Identification of essential oils with activity against stationary phase *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is the most dominant human pathogen, responsible for a variety of chronic and severe infections. There is mounting evidence that persisters are associated with treatment failure and relapse of persistent infections. While some essential oils were reported to have antimicrobial activity against growing *S. aureus*, activity of essential oils against the non-growing stationary phase *S. aureus* enriched in persisters has not been investigated. In this study, we evaluated the activity of 143 essential oils against stationary phase *S. aureus* and identified 39 essential oils (Cinnamon bark, Oregano, Thyme white, Bandit "Thieves", Lemongrass (*Cymbopogon flexuosus*), Sandalwood oil, Health shield, Allspice, Amyris, Palmarosa, Cinnamon leaf, Clove bud, Citronella, Geranium bourbon, Marjoram, Peppermint, Lemongrass (*Cymbopogon citratus*), Cornmint, Elemi, Ho wood, Head ease, Lemon eucalyptus, *Litsea cubeba*, Myrrh, Parsley seed, Coriander oil, Dillweed, Hyssop, Neroli, Rosewood oil, Tea tree, Cajeput, Glove bud, Lavender, Sleep tight, Vetiver, *Palo santo*, Sage oil, Yarrow) at 0.5% concentration, 10 essential oils (Cinnamon bark, Oregano, Thyme white, Bandit "Thieves", Lemongrass (*Cymbopogon flexuosus*), Sandalwood oil, Health shield, Allspice, Amyris, Palmarosa) at 0.25% concentration, and 7 essential oils (Cinnamon bark, Oregano, Thyme white, Lemongrass (*Cymbopogon flexuosus*), Allspice, Amyris, Palmarosa) at 0.125% concentration to have high activity against stationary phase *S. aureus* with no visible growth on agar plates after five-day exposure. Among the 10 essential oils which showed high activity at 0.25% concentration, 9 (Cinnamon bark, Oregano, Thyme white, Bandit "Thieves", Lemongrass (*Cymbopogon flexuosus*), Health shield, Allspice, Palmarosa, Amyris) showed higher activity than the known persister drug tosufloxacin, while the other one (Sandalwood oil) was found to be active at a higher concentration. In Oregano essential oil drug combination studies with clinical antibiotics, Oregano plus quinolone drugs (tosufloxacin, levofloxacin, ciprofloxacin) and rifampin completely eradicated all stationary phase *S. aureus* cells, but had no apparent enhancement for linezolid, vancomycin, sulfamethoxazole, trimethoprim, azithromycin and gentamicin. Our findings may facilitate development of more effective treatment for persistent *S. aureus* infections.

Introduction

Staphylococcus aureus is the leading cause of nosocomial and community-associated infections, which is responsible for a wide variety of infections that include mild superficial skin infections, osteomyelitis, implant-associated heart valve, native valve endocarditis, severe sepsis and bacteremia [1]. Although antibiotic resistance is a major problem in treatment of infections caused by *S. aureus*, drug-tolerant persisters are demonstrated to be significant contributors of chronic persistent infections and recurrent infections [2]. Clinically, infections caused by *S. aureus* such as soft tissue infections, endocarditis, osteomyelitis, prosthetic joint infections, and biofilm-related infections on indwelling device is difficult to cure with the current antibiotics, which are mainly active against the growing bacteria but have poor activity against the non-growing persisters [3]. Recently, it has been shown that a drug combination approach using drugs targeting both log phase growing bacteria and the non-growing stationary phase bacteria could more effectively eradicate a persistent urinary tract infection and a biofilm skin infection in the mouse models [4, 5]. However, the choice of persister drugs is limited at present, and treatment of persistent infections remains a challenge. Although some essential oils were found to be active against growing *S. aureus* [6-8], the activity of essential oils against non-growing stationary phase *S. aureus* has not been studied. Because activity against non-growing persisters or stationary phase bacteria correlates with in vivo activity against persistent infections in the context of drug combination in the case of uropathogenic *E. coli* and *B. burgdorferi* persistent infections [4, 9, 10], here, we evaluated a panel of 143 essential oils for their activity against stationary phase *S. aureus* as a model for activity against *S. aureus* persisters. We identified a range of highly potent essential oils with excellent activity against non-growing stationary phase *S. aureus.*

Materials and Methods

Bacterial strain

S. aureus Newman, a commonly used pan-susceptible strain isolated from a patient suffering from osteomyelitis [3] was used in this study. The strain was incubated in Tryptic Soy Broth (TSB) medium overnight to stationary phase without shaking at 37 ^{o}C, 5% CO_2. The stationary phase *S. aureus* culture (~10^9 CFU/mL) was used directly without dilution for essential oil screens and drug exposure tests.

Antibiotics and essential oils

Tosufloxacin, ciprofloxacin, levofloxacin, rifampin, linezolid, vancomycin, sulfamethoxazole, trimethoprim, azithromycin and gentamicin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) or H_2O to form stock solutions. All antibiotic stocks (except DMSO stocks) were filter-sterilized by 0.2 μm filter and stored at −20°C.

Commercially available essential oils were purchased from Natural Acres (MO, USA), Plant Therapy (ID, USA) and Plant Guru (NJ, USA). More information about the essential oils can be found on their websites (http://www.theplantguru.com/gc-ms-testing, http://www.planttherapy.com/essential-oils, http://naturalacresoils.com/collections/all).

DMSO-soluble essential oils were dissolved in DMSO at 5% (v/v). DMSO-insoluble essential oils were directly added to *S. aureus* cultures, then vortexed to form aqueous suspension. The 5% essential oils or aqueous suspension were further diluted into the bacterial cultures to achieve desired dilution in the following drug exposure or MIC experiments to evaluate their activity against non-growing stationary phase or growing log phase *S. aureus*.

