The *in vitro* antimicrobial activity of *Cymbopogon* essential oil (lemon grass) and its interaction with silver ions

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a b s t r a c t

Background: It is well known that *Cymbopogon* (lemon grass) essential oil exhibits antimicrobial activity while the efficacy of silver ions as a disinfectant is equally well reported.
Hypothesis: The antimicrobial activity of CEO and Ag^+ and their synergistic combinations will be useful in improving the current treatment strategies for various infections.
Study design: In the present study, we determined the chemical composition and *in vitro* antimicrobial activity of six different *Cymbopogon* essential oils (CEO's) alone and in combination with silver ions (Ag^+) against two Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*), two Gram-negative (*Escherichia coli* and *Moraxella catarrhalis*) and two yeast species (*Candida albicans* and *Candida tropicalis*). The nature of potential interactions was determined by fractional inhibitory concentration indices (FICIs) for CEO's and Ag^+ calculated from microdilution assays and time–kill curves.
Results: Gas chromatography–mass spectrometry results confirmed the presence of nerol, geranial and geraniol as major volatile compounds. Minimum inhibitory concentration (MIC) values confirmed that all the tested pathogens are variably susceptible to both CEO's as well as Ag^+. The MIC of CEO's and Ag^+ against all the tested pathogens ranged from 0.032 mg/ml to 1 mg/ml and 0.004 and 0.064 mg/ml respectively, whereas when assayed in combination the FICI values were drastically reduced to range between 0.258 and 2.186, indicating synergy, additive and indifferent interactions. The most prominent interaction was observed between *Cymbopogon flexuosus* essential oil and Ag^+ against *C. albicans* with 2:FIC = 0.254. The synergistic interactions were further confirmed through the construction of isobolograms and time–kill plots. Transmission electron microscopy showed disturbance in the cell envelope upon the concomitant treatment of CEO's and Ag^+, which ultimately leads to cell death.
Conclusion: Results suggest that CEO's and Ag^+ when used in combination offers an opportunity to the formulation scientist to produce novel combinations acting synergistically in the continued quest to control important infectious pathogens.

Introduction

Over the past 70 years, several antimicrobial agents have been discovered and synthesized by medicinal chemists to combat microbial pathogens including bacteria, fungi, viruses, parasites, etc. However, with the passage of time many pathogens have adapted to these drugs, leading to drug resistances. The incidences of antimicrobial resistance have steadily increased globally. Besides increasing the morbidity and mortality rates, resistance to antimicrobial agents has resulted in treatment failures and increased health care costs (Howard et al. 2003). This problem has ignited the interest of researchers and clinicians in the use of natural products as antimicrobial agents. Among the plethora of natural products used as antimicrobial agents, essential oils and metal ions gained interest because of their broad spectrum activity (Chopra 2007; Low et al. 2011). Several studies have demonstrated that essential oils have an appreciable antibiotic spectrum with multiple drug targets (Khan et al. 2010; Lu et al. 2012). *Cymbopogon* species are commonly used in folk medicines for the treatment of infectious diseases and several other diseases and disorders (Santin et al. 2009). Among all the inorganic metal ions, the antimicrobial properties of silver have been investigated most extensively (Guggenbichler et al. 1999). Since the 19th century, silver ions have been identified to be effective against a broad range of microorganisms and during the early 20th century these ions have been approved for use as antimicrobial agents (Hugo and Russell 1982; Chopra 2007). In addition, these ions are also used to control bacterial growth in a variety of medical applications, including

dentistry, catheters, and the healing of burn wounds (Klasen 2000). However, with the discovery of new antibiotics the interest in Ag^+ has diminished but interest has recently resurfaced due to the growing concern of multidrug resistance.

In view of the lack of new classes of drugs emerging and the increase in antibiotic resistances, combination therapies might be considered a viable strategy, considering the popularity of a multiple microbial drug target approach (Mukherjee et al. 2005; Ahmad et al. 2010a). Among the numerous advantages of combinational strategies is a synergistic interaction, in which the antimicrobial activity is greater than the individual contribution of each agent. In the present study, we have evaluated the *in vitro* antimicrobial activity of CEO's against two Gram-positive, two Gram-negative and two yeast isolates. We have explored the possibility of synergistic interaction between CEO's and Ag^+ against a panel of microbes using broth microdilution assays, isobolograms and time–kill studies. We also generated images with transmission electron microscopy (TEM) to observe the possible mechanism of action of CEO/Ag^+ alone and in combination against different tested pathogens.

Materials and methods

Essential oils

Six *Cymbopogon* essential oils were obtained from a commercial supplier (Paranom, Belgium); *Cymbopogon giganteus* Chiov. (CgEO), *Cymbopogon winterianus* Jowitt ex Bor (CwEO), *Cymbopogon flexuosus* (Nees ex Steud.) W. Watson (CfEO), *Cymbopogon martinii* (Roxb.) W. Watson (CmEO), *Cymbopogon nardus* (L.) Rendle (CnEO) and *Cymbopogon citratus* (DC.) Stapf (CcEO).

Strains, media and chemicals

All strains were initially grown in Tryptone Soya Broth (TSB). Prior to assays, pure cultures from Tryptone Soya Agar (TSA) plates were sub-cultured and incubated for their respective incubation periods (bacteria for 24 h and fungi for 48 h) at 37 °C. All other chemicals and media were of analytical grade and were procured from Oxoid, England. DMSO, ciprofloxacin (CFL) and amphotericin B (AmB) and $AgNO_3$ (99.99%) was purchased from Sigma Fluke.

