Cymbopogon giganteus Chiov. essential oil: Direct effects or activity in combination with antibiotics against multi-drug resistant bacteria

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ABSTRACT

The discovery of new antimicrobial agents is necessary due to the emergence of multi-drug bacterial resistance. The aim of this work was to study the direct and indirect antimicrobial activity of a Beninese sample of Cymbopogon giganteus essential oil (EOCG) on multi-drug resistant clinical bacteria, its chemical composition, and its cytotoxicity. Direct antimicrobial activity was tested by determination of minimal inhibitory concentration (MIC), and indirect activity, by determining Fractional Inhibitory Concentration Index using checkerboard [fractional inhibitory concentration indices (FICI); synergy: FICI ≤ 0.5; additivity: 0.5 < FICI ≤ 1]. EOCG composition was determined by GC-MS and GC-FID and cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphényltetrazolium bromide (MTT) assay. p-Menthane derivatives (54.87%) and limonene (12.07%) were detected as major compounds by GC analysis. Our results confirmed the direct antimicrobial activity of EOCG, but here on clinical resistant strains (MIC from 0.125% v/v to 0.5% v/v). We also show synergistic effects between EOCG and amoxicillin with FICI ranges of 0.12–0.5 against two Escherichia coli resistant clinical strains, synergistic to additive effects between EOCG and colistin or oxacillin/ampicillin, respectively, against Pseudomonas aeruginosa PA544 and Staphylococcus epidermidis SECN361 (two resistant clinical isolates). Our results also indicate that EOCG had low cytotoxicity (IC50: 67.06 ± 2.694 μg/ml).

1. INTRODUCTION

The emergence of multi-drug resistant bacteria (MDR) over the last years constitutes nowadays a major issue of national and international concern. This emergence of antimicrobial resistance is due to incorrect and irrational use of antibiotics [1]. Bacterial strains have developed resistance to most useful antibiotic classes by using different strategies such as elimination by efflux pumps or reduction in outer membrane permeability, production of antibiotic-modifying or -hydrolyzing enzymes, and mutation in antibiotic targets [2,3]. The discovery of new antimicrobial agents with a new mechanism of action represents a major challenge.

Medicinal herbs and their related products are usually employed in developing countries to treat a wide variety of diseases. Essential oils (EOs) are complex odoriferous mixtures of volatile and scent-laden compounds like monoterpene skeletons, sesquiterpenes, and their derivatives such as aldehydes and phenols [4]. The composition differs between species and seasons of the year [5]. They are known for their antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties [6]. Some studies also showed that some EOs may restore the antibacterial efficacy of antibiotics on resistant strains and represent an attractive and commercially interesting alternative in fighting these multi-resistant bacteria [7].

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2. MATERIALS AND METHODS

2.1. Antimicrobial Assays

2.1.1. Culture media and bacterial strains

The 11 clinical and references strains tested are listed in Table 1. Minimal inhibitory concentrations (MICs) were determined by broth microdilution method following Clinical and Laboratory Standards Institute recommendations in cation-adjusted Mueller-Hinton broth (CA-MHB, Becton, Dickinson and Company, Franklin Lakes, NJ). Susceptibility was categorized according to the European Committee on Antibiotic Susceptibility Testing (EUCAST version 6.0) interpretive criteria (http://www.eucast.org/clinical_breakpoints/) assessed 12 December 2016). All organisms were maintained in CA-MHB containing 20% (v/v) glycerol at −80°C. Before testing, the suspensions were transferred to Mueller Hinton Agar and aerobically grown overnight at 37°C.