Screening of essential oils for their activity against stationary phase *S. aureus*

To evaluate the effect of essential oils on stationary phase bacteria, the essential oils and drugs were added to the 96-well plates containing stationary phase bacteria, leaving the first and last columns in each plate blank for control. In the primary screen, each essential oil was assayed at three concentrations: 0.5%, 0.25% and 0.125% (v/v). Tosufloxacin, ciprofloxacin, levofloxacin, rifampin, linezolid, vancomycin, sulfamethoxazole, trimethoprim, azithromycin and gentamicin were used at 50 μM as control antibiotics. The plates were incubated at 37 ^{o}C, 5% CO_2 without shaking. After three days and five days of exposure to essential oils or drugs, the bacterial suspension was transferred to TSB plates with a 96-pin replicator to monitor the bacterial survival and regrowth after further incubation at 37 ^{o}C. All tests were run in triplicate.

Antibiotic susceptibility test

The minimum inhibitory concentrations (MICs) were determined using microdilution method according to the CLSI guideline [11]. Essential oils were 2-fold diluted from 1% to 0.0075%. Gentamicin was 2-fold diluted from 512 μg/mL to 0.25 μg/mL as a control antibiotic. The 96-well plates were sealed and incubated at 37 ^{o}C overnight without shaking. All experiments were run in triplicate.

Validation of active essential oils by colony forming unit (CFU) assay

The stationary phase bacteria were transferred into Eppendorf tubes. Essential oils were added at 0.25% and 0.125% concentrations. Tosufloxacin, ciprofloxacin, levofloxacin, rifampin, linezolid, vancomycin, sulfamethoxazole, trimethoprim, azithromycin and gentamicin were added to bacterial suspensions at the final concentration of 20 μM, respectively. At different time points, 100 μL bacterial suspensions were collected by centrifugation, washed and resuspended in PBS. After serial dilutions, 10 μL of each dilution was plated on TSB plate for CFU count.

Drug combination assay on stationary phase *S. aureus*

In this study, we used Oregano as the common element to test the activity of various two-drug combinations in killing *S. aureus* Newman stationary phase cells. We evaluated tosufloxacin, levofloxacin, ciprofloxacin, rifampin, linezolid, vancomycin, sulfamethoxazole, trimethoprim, azithromycin and gentamicin at the final concentration of 5 μg/mL in combination with Oregano (0.025%). The designed drug combinations or single drug controls were added directly to stationary phase culture and CFU count was performed at different time points.

Results

Identification of active essential oils against stationary phase *S. aureus*

Consistent with our previous study [3], tosufloxacin was shown to have high activity against stationary phase *S. aureus*, while other clinical drugs including ciprofloxacin, levofloxacin, rifampin, linezolid, vancomycin, sulfamethoxazole, trimethoprim, azithromycin and gentamicin were not able to completely kill stationary phase *S. aureus* at 50 μM after five-day drug exposure [8]. Interestingly, after three-day exposure, 30 (Cinnamon bark, Oregano, Thyme white, Lemongrass (*Cymbopogon flexuosus*), Bandit "Thieves", Sandalwood oil, Health shield, Allspice, Amyris, Palmarosa, Cinnamon leaf, Clove bud, Citronella, Geranium bourbon, Marjoram, Peppermint, Lemongrass (*Cymbopogon citratus*), Cornmint, Elemi, Ho wood, Head ease, Lemon eucalyptus, Litsea cubeba, Myrrh, Parsley seed, Coriander oil, Dillweed, Hyssop, Neroli, Rosewood oil), 6 (Cinnamon bark, Oregano, Thyme white, Bandit "Thieves", Lemongrass (*Cymbopogon flexuosus*), Sandalwood oil) and 7 (Cinnamon bark, Oregano, Thyme white, Lemongrass (*Cymbopogon flexuosus*), Allspice, Amyris, Palmarosa) essential oils were found to have high activity against stationary phase *S. aureus* at 0.5%, 0.25% and 0.125% concentrations, respectively. When the drug exposure was extended to five days, additional 9 essential oils (Tea tree, Cajeput, Glove bud, Lavender, Sleep tight, Vetiver, Palo santo, Sage oil, Yarrow) and 4 essential oils (Health shield, Allspice, Amyris, Palmarosa) were found to be active at 0.5% and 0.25% concentration, respectively (Table 1). The top 10 essential oils (Cinnamon bark, Oregano, Thyme white, Lemongrass (*Cymbopogon flexuosus*), Bandit "Thieves", Sandalwood oil, Health shield, Allspice, Amyris, Palmarosa), which showed high activity at 0.25% concentration, were used in the subsequent testing to confirm their activity in inhibiting growth of *S. aureus* in MIC test and in CFU drug exposure assay for their activity against non-growing stationary phase *S. aureus*.

MIC determination of the top active essential oils

We carried out antibiotic susceptibility testing to determine the activity of the top 10 active essential oils against growing *S. aureus*. As shown in Table 2, Oregano, Amyris and Sandalwood oil were the most active agents in inhibiting the growth of *S. aureus*, with the lowest MIC of 0.015% in our study. The growth of *S. aureus* was efficiently suppressed by Cinnamon bark at 0.03%. Allspice could inhibit the growth of *S. aureus* with an MIC of 0.06%, while Thyme white, Health shield, Bandit "Thieves", Lemongrass (*Cymbopogon flexuosus*) and Palmarosa had the same MIC of 0.125% against *S. aureus*. Clinical drug gentamicin included as a control inhibited the growth of *S. aureus* with an MIC of 1 μg/mL.

Comparison of active essential oils in their ability to kill stationary phase *S. aureus*

We first tested the activity of tosufloxacin and other clinically used drugs against stationary phase *S. aureus* at 20 μM. As previously described [3], tosufloxacin could kill all stationary phase *S. aureus* cells after seven-day drug exposure, with no visible colonies remaining on TSB plate. Levofloxacin, ciprofloxacin and rifampin had weak activity with 10^4~10^5 CFU/mL cells remaining after seven-day exposure. In contrast, other clinical drugs including linezolid, vancomycin, sulfamethoxazole, trimethoprim, azithromycin and gentamicin did not

show obvious activity against stationary phase *S. aureus* even when the drug exposure was extended to seven days (Figure 1). In contrast, eight essential oils (Cinnamon bark, Oregano, Thyme white, Bandit "Thieves", Lemongrass (*Cymbopogon flexuosus*), Health shield, Allspice, Palmarosa) at 0.25% concentration could eradicate all stationary phase cells after one-day exposure. Meanwhile, Amyris could clear all the cells after three-day exposure whereas Sandalwood oil could not wipe out the stationary phase *S. aureus* cells after seven-day exposure. At a lower concentration of 0.125%, we noticed that Oregano, Lemongrass (*Cymbopogon flexuosus*) and Thyme white still exhibited strong activity against stationary phase *S. aureus*, and no CFU could be detected after one-day exposure (Figure 2). Meanwhile, Cinnamon bark, Allspice, Amyris and Palmarosa could eradicate stationary phase *S. aureus* cells after three-day exposure. On the other hand, Bandit "Thieves", Sandalwood oil and Health shield could not eradicate the stationary phase *S. aureus* culture even after seven-day exposure.

