SPECIAL GUEST EDITOR SECTION

TLC-Direct Bioautography for Determination of Antibacterial Activity of *Artemisia adamsii* Essential Oil

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The aim of the present study was the chemical characterization of the essential oil of a Mongolian medicinal plant, *Artemisia adamsii* Besser, and the investigation of the antibacterial effect of its oil on different human pathogenic bacteria (*Staphylococcus aureus*, methicillin-resistant *S. aureus*, and *S. epidermidis*). The chemical composition of the oil was established by GC and GC/MS. Direct bioautography was used for detecting the antibacterial activity of the essential oil. The result of GC experiments showed that α-thujone was the main component (64.4%) of the oil, while the amount of β-thujone was 7.1%. 1,8-Cineole seemed to be the other relevant component (15.2%). The antibacterial activity of the *A. adamsii* essential oil against all three investigated bacteria was observed in the bioautographic system, but this effect was not proportional to the concentrations of α- or β-thujone; therefore, from a microbiological aspect, thujone content does not determine the medicinal value of this oil. On the whole, the combination of TLC separation with biological detection is an appropriate method for evaluating multicomponent and hydrophobic plant extracts, for instance, essential oils, and it provides more reliable results than traditional microbiological methods (e.g., disc diffusion and agar plate techniques).

Many classes of bioactive compounds can be separated by TLC. A special method of detection, direct bioautography (DB), is suitable for studying the antimicrobial activity of plant extracts of natural origin when linked to TLC. Paper chromatography and the microbial detection of antibiotics were combined for the first time in 1946 by Goodall and Levi to determine the purity of penicillin in a mixture (1). Later, other researchers applied TLC plates to detect antibiotic activity (2, 3). DB belongs to a large group of antimicrobial screening methods based on layer separations [TLC, HPTLC, and over pressured layer chromatography (OPLC)]. In the TLC-DB method, a developed TLC plate is dipped in the suspension of microorganisms growing in a suitable broth. The plate is incubated, and microorganisms grow directly on it. For location and visualization of antibacterial substances, tetrazolium salts are usually used, which are converted by the dehydrogenases of living microorganisms to intensely colored formazan (4–8).

The fact of antibiotic resistance contributes to the increase in the number of studies focusing on the application of essential oils as potential, natural antimicrobial agents against plant and human microorganisms (9–12). Essential oils are volatile, hydrophobic substances that are insoluble in water; therefore, the application of common screening methods (e.g., disc diffusion, agar absorption, and agar or broth dilution) may not provide reproducible and reliable experimental results (13). Therefore, there is a need for optimized assays to evaluate the antimicrobial activity of essential oils.

Artemisia adamsii Besser (*Asteraceae*) is a perennial plant native to Mongolia, the Russian Federation, and China. The whole herb of *A. adamsii* has been used as a stomachic agent in Traditional Chinese Medicine for ages (14). While more than 60 *Artemisia* species are used for medicinal purposes, the active compounds of most of the species are still unknown. To date, only a few phytochemical results of this plant have been published. The aerial parts of *A. adamsii* afforded the sesquiterpene arborescin as the main constituent. Bohlmann et al. (15) described 11 new guaianolides, which are constituents of the plant related to arborescin. Only one paper described the essential oil components of *A. adamsii* (16). Therefore, our study was aimed at the phytochemical characterization of the essential oil extracted from *A. adamsii* by GC and GC/MS, and the microbiological evaluation of the essential oil and its components using DB. This method combines TLC separation with microbiological detection. It can be used for detection of antimicrobial activity of plant extracts (e.g., essential oils) after TLC separation. To the best of our knowledge, the present study is the first to investigate the biological activity of the above-mentioned essential oil against different *Staphylococcus* strains using TLC-bioautography.

Experimental

Chemicals

Thujone and 1,8-cineole (eucalyptol) were purchased from Sigma-Aldrich Ltd (Budapest, Hungary). TLC was performed on silica gel 60 F_{254} foil-backed TLC plates (Merck, Darmstadt, Germany). The mobile phase for TLC was prepared from analytical-grade solvents. These types of solvents obtained from VWR International Ltd (Debrecen, Hungary) were used in all experiments. Analytical-grade dye reagent MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich Ltd. Mueller-Hinton agar was obtained from Oxoid Ltd (London, UK) for the microbiological experiments.

