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THE MODE OF ANTIMICROBIAL ACTION OF *Cinnamomum burmannii's* ESSENTIAL OIL & CINNAMALDEHYDE

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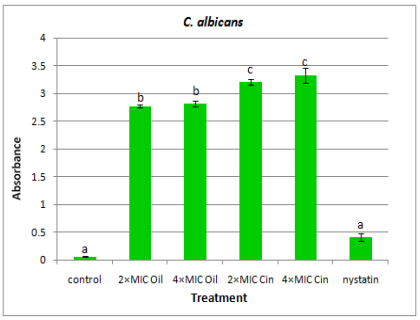
Graphical abstract



Abstract

The aim of this study was to postulate the mode of antimicrobial actions of both essential oil and cinnamaldehyde from *Cinnamomum burmannii* on the cell membrane of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The essential oil was extracted by steam distillation and followed by the isolation of cinnamaldehyde. Four modes of action were tested including time-killing assay, salt tolerance assay, crystal violet assay and leakage of cellular metabolites. The antimicrobial effect on the cell membrane was dose-dependent whereby stronger antimicrobial action was observed by cinnamaldehyde at concentration equal to 4×MIC (1.33 mg/mL) compared to the essential oil. The potential of cinnamaldehyde as an antimicrobial compound of the cinnamon essential oil was discovered and proven to act on the cell membrane of tested microorganisms particularly against *C. albicans*.

Keywords: *Cinnamomum burmannii*, antimicrobial, mode of action, cinnamaldehyde, essential oil

1.0 INTRODUCTION

The use of natural-based antimicrobial products are currently captured the research interest in an effort to overcome the widespread of infectious diseases caused by the MDROs. The infectious diseases may include bacteriaemia, urinary tract infections, iatrogenic infections and pneumonia [1, 2]. The discovery of plants activity such as antibacterial and antifungal activity has led many researchers to further study on their mode of action since screening alone would not be sufficient to determine the target sites or effects of the drugs.

Cinnamomum burmannii (CB) is one of the cinnamon species from the family of Lauraceae and native to Southeast Asia. There had been many reports on the in-vitro antimicrobial activity of the cinnamon essential oil (EO) [3-5] and cinnamaldehyde (CD) [6-8] but less on their mode of action. Therefore, the present study is designed to postulate the mode of action on the cell membrane of microorganism upon treatment with the EO as well as its major compound, CD.

2.0 MATERIAL AND METHODS

2.1 Plant Collection

CB barks were collected from the foothills of Singgalang Mountain (0°23'24"S 100°19'5"E) at West Sumatra, Indonesia in March 2012.

2.2 Extraction and Isolation

The extraction of EO, isolation of CD and characterization procedures were done as described by the previous study [9].

2.3 Microorganisms

Three microbial strains used in this study, a Gram-positive (*Staphylococcus aureus* ATCC 25923), a Gram-negative (*Escherichia coli* ATCC 8739) and a fungus strain (*Candida albicans* IMRC 533/11A).

2.4 Time-killing Assay

The time-kill assay was performed on the killing ability of EO and CD over time intervals of 0, 30, 60 and 120 minutes of treatments [10]. The test was done on the exponential phase culture at concentration equal to 4×MIC value. 100 µL of treated and untreated culture after time interval was transferred on the Mueller Hinton Agar/Sabouraud Dextrose Agar (MHA/SDA) plate and incubated at 37 °C for 24 h (bacteria) or at 25-27 °C for 48 hours (fungus). The numbers of colonies were counted for every time interval after incubation.

2.5 Salt Tolerance Assay

This test was done according to Carson *et al*. [11] with some modifications. Suspensions of culture were treated with EO and CD at concentration of their MIC and 2×MIC values while untreated culture was used as a control. The treated and untreated cultures were incubated for 30 min, serially diluted after the incubation and inoculated by transferring 100 µL of treated and untreated cultures onto Nutrient Agar (NA)-NaCl (bacteria strain) or SDA-NaCl (fungi strain) as well as NA or SDA without NaCl (control). After incubation, the numbers of CFU/mL on each plate was counted and compared to the control plate.

