Essential oils from tropical medicinal herbs and food plants inhibit biofilm formation in vitro and are non-cytotoxic to human cells

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Abstract

The biofilm inhibition and eradication potential of essential oils (EOs) extracted from six tropical medicinal herbs and food plants [*Psiadia arguta* (PA), *Psiadia terebinthina* (PT), *Citrus grandis* (CGp), *Citrus hystrix* (CH), *Citrus reticulata* (CR), and *Cinnamomum zeylanicum* (CZ)] were assessed. The mechanism of inhibition was studied via quenching of efflux pump. Cytotoxicity was evaluated using *Artemia salina* assay and cell lines [human cervix carcinoma (HeLa), human lung fibroblast (MRC-5), and murine melanoma (B16F10)]. EOs of CH, CR, PA, and PT were found to be prospective antibiofilm agents (IC₅₀ of 0.29, 0.59, 0.22, and 0.11 mg/mL against *Staphylococcus epidermidis*; 0.39, 0.54, 0.09, and 0.13 mg/mL against *Escherichia coli*; and 0.54, 0.90, 0.44 and 0.51 mg/mL against *Candida albicans* for CH, CR, PA, and PT, respectively). The simultaneous actions of the EOs and efflux pump inhibitor impacted on the resistance of the biofilms. LC₅₀ of the EOs ranged from 223 to 583 µg/mL against *A. salina*. The non-cytotoxic concentration of the EOs varied from 200 to 300 µg/mL (HeLa and MRC-5), and 150–200 µg/mL (B16F10). EOs from these tropical medicinal herbs and food plants are useful sources of new antimicrobials with low cytotoxicity which could open new horizons in the drug development process.

Keywords Antibacterial activity · Cytotoxicity · Herbal medicine · Essential oils · Medicinal plants · Traditional medicine · Efflux pump inhibitor

Introduction

Biofilms, which are surface-associated communities of microorganisms, have long been associated with persistent infections. They are widespread, occurring in different environmental systems including medical devices in healthcare facilities, where they can be life threatening (biomedical implant infection, haemodialysis complications, and catheter-associated infection amongst others) (Donlan 2002).

Bacteria can be found in at least two discrete states, which are the planktonic form and the sessile cells. Planktonic cells are referred to as the free-flowing bacteria in suspension. On the other hand, sessile cells are the consortium-forming bacteria, that is, these microorganisms establish a structured community of bacterial cells, surrounded by a self-produced matrix, adherent to an inert or living surface. Biofilms are composed of these sessile adherent cells (Donlan 2002). Their occurrence in nature, in specific environments and in hosts has been reported to be a mean of optimum colonisation and appropriation of a nutrient-rich environment (Poole 2009; Roilides et al. 2015).

Currently, the concept of biofilm is associated with the irreversible cell attachment that is the leading cause of recurrent infections, as well as to new attributes such as modification in growth rate and gene transcription (Poole 2009). Research on the link existing between pathogenic bacteria and their host has shown that there are multiple tactics employed by the former to bypass the innate defence mechanism. These tactics involved primarily the production of cytotoxic enzymes as well as other molecules, to allow

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their invasion in the host tissue, along with the formation of protective shields. Pathogenic bacteria that form biofilms are often referred as the chronic bacterial forms. Unlike the free-floating planktonic bacteria that cause acute infections, those chronic bacterial forms have evolved and form communities to combat the cells of the host's immune system that are focused upon their destruction but also as a means of protection against biotic and abiotic stresses. It has been argued that within a biofilm matrix, bacteria are able to resist conventional antibiotics at concentrations up to 1000–1500 times higher than that clinically used (Roilides et al. 2015).

Antibiofilm potential of natural products including EOs and their isolated compounds have been widely studied over the past few years. Due to the lipophilic character of EOs, they are able to slither through cell membranes, dislocating the different phospholipids, polysaccharides, and fatty acid layers. In this process, the EO components lead to a change in cell permeability, triggering a loss of integrity (Nazzaro et al. 2013).

The high incidence of infections increases the importance for new alternative therapeutic strategy. Natural products derived from medicinal plants have shown great potential in research on the management of infectious diseases (Boto 2010). Various classes of substances such as alkaloids, neolignans, terpenes, lignans, phenolics, and EOs have been isolated from plants, demonstrating potent bioactivity (Budzynska et al. 2011). An EO is a blend of bioactive molecules which offers potent antibiofilm properties which can be exploited by the pharmaceutical industry.