Guest edited as a special report on "Thin-Layer Chromatography Hyphenated with Bioassays" by Irena Choma.
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DOI: 10.5740/jaoacint.SGEHorvath

Gentamicin for injection (containing 80 mg/2 mL gentamicin; Sanofi-Aventis, Budapest, Hungary) and vancomycin (50 mg; Teva, Debrecen, Hungary) antibiotics were used as positive controls in the bioautographic method. In the case of vancomycin, 1 mg/mL stock solution was prepared using sterile distilled water.

Plant Material

The aerial parts of *A. adamsii* Besser were collected in Mongolia (near Ulan Bator) in July 2010. The plant was identified by a biologist, Dr. Tserennadmid Rentsenkhand (National University of Mongolia). The plant sample was air dried at room temperature (22°C) and pulverized just before distillation. A voucher specimen (*A. adamsii*) was prepared and deposited in the Herbarium of the Department of Pharmacognosy, University of Pécs, Hungary.

Essential Oil Isolation

The essential oil was isolated by steam distillation for 3 h using 25 g dried, powered plant material, according to the Hungarian Pharmacopoeia, 7th edition (17). Its oil content was measured directly by the volumetric method.

GC and GC/MS Measurements

A Fisons GC 8000 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame ionization detector (FID) was used for the analysis of the essential oil components. The capillary column used was Rt-β-DEXm (Restek Corp., Bellefonte, PA), 30 m × 0.25 mm id, 0.25 μm film thickness. Nitrogen was the carrier gas (6.86 mL/min flow rate); 0.2 mL 0.1% solution was injected (1 μL essential oil in 1 mL chloroform). A splitless injection was made. The temperatures of the injector and detector were 210 and 240°C, respectively. The column oven temperature was increased at a rate of 8°C/min from 60 to 230°C, with a final isothermal hold at 230°C for 5 min. Identification of peaks was made by retention time and standard addition; percentage evaluation was carried out by area normalization. All measurements were made in triplicate; RSD was below 4.5%.

GC/MS was performed with a coupled system comprising an Agilent (Santa Clara, CA) 6890N gas chromatograph, 5973N mass selective detector; Chrom Card Server version 1.2, and HP-5MS capillary column, 30 m long, 0.25 mm id., and 0.25 μm film thickness. Helium was the carrier gas (pressure, 0.20 MPa) at 1 mL/min flow rate; 1 μL (10 μL/mL essential oil in ethanol) was injected at 0.7 mg/mL velocity, splitless mode, with an Agilent 7683 autosampler. Injector temperature was 280°C, and the temperature of the transfer line 275°C. The oven temperature was held initially at 60°C for 3 min, increased at a rate of 8°C/min to 200°C, kept at 200°C for 2 min, and increased at a rate of 10°C/min to 250°C, with a final isothermal hold at 250°C for 15 min. MS conditions were: ionization energy, 70 eV; mass range, *m*/*z* 40–500; and 1 analysis/min. Peaks were identified based on the National Institute of Standards and Technology (Rockville, MD) spectral library and literature data (18).

TLC-DB

The process of TLC-DB can be divided into three parts: cultivation of test microorganisms for dipping; TLC of test materials (with or without separation); and postchromatographic microbial detection, i.e., DB.

(**a**) *Bacterial strains*.—*S. aureus* (ATCC 29213), *S. epidermidis* (1118), and methicillin-resistant *S. aureus* (MRSA 4262) were involved in the microbiological experiments. *S. epidermidis* and MRSA strains were isolated from blood cultures. All the examined test bacteria were maintained on Mueller-Hinton agar in the Institute of Medical Microbiology and Immunology, University of Pécs, Hungary

(**b**) *Cultivation of test bacteria for dipping*.—Bacteria used in this study were grown in 100 mL Mueller-Hinton nutrient broth (pH 7.3) at 37°C in a shaker incubator (Psycro Therm, New Brunswick Scientific Co., NJ) 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 CFU/mL.

(**c**) *TLC*.—Chromatography was performed on 5 × 10 and 10 × 10 cm aluminium foil backed silica gel 60 F_{254} plates. Before use, the plates were preconditioned by heating at 120°C for 2 h. First, the antibacterial activity of the essential oil and antibiotics was investigated without TLC separation. Then, the antibacterial effect of the essential oil components was studied after separation.

(*1*) *Preparing plates for TLC-DB without separation*.—Essential oil was dissolved in ethanol to give a 200 μL/mL solution, then 3–5 μL (equivalent to 0.6–1 μL undiluted oil) was applied to the 5 × 10 cm plates with Minicap capillary pipets (Hirschmann Laborgerate GmbH & Co. KG, Eberstadt, Germany). Antibiotic solutions (equivalent to 0.004 mg vancomycin and 0.16 mg gentamicin) were applied to the plates (4 μL each). The position of the standards and essential oil spots was 2 cm from the left side in one line, and between the spots there was a distance of 3–3 cm. TLC separation was not performed.

