with TLC separation has a novel and expanding field of application and practical advantage in research on antibiotic substances. Bioautography is a valuable technique which can quickly determine which component of an essential oil imparts antibacterial activity. Further investigations need to focus on modelling complex antimicrobial effects *in vitro* and *in vivo*.

Experimental

Chemicals: Thymol, carvacrol, eucalyptol and eugenol were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary). TLC was performed on silica gel 60 F_{254} aluminum sheet TLC plates (Merck, Darmstadt, Germany). The mobile phase for TLC was prepared from analytical-grade solvents from Spektrum-3D Ltd. (Debrecen, Hungary). Analytical-grade solvents obtained from Spektrum-3D Ltd. were used in all experiments. Analytical-grade dye reagent MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich Ltd. (Budapest, Hungary).

Essential oil samples: The essential oils of thyme, clove, eucalyptus, tea tree, and cinnamon bark (*Cinnamomum ceylanicum* Nees.) were obtained from a Hungarian pharmacy (Herbaria, Hungary). The quality of the essential oils used met the standards described in the 4th edition of the European Pharmacopoeia.

Quantification and identification of the oils: The 5 essential oils were analyzed using a Fisons GC 8000 gas chromatograph (Carlo Erba, Milano), equipped with a flame ionization detector (FID). An Rt-*β*-DEXm (Restek) capillary column, 30 m long, 0.25 mm id., 0.25 μm film thickness was used. The carrier gas was nitrogen at 6.86 mL/min flow rate; 0.2 μL of a 0.25% solution was injected (2 μL of essential oil in 1 mL of chloroform). Splitless injection was made. The temperatures of the injector and detector were 210˚C and 240˚C, respectively. Oven

temperature increased at a rate of 8˚C/min from 60 to 230˚C, with a final isotherm at 230˚C for 5 min. Percentage evaluation of compounds was carried out by area normalization; identification of peaks was made by comparison of retention times of standards and co-addition of standards. All measurements were made in duplicate.

GC/MS was performed with a coupled system Agilent 6890N GC, 5973N mass selective detector. The Chrom Card Server ver. 1.2. equipped with a HP-5MS capillary column, 30 m long, 0.25 mm id., 0.25 μm film thickness was used. The carrier gas was helium (p_{He} was 0.20 MPa), at 1 mL/min flow rate; 1 μL (2 μL/mL essential oil in chloroform) was injected at 0.7 mg/mL velocity, split type (1:30 split ratio) with an Agilent 7683 autosampler. The temperature of the injector was 280°C, and the temperature of the transfer line was 275°C. Oven temperature was programmed initially at 60°C for 3 min, then increased at a rate of 8°C/min to 200°C, then kept at 200 °C for 2 min, before increasing at a rate of 10°C/min to 250°C with a final isotherm at 250°C for 15 min. MS conditions: ionization energy 70 eV; mass range m/z 40-500; 1 analysis/min. Identification of peaks was carried out by comparison with MS and retention data of standards and spectra from the NIST library [30]. These analyses were meant to check only the main components and the composition of the essential oils examined in this study.

Bacterial strains: Four MRSA (MRSA 6, 13, 14, 15) and four MSSA (MSSA 70, 86, 10, 107) strains were involved in our study. All the examined *S. aureus* strains were isolated from blood cultures and were obtained from the Institute of Medical Microbiology and Immunology, University of Pécs (Hungary). The test organisms were maintained on Mueller-Hinton agar (Oxoid, UK).

Microbiological identification of* S. aureus *strains: Phenotypic identification was achieved by classical microbiological methods, such as Gram-staining, and catalase and clumping-factor tests (bioMerieux). The phenotypic results were confirmed by polymerase chain reaction (PCR) by detecting the genes encoding thermostabile endonuclease (*nucA*) and 23S rRNA, and methicillin resistance was examined by the presence of the *mecA* gene, as previously described [31].

Direct bioautography: This process can be divided into 3 parts: cultivation of test bacteria for dipping; planar chromatographic separation and detection; and treatment for post-chromatographic detection. The antibacterial activity of the essential oils examined in this study was investigated by direct bioautography.

