Introduction

In 1928, the advent of penicillin dramatically impacted how bacterial infections were treated. Previous rates of high mortality associated with *Staphylococcus aureus* and other bacterial infections were greatly reduced following antibiotic discovery [1][2]. However, with antibiotic pharmaceutical discovery came new evolutionary selective pressures for bacterial pathogens which has over the decades, reduced the efficacy of these once novel treatments. [3] In modern medicine, there has been a rise in multidrug-resistant strains (MDR) of a number of bacterial pathogens, including *S. aureus.* By circumventing the mechanisms of action of many common antibiotics, resistant *S. aureus*, popularized as MRSA (methicillin-resistant *S. aureus*), has become synonymous with failed treatments often resulting in mortality and thus become a pressing public health concern for both the medical and greater scientific community. [6, 7] These concerns are not unfounded with epidemiological predictions suggesting that by the year 2050, without new antibiotic introduction and research into the mechanisms of MDR, mortalities from cancer will be surpassed by infections due to MDR pathogens. [6]

The mechanistic explanation behind this emerging health concern lies both with evolution and by chance. Adaptive resistance to antibiotics develops in part because of random mutations occurring with each bacterial division cycle, thereby imparting bacteria with improved mechanisms of defense. [8] Adaptive resistance can be further exacerbated during antibiotic misuse and subsequent non-fatal exposures that provide exogenous selection for antibiotic tolerability. [9] Bacterial resistance is developing at an alarmingly rapid pace, and as antibiotic mechanisms of action are exploited, the necessity of new antibiotic sources becomes apparent. [13] [14] Botanicals provide a potential source of novel antibiotics likely developed by plants as a self defense mechanism against environmental bacterial plant pathogens. [15] [16] Many hundreds of plants worldwide are used in traditional medicine as treatments for bacterial infections. [25] It is very likely that in the future, more botanical-based therapies and phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians. Worldwide research on finding new anti-infective agents, including plant sources, are being investigated. [25] In addition, the public is becoming increasingly aware of problems with the over-prescription and misuse of traditional antibiotics. Many people are interested in having more autonomy over their medical care. [25] A multitude of plant-based therapeutics are readily available over-the-counter from herbal suppliers and natural-food stores, and self-medication with these substances is commonplace. The use of plant extracts, as well as other alternative forms of medical treatments, is increasing in popularity since the late 1990s. [25] With increased interest and use of therapeutic antimicrobial botanicals, prudence is necessary regarding the evaluation of these botanical antimicrobials for susceptibility to the development of bacterial resistance. Since therapeutic botanical extracts may contain multiple antimicrobial constituents as well as potentially novel mechanisms of action, the question remains as to whether or not bacteria can develop resistant to these extracts. In this study, we investigated the potential of *S. aureus* to develop resistance to five specific botanical antimicrobials including *Arctostaphylos uva-ursi*, *Coptis chinensis*, *Eucalyptus globulus*, *Larrea tridentata*, and *Salvia officinalis.*

Materials and Methods

Bacterial strains and stock preparation. Antibiotics were obtained from Sigma-Aldrich (St. Louis, MO). The stock solution of tetracycline was prepared at 5 mg/mL in ethanol. A working solution was prepared by dilution in water to 50 µg/ml. Vancomycin and levofloxacin were prepared and used as working solutions at 50 µg/ml in water. Tryptic soy broth (TSB) and the bacterial culture Staphylococcus aureus ATCC 11632 were obtained from Hardy Diagnostics (Santa Monica, CA).

Botanical extractions. Plant material was obtained from reputable sources with documentation of authenticity (Starwest Botanicals, Sacramento, CA). All plant material was subsequently verified by qualified botanical specialists using herbal pharmacopoeia monographs and reference keys. A voucher specimen of all plant material was deposited in our repository. For extraction, the botanicals were ground to a fine powder, re-suspended in extraction solution, and incubated for 2 days at room temperature. The plant-to-liquid ratio of ethanol/distilled water/glycerol included dried *Salvia officinalis* 1:3 (58/35/07), dried *Eucalyptus globulus* 1:3 (53/42/05), dried *Arctostaphylos uva-ursi* 1:3 (42/48/10), dried *Coptis chinensis* 1:4 (58/37/05), and dried *Larrea tridentata* 1:3 (79/15/06). Following extraction, the liquid was pressed from the solid botanical material, filtered through unbleached paper filters, and sterilized by 0.22 µm filtration. Since the specific antibacterial constituents present in these extracts have not been identified, the extracts were normalized based on drying of the extracts and measurement of the remaining material. A sample of each extract was dried, and all extracts were found to contain similar concentrations of non-volatile solutes. *C. chinensis* 26.7 mg/ml, *S. officinalis* 25.9 mg/ml, *E. globulus* 28.6 mg/ml, *A. uva-ursi* 31.0 mg/ml and *L. tridentata* 35.3 mg/ml. These values are not meant to imply that the active constituents include only non-volatile solutes, but rather to provide a value of standardization, normalization, and comparison.