2.6 Crystal Violet (CV) Assay

Crystal violet assay was done according to Devi *et al*. [12] with some modifications. Cells were harvested upon obtaining the exponential growth at 4500×g for 5 min (4 °C) and washed twice before re-suspended in PBS (pH-7.4). Different concentrations of EO and CD were added to the cell suspension (2×MIC and 4×MIC values). Cell suspension without sample was treated with 0.25 M EDTA and used as positive control. An antibiotic (chloramphenicol) was also used in the treatment. The cells were harvested at 9300×g for 5 min after incubation for 30 min and re-suspended in PBS containing 10 µg/mL of CV and incubated for 10 min. Then, the suspensions were centrifuged at 13,400×g for 15 min. The OD value of supernatant was measured by UV spectrophotometer at 600 and 494 nm for bacteria and fungus, respectively. The percentage of CV uptake of all cells was calculated by the following formula:

(OD value of the sample/ OD value of crystal violet solution) × 100%

2.7 Leakage of 260 & 280 nm Absorbing Materials

The leakage of UV-absorbing material was analysed according to Zhou *et al*. [13] with some modification. Cells were harvested upon obtaining exponentially growing cultures, washed twice, and then re-suspended in PBS (pH 7.4). Different concentrations of samples (2×MIC and 4×MIC values) were added to 1 mL cell suspension. Chloramphenicol was used as positive control and untreated cells were used as negative control. All of the cells were incubated for 60 min and then centrifuged at 13,400×g for 15 min. OD_{260} or OD_{280} value of the supernatant was considered as a percentage of the extracellular UV-absorbing materials released by cells.

2.8 Statistical Analysis

All of the experiments were carried out in triplicate, and the data were expressed as mean with standard deviation (SD). Significant differences between groups were examined using one-way ANOVA (IBM SPSS statistic version 20). A p-value less than 0.05 ($p<0.05$) denoted the presence of statistically significant difference.

3.0 RESULTS AND DISCUSSION

3.1 Time-Killing Assay

The growth reduction of treated *E. coli, S. aureus* and *C. albicans* (Figure 1) cultures were significantly increased over time interval as compared to control. *E. coli* and *C. albicans* treated with 4×MIC (1.33 mg/ml) of CD showed more growth reduction after 30 min compared to cells treated with 4×MIC of EO. However, the growth reduction of *S. aureus* treated with 4×MIC of CD was observed to be equally similar or significantly no difference as compared to *S. aureus* treated with 4×MIC of EO. Nevertheless, loss of viability of all treated cells was observed after 1 h treatment of essential oil as well as CD at 4×MIC. The antimicrobial activity of CD might be explained by the high electro-negativity arrangement of the conjugation of aldehyde to carbon double bond whereby increased of electro-negativity caused increased of antimicrobial activity [14].

3.2 Salt Tolerance Assay

The salt tolerance assay results by untreated and treated *E. coli*, *S. aureus* and *C. albicans* were shown in Figure 2. The ability of cell membrane to osmoregulate and exclude the toxic or unwanted materials was investigated in order to reveal the sub-lethal damage of the microorganisms. Concentration (MIC and 2×MIC) that able to reduce the surviving numbers of colonies were used in this assay.