In this study, we evaluated antibiofilm effects of EOs extracted from common medicinal herbs and food plants from Mauritius, against planktonic and biofilm bacterial species. In addition, cytotoxicity evaluation was performed against three cell lines and using *Artemia salina* model as there is currently a dearth of data on the cytotoxicity of these EOs.

Materials and methods

Plant materials

Exotic and endemic plants included in the present study are those used traditionally by the local people as medicinal herbs and food plants (Aumeeruddy-Elalfi et al. 2015; Aumeeruddy-Elalfi and Mahomoodally 2016). The leaves of *Cinnamomum zeylanicum* Nees (CZ), *Psiadia arguta* Pers. (PA), *Psiadia terebinthina* A.J. Scott (PT), as well as the fully ripened fruits of three *Citrus* species, namely *Citrus grandis* L. (CGp), *Citrus hystrix* D.C. (CH), and *Citrus reticulata* Blanco (CR) were collected from the University farm. Each plant was identified by a local botanist. Voucher specimens were set for all the plants and deposited at the

Herbarium for identification and certified against existing samples at the National Herbarium, Mauritius Sugar Industry Research Institute, (MSIRI), Réduit, Mauritius.

Extraction of essential oils

The leaves of the plants were gently plucked, washed, and finely cut into pieces. Fruits were peeled off carefully with the use of a sharp knife to avoid any damage of the oil glands and finely reduced to uniform size. The plant materials were then subjected to the hydrodistillation process for a period of 3 h using a Clevenger type apparatus (Kulisic et al. 2004). The distillates of the EOs thus yielded were then dried over anhydrous magnesium sulphate, filtered and stored at -4°C until further analysis (Hussain et al. 2008).

Biofilm inhibition assay

Staphylococcus epidermidis (ATCC 35984), *Escherichia coli* (ATCC 35218) and *Candida albicans* (ATCC 10231) biofilms were grown 24 h prior to experiment in flasks containing yeast peptone dextrose (YPD) (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose, 1.5% agar, US Biological, cat. no. Y2076). For *S. epidermidis* (ATCC 35984), *E. coli* (ATCC 35218), brain heart infusion broth (BHI) was used. Twenty millilitres of YPD medium and BHI broth was inoculated, respectively, with a loopful of cells from stock. The flasks were allowed to incubate overnight in the orbital shaker at 180 rotations per minute (rpm) and a temperature of 30°C . Biofilms were grown on pre-sterilized, polystyrene, flat-bottom 96-well microtitre plates by pipetting $100\ \mu\text{L}$ of 10^6 cells mL^{-1} (for *C. albicans*) and 10^8 cells mL^{-1} (for *S. epidermidis* and *E. coli*) standardised cell suspensions [in RPMI-1640 solution, supplemented with L-glutamine (Cellgro, cat. no. 50–020-PB) and buffered with morpholine-propanesulfonic acid (Fisher, cat. no. BP308) to pH 7] into the wells of the 96-well microtiter plates. Fifty microlitres of diluted EOs was added immediately into the microplate containing the biofilm suspension. Plates were incubated for 20 h at 37°C on an orbital shaker at 200 rpm. Biofilm formation was calculated using a 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5 carboxanilide (XTT) reduction assay (Nett et al. 2011). One hundred millilitres of sterile distilled water were added to each of the prewashed biofilm wells and into the control wells. One hundred millilitres of the XTT solution, 0.5 g/L, was then added to the wells. The microplates were incubated in the dark for 2 h at 37°C . A colorimetric change resulting from XTT reduction and representing a direct correlation of metabolic activity of the biofilm was measured in a microtitre plate reader (ELx800, Biotek Instruments) at 490 nm. The inhibitory percentage was calculated as follows: $\%I = [(A_c - A_s)/A_c] \times 100$, where A_c is the absorbance of the control well, and A_s is the

absorbance of the well treated with EO. Assays were performed in three independent experiments.

