Krameria erecta extract increases density of *Staphylococcus epidermidis* biofilm

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1. Abstract

BIOFILMS are communities of microorganisms that exhibit properties distinct from planktonic cultures. They commonly infect medical devices and are very difficult to remove. *Krameria*, also known as Rhatany, plant species has been used extensively for medicinal purposes in the past and was recently reported to inhibit growth of planktonic *Staphylococcus aureus*. *Oenothera biennis*, or evening primrose, has been reported to inhibit growth of various bacteria, including *Staphylococcus epidermidis*. In our experiments, extracts of *Krameria erecta* (little-leaf rathany) and *Oenothera biennis* showed a tendency to increase the density of biofilm formed by *Staphylococcus epidermidis*, a common nosocomial pathogen.

2. Introduction

Staphylococcus epidermidis biofilms, structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adherent to a surface [5], are known to cause a variety of persistent infections. They include those found on mechanical heart valves, sutures, exit sites, orthopedic devices and other surfaces [5]. Once a bacterial biofilm is formed, the bacteria become more resistant to therapy, thus often causing chronic disease [5]. Moreover, biofilms are ubiquitous in the environment, which eases the spread of pathogenicity islands [6].

Krameria preparations have been used in Europe and North and South America for a wide variety of medicinal purposes, including for ulcers, as tooth powder and as to cure diarrhea [11]. Extracts of *Krameria trianda* [9] and *Krameria erecta* [12] have been reported to ihibit growth of *S. aureus* and *S. epidermidis*, respectfully. *Oenothera biennis* has been known for a long time and was reported to reduce growth of planktonic *Staphylococcus epidermidis* [10], *S. mutans* [10], and *Eschirichia coli* [7].

To evaluate effects of *Krameria erecta* and *Oenothera biennis* on *S. epidermidis* biofilm formation, we used two methods of biofilm growth and quantification, the glass coupon method and the microtiter plate method.

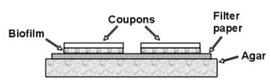
3. Materials and methods

Extracts Plants were collected at Spring Mountain State Park near Bonnie Springs, NV and CH_3OH/H_2O *K. erecta* and CH_3Cl_2 *O. bienis* extracts were obtained as described by [12]. The extracts were left in open containers for several days in order to increase their concentration. Then, they were diluted with corresponding solvents to the desired concentrations.

Bacterial strains and media A pure culture of *S. epidermidis* (ATCC# 49134) was used. For each experiment the organism was cultured overnight at 37C in 5ml of Triptic Soy Broth (TSB). Bioflims were grown in 1/10 TSB and 96-well PVC Microtiter or Microplate™with 12-Well Strips (Bio-RAD# 166-2405EDU) plates.

The static glass coupon reactor and the drop plate method Detailed glass coupon reactor procedure has been outlined elsewhere [4, 1, 2, 3]. Planktonic cultures of *S. aureus* were grown overnight in 5 ml of TSB. During inoculation, the appropriate concentrations of water and methanol extract of *K. erecta* or *O. bienis* or methanol control added. Next, the cultures were standardized to a 0.5 McFarland standard. The glass coupon reactors were set up on TSA plates as shown on Fig. 1. After 24 hours, plates were remoistened with 1 ml of 1/10 strength TSB and collected 24 hours later. Glass coupons were then scraped for 30 to 60 seconds, and rinsed with 1 ml of phosphate buffered saline (PBS) to remove the biofilm from glass slides. Next, PBS containing the biofilms was sonicated for 5 minutes and vortexed to homogenize the mixtures. Dilutions were made in PBS, and a small amount of the diluted culture was dropped on R2A plates and allowed to grow overnight. The number of colony-forming units was then counted.

(a) A side view of the static glass coupon reactor



(b) The resulting R2A plates

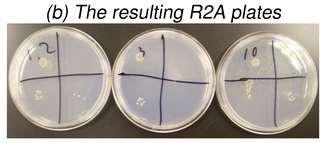


Fig. 1: The major parts of the experimental apparatus

The microtiter plate method and colorimetric measurement of biofilm density To grow the microorganisms using the microtiter plate method [8], 1 ml of an overnight culture was diluted with 9 ml of 1/10 TSB to a 0.5 McFarland standard. The microtiter plate was sterilized by immersing in ethyl alcohol, rinsing with sterile distilled water, and air drying for 15 minutes at room temperature within a laminar flow cabinet. Each well of the microtiter plate was then inoculated with 100 µl of the culture, coinciding with or followed by the treatment. The plate was either sealed within a polyethylene bag or using a stackable microtiter plate as an improvised lid and subsequently incubated at 37C for 48 or 96 hours.

