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ESSENTIAL OILS EFFECT IN COMBINATION WITH ANTIBIOTICS AGAINST *STAPHYLOCOCCUS AUREUS* ATCC 29213 BIOFILM SUSCEPTIBILITY

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ABSTRACT: *Staphylococcus aureus* biofilm has been known as one inducing factor for the bacteria's resistance to various antibiotics. One strategy which may increase the efficacy of the antibiotic is by combining the antibiotic therapy with antiinfective from natural resources. This research was evaluating the potency of three known antiinfective essential oil derived from leaves of *Piper betle* L., *Ocimum basilicum* L. forma *citratum* Back and *Cymbopogon citratus* L., in a combination with antibiotics i.e. chloramphenicol, streptomycin, and erythromicin towards *S. aureus* ATCC 29213. The essential oils were obtained by steam-hydrodistillation of the fresh raw materials. Microdillution technique combined with colorimetric was used to determine the biofilm inhibition. Crystal violet was used for biofilm staining of which the reading was performed on a microplate reader. Fractional Inhibitory Concentration Index (FICI) values was evaluated based on the comparison of % inhibitory obtained from the essential oils and the antibiotics in a single and in a combination. The essential oils alone has the $PMIC_{50}$ (planktonic) values as follows, 0.2% (*P. betel*), 0,3% (*C. citratus*) and 0.84% v/v (*O. basilicum*). However, all essential oils has FICI values of > 2 indicates that instead of causing a synergistic effect, the essential oils seems to be antagonist to the antibiotics' biofilm formation inhibition activities.

INTRODUCTION: Biofilm is a form of microorganism attaching to a surface in aquoeus environment by forming extracellular polymeric substance (EPS) matrix [1]. This microbial form reduces susceptibility towards chemical, physical and biological threat, including towards existing antibiotics and host immune system. Dental plaque, implanted device related infection and cystic fibrosis are some of biofilm related health problems found in human [2]. *Staphylococcus aureus* is one of human normal flora which has been related to several biofilm related infection.

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Kamath et al.[3] reported that *S. aureus* found in 43% of biopsi paranasal sinus biopsy/swab of Chronic Rhino Sinusitis (CRS) patients. Sinus tissue of CRS patients contain intraepithelial *Staphylococcus aureus* (IESA), of which 100% of the tissue with IESA contains biofilm. *S.aureus* biofilm is correlated with more severe diseases and slower recovery process after surgery [4].

In other report, Rebiahi and collaborators [5] showed that *S. aureus* biofilm plays role in neonatus nocosomial incidence and is proven to be 100 times more resistance to antibiotic dosage regimen. *S. aureus* is known to have the ability to develop resistance to all kind of antibiotics [6]. Biofilm formation supports the ability by creating persistance cells which are adapted to antibiotics by reducing their dependency to the respective cell part target or by shutting down the target production [7].

Other possibility is that the complex matrix can reduce the antibiotic permeability into the target cells. Cells in biofilm state have slower metabolisme which is in turn influence antibiotic efficacy for those targeting fast growing microbes [8].

Despite several side effects may occur following a long term of antibiotics usage, several antibiotics which can be used against *S. aureus* infection are erythromycin, streptomycin, and chloramfenicol [9]. Leaves of *Piper betel* L. (Piperaceae), *Ocimum basilicum* L. forma *citratus* (Labiatae) and herbs of *Cymbopogon citratus* L. (Poaceae) have been reported as potential antibacterial from plants [10-11]. One of potential usage of herbal medicine is to complement the antibiotics in order to increase the efficacy and or to prevent the bacterial resistance to the respective antibiotics [12]. The antibiotics chosen to be studied were erythromycin, streptomycin and chloramphenicol which are known to be less effective towards microbial in biofilm state [13].

MATERIALS AND METHODS: Raw materials were collected from Yogyakarta and surroundings, Indonesia. Taxonomy identification was performed in Pharmacognosy Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia under registration Nr. BF/32/Ident/Det/I/ 2015, Nr. BF/33/Ident/Det/I/ 2015, and Nr. BF/35/ Ident/ Det/ I/2015.