Biofilm eradication assay

A time-dependent inhibitory assay was performed to assess the biofilm eradication potential of active EOs. The biofilm was cultured as described in the previous experiment, in polystyrene 96-well culture microplate. Biofilms of *S. epidermidis* (ATCC 35984) and *E. coli* (ATCC 35218) were used for this experiment. One hundred millilitres of inoculum at a concentration of 10^6 CFU/mL was incubated for 24 h at 37 °C. The different inocula were treated with 50 µL of EO at different concentrations (0.25, 0.50, 1.00, and 2.00 mg/mL). The evolution of the biofilm in the wells was monitored after 1, 4, 6, and 24 h of treatment with the EOs. EO concentration leading to 90% of reduction in biomass metabolic activity (MBEC₉₀) at the different time was assessed using crystal violet solution. Biofilm formed by bacteria adherent to the wells were fixed using 2% sodium acetate and stained by crystal violet (0.1%) in 200 µL of distilled water. Ethanol was added to the wells and the OD at wavelength 550 nm was read. Optical density of adherent biofilm was obtained using micro-ELISA microplate reader (ELx800, Biotek Instruments). The experiment was performed in triplicate ($n=3$). The interpretation of biofilm production was done according to Budzynska et al. (2011). The relative inhibition of biofilm (expressed as mean percentage) was determined as follows: $100 - [(OD_{550} \text{ of treated well} / OD_{550} \text{ of reference well}) \times 100]$ (Budzynska et al. 2011).

Efflux pump inhibition assay

The assay was performed as described by Laudy et al. (2016). Biofilm was cultured as described in the previous experiment, in polystyrene 96-well culture microplate. Biofilms of *S. epidermidis* (ATCC 35984), *E. coli* (ATCC 35218) as well as non-biofilm forming *S. epidermidis* (ATCC 12228) and *E. coli* (ATCC 25922) were used for this experiment. One hundred millilitres of inoculum at a concentration of 10^6 CFU/mL was incubated for 24 h at 37 °C and treated with 50 µL of EO. Twenty microlitres of efflux pump inhibitor, Phe-Arg-β-naphthylamide (PaβN), at a concentration of 80 mg/L was used as described by Laudy et al. (2016, 2015). To illustrate the ability of the strains to remove antimicrobial agents by multi-drug-resistant efflux pumps, the MIC was evaluated using different combinations: with or without EO and PAβN.

Microwell cytotoxicity using *Artemia salina*

Cytotoxicity assays using *A. salina* were carried out as described by Solis et al. (1993). *Artemia* nauplii has been

used in several previous publications for preliminary assessments of pharmacological and toxicological activities of complex compounds (Dvorak et al. 2010). The eggs of *A. salina* (brine shrimp) can be easily purchased and at low cost. Brine shrimps are not only commonly used for the screening of plant extracts but have been reported in the screening of pesticide residues, mycotoxins, streams pollutants, and many others.

EOs were dissolved in methanol (50 µL) and made up to 1 mg/mL in artificial sea water. Serial dilutions were made in 96-well microplates in 100 µL artificial sea water to have wells of varying EO concentrations (1000–7.185 µg/mL). A suspension of nauplii containing ten organisms representing 100 µL was added to each well. The plate was covered and allowed to incubate at 25 °C for 24 h. The number of dead (non-motile) nauplii in each well was counted. LC₅₀ values were calculated. Methanol was used in the control wells. All experiments were independent and performed in triplicate.

For the endpoint validation of the cytotoxicity of the EOs, Clarkson's toxicity criterion for the toxicity assessment of the EOs was used and expressed as lethal concentration (LC₅₀). The classification used was LC₅₀ > 1000 µg/mL: non-toxic, 500 < LC₅₀ < 1000 µg/mL: low toxicity, 100 < LC₅₀ < 500 µg/mL: moderately toxic, and 0 < LC₅₀ < 100 µg/mL: highly toxic (Nunkoo and Mahomoodally 2012).

