

Synergistic Benefit of *Eugenia Caryophyllata* L. and *Cinnamomum Zeylanicum* Blume Essential Oils against Oral Pathogenic Bacteria

Siti Nurazwa Zainol^a, Shahida Mohd Said^b, Zamirah Zainal Abidin^b, Nuramirah Azizan^d, Fadzilah Adibah Abdul Majid^{*,c}, Ibrahim Jantan^d

^aDepartment of Bioprocess Engineering, Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, 81310 Johor Bahru, Malaysia

^bFaculty of Dentistry, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia

^cInstitute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

^dFaculty of Pharmacy, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia
f.adibah@umt.edu.my

Eugenia caryophyllata L. and *Cinnamomum zeylanicum* Blume essential oils offer great potential against the pathogens of oral cavity. Both of these essential oils have recently being used as antibiotics and antiviral agents. This study investigates the synergistic effect of *E. caryophyllata* and *C. zeylanicum* oils against known resistant bacteria in oral infection namely *Enterococcus faecalis*, *Aggregatibacter actinomycetemcomitans*, *Streptococcus mutans* and *Streptococcus salivarius*. The combination effect *E. caryophyllata* and *C. zeylanicum* were evaluated using checkerboard assays. Amoxicillin at concentration 0.1 mg/mL was used as positive control while each bacteria suspension (10^5 CFU/mL) was used as negative control. The minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FIC) were calculated to characterised interaction between the combinations. Both *E. caryophyllata* and *C. zeylanicum* oils possess antimicrobial activity against all the four bacteria when used on its own or in combination. In combination, the MIC values were reduced for all bacteria compared when in single form. The synergistic antibacterial effect was significant toward *E. faecalis*, *A. actinomycetemcomitans* and *S. mutans* with FIC index is 0.5 and below. Partial synergy was produced in *S. salivarius*. The acceptable range for synergistic effect is between 0.16 mg/mL and 0.31 mg/mL. Above from 0.63 mg/mL are considered as additive and antagonistic effect. The combinations of these essential oils gave a stronger antibacterial activity and directly can replace the common use of antibiotics like Amoxicillin. This finding suggests a potential therapeutic benefit using the combination of *E. caryophyllata* and *C. zeylanicum* oils in the future management of oral infection.

1. Introduction

Oral disease including tooth decay and periodontal disease is a major health problem especially in the developing countries and about 60 - 90 % of children and adults are affected (Petersen, 2004). Although there are some successful conventional clinical approaches, herbal medicine is also popularly used as preventive therapy. Essential oils has been used especially as mouth rinse either traditionally or clinically to treat oral diseases including periodontal disease (Moran, 2008). Natural antimicrobial agents has been used in recent years as a strategies to control the resistant food-borne bacteria and other pathogenic microorganism (Yap et al., 2014). Interaction between essential oils may lead to synergistic, additive, indifferent (non-interaction) or antagonistic effect. Synergistic effect is observed when the effect of combined substances is greater than the sum of the individual effects. An additive is when the combined effect is equal to the sum of the individual effects while the absence of interaction is defined as indifference. Antagonistic is defined when the effect of one or both substances is less when they applied together than when individually applied (Bassolé and Juliani, 2012). Clove (*Eugenia caryophyllata* L.) oil has a pleasant aroma and is useful as an essential food flavour, pharmaceutical and dentistry. It has been used for a long time by dentists as its main active ingredient, eugenol,

is primarily responsible either as a bacteriocidal or bacteriostatic agent to fight dental infection on teeth (Bankar et al., 2012). It is also used traditionally in many cultures for relieving toothache, sore gums and oral ulcers beside as a dressing in dentistry for minor wounds (Aneja and Joshi, 2010).

Cinnamon (*Cinnamomum zeylanicum* Blume) oil on the other hand, has been reported to have high antioxidant activity and antimicrobial properties (Li et al., 2013;). It is also been used in toothpastes, mouthwash or chewing gum for dental caries prevention (Pandita et al., 2014).