| Bacterial strains          | Resistance phenotype                                                                 | Origin                        |
|----------------------------|--------------------------------------------------------------------------------------|-------------------------------|
| *P. aeruginosa* (PA344)    | Resistant to colistin                                                                 | Erasme Hospital, Belgium      |
| *P. aeruginosa* (PA384)    | Resistant to ciprofloxacin, meropenem, and tobramycin                                | St-Luc Hospital, Belgium      |
| *P. aeruginosa* (PA434)    | Resistant to ciprofloxacin and tobramycin                                            | Military Hospital, Belgium    |
| *P. aeruginosa* (PA372A)   | Resistant to ciprofloxacin, meropenem and tobramycin                                 | St-Luc Hospital, Belgium      |
| *P. aeruginosa* (PA413)    | Intermediate to meropenem, sensitive to ciprofloxacin, tobramycin, and colistin       | Erasme Hospital, Belgium      |
| *P. aeruginosa* (PA417)    | Intermediate to meropenem, sensitive to ciprofloxacin, tobramycin, and colistin       | Erasme Hospital, Belgium      |
| *S. aureus* (SA6138Bis)    | Resistant to ampicillin, oxacillin, vancomycin, moxifloxacin, and ciprofloxacin       | P. Appelbaum, Hershey Medical Center, Hershey, PA. |
| *Staphylococcus epidermidis* (SE CN361) | Resistant to ampicillin, oxacillin, linezolid, moxifloxacin, and ciprofloxacin       | P. Appelbaum, Hershey Medical Center, Hershey, PA. |
| *Staphylococcus epidermidis* (SE CN362) | Resistant to ampicillin, oxacillin, moxifloxacin, and ciprofloxacin       | P. Appelbaum, Hershey Medical Center, Hershey, PA. |
| *Escherichia coli* (EC 06AB003) | Resistant to amoxicillin                                                             | Laboratory of Bacteriology, Cliniques Universitaires UCL de Mont-Godinne |
| *Escherichia coli* (EC G5)  | Resistant to amoxicillin                                                             | Laboratory of Bacteriology, Cliniques Universitaires UCL de Mont-Godinne |
| *S. aureus* (MSSA ATCC 25923) | Sensitive to β-lactams                                                             | American Type Culture collection |
| *S. aureus* (MRSA ATCC 33591) | Resistant to β-lactams by production of PBP_2 and β-lactamases                     | American Type Culture collection |
| *P. aeruginosa* PA01        | Wild type                                                                            | American Type Culture collection |
| *P. aeruginosa* PA01pump (PA0509) | PA01 deleted for expression of MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM efflux pumps | [28] |
50 μl of sterile MHB-CA. An aliquot of 50 μl of the inoculum (10^6 cfu/ml) was added to each well, which contains diluted EO and/or antibiotic. Positive and negative growth controls were performed for each plate. The plates were incubated aerobically at 37°C for 16—20 hours. After that, 30 μl of a 0.02% resazurin (Sigma-Aldrich, St Louis MO) aqueous solution was added in each well, which allows to easily identify conditions in which bacteria had grown (metabolization of blue resazurin into pink resorufin). The MIC was considered as the lowest concentration where the well did not change color to pink. The checkerboard synergy test was done in 96-well plates by multi-fold dilutions of antibiotics horizontally with the highest concentration of 2 × MIC while EOCG was diluted vertically with the highest concentration of 1%. The FICI used to determine the checkerboard test was obtained by calculating the sum of the MICs using this formula: FICI = MIC A + MIC E.

FIC A is the MIC of antibiotic in combination/MIC of antibiotic alone while FIC E is the MIC of EO in combination/MIC of EO alone [15]. Different results can be observed: synergistic for FICI ≤ 0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1 < FICI ≤ 4, and antagonistic for FICI > 4 according to the European Committee for Antimicrobial Susceptibility Testing [16]. All experiments were done in triplicate.

2.2. Plants

2.2.1. Collection
CG Chiov was collected in Parakou areas (9°20ʹN, 2°37ʹE) in November 2016. Crops were identified by Herbier National du Bénin (Université Abomey-Calavi) where a voucher specimen was deposited under number AA6680/HNB.

2.2.2. EO and extraction
200 g air-dried leaves were submitted to hydro-distillation using a Clevenger steam-distillation apparatus for 3 hours. EOs were stored at 4°C prior use. The yields were calculated according to the starting weight of the plant material before hydro-distillation (expressed as percentage w/w of the dry vegetable material).

2.2.3. GC/FID Analysis
Gas chromatography analysis was performed on a FOCUS GC (Thermo Finigan, Milan, Italy) equipped with a flame ionization detector and a DB-wax column (30 m × 0.25 mm; 0.25 μm film thickness; Agilent, Palo Alto, CA). Carrier gas: helium in constant flow mode (1.3 ml/min) and the oven temperature program was: 5 minutes at 45°C, 45°C–250°C (3°C/minute) and 5 minutes at 250°C. 1 μl of EO diluted in TBME (1%) was injected at 230°C for the front inlet and at 260°C for detection. The split ratio was 1:50. Calculation of peak area percentage was performed by ChromCard (Interscience Technology) using the normalization method.

