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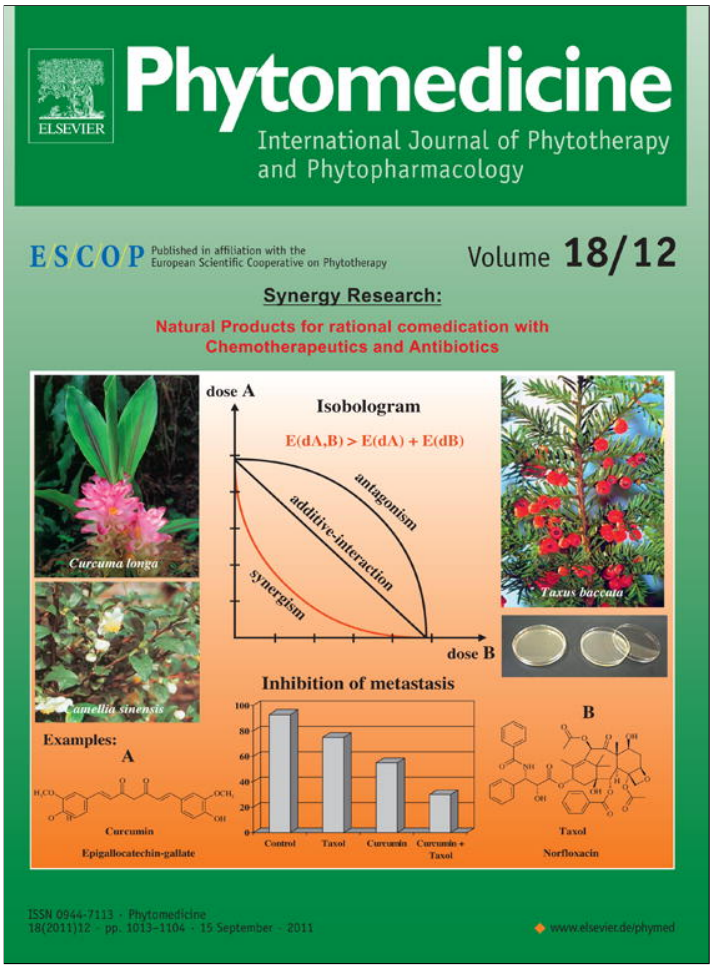

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Chemical composition and antimicrobial activity of *Cymbopogon citratus* and *Cymbopogon giganteus* essential oils alone and in combination

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ABSTRACT

As part of ongoing research on the chemical composition and the antimicrobial properties of Burkinabe plants essential oils alone and in combination, essential oils (EOs) from leaves of *Cymbopogon citratus* and *Cymbopogon giganteus* from Burkina Faso were analyzed by GC–FID and GC–MS. Five constituents, which accounted for 96.3% of the oil, were identified in the EO of *C. citratus*. Geranial (48.1%), neral (34.6%) and myrcene (11.0%) were the major constituents. For *C. giganteus* a total of eight compounds were identified which represented 86.0% of the oils extracted. The dominant compounds were limonene (42%) and a set of monoterpene alcohols: trans-p-mentha-1(7),8-dien-2-ol (14.2%), cis-p-mentha-1(7),8-dien-2-ol (12%), trans-p-mentha-2,8-dien-1-ol (5.6%) and cis-p-mentha-2,8-dien-1-ol (5.2%). The EOs were tested against nine bacteria by using disc diffusion and microdilution methods. *C. giganteus* EO showed antimicrobial effects against all microorganisms tested whereas *C. citratus* EO failed to inhibit *Pseudomonas aeruginosa*. The antimicrobial activity of combinations of the two EOs was quantified by the checkerboard method. Combinations of the two EOs exerted synergistic, additive and indifferent antimicrobial effects. Results of the present investigation provide evidence that the combinations of plant EOs could be assessed for synergistic activity in order to reduce their minimum effective dose.