Inoculum preparation

All bacterial strains were initially sub-cultured aseptically on the TSA and single colonies of all the cultured bacteria were subsequently grown to exponential phase in TSB at 37 °C for 24 h and adjusted to a final density of 10^8 colony forming units (CFU/ml) by diluting fresh cultures and comparing with McFarland scale. Fungal cells were also cultured on TSA plates and incubated at 35 ± 2 °C for 48 h until white round colonies were observed. A fresh single colony of each fungal strain was then suspended in 5 ml of sterilized saline to yield a final concentration 10^6 CFU/ml, when compared with the McFarland solution.

Gas chromatography–mass spectrometry analysis

All six *Cymbopogon* essential oils (CEO's) were subjected to GC–MS analysis using a gas chromatograph coupled to a mass spectrometer and flame ionization detector (GCMS-FID) as described previously (de Rapper et al. 2013). The Agilent (6890N) GC system was equipped with a HP-Innowax polyethylene glycol column (60 m × 250 μm i.d × 0.25 μm film thickness). The chemical components were identified by comparing mass spectra from the total ion chromatogram, and retention indices using NIST and Mass Finder GC–MS libraries.

Antimicrobial susceptibility

Determination of minimal inhibitory concentration (MIC)

The MICs of all six *Cymbopogon* essential oils and Ag^+ for bacteria and fungi were determined by the Clinical and Laboratory Standards Institute recommended broth microdilution methods M7-A6 and M27-A3, respectively (CLSI, 2003 , 2008). Essential oils and Ag^+ were diluted to yield a concentration of 8 mg/ml using 1% DMSO and sterile distilled water as diluents, respectively. The positive control ciprofloxacin (0.01 mg/ml) for bacteria and amphotericin B (0.1 mg/ml) for yeasts and the negative vehicle control (1% DMSO) were also included in every set of experiments. Media and culture controls were included to confirm the sterility and viability, respectively. The reference test organisms, from the initial densities were adjusted to obtain an approximate final inoculum size of 5×10^5 CFU/ml for bacteria and 1×10^3 CFU/ml for fungi, were then added to each well, at a volume of 0.1 ml. The microtitre plates were sealed with a sterile adhesive film to prevent any essential oil loss due to their inherent volatility. The microtitre plates were incubated under optimal conditions (37 °C for 24 h for bacteria and 37 °C for 48 h for yeasts). After incubation, 0.4 mg/ml of *p*-iodonitrotetrazolium violet solution (INT) was added to each well (0.04 ml). Viable microorganisms interact with INT to create a color change from clear to a red-purple color. Thus, the lowest dilution with no color change was considered as the MIC for that CEO (de Rapper et al. 2013). All the results were calculated as a mean of the experiments done in duplicate.

Assessment of the FIC index

To determine the interaction of the essential oils with the Ag^+, microdilution assays were performed in 96-well microtitre plates as described previously (Ahmad et al. 2014). Briefly, CEO's and Ag^+ were added to the microtitre plates in 1:1 volumes together with 0.1 ml media and were serially diluted. To assess the interactions, the data obtained were further analyzed using the fractional inhibitory concentration index (FICI), which is based on the zero-interaction theory of Loewe additivity. The FICI was calculated as follows:

$$\mathrm{FICI} = \mathrm{FICa} + \mathrm{FICb} = \frac{\mathrm{MICa\ in\ combination}}{\mathrm{MICa\ tested\ alone}} + \frac{\mathrm{MICb\ in\ combination}}{\mathrm{MICb\ tested\ alone}}$$

where MICa and MICb are the MICs of the CEO's and Ag^+ respectively. A FICI value was interpreted as synergy when FICI is $\leq$ 0.5 and antagonism when FICI is $>$ 4. A FICI result between 0.5 and 1.0 was considered additive and a value between 1.0 and 4.0 was considered as indifferent (Van Vuuren and Viljoen 2011).

Varied ratio combinations and isobolograms

On the basis of the promising synergistic interactions between the CEO's and Ag^+ observed in the microdilution assays, isobolograms were constructed. Nine ratios (9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; and 1:9) of the CEO's and Ag^+ were mixed and thereafter the MIC values were determined for these combinations, as well as for the essential oils and Ag^+ independently. Isobolograms were plotted using GraphPad Prism, version 5 software, to present the mean MIC values of the combinations as ratios (Ahmad et al. 2014). The isobolograms were interpreted by examining the data points for each ratio in relation to the MIC values for the oils independently. All points between the 1.0:1.0 line and 4.0:4.0 line were classified as non-interactive. Points between the 0.5:0.5 and 1.0:1.0 line were interpreted as additive and points below or on the 0.5:0.5 line on the isobologram were interpreted as synergistic. Antagonism was identified as data points above the 4.0:4.0 line (Van Vuuren and Viljoen 2011).

Time–kill curves

In order to further confirm the possible synergy of the CEO's and Ag^+, killing assays were performed with one Gram-positive, one

Gram-negative and one yeast strain, according to the standard protocol as per the guidelines of CLSI (NCCLS 1999). Briefly, the cell suspension of 5 × 10^6 CFU/ml was diluted 1:10 in media to yield a final inoculum concentration of 5 × 10^5 CFU/ml. Final concentrations of CEO and Ag^+ were ¼ MIC values for each strain. Cultures (5 ml final volume) were incubated at 37 °C with agitation (200 rpm). At predetermined time points (0, 2, 4, 8, 12 and 24 h) 100 µl aliquots were removed and transferred to Eppendorf tubes, centrifuged (4000 *g* at 4 °C for 1 min) and rinsed twice with 1 ml of sterile distilled water to obtain compound-free cells. Pellets were suspended in 0.1 ml of sterile distilled water and serially diluted. 20 µl was spread onto TSA plates and incubated at 37 °C for their respective incubation periods (until the colonies were seen on the plates) to determine the numbers of CFU/ml.