2.2.4. GC/MS analysis
The GC/MS (Trace GC 2,000 series Thermo Quest, Rodano, Italy) was interfaced with Trace MS (Thermo Quest) operating in the impact electronic mode at 70 eV and was equipped with DB-wax column (30 m × 0.25 mm; 0.25 μm film thickness; Agilent Palo Alto, CA). Carrier gas: helium in constant flow mode (1.3 ml/

| Compounds                     | KI  | Composition (%) |
|-------------------------------|-----|-----------------|
| Limonene                      | 710 | 12.07           |
| trans-p-mentha 2,8-dien-1-ol   | 1,131 | 13.79          |
| cis-p-mentha 2,8-dien-1-ol     | 1,172 | 8.53         |
| cis-carveol                   | 1,250 | 9.12          |
| trans-carveol                 | 1,357 | 3.44         |
| Carvone                       | 1,225 | 3.75          |
| trans-p-Mentha-1(7), 8-dien-2-ol | 1,294 | 15.52         |
| cis-p-Mentha-1(7), 8-dien-2-ol | 1,382 | 17.03         |
| 3,9-Epoxy-p-mentha-1,8(10)-diene | 1,057 | 1.83          |
| Total identified              |     | 85.08          |
Sixteen components were detected with cis-p-mentha-1(7), 8-dien-2-ol (17.03%), trans-p-mentha-1(7),8-dien-2-ol (15.52%), trans-p-mentha 2,8 dien-1-ol (13.79%), limonene (12.07%), cis-carveol (9.12%), and cis-p-mentha 2,8 dien-1-ol (8.53%) as major constituents. Comparison of the compositions of our sample of EOCG and those described in the literature (from Benin or other countries) showed mostly quantitative differences in major constituents. Indeed, Kpoviessi et al. [20] obtained cis-p-mentha-1(7),8-dien-2-ol (8.9%), trans-p-mentha-1(7),8-dien-2-ol (18.3%), trans-p-mentha 2,8 dien-1-ol (15.5%), limonene (8.3%), cis-carveol (7.3%), and cis-p-mentha 2,8 dien-1-ol (11.3%) while Alitonou et al. [18] reported cis-p-mentha-1(7),8-dien-2-ol (17.34%), trans-p-mentha-1(7),8-dien-2-ol (13.95%), trans-p-mentha 2,8 dien-1-ol (13.91%), limonene (19.33%), and cis-p-mentha 2,8 dien-1-ol (8.10%) as major compounds. However, Bassole et al. [11] reported composition of EOCG from Burkina-Faso with limonene as the dominant compound (42%). It has further been shown that the composition of EOCG (particularly, the limonene content) can differ with the extraction method [21] and collection period or place [20].

Table 3 summarizes the MIC of the EOCG against four reference strains (Staphylococcus aureus MSSA ATCC 25923, S. aureus MRSA ATCC 33591, Pseudomonas aeruginosa PAO1, and its deletion mutant PAO509 (which does not express anymore the main RND multidrug efflux pumps) and a series of clinical isolates of S. aureus, S. epidermidis, P. aeruginosa, or Escherichia coli harboring different resistance phenotypes (see Table 1). The results indicate that EOCG was active with an MIC of about 0.0625%–0.125% v/v against S. aureus MSSA ATCC 25923.