Materials used were ethanol, methanol (technical grade, General, Indonesia), Luria-Bertani broth (Sigma-Aldrich, Germany), *Brain Heart Infusion* (Oxoid), Muëller-Hinton (Sigma-Aldrich, Germany), Crystal violet (Merck, Germany), chloramfenicol (Sigma-Aldrich, Germany), erythromycin (Sigma-Aldrich, Germany), streptomycin (Sigma-Aldrich, Germany). *S. aureus* ATCC 29213 was obtained from the stock culture of Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada.

Fresh samples were washed under flowing water, drained, and cut into 8-10 cm parts, and distilled for ± 6 hours. The resulted essential oils were kept in a dark vial inside a refrigerator. Luria Bertani broth media (pH 7) was prepared by diluting 10 g tryptone, 10 g NaCl, 5 g yeast extract in 1000 mL distilled water. NaOH or KCl was used to adjust the pH. Muëller-Hinton was prepared by diluting 23 g of the media 1 L of distilled water, while the Brain Heart Infusion (BHI) was prepared by diluting 37 g of the media into 1 L of distilled water. The media were sterilized by using autoclave at 121° C for 20 min.

Streptomycin 50 mg/mL was prepared by diluting 500 mg of the powder in 10 mL of sterile distilled water and then filtered by a 0.22 µm filter. Chloramphenicol by 30 mg/mL was prepared by diluting 300 mg powder in 10 mL ethanol. Erythromycin was prepared by diluting 100 mg powder in 10 mL ethanol. All antibiotic solutions were kept in a 4°C refrigerator.

PMIC values determination of the essential oils and antibiotics were performed by microdilution technique. Samples were prepared in LB media as double dilution in a range of concentration as follows:

a. The essential oils: 2 – 0.06 % v/v in methanol.
b. Chloramphenicol: 1.0 – 0.05 %v/v in ethanol.
c. Streptomycin: 1.0 – 0.05 %v/v in ethanol
d. Erythromycin: 1.0 – 0.05 %v/v in ethanol

S. aureus fresh culture was prepared on a OD_{600} of 0.1 (approx. 10^8 cell/mL). The test samples in amount of 200 µL each, were put into the 96 wells-microplate, added with 5 µL of *S. aureus* culture to obtain a bacterial concentration of 2.05×10^7. The microplate was put into a box containing wet tissue to keep the moisture, incubated for 18-24 h at 37°C incubator. Afterwards, the OD was measured by a microplate reader at 595 nm. The results were quantified as % inhibitory (formula 1) of which the $PMIC_{50}$ values are the smallest concentration of the samples which can inhibit the planktonic growth by 50% in comparison to the negative control.

$$\% \text{ Inhibitory} = \frac{OD_{\text{negative control}} - OD_{\text{test sample}}}{OD_{\text{negative control}}} \times 100\%$$

The resulted $PMIC_{50s}$ were used to calculate the sub-PMIC: $\frac{1}{2}$PMIC, in BHI. Each samples and control of 95 µL was put into the wells and 5 µL of *S. aureus* culture was added, incubated for 48 h at

37° C with moisture kept with the wet tissue. Crystal violet 1% was used for staining the biofilm. After discarded the media, and thoroughly rinse with water three times, each wells were given of 125 µL of the stain and left for 15 min. Afterwards, the staining solution was discarded and the wells were rinsed with 175 µL of distilled water, and drained. Each wells were added with 175 µL of ethanol absolute and left for 15 minutes and then read at 595 nm by a microplate reader.

A Fractional Inhibitory Concentration Index (FICI) was calculated according to the formulas as follows:

$$\text{FIC oil} = \frac{\text{MIC of oil in combination}}{\text{MIC of oil alone}} \quad \text{..... (2)}$$

$$\text{FIC antibiotic} = \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}} \quad \text{..... (3)}$$

$$\text{FICI} = \text{FIC oil} + \text{FIC antibiotic} \quad \text{..... (4)}$$

FICI ≤ 0.5 is considered as synergist, additive if the FICI > 0.5 and ≤ 1, neutral if FICI > 1 and ≤ 2, while antagonism is considered in the case of FICI >2 [14, 15].

Statistical methods: Statistical significance of the data was determined using ANOVA, followed by Dunnett's test. Differences were considered significant with *P* values of 0.05 or less.