Transmission electron microscopy

To determine the mechanism of action of CEO's and Ag^+ in combination, TEM imaging was performed using CmEO and Ag^+ against *E. coli*, *S. aureus* and *C. albicans*. All the cells at the final concentration of 1 × 10^6 CFU/ml were exposed to MIC values of CmEO and Ag^+ for 1 h with shaking at 37 °C. Post exposure, to determine the ultrastructural changes, cells were washed twice for 15 min in 0.1 M sodium phosphate buffer (pH 6.0) and fixed for 2 h in 2.5% (v/v) glutaraldehyde/formaldehyde in 0.075 M phosphate buffer (pH 7.4) at room temperature. Cells were then rinsed three times with the same buffer for a period of 10 min. The specimens were post-fixed with 0.5% (w/v) aqueous osmium tetroxide for 1 h and rinsed thrice with the same buffer for 10 min. The post-fixed specimens were dehydrated in a graded ethanol series (once in 30, 50, 70, 80, and 95% and three times in 100% for 10 min each). All the specimens were then infiltrated with 50% quetol in ethanol for 1 h followed by an infiltration in 100% quetol for 4 h. All the samples were then polymerized at 60 °C for 39 h. After polymerization ultrathin sections (0.1 µm) were cut using a Reichert-Jung Ultracut E microtome (Vienna, Austria) and then transferred to a copper grid. Samples were stained for 10 min in 4% aqueous uranyl acetate followed by Reynolds' lead citrate for 2 min. Samples were washed three times in Milli-Q water and dried by touching Whatman filter paper. Sections were examined with a Jeol (Tokyo, Japan) JEM-2100F transmission electron microscope at 120 kV.

Results

GC–MS analysis

Aromatic plants are known to be highly variable in terms of essential oil composition, therefore the composition of each studied essential oil was determined by GC–MS. The major compounds identified in each of the six oils are given in Table 1. From these results, it was observed that the oil composition of CgEO is qualitatively similar to previous reports (e.g. Bassolé et al. 2011), however, quantitative differences were observed e.g. limonene (42% vs. 11%). Concerning CcEO, the chemical composition was also in congruent with that published by Bassolé et al. (2011), who reported neral (34.6%) as the major constituent which is similar to our study (30.5%). Kpoviessi et al. (2014) reported the major component in CgEO to be *trans*-*p*-mentha-1(7),8-dien-2-ol (18.3%) which is similar to our results (16.2%). For CwEO, the qualitative composition compares favorably with that published by Gonçalves et al. (2010), however, quantitative differences were observed for citronellal, geraniol, citronellol and limonene. Interestingly, Malele et al. (2007) confirmed linalool (27%) as a lead constituent of CwEO, while in our study linalool was not detected. Aromatic plants are notoriously variable in their essential oil composition. Hence, it is imperative that studies reporting the biological properties of an essential oil are accompanied by a chemical fingerprint to document the

Table 1.
Chemical composition of different *Cymbopogon* essential oils.

Compound	RRI	%
Cymbopogon giganteus (CgEO)		
Limonene	1194	11.8
cis-*p*-Mentha-2,8-dien-1-ol	1613	21.3
1,3,8-*p*-Menthatriene	1664	17.8
Carveol (isomer)	1713	8.8
Carvone	1741	3.3
trans-*p*-Mentha-1(7),8-dien-2-ol	1802	16.2
6-Acetoxy-*p*-mentha-1(7),8-diene	1808	5.6
Carveol (isomer)	1839	3.1
Total		88.9
Cymbopogon winterianus (CwEO)		
Limonene	1194	3.6
Isopulegol	1340	1.3
Citronellal	1482	36.7
β-Elemene	1591	2.1
Citronellyl acetate	1662	2.4
Bicyclo-sesquiphellandrene	1723	2.1
Citronellol	1765	13.1
Cadinene	1808	4.9
Geraniol	1822	21.8
Elemol	2087	2.9
Eugenol	2188	1.5
Total		92.4
Cymbopogon flexuosus (CfEO)		
Limonene	1194	1.8
Linalyl acetate	1563	2.1
β-Caryophyllene	1596	1.0
Neral	1689	32.9
Geranial	1740	46.1
Cadinene	1808	3.5
Geraniol	1822	6.1
Total		93.5
Cymbopogon martinii (CmEO)		
λ-Terpinene	1242	1.5
Linalool	1541	3.1
β-Caryophyllene	1596	1.9
Nerol	1800	8.9
Geraniol	1822	79.7
Total		95.1
Cymbopogon nardus (CnEO)		
Tricyclene	1005	1.0
α-Pinene	1016	1.2
Camphene	1057	7.5
Limonene	1194	7.5
Z-β-Ocimene	1232	1.9
E-β-Ocimene	1250	1.0
Citronellal	1482	3.7
β-Elemene	1591	0.9
β-Caryophyllene	1596	1.9
Citronellyl acetate	1662	0.9
α-Farnesene	1665	3.9
α-Terpineol	1701	1.4
Borneol	1702	5.9
Geranyl acetate	1758	7.7
Cadinene	1763	1.2
Citronellol	1765	3.1
Geraniol	1822	25.9
Geranyl butyrate	1893	1.3
Methyl isoeugenol	2021	7.3
Elemol	2087	1.4
Total		86.6
Cymbopogon citratus (CcEO)		
Limonene	1194	5.1
6-Methyl-5-hepten-2-one	1339	1.3
Linalyl acetate	1563	1.2
β-Caryophyllene	1596	1.9
Neral	1689	30.5
Geranial	1740	42.1
Geranyl acetate	1758	4.7
Cadinene	1808	1.2
Geraniol	1822	7.1
Total		95.1

Table 2
Minimum inhibitory concentrations and fractional inhibitory concentration index of *Cymbopogon* essential oils and silver ions.