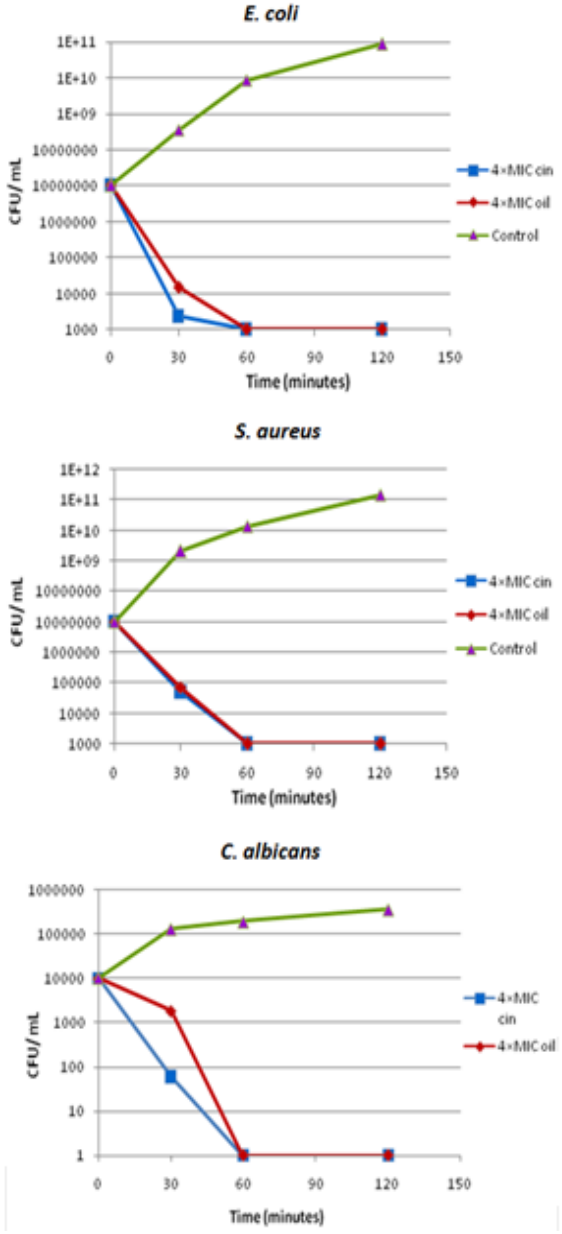


Figure 1 Time-killing curves of untreated (□) and treated *E. coli*, *S. aureus* and *C. albicans* at concentration equal to 4×MIC of essential oil (**O**) and cinnamaldehyde (Δ). The difference was considered significant in comparing with control and between samples at $p < 0.05$.

The sub-lethal damage of cell membrane was evident by the loss of salt tolerance or reduction in the ability to form colonies on the NA supplemented with NaCl. After the treatment, all the treated cells appeared to be reduced in colony-forming ability as the concentration of the treatment increased. However, there were only small decreased of colonies survivors on the NA-NaCl with no significant difference at MIC value (0.33 mg/ml) of CD (*E. coli* and *C. albicans*) as well as MIC value of EO (*S. aureus* and *C. albicans*) compared to survivors of colonies on the NA or SDA. According to a study done on tea tree oil, there was also no significant effect observed upon the salt tolerance test at half MIC against *S. aureus* as compared to treated *S. aureus* without NaCl supplement. This finding may be possibly due to the killing of the most susceptible cells and only more sturdy or salt-tolerant cells survived [11].

Nonetheless, EO and CD treated cells possessed significant ability especially at 2×MIC value compared to the untreated cells (control). The survivors of colonies grown on the NA/SDA-NaCl by *E. coli*, *S. aureus* and *C. albicans* treated with 2×MIC (0.66 mg/mL) of CD were observed to be equal to 44×104, 21×104 and 14×103 CFU/mL, respectively, whereas E. coli (10×105), *S. aureus* (50×104) and *C. albicans* (22×103 CFU/mL) treated with 2×MIC of EO appeared to have more survivors of colonies on the NA/SDA-NaCl. The present study had revealed that CD significantly reduced the ability of cells to form colonies on NA-NaCl at concentration equal to 2×MIC.

3.3 Crystal Violet (VC) Assay

As shown in Figure 3, the percentage of CV uptake by *E. coli*, *S. aureus* and *C. albicans* was increased as the concentration of EO and CD increased. The percentage of CV uptake by cells treated with CD was significantly higher than cells treated with EO. The percentage of CV uptake by *E. coli*, *S. aureus* and *C. albicans* treated at 2×MIC of CD were 58.28 %, 35.88 % and 26.92 %, respectively. Meanwhile, for *E. coli*, *S. aureus* and *C. albicans* treated with 4×MIC of CD, the percentages of CV uptake were 68.98 %, 56.56 % and 56.59 %, respectively. Lower percentage of CV uptake was seen for *E. coli*, *S. aureus* and *C. albicans* treated with EO which were about 49.66 %, 18.60 % and 21.44 %, respectively, at 2×MIC, whereas, the percentage of CV uptake at 4×MIC were 63.22 %, 33.55 % and 37.55 %, respectively. *E. coli* and *S. aureus* treated with 0.25 M EDTA and 20 µg/ml of chloramphenicol was observed to be significantly affected compared to control (untreated cell). However, *C. albicans* treated with 0.25 M EDTA (13.55 %) and nystatin (12.14 %) had no significant difference upon the treatment compared to the untreated cell (9.77 %). Dose dependent effect was observed by the significant increase of CV uptake when higher concentration of treatment was applied. The significant uptake of CV by *E. coli*, *S. aureus* and *C. albicans* treated with CD had suggested the great capability of CD to alter the membrane permeability which made the cells highly permeable to solutes. This statement was in line with the previous study done by Devi *et al.* [12].