MTT assay

Cytotoxicity of the EOs was also assessed using the human cervix carcinoma (HeLa), human lung fibroblast (MRC-5), and the murine melanoma (B16F10) cell lines. The principle of this assay is to investigate the change in mitochondrial/non-mitochondrial dehydrogenase activity using 3-(4,5-dimethylthiazol-2,5)-diphenyltetrazolium bromide (MTT) as described by Melo et al. (2014), with slight modifications. Cells at 90% confluence were sub-cultured on 96-well microplates. One hundred microlitre of cells suspensions at a concentration of 1×10^4 was seeded in 96-well microplates and cultured in RPMI 1640 containing 10% FBS, under 5% CO₂ and at a temperature of 37 °C, for 24 h to allow the cells to attach to the wells. Following incubation, the cells were treated with EOs dissolved in DMSO 0.5% (V/V) at different concentrations (2, 10, 20, 100, 200, 400 µg/mL) for a period of 48 h. Twenty microlitres of MTT solution at a concentration of 5 mg/mL was added to the wells. The microplate was incubated for another 4 h. The cells were centrifuged at 1500 rpm for a period of 5 min. The supernatant was removed and 200 µL of DMSO was added. For the wells containing viable cells, a reduction of the soluble yellow MTT salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by mitochondrial succinate dehydrogenase will be observed, yielding the insoluble purple product formazan. The purple formazan crystals are

solubilized using DMSO. End-point parameter was calculated using the absorbance parameter, which was measured at 550 nm in a microplate reader (Biotek, Synergy HT). Cell survival (expressed as a percentage) was computed as the ratio of absorbance from the treated well to the absorbance from the non-treated well, as follows: % Cell viability = $\frac{\text{Abs}_{550\text{S}}}{\text{Abs}_{550\text{B}}} \times 100$, where $\text{Abs}_{550\text{S}}$ is the mean value of the absorbance of the test item or control and $\text{Abs}_{550\text{B}}$ is the mean value of the absorbance of the blank. The cytotoxicity was expressed in terms of lethal concentration (LC). Cell viability < 70% compared to untreated cells was taken as the criterion for cytotoxicity.

Statistical analysis

To ensure the reproducibility of the results, each experimental procedure was carried out three times. The end-point values, inhibition % and 50% inhibition concentration (IC_{50}) of the EOs were expressed as mean $\pm$ SEM. Pairwise comparisons among EOs were assessed using paired sample *T* tests (5%, one sided) to test for superiority.

To investigate how mortality rates changed with respect to the concentration levels, a probit model was fitted to the dose-quantal collected data. For each essential oil, the median lethal concentration (LC_{50}) was estimated from the fitted model. This analysis, in other words, modelled the probability of shrimp's positive response (death) as a function of the concentration level. Comparisons among EOs were performed by an ANOVA procedure with the mean number of dead shrimp as response variable and EO as fixed factor. Non-cytotoxic concentrations of EOs (MTT assay) were compared using an ANOVA, followed by Dunnet's procedure for pairwise comparisons versus doxorubicin.

The MIC from EO combinations were compared to non-EO combinations using a one-sample *T* test (also verified and confirmed using non-parametric alternatives to cater for deviations from normality), testing whether the average obtained from EOs were significantly different from non-EO means. GraphPad Prism 6.0, SPSS 19.0 and Microsoft Excel 2010 were used as software applications for statistical analyses.

Results

Biofilm inhibition

The activity of EOs on biofilm formation was first addressed by the determination of the inhibition capacity and minimum inhibition concentration. Results showed that EOs of CH, CR, PA, and PT are potential antibiofilm agents (Fig. 1a–c and Table 1). PT being the most active

was found to exhibit an IC_{50} of 0.11 ± 0.01 , 0.13 ± 0.02 , and 0.51 ± 0.02 mg/mL against *S. epidermidis*, *E. coli*, and *C. albicans*, respectively. It was followed by PA, with IC_{50} values of 0.22 ± 0.03 , 0.09 ± 0.01 , and 0.44 ± 0.05 mg/mL against *S. epidermidis*, *E. coli*, and *C. albicans*, respectively and that of CH with IC_{50} values of 0.29 ± 0.06 , 0.39 ± 0.08 , and 0.54 ± 0.02 mg/mL against *S. epidermidis*, *E. coli*, and *C. albicans*, respectively (Table 1). However, it can also be noted that all the three pathogens tested were not sensitive to the EOs to the same extent.

Biofilm eradication assay

As shown in Table 2, results obtained from this experiment confirmed the potential of the EOs of CH, CR, PA, and PT as potential antibiofilm agents. CH showed a relative 50% inhibition of *S. epidermidis* biofilm at 0.25 mg/mL while PT reached a relative inhibition of 76.67% at 0.25 mg/mL. PA, CR, and CH showed a relative inhibition of 68.97, 68.97, and 67.86%, respectively, at 0.5 mg/mL. With the aim being to eradicate biofilm, one can conclude that PT and PA were the most active reaching a maximum of 90% relative inhibition against both *S. epidermidis* and *E. coli* as shown in Table 2. The EO concentrations causing 50% or at least 90% reduction in biomass metabolic activity (MBEC_{50} and MBEC_{90}) are shown in Fig. 2a–l. MBEC_{50} can be observed at MIC having a decrease in absorbance to half of that obtained for the negative control (3.0), while MBEC_{90} can be observed at MIC having a decrease in absorbance from 3.0 to 0.3.