While the antimicrobial of *E. caryophyllata* and *C. zeylanicum* oils has been intensively studied only a few studies have been reported on antimicrobial effect of combined application of *E. caryophyllata* and *C. zeylanicum* oils. However, there are only few studies demonstrated the activity of combined *E. caryophyllata* or *C. zeylanicum* oils with other essential oils. For example, the combination of *E. caryophyllata* with rosemary oils (Fu et al., 2007), *C. zeylanicum* with Thyme or clove oil (Lu et al., 2011) and *C. zeylanicum* with *Lavandula angustifolis* oils (de Rapper et al., 2013). All of these studies shown antimicrobial effect on Gram-positive and Gram-negative strains. Moreover, they also limited information on the scientific data of essential oils including *Eugenia caryophyllata* L. and *Cinnamomum zeylanicum* Blume oils on oral pathogens. Thus, synergistic effect of *Eugenia caryophyllata* L. and *Cinnamomum zeylanicum* Blume oils is carried out as an alternative treatment for potential treatment of oral infections.

2. Methodology

2.1 Essential oils and chemicals

Eugenia Caryophyllata L. and *Cinnamomum Zeylanicum* Blume essential oils were provided by Department of Periodontology, Faculty of Dentistry, Universiti Kebangsaan Malaysia (UKM). Dimethylsulfoxide (DMSO, 100 %) (Sigma), ethanol (75 %) (Merck, Germany)

2.2 Bacteria cell and culture media

All bacteria strains used were provided by Department of Periodontology, Faculty of Dentistry, Universiti Kebangsaan Malaysia (UKM) namely *Enterococcus faecalis* (ATCC® 29212™), *Streptococcus mutans* (ATCC® 25175™), *Streptococcus salivarius* (ATCC® 13419™) and *Aggregatibacter actinomycetemcomitans* (ATCC® 29522™). These are anaerobic and facultative anaerobic bacterial commonly found in periodontal tissue and associate with periodontal disease. Brain Heart Infusion (BHI) agar and broth was used as a media culture. Mitis salivarius (MS) agar was used to maintain the growth of streptococci spp. and tryptic soy-serum-bacitracin-vancomycin (TSBV) agar was prepared for *A. actinomycetemcomitans* culture.

2.3 Antibacterial assay

2.3.1 Minimal inhibitory concentration (MIC)

The microdilution minimum inhibitory concentration assay was carried out as described by de Rapper et al. (2013) with slight modifications. This method was used to determine the lowest concentration that inhibit the bacteria and used as references to calculate fractional inhibition concentration (FIC) index. The *E. caryophyllata* and *C. zeylanicum* oils were diluted to a concentration 250 mg/mL using dimethylsulfoxide (DMSO) as a diluent. Both essential oils were serially diluted to concentrations 1.25 mg/mL, 0.62 mg/mL, 0.31 mg/mL, 0.15 mg/mL, 0.078 mg/mL, 0.039 mg/mL, 0.019 mg/mL and 0.00 mg/mL. The volume of essential oils was added at a same volume 50 : 50 µL for combination test and dispended in 96-microtiter plate. The selected bacteria suspension was prepared by diluting it in broth media culture to get an approximate concentration of 1×10^5 colony forming units (CFU)/mL. Bacteria suspensions were added to all well of the microtiter plates at a volume of 100 µL. Then, the microtiter plates were sealed with parafilm, to prevent any loss of essential oils due to their inherent volatility. The plates were incubated at 37 °C in anaerobic environment for 24 h. The reading of absorbance was taken using a plate reader at wavelength 590 nm. The negative control used was bacteria suspension in broth only while the positive control was Amoxicillin (0.1 mg/mL). All assays were done in triplicates in three independent experiment.

The percentage of bacterial inhibition was calculated using Eq(1):

$$\begin{aligned} & \% \text{ inhibition of concentration} \\ & = \left[\frac{\text{Mean OD}_{590} \text{ control well} - \text{Mean OD}_{590} \text{ sample well}}{\text{Mean OD}_{590} \text{ control well}} \right] \times 100 \% \end{aligned} \quad (1)$$

where Control well = bacteria suspension in broth medium only , and Sample well = concentration of essential oils combination in broth medium