Essential oils	MIC (mg/ml)	*E. coli* ATCC8739	*M. catarrhalis* ATCC 23246	*S. aureus* ATCC126000	*E. faecalis* ATCC29212	*C. albicans* ATCC 10231	*C. tropicalis* ATCC 201380
CcEO	MIC (mg/ml) alone	0.063	0.250	0.125	0.125	0.063	0.016
	MIC_{CcEO} (mg/ml) in combination	0.063	0.016	0.128	0.064	0.063	0.063
	MIC_{Ag+} (mg/ml) in combination	0.500	0.500	0.250	0.250	0.500	0.500
	FICI	0.563	0.516	0.378	0.314	0.563	0.563
	Interpretation	ADD	ADD	SYN	SYN	ADD	ADD
CmEO	MIC (mg/ml) alone	0.125	0.125	0.125	0.063	0.063	0.032
	MIC_{CmEO} (mg/ml) in combination	0.016	0.016	0.500	0.254	0.032	0.032
	MIC_{Ag+} (mg/ml) in combination	0.250	0.250	0.977	0.500	0.250	0.250
	FICI	0.266	0.266	1.477	0.754	0.282	0.282
	Interpretation	SYN	SYN	ADD	ADD	SYN	SYN
CwEO	MIC (mg/ml) alone	0.500	0.500	0.125	0.125	0.125	0.063
	MIC_{CwEO} (mg/ml) in combination	0.016	0.008	0.500	0.252	0.032	0.063
	MIC_{Ag+} (mg/ml) in combination	1.000	0.500	0.977	0.984	0.500	1.000
	FICI	1.016	0.508	1.477	1.236	0.532	1.063
	Interpretation	IND	ADD	IND	IND	ADD	IND
CnEO	MIC (mg/ml) alone	0.125	0.250	0.250	0.250	0.125	0.063
	MIC_{CnEO} (mg/ml) in combination	0.016	0.008	0.128	0.064	0.032	0.032
	MIC_{Ag+} (mg/ml) in combination	0.250	0.250	0.500	0.500	0.500	0.500
	FICI	0.266	0.258	0.628	0.564	0.532	0.532
	Interpretation	SYN	SYN	ADD	ADD	ADD	ADD
CfEO	MIC (mg/ml) alone	1.000	1.000	0.500	0.250	0.250	0.063
	MIC_{CfEO} (mg/ml) in combination	0.008	0.008	0.064	0.032	0.004	0.127
	MIC_{Ag+} (mg/ml) in combination	1.000	1.000	0.500	0.250	0.250	2.000
	FICI	1.008	1.008	0.564	0.282	0.254	2.127
	Interpretation	IND	IND	ADD	SYN	SYN	IND
CgEO	MIC (mg/ml) alone	1.000	0.500	0.500	0.250	0.125	0.125
	MIC_{CgEO} (mg/ml) in combination	0.008	0.016	0.032	0.032	0.016	0.016
	MIC_{Ag+} (mg/ml) in combination	1.000	1.000	0.250	0.250	0.500	0.500
	FICI	1.008	1.016	0.282	0.282	0.516	0.516
	Interpretation	IND	IND	SYN	SYN	ADD	ADD
Ag^+	MIC alone	0.008	0.008	0.064	0.032	0.008	0.004
Positive controls[a]	0.0005	0.001	0.0005	0.001	0.001	0.001	

[a] Controls are ciprofloxacin for bacteria and amphotericin B for the yeasts.

specific chemotype which will allow the results to be appropriately discussed and provide a basis for future research.

Antimicrobial activity

The MIC values obtained for all the CEO's and Ag^+ as well as for the positive controls against the six microbial species are shown in Table 2. Evaluation of MIC data showed that both CEO's as well as Ag^+ were active against all the tested pathogens with MIC values ranging from 0.016 to 1.000 mg/ml for CEO's and 0.004–0.64 mg/ml for Ag^+. The order of sensitivity on the basis of MIC values of the CEO's against all the pathogens tested was CcEO > CmEO > CwEO > CnEO > CfEO > CgEO. Sensitivity to Ag^+ to different pathogens was observed in the order of *C. tropicalis* (0.004 mg/ml) > *C. albicans* (0.008 mg/ml) ::: *E. coli* (0.008 mg/ml) ::: *M. catarrhalis* (0.008 mg/ml) > *E. faecalis* (0.032 mg/ml) > *S. aureus* (0.064 mg/ml). Yeast strains are highly sensitive to both CEO's and Ag^+, but a difference in sensitivity was observed with the bacterial strains where Gram-positive pathogens were more sensitive to CEO's than Gram-negative pathogens while the Gram-negative pathogens were more sensitive to Ag^+ than Gram-positive pathogens. Differences in sensitivity by Gram-positive and Gram-negative bacteria against the hydrophobic EO's and hydrophilic Ag^+ are in agreement with previous findings (Low et al. 2011).