Efflux pump inhibition assay

The efflux pump inhibitory assay resulted in several end-point effect in terms of MIC (Table 3). Significant inhibitory activities were observed for all the EOs in the presence of PA β N. The simultaneous actions of the EOs and efflux pump inhibitor impacted on the resistance of the organisms within the biofilms as well as the non-biofilm-forming pathogens.

Microwell cytotoxicity using *Artemia salina*

The results deducted by a probit analysis for this experiment tend to classify the EOs CGp (201.06 $\mu\text{g/mL}$), CH (140.23 $\mu\text{g/mL}$), CR (144.42 $\mu\text{g/mL}$), PA (176.79 $\mu\text{g/mL}$) and PT (242.46 $\mu\text{g/mL}$) as moderately toxic on *A. salina* as per Clarkson's toxicity criterion for natural products with $100 < \text{C}_{50} < 500$ $\mu\text{g/mL}$ (Table 4). Figure 3 shows the percentage of dead shrimp with respect to variation in concentration of the different EOs.

Fig. 1 Inhibition of *E. coli* (a), *S. epidermidis* (b) and *C. albicans* (c) biofilms. CGp: *Citrus grandis* (peel); CH: *Citrus hystrix*; CR: *Citrus reticulata*; CZ: *Cinnamomum zeylanicum*; PA: *Psiadia arguta*; PT:

Psiadia terebinthina. **p* value ≤ 0.05 (statistical analysis: ANOVA with mean inhibition as response variable and concentration as fixed factor)

MTT assay

The cytotoxic activities of CH, CR, CGp, CZ, PA, and PT were investigated using tumor and normal cell lines (Fig. 4). Among the EOs tested, it was found that CH exhibited a lower cytotoxic concentration compared to the other EOs. For instance, the non-cytotoxic concentration of CH (at which $\geq 70\%$ of B16F10 cells were viable) was expressed at a concentration

of 150 µg/mL, while for the other EOs the non-cytotoxic concentration towards B16F10 cells were observed at 200 µg/mL as illustrated in Fig. 4.

Table 1 EO concentration at 50% biofilm inhibition

Essential oil	IC ₅₀ (mg/mL)		
	<i>S. epidermidis</i>	<i>E. coli</i>	<i>C. albicans</i>
CH	0.29 ± 0.06 ^a	0.39 ± 0.08	0.54 ± 0.02 ^c
CR	0.59 ± 0.02 ^b	0.54 ± 0.01	0.90 ± 0.02
CGp	1.12 ± 0.01	1.10 ± 0.05	1.42 ± 0.08
CZ	0.98 ± 0.05	2.35 ± 0.09	2.06 ± 0.03
PA	0.22 ± 0.03	0.09 ± 0.01 ^c	0.44 ± 0.05 ^c
PT	0.11 ± 0.01	0.13 ± 0.02	0.51 ± 0.02

Values are expressed in means ± SEM

Values with no superscript indicate a significant difference compared to other EOs

Significance: *p* value ≤ 0.05 (paired Student's *T* test)

^aNot significantly different compared to PA

^bNot significantly different compared to CGp and CZ

^cNot significantly different compared to PT

Table 2 Relative inhibition of biofilm formation by EOs

EO	Concentration (mg/mL)	Relative inhibition % at 24 h	
		<i>S. epidermidis</i>	<i>E. coli</i>
CZ	0.25	33.33	37.93
	0.50	64.29	44.83
	1.00	76.67	75.86
	2.00	83.33	82.76
CGp ^a	0.25	23.33	20.69
	0.50	28.57	27.59
	1.00	36.67	44.83
	2.00	46.67	58.62
CH ^b	0.25	50.00	41.38
	0.50	67.86	44.83
	1.00	76.67	68.97
	2.00	83.33	75.86
CR	0.25	33.33	62.07
	0.50	68.97	75.86
	1.00	76.67	75.86
	2.00	83.33	82.76
PA	0.25	46.67	70.00
	0.50	68.97	72.41
	1.00	76.67	76.67
	2.00	90.00	90.00
PT ^c	0.25	76.67	80.00
	0.50	86.21	82.76
	1.00	90.00	85.00
	2.00	90.00	93.67