RESULTS AND DISCUSSION: The essential oils yields were 0.27% v/w (*C. citratus*), 0.25%v/w (*O. basilicum*) and 0.25% (*P. betel*). Chemical analyses for the essential oils used was performed by a GCMS of which the results as described in **Fig. 1, 3-4** as the GC chromatogram and the tables I-III for the chemical contents prediction based on the MS data.

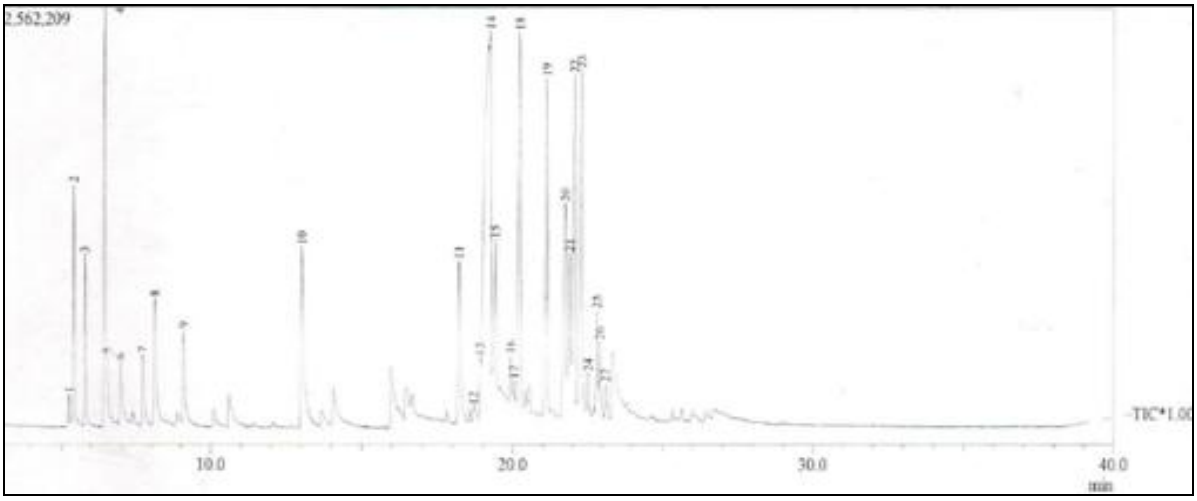

FIG.1: GC CHROMATOGRAM OF THE *PIPER BETLE* ESSENTIAL OIL

TABLE 1: CHEMICAL CONTENTS PREDICTION OF MAIN PEAKS DETECTED BY GC-MS OF THE *PIPER BETLE* ESSENTIAL OIL. LIBRARY USED WAS WILEY229 AND NIST62

Peak Number	tR	% Total	MW	Compounds
14	19.281	24.41	164	Eugenol
18	20.239	8.55	204	beta-caryophylene
19	21.147	6.45	204	alpha-humulene
22	22.072	6.84	204	Germacrene A
23	22.309	8.38	204	Germacrene A

Chavibetol is the main constituent of the *P. betel* essential oil [10]. Nevertheless, several literatures steted eugenol as the main constituent [16]. Chavibetol (meta-eugenol) itself is an isomer of eugenol **(Fig. 2).** Eugenol and caryophylene were reported elsewhere as antimicrobial constituents.

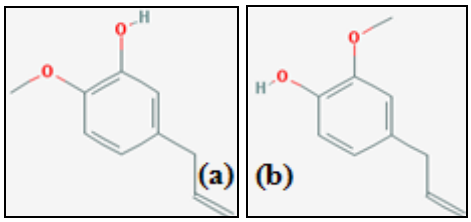

FIG. 2: CHAVIBETOL (a) AND EUGENOL (b) (source: http://pubchem.ncbi.nlm.nih.gov)