Cultivation of test bacteria for dipping: For bioautographic assay, bacteria were grown in 100 mL Mueller-Hinton nutrient broth (pH 7.3) at 37˚C in a shaker incubator at a speed of 60 rpm for 24 h. The bacterial suspension was diluted with fresh nutrient broth to an OD_{600} of 0.4, which corresponds to approximately 4×10^7 colony-forming units (cfu) mL.

Planar chromatographic separation and detection: Chromatography was performed on 10 cm × 10 cm silica gel 60 F_{254} aluminum sheet TLC plates. Before use the plates were preconditioned by heating at 120˚C for 3 h.

A) Investigation of antibacterial effect of essential oils (without TLC separation): Essential oils were dissolved in ethanol to give solutions containing 100 μL oil/5 mL ethanol, and 1-5-10 μL of it (equivalent to 0.02-0.1-0.2 μL undiluted oil) were applied to the TLC plate with Minicaps capillary pipettes (Germany). Absolute ethanol was the control, from which 1-5-10 μL were applied to the TLC plate with capillary pipettes. The position of the control and sample spots was 1.5 cm from the left side in 3 lines, and between the spots was a distance of 1.5-1.5. In this case, TLC separation was undertaken, but a bioautographic detection of antibacterial activity was possible.

B) Investigation of antibacterial effect of essential oils and their components (after TLC separation): The antibacterial activity of the characteristic components (thymol, carvacrol, eucalyptol and eugenol) of the essential oils was also investigated by direct bioautography. Pure samples of these chemicals were dissolved in ethanol to give solutions containing 1 mg/mL. From the solution of the standards and the essential oils 5-5 μL were applied to the plates using Minicaps capillary pipettes, respectively. The position of the starting line was 1.5 cm from the bottom and 1.5 cm from the left side. The standards were applied to the TLC plates next to the spots of the oils. After sample application, the plates were developed with the previously optimised mobile phase. For the separation of essential oils in the literature, toluene – ethyl acetate (93: 7) was recommended as the mobile phase [25]. Ascendant development chromatography was used, in a saturated twin trough chamber (Camag, Switzerland). All TLC separations were performed at room temperature (20˚C). After chromatographic separation the absorbent layers were dried at 90˚C, for 5 min to remove the solvent completely.

B/1.) Post-chromatographic detection of oil components by chemical reaction: Ethanolic vanillin – sulfuric acid reagent [25] was used to visualize the separated compounds. The developed layers were dipped into this reagent and heated for 5 min at 100˚C. Detection of the separated compounds was performed from R_f value and color of the standards. Although the TLC plate was fluorescent, evaluation of the separated compounds by UV was not done. At UV-365 nm no characteristic fluorescence of terpenoids and propylphenols is noticed [25]. It should be noted that the TLC plate for bioautography was processed in parallel, without final development with a reagent, because this does not affect the success of the subsequent microbial detection process in bioautography.

B/2.) Post-chromatographic detection of antibacterial activity by bioautography: For bioautography, the developed plates were dipped for 10 s in approximately 50 mL culture medium containing inocula and then dried under air flow for 2 min. The purity of the culture was checked by cultivation on Mueller-Hinton agar plates. TLC plates were incubated in a water-vapor chamber (chamber dimension: 20 cm × 14.5 cm × 5 cm) at 37˚C for 17 h, then dipped for 10 s in an aqueous solution of MTT (0.1 g/60 mL). Treated plates were incubated at 37˚C for 3 h, then dipped in 70% ethanol for 10 s and dried at room temperature.

The inhibition zones of the separated compounds were visualized by detecting dehydrogenase activity in living test bacteria with tetrazolium salt-based reagents. On the TLC plate, metabolically active living bacteria convert the tetrazolium salt, MTT, into blue formazan dye. Thus, the inhibition zones were visible as pale spots against a dark-blue background. The diameters of zones of inhibition were measured in mm. All measurements were performed in triplicate. TLC plates were photographed with a Canon PowerShot A95 camera.

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