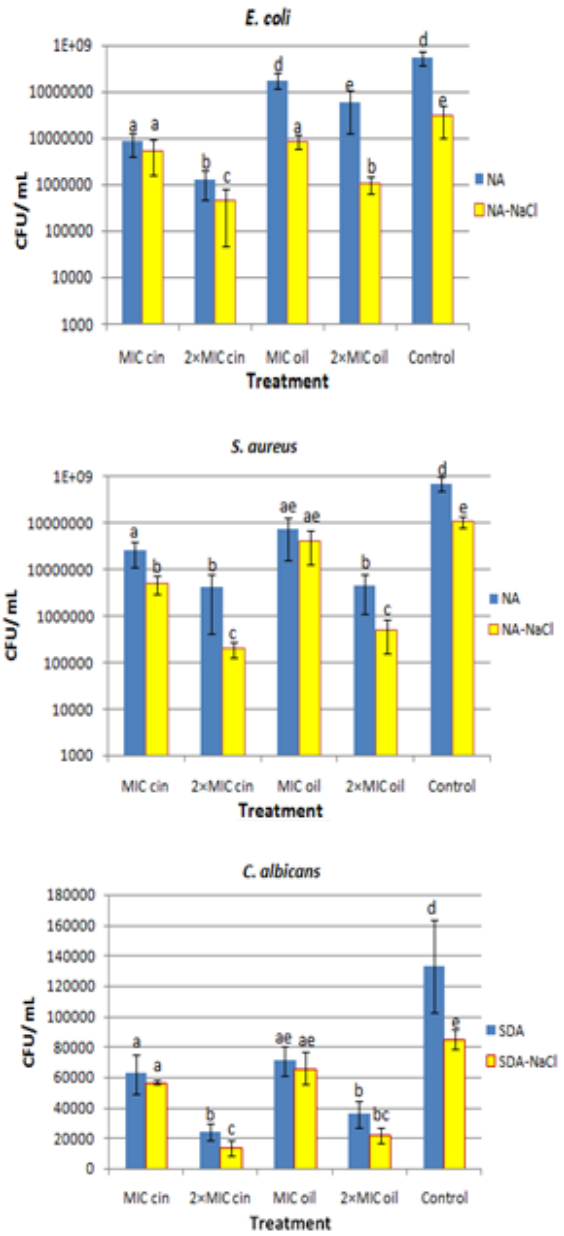


Figure 2 Salt tolerances by untreated (control) and treated E. coli, S. aureus and C. albicans at concentration equal to MIC and 2×MIC of EO and CD. Data represent the mean of triplicate readings and expressed as Mean±SD. The Mean was considered significantly different by different alphabets at $p<0.05$.

3.4 Leakage of 260 nm Absorbing Materials

There was significant leakage of extracellular materials by the high absorbance value from cells treated with CD compared to the cells treated with EO. Though, only small increased in absorbance value or no significant difference was observed with increasing concentration of treatments (Figure 4). At 4×MIC (1.33 mg/mL) of CD, the absorbance readings of *E. coli*, *S. aureus* and *C. albicans* were equal to 3.613, 3.713 and 3.398, respectively. As compared to absorbance readings by *E. coli*, *S. aureus* and *C. albicans* treated with 4×MIC of EO, lower absorbance value was observed which were 3.002, 3.031 and 2.918, respectively. *E. coli* (A260 equal to 1.163) and *S. aureus* (A260 equal to 1.215) treated with chloramphenicol had also indicated significant leakage of cellular materials as compared to the untreated cells. However, *C. albicans* treated with nystatin (A260 equal to 0.205) was observed to give no significant difference of cellular leakage as compared to untreated *C. albicans* (A260 equal to 0.075). It was suggested that CD caused damage on the cell membrane which subsequently resulted in significant leakage of intracellular components. The damage of cell membrane might be due to cell lysis or non-selective pores formation in the cell membrane resulting in the leakage of vital cell components as suggested by a study done by Xing *et al*. [15] which then further support the finding of this study.