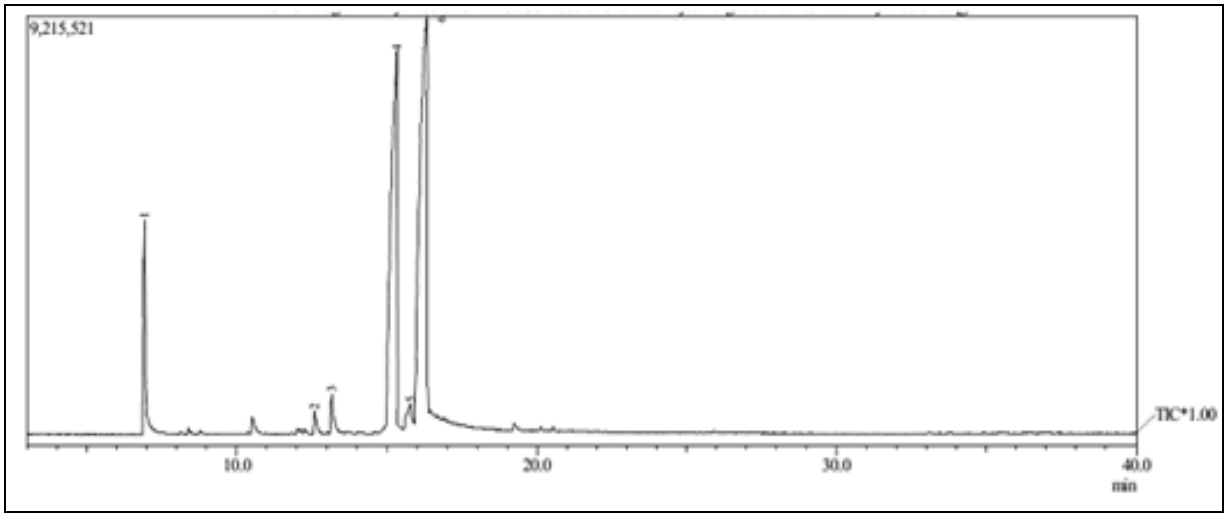

FIG. 3: GC CHROMATOGRAM OF THE *C. CITRATUS* ESSENTIAL OIL

TABLE 2: CHEMICAL CONTENTS PREDICTION OF MAIN PEAKS DETECTED BY GC-MS OF THE *C. CITRATUS* ESSENTIAL OIL. LIBRARY USED WAS WILEY229 AND NIST62

Peak Number	tR	% Total	MW	Compounds
1	136	6.79	136	Beta myrcene
4	152	38.69	152	Z-citral
5	154	1.77	154	Geraniol
6	152	50.14	152	E-citral

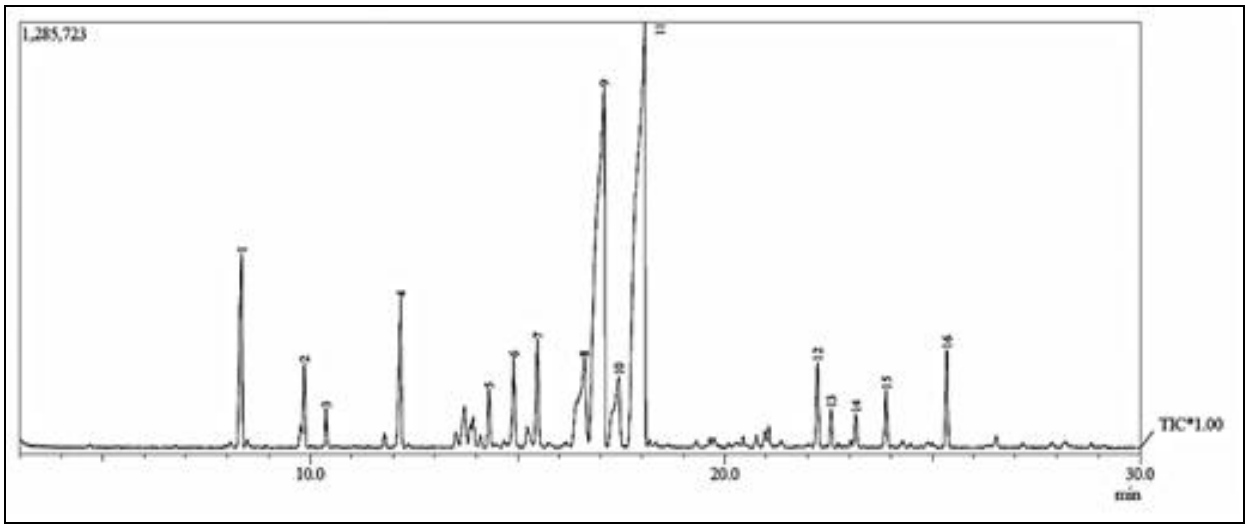

FIG.4: GC CHROMATOGRAM OF THE *O. BASILICUM*, ESSENTIAL OIL

TABLE 3: CHEMICAL CONTENTS PREDICTION OF MAIN PEAKS DETECTED BY GC-MS OF THE *O.BASILICUM* ESSENTIAL OIL. LIBRARY USED WAS WILEY229 AND NIST62

