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ORIGINAL RESEARCH PAPER

Antibacterial compounds of *Cymbopogon nardus* essential oil exposed by high-performance thin-layer chromatography–direct bioautography

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Abstract
Essential oil of citronella grass (*Cymbopogon nardus*) was screened for antibacterial compounds by high-performance thin-layer chromatography (HPTLC) combined with direct bioautography using soil bacterium *Bacillus subtilis*, marine bacterium *Aliivibrio fischeri*, and plant pathogens *Pseudomonas syringae* pv. *maculicola* and *Xanthomonas euvesicatoria*. The parallel derivatization using HPTLC–anisaldehyde reagent also revealed the bioactive compounds separated with *n*-hexane–isopropyl acetate (9:1, *v/v*), which were analyzed by offline solid-phase microextraction–gas chromatography–electron ionization-MS (SPME–GC–EI-MS) after scraping off and elution. The compounds responsible for the antibacterial effect were identified as citronellal, geranial, neral, geraniol, α-cadinol, and elemol. These compounds inhibited all studied bacterial strains except elemol that demonstrated activity only against *B. subtilis* and *X. euvesicatoria*.

Keywords Citronella essential oil · HPTLC–direct bioautography · SPME–GC–MS · Antibacterials

1 Introduction

Cymbopogon nardus, known as citronella grass, is a tropical plant from the sweet grass family (Poaceae). It originates from Southeast Asia and is highly valued for its aromatic essential oil (EO) extracted from the leaves. The EO is rich in citronellal, citronellol, and geraniol, contributing to its characteristic fresh, lemony scent [1]. Therefore, it is utilized in the perfume and cosmetics industry and aromatherapy for its calming and stress-relieving effects. Ayurvedic and traditional Chinese medicine employs the plant to relieve fever, pain, colds, inflammation, infections, and digestive problems [2, 3]. In some regions, leaves and extracts are used as a poultice to heal wounds and treat skin infections. Citronella EO is known for its insecticidal properties and is used in candles, sprays, and skin protection products to keep mosquitoes and other insects away [2]. In addition to its repellent effect, the EO has anti-inflammatory, antioxidant, and antimicrobial properties [4]. *C. nardus* EO was effective against various bacterial [5] and fungal [6] species. It also has potential antispasmodic and analgesic effects, which could be beneficial in treating muscle tension and rheumatic complaints.

High-performance thin-layer chromatography (HPTLC) combined with direct bioautography is a powerful tool for screening antimicrobial natural products, such as EOs [7–9]. Further analysis of the volatile compounds in the inhibition zones can be conducted, e.g., by scanning *in situ* using HPTLC–direct analysis in real-time mass spectrometry (HPTLC–DART-MS) [8] or by gas chromatography–electron ionization–MS (GC–EI-MS) after eluting the components from the layer, e.g., by using overpressured-layer chromatography (OPLC) [9]. Using conventional HPTLC followed by offline scraping off and elution approach, solid-phase microextraction GC–EI-MS (SPME–GC–EI-MS) is preferred as it discards from the analysis non-volatile compounds originating from the adsorbent [8, 10]. *Cymbopogon* species, including *C. nardus,* have been studied by TLC–direct bioautography using methicillin-resistant

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Staphylococcus aureus (MRSA) bacterial strain and *n*-hexane–ethyl acetate (9:1, *v/v*) as the mobile phase [5]. The parallel GC–MS analysis of the EOs identified geraniol/citronellol in the same zone as the compounds responsible for the characteristic inhibition zone in *C. nardus* EO.

The study aimed at the screening, characterization, and identification of antibacterial *Cymbopogon nardus* EO components by the combination of HPTLC–direct bioautography assays using *Bacillus subtilis*, *Aliivibrio fischeri*, *Xanthomonas euvesicatoria*, and *Pseudomonas syringae* pv. *maculicola*, and SPME–GC–EI-MS of the eluted compounds from the inhibition zones.

2 Experimental

2.1 Materials

The 20 cm × 10 cm aluminum foil-backed HPTLC silica gel 60 F_{254} layers (#1.05548) were acquired from Merck (Darmstadt, Germany). Analytical-grade isopropyl acetate was obtained from Sigma-Aldrich (Budapest, Hungary), and all other solvents used were of analytical grade from Molar Chemicals (Halásztelek, Hungary). Vanillin was purchased from Reanal (Budapest, Hungary). Dye reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Carl Roth (Karlsruhe, Germany) and concentrated sulfuric acid (96%) from Carlo Erba (Milan, Italy). Citronella (*Cymbopogon nardus*) EO was obtained from a Hungarian drug store chain (Aromax Ltd., Budapest, Hungary). Test substances citronellal and citral (mixture of neral and geranial) were purchased from Sigma-Aldrich.