Susceptibility in combination with silver ions

The FICI values of CEO's combined with Ag^+ were calculated to determine their possible interactions against all the tested microorganisms using the microbroth dilution method as described previously (Ahmad et al. 2014). The data, shown in Table 2, for the six CEO's in combination with Ag^+ in a 1:1 ratio, indicates synergistic, additive or indifferent interactions, while no antagonistic interaction was observed. Of the 36 combinations produced (Table 2), the FICI values against all the tested pathogens ranged from 0.254 to 2.127. For all the combinations tested (n = 36), 33% were found to be synergistic, 42% additive and 25% indifferent (Table 2). The most pronounced synergistic interaction was observed between CfEO and Ag^+ against *C. albicans* (FICI = 0.254). Most of the synergistic interactions were observed between CmEO and Ag^+ (67%), while no synergistic interaction was observed between CwEO and Ag^+. For 12 of the synergistic combinations observed in the 1:1 ratio, further in depth studies were carried out through the construction of isobolograms. For isobolograms, nine different ratios of CEO's and Ag^+ were blended and antimicrobial efficacies were determined (Fig. 1). From these isobolograms, it is evident that most of the combinations, regardless of the ratio, display synergistic interactions. The combinations of CnEO and Ag^+ against *M. catarrhalis* and CcEO and Ag^+ against *E. faecalis* showed synergistic interactions for all nine ratios examined. CmEO and CfEO in combination with Ag^+ against these pathogens (*M. catarrhalis* and *E. faecalis*, respectively) showed synergy for eight of the nine ratios assayed. In other CEO combinations with Ag^+, most of the ratios showed synergy while all other ratios exhibit additive effects.

Time–kill curves

The synergism observed using the broth microdilution assay and isobolograms were further confirmed by time–kill curves. As shown in Fig. 2, CEO's and Ag^+ at their respective ¼ MIC values did not affect the growth of tested microorganisms. Both the agents alone at lower concentrations had a weak antimicrobial effect. In contrast, the combination of CEO's with Ag^+ showed potent antimicrobial activity. In the case of *E. coli* and *C. albicans*, after 6 h of incubation, the CmEO–Ag^+

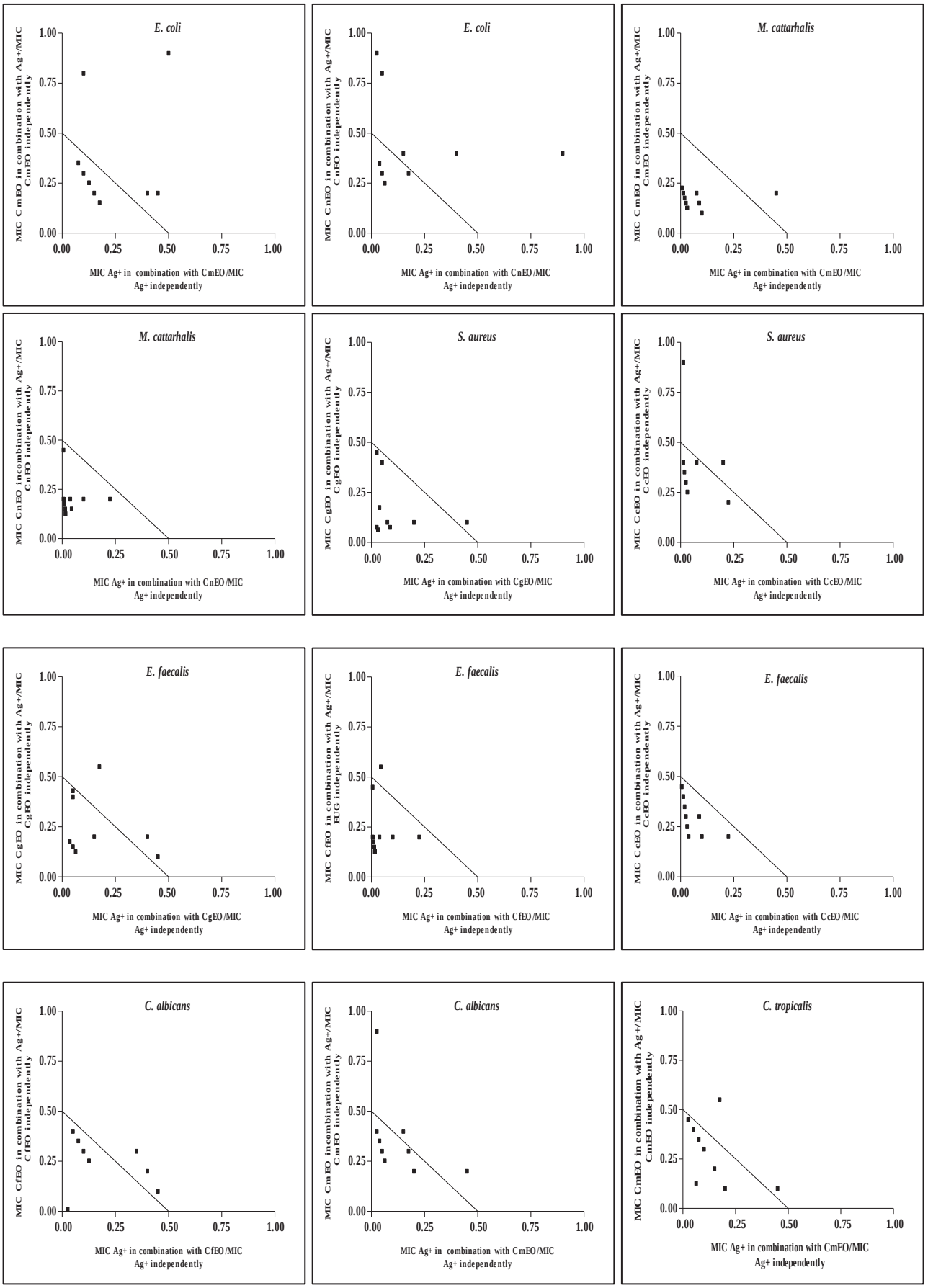

Fig. 1. Isobolograms of CEO and Ag^{+} in nine different ratios against selected pathogens.

