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Alkaloid-based chemical defenses in the Brazilian poison frog Melanophryniscus simplex inhibit mycobacterial growth

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Introduction

Throughout evolutionary history, the process of natural selection has resulted in a remarkable diversity of defensive adaptations that help organisms deter or avoid predation. One such adaptation is chemical defenses. Organisms that use chemical defenses possess unpalatable or toxic compounds that discourage attack from potential predators. While most species synthesize their own chemical defenses, some sequester compounds from their diet (Saporito et al. 2012; Savitzky et al. 2012). For instance, certain organisms acquire toxins from their food and store them within their tissues, typically in specialized structures such as glands or spines (Saporito et al. 2012; Savitzky et al. 2012). A variety of organisms have the ability to sequester chemical defenses, including arthropods, snakes, and birds, but this mechanism has been best studied in amphibians, specifically poison frogs (Daly et al. 1994; Saporito et al. 2012; Savitzky et al. 2012).

Poison frogs in the families Dendrobatidae, Bufonidae, Mantellidae, Myobatrachidae, and Eleutherodactylidae sequester lipophilic alkaloids from a diet of arthropods such as mites, ants, beetles, and millipedes (for review, see Saporito et al. 2009). Many types of alkaloids have been identified in poison frog species, which include over 850 unique alkaloid compounds categorized into more than 20 structural classes. In general, arthropods possess different types and classes of alkaloids, so the specific set of alkaloids sequestered by an individual frog varies based on the arthropods in each frog's diet. As such, there is significant variation in the types and quantities of alkaloids between individual frogs based on their sex, age, and other characteristics (Saporito et al. 2009, 2012). Furthermore, alkaloid composition varies among populations of frogs, and several studies have shown that frog populations from geographically close locations have similar alkaloid profiles while more distant populations have greater differences in alkaloid composition (Daly et. al 1994; Saporito et al. 2007; Saporito et al. 2012; Stuckert et al. 2014; Mina et al. 2015).

In addition to serving as a chemical defense from predators, recent research has shown that alkaloids may defend poison frogs against infection from microorganisms (MacFoy et al. 2005; Mina et al. 2015). Frogs have moist skin and often live in environments that are ideal for the growth of potentially pathogenic microbes, such as decaying leaf-litter, rotting logs, or slow moving-bodies of water (Pough et al. 2003). These conditions would presumably make frogs highly susceptible to infection; however, frogs appear to have some defenses that are effective

against microbes (Daly et al. 1987; Clarke 1996). Frog skin secretions contain a wide variety of biologically active chemicals, including proteins, peptides, steroids, and alkaloids (Daly et al. 1987; Daly et al. 2005; Macfoy et al. 2005). Some of these – especially peptides, which are manufactured *de novo* by various frog species – have been found to possess significant antimicrobial properties (Bevins and Zasloff 1990; Clarke 1996; Rinaldi 2002).

More recently, researchers have determined that individual alkaloids such as pumiliotoxins and indolizidines found in dendrobatids and other poison frogs are effective antimicrobial agents against the bacteria *Bacillus subtilis* and *Escherischia coli* as well as the fungus *Candida albicans* (Macfoy et al. 2005). During the last few years, researchers have begun to examine the antimicrobial effects of alkaloid cocktails, using the entire complement of alkaloids extracted from a frog, rather purified individual alkaloids. Alkaloid cocktails from geographically isolated populations of the dendrobatid frog *Oophaga pumilio* and the bufonid toad *Melanophryniscus simplex* were found to inhibit the growth of *B. subtilius, E. coli,* and *C. albicans* to varying degrees (Gronemeyer 2013; Mina et al. 2015). Gronemeyer (2013) reported that the alkaloid cocktails in *M. simplex*, which had greater alkaloid diversity (i.e., more types of alkaloids) than that of *O. pumilio* were more effective at inhibiting microbial growth. This may indicate that alkaloids have a synergistic effect; that is, greater alkaloid diversity results in greater microbial growth inhibition (Gronemeyer 2013). Mina et al. (2015) tested alkaloids from five populations of *O. pumilio* against the same microbes and found that each population inhibited growth of *E. coli* and *B. subtilis*, while only one population inhibited the growth of *C. albicans*. This suggests that there are differences in inhibition among frog populations and between microbe species.