The resulting biofilms were quantified by staining. The microtiter plate was washed with distilled water or saline six times to remove planktonic bacteria. For colorimetric quantification, the wells were then dried at room temperature and stained with 125 µl of 1% crystal violet. The stained biofilms were then rinsed with distilled water, dried, and extracted with 200 l of 95% ethanol. The OD_{550} [1] was estimated using a Thermo Scientific GENESYS 20 Visible Spectrophotometer after bringing the volume to 1 ml with 800 l of distilled water.

Statistics All data were analyzed using statistical package R. Counts of colony forming units (CFU) from the drop plate method and absorbance for the colorimetric assay were transformed to the power of $\frac{1}{4}$ to reduce variability. Next, linear regression was performed for a model that took into account effects of extract concentration and differences in means between the experimental sessions.

4. Results

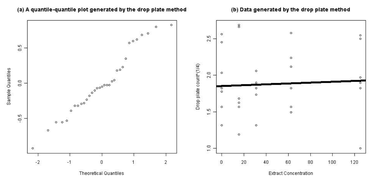
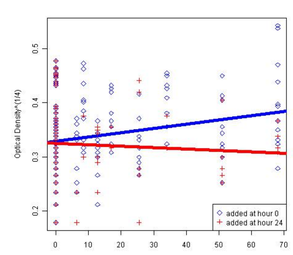


Fig. 2: The drop plate method produced inconclusive results with non-normal distribution (a) and much variability (b)

(a) Effect of CH_3Cl_2 O. bienis extract



(b) Effect of CH_3OH/H_2O K. erecta extract

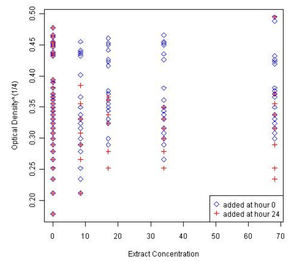


Fig. 3: Results of the microtiter plate experiments. Both extracts of *O. bienis* (a) and *K. erecta* (b) significantly increased biofilm density when added simultaneously with the bacterial culture. For *O. bienis*, r=.5084, β_1i.001 when the extract was added simultaneously with the culture (hour 0) and r=.3671, β_1=.95847 when the extract was added 24 hour following incubation (hour 24). For *K. erecta*, r=.4946 and β_1 <.001 (hour 0), r=0.3525 and β_1=.71839 (hour 24).

5. Discussion

The data collected from the drop plate experiment had smaller number of observations (n=30) than those from the microtiter well method. Moreover, the distribution was not normal, as is evident from the quantile-quantile(Q-Q) plot (Fig. 1a), and had significant (p<.05) difference in means between days on which experiments were carried out (data not shown). A possible explanation for these observations may be that the process of scraping the slides (see Methods) is inherently imprecise. Therefore, drop plate data were considered inconclusive in this study.

On the other hand, the microtiter plate method yielded useful results. The size of the dataset generated was considerably larger (n=167 for *O. bienis* and n=169 for *K. erecta*). Q-Q plots (not shown) indicated reasonable normality for both *K. erecta* and *O. bienis* data distributions. Although linear regression is fairly weak (r=.50 for *O. bienis* and r=.55 for *K. erecta*), the probability that observed effects occured simply by chance is less than .01 (p<.01 between for both datasets).

The striking disparity between the previously described inhibitory effect in disk diffusion testing and the present result underscores the necessity of including biofilms in any antimicrobial screening protocol. This is especially important because most microorganisms of clinical significance exhibit the biofilm phenotype in nature, and any putative antibotic should be evaluated for effectiveness against the phenotype most likely to be encountered. Additional follow-up experiments of increased rigor may help elucidate the mechanism responsible for this unexpected result.

6. Acknowledgements

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