2.4 Fractional inhibition concentration (FIC)

The Fractional inhibition concentration was used to determine the interaction between the oils by calculating the fractional inhibitory concentration index (Σ FIC) (Sadiki et al., 2014) using the following formula:

$$\Sigma\text{FIC} = \text{FIC (E. caryophyllata L)} + \text{FIC (C. zeylanicum Blume)} \quad (2)$$

$$\text{FIC (E. caryophyllata L)} = \frac{\text{MIC (E.caryophyllata L.)in combination (mg/mL)}}{\text{MIC (E. caryophyllata L.) alone (mg/mL)}} \quad (3)$$

$$\text{FIC (C. zeylanicum Blume)} = \frac{\text{MIC (C.zeylanicum Blume)in combination (mg/mL)}}{\text{MIC (C. zeylanicum Blume) alone (mg/mL)}} \quad (4)$$

Σ FICI values interpreted as follows:

- ≤ 0.5 = synergistic;
- 0.5 - 0.75 = partial synergy;
- 0.76 - 1.0 = additive;
- > 1.0 - 4.0 = non-interactive;
- > 4.0 = antagonistic

2.5 Gas chromatography test

The sample preparation was done by diluted 250 μL of each essential oils in 250 μL ethyl acetate and 1.0 μL was injected into the column. Shimadzu GC2000 gas chromatography equipped with column DB-5 (1 μm thickness, 30.0 m length, 0.25 mm diameter), was used for the analysis of oils. Injector and flame ionisation detector (FID) temperature were maintained at 250 $^{\circ}\text{C}$. Nitrogen was the carrier gas used with a flow rate of 1.0 mL/min. Initial temperature of the oven was programmed at 75 $^{\circ}\text{C}$ for the initial 10 min, then 3 $^{\circ}\text{C}/\text{min}$ to 230 $^{\circ}\text{C}/\text{min}$ for 5 min. Total program time was 73.33 min (Ahmad et al., 2010).

3. Result and discussion

The synergistic effect of *E. caryophyllata* and *C. zeylanicum* oils was shown in Table 1. In combination, the MIC values were reduced for all bacteria and showed synergistic effect, defined FICI as ≤ 0.5 . This was shown when the combination oils interacted with *E. faecalis*, *A. actinomycetemcomitans* and *S. mutans*. However, only partial synergy effect was shown for *S. salivarius*, *E. faecalis* and *S. mutans* with the FICI values 0.38 and 0.31. The finding from our study was similar to other reported studies where *E. caryophyllata* and *C. zeylanicum* oils was found gave a greater inhibition of some Gram positive and Gram negative bacteria when tested alone (Hussein et al., 2014). We also found similar finding which showed *E. caryophyllata* having lesser antibacterial activity on *S. mutans* than other species (Cai and Wu, 1996). Meanwhile, a different finding was seen with *C. zeylanicum*, where *S. mutans* were observed to be the most sensitive at a lower MIC value of 12.8 $\mu\text{g}/\text{mL}$ (Kohanteb and Fani, 2011). When in combinations, the synergy effects were achieved against *S. mutans* in equal volume ratios even though a predominant non-interactive effect was noted. Here, *S. salivarius* antibacterial activity was less sensitive towards combination of *E. caryophyllata* and *C. zeylanicum* oils.

Table 1: Synergistic effect of *E. caryophyllata* and *C. zeylanicum* oils against 4 selected bacteria

Strains	Essential Oils	MIC (mg/mL)		FIC	FICI	Outcome
		Alone	Combination			
<i>E. faecalis</i>	<i>E. caryophyllata</i>	1.25	0.16	0.13	0.38	Synergistic
	<i>C. zeylanicum</i>	1.25	0.31	0.25		
<i>A. actinomycetemcomitans</i>	<i>E. caryophyllata</i>	1.25	0.31	0.25	0.5	Synergistic
	<i>C. zeylanicum</i>	0.63	0.16	0.25		
<i>S. mutans</i>	<i>E. caryophyllata</i>	1.25	0.08	0.06	0.31	Synergistic
	<i>C. zeylanicum</i>	0.63	0.16	0.25		
<i>S. salivarius</i>	<i>E. caryophyllata</i>	1.25	0.63	0.5	0.75	Partial synergy
	<i>C. zeylanicum</i>	1.25	0.31	0.25		

Figure 1 showed a summary of synergistic study for *E. caryophyllata* and *C. zeylanicum* oils, the acceptable range concentration when to do a further study in term of product development. The synergistic range was between 0.16 mg/mL and 0.31 mg/mL. The partial synergy was produced when the concentration of the oils in between 0.31 mg/mL and 0.63 mg/mL. Beyond this outcome, results were recorded as additive or antagonistic effects. The antibacterial activity of this combination was good as antibiotics, like Amoxicillin. The antibacterial activity of the Amoxicillin was reported when the concentration at 0.1 mg/mL.