Gram-positive *Bacillus subtilis* soil bacterium (strain F1276) was a gift from József Farkas (Central Food Research Institute, Budapest, Hungary). Gram-negative, naturally luminescent marine bacterium *Aliivibrio fischeri* (DSM 7151) was obtained from Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Berlin, Germany, the Hungarian paprika pathogen *Xanthomonas euvesicatoria* from János Szarka (Primordium Kft., Budapest, Hungary) and *Arabidopsis* pathogen *Pseudomonas syringae* pv. *maculicola* from Jun Fan (John Innes Center, Department of Disease and Stress Biology, Norwich, UK [11]).

2.2 High-performance thin-layer chromatography

HPTLC separation was achieved in a 20 cm × 10 cm unsaturated chamber (CAMAG, Muttenz, Switzerland) with *n*-hexane–isopropyl acetate (9:1, *v/v*) as the mobile phase. CEO (30 mg/mL), citronellal (5 mg/mL), and citral (5 mg/mL) dissolved in ethanol were applied manually in the range of 2–5 µL at 8 mm height from the bottom edge in 6 mm bands by a 10 µL syringe (Hamilton, Bonaduz, Switzerland). The chromatoplates developed up to 8 cm from the lower edge were dried by a cold air stream using a hair dryer (5 min) and documented with a digital camera (Cybershot DSC-HX60, Sony, Neu-Isenberg, Germany) under an ultraviolet (UV) lamp ($\lambda = 254$ nm) (CAMAG) and at Vis after derivatization with vanillin–sulfuric acid reagent (200 mg vanillin + 50 mL ethanol + 1 mL concentrated sulfuric acid; the dipped plates were heated to 110 °C for 5 min).

For isolation, 150 µL of citronella EO solution (30 mg/mL) was applied manually as a 170 mm band by a 100 µL syringe and developed with the mobile phase *n*-hexane–isopropyl acetate (4:1, *v/v*). Then, zones of interest, determined by vanillin-sulfuric acid reagent using the left side of the chromatogram (0.5 cm), were scraped off from the remaining underivatized part into a syringe with a Teflon filter (0.22 µm, Phenomenex) and eluted with 500 µL of ethanol. The eluates were analyzed by SPME–GC–MS.

2.3 HPTLC–bioassay

The bioassays were performed using *B. subtilis*, *A. fischeri*, *P. syringae* pv. *maculicola*, and *X. euvesicatoria* bacterial strains based on previously published methods [8]. Briefly, the dried HPTLC plates developed for *B. subtilis* and *X. euvesicatoria* bioassays were immersed into the appropriate cell suspension, incubated for 2 h in a vapor chamber at 37 °C and 28 °C, respectively, stained with aqueous MTT solution (100 mg in 100 mL of water) by immersion, and after a 15–20 min incubation, the bioautograms were documented with the Cybershot DSC-HX60 digital camera. The bright spots against the bluish background indicate the zones of antibacterials.

In the cases of luminescent *A. fischeri* and *P. syringae* pv. *maculicola,* the developed layers were dipped into the cell suspensions and immediately put into a transparent glass cage under a low-light camera (iBright FL1500 Imaging System, Thermo Fisher Scientific, Budapest, Hungary). The exposure time was 40–80 s for *A. fischeri* and 2–3 min for *P. syringae* pv. *maculicola*. The dark zones lacking luminescent viable cells indicate antibacterial activity.

2.4 SPME–GC–MS conditions

The analysis of the EO and its compounds was carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with a Supelco (Sigma-Aldrich) SLB-5MS capillary column (30 M × 250 µm × 0.25 µm). The GC oven temperature increased from 60 °C (3 min isothermal) to 250 °C at 8 °C/min (1 min isothermal). High-purity helium (6.0) was used as a carrier gas at 1.0 mL/min (37 cm/s) in constant flow mode. Static headspace