3.5 Leakage of 280 nm Absorbing Materials

As shown in Figure 5, cells treated with CD at concentration equal to 2×MIC (0.66 mg/mL) and 4×MIC (1.33 mg/mL) values were observed to possess significantly higher absorbance value as compared to cells treated with EO. It was also observed that there was slightly increased in absorbance value with no significant difference upon increased of treatment concentration. *E. coli*, *S. aureus* and *C. albicans* treated with 4×MIC of CD have the absorbance value equal to 3.483, 3.582 and 3.318, respectively. Meanwhile, the absorbance value of *E. coli*, *S. aureus* and *C. albicans* treated with 4×MIC of EO were equal to 2.856, 2.91 and 2.818, respectively. Chloramphenicol also caused significant leakage of cellular materials from *E. coli* (A280 equal to 1.092) and *S. aureus* (A280 equal to 1.195) whereas nystatin only caused small amount of cellular leakage from *C. albicans* (A280 equal to 0.408) as compared to the untreated *C. albicans* (A280 equal to 0.058). In the present study, the bactericidal or fungicidal effect of EO and CD was observed upon the time interval. The population of all of the tested microorganisms was dramatically vanished by the loss of viability upon prolong exposure. CD at concentration equal to 4×MIC value (1.33 mg/mL) was observed to have much stronger effect upon 30 min of treatment especially against *C. albicans* which suggested that CD possessed a good fungicidal activity and had the capability of reducing the re-population as compared to its crude, EO. The fungicidal effect of CD may be explained by severe alteration of the cell membrane as been demonstrated by a morphological study done on *C. albicans* treated with CD [16].

5.0 CONCLUSION

The mode of action of EO and CD was time and dose-dependent. CD had stronger antimicrobial actions against *C. albicans* compared to EO.

These results may imply that CD exhibited the antimicrobial action by targeting on the cell membrane of *E. coli*, *S. aureus* and *C. albicans*.