Introduction

Cymbopogon citratus and *Cymbopgon giganteus*, belongs to the Family *Gramineae*, which has about 660 genera and 9000 species (Clayton 1968). *C. citratus* is commonly used in folk medicine for treatment of nervous and gastrointestinal disturbances, and as an antispasmodic, analgesic, anti-inflammatory, anti-pyretic, diuretic and sedative (Santin et al. 2009). Decoctions of the leaves and flowers of *C. giganteus* are used as an effective treatment against skin disorders, conjunctiva, migraine and hepatitis (Adjanohoun et al. 1979; Adjanohoun and Aké Assi 1979, 1985). Essential oil compositions of both species have previously been investigated. The main constituents of the investigated *C. citratus* oils were identified as neral (cis-citral, citral B), geranial (trans-citral, citral A) and myrcene (Chisowa et al. 1998; Kasali et al. 2001; Menut et al. 2000; Olivero-Verbel et al. 2010; Sacchetti et al. 2005). Limonene and p-menthane derivatives such as cis- and trans-p-mentha-2,8-dien-1-ols, cis- and trans-p-mentha-1(7),8-dien-2-ols cis- and trans-isopiperitenols have been identified as the main components of *C. giganteus* essential oil of from various origins (Boti et al. 2006; Jirovetz et al. 2007; Sidibé et al. 2001). A number of studies have demonstrated the antimicrobial properties of both specie oils against a wide range of microorganisms (Jirovetz et al. 2007; Onawunmia et al. 1984). As a part of our ongoing project devoted to explore the chemical composition and the antimicrobial properties of Burkinabe plants essential oils alone and in combination, we present here the chemical composition and the antimicrobial activity of *C. citratus* and *C. giganteus* essential oils alone and in combination against a range of microorganisms.

Materials and methods

Plant material

Leaves of *Cymbopogon citratus* and *Cymbopogon giganteus* were collected during June 2009 from the botanical garden at the Institut de Recherche en Sciences Appliquées et Technologies, Ouagadougou, Burkina Faso. Plants were identified at the Laboratoire de Biologie et d'Ecologie (Université de Ouagadougou), where a

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voucher specimen is deposited under numbers 13183 and 13184 for *Cymbopogon citratus* and *Cymbopogon giganteus*, respectively.

Essential oils

Fractions of 200 g dried plant material were submitted to hydrodistillation using a Clevenger-type apparatus for 3 h. Anhydrous sodium sulphate was used to remove water after extraction. Essential oils were stored in airtight containers in a refrigerator at 4 °C. The yields were calculated according to the weight of the plant material before distillation (expressed in percent, w/w of the dry vegetable material). Five microliters of essential oil was diluted to 1 ml with dichloromethane containing 0.1 mg/ml of biphenyl as internal standard, prior to GC–FID.

GC/FID analysis

Gas chromatographic analysis was performed on an Agilent 6890N instrument equipped with a flame ionization detector and a DB-5 narrow bore column (length 10 m × 0.1 mm ID, 0.17 μm film thickness; Agilent, Palo Alto, CA, USA). Helium (average velocity 42 cm/s) was used as carrier gas and the oven temperature program was: 60–165 °C (8 °C/min) and 165–280 °C (20 °C/min) with 1 min post run at 280 °C. Samples (1 μl) were injected at 260 °C front inlet temperature and the split ratio was 100:1. Calculation of peak area percentage was performed on the basis of the FID signal using the GC HP-Chemstation software (Agilent Technologies).

GC/MS analysis

The GC–MS (HP 6890 coupled to HP 5972 MSD; Hewlett Packard, Palo Alto, CA, USA) was equipped with a ZB-5MS Zebron capillary column (length 30 m × 0.25 mm ID, 0.25 μm film thickness; Agilent). Helium (average velocity 39 cm/s) was used as carrier gas and the oven temperature was hold 45 °C for 2 min and increased from 45 to 165 °C (4 °C/min), 165 to 280 °C (15 °C/min). Samples (1 μl) were injected at 250 °C and the split ratio was 50:1.

Identification of components

The constituents were identified by comparison of their retention indices with those of the literature. The retention indices were determined in relation to a homologous series of *n*-alkanes (C8–C32) under the same operating conditions. Further identification was made by comparison of their mass spectra with those stored in NIST library or with mass spectra from literature (Adams 2007; Stein et al. 2002). Component relative percentages were calculated based on GC peak areas without using correction factors.

Microbial strains

The microorganisms used were: Escherichia coli CIP 105182, Enterobacter aerogenes CIP 104725, Enterococcus faecalis CIP 103907, Listeria monocytogenes CRBIP 13.134, Pseudomonas aeruginosa CRBIP 19.249, Salmonella enterica CIP 105150, Salmonella typhimurium ATCC 13311, Shigella dysenteriae CIP 54.51 and Staphylococcus aureus ATCC 9144.

