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Antimicrobial activity of essential oils-derived volatile compounds against several nosocomial pathogens including representative multidrug-resistant A. baumannii clinical isolates

Blanca A. Alanís-Garzaa, Paola Bocanegra-Ibariasb, Noemí Waksman de Torresa, Ricardo Salazar-Arandaa, Soraya Mendoza-Olazaránb, Luis A. Pérez-Lópeza, Samantha Flores-Treviñob and Elvira Garza-Gonzálezb

aFacultad de Medicina, Departamento de Química Analítica, Universidad Autónoma de Nuevo León, Monterrey, México; bServicio de Gastroenterología, Hospital Universitario Dr. José Eleuterio González, Universidad Autónoma de Nuevo León, Monterrey, México

ABSTRACT

The aim was to evaluate the antimicrobial activity of essential oils-derived volatile compounds against nosocomial pathogens, including representative multidrug-resistant (MDR) Acinetobacter baumannii clinical isolates. Minimum inhibitory dose (MID) values for the compounds were determined by the gaseous contact assay. A. baumannii representative clones were selected by pulsed-field gel electrophoresis. MDR profiles were determined by microdilution assay. Drug-resistant genes were detected by PCR. Biofilm production was determined by the crystal violet method. From all tested compounds, carvacrol had markedly lower MIDs (3.89–48.8 mg/L) against A. baumannii than against the other nosocomial MDR pathogens. The lowest MID was detected against three strains, which were obtained from different specimen types, had high drug resistance profiles, and showed variable biofilm production. The work herein provides evidence that carvacrol may have therapeutic potential as a treatment for MDR A. baumannii infections.

Introduction

The development of antibiotic resistance in pathogenic bacteria increases mortality rates in patients with infectious diseases, representing a major threat to global health. The emergence of antibiotic-resistant bacterial strains has been accelerated both by antibiotic overuse and by reduced prevention and infection control (1). Pathogenic species with commonly encountered antibiotic-resistant profiles include Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Acinetobacter baumannii (2).

Drug resistance in nosocomial A. baumannii is a global concern (3); this bacteria has been given critical priority in the global priority pathogens list of antibiotic-resistant bacteria that urgently require the research and development of new and effective antibiotic treatments (6). Vulnerable patients at hospitals are at risk of contracting serious infections with this opportunistic microorganism. In fact, pneumonia caused by A. baumannii is the most commonly acquired infection in hospitalized patients (5). A. baumannii infections can affect various tissues, including the skin, soft tissues, central nervous system and even bone (4). Carbapenem resistance of A. baumannii has been associated with the production of class B and D β-lactamases; the genes encoding these resistance-conferring enzymes may be chromosomal or plasmid. A plasmid location facilitates resistance dissemination, particularly in hospital settings (3).

The increased prevalence of antibiotic-resistant bacteria has triggered interest in plant products as alternative antimicrobial agents. Some essential oils (EO) and other plant extracts have antibacterial activity and are being evaluated as potential sources of new antimicrobial compounds, which may be considered as an alternative for treating infectious diseases (7–10). The EO are aromatic oily fluids obtained from any part of the plant. The variety and number of volatile components present in plant tissues vary, not only across species but also across parts of the same plant.

The activities of EOs have mainly been evaluated in a liquid medium (11). However, EO activities can also be evaluated by a gaseous contact assay in which a highly volatile compound deposited on a paper disc is exposed
to organisms on plates, placed upside down, to determine the minimum inhibitory dose (MID) (12). The aim of this study was to evaluate the antimicrobial activity of EO-derived volatile compounds against nosocomial pathogens, including representative multidrug-resistant A. baumannii clinical isolates.

Materials and methods

Chemical compounds

The chemicals (-)-bornyl acetate, (-) terpinen-4-ol, (-) trans-caryophyllene, (-) α-gurjunene, (-)-β-pinene, (+)-camphene, (R)-(+) limonene, 2-nonanone, camphor, carvacrol, eucalyptol, eugenol, α-humulene, α-pinene and γ-terpinene were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of these chemicals was ≥ 95%.