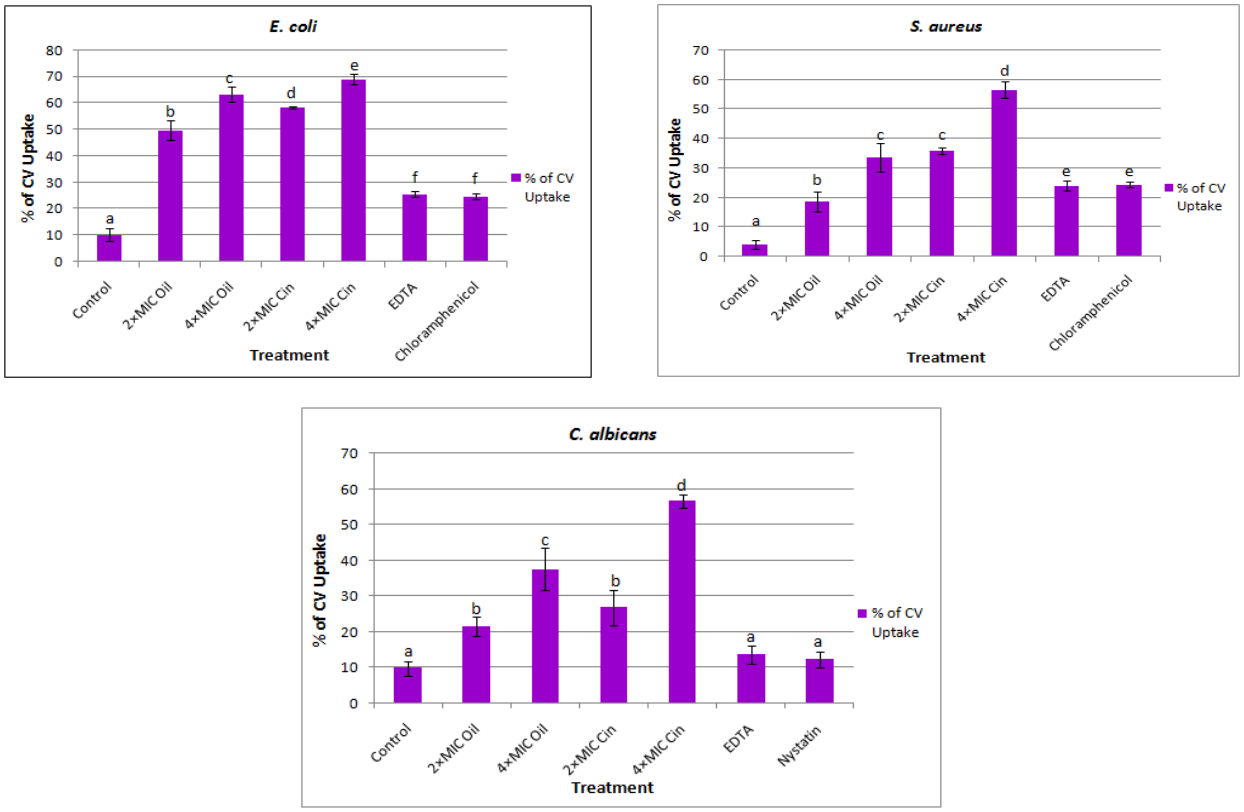
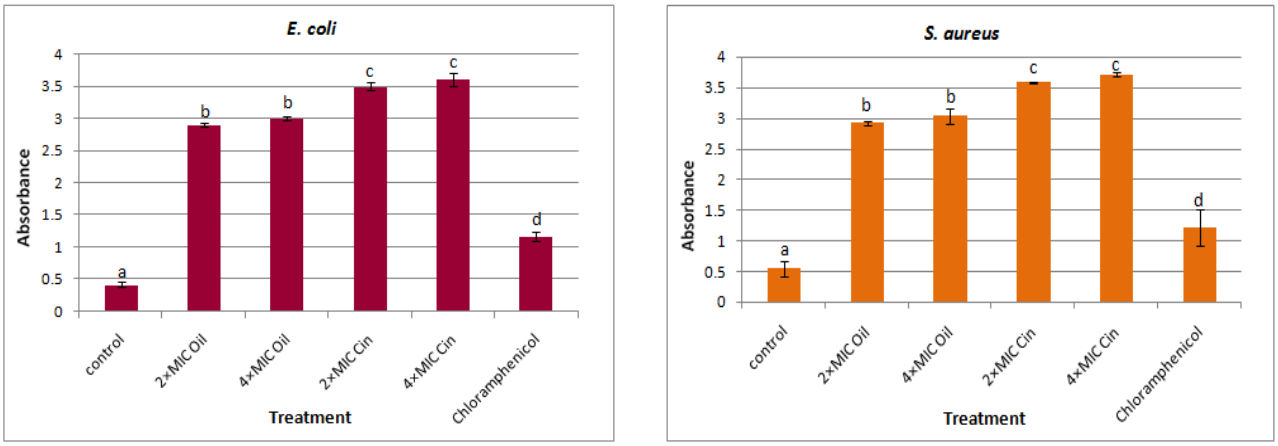


Figure 3 Percent of crystal violet uptake by untreated and treated *E. coli*, *S. aureus*, and *C. albicans* at concentration equal to 2×MIC and 4×MIC of EO and CD, 0.25 M EDTA, 20 µg/mL chloramphenicol and nystatin. Data represent the mean of triplicate readings and expressed as Mean±SD. The Mean was considered significantly different by different alphabets at $p<0.05$.



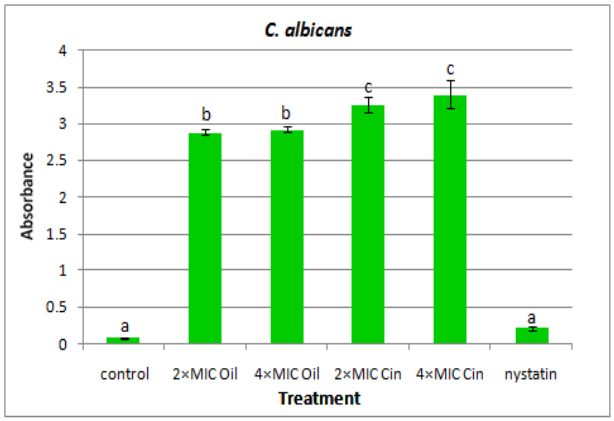


Figure 4 Leakage of extracellular 260 nm-absorbing materials from untreated and treated *E. coli*, *S. aureus* and *C. albicans* with EO and CD at concentration equal to 2×MIC and 4×MIC, 20 µg/mL chloramphenicol and nystatin. Data represent the mean of triplicate readings and expressed as Mean±SD. The Mean was considered significantly different by different alphabets at p<0.5.