Antimicrobial assay

Disk diffusion assay

The agar disk diffusion method was employed for the screening of antimicrobial activities of the essential oils (Bassole et al. 2005). The test was performed in sterile Petri dishes (90 mm diameter) containing solid and sterile Mueller–Hinton agar medium (Becton, Dickinson, USA). The essential oils absorbed on sterile paper discs (5 μl per Whatman disc of 6 mm diameter), were placed on the surface of the media previously inoculated with 100 μl of overnight microbial suspension (10^8 CFU/ml). One filter paper disc was placed per Petri dish in order to avoid a possible additive activity. Every dish was sealed with laboratory film to avoid evaporation, then incubated aerobically at either 30 °C or 37 °C according to bacteria for 18–24 h, followed by measurement of the zone diameter of the inhibition expressed in mm. Antibiotic discs of erythromycin (15 μg/disc) and tetracycline (30 UI) were used as positive controls.

Determination of minimal inhibitory concentration (MIC)

The minimal inhibition concentration (MIC) values were studied for the bacterial strains which were sensitive to the essential oil in disc diffusion assay. Minimal inhibition concentration (MIC) values were determined using micro-well dilution assay method (Carson et al. 1995). A serial doubling two-fold dilution of either essential oil was prepared in a microtiter tray over the range 10–0.075 mg/ml in 100 μl Mueller–Hinton broth. The broth was supplemented with ethanol absolute at a concentration of 0.5% in order to enhance essential oils solubility. Overnight broth cultures of each strain were prepared from 18 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. An aliquot 100 μl of the inoculum was added to diluted essential oil. The final volume in each well was 200 μl. The plate was covered with a sterile plate sealer. Positive and negative growth controls were included in every test. The tray was incubated aerobically at either 30 °C or 37 °C according to bacteria for 18–24 h. The MIC is defined as the lowest concentration of the essential oil at which the microorganism tested does not demonstrate visible growth in the broth. Bacterial growth was indicated by turbidity.

The checkerboard method

The checkerboard method was performed using 96-well microtitre plates as described previously (Moody 2003; Schelz et al. 2006), to obtain the FIC index. The microplate assay was arranged as follows: Essential oil A (EOA) was diluted two-fold along the *x*-axis, whilst EOB B was diluted two-fold along the *y*-axis. The final volume in each well was 100 μl comprising 50 μl of each EO dilution. Subsequently, 100 μl of media containing 2×10^6 CFU/ml of the indicator strain were added to all wells. The plates were then incubated at 30 °C or 37 °C for 18 h. The FIC indices were calculated as FICA + FICB, where FICA and FICB 18 are the minimum concentrations that inhibited the bacterial growth for EOs A and B, respectively. Thus, FICs were calculated as follows: FICA = (MICA combination/MICA alone) and FICB = (MICB combination/MICB alone). The results were interpreted as synergy (FIC < 0.5), addition ($0.5 \leq$ FIC ≤ 1), indifference ($1 <$ FIC ≤ 4) or antagonism (FIC > 4). All experiments were done in triplicate.

Statistical analysis

The data were analyzed with Student's *t*-test or one-way ANOVA followed by Bonferroni test (GraphPad Prism 5.01; GraphPad Software Inc., San Diego, USA). The criterion for statistical significance was taken as $p < 0.05$.

Results and discussion

Chemical composition of the essential oils

Air-dried leaves of the plants were subjected to hydrodistillation using a Clevenger-type apparatus and the yellow-coloured oils were obtained in the yield of 1.52% (w/w) and 0.60% (w/w) for

C. citratus and *C. giganteus*, respectively. Both essential oils consisted exclusively in monoterpenoids. Oxygenated terpenes were the most dominant in the essential oil of *C. citratus* whereas *C. giganteus* had a balanced content of terpenes hydrocarbons and oxygenated terpenes. Five constituents, which accounted for 96.3% of the oil, were identified in the essential oil of *C. citratus*. Geranial (48.1%), neral (34.6%) and myrcene (11.0%) were the major constituents, thus making this a citral type of oil. Geraniol (1.9%) and linalool (0.7%) were the minor constituents. To the best of our knowledge, there are many reports on the chemical composition of the oils from the plants belonging to the specie *C. citratus* (Chisowa et al. 1998; Kasali et al. 2001; Menut et al. 2000; Olivero-Verbel et al. 2010; Sacchetti et al. 2005; Sidibé et al. 2001). Most of these reports indicate that neral and geranial are the main characteristic constituents of *C. citratus*. The findings on the composition of EO from the leaves of *C. citratus* were similar to those previously reported except for methyl-5-epten-2-one which was not found in our sample (Sacchetti et al. 2005). A total of eight compounds were identified from the essential oils of *C. giganteus*, which represented 86.0% of the oils extracted. The dominant compounds were limonene (42%) and a set of monoterpene alcohols: trans-p-mentha-1(7),8-dien-2-ol (14.2%), cis-p-mentha-1(7),8-dien-2-ol (12%), trans-p-mentha-2,8-dien-1-ol (5.6%) and cis-p-mentha-2,8-dien-1-ol (5.2%). The minor compounds were trans-carveol (4%), carvone (2.5%) and 1,3,8-p-menthatriene (0.5%). Regarding the previously reported content of *C. giganteus* essential oil (Boti et al. 2006; Jirovetz et al. 2007; Sidibé et al. 2001), it is interesting to point out that there are important quantitative differences suggesting that the environmental factors strongly influence its chemical composition. However, our sample exhibited the highest content of limonene (42%) which contributed strongly to the aroma of the oil (Table 1).