Peak Number	tR	% Total	MW	Compounds	Smilarity Index
1	12.183	3.84	136	α-terpinolone	95
2	17.092	29.22	152	Neral or Z-citral	94
3	18.067	36.85	154	Geranial or E-citral	95
4	22.558	0.77	204	Trans-α-bergamoten	91
5	16.611	5.61	154	Nerol	93
6	17.440	4.20	154	Geraniol	95
7	9.863	1.45	154	Eucalyptol	94

$PMIC_{50}$ determination results supports reports of the essential oils potency as planktonic growth inhibitors towards *S. aureus* [16-18]. Table IV describes the $PMIC_{50}$ values of each essential oils and the antibiotics used.

TABLE 4: $PMIC_{50}$ AND $SMIC_{50}$ VALUES OF THE ESSENTIAL OILS AND ANTIBIOTICS

Samples	$PMIC_{50}$
P. betel	0.2%
C. citratus	0.3% v/v
O. basilicum forma *citratus*	0.84% v/v
Erythromycin	0.16 mg/mL
Streptomycin	0.08 mg/mL
Chloramphenicol	0.011 mg/mL

Table 5 describes the effects of the essential oils combined to the antibiotics to the biofilm formation of *S. aureus* which can be determined by the value of the FICI.

TABLE 5: FIC AND FICI DATA RESULTS FROM THE COMBINATION OF THE ESSENTIAL OILS AND ANTIBIOTICS

Essential oil	Antibiotics	FIC EO	FIC AB	FICI	Interpretation
$SMIC_{50}$ *P. betel*	$SMIC_{50}$ Erythromycin	3.13	1.93	5.07	Antagonist
	½ $SMIC_{50}$ Erythromycin	88.17	38.53	126.70	Antagonist
½ $SMIC_{50}$ *P. betel*	$SMIC_{50}$ Erythromycin	1.23	1.08	2.31	Antagonist
	½ $SMIC_{50}$ Erythromycin	5.08	3.17	8.25	Antagonist
$SMIC_{50}$ *P. betel*	$SMIC_{50}$ Streptomycin	24.05	22.82	46.86	Antagonist
	½ $SMIC_{50}$ Streptomycin	2.62	0.83	3.45	Antagonist
½ $SMIC_{50}$ *P. betel*	$SMIC_{50}$ Streptomycin	3.50	4.74	8.24	Antagonist
	$SMIC_{50}$ Streptomycin	2.81	1.27	4.08	Antagonist
$SMIC_{50}$ *P. betel*	$SMIC_{50}$ Chloramphenicol	1.62	0.86	2.48	Antagonist
	½ $SMIC_{50}$ Chloramphenicol	2.56	0.73	3.29	Antagonist
½ $SMIC_{50}$ *P. betel*	$SMIC_{50}$ Chloramphenicol	14.96	11.33	26.29	Antagonist
	½ $SMIC_{50}$ Chloramphenicol	3.43	1.39	4.82	Antagonist
$SMIC_{50}$*C. citratus*	$SMIC_{50}$ Erythromycin	1.06	1.01	2.07	Antagonist
	½ $SMIC_{50}$ Erythromycin	0.94	0.41	1.35	Neutral
½ $SMIC_{50}$ *C. citratus*	$SMIC_{50}$ Erythromycin	0.89	1.21	2.09	Antagonist
	½ $SMIC_{50}$ Erythromycin	0.95	0.66	1.62	Neutral
$SMIC_{50}$ *C. citratus*	$SMIC_{50}$ Streptomycin	1.19	1.19	2.38	Antagonist
	½ $SMIC_{50}$ Streptomycin	0.87	0.63	1.50	Neutral
½ $SMIC_{50}$ *C. citratus*	$SMIC_{50}$ Streptomycin	1.35	1.91	3.26	Antagonist
	$SMIC_{50}$ Streptomycin	1.08	1.20	2.28	Neutral
$SMIC_{50}$ *C. citratus*	$SMIC_{50}$ Chloramphenicol	1.02	0.97	1.99	Neutral
	½ $SMIC_{50}$ Chloramphenicol	1.33	0.99	2.33	Antagonist
½ $SMIC_{50}$ *C. citratus*	$SMIC_{50}$ Chloramphenicol	0.68	0.90	1.58	Neutral
	½ $SMIC_{50}$ Chloramphenicol	1.83	1.35	3.18	Antagonist
$SMIC_{50}$ *O. basilicum*	$SMIC_{50}$ Erythromycin	0.83	0.77	1.60	Neutral
	½ $SMIC_{50}$ Erythromycin	1.07	0.91	1.98	Neutral
½ $SMIC_{50}$ *O. basilicum*	$SMIC_{50}$ Erythromycin	0.77	0.87	1.64	Neutral
	½ $SMIC_{50}$ Erythromycin	0.93	0.96	1.89	Neutral
$SMIC_{50}$ *O. basilicum*	$SMIC_{50}$ Streptomycin	1.37	0.91	2.28	Antagonist
	½ $SMIC_{50}$ Streptomycin	4.91	2.92	7.83	Antagonist
½ $SMIC_{50}$ *O. basilicum*	$SMIC_{50}$ Streptomycin	0.88	0.71	1.59	Neutral
	$SMIC_{50}$ Streptomycin	0.97	0.70	1.67	Neutral
$SMIC_{50}$ *O. basilicum*	$SMIC_{50}$ Chloramphenicol	0.80	0.82	1.62	Neutral
	½ $SMIC_{50}$ Chloramphenicol	0.89	0.85	1.74	Neutral
½ $SMIC_{50}$ *O. basilicum*	$SMIC_{50}$ Chloramphenicol	1.56	1.97	3.53	Antagonist
	½ $SMIC_{50}$ Chloramphenicol	2.85	3.29	6.14	Antagonist