Melanophryniscus simplex, commonly known as the red-belly toad, is a Brazilian poison frog. Recent research has shown that the red-belly toad possesses alkaloids not only in its skin, but also in muscle, liver, and mature oocytes (Grant et al. 2012). This discovery supports the hypothesis that alkaloids play a role in "help[ing] prevent or suppress infection by non-skin penetrating parasites" (Grant et al. 2012). Sixteen different classes of alkaloids have been detected in *M. simplex* skins, and each individual frog has an average of 38 unique alkaloid compounds (Daly et al. 2007; Daly et al. 2008; Grant et al. 2012). This represents a relatively diverse alkaloid cocktail, which may result in greater antimicrobial effects. The goals of the

present study were to test the effectiveness of alkaloid cocktails from six geographically isolated populations of *M. simplex* against two bacteria in the genus *Mycobacterium*.

The genus *Mycobacterium* is comprised of over 150 species of Gram-positive bacteria, most of which are harmless and commonly found in the environment (Balazova et al. 2014). However, some species of *Mycobacterium* are pathogenic in humans and other animals. For example, *M. tuberculosis* and *M. leprae* cause tuberculosis and leprosy (respectively) in humans. Additionally, *M. bovis* and *M. avium* cause disease in cattle and other mammals (Rue-Albrecht et al. 2014). Several species have also been known to cause infection in frogs, including *M. marinum, M. ulcerans*, and *M. lifelandii* (Haridy et al. 2014; Tobias et al. 2013; Suykerbuyk et al. 2007). Several previous studies support the idea that some alkaloids from other sources – plants, sponges, and tunicates – are effective antimycobacterial agents (Houghton et al. 1998; Arai et al. 2014; Sureram et al. 2012; Appleton et al. 2010); however, it is not known if alkaloids from frog skins could provide protection against mycobacteria.

This study investigated the antimycobacterial properties of *M. simplex* alkaloids from geographically isolated populations against two species of mycobacteria, *M. smegmatis*, a soil-dwelling species, and *M. phlei,* a saprophytic species (Berney et al. 2014; Egamberdieva 2011). Both of these species are fast-growing, commonly found in the environment, and typically nonpathogenic in healthy animals (Balazova et al. 2014, Berney et al. 2014, Egamberdieva 2011). Both *M. smegmatis* and *M. phlei* are commonly used as model strains for the study of antimycobacterial agents (Zhang et al. 2014, Bruce-Micah et al. 2009). Optical density and colony-forming unit assays were used to evaluate the antimycobacterial effect of these alkaloids. Optical density was used as a measure of growth inhibition, i.e. the alkaloids' ability to prevent the bacteria from growing. Colony-forming unit assays were used to measure the decrease in viability of the bacteria, i.e. the alkaloids' ability to kill the bacteria. Ultimately, this study will provide useful information regarding the role alkaloids play in the microbial defense of poison frogs, especially against mycobacterial pathogens in *M. simplex*.

Methods

Frog samples. In this study, alkaloids were extracted from *M. simplex* skins preserved in methanol. Alkaloid cocktails were isolated from 18 individual frogs from 6 geographically isolated Brazilian populations (Figure 1). The frogs used in the present study were a part of a

larger study aimed at understanding the link between alkaloid variation and arthropod diet among populations of *M. simplex* (see Grant et al. 2012). Three frogs were randomly selected from each of the six populations, and represent a sub-sample of the original collection of *M. simplex*.

Alkaloid fractionation. The method of alkaloid fractionation was described in Saporito et al. 2009 and Grant et al. 2012. Each frog skin was preserved in 4 mL of methanol in a glass vial with a Teflon-lined ® cap (methanol-skin solution). First, 1 mL of the methanol-skin solution was added to a conical glass vial with 50 µL of HCl. After mixing, each sample was concentrated with nitrogen gas to a volume of 100 µL. Next, 200 µL of water was added to the sample. The sample was then extracted four times with 300 µL portions of hexane, and the hexane layer was removed. The resulting solution was then basified with saturated $NaHCO_3$ and tested with pH paper to confirm basicity. Then, three extractions with 300 µL portions of ethyl acetate were carried out. The ethyl acetate alkaloid layer was removed and dried with anhydrous Na_2SO_4. The sample was then concentrated to dryness with nitrogen gas. The remaining pure alkaloids were suspended 100 µL of methanol and stored at -15°C until use in optical density assays.