Development of essential oil drug combinations to eradicate stationary phase *S. aureus* in vitro

It has been reported that synergistic activity between antibiotic and essential oil could occur, which achieved better bactericidal effect against growing *S. aureus* [12]. It is of great importance to include drugs that target persister bacteria in the treatment of infection diseases [4]. Based on our results, Oregano demonstrated high activity against not only log phase growing *S. aureus* with a low MIC but also stationary phase non-growing bacteria. Meanwhile, clinically used drugs had limited activity to kill *S. aureus* persisters. To more effectively eradicate the stationary phase *S. aureus*, we evaluated essential oil drug combinations using clinical drugs in combination with Oregano (0.025%). We found that some essential oil drug combinations were indeed much more effective than single drugs (Figure 3). Among them, rifampin + Oregano could completely eradicate all the stationary phase *S. aureus* after just one-day exposure. Tosufloxacin + Oregano could wipe out all stationary phase cells after three-day exposure. Meanwhile, levofloxacin + Oregano and ciprofloxacin + Oregano could kill all the stationary phase *S. aureus* after five-day exposure. These drug combinations showed much better activity than respective single drugs (10^4 ~ 10^6 CFU/mL cells remaining) and somewhat better activity than single Oregano (10^4 CFU/mL remaining). In contrast, other essential oil drug combinations such as linezolid + Oregano, vancomycin + Oregano, sulfamethoxazole + Oregano, trimethoprim + Oregano, azithromycin + Oregano and gentamicin + Oregano had limited activity against stationary phase cells, with 10^4 CFU/mL bacterial cells remaining even after five-day exposure, suggesting these combinations were not significantly better than Oregano alone (10^4 CFU/mL remaining).

Discussion

S. aureus is known to give rise to a diverse range of infections from mild skin infections to serious diseases such as endocarditis and osteomyelitis and biofilm infections. Persisters are dormant phenotypic variants of bacterial cells that are tolerant to antibiotics and genetically identical drug susceptible kin [2]. Since persisters were first identified in 1944 [13], there is considerable evidence that drug-tolerant persisters are the contributors to *S. aureus* persistent and relapsing infections [2, 14, 15], Meanwhile, treatment of persistent *S. aureus* infections

has remained a challenge. It has been proposed to use persister drugs in the context of drug combination as in Yin-Yang model for more effective treatment of persistent infections [16]. Although previous study has screened FDA-approved drug library to identify agents that have good activity against stationary phase *S. aureus* [3], only few useful hits such as tosufloxacin and clinafloaxin were identified. Although ADEP4, an experimental acyldepsipeptide antibiotic killing *S. aureus* persisters in combination with rifampin has been reported to cure a deep wound infection in a mouse model [17], its validity in treating other persistent infections in other disease models remains to be confirmed. Thus, while it is of great importance to include drugs targeting persister bacteria in the treatment of *S. aureus* infections, the choice of persister drugs that may be useful is quite few. Since most studies of essential oil activity on *S. aureus* were performed on log phase growing bacteria [6, 7], here we set out to determine the activity of a large panel of essential oils against stationary phase *S. aureus* cultures enriched in persister bacteria. Interestingly, we identified a range of essential oils that have strong activity against stationary phase cultures of *S. aureus* that may be useful for more effective treatment of persistent *S. aureus* infections.

Essential oils, a widely studied alternative against antibiotic resistant bacteria, are concentrated volatile liquids extracted from plants. While there are some reports on activity of essential oils against log phase *S. aureus*, the number of evaluated essential oils is small (just one or two kinds of essential oils) [6, 8], and their activity against stationary phase *S. aureus* cultures has not been studied [6, 8]. In this study, we evaluated a panel of 143 essential oils for their activity against stationary phase *S. aureus*. We identified 9 essential oils (at 0.25% concentration) that are more active than persister drug tosufloxacin (20 μM), a quinolone drug control that could eradicate stationary phase *S. aureus*. Among them, 7 essential oils (Cinnamon bark, Oregano, Thyme white, Lemongrass (*Cymbopogon flexuosus*), Allspice, Amyris, Palmarosa) showed outstanding activity against stationary phase *S. aureus* at 0.125% concentration (Figure 2). Meanwhile, all of the top 9 essential oils showed high activity against growing *S. aureus* (Table 2), of which some essential oils such as Thyme white, Oregano and Cinnamon bark have been reported to have high activity against log phase *S. aureus* in previous studies [7, 18, 19], but Lemongrass (*Cymbopogon flexuosus*), Bandit "Thieves", Health shield, Allspice, Amyris, Palmarosa were first reported in this study. Compared with our previous work on activity of essential oils against stationary phase *E. coli*, some essential oils including Cinnamon bark, Oregano, Bandit "Thieves", Health shield and Allspice exhibited outstanding activity against both Gram-positive *S. aureus* and Gram-negative *E. coli* [20], while it seems that Thyme white, Lemongrass (*Cymbopogon flexuosus*), Amyris, Palmarosa just showed high activity against *S. aureus* [20], while Cinnamon leaf, Clove bud and *Syzygium aromaticum* were only active against *E. coli* [20]. Moreover, although some studies indicate that certain active essential oils including their main active components such as carvacrol or eugenol could induce membrane damage by causing loss of cellular contents [9, 21, 22], there are limited studies available that focus on the active components and the mechanisms of antimicrobial action of essential oils in general. Here, we identified some active essential oils against stationary phase *S. aureus*. Further studies are needed to determine the main active components and the mechanisms of action of the active essential oils identified in this study.