Bacterial strains

The tested microorganisms included the following clinical isolates: an extended spectrum beta-lactamase producer (ESBL) Klebsiella pneumoniae (14-1694), an ESBL Escherichia coli (14-2148), a Pseudomonas aeruginosa (15-2050) and ten A. baumannii (4676, 8366, 8496, 9700, 10342, 11325, 11391, 12228, 12445 and 12565) clinical isolates. The ATCC strains: methicillin-resistant Staphylococcus aureus (MRSA) 29213 and A. baumannii 15308 were also included in the study. The A. baumannii clinical isolates were collected at the Hospital Universitario Dr. José Eleuterio González in Nuevo Leon, Mexico over a five-year period (2007–2012). All isolates were maintained in 15% glycerol at −70 °C and subcultured on blood agar before testing.

Identification of isolates

The identities of all clinical isolates were determined in sensititre panels (TEK Diagnostic Systems Inc, Cleveland, OH) according to the recommendations of the manufacturer. Identification of the A. baumannii complex was confirmed by growth on MacConkey agar and triple sugar agar, an oxidase test, a catalase test and assessment of mobility and growth at 42 °C. A. baumannii strains were identified by sequence analysis of the recA gene and the 16S-23S rRNA intergenic spacer as described elsewhere (11).

Gaseous contact assay

The gaseous contact assay was performed as previously described (12). Briefly, a bacterial suspension was prepared from a 24-h culture in Mueller-Hinton agar. Some colonies were suspended in saline to achieve turbidity equal to McFarland’s standard 0.5. The suspension was diluted 1:100 in Mueller-Hinton broth and Mueller-Hinton agar plates were inoculated (5 μL of 10⁶ CFU/mL). Paper discs were soaked with a range of volumes (5 μL, 4 μL, 3 μL, 2 μL, 1.5 μL, 1 μL, 0.5 μL, 1.0 μL, 0.5 μL, 0.3 μL and 0.2 μL) of each of the highly volatile compounds and placed in inoculated plates upside down. Plates were double-sealed with parafilm sealing film and incubated for 24 h at 37 °C. Compound concentrations were tested in the range of 3.89–230 mg/L air. All assays were performed twice. The Minimum Inhibitory Dose (MID) of each compound was determined as the lowest concentration of that compound that completely inhibited microbial growth (100%).

Pulsed-field gel electrophoresis (PFGE) of A. baumannii isolates

PFGE analysis was performed on 232 A. baumannii clinical isolates that had been collected from 2006 to 2012. Chromosomal DNA was prepared according to the suggested methodology for S. aureus (13) with modifications recommended for A. baumannii (14). DNA samples from the strains were digested with 10 U of SmaI (Takara Bio Inc., Shiga, Japan). PFGE was performed with a CHEF-DRIII instrument (BioRad Laboratories, Hercules, CA) and band patterns were interpreted as described by Seifert et al. (15). Isolates with 100% similar band restriction patterns were classified as belonging to the same clone.

Antimicrobial susceptibility testing in A. baumannii isolates

The minimum inhibitory concentration (MIC) values of antibiotics were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines (16). For all assays, the quality control organisms used were E. coli ATCC 25922, E. coli ATCC 35218 and P. aeruginosa ATCC 27853. The following antibiotics were included: ciprofloxacin, levofloxacin, gentamicin, amikacin, cetazidime, cefotaxime, imipenem, meropenem, colistin, tetcyclacine and tigecycline (Sigma-Aldrich, Toluca, Mexico). The breakpoint for tigecycline was > 2 as indicated by the British Society for Antimicrobial Chemotherapy (17). Strains resistant to at least three classes of antimicrobials (such as fluoroquinolones, cephalosporins and tetracyclines) were defined as multidrug-resistant (MDR). MDR-confirmed isolates that also exhibited resistance to carbapenems were defined as extreme drug-resistant (XDR) (1).
Characterization of carbapenemase genes in A. baumannii isolates

The carbapenemase genes encoded metallo-β-lactamases (VIM, IMP, NDM-1) and OXA-type carbapenemases (OXA-23, OXA-24, OXA-51 and OXA-58), determined as previously reported (18–21). All OXA-positive PCR products were purified, sequenced by the chain termination method with a Big-Dye Terminator kit (Applied Biosystems Foster City, CA) and analysed in an ABIPRISMA 3100 genetic analyser (Applied Biosystems).

Biofilm production in A. baumannii isolates

Semi-quantitative determination of biofilm formation was performed by crystal violet staining as previously described (22). Stained biofilm was measured at 595 nm (OD595). The biofilm index (OD595/OD600) was used to normalize formed biofilms by the number of cells of each strain. S. aureus ATCC 29213 (a strong biofilm producer) and S. aureus ATCC BAA-44 (a weak biofilm producer) were used as quality control standards.