Essential oils have been used for many years to treat human health from infectious diseases and against notorious pathogens. More recently, there are increasing evidence indicating that essential oils and plant extracts are useful for specific medical treatment. The antibacterial activity of *E. caryophyllata* and *C. zeylanicum* have been reported, but in the present study tested on combination of these two essential oils have not reported yet especially on oral health perspective. Previous study reported that *E. caryophyllata* and *C. zeylanicum* exhibited strong antibacterial activity against for both gram-positive and gram-negative bacteria (Hussein et al., 2014). The investigation on the essential oils is necessary to improve the quality of healthcare because there are potentially useful source for antimicrobial compounds, especially against bacterial pathogens (Prabuseenivasan et al., 2006).

Our finding has the potential to assist researchers and clinicians to explore further the use of herbal oils as alternative to the commonly used antibiotics. Antibiotics are commonly prescribed in dental practice for oral infections and the total percentage of global antibiotic costs was 75 % (Roda et al., 2007). It has been reported that there are about 5 % of hypersensitivity and diarrhoea occurred when patients were prescribed with amoxicillin (Jorger and Miriam, 2002). The combinations of these essential oils gave stronger antibacterial effect and may be beneficial to replace common use of antibiotics like Amoxicillin in the clinic.

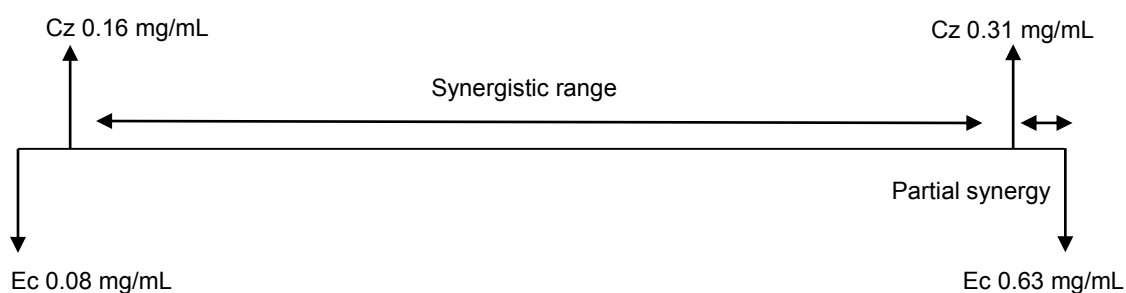


Figure 1: Summary for synergistic study for *E. caryophyllata* (Ec) and *C. zeylanicum* (Cz) oils

It is believed the chemical composition of the *E. caryophyllata* L. and *C. zeylanicum* Blume oils contributed to the anti-microbial properties and been evaluated. About 42 and 24 compounds of the oils were identified with the aid of gas chromatography (GC) and literature comparison. In this study, (Z)- Methyl cinnamate was detected as major compound in *C. zeylanicum* Blume oils with amount 38.57 % followed by (E)-cinnamaldehyde (16.71 %). The other constituents are bornyl acetate (6.33 %), methyl nonanate (1.25 %), benzaldehyde (1.29 %), benzene acetaldehyde (1.48 %), borneol (0.95 %), 1, 8-cineole (0.19 %), (E)-isoeugenol (0.41 %) and (Z)-cinnamyl acetate (0.059 %). As reported from previous study, (E)-cinnamaldehyde was found as major compounds with the percentage amount 68.95 % (Unlu et al., 2010) and 97.7 % (Singh et al., 2007). However, in this study the percentage amount (E)-cinnamaldehyde detected is low. This is because cinnamaldehyde was easily oxidised and has high volatility properties (Gunkel et al., 2011). The composition in *C. zeylanicum* oil shows the noticeable amount of aromatic components.