(*2*) *TLC separation preceding derivatization or DB*.—The antibacterial activity of the essential oil, as well as of its characteristic components (thujone and 1,8-cineole), were also investigated after TLC separation. Thujone and 1,8-cineole standards were dissolved in ethanol to give solutions of 10 μL/mL. Essential oil samples of 1, 3, and 5 μL and 2 μL aliquots of standard solutions were applied to the 10 × 10 cm plates, in duplicate, one plate for derivatization with vanillin–sulfuric acid reagent and the other for bioautography (Figures 1 and 2). The position of the starting line was 1.5 cm from the bottom and 1.5 cm from the left side. After sample application, the plates were developed with toluene–ethyl acetate (93 + 7, v/v; 19). Ascending development was used in a saturated twin trough chamber (CAMAG, Muttenz, Switzerland). All TLC separations were performed at room temperature (23°C). The separation distance was 8 cm, and development time approximately 20 min. After chromatographic separation, the adsorbent layers were dried at room temperature under air flow for 10 min to remove the mobile phase completely. Ethanolic vanillin–sulfuric acid reagent (19) was used to detect the separated compounds. The developed layers were dipped into this reagent and heated at 90°C for 3 min. Detection and identification of the separated compounds was performed on the basis of Rf values and colors of the standards. *Note*: The TLC plate for bioautography was prepared without the derivatization step.

(*3*) *Postchromatographic microbial detection–DB*.—The developed plates were dipped in approximately 80 mL bacterial suspension for 10 s, then dried under air flow for 2 min. The purity of the culture was checked by spreading out and cultivation

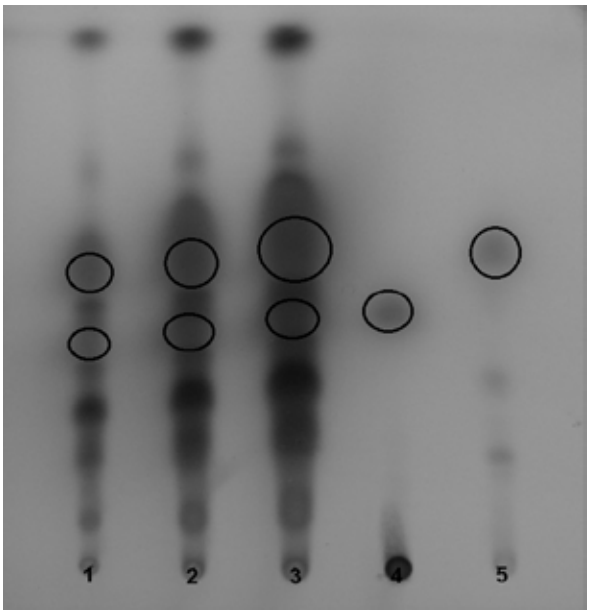

Figure 1. TLC separation of the essential oil of *A. adamsii* on silica gel 60 F_{254}. Solvent: toluene–ethyl acetate (93+7, v/v). Detection: alcoholic vanillin–sulfuric acid reagent. Sample application: lane 1: 1 µL oil, lane 2: 3 µL oil, lane 3: 5 µL oil, lane 4: 1,8-cineole standard (2 µL), and lane 5: thujone standard (2 µl). The black circles show the presence of 1,8-cineole and thujone in the oil sample.