Significance: *p* value ≤ 0.05 (paired Student's *T* test)

^aSignificantly different compared to all other EOs except CH and CZ for *E. coli*

^bSignificantly different from PA for *E. coli*

^cSignificantly from CZ and CR for *E. coli* and from CH for both *S. epidermidis* and *E. coli*

Discussion

Antibiofilm potential of EOs

It is well known that the source of multiple human microbial infections is based on the genetic evolution of the pathogens and the formation of resistance mechanisms such as biofilms structures (Davies 2003). Although many reports have been dedicated to the antimicrobial properties of EOs, few have been geared towards the antibiofilm activities of these natural compounds. Following this concept, antimicrobial evaluation of EOs extracted from medicinal plants was devised to assess their possible biofilm inhibition and eradication capacity. From these experiments we concluded that EOs of CH, CR, PA, and PT have the capacity to enhance the efficiency of conventional antibiotics and can also inhibit the formation of biofilm.

These EOs presented as major components vanillin, limonene, α-curcumen, β-pinene, and isoeugenol, which are known for their antimicrobial properties (Cava-Roda et al. 2012; Sonboli et al. 2006). In addition, limonene has also been reported for its antibiofilm properties with inhibition percentage of up to 75–95% which can partly explain the results observed in this study for the EOs of CH and CR rich in limonene (Subramenium et al. 2015). Recently, the use of EOs as efficient natural products with antibiofilm properties has been argued (Kavanaugh and Ribbeck 2012). Biofilm incubation period has been reported as a factor influencing biofilm inhibition (Budzynska et al. 2011). This was explained by the fact that microorganisms within a biofilm react differently to natural products. For instance, natural products can induce a delay in the cycles of the biofilm evolution from planktonic form to sessile cells. In addition, natural products such as EOs are complex blends of biologically active molecules. Each EO depicts a different identity with a specific chemical composition, which tends to vary with extraction method, as well as with environmental parameters such as altitude and season (Aumeeruddy-Elalfi and Mahomoodally 2016). Thus, the effect of EO on biofilm tends to vary depending on the composition of the EOs.

Thus, assessing the antibiofilm potential at different time points during formation of the biofilm is warranted. For this reason, an assay was designed to monitor biofilm formation following treatment with EOs and at different time intervals. The result showed that the action of EOs on biofilm evolved with time and concentrations of the EO. Partial destruction at MBEC₅₀ was observed after 24 h as from a concentration of 0.5 mg/mL when *S. epidermidis* biofilm was treated with CZ, CH, and CR, while for PA and PT, MBEC₅₀ was observed after 24 h at 0.25 mg/mL. The experiment repeated with *E. coli* also

Fig. 2 Antibiofilm activity of CGp: *Citrus grandis* (peel); CH: *Citrus hystrix*; CR: *Citrus reticulata*; CZ: *Cinnamomum zeylanicum*; PA: *Psiadia arguta*; PT: *Psiadia terebinthina* against *E. coli* (b, d, f, h, j, l), and *S. epidermidis* (a, c, e, g, i, k). *Limit significance ($0.1 < p$

value); **significant (p value ≤ 0.05); statistical analysis: ANOVA with mean absorbance as response variable and concentration as fixed factor

showed variability in concentration. In this case, MBEC₅₀ was observed as from a concentration of 0.5 mg/mL for CZ, 1 mg/mL for CGp, and CH while for CR, PA, and PT MBEC₅₀ was observed at 0.25 mg/mL. From these data, one would conclude that there is a relationship between biofilm mass production and eradication with respect to the time intervals following contact of EOs with the biofilm.

However, it can also be noted that this relationship is variable from strain to strain. For instance, MBEC₉₀ was observed only with PA and PT, at 0.5 and 1 mg/mL,

respectively, for the biofilm of *S. epidermidis* and at 2 mg/mL for *E. coli*. Biofilm structure varies in composition and thickness from one strain to another. Differential gene expression has been evaluated to result in a change in the morphology and physiology of biofilm cells. These gene expressions vary within species. Another possibility for this variation in data can be linked to the penetration rate of EOs within the biofilm. This penetration rate has been reported to be different from one biofilm to another and from one antimicrobial agent to another (Ouhayoun 2003).