Results

Antimicrobial activity of highly volatile compounds

The antibacterial activities of fifteen highly volatile compounds in the gaseous state were evaluated. The MIDs of five of these compounds against ESBL-K. pneumoniae (14-1694), ESBL-E. coli (14-2148), MDR-A. baumannii (12445) and P. aeruginosa (15-2050) clinical isolates and ATCC MRSA (29213) are reported in Table 1. Two compounds showed antimicrobial activity against only one of the five strains tested: (+)-camphene inhibited P. aeruginosa and (-)trans-caryophyllene inhibited ESBL E. coli. Meanwhile, (-)-bornyl acetate inhibited two test strains, MRSA and ESBL-E. coli (Table 1). Two compounds, (-) terpinen-4-ol and carvacrol, showed activity against all five strains tested. Notably, carvacrol had markedly lower MIDs against MRSA and A. baumannii than against ESBL-K. pneumoniae, ESBL-E. coli and P. aeruginosa (Table 1).

A. baumannii representative isolates

The PFGE assays of the A. baumannii isolates yielded 54 different restriction patterns and 10 clones, which were clone A (n = 36, 15.5%), B (n = 14, 6%), C (n = 54, 23.3%), D (n = 6, 2.6%), F (n = 4, 1.7%), H (n = 12, 5.2%), K (n = 2, 0.9%), L (n = 2, 0.9%), M (n = 2, 0.9%) and N (n = 3, 1.3%). One isolate of each clone was selected to be included in the following assays.

The clinical characteristics of the patients from which the A. baumannii isolates were obtained are listed in Table 2. The clinical specimens included bronchoalveolar lavage (BAL) (n = 5, 50%), blood (n = 4, 40%) and abscess (n = 1, 10%) and were from patients treated at the intensive care unit (ICU) (n = 9, 90%) and the ambulatory surgery (n = 1, 10%).

The drug resistance profiles of the isolates included MDR (n = 4, 40%), XDR (n = 4, 40%) and non-MDR (n = 2, 20%). All 10 isolates were negative for metallo-β-lactamases genes (VIM, IMP and NDM) and OXA-23; they all were positive for the housekeeping gene OXA-51. OXA-24 and OXA-58 genes were positive in 50% (n = 5) and 40% (n = 4) of the isolates, respectively. Most of the isolates were biofilm producers (n = 7, 70%) and of those, the majority were strong biofilm producers (n = 6, 60%).

Antimicrobial activity of carvacrol

Because of its particularly low MID against A. baumannii (Table 1), carvacrol was selected for further exploration of its activity against individual clinical MDR A. baumannii isolates representative of each of the 10 clones. The drug susceptibility results obtained by gaseous contact assays for the 10 selected A. baumannii isolates are shown in Table 3. The MIDs for carvacrol against these clinical isolates ranged from 3.89 and 48.8 mg/L, with the best results (lowest MID) being obtained against A. baumannii strains 9700, 10342 and 12565. These three strains were obtained from three different specimen types, had MDR or XDR profiles, and were either OXA-24 or OXA-58 positive. They had highly variant biofilm production properties.

Table 1. Minimal inhibitory dose (MID) of five highly volatile compounds against five clinically relevant bacterial species.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ESBL K. pneumoniae 14-1694</th>
<th>MRSA 29213</th>
<th>ESBL E. coli 14-2148</th>
<th>MDR A. baumannii 12445</th>
<th>P. aeruginosa 15-2050</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) Terpinen-4-ol</td>
<td>174.15</td>
<td>217.69</td>
<td>217.69</td>
<td>130.61</td>
<td>217.69</td>
</tr>
<tr>
<td>(+)-Camphene</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>156.43</td>
</tr>
<tr>
<td>(-)-Bornylacetate</td>
<td>NA</td>
<td>229.81</td>
<td>229.81</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>227.47</td>
<td>24.4</td>
<td>227.47</td>
<td>4.88</td>
<td>227.47</td>
</tr>
<tr>
<td>(-)trans-caryophyllene</td>
<td>NA</td>
<td>210.23</td>
<td>210.23</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Notes: NA = not active at 230 mg/L of air.

(-)β-Pinene, α-Pinene (R), Eugenol, (-)(+)-Limonene, 2-Nonanone, Eucalyptol, Camphor, γ-Terpinene, (-)α-Gurjunene and α-Humulene had no activity against the strains evaluated.