Table 1
Percentage composition of *C. citratus* and *C. giganteus* essential oils obtained by hydrodistillation.

Compounds	RI	Composition (%)	
		C. citratus	*C. giganteus*
β-Myrcene	988	11.0	–
1,3,8-p-Menthatriene	1026	–	0.5
Limonene	1029	–	42.0
Linalool	1097	0.7	–
trans-p-Mentha-2,8-dienol	1123	–	5.6
cis-p-Menth-2,8-dienol	1138	–	5.2
trans-p-Mentha-1(7),8-dien-2-ol	1189	–	14.2
trans Carveol	1215	–	4.0
cis-p-Mentha-1(7),8-dien-2-ol	1231	–	12.0
Neral	1238	34.6	–
Carvone	1243	–	2.5
Geraniol	1253	1.9	–
Geranial	1267	48.1	–
Total		96.3	86.0
Monoterpenoids		96.3	86.0
Terpenes hydrocarbons		11.0	42.5

Antimicrobial activity

The antimicrobial activity of *C. citratus* and *C. giganteus* essential oil were evaluated against a set of nine microorganisms and their potency were assessed qualitatively and quantitatively by the presence or absence of inhibition zones, zone diameters (ZDs) and MIC values. The correlation between two different screening methods examined was generally larger ZDs correlated with lower MICs. The results are given in Table 2 and indicate that essential oils displayed a variable degree of antimicrobial activity against the different strains tested. *E. faecalis* CIP 103907 was the most sensitive microorganism to the essential oils while *P. aeruginosa* CRBIP19.249 was the most resistant. The fact of being Gram-positive or Gram-negative appears to have little influence on the sensitivity to the oils. The antimicrobial activity of the essential oils displayed considerable variations among two *Cymbopogon* species. The essential oil of *C. citratus* showed the highest activity against *E. faecalis* CIP 103907, *L. monocytogenes* CRBIP13.134, *S. enterica* CIP 105150, *S. typhimurium* ATCC 13311 and *S. dysenteria* 5451 CIP. *C. giganteus* exhibited a considerably stronger activity than that of *C. citratus* when tested against *S. aureus* ATCC 9144, *E. coli* CIP 105182 and *P. aeruginosa* CRBIP19.249. Both *Cymbopogon* species showed similar activity against *E. aerogenes* CIP 104725. Essential oils from *C. citratus* showed no inhibitory activity against *P. aeruginosa* CRBIP19.249. The antimicrobial activity of the essential oils was compared with that of the standard antibiotics by disc diffusion method. The essential oils were more active than standard antibiotics against *E. faecalis* CIP 103907, *L. monocytogenes* CRBIP13.134, *S. typhimurium* ATCC 13311, *S. dysenteria* 5451 CIP and *P. aeruginosa* CRBIP19.249. *P. aeruginosa* was found to be the most resistant to the essential oil of *C. citratus*. Our results are in agreement with those previously reported on the antimicrobial activity of *C. citratus* essential oil and its main components (Cimanga et al. 2002; Onawunmia et al. 1984). This tolerance is not surprising, since *P. aeruginosa* possesses an intrinsic resistance, which is associated with the nature of its outer membrane, to a wide range of biocides (Cox and Markham 2007; Walsh et al. 2003). The relatively high antimicrobial activity of *C. giganteus* essential oil against *P. aeruginosa* and others microbial strains could be due to its high content in limonene. A disc diffusion study on the antimicrobial activity of different volatile oil components indicated on the one hand moderate to no inhibition for (+)-limonene, depending on the pathogen studied and the other hand high antimicrobial activity for (+)-limonene (Aggarwal et al. 2002; Geda 1995; Jirovetz et al. 2004; Neirotti et al. 1996). Jirovetz et al. (2004) reported sensitivities only for (+)-limonene, with no values given for (−)-limonene. However, minor compo-

Table 2
Diameter of inhibition zone expressed (including paper disc diameter) in mm of two investigated essential oils and antibiotics used as a positive control.