| Strains    | MIC EOCG (%v/v) | Antibiotics | MIC (mg/l) | Susceptibility according to EUCAST | FICI |
|------------|----------------|-------------|------------|-----------------------------------|------|
| SA ATCC 25923 | 0.0625–0.125  | -           | -          | -                                 | -    |
| SA ATCC 33591 | 0.0625–0.125  | -           | -          | -                                 | -    |
| PAO1       | 0.5           | -           | -          | -                                 | -    |
| PAO509     | 0.0625–0.5    | -           | -          | -                                 | -    |
| SA618Bis   | 0.5–0.25      | Ampicillin  | 32         | R                                 | 0.52–1 |
|            |               | Oxacillin   | 256        | R                                 | 0.55–1 |
|            |               | Moxifloxacin| 4          | R                                 | 1–2   |
|            |               | Ciprofloxacin| 64        | R                                 | 0.62–1 |
| SE361      | 0.25–0.125    | Ampicillin  | 16         | R                                 | 0.37–1 |
|            |               | Oxacillin   | 64         | R                                 | 0.12–1 |
|            |               | Linzolid    | 32         | R                                 | 1     |
|            |               | Moxifloxacin| 32         | R                                 | 1–2.5 |
|            |               | Ciprofloxacin| 32       | R                                 | 1     |
| SE362      | 0.25–0.125    | Ampicillin  | 8          | R                                 | 0.56–1 |
|            |               | Oxacillin   | 4          | R                                 | 0.53–1 |
|            |               | Moxifloxacin| 2          | R                                 | 1–2   |
|            |               | Ciprofloxacin| 64       | R                                 | 0.62–1 |
| PA544      | 0.125         | Colistin    | 256        | R                                 | 0.31–1 |
| PA417      | 0.5–0.25      | Meropenem   | 4          | I                                 | 1     |
| PA413      | 0.5           | Meropenem   | 4          | I                                 | 0.62–1 |
| PA372A     | 0.5           | Ciprofloxacin| 4        | R                                 | 1–2   |
|            |               | Meropenem   | 16         | R                                 | 1     |
|            |               | Tobramycin  | 64         | R                                 | 1     |
| PA434      | 0.5           | Ciprofloxacin| 32       | R                                 | 0.56–1 |
|            |               | Tobramycin  | 64         | R                                 | 1     |
| PA384      | 0.5           | Ciprofloxacin| 2        | R                                 | 0.53–1 |
|            |               | Meropenem   | 8          | I                                 | 0.75–1 |
|            |               | Tobramycin  | 32         | R                                 | 0.62–1 |
| ECU6AB003  | 0.25          | Amoxicillin | 4,096      | R                                 | 0.12–0.5 |
| EC5        | 0.25          | Amoxicillin | 8,192      | R                                 | 0.15–0.5 |

PA: Pseudomonas aeruginosa, SA = Staphylococcus aureus, SE = Staphylococcus epidermidis, EC = Escherichia coli; R = Resistant; S = Susceptible; I = Intermediate; *MIC = Minimal Concentration Inhibitory
*EUCAST breakpoints: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_6.0_Breakpoint_table.pdf
*aFICI: Synergistic for FICI ≤ 0.5, additive for 0.5 < FICI ≤ 1, indifferent for 1 < FICI ≤ 4, and antagonistic for FICI > 4 according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST 2000)
and \textit{S. aureus} MRSA ATCC 33591, suggesting that this activity is not modified by the production of \(\beta\)-lactamases and modified PBP target (PBP\(_2\)). EOCG showed a greater effect on PA0509 (0.06\% v/v) than PA01 (0.5\% v/v) suggesting its sensitivity to efflux pumps, as antibiotics. Against clinical isolates, EOCG showed activity against Gram-positive bacteria (0.125\%–0.25\% v/v) and Gram-negative bacteria (0.125\%–0.5\% v/v), whatever their resistance mechanisms. Antimicrobial activity of EOCG was also reported against other reference bacterial strains but not on clinical isolates strains. Indeed, Bassole et al. \cite{11} reported an MIC of 2.1 mg/ml against \textit{S. aureus} ATCC 9144 with lower activities for \textit{P. aeruginosa} CRBIP19.249 (70 mg/ml) being the most resistant strain. Several studies have reported that \textit{P. aeruginosa} is the least sensitive bacteria to EOs \cite{22}. The direct antimicrobial activity observed could perhaps be explained, at least in part, by the presence of a consequent percentage (71.86\%) of oxygenated compounds such as \(p\)-menthane derivatives, carvone, and carveol possessing antimicrobial activities \cite{23}. Other essentials oils rich in \(p\)-mentha-1(7), 8-dien-2-ol which is the major component of EOCG have also demonstrated interesting antibacterial activities \cite{24}.

The checkerboard test was used to analyze the combination of EOCG with different classes of antibiotics against MDR bacteria. The improvement of antibacterial activity of these antibiotics can be due to a possible action of the EOCG on the mechanism of resistance to a specific antibiotic, an increased effect due to the combination of different actions on the bacteria, or an improvement of antibiotic concentration at the target site in the presence of EO. The results of FICI values obtained for combinations of EOCG and antibiotics are given in Table 3. We only analyzed combinations between EOCG and antibiotics to which our 11 different strains were resistant, or at least intermediate (ampicillin, oxacillin, vancomycin, linezolid, moxifloxacin, ciprofloxacin, meropenem, tobramycin, colistin, and amoxicillin, see Table 1).