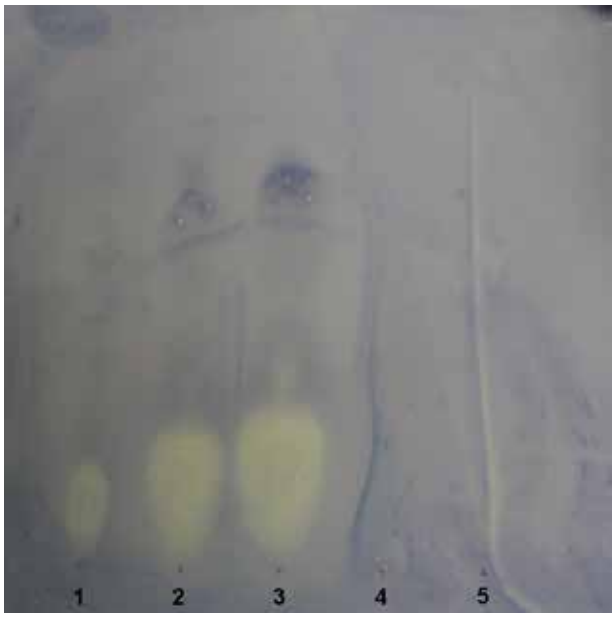

Figure 2. Detection of antibacterial activity of essential oil of *A. adamsii* by DB. Test bacterium: *S. epidermidis* (1118). Adsorbent: silica gel 60 F_{254}. Mobile phase: toluene–ethyl acetate (93 + 7, v/v). Sample application: lane 1: 3 µL oil, lane 2: 3 µL oil, lane 3: 5 µL oil, lane 4: 1,8-cineole standard (2 µL), and lane 5: thujone standard (2 µL).

on Mueller-Hinton agar plates. The TLC layers were incubated in a water vapor-saturated chamber (chamber dimension: 20×14.5×5 cm) at 37°C for 17 h, then dipped in an aqueous solution of MTT (50 mg/80 mL) for 10 s. Then, the plates were incubated at 37°C for 2 h, and photographed with a Canon PowerShot A95 camera. The inhibition zones of microbiologically active components were visualized by detecting dehydrogenase activity in living test bacteria with tetrazolium salt-based reagents (5, 6). On the TLC plate, metabolically active living bacteria converted MTT into blue formazan dye; therefore, the inhibition zones appeared as pale spots against a blue background. The diameters of the zones of inhibition were measured in mm. All bioautographic experiments were performed in triplicate.

Results and Discussion

Essential Oil Content and Composition

The essential oil yield was 0.56 mL/100 g of dried plant material. The color of the oil obtained by steam distillation was pale yellow. Using GC and GC/MS analysis, the presence of thujones in the oil was confirmed; α-thujone was the main component (64.4%), while the amount of β-thujone was much lower (7.1%.). The presence of 1.8-cineole (15.2%) was also confirmed by GC/MS. Other components were *p*-cymene (1.5%), terpinen-4-ol (1.5%), linalool (0.4%), and spatulenol (1.8%). The occurrence of these components in the oil could also be confirmed by GC/MS (Table 1). In the future, we plan to examine the composition of this oil by an SPME (solid phase microextraction) technique, which is frequently used in the analysis of essential oil composition.

TLC

In the essential oil, thujone could be identified as a single compound at R_f 0.56 compared to the thujone standard (Figure 1). α- and β-Thujone could not be separated by TLC with the mobile phase toluene–ethyl acetate (93+7, v/v) due to their similar R_f values. The spot of 1,8-cineole was identified at approximately R_f 0.45 compared to the 1,8-cineole standard. The small differences between the R_f values can be seen on the TLC layer because of the increasing concentration of the oil. According to ref. 19, the terpene zones can be found in the 0.15–0.45 R_f range in the essential oil of *Artemisia* species. The terpinen-4-ol component was identified at R_f 0.37, according to ref. 19. Linalool (R_f 0.33) was also determined based on this reference. The presence of these components in the oil was confirmed by GC and GC/MS.

TLC-DB

The antibacterial activity of the essential oil and its separated compounds was estimated by DB. Initially, TLC separation of the essential oil was not performed so that the effect of control antibiotics could be compared with the oil. The antibacterial activity was expressed as the diameter (mm) of inhibition zones (Table 2). All bacterial strains used in this study were sensitive to one of the antibiotics (gentamicin or vancomycin) and the oil of *A. adamsii*. *S. epidermidis* was the most sensitive strain. Compared to antibiotics, a higher concentration of the oil must be applied to achieve the effectiveness of the antibiotics used in this study (Figure 3). After TLC separation, the antibacterial effect of the essential oil components could be detected. In every case, the terpene components showed antibacterial activity in the 0.15–0.37 R_f range, and the inhibition zones appeared as pale white spots around the separated constituents. In this case, *S. epidermidis* was

Table 1. Composition of *A. adamsii* essential oil[a]

Compounds	Retention time, min		Composition, %	
	FID	MS	FID	MS
p-Cymene	7.68	8.08	1.5	1.0
1,8-Cineole	7.95	8.31	15.2	15.9
Linalool	9.84	7.84	0.4	0.9
α-Thujone	10.38	10.66	64.4	77.6
β-Thujone	10.66	11.02	7.1	6.5
Terpinen-4-ol	11.69	13.14	1.5	1.3
Spatulenol	18.01	24.95	1.8	1.5

[a] All measurements were made in triplicate; RSD values were below 4.5%.