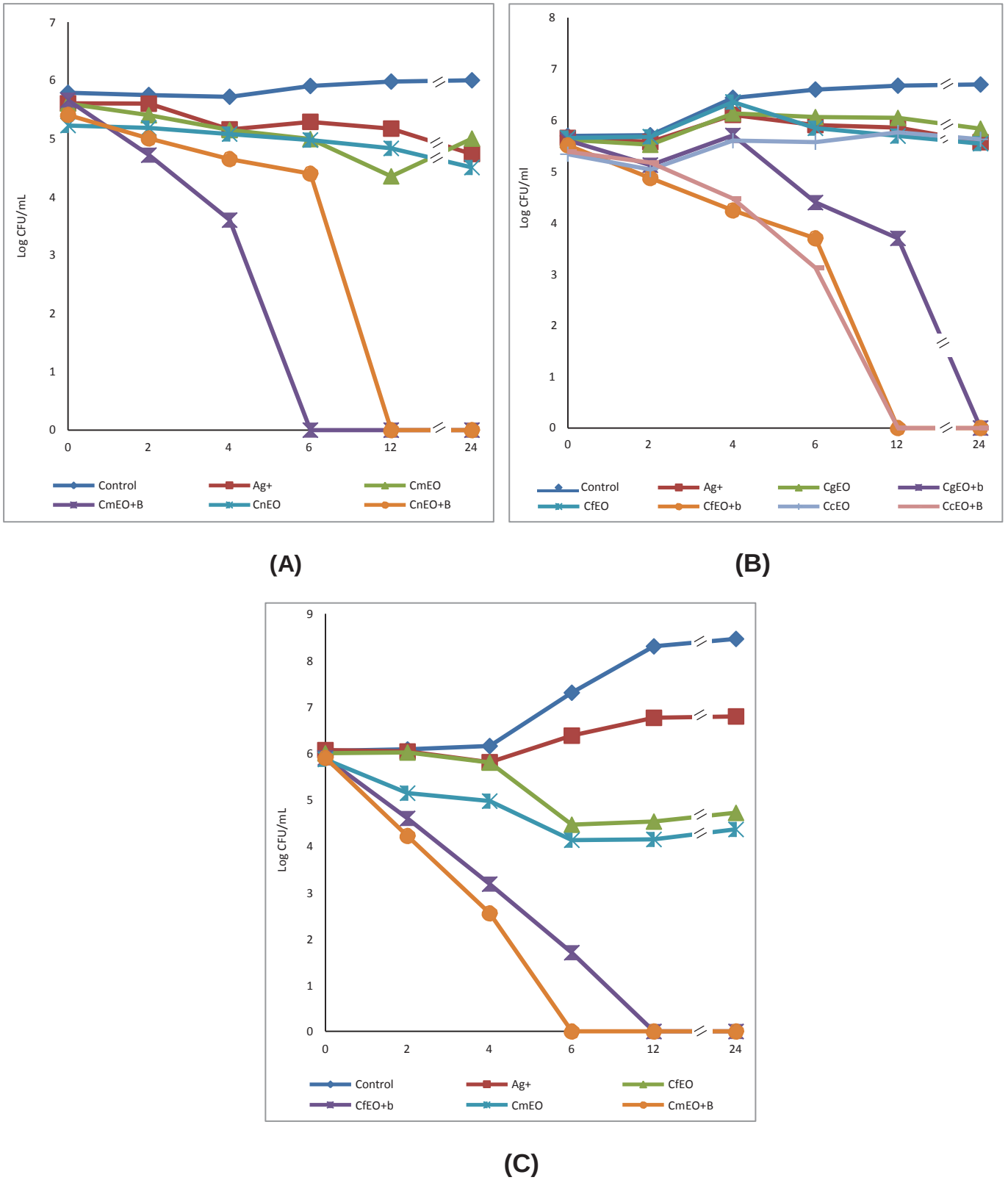

Fig. 2. Time–kill curves of *E. coli* (A), *S. aureus* (B) and *C. albicans* (C) isolates following exposure to ¼ MIC of CEO, ¼ MIC of Ag^+ and ¼ MIC of CEO combined with ¼ MIC of Ag^+. Curves (blue with stars) represent the control cells without any treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