Our result on the multi-resistant staphylococci clinical species (SA618Bis, SECN361, and SECN362) showed that the addition of EOCG does not lower the MIC of moxifloxacin.

All other tested combinations on \textit{Staphylococcus} species (\textit{aureus} and \textit{epidermidis}) indicated some additive effect, except combinations with \(\beta\)-lactams drugs such as ampicillin and oxacillin on SECN361 showing a synergistic/additive effect (FICI: 0.12–1). Nevertheless, this synergistic activity is not sufficient to reverse resistance of this strain to these antibiotics (Table 4).

The results of checkerboard test on multi-resistant \textit{P. aeruginosa} clinical species reveal in general, some additive effects except for combination with ciprofloxacin on PA372A where it can be considered that no effect was detected, and combination of colistin against PA544, a colistin-resistant strain which shows a synergistic/additive effect (FICI: 0.31–1) with a significant decrease of the MIC of colistin, but not enough to revert resistance (Table 4).

For amoxicillin-resistant \textit{E. coli} clinical isolates, a synergistic effect was observed with amoxicillin on both strains, reversing resistance to amoxicillin on EC06A003, but not ECG5 (Table 4). Several studies also reported a synergistic action when an EO was combined with \(\beta\)-lactams drugs against \textit{E. coli} clinical isolated strains \cite{25} but it is the first time that it was shown with EOCG.

The mechanism of synergy between antibiotics and EOs is not elucidated yet and is difficult to establish due to the possible multtarget actions of EOs and their complex compositions \cite{26}. Furthermore, EOs could act non-specifically, affecting membrane integrity which could improve the antibiotics uptake \cite{27}. The best improvement of antibiotic efficacy was obtained with a combination of EOCG and amoxicillin on amoxicillin-resistant clinical isolates of \textit{E. coli} reducing amoxicillin MIC from 32 to about 4,000-fold.

We also analyzed the cytotoxicity of EOCG on a human non-cancer fibroblast cell line (WI38) by the MTT tests and showed its low cytotoxicity (Table 5). This result was in accordance with the report of Kpoviessi et al. \cite{20} who found an IC\(_{50}\) higher than 50 \(\mu\)g/ml on Chinese hamster ovary cells and the WI38 cell line.

### 4. CONCLUSION

In conclusion, we showed synergistic effects between EOCG and amoxicillin against two amoxicillin-resistant \textit{Escherichia coli} strains, synergistic/additive effects between EOCG and colistin and oxacillin/ampicillin, respectively, against \textit{P. aeruginosa} PA544 and \textit{Staphylococcus epidermidis} SE361. However, in order to assess the potential of these combinations, further work will be essential to understand the mode of action of EOCG and/or its constituents, its toxicity/safety profile, and the molecular mechanisms of observed synergy.

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### Table 4: Impact of synergy on MIC of antibiotic and EOs.

| Antibiotics | Strains/FICI | MIC HE\(_{ALONE}\) Combination (\% v/v) | MIC ATB\(_{ALONE}\) Combination (mg/l) | Susceptibility/Resistance ALOINE Combination (mg/l) |
|-------------|-------------|----------------------------------------|----------------------------------------|-----------------------------------------------|
| Amoxicillin | SECN361/0.37-1 | 0.25/0.03125 | 16/4 | R/R |
| Oxacillin | SECN361/0.12-1 | 0.25/0.015 | 64/4 | R/R |
| Colistin | PA544/0.15-0.5 | 0.125/0.03125 | 256/16 | R/R |
| Amoxicillin | ECG5/0.15-0.5 | 0.25/0.03125 | 8192/256 | R/R |
| Amoxicillin | EC06AB003/0.12-0.5 | 0.25/0.03125 | 4096/1 | R/S |

Note: MIC = Minimal Concentration Inhibitory, PA = Pseudomonas aeruginosa, SA = Staphylococcus aureus, SE = Staphylococcus epidermidis, EC = Escherichia coli, R = Resistant, S = Susceptible, I = Intermediate.

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### Table 5: Cytotoxic effect of EOCG.

| C. giganteus essential oil | 67.06 ± 2.694 |
|---------------------------|----------------|
| Camptothecin | 0.04 ± 0.001 |

IC\(_{50}\) = Concentration causing 50\% inhibition of mitochondrial activity.
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