solid-phase microextraction (sHS-SPME) technique was performed with an automatic multipurpose sampler (CTC Combi PAL, CTC Analytics AG, Zwingen, Switzerland) using a 65 µM StableFlex polydimethyl siloxane/carboxene/divinyl benzene (CAR/PDMS/DVB) SPME fiber (Supelco, Bellefonte, PA, USA) and 20 mL headspace vials. Extraction was performed after a 5 min incubation at 100 °C by exposing the fiber to the headspace for 10 min. Then, the fiber was immediately transferred to the injector port and desorbed for 1 min at 250 °C. Cleaning and conditioning of the SPME fiber was carried out in a Fiber Bakeout Station (Agilent) in a pure nitrogen atmosphere at 250 °C for 15 min. The mass selective detector was equipped with a quadrupole mass analyzer and was operated in electron ionization mode at 70 eV in full scan mode (41–500 a.m.u. at 3.2 scan/s). MSD ChemStation D.02.00.275 software (Agilent) was used for data analysis. Compound identification was carried out by comparing retention data and the recorded spectra with the data of the NIST 2.0 library. Percentage evaluation included area normalization.

3 Results and discussion

Antibacterial compounds of citronella EO were separated on HPTLC layers using *n*-hexane–isopropyl acetate (9:1, *V*/*V*) as the mobile phase and detected at UV 254 nm and after derivatization with vanillin–sulfuric acid reagent and via direct bioautographic antibacterial assays using Gram-positive *B. subtilis* and Gram-negative *A. fischeri*, *P. maculicola*, and *X. euvesicatoria* (Fig. 1). Six chromatographic zones at hR_F 22 (c1), 27 (c2), 31 (c3), 52 (c4), 57 (c5), and 80 (c6) that showed antibacterial effect were marked (Fig. 1). Derivatization with vanillin–sulfuric acid reagent showed all indicated zones in color (Fig. 1b). However, at 254 nm (Fig. 1a), only zones c4 and c5 were detectable. Inhibition by zones c1, c2, and c6 of the EO was visible against all bacterial strains (Fig. 1c–f). Still, zone c3 exhibited strong activity against *B. subtilis* and *X. euvesicatoria*, while it had a weak effect against *A. fischeri* and *P. maculicola*. The EO seems to contain zones c4 and c5 (Fig. 1a), but their ability for characteristic inhibition against *B. subtilis* was low (Fig. 1c). In the cases of other strains, the minimum inhibitory amounts were not reached (Fig. 1d–f).

Using standard compounds, the presence of geranial, neral, and citronellal was confirmed in zones c4–c6, respectively (Fig. 1a, b). These compounds inhibited all strains and were the constituents of the citronella EO, as confirmed by SPME–GC–MS analysis (Fig. 2a). The main components of the citronella EO are listed in Table 1.

The compounds in zones c1–c3 responsible for the antibacterial effect (Figs. 1 and 3) were identified by offline SPME–GC–MS after scraping off and eluting with ethanol. HPTLC–vanillin-sulfuric acid reagent (Fig. 3a) and HPTLC–*B. subtilis* assay (Fig. 3b) confirmed the purity and the bioactivity of the eluates at the appropriate hR_F and based on SPME–GC–MS analysis (Fig. 2), geraniol, α-cadinol, and elemol were present in the inhibition zones c1–c3 (Fig. 2b–g), respectively.

Citronellal, geranial, neral, geraniol, α-cadinol, and elemol have been described as constituents of citronella EO [4, 5, 12, 13], all displaying a cytotoxic effect [14–16]. Moreover, anti-inflammatory activities of α-cadinol [17], elemol [18], and geraniol [19] have been reported. The antibacterial effect of citronellal, citral, and geraniol has been documented against diverse strains, among others, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* [20–23]. Geraniol and