The major components of *E. caryophyllata* L. oil was isobornyl propanoate (53.15 %), (E)-isoeugenol (11.39 %), γ -muurolene (1.26 %), (E)- γ bisbolene (1.35 %), cis-beta-elemenone (2.99 %), dihydro-eugenol acetate (0.60 %), (Z)-isoeugenol (0.25 %), (E)-methyl isoeugenol (0.19 %), (E)-caryophyllene (0.09 %) and eugenol (0.07 %). In contrast, Nassar et al. (2007) identified eugenol (71.56 %) and eugenol (8.99 %) acetate as a major volatile constituents in *E. caryophyllata* oil with the same approaches. Based on literature, eugenol was claimed as a major compound in *E. caryophyllata* oil but in this study, eugenol is a minor compound. Since *E. caryophyllata* oil is volatile oil, this could be a one of the reason the decline of eugenol compounds during analysis. The characteristic of volatile oils is easily to oxidise when the oils exposed to light and air. The oils also will evaporate when subjected to heat.

4. Conclusions

E. caryophyllata L. and *C. zeylanicum* Blume oils when used in combination showed a potential benefit in inhibiting oral infection involving *E. faecalis*, *A. actinomyces comitans*, *S. mutans* and *S. salivarius* from the oral cavity. These essential oils combination at suitable concentration could be an alternative to use of synthetic antimicrobial agents and may lead a new research on natural products in future management of oral infection.

Acknowledgements

This study was supported by Young Investigator Initiative Grant (GGPM-2014-020) from UKM, UTM-Research University Grant (RUG) (03H95) and Ministry of Agriculture Grant (MOA-NRGS) (4H016).