Selective pressure growth. The MIC (minimum inhibitory concentration) of each antibiotic and botanical extract on *S. aureus* cultures was determined by standard procedures. Briefly, 1 x10^6 colony forming units (cfu) of *S. aureus* was added to 1ml TSB in the presence of serially diluted antibiotic or botanical extract. The cultures were incubated for 24 hours with continuous aeration at 37^{o}C. The MIC was determined as

the dose of the antimicrobial where growth was no longer visible.

Subsequently, for selective pressure growth, *S. aureus* cultures (1 x10^6 cfu/ml) in TSB were treated with a 75% MIC dose of the indicated antibiotic or botanical extract. The cultures were incubated at 37^{o}C with continuous aeration. Every 24 hours, the bacterial culture was transferred to five different vials of fresh TSB media containing increasing amounts of the antimicrobial. The vial that demonstrated bacterial growth at the highest dose of antimicrobial was selected to continue the selection process. This process was repeated for a total of 15 days. After completion, the MIC dose was determined for the original (Day 0) culture, intermediate cultures on days 9 and 12, and the final selected (Day 15) culture.

Chemical mutagenesis. *S. aureus* cultures were treated with a chemical mutagen, 200 mM ethyl methanesulfonate for 60 min at 37^{o}C. The mutagenized bacterial stock was then treated with increasing concentrations of the indicated antibiotic or botanical extract to determine the MIC value.

Results

In this study, *S. aureus* was grown in the presence of individual traditional antibiotics or botanical extracts at sub lethal doses for a period of 15 days. The bacteria were exposed to eight different antimicrobial agents, three of them being pharmaceutical antibiotics and five of them being of botanical origin. The original minimum inhibitory concentration (MIC) at day 0 of these antimicrobials ranged from 0.06 to 2.0 μg/ml for the pharmaceutical antibiotics and 90-150 μg/ml for the botanical extracts (Table 1). For the botanicals used in this study, the specific antibacterial active constituents have not been conclusively identified. Since the botanical extracts contain thousands of various compounds, the concentration of the active antimicrobial constituents could not be determined. When *S. aureus* was grown under the selective pressure of a 75% MIC dose for each of the antimicrobials followed by re-assessment of the MIC, it was observed that the bacteria was able to develop resistance to all the antimicrobials tested, including the botanical extracts (Table 1 and Fig. 1). For the conventional antibiotics, this resulted in a 2 to 11-fold increase in the MIC dose with an average increase of 2.73-fold for the three antibiotics tested (Table 1 and Fig. 1). For the botanical extracts, this resulted in a similar 2.5 to 7.5-fold increase in MIC dose, with an average of 3.86-fold increase for the five botanical extracts tested (Table 1 and Fig. 1). This fold-change in the MIC values was statistically significant using a paired t-test ($p < 0.05$) for all samples tested (levofloxacin, $t(5) = -6.61$, $p = 0.0131$; tetracycline, $t(5) = -20.68$, $p = 0.0040$; *Arctostaphylos*, $t(5) = -9.64$, $p = 0.0236$; *Coptis*, $t(5) = -26.76$, $p = 0.0016$; *Eucalyptus*, $t(5) = -12.25$, $p = 0.0052$; *Larrea,* $t(5) = -11.73$, $p = 0.0033$; *Salvia*, $t(5) = -12.67$, $p = 0.0131$), except for vancomycin with a $t(5) = -2.71$, $p = 0.0669$.