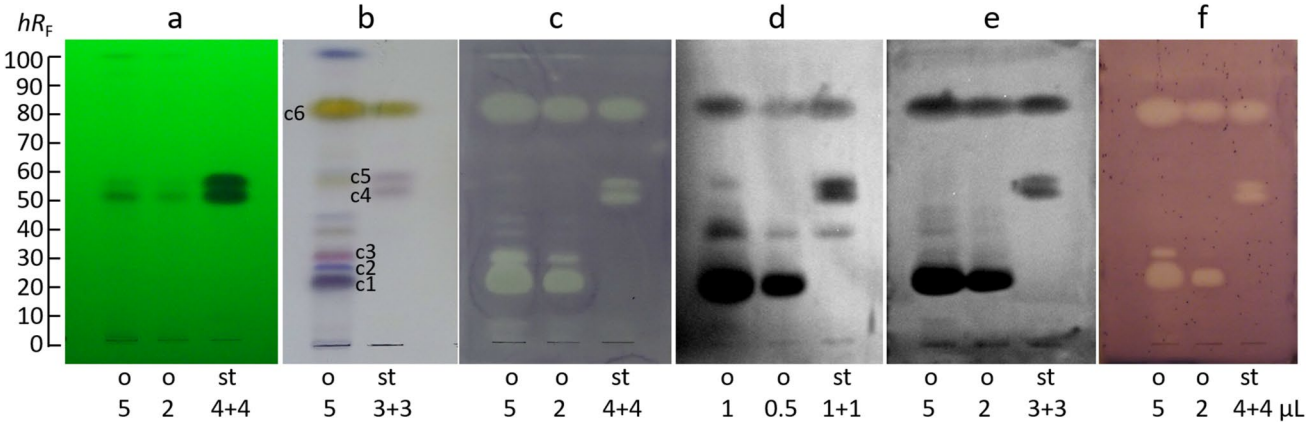

Fig. 1 HPTLC chromatograms of citronella essential oil (o) and standards (st) citronellal (c6), neral (c5), and geranial (c4), developed with *n*-hexane–isopropyl acetate (9:1, *V*/*V*) and detected at UV 254 nm (**a**), at white light illumination after derivatization with vanillin-sulphuric acid reagent (**b**) and bioautograms after *Bacillus subtilis* (**c**), *Aliivibrio fischeri* (**d**), *Pseudomonas syringae* pv. *maculicola* (**e**), and *Xanthomonas euvesicatoria* (**f**) bioassays. The compound zones are indicated as c1–c6