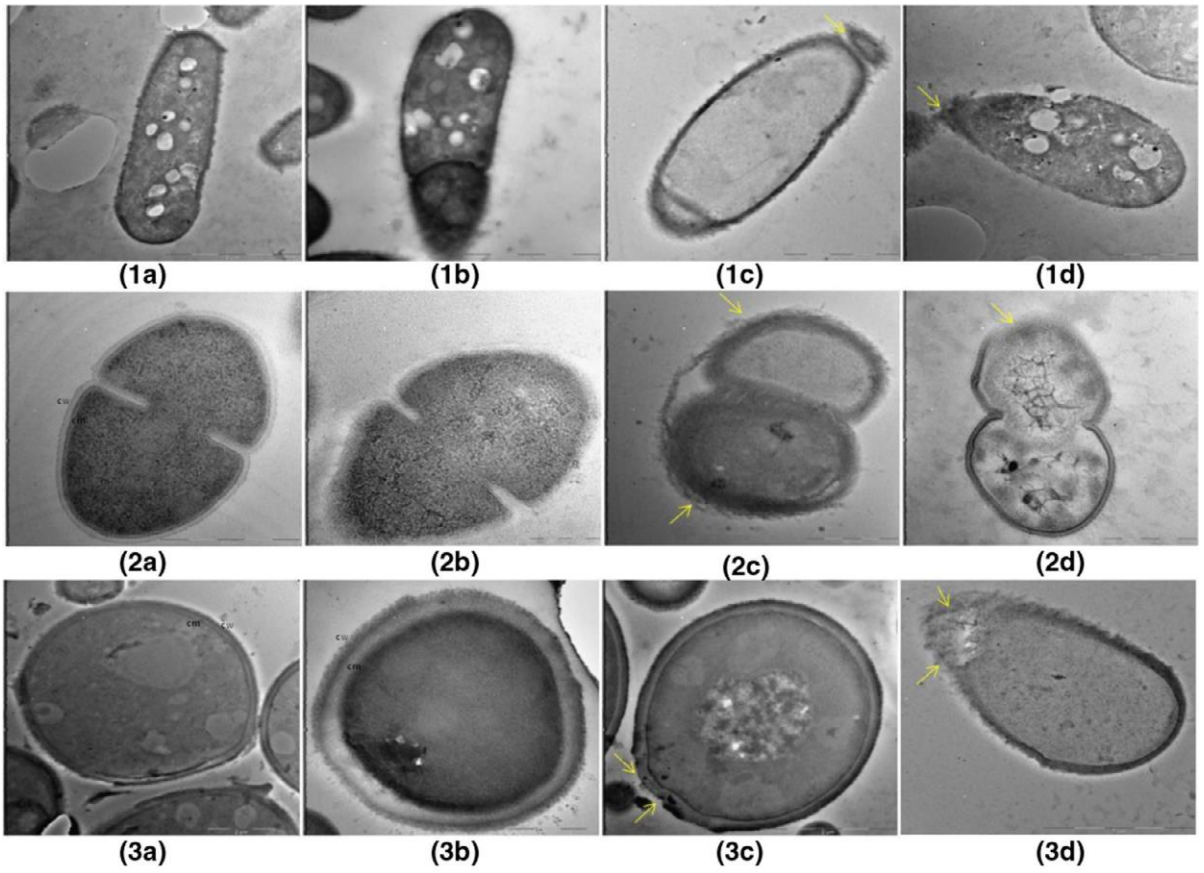

Fig. 3. Representative transmission electron micrographs of *E. coli* (1), *S. aureus* (2) and *C. albicans* (3) cells exposed to Ag^+ alone (b), CmEO alone (c) and Ag^+ plus CmEO (d). In all the three cases untreated controls are represented by (a). Morphological alterations in the exposed cells are shown with yellow arrows. Abbreviation 'cw' refers to cell wall and 'pm' refers to plasma membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

combination yielded a 5.9-log-CFU/ml and 5.6-log-CFU/ml decrease compared with a CmEO treated alone (Fig. 2A and C). The decrease for *S. aureus* after 12 h was observed to be 5.3-log and 5.6-log in viable counts compared with the number of CFU/ml produced in CcEO and CfEO treatment, respectively (Fig. 2B). From these results, it can also be concluded that CmEO when combined with Ag^+ against *E. coli* and *C. albicans*, the end point was attained after 6 h of incubation while as for the other CEO's the end point was only reached after 12 h of incubation. These results are congruent with the FICI values obtained from the microbroth dilution assays.

Transmission electron microscopy

Transmission electron microscopy (TEM) revealed the possible mechanisms of action for the synergistic combinations of CEO's and Ag^+ by observing the morphological features of the tested strains. The untreated cells retained their normal morphology with intact cell wall and cell membranes (Fig. 3). Untreated *E. coli* cells showed normal morphology having small fimbriae on their membranes (Fig. 3(1a)). Control *S. aureus* cells retained their coccal shape (Fig. 3(2a)) and untreated yeast cells also showed intact cellular morphology with a bound plasma membrane and cell wall (Fig. 3(3a)). In contrast the cells which are exposed to CmEO alone and CmEO in combination with Ag^+ appeared to undergo cell wall and membrane disruptions which results in the release of their cellular contents into the surrounding environment. The treated cells appeared to be damaged with aberrant morphology. Precipitates are clearly visible around the cells which are exposed either to CmEO alone or to CmEO in combination with Ag^+ (Fig. 3(1c–d), (2c–d) and (3c–d)). The bacterial cells, on the other hand, exposed to Ag^+ alone showed an intact morphology which describes the involvement of the other target sites as a mechanism of antimicrobial action of Ag^+ (Fig. 3(1b) and (2b)). Interestingly, in the case of *C. albicans* cells treated with Ag^+ alone, a complete separation of the cell membrane from the cell wall was observed which could be a cellular shrinkage (Fig. 3(3b)), which is a characteristic marker of apoptosis (Khan et al. 2014).