Table 1

Treatment	Day 0 MIC (μg/ml)	Day 15 MIC (μg/ml)	Post-mutagen MIC (μg/ml)
Levofloxacin	1.0 (+/- 0.1)	2.0 (+/- 0.2)	ND
Tetracycline	0.06 (+/- 0.01)	0.26 (+/- 0.05)	ND
Vancomycin	2.0 (+/- 0.2)	4.1 (+/- 0.2)	4.0 (+/- 0.2)
Arcostaphylos uva-ursi	90 (+/- 6)	272 (+/- 12)	ND
Coptis chinensis	121 (+/- 10)	910 (+/- 36)	720 (+/- 20)
Eucalyptus globulus	118 (+/- 9)	228 (+/- 18)	180 (+/- 12)
Larrea tridentata	60 (+/- 5)	183 (+/- 14)	ND
Salvia officinalis	150 (+/- 16)	614 (+/- 28)	453 (+/- 22)

Table 1: Minimum inhibitory concentration (MIC) of S. aureus to conventional antibiotics and botanical extracts. S. aureus cultures were treated with increasing concentrations of the indicated antimicrobial for 24 hours. The cultures were incubated at 37^{o}C with continuous aeration (by rotation). The MIC (μg non-volatile constituents/ml) was determined as the dose of the botanical extract required to completely inhibit replication of the bacteria as measured by a lack of visual turbidity. Values shown are the mean (+/- SEM). Day 15 MIC and Post-mutagen MIC were determined from S. aureus cultures which were grown under selective pressure or in the presence of a mutagenic agent as described in the Methods section. ND values were not determined.

Figure 1

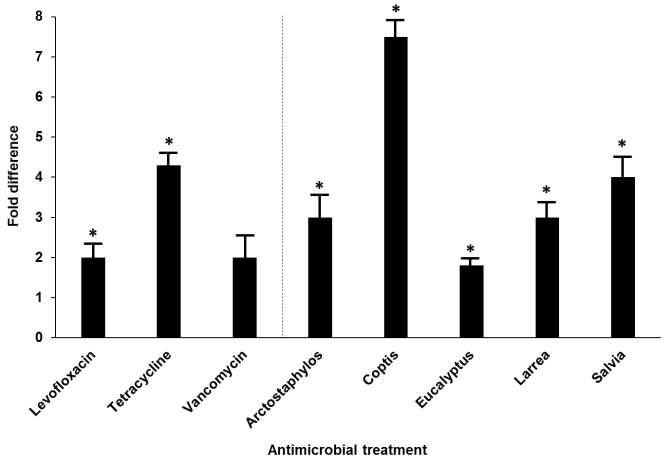

Figure 1. Change in minimum inhibitory concentration (MIC) of anti-S. aureus to conventional antibiotics and botanical extracts following selective pressure growth. S. aureus cultures were grown in the presence of a 75% MIC concentration of each of the antimicrobials indicated for a period of 15 days. Every 24 hours, the cultures were treated with appropriate increases in the antimicrobial to maintain a 75% MIC dose. After 15 days, the MIC of the cultures were determined and compared to the original bacterial culture. The graph represents the fold change in MIC value of the day 15 culture compared to the original culture. Values shown with error bars (mean +/- SEM) represent the values from three separate experiments. * denotes significant differences ($p < 0.05$) in the change in MIC values for the Day 0 to the Day 15 cultures using a

paired t-test. For individual samples: Levofloxacin: Range 1.7-2.3, 95% CI 2.0+/-0.34; Tetracycline: Range 4.0-4.6, 95% CI 4.3+/-0.34; Vancomycin: Range 1.5-2.5, 95% CI 2.0+/-0.56; Arctostaphylos: Range 2.5-3.5, 95% CI 3.0+/- 0.56; Coptis: Range 7.0-8.0, 95% CI 7.5+/-0.07; Eucalyptus: Range 1.6-2.0, 95% CI 1.8+/-0.0.23; Larrea: Range 2.7-3.3, 95% CI 3.0+/-0.0.34; Salvia: Range 3.6-4.4, 95% CI 4.0+/-0.45.