Fig. 2 (continued)

Further experiment was thus carried out to investigate on the possible mechanism of biofilm inhibition by EO, via the quenching effect of efflux pump. Efflux pumps function to regulate the uptake of molecules within the consortium of the biofilm. In this experiment, phenylalanine-arginine- β -naphthylamide (PA β N) was used as efflux pump inhibitor as it is classified as broad-spectrum antibiotic and has been reported to reduce fluoroquinolone (synthetic antibiotics such as ciprofloxacin and others) resistance in bacteria (Lomovskaya et al. 2001). It was found that PA β N showed a concentration-dependent effect on the potency of ciprofloxacin. The experiment was carried out at a concentration of 50 μ g/mL of PA β N. At

this concentration, PA β N in association with ciprofloxacin significantly decreased the biofilms of *E. coli* and *S. epidermidis*. Lamers et al. (2013) reported the effect of PA β N in permeabilising the inner and outer membranes of bacterial cells, which can contribute to explain the concentration-dependent action of PA β N towards *E. coli* and *S. epidermidis*. Efflux system present in bacterial cell has been documented as one of the leading causes of multidrug resistance in pathogenic bacteria (Lamers et al. 2013; Lomovskaya et al. 2001), as it creates a gradient of high concentration of antibiotics from the outer membrane to the intra-membrane, thus accentuating the action of the antibiotics on the bacteria.

Table 3 Action of EO in the presence and absence of PAβN

Combinations	MIC (mg/mL)							p value
	CH	CR	CGp	CZ	PA	PT	Ciprofloxacin	
EO/ciprofloxacin + <i>E. coli</i> ^a	1	0.5	1	0.5	1	0.5	4	<0.001
PAβN + EO/cipro + <i>E. coli</i> ^a	0.5	0.13	0.5	0.25	0.5	0.5	2	<0.001
EO/ciprofloxacin + <i>E. coli</i> ^b	2	1	1.5	1	2	1	16	<0.001
PAβN + EO/ciprofloxacin + <i>E. coli</i> ^b	0.5	0.25	1	0.5	0.25	0.13	4	<0.001
EO/ciprofloxacin + <i>S. epidermidis</i> ^a	1	1	1	2	0.25	0.25	8	<0.001
PAβN + EO/ciprofloxacin + <i>S. epidermidis</i> ^a	1	1.5	1	1	0.25	0.25	4	<0.001
EO/ciprofloxacin + <i>S. epidermidis</i> ^b	2	1	2	4	1	0.5	16	<0.001
PAβN + EO/ciprofloxacin + <i>S. epidermidis</i> ^b	2	1	2	4	0.5	0.5	8	<0.001

CGp: *Citrus grandis* (peel); CH: *Citrus hystrix*; CR: *Citrus reticulata*; CZ: *Cinnamomum zeylanicum*; PA: *Psiadia arguta*; PT: *Psiadia terebinthina*

^aATCC strains

^bATCC biofilm strains

Table 4 Lethal concentration of EOs on *Artemia salina*

EO	LC50 (μg/mL)*
CR	144.42
CZ	404.36
CH	140.23
CG	201.06
PA	176.79
PT	242.46

*No significant difference between EOs (statistical test: one-way ANOVA with mean number of dead shrimp as response and EO as fixed factor)

PAβN was found to significantly increase the activity of the EOs by a minimum twofold to a maximum fourfold observed in PA (from 2 to 0.25 mg/mL). It is noteworthy to highlight that PAβN was found to be more efficient against Gram-negative *E. coli* than the Gram-positive *S. epidermidis*. This can be correlated to factors which are known to influence Gram-negative bacteria such as membrane permeability and the activity of efflux pumps. Microorganisms inside a biofilm are thought to be more susceptible to the activity of EOs and component which can penetrate the consortium and accumulate inside. On the same note, Kavanaugh and Ribbeck (2012) showed that active components of EOs are able to be absorbed by the extracellular matrix of the biofilm and in so doing, increase the local concentration of these EO components.