DISCUSSION: The activity of *P. betel* essential oils in inhibiting the growth of the *S. aureus* growth either in planktonic or biofilm can be trace back to eugenol as its main constituent. This compound has been reported to have an ability to permeate through the cell walls and interact with the cell protein. The non-specific permeability can induce the increase of the potassium ion and ATP transport out. The eugenol hydroxyl bond with protein is expected to contribute in the antimicrobial effect as well [16, 19-21]. Eugenol was also reported to inhibit the bacterial quorum sensing which may contribute to its effect on biofilm formation inhibition [22]. Caryophyllene, other *P. betel* constituent, was also

reported elsewhere as a potential antimicrobials [23]. In general, antibiofilm activity of essential oils is correlated to their property to destabilize the cell membrane due to them being highly lipophilic and small molecule properties. The interaction of the essential oil with the cell wall components can also lead to leakage which lead to cell death [24-26].

Despite promising antimicrobial properties of the essential oils as single component, the experiments displayed unexpected results in the combination with the antibiotics. An antagonistic effect occured following combination of all antibiotics with the *P. betel*. However, different results were displayed by the combinations of the same antibiotics with the essential oils of *O. basilicum* and *C. citratus*, of which neutral results were observed in several treatments depending on the concentration used.

Antagonisms can occur due to several causes. The active constituents of the essential oil and the antibiotics can chemically interact which further causing structural changes into inactive compounds or prevent to interact with target. Other possibility is that the active constituents and the antibiotics work on different target of action of which diminishing the activity of the other. Neutral results can be achieved by either the antibiotics or the essential oils has higher activity in comparison to the other so then addition of the less active agent will not significantly increase the activity.

CONCLUSION: All the tested essential oils showed potential inhibition towards the planktonic and biofilm formation of *Staphylococcus aureus*. However, *P. betel* essential oil showed antagonistic effect on the biofilm inhibition activities of all antibiotics tested i.e. erythromycin, streptomycin and chloramphenicol. Nevertheless, the essential oils of *C. citratus* and *O. bacilicum* showed neutral and antagonist effect in combination with the antibiotics, which depends on the concentration tested.

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CONFLICT OF INTEREST: The authors declare that they are no conflict of interest regarding this manuscript.

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