Bacteria strains	Reference strains	Essential oils		Standard Antibiotics	
		C. citratus	*C. giganteus*	Tetracycline (30 UI)	Erythromycin (15 μg/disc)
E. faecalis	CIP 103907	34 ± 1.3 a	24 ± 0 b	16 ± 1 c	8 ± 0 d
S. aureus	ATCC 9144	24.3 ± 0.4 a	28.3 ± 1.6 b	34 ± 0 c	30.5 ± 0.5 d
L. monocytogenes	CRBIP13.134	29.3 ± 0.9 a	20.3 ± 5.1 b	19 ± 0 b	8 ± 0 c
E. aerogenes	CIP 104725	11.3 ± 0.9 a	11.7 ± 0.9 a	21 ± 0 b	0
E. coli	CIP 105182	15.3 ± 1.1 a	24 ± 0 b	24 ± 1 b	10 ± 0 c
P. aeruginosa	CRBIP19.249	0	20.3 ± 0.4	0	0
S. enterica	CIP 105150	24 ± 0.7 a	19.3 ± 1.3 b	23 ± 1 a	31 ± 1 c
S. typhimurium	ATCC 13311	31.7 ± 0.4 a	23.7 ± 1.6 b	25 ± 0 c	24.5 ± 4.5 c
S. dysenteria	CIP 5451	26 ± 0.7 a	10.3 ± 0.4 b	12 ± 0 c	9.5 ± 0.5 b

Data in the same line followed by different letters are statistically different by Fisher's test ($p < 0.05$).
Values are means ± standard deviation of three separate experiments.

Table 3
MIC (mg/ml) and FIC indices of *C. citratus* and *C. giganteus* combinations.

Bacteria	Reference strains	*C. citratus*	*C. giganteus*	FIC value	Interaction
E. faecalis	CIP 103907	1 ± 0 a	6.7 ± 0.4 b	1.6	I
S. aureus	ATCC 9144	2.5 ± 0 a	2.1 ± 0 a	0.4	S
L. monocytogenes	CRBIP13.134	8.3 ± 0.2 a	13.3 ± 0.2 b	0.1	S
E. aerogenes	CIP 104725	13.3 ± 0.4 a	12 ± 0 b	0.1	S
E. coli	CIP 105182	10 ± 0 a	6.3 ± 0.2 b	0.5	A
P. aeruginosa	CRBIP19.249	>80	70 ± 0.8	nd	nd
S. enterica	CIP 105150	2.1 ± 0.1 a	10 ± 0 b	1.1	I
S. typhimurium	ATCC 13311	2.5 ± 0 a	8.3 ± 0.1 b	0.2	S
S. dysenteria	CIP 5451	8.3 ± 0.2 a	25 ± 0.4 b	0.8	A

S, synergism; A, addition; I, indifference; nd, non-determined.
Data in the same line followed by different letters are statistically different by Fisher's test ($p < 0.05$).

nents such as p-menthadienol could also contribute to the activity (Onawunmia et al. 1984).

Interaction between essential oils

The quantitative effects of *C. citratus* and *C. giganteus* in combination are described in terms of FIC indices. The FIC indices ranging from 0.1 to 1.6 are listed in Table 3. Combinations of the two essential oils exerted synergistic antimicrobial effect against *S. aureus* ATCC 9144, *L. monocytogenes* CRBIP13.134, *E. aerogenes* CIP 104725 and *S. typhimurium* ATCC 13311. An additive effect was observed for *E. coli* CIP 105182 and *S. dysenteria* 5451 CIP. An indifferent interaction was found for *E. faecalis* CIP 103907 and *S. enterica* CIP 105150. None antagonistic effect was observed. In the most cases combinations of *C. citratus* and *C. giganteus* essential oils exhibited synergistic and additive effects against all the test strains. These results can be explained considering the chemical composition of EOs individually. Individual essential oils contain complex components which, when combined with each other, may lead to indifferent, additive, synergistic or antagonistic effects (Burt 2004). To the best of our knowledge, the antimicrobial activity of combinations of *C. citratus* and *C. giganteus* essential oils has not been reported before. The mechanism of antimicrobial activity of mixed essential oils is still not clear and further studies in this area are needed. This result may be useful for the combination of *C. citratus* and *C. giganteus* essential oils against pathogen bacteria. The *in vitro* results of the present investigation demonstrated the potential of the combination of *C. citratus* and *C. giganteus* essential oils to improve their antimicrobial properties. The combinations of the two EOs may be used in order to provide better efficacy for combating various infections and drug resistance.

Conflict of interest statement

The authors declare there is no conflict of interest.

Author agreement

All authors have made substantial contributions and final approval of the conceptions, drafting, and final version.

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