Table 2. Antibacterial activity (expressed as the diameter in mm of inhibition zones) of the essential oil of *A. adamsii* and antibiotic standards detected by DB[a]

Bacterial strains	Essential oil		Gentamicin	Vancomycin
	3 µL	5 µL	4 µL	4 µL
S. aureus ATCC 29213	4	5	7	—
Methicillin-resistant *S. aureus* (MRSA 4262)	4.5	5.5	—	7
S. epidermidis (1118)	5	6	9	—

[a] The values are averages of three parallel measurements. 3–5 µL oil (equivalent to 0.558–0.903 mg undiluted oil) and 4 µL antibiotics (equivalent to 0.004 mg vancomycin and 0.16 mg gentamicin) were applied to TLC plates.

also the most sensitive bacterium (Figure 2). According to ref. 19 and our GC results, we supposed that terpinen-4-ol and linalool were the bioactive compounds in the oil of *A. adamsii*. Thujone as the main component of the oil had no apparent antibacterial activity at the concentration at which it was present in the oil. The standards of thujone and 1,8-cineole did not show activity in the bioautographic system, as well.

The antibacterial activity of different *Artemisia* essential oils belonging to thujone chemotype has already been published (20, 21). In these experiments the agar diffusion method was used. According to results presented in the references, the essential oil samples showed antimicrobial activity. It should be noted that the conventional microbiological assays used for determination of antibacterial effect of essential oils do not provide information about the activity of the individual components, including the synergistic effect. Our results confirmed that the antibacterial activity of the essential oil obtained from *A. adamsii* is not proportional to the concentrations of α-, β-thujone, and 1,8-cineole; therefore, from a microbiological aspect, these components do not determine the medicinal value of this oil.

This study showed that DB is an appropriate method for detection of plant products (e.g., essential oils) with antibacterial activity against human pathogenic bacteria. Interest in the idea of using volatile oils against pathogenic microbes keeps growing because, in contrast to antibiotics, resistance rarely develops due to the large number of their components. Bioautography facilitates the investigation of microbiologically active hydrophobic plant extracts containing a large number of compounds.

In our experiments, the oil of this plant showed antibacterial activity in the bioautographic system; however, its medical application for internal use is not recommended because of the thujone content. The essential oil of *A. adamsii* might be useful as a disinfectant in controlling hospital infections caused by antibiotic-resistant bacteria, e.g., MRSA. Bouaziz et al. published the disinfectant properties of thujone-type essential oil obtained from *Salvia officinalis* (22). Vaporization of 0.25 mL/m^3 of *S. officinalis* oil reduced the total microbial count after residence times of 1, 6, and 24 h in a selected testing room.

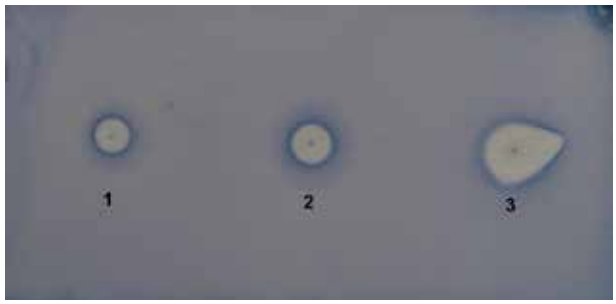

Figure 3. Detection of antibacterial activity of essential oil of *A. adamsii* by DB. Test bacterium: MRSA 4262. Adsorbent: silica gel 60 F_{254}. Sample application: lane 1: 3 µL oil (equivalent to 0.558 mg undiluted oil), lane 2: 5 µL oil (equivalent to 0.903 mg undiluted oil), lane 3: and 4 µL vancomycin solution (equivalent to 0.004 mg antibiotic)

In our further studies, we will investigate the composition of *A. adamsii* oil by an SPME technique and determine the mode of action of the oil and its individual components using different microbiological and chromatographic methods. Undoubtedly, TLC-bioautography allows a rapid identification of the active compound or compounds in a complex mixture.

Acknowledgments

We thank Tserennadmid Rentsenkhand (National University of Mongolia, School of Biology) for supplying and identifying the plant material, Erika Kocsis for technical support in the microbiological experiments, and Andrea Böszörményi, Éva Lemberkovics, and Szabolcs Szarka for the GC analyses. This work was financially supported by the OTKA PD 104660 grant.

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