The results obtained suggest that there are active components in the EOs such as that of PA, which are acting inside the bacterial cell, as a substrate of the efflux pump system present in the pathogenic bacteria. From this reflection, the hypothesis of the inhibition of efflux pump as mode of action of EOs on biofilms forming bacteria can be argued. However, it is wise to state that in to target the appropriate

concentrations and endpoint values, experiments dealing with pharmacokinetics and pharmacodynamics (Pai and Pai 2007) must be considered, along with clinical testing.

Cytotoxicity evaluations

The cytotoxic effect of the EOs assessed on the human cervix carcinoma, human lung fibroblast, and the murine melanoma cell lines in the MTT assay revealed the concentration at which the cell viability was greater or equal to 70%. A pattern in the activities was observed between the two toxicity assays, that is, microwell cytotoxicity using *A. salina* and the cytotoxicity evaluation using the three different types of cell lines. Such correlation has been previously described in the literature. The toxicity obtained in the microwell cytotoxicity using *A. salina* identified the EOs as exhibiting a moderate toxicity. The EO of CZ, for instance, was less toxic among the EOs tested against *A. salina* with an LC₅₀ of 404.36 μg/mL. However, the non-cytotoxic concentration observed for CZ was of 200, 250, and 300 μg/mL for the cell lines B16F10, MRC-5, and HeLa, respectively, in the MTT assay.

The performance of CH when tested on the cells was different from one cell line to the other. A notable change in pattern was observed for CH with respect to the other *Citrus* species. CH was found to be more cytotoxic than the other *Citrus* species with lethal concentration observed at concentrations greater than 150, 200 and 200 μg/mL for B16F10, MRC-5 and HeLa, respectively, while for CR the lethal concentrations were observed for concentrations greater than 200, 250, and 300 μg/mL for B16F10, HeLa, and MRC-5, respectively. CGp was found to be cytotoxic at concentrations greater than 200, 250 and 250 μg/mL for B16F10, MRC-5, and HeLa, respectively. When attempting to justify these results with the phytochemical profile, it was found that the abundance of limonene in CH (85.39%)

Fig. 3 Effect of the variation of EO [CGp: *Citrus grandis* (peel); CH: *Citrus hystrix*; CR: *Citrus reticulata*; CZ: *Cinnamomum zeylanicum*; PA: *Psiadia arguta*; PT: *Psiadia terebinthina*] concentration on the % of dead shrimp

Fig. 4 Non-cytotoxic concentrations of EOs (CGp: *Citrus grandis* (peel); CH: *Citrus hystrix*; CR: *Citrus reticulata*; CZ: *Cinnamomum zeylanicum*; PA: *Psiadia arguta*; PT: *Psiadia terebinthina*) representing a viability $\geq 70\%$ of the cells. Statistical test: ANOVA followed by Dunnet's procedure to compare versus Doxorubicin

was more substantial than in the other *Citrus* species (CGp: 75.43% and CR: 37.55%). It has also been described in the literature that limonene can induce apoptosis (Crowell 1999; Jomaa et al. 2012). One would tend to connect these apoptotic properties of limonene to the cytotoxicity activity observed against the cell lines, that is, higher the concentration of limonene higher is the cytotoxicity.

It is a known fact that the toxicity of EOs is loosely related to either the components on their own or the synergistic effect of different compounds present in the EOs. However, it can also be an antagonist response of the components with other constituents present in their immediate environment. Significant variation in EO composition within a family as well as within species has been identified in a previous study

for CH, CR, CGp, CZ, PA, and PT (Aumeeruddy-Elalfi et al. 2015; Aumeeruddy-Elalfi and Mahomoodally 2016). This variation in physico-chemical composition can account for the variable cytotoxicity profile observed.

Findings of this study highlighted the EOs of *P. terebinthina* and *P. arguta* as potent, rich in bioactive components, antibiofilm agents. These results tally with the use of *P. terebinthina* and *P. arguta* in traditional medicine for the treatment of infections and for wound healing. The interesting biofilm inhibitory and eradication activity found against *E. coli* and *S. epidermidis* biofilm makes these ethnomedicinal plant outstanding candidates for the management of nosocomial infections. Among the citrus species tested, *C. hystrix* stood out in terms of its anti-biofilm inhibitory potential which can also be associated with its ethnobotanical use in the treatment of skin infections. This information will be useful when developing strategies to improve microbiological safety as well as for the development of new antimicrobials.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest with the publication.

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