Reference

- Ahmad F., Moharm B.A., Jantan I., 2010, A Comparative study of the constituents of the essential oils of *Goniothalamus tapis* Miq. and *G. tapisoides* Mat Salleh from Borneo, *Journal of Essential Oil Research* 22 (6), 499-502.
- Aneja K.R., Joshi R., 2010, Antimicrobial Activity of *Syzygium aromaticum* and Its Bud Oil Against Dental Cares Causing Microorganisms, *Ethnobotanical Leaflets* 14, 960-75
- Bankar R., Kumar A., Puri S., 2011, Phytochemical Constituent *Syzygium aromaticum* L., *International Journal of Current Research* 3 (7), 215–217.
- Bassolé I.H.N., Juliani H.R., 2012, Essential oils in combination and their antimicrobial properties, *Molecules* 17 (4), 3989–4006.
- Cai L., Wu C.D., 1996, Compounds from *Syzygium aromaticum* possessing growth inhibitory activity against oral pathogens, *Journal of Natural Products* 59 (10), 987–990.
- de Rapper S., Kamatou G., Viljoen A., van Vuuren S., 2013, The In Vitro Antimicrobial Activity of *Lavandula angustifolia* Essential Oil in Combination with Other Aroma-Therapeutic Oils, *Evidence-Based Complementary and Alternative Medicine*, Hindawi Publishing Corporation, Cairo, Egypt.
- Dorman H.J., Deans S.G., 2000, Antimicrobial agents from plants: antibacterial activity of plant volatile oils, *Journal of Applied Microbiology* 88 (2), 308–316.
- Fu Y., Zu Y., Chen L., Shi X., Wang Z., Sun S., Efferth T., 2007, Antimicrobial Activity of Clove and Rosemary Essential Oils Alone and in Combination, *Phytotherapy Research* 21, 989–994.
- Gunkel W., Fraser L.C., Bhatia S.C., 2011, *Essential Oils Handbook*, 1st Edition, CBS Publishers and Distributor Pvt. Ltd, Alkem Company (S) Pte Ltd., New Delhi, India.
- Goñi P., López P., Sánchez C., Gómez-Lus R., Becerril R., Nerín C., 2009, Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils, *Food Chemistry* 116 (4), 982–989.
- Hussein H.A., Abaas I.S., Hussain R., 2014, Innovare Academic Sciences Antibacterial Activities Of Cinnamon *Zelanicum Syzygium Aromaticum* Essential Oil, *International Journal of Pharmacy and Pharmaceutical Sciences* 6 (5), 165-168.
- Jakhetia V., Patel R., Khatri P., Pahuja N., Garg S., Pandey A., Sharma S., 2010, J Cinnamon: a pharmacological review, *Journal of Advanced Scientific Research* 1 (2), 19–23.
- Jorger S., Miriam T., 2002, Systemic antibiotics in the treatment of periodontal disease, *Periodontology* 2000 28, 106–176.
- Kohanteb J., Fani M.M., 2011, Inhibitory Activity of Cinnamon *Zelanicum* and *Eucalyptus Globulus* Oils on *Streptococcus Mutans*, *Staphylococcus Aureus*, and *Candida* Species Isolated From Patients With Oral Infections, *Shiraz University Dental Journal* 11, 14–22.
- Li Y.Q., Kong D.X., Wu H., 2013, Analysis and evaluation of essential oil components of cinnamon barks using GC-MS and FTIR spectroscopy, *Industrial Crops and Products* 41 (1), 269–278.
- Lu F., Ding Y., Ye X., Ding Y., 2011, Antibacterial Effect of Cinnamon Oil Combined with Thyme or Clove Oil, *Agricultural Sciences in China* 10 (9), 1482–1487.
- Moran J.M., 2008, Home-use oral hygiene products: Mouthrinses, *Periodontology* 2000 48 (1), 42–53.
- Nassar M.I., Gaara A.H., El-Ghorab A.H., Farrag A.-R. H., Shen H., Huq E., Mabry T.J., 2007, Chemical constituents of clove (*Syzygium aromaticum*, Fam Myrtaceae) and their antioxidant activity, *Revista Latinoamericana de Quimica* 35 (3), 47–57.
- Núñez L., D'Aquino M., 2012, Microbicide activity of clove essential oil (*Eugenia Caryophyllata*), *Brazilian Journal of Microbiology* 43 (4), 1255–1260.
- Pandita V., Patti V., Singla A., Singh S., Malhi R., Vashishtha V., 2014, Dentistry meets nature-role of herbs in periodontal care: systematic review. *Journal of Indian Association of Public Health Dentistry* 12 (3), 148-156.
- Petersen P.E., 2004, Challenges to improvement of oral health in the 21st century--the approach of the WHO Global Oral Health Programme, *International Dental Journal* 54, 329–343.
- Prabuseenivasan S., Jayakumar M., Ignacimuthu S., 2006, In vitro antibacterial activity of some plant essential oils, *BMC Complementary and Alternative Medicine* 6, Article no 39.
- Roda R.P., Bagán J.V., María J., Bielsa S., Pastor E.C., Roda R.P., 2007, Antibiotic use in dental practice, A review, *Med Oral Patol Oral Cir Bucal* 12, 186–192.

- Sadiki M., Balouiri M., Barkai H., Maataoui H., Koraichi S.I., Elabed S., 2014, Synergistic antibacterial effect of *Myrtus communis* and *Thymus vulgaris* essential oils fractional inhibitory concentration index, *International Journal of Pharmacy and Pharmaceutical Sciences* 6 (6), 121–124.
- Singh G., Maurya S., DeLampasona M.P., Catalan C.A., 2007, A comparison of chemical, antioxidant and antimicrobial studies of cinnamon leaf and bark volatile oils, oleoresins and their constituents. *Food and Chemical Toxicology* 45 (9), 1650–1661.
- Sweeney L.C., Dave J., Chambers P.A., Heritage J., 2004, Antibiotic resistance in general dental practice — a cause for concern, *Journal of Antimicrobial Chemotherapy* 53 (4), 567–576.
- Unlu M., Ergene E., Unlu G.V., Zeytinoglu H.S., Vural N., 2010, Composition, antimicrobial activity and in vitro cytotoxicity of essential oil from *Cinnamomum zeylanicum* Blume (Lauraceae), *Food and Chemical Toxicology* 48 (11), 3274–3280.
- Wang R., Wang R., Yang B., 2009, Extraction of essential oils from five cinnamon leaves and identification of their volatile compound compositions, *Innovative Food Science and Emerging Technologies* 10 (2), 289–292.
- Yap P.S.X., Yiap B.C., Ping H.C., Lim S.H.E., 2014, Essential oils, a new horizon in combating bacterial antibiotic resistance, *The Open Microbiology Journal* 8, 6–14.