In order to assess the rate of the development of resistance, the MIC values of the cultures grown under selective pressure were assessed on days 9, 12 and 15. As shown in Figure 2, the pharmaceutical antibiotics developed statistically significant resistance (paired t-test, $p < 0.05$) by day 9 for tetracycline ($t(3) = -4.24$, $p = 0.0340$) and to levofloxacin ($t(3) = -7.07$, $p = 0.0131$) and vancomycin ($t(3) = -10.60$, $p = 0.0015$) by Day 12 (Fig. 2). When compared to the botanicals, statistically significant resistance to *Coptis* ($t(3) = -14.78$, $p = 0.0010$) was developed by day 9 and by day 12 for *Arctostaphylos* ($t(3) = -7.80$, $p = 0.0110$), *Larrea* ($t(3) = -12.98$, $p = 0.0008$), *Salvia* ($t(3) = -15.99$, $p = 0.0002$), and *Eucalyptus* ($t(3) = -8.49$, $p = 0.0033$). These results support similar rates of resistance development for *S. aureus* against both pharmaceutical antibiotics and antimicrobial botanical extracts.

Figure 2

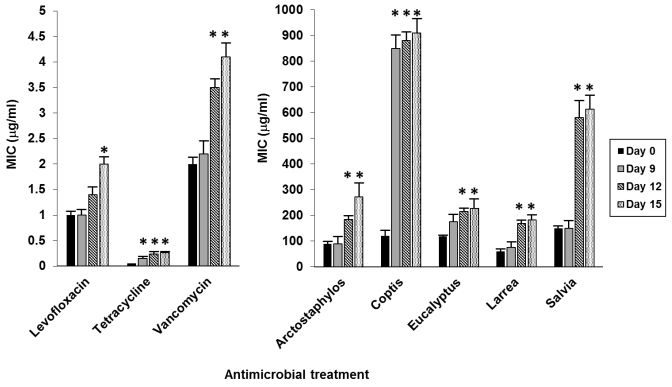

Figure 2. Rate of change in minimum inhibitory concentration (MIC) of anti-S. aureus to conventional antibiotics and botanical extracts following selective pressure growth. S. aureus cultures were grown in the presence of a 75% MIC concentration of each of the antimicrobials indicated for a period of 15 days. Every 24 hours, the cultures were treated with appropriate increases in the antimicrobial to maintain a 75% MIC dose. After 0, 9, 12 and 15 days, the MIC of the cultures were determined. The graph represents the MIC value for each antibiotic and botanical extract (n=2 for each sample). * denotes significant differences ($p < 0.05$) in the change in MIC values when comparing the MIC value for the Day 0 to that of Day 9, 12 or 15 using a paired t-test.

To assess the development of resistance through direct mutagenesis rather than selective pressure, *S. aureus* was exposed to the mutagenic compound, ethyl methanesulfonate, followed by immediate measurement of MIC values. Similarly, to when *S. aureus* was grown for several days under selective pressure, the bacteria was also able to develop resistance to all the antimicrobials, including the botanical extracts, following mutagenesis (Table 1 and Fig. 3). For the antibiotic vancomycin, this resulted in a 2-fold increase in the MIC dose which was the same as that observed in Figure 1 following selective pressure growth. For the botanical extracts tested, this resulted in a similar 1.5 to 6-fold increase in MIC dose. This fold-change in the MIC values was statistically significant (paired t-test, $p < 0.05$) for all samples tested (vancomycin, $t(5) = -9.13$, $p = 0.0010$; *Coptis*, $t(5) = -14.08$, $p = 0.0048$; *Eucalyptus*, $t(5) = -6.12$, $p = 0.0072$; *Salvia*, $t(5) = -10.95$, $p = 0.0171$). The individual MIC values for each botanical extract increased to similar levels as that observed in Figure 1. These results support that *S. aureus* was able to develop rapid resistance to the anti-bacterial botanical extracts when exposed to a mutagenic compound.