Discussion

Cymbopogon has been used as a folk medicine in tropical and subtropical regions and is known to possess pharmacological activity, including antimicrobial and disinfectant properties (Hanaa et al. 2012; Jeong et al. 2009). The oligodynamic effect of metals on biological systems (including the antimicrobial properties) was already described in 1893 by Nageli (Nageli 1893). Among all the metals, silver ions are known to be most efficient antimicrobial agent and have been used for many years in the medical field for antimicrobial purposes (Guggenbichler et al. 1999; Fu et al. 2006). Drug combination is now emerging as the therapy of choice to treat many infections e.g. tuberculosis, malaria etc. The main aim of combination therapy is to achieve synergy, reducing drug dosage and minimizing or delaying the onset of drug resistance (Van Vuuren and Viljoen 2011; Ahmad et al. 2014).

The differences in chemical compositions of the CEO, irrespective of qualitative and/or quantitative differences with the previously published papers, restrict the validity of these interesting biological results to the CEO's with similar chemical compositions. With main lead essential oil molecules identified as geraniol, geranial and neral as confirmed by GC–MS analysis; it has already been elucidated that these compounds exhibit high antimicrobial activities against various Gram-positive and Gram-negative bacteria, as well as against

the yeast *Candida* (Jirovetz et al. 2007; Korenblum et al. 2013). The antimicrobial activity of CEO's may be attributed to the presence of these chemical components. The antimicrobial efficacies of CEO's and Ag^+ were determined against a panel of pathogens. These results depict differences in the chemical composition of the different species of CEO's which significantly influences the antimicrobial potency of the different essential oils. The MIC values for the CEO's against Gram-negative bacteria are comparatively higher than for the Gram-positives and yeasts. As already demonstrated, our results also showed that the activity of the agents against each microbe varies due to structural differences between the microorganisms. In addition, the permeability differences due to the hydrophobic (CEO's) and hydrophilic (Ag^+) nature of agents across the cell membrane may influence the extent of their antimicrobial efficacies. The higher content of lipopolysaccharides in the outer membrane of the Gram-negative bacteria may hinder the penetration of the hydrophobic CEO's into the cells. Our results further demonstrate that Gram-negative bacteria are more susceptible to Ag^+ than Gram-positive bacteria, which are in alignment with previous findings (Kawahara et al. 2000). In the case of Gram-positive bacteria, it is hypothesized that the positively charged Ag^+ are trapped by the negatively charged peptidoglycans in the membrane, thus restricting their entry into the cell to reach their target. Gray et al. (2003) also reported this action due to the thin cell walls of Gram-negative bacteria, which may allow rapid absorption of Ag^+ ions into the cells.

Several reports have been published illustrating that the combination of natural products and silver ions collectively improve antimicrobial activity (Low et al. 2011). It has also been observed that safe and tolerant levels of silver in combination with essential oil components are effective in controlling bacterial infections (Ghosh et al. 2013). In the present work, we have focused on the combinations of the CEO's with Ag^+ and observed that combined agents showed an increase in antimicrobial activities as measured by FICI and time–kill curves. The mechanism of action was determined by TEM imaging of untreated and treated cells. Following the CEO's and Ag^+ treatment, the cell envelope ruptures resulting in the leakage of cellular contents which ultimately leads to cell death. Essential oils are already known for impairing membrane structures and functions and can bind to proteins and sterols and augment structural changes in the cell wall and membrane, leading to cell distortion and death (Khan et al. 2014). Silver ions, on the other hand, did not disrupt the cellular morphology and therefore the mechanisms for antimicrobial properties of Ag^+ are considered different from the membrane disintegrations. To achieve the efficient bactericidal activity of silver, the ions need to enter the bacterial cell to block DNA replication when DNA is in its condensed form and also deactivates vital enzymes of the cell (Huang et al. 2011). For the synergistic antimicrobial activity of CEO's and Ag^+, it is plausible to reason that the CEO's interact with the cell membranes resulting in a compromised and porous structure, thus altering the membrane integrity which increases the penetration of Ag^+ into the cells to reach their targets. The most prominent synergy (42%) was observed with the Gram-positive bacteria, consistent with these bacteria being considered to have a high intrinsic permeability barrier for Ag^+. The Gram-negative and yeast species have intermediate and the lowest (although significant) degree of synergies due to the comparatively lower membrane permeability for Ag^+. This dual killing effect on the microbes gives rise to the observed enhanced antimicrobial activity of CEO's and Ag^+ at the sub-MIC values.

The *in vitro* hemolytic assay is a possible screening tool to gauge *in vivo* toxicity to host cells (Ahmad et al. 2010b). Silver ions have no or negligible cytotoxicity and hemolytic activity to humans over a different range of concentrations which exceeds the MIC values determined in this study by a factor several times larger (Kawahara et al. 2000). CEO's have also been reported to possess negligible toxicity effect on humans (Olorunnisola et al. 2014) and some studies have shown the use of CEO for maize storage and herbal teas at prescribed concentrations (Leite et al. 1986; Fandohan et al. 2008). At higher concentrations, although, some biochemical disturbances were observed in some of the volunteers, however these changes did not exhibit any medical implication (Olorunnisola et al. 2014).

To our knowledge, this is the first attempt to study the synergistic interaction between CEO's and silver ions. CEO's may prove to be a potent phytotherapeutic and/or combination agent with silver ions. Since the CEO's concentration effective *in vitro* is achievable *in vivo*, the combination of this agent with Ag^+ represents an attractive prospect for the development of new strategies to target opportunistic pathogenic microorganisms, and should be investigated further using *in vivo* models. With this synergistic combinatorial approach many antimicrobial agents may find even broader therapeutic applications, especially in the formulation of environmentally friendly cleaning agents, sanitizers and herbal gels.

Conflicts of interest

The authors declare no conflict of interest.

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