Oregano is known to be one of the most effective essential oils against a wide variety of pathogens, including *Pseudomonas sp*., *Salmonella sp*., *Escherichia coli* and *Borrelia burgdorferi* [7, 23]. In this study, Oregano exhibited high activity against not only log phase growing *S. aureus* with a low MIC of 0.015% but also stationary phase non-growing bacteria with complete clearance without any regrowth at 0.125% concentration. Remarkably, when combined with some currently recommended antibiotics for *S. aureus* infections, Oregano showed a positive enhancement effect in increasing the activity of some antibiotics (quinolones, rifampin) against stationary phase *S. aureus* (Figure 3). When combined with rifampin, the combination showed outstanding activity with 100% clearance after just one-day exposure. When combined with tosufloxacin and two other quinolone drugs (levofloxacin and ciprofloxacin), the combinations could wipe out all stationary phase cells after three-day or five-day exposure. The synergistic effect of Oregano and the four drugs may have implications for improved treatment of *S. aureus* persistent infections. Further studies should be carried out to confirm if such combination approaches are useful in animal models.

Additionally, we found Cinnamon bark, Thyme white, Lemongrass (*Cymbopogon flexuosus*), Allspice, Amyris and Palmarosa showed excellent activity against stationary phase *S. aureus* at a low concentration of 0.125% (Figure 2). Cinnamon bark was reported to have activity against bacteria, fungi, inflammation, cancer and diabetes [24]. Also, it was demonstrated to be a potential antibacterial agent that exhibited strong activity against methicillin resistant *S. aureus* (MRSA) [25]. In this study, Cinnamon bark also showed its remarkable activity against non-growing stationary phase *S. aureus*. Thyme white, extracted from *Thymus zygis*, showed great activity against both log phase and stationary phase *S. aureus* at the same concentration of 0.125%. Essential oils obtained from *Thymus* species were often compared for their antibacterial and antioxidant activity. And oil from *Thymus zygis* was the most active one towards log phase Gram-positive and Gram-negative bacteria [18, 26]. Our results highlighted its antibacterial activity not only against growing *S. aureus* bacteria but also non-growing stationary phase cells. Lemongrass from two different plants (*Cymbopogon flexuosus* and *Cymbopogon citratus*) were evaluated in this study. While Lemongrass from *Cymbopogon flexuosus* could kill all the stationary phase *S. aureus* in just one day at 0.125%, Lemongrass from *Cymbopogon citratus* showed obvious activity only at a high concentration (0.5%). This provides the basis for further testing of *Cymbopogon flexuosus* in animal models of infection. Allspice is widely known as a popular spice in food processing [27], here, its activity against *S. aureus* may facilitate its usage for antibacterial purpose. Compared with other essential oils, there are few studies discussing bioactivity of Amyris. One study revealed that vapor delivery of Amyris could alter pyrethroid efficacy and detoxification enzyme activity in mosquitoes [28]. Our new finding of Amyris activity on *S. aureus* in this study may contribute to more bioactivity of Amyris and therapeutic use of *Amycris balsamifera*. Palmarosa, known as *Cymbopogon martini*, is used in Ayurvedic medicine to relieve nerve pain for skin problems and as a skin tonic in aromatherapy due to its antimicrobial properties [29]. While its immunomodulatory activity is based on geraniol [29], the main component of Palmarosa active against stationary phase *S. aureus* is unknown

and will be determined in the future.

Along with the essential oils which showed strong activity against *S. aureus* persisters at 0.125%, there were three essential oils (Bandit "Thieves", Sandalwood oil and Health shield) that showed obvious activity only at higher concentrations. Bandit "Thieves" and Health shield could eradicate all stationary phase cells after one-day exposure at 0.25% concentration. Sandalwood oil exhibited obvious activity at 0.5% concentration. Both Bandit "Thieves" and Health shield are synergy blend of essential oils. While Bandit "Thieves" contains clove, cinnamon, lemon, rosemary and eucalyptus oils, Health shield is a mixture of essential oils from cassia, clove, eucalyptus, lemon and rosemary. They were active against the growing of *S. aureus* with the same MIC value of 0.125% and showed similar activity against stationary phase *S. aureus* cells (Table 2 and Figure 2). Sandalwood oil in this study is obtained from *Santalum spicatum* (Australian Sandalwood). There are two other kinds of Sandalwood oil: East Indian Sandalwood oil extracted from *Santalum album* and New Caledonian Sandalwood oil prepared from the wood of *Santalum austrocaledonicum* [30]. Sandalwood oil from East Indian is widely studied as an attractive natural therapeutic for inflammatory skin diseases [31]. On the other hand, Sandalwood oil from *Santalum spicatum* has high commercial value for applications in aromatherapy and for the production of cosmetics such as soaps, creams and powder [30]. In this study, Sandalwood oil extracted from *Santalum spicatum* showed high activity against *S. aureus*, which demonstrated that it may be a promising antibacterial agent.

In summary, this is the first study of a large collection of 143 essential oils for activity against stationary phase *S. aureus* where we identified several promising essential oils. The top hits are Oregano, Cinnamon bark, Thyme white, Lemongrass (*Cymbopogon flexuosus*), Bandit "Thieves", Sandalwood oil, Health shield, Allspice, Amyris, Palmarosa. Meanwhile, we found in drug combination study with essential oil (Oregano) and antibiotics that some potent combinations such as Oregano plus quinolones or rifampin could effectively eradicate *S. aureus* persisters in vitro. Further studies should be carried out to identify the active components, evaluate safety, pharmacokinetics, and their activity to eradicate *S. aureus* persistent infections in animal models.

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References

1. Springer MT, Singh VK, Cheung AL, Donegan NP, Chamberlain NR. Effect of clpP and clpC deletion on persister cell number in Staphylococcus aureus. J Med Microbiol. 2016;65(8):848-57. Epub 2016/07/05. doi: 10.1099/jmm.0.000304. PubMed PMID: 27375177.

2. Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, et al. Persister formation in

349 Staphylococcus aureus is associated with ATP depletion. Nature Microbiology. 2016;1(5). doi:
350 10.1038/nmicrobiol.2016.51.
351 3. Niu H, Cui P, Yee R, Shi W, Zhang S, Feng J, et al. A Clinical Drug Library Screen Identifies
352 Tosufloxacin as Being Highly Active against Staphylococcus aureus Persisters. Antibiotics (Basel).
353 2015;4(3):329-36. Epub 2015/01/01. doi: 10.3390/antibiotics4030329. PubMed PMID: 27025627;
354 PubMed Central PMCID: PMCPMC4790289.
355 4. Cui P, Niu H, Shi W, Zhang S, Zhang H, Margolick J, et al. Disruption of Membrane by Colistin
356 Kills Uropathogenic Escherichia coli Persisters and Enhances Killing of Other Antibiotics. Antimicrob
357 Agents Chemother. 2016;60(11):6867-71. Epub 2016/09/08. doi: 10.1128/AAC.01481-16. PubMed
358 PMID: 27600051; PubMed Central PMCID: PMCPMC5075097.
359 5. Yee R, Yuan Y, Tarff A, Brayton C, Gour N, Feng J, et al. 2019. doi: 10.1101/686097.
360 6. Bouhdid S, Abrini J, Zhiri A, Espuny MJ, Manresa A. Investigation of functional and morphological
361 changes in Pseudomonas aeruginosa and Staphylococcus aureus cells induced by Origanum
362 compactum essential oil. J Appl Microbiol. 2009;106(5):1558-68. Epub 2009/02/20. doi:
363 10.1111/j.1365-2672.2008.04124.x. PubMed PMID: 19226402.
364 7. Caillet S, Ursachi L, Shareck F, Lacroix M. Effect of gamma radiation and oregano essential oil on
365 murein and ATP concentration of Staphylococcus aureus. J Food Sci. 2009;74(9):M499-508. Epub
366 2010/05/25. doi: 10.1111/j.1750-3841.2009.01368.x. PubMed PMID: 20492121.
367 8. de Morais Oliveira-Tintino CD, Tintino SR, Limaverde PW, Figueredo FG, Campina FF, da Cunha
368 FAB, et al. Inhibition of the essential oil from Chenopodium ambrosioides L. and alpha-terpinene on
369 the NorA efflux-pump of Staphylococcus aureus. Food Chem. 2018;262:72-7. Epub 2018/05/13. doi:
370 10.1016/j.foodchem.2018.04.040. PubMed PMID: 29751924.

9. Feng J, Shi W, Miklossy J, Tauxe GM, McMeniman CJ, Zhang Y. Identification of Essential Oils with Strong Activity against Stationary Phase Borrelia burgdorferi. Antibiotics (Basel). 2018;7(4). Epub 2018/10/20. doi: 10.3390/antibiotics7040089. PubMed PMID: 30332754; PubMed Central PMCID: PMCPMC6316231.

10. Feng J, Li T, Yee R, Yuan Y, Bai C, Cai M, et al. Stationary phase persister/biofilm microcolony of Borrelia burgdorferi causes more severe disease in a mouse model of Lyme arthritis: implications for understanding persistence, Post-treatment Lyme Disease Syndrome (PTLDS), and treatment failure. Discovery medicine. 2019;27(148):125-38. Epub 2019/04/05. PubMed PMID: 30946803.

11. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. CLSI document M100-S17. 2007;27(1):154-61.

12. Buldain D, Buchamer AV, Marchetti ML, Aliverti F, Bandoni A, Mestorino N. Combination of Cloxacillin and Essential Oil of Melaleuca armillaris as an Alternative Against Staphylococcus aureus. Front Vet Sci. 2018;5:177. Epub 2018/08/18. doi: 10.3389/fvets.2018.00177. PubMed PMID: 30116728; PubMed Central PMCID: PMCPMC6082948.

13. Conlon BP. Staphylococcus aureus chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in S. aureus disease. Bioessays. 2014;36(10):991-6. Epub 2014/08/08. doi: 10.1002/bies.201400080. PubMed PMID: 25100240.

14. Bojer MS, Lindemose S, Vestergaard M, Ingmer H. Quorum Sensing-Regulated Phenol-Soluble Modulins Limit Persister Cell Populations in Staphylococcus aureus. Front Microbiol. 2018;9:255. Epub 2018/03/09. doi: 10.3389/fmicb.2018.00255. PubMed PMID: 29515541; PubMed Central PMCID:

393 PMCPMC5826201.

394 15. Guo L, Xu R, Zhao Y, Liu D, Liu Z, Wang X, et al. Gas Plasma Pre-treatment Increases Antibiotic
395 Sensitivity and Persister Eradication in Methicillin-Resistant Staphylococcus aureus. Front Microbiol.
396 2018;9:537. Epub 2018/04/10. doi: 10.3389/fmicb.2018.00537. PubMed PMID: 29628915; PubMed
397 Central PMCID: PMCPMC5876240.

398 16. Zhang Y. Persisters, persistent infections and the Yin-Yang model. Emerg Microbes Infect.
399 2014;3(1):e3. Epub 2014/01/01. doi: 10.1038/emi.2014.3. PubMed PMID: 26038493; PubMed Central
400 PMCID: PMCPMC3913823.

401 17. Mina EG, Marques CN. Interaction of Staphylococcus aureus persister cells with the host when in
402 a persister state and following awakening. Sci Rep. 2016;6:31342. Epub 2016/08/11. doi:
403 10.1038/srep31342. PubMed PMID: 27506163; PubMed Central PMCID: PMCPMC4979210.

404 18. Afonso AF, Pereira OR, Valega M, Silva AMS, Cardoso SM. Metabolites and Biological Activities
405 of Thymus zygis, Thymus pulegioides, and Thymus fragrantissimus Grown under Organic Cultivation.
406 Molecules. 2018;23(7). Epub 2018/06/23. doi: 10.3390/molecules23071514. PubMed PMID:
407 29932435; PubMed Central PMCID: PMCPMC6099726.