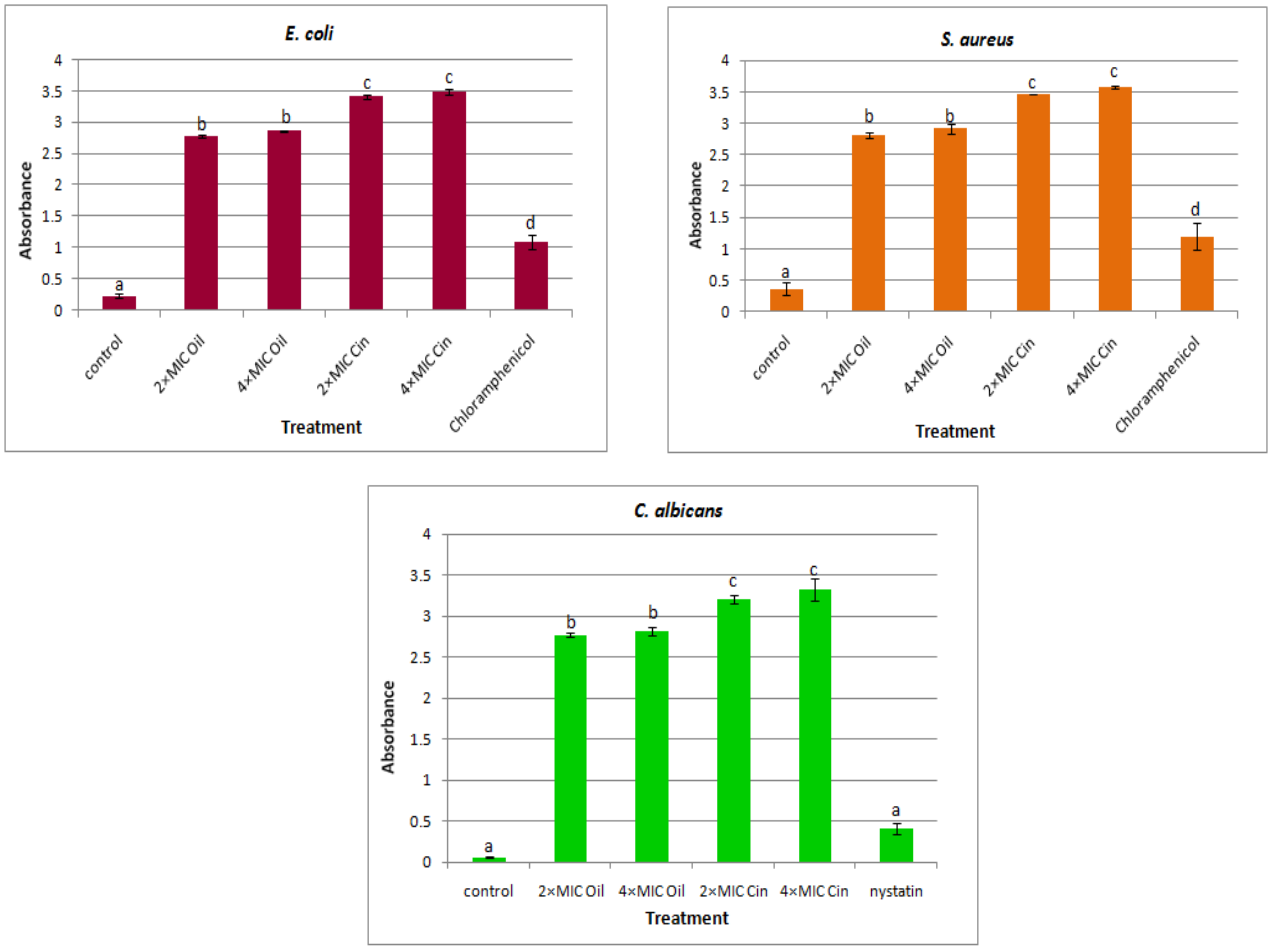


Figure 5 Leakage of extracellular 280 nm-absorbing materials from untreated and treated *E. coli* (a), *S. aureus* (b) and *C. albicans* (c) with EO and CD at concentration equal to 2×MIC and 4×MIC, 20 µg/mL chloramphenicol and nystatin. Data represent the mean of triplicate readings and expressed as Mean±SD. The Mean was considered significantly different by different alphabets at p<0.05.

Acknowledgment

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