Figure 3

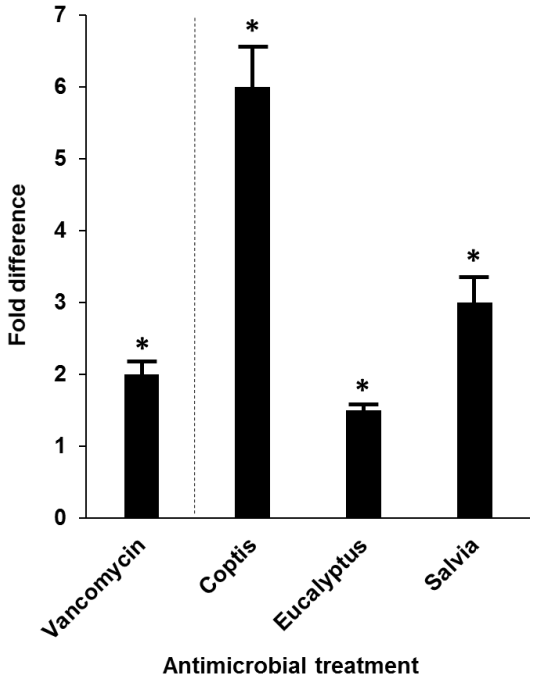

Figure 3. Change in minimum inhibitory concentration (MIC) of anti-S. aureus to conventional antibiotics and botanical extracts following direct mutagenesis. S. aureus cultures were treated with ethyl methanesulfonate for 60 min at 37°C. The mutagenized bacterial stock was then treated with increasing concentrations of the indicated antibiotic or botanical extract to determine the MIC. The graph represents the fold change in MIC value of the mutagenized culture compared to the original culture. Values shown with error bars (mean +/- SEM) represent the values from three separate experiments. * denotes significant differences ($p < 0.05$) in the change in MIC values for untreated to the mutagenized cultures using a paired t-test. For individual samples: Vancomycin: Range 1.9-2.1, 95% CI 2.0+/-0.11; Coptis: Range 5.4-6.6, 95% CI 6.0+/-0.68; Eucalyptus: Range 1.45-1.55, 95% CI 1.50+/-0.01; Salvia: Range 2.8-3.2, 95% CI 3.0+/-0.31.

Discussion

The results presented demonstrate that *S. aureus* was able to form resistance to antimicrobial botanical extracts including *Arctostaphylos uva-ursi, Coptis chinensis, Eucalyptus globulus, Larrea tridentata* and *Salvia officinalis*. These botanicals have previously been shown to have anti-*S. aureus* antimicrobial activity and have a strong history of traditional use against bacterial infections [20] [21] [22] [23]. Medically, bacteria continue to develop various mechanisms of resistance to circumvent the action of pharmaceutical drugs [18]. Botanicals may offer the medical and scientific community potential sources of novel antibiotics, since plants have a long history as sources of medicine [19]. Such botanicals may be a source of novel antibiotics which could be used for the treatment of multi-drug resistant bacterial infections. For example, it has previously been shown that corilagen, a compound within *Arctostaphylos*, is able to decrease the MIC of beta-lactam antibiotics for methicillin-resistant *Staphylococcus aureus*. [24] When therapeutic botanical extracts are prepared, they contain potentially thousands of active constituents. How many of these constituents will have antibacterial activity is unknown and will likely vary between botanical species. Based on this, it is often assumed that bacteria will have difficulty developing resistance to unpurified therapeutic botanical extracts since multiple, and potentially synergistic, active compounds may be present.

In general, botanical antimicrobial mechanisms of action and active constituents involved have not been widely studied. In this study, *S. aureus* was demonstrated to be capable of developing resistance to five commonly used botanical extracts at a similar rate and level to conventional antibiotics. These results may suggest that these botanical extracts do *not* contain multiple antibacterial constituents since resistance would likely occur at a slower rate if multiple mechanisms of antibacterial activity were present in the extract. This concept is further corroborated by the mutagenic experiment were similar levels of resistance were obtained between the botanical extracts and the pharmaceutical antibiotics.

In the future, understanding the mechanism of action of botanical constituents will make treatments more specific to the infection and aid practitioners in selecting the best botanicals and formulations for optimal therapeutic efficacy. As part of future studies, we have been able to demonstrate that for several of the bacteria that developed botanical resistance, cross-resistance to standard pharmaceutical antibiotics concomitantly occurred (data not shown). These results may support the development of multi-drug resistance emphasizing the need for proper use of antimicrobial botanicals. With the substantiated antibacterial therapeutic value of botanicals, practitioners should be aware of and practice proper use of botanicals in order to avoid the development of bacterial resistance. Due to their effectiveness, increased use, potential side effects and contraindications, botanical antimicrobials should be used under the guidance and supervision of a trained professional.

Current literature is very limited on the detailed use of antimicrobial botanicals where there is no clear standard on duration, dose, or course of treatment when using botanicals. As our results have shown, bacteria can develop resistance to the antibacterial activity of botanical extracts and therefore proper use is paramount in order to maintain the long-term use of these therapies for generations to come.

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Conflict of Interest Disclosure: The authors declare no conflict of interest.

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