408 19. Song X, Sun Y, Zhang Q, Yang X, Zheng F, He S, et al. Failure of Staphylococcus aureus to
409 Acquire Direct and Cross Tolerance after Habituation to Cinnamon Essential Oil. Microorganisms.
410 2019;7(1). Epub 2019/01/16. doi: 10.3390/microorganisms7010018. PubMed PMID: 30641977;
411 PubMed Central PMCID: PMCPMC6352263.

412 20. Xiao S, Cui P, Shi W, Zhang Y. 2019. doi: 10.1101/702951.

413 21. Mith H, Clinquart A, Zhiri A, Daube G, Delcenserie V. The impact of oregano (Origanum
414 heracleoticum) essential oil and carvacrol on virulence gene transcription by Escherichia coli O157:H7.

415 FEMS Microbiol Lett. 2015;362(1):1-7. Epub 2015/03/20. doi: 10.1093/femsle/fnu021. PubMed PMID:
416 25790499.
417 22. Teixeira B, Marques A, Ramos C, Serrano C, Matos O, Neng NR, et al. Chemical composition and
418 bioactivity of different oregano (Origanum vulgare) extracts and essential oil. J Sci Food Agric.
419 2013;93(11):2707-14. Epub 2013/04/05. doi: 10.1002/jsfa.6089. PubMed PMID: 23553824.
420 23. Feng J, Zhang S, Shi W, Zubcevik N, Miklossy J, Zhang Y. Selective Essential Oils from Spice or
421 Culinary Herbs Have High Activity against Stationary Phase and Biofilm Borrelia burgdorferi. Front
422 Med (Lausanne). 2017;4:169. Epub 2017/10/28. doi: 10.3389/fmed.2017.00169. PubMed PMID:
423 29075628; PubMed Central PMCID: PMCPMC5641543.
424 24. Han X, Parker TL. Antiinflammatory Activity of Cinnamon (Cinnamomum zeylanicum) Bark
425 Essential Oil in a Human Skin Disease Model. Phytother Res. 2017;31(7):1034-8. Epub 2017/04/27.
426 doi: 10.1002/ptr.5822. PubMed PMID: 28444928; PubMed Central PMCID: PMCPMC5518441.
427 25. Vong AT, Chong HW, Lim V. Preliminary Study of the Potential Extracts from Selected Plants to
428 Improve Surface Cleaning. Plants (Basel). 2018;7(1). Epub 2018/03/07. doi: 10.3390/plants7010017.
429 PubMed PMID: 29509658; PubMed Central PMCID: PMCPMC5874606.
430 26. Ballester-Costa C, Sendra E, Fernandez-Lopez J, Viuda-Martos M. Evaluation of the antibacterial
431 and antioxidant activities of chitosan edible films incorporated with organic essential oils obtained from
432 four Thymus species. J Food Sci Technol. 2016;53(8):3374-9. Epub 2016/10/28. doi:
433 10.1007/s13197-016-2312-y. PubMed PMID: 27784932; PubMed Central PMCID: PMCPMC5055899.
434 27. Yilmaz S, Ergun S. Dietary Supplementation with Allspice Pimenta dioica Reduces the
435 Occurrence of Streptococcal Disease during First Feeding of Mozambique Tilapia Fry. J Aquat Anim
436 Health. 2014;26(3):144-8. Epub 2014/09/18. doi: 10.1080/08997659.2014.893459. PubMed PMID:

437 25229484.

438 28. O'Neal ST, Johnson EJ, Rault LC, Anderson TD. Vapor delivery of plant essential oils alters
439 pyrethroid efficacy and detoxification enzyme activity in mosquitoes. Pestic Biochem Physiol.
440 2019;157:88-98. Epub 2019/06/04. doi: 10.1016/j.pestbp.2019.03.007. PubMed PMID: 31153481.

441 29. Murbach Teles Andrade BF, Conti BJ, Santiago KB, Fernandes Junior A, Sforcin JM.
442 Cymbopogon martinii essential oil and geraniol at noncytotoxic concentrations exerted
443 immunomodulatory/anti-inflammatory effects in human monocytes. J Pharm Pharmacol.
444 2014;66(10):1491-6. Epub 2014/06/18. doi: 10.1111/jphp.12278. PubMed PMID: 24934659.

445 30. de Groot AC, Schmidt E. Essential Oils, Part VI: Sandalwood Oil, Ylang-Ylang Oil, and Jasmine
446 Absolute. Dermatitis. 2017;28(1):14-21. Epub 2016/12/22. doi: 10.1097/DER.0000000000000241.
447 PubMed PMID: 28002230.

448 31. Sharma M, Levenson C, Clements I, Castella P, Gebauer K, Cox ME. East Indian Sandalwood Oil
449 (EISO) Alleviates Inflammatory and Proliferative Pathologies of Psoriasis. Front Pharmacol.
450 2017;8:125. Epub 2017/04/01. doi: 10.3389/fphar.2017.00125. PubMed PMID: 28360856; PubMed
451 Central PMCID: PMCPMC5352686.

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454 **Table 1. Effect of essential oils on stationary phase *Staphylococcus aureus***