ESBL, extended spectrum beta-lactamase producer; MRSA, methicillin-resistant Staphylococcus aureus; MDR, multidrug resistant.
relative to one another (one strong, one weak and one non-producer).

**Discussion**

Natural products are an important source of drugs for the treatment of different diseases. There are numerous studies that describe the antimicrobial activity of essential oils of many plants, including individual oil components (2000, HJD Dorman and SG Deans), against various bacteria (2013 Nicholas, 2017 Sonam). The compounds studied in this work were chosen because they are commonly present in essential oils that show antimicrobial activity. In this study, examination of the antimicrobial activities of fifteen EO compounds against five different clinically relevant bacterial species indicated that two compounds, carvacrol and terpinen-4-ol, have broad antimicrobial properties affecting all five pathogenic species. Because carvacrol shows uniquely strong antimicrobial activity, characterized by very low MIDs for both MRSA and MDR A. baumannii, its activity was tested against ten clinical A. baumannii isolates, selected from representative clones affecting patients in our hospital. The selected strains, originally obtained from blood, bronchoalveolar lavage, or abscess specimens, were carbapenem-resistant, carbapenemase producers; all MDR but two were XDR and a majority were strong biofilm producers. The presently observed carvacrol MIDs for these isolates were lower than those reported for other Gram-negative species (23).

The high prevalence of carbapenem resistance among the presently tested representative clones is noteworthy, given that carbapenem is the treatment of choice for A. baumannii infections. Indeed, we obtained the most promising antimicrobial activity results for carvacrol against three strains that were MDR or XDR, positive for carbapenemase-encoding genes and variable in biofilm production (high, low and non-producer). Hence, carvacrol represents an interesting candidate as an anti-infection molecule, particularly for carbapenem-resistant A. baumannii. It is important to highlight that the bacteria studied in this work were challenged with the compounds in the gaseous phase, demonstrating that in this phase the carvacrol also presents activity.

Our finding that carvacrol, a monoterpenoid phenol, emerged as the compound with the highest antimicrobial activity of the volatile compounds examined here is consistent with the possibility that phenolic compounds may be mainly responsible for the antibacterial activity of Eos (24). The antibacterial action of carvacrol (23) may be due to its ability to damage bacterial membranes (25). Carvacrol is a predominant EO compound in plants of the Labiatae family, including oregano (Origanum vulgare) and thyme (Thymus vulgaris) (25). It is defined as GRAS (Generally Recognized As Safe) and has been approved for food use (26), making it a plausible alternative for use in humans. Furthermore, carvacrol may be useful for controlling foodborne pathogens, such as Enterococcus faecalis, Listeria monocytogenes, S. aureus, E. coli O157:H7, Pseudomonas fluorescens, Salmonella typhimurium, Vibrio cholerae and Vibrio vulnificus (23,27).

Although the antimicrobial activity of carvacrol against A. baumannii has been studied before (28–30), this study was the first to analyse carvacrol in its gaseous state, which is important for its potential use against respiratory infections.

Synergism between carvacrol and established antibiotics (such as ampicillin, bacitracin, chloramphenicol, doxycycline, erythromycin, nalidixic acid, nitrofurantoin, novobiocin, penicillin, streptomycin, sulfamethoxazole, tetracycline, colistin) against both Gram-positive and Gram-negative bacterial species (29, y Zanini 2014), including A. baumannii, has been reported (27). Synergy with carbenapens has not been reported for any bacterial species. Thus, carvacrol-carbapenem synergism assays for
activity against *A. baumannii* and other clinically relevant species remain a major issue to be addressed.

In conclusion, the present results indicate that further exploration of carvacrol as a potential alternative treatment agent for use against MDR *A. baumannii* strains is warranted. In particular, the high volatility of carvacrol makes it conducive to the treatment of respiratory tract and pulmonary infection via an aerosol therapy route.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Blanca A. Alanís-Garza [http://orcid.org/0000-0002-4760-2223](http://orcid.org/0000-0002-4760-2223)

Ricardo Salazar-Aranda [http://orcid.org/0000-0001-7448-5256](http://orcid.org/0000-0001-7448-5256)

Luis A. Pérez-López [http://orcid.org/0000-0001-6865-4914](http://orcid.org/0000-0001-6865-4914)

Elvira Garza-González [http://orcid.org/0000-0001-5831-9661](http://orcid.org/0000-0001-5831-9661)

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