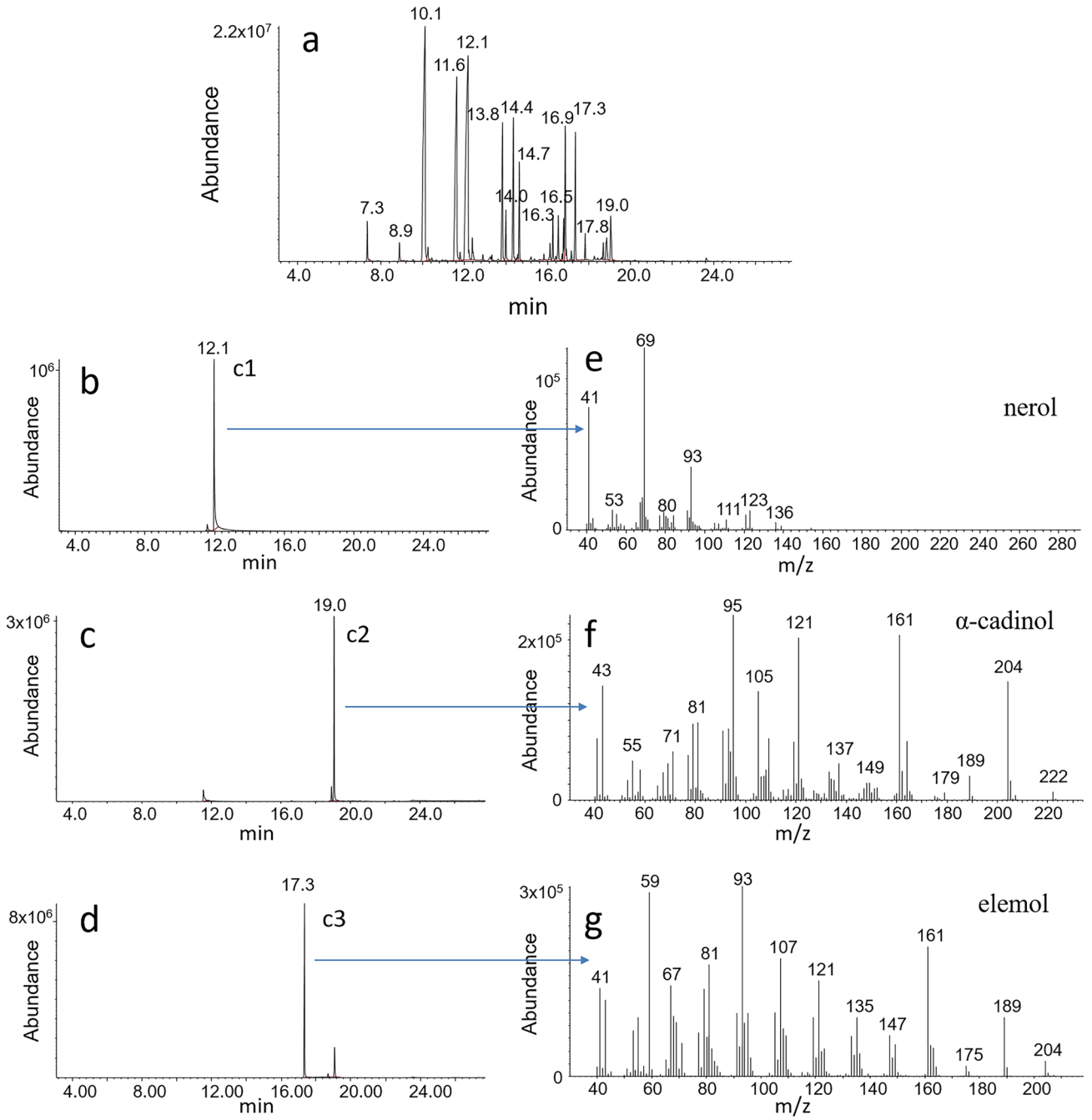

Fig. 2 SPME–GC–MS total ion chromatograms of the citronella essential oil (**a**) and the isolates c1 (**b**), c2 (**c**), and c3 (**d**) and the EI-MS spectrum of the isolated compounds (**e**–**g**, respectively)

citral displayed anti-yeast activity against *Candida albicans* [24, 25] and citronellal and citral inhibited some filamentous fungi, *e.g.,* various *Aspergillus* strains [6, 26]. Among the four studied bacterial strains, only the anti-*Bacillus subtilis* activity of citral has been reported previously [27].

4 Conclusions

The combination of HPTLC–direct bioautography with SPME–GC–MS enabled efficient screening and identification of antibacterial compounds of citronella essential oil, which were identified as citronellal, citral, geraniol, α-cadinol, and elemol. To the best of our knowledge,

Table 1 Main components of the citronella (*Cymbopogon nardus*) essential oil and their percentage based on the peak area obtained by SPME–GC–MS

Component	t_R (min)	Percent area (%)
Limonene	7.3	1.3
Citronellal	10.1	21.5
Citronellol	11.6	15.0
Neral	11.8	0.3
Geraniol	12.1	22.7
Geranial	12.4	1.0
Citronellyl acetate	13.8	5.7
Eugenol	14.0	1.6
Neryl acetate	14.4	5.5
β-Elemene	14.7	3.1
γ-Muurolene	16.3	1.4
α-Muurolene	16.5	1.6
γ-Cadinene	16.8	1.3
β-Cadinene	16.9	4.9
Elemol	17.3	4.6
α-Cadinol	19.0	2.2

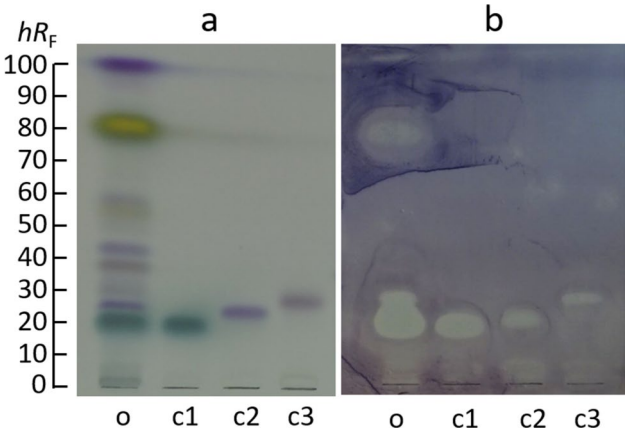

Fig. 3 HPTLC chromatogram (**a**) and bioautogram (**b**) of citronella essential oil (o) and isolates (c1-c3) developed with *n*-hexane–isopropyl acetate (9:1, *V/V*) documented at white light after derivatization with vanillin-sulfuric acid reagent (**a**) and after *Bacillus subtilis* (**b**) bioassay

among the antibacterial effects demonstrated in this study, only the anti-*Bacillus subtilis* activity of citral has been previously known. Thus, this is the first report also about the inhibition effect of citronella essential oil components against plant pathogens *P. maculicola* and *X. euvesicatoria*, which can adumbrate the use of these compounds as agrochemical agents after appropriate formulation.

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Author contributions Conceptualization, methodology, resources, supervision, and writing—original draft preparation: Á.M.M. Formal analysis and investigation: Á.M.M., P.G.O., M.B., and A.C. Writing—review and editing: Á.M.M., P.G.O., and M.B.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest The first and corresponding author, Á.M.M., is a member of the Editorial Board of the journal. Therefore, the submission was handled by a different member of the editorial board, and she did not take part in the review process in any capacity.

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