EO[a]	Plant	Viability of bacteria after 3 or 5 days of exposure[b]					
		0.5% EO[a]		0.25% EO[a]		0.125% EO[a]	
		3 days	5 days	3 day	5 days	3 day	5 days
Cinnamon bark	*Cinnamomum zeylanicum*	-	-	-	-	-	-
Oregano	*Origanum vulgare*	-	-	-	-	-	-
Thyme white	*Thymus zygis*	-	-	-	-	-	-
Lemongrass	*Cymbopogon flexuosus*	-	-	-	-	-	-
Bandit "Thieves"	Synergy blend	-	-	-	-	+	+
Sandalwood oil	*Santalum Spicatum*	-	-	-	-	+	+
Health shield	Synergy blend	-	-	+	-	+	+
Allspice	*Pimenta officicalis*	-	-	+	-	-	-
Amyris	*Amycris balsamifera*	-	-	+	-	-	-
Palmarosa	*Cymbopogon martinii*	-	-	+	-	-	-
Cinnamon leaf	*Cinnamomum zeylanicum*	-	-	+	+	+	+
Clove bud	*Eugenia caryophyllata*	-	-	+	+	+	+
Citronella	*Cymbopogon winterianus*	-	-	+	+	+	+
Geranium bourbon	*Pelargonium graveolens*	-	-	+	+	+	+
Marjoram (Sweet)	*Origanum marjorana*	-	-	+	+	+	+
Peppermint	*Mentha piperita*	-	-	+	+	+	+
Lemongrass	*Cymbopogon citratus*	-	-	+	+	+	+
Cornmint	*Menta arvensis*	-	-	+	+	+	+
Elemi	*Canarium iuzonicum*	-	-	+	+	+	+
Ho wood	*Cinnamomum camphora*	-	-	+	+	+	+
Head ease	Synergy blend	-	-	+	+	+	+
Lemon eucalyptus	*Eucalyptus citriadora*	-	-	+	+	+	+
Litsea cubeba	*Litsae cubeba*	-	-	+	+	+	+
Myrrh	*Commiphora myrrha*	-	-	+	+	+	+
Parsley seed	*Petroselinum sativum*	-	-	+	+	+	+
Coriander oil	*Coriandrum sativum*	-	-	+	+	+	+
Dillweed	*Anethum graveolens*	-	-	+	+	+	+
Hyssop	*Hyssopus officinalis*	-	-	+	+	+	+
Neroli	*Citrus aurantium*	-	-	+	+	+	+
Rosewood oil	*Aniba rosaeodora*	-	-	+	+	+	+
Tea Tree	*Melaleuca alternifolia*	+	-	+	+	+	+
Cajeput	*Melaleuca cajeputi*	+	-	+	+	+	+
Glove Bud	*Syzygium aromaticum*	+	-	+	+	+	+
Lavender	*Lavendula officianalis*	+	-	+	+	+	+
Sleep tight	Synergy blend	+	-	+	+	+	+
Vetiver	*Vetiveria zizanoides*	+	-	+	+	+	+
Palo Santo	*Bursera graveolens*	+	-	+	+	+	+
Sage oil	*Salvia officinalis*	+	-	+	+	+	+
Yarrow	*Achillea millefolium*	+	-	+	+	+	+

455 [a] "EO" essential oil.

[b] "-" No obvious colonies grew on TSB plate after drug exposure; "+" Obvious colonies were found on TSB plate after drug exposure.

460 **Table 2. Activity of top 10 essential oils that are active against stationary phase *Staphylococcus aureus* in terms**
461 **of their activity against growing bacteria (MIC) and non-growing bacteria in drug exposure**

Drug /essential oil	Plant	MIC (μg/mL /%)	Viability of bacteria after 3 or 5 days of EO [b] exposure (0.25%) [c]	
			3 day	5 days
Gentamicin [a]	-	1	+	+
Sandalwood oil	*Santalum spicatum*	0.015	-	-
Amyris	*Amycris balsamifera*	0.015	+	-
Oregano	*Origanum vulgare*	0.015	+	-
Cinnamon bark	*Cinnamomum zeylanicum*	0.03	-	-
Allspice	*Pimenta officicalis*	0.06	+	-
Thyme white	*Thymus zygis*	0.125	-	-
Health shield	*Cassia, clove, eucalyptus, lemon and rosemary*	0.125	+	-
Bandit "Thieves"	*cloves, cinnamon, lemon, rosemary and eucalyptus*	0.125	-	-
Lemongrass	*Cymbopogon flexuosus*	0.125	-	-
Palmarosa	*Cymbopogon martinii*	0.125	+	-

462 [a] The concentration of gentamicin used in drug exposure was 50 μM; [b] "EO" essential oil; [c] "-" No obvious
463 colonies grew on TSB plate after drug exposure; "+" Obvious colonies were found on TSB plate after drug
464 exposure.
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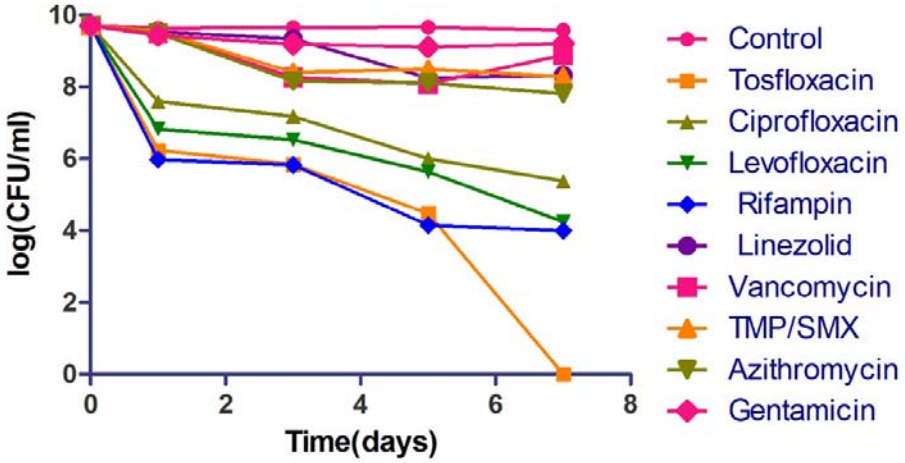

466

467 **Figure 1. Activity of tosufloxacin and commonly used antibiotics against stationary phase *S. aureus*.**
468 Tosufloxacin had good anti-persister activity against *S. aureus*. Antibiotics commonly used to treat infections
469 caused by *S. aureus* had poor activity against the stationary phase bacteria. The final concentration of antibiotics
470 including tosufloxacin, ciprofloxacin, levofloxacin, rifampin, linezolid, vancomycin, sulfamethoxazole,
471 trimethoprim, azithromycin and gentamicin, was all 20 μM. Sulfamethoxazole-trimethoprim is the combination
472 of trimethoprim and sulfamethoxazole in a ratio of 5:1.

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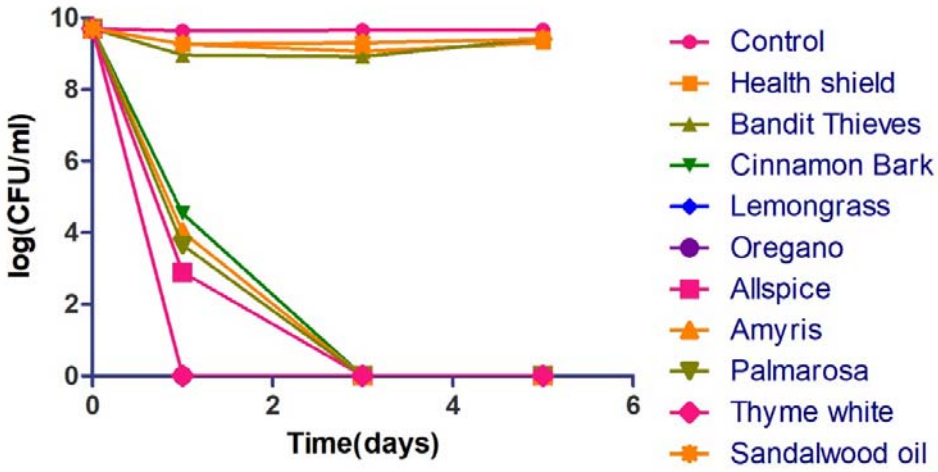

474

475 **Figure 2. Activity of active essential oil candidates (0.125%) against stationary phase *S. aureus*.** Oregano,
476 Lemongrass (*Cymbopogon flexuosus*) and Thyme white could eradicate all stationary phase cells after one-day oil
477 exposure. Cinnamon bark, Allspice, Amyris and Palmarosa could eradicate stationary phase *S. aureus* cells after
478 three-day exposure. Bandit "Thieves", Sandalwood oil and Health shield still could not wipe out the *S. aureus*
479 stationary phase culture even after five-day exposure.

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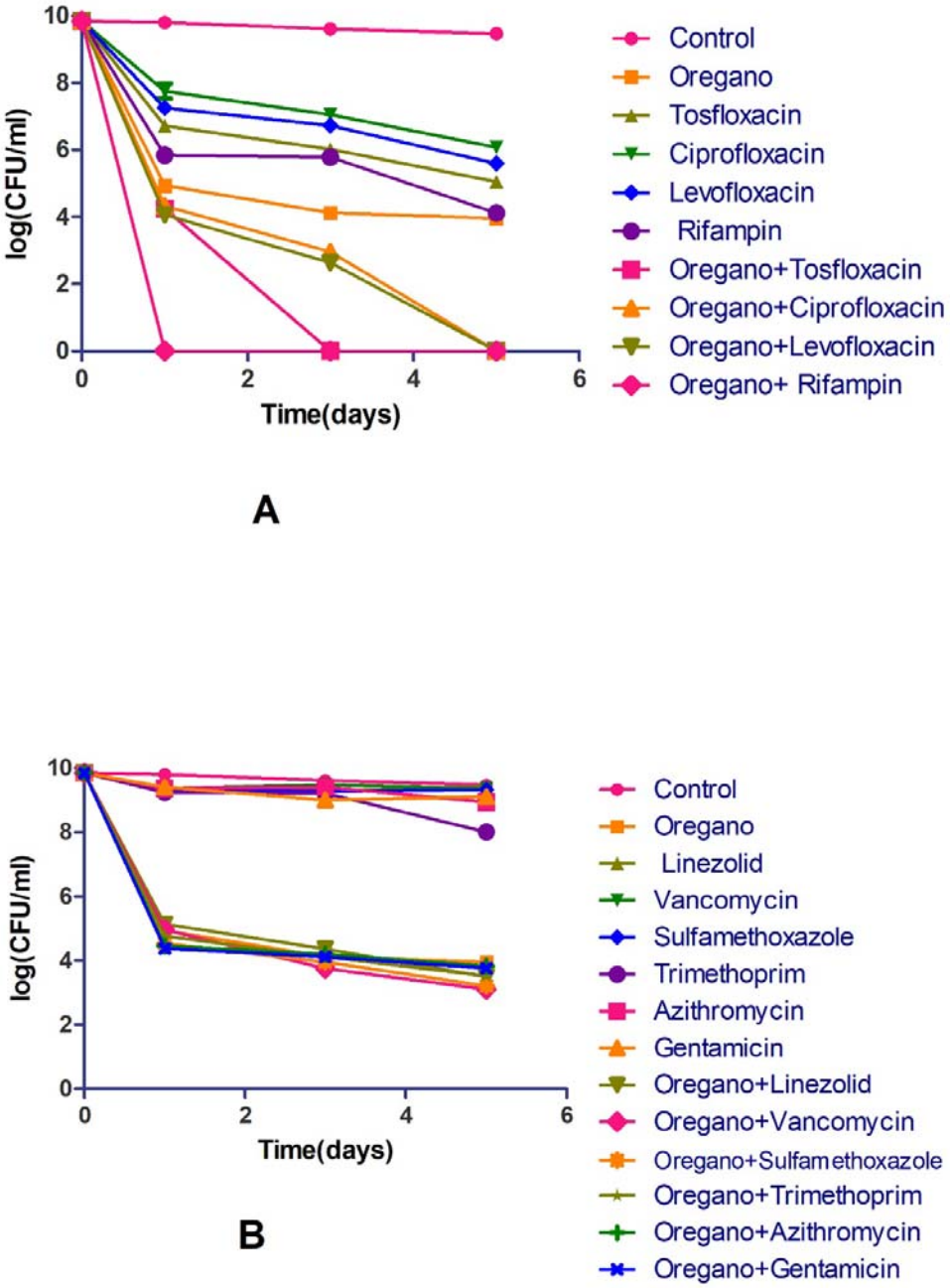

482

483 **Figure 3. Comparison of the activity of Oregano in combination with different antibiotics against stationary**
484 **phase *S. aureus*.** Effects of ciprofloxacin, levofloxacin, tosufloxacin, rifampin alone and their combinations with
485 Oregano are presented in (A). Effects of linezolid, vancomycin, sulfamethoxazole, trimethoprim, azithromycin,
486 gentamicin and their combinations with Oregano are presented in (B). The final concentration of antibiotics is 5
487 μg/mL and the concentration of Oregano is 0.025%.

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