Pilot studies. To determine the optimal growth period for accurate evaluation of inhibition and lethality, pilot studies were completed for *M. smegmatis* and *M. phlei*. Based on previous research using mycobacteria (Houghton et al. 1998; Arai et al. 2014; Sureram et al. 2012; Appleton et al. 2010) as well as optical density measurements of the growth of untreated *M. smegmatis* and *M. phlei*, a liquid culture of each bacterium was grown at 37°C with agitation in a 7H9 nutrient broth. The culture was filtered through a 0.5 µm filter. Optical density measurements of 200 µL samples of the broth were taken every 30 minutes at 620 nm with five seconds of agitation before each measurement. Growth curves were plotted for each bacterium over various periods of time, and it was determined that a 48 hour optical density measurement period would best capture the lag, exponential, stationary, and decline phases of growth for both bacteria.

Optical density assay. For both *Mycobacterium smegmatis* and *Mycobacterium phlei*, a liquid culture was grown for 48 hours in a 7H9 nutrient broth at 37°C with agitation. Two hundred microliters of filtered culture were mixed with either 4.8 µL or 9.6 µL of fractionated alkaloid samples. These volumes were based upon the findings of Mina et al. (2015), in which 4.8 µL was shown to be effective against other bacteria as a minimal inhibitory dose. To determine whether a dose-response relationship existed in comparison to the 4.8 µL treatments, replicates were treated with 9.6 µL of alkaloid as well. Three replicates were completed for each individual at each volume. Additionally, three replicates were treated with 4.8 µL and 9.6 µL of methanol alone to determine whether the methanol in which the alkaloids were suspended had an effect on the growth of the bacteria. Using a 96-well plate spectrophotometer, optical density measurements were taken at 620 nm every 30 minutes over 48 hours at 37°C. The plate was agitated for five seconds prior to each measurement. Based on the normal growth curve of each bacteria, inhibition in optical density assays was evaluated at the endpoint of the measurements.

Colony-forming unit (CFU) assay. Once the optical density measurements were complete, dilutions of broth cultures from each treated well were spotted in 10 µL aliquots onto 7H11 agar plates. The first column was a pure sample from the treated well. From the pure sample, tenfold dilutions were performed in 7H9 nutrient broth and spotted onto the plates (Figure 2). These cultures were grown at 37°C for 48 hours. Following the growth period, the colonies were counted and the number of colony-forming units per milliliter of broth were determined using the following formula:

$$\frac{CFUs}{mL} = \frac{number\ of\ colonies * aliquot\ volume}{dilution\ factor\ of\ broth}$$

Statistical analyses. Mean optical densities and mean colony-forming units were compared among populations and methanol controls with a one-way Analysis of Variance (ANOVA). Pair-wise comparisons were analyzed using Tukey post-hoc tests. All statistical analyses were completed using GraphPad Prism 6.

Results

Mycobacterium smegmatis. Optical density assays of *M. smegmatis* treated with 4.8 μL of alkaloid (Figure 3A) demonstrated that alkaloids from Site 1 (p-value ≤ 0.001), Site 3 (p-value = 0.002), and Site 6 (p-value = 0.050) inhibited the growth of the bacteria significantly more than the methanol control. There were no significant differences in inhibition between the alkaloids from sites 1, 3, and 6 (p-value > 0.05 in all comparisons). Colony-forming unit assays of *M. smegmatis* treated with 4.8 μL of alkaloid (Figure 3B) showed that alkaloids from Site 1 (p-value ≤ 0.001), Site 2 (p-value ≤ 0.001), and Site 3 (p-value ≤ 0.001) were significantly more lethal to the bacteria than the methanol control. There were no significant differences in lethality between populations 1, 3, and 6 (p-value > 0.05 in all comparisons).

According to optical density assays of *M. smegmatis* treated with 9.6 μL of alkaloid (Figure 3C), alkaloids from Site 2 (p-value = 0.014), Site 3 (p-value = 0.002), Site 4 (p-value ≤ 0.001), Site 5 (p-value ≤ 0.001), and Site 6 (p-value ≤ 0.001) inhibited bacterial growth significantly more than the methanol control. Between these populations, there were significant differences in inhibition between Sites 2 and 6 (p-value = 0.037), Sites 4 and 6 (p-value = 0.003), and Sites 5 and 6 (p-value = 0.004). Colony-forming unit assays of *M. smegmatis* treated with 9.6 μL of alkaloid indicated that alkaloids from Site 1 (p-value ≤ 0.001), Site 2 (p-value = 0.011), Site 3 (p-value ≤ 0.001), and Site 4 (p-value = 0.044) were significantly more lethal to the bacteria than the methanol control. Among these populations, there were significant differences in inhibition between Sites 1 and 2 (p-value = 0.021), Sites 1 and 4 (p-value ≤ 0.001), Sites 2 and 3 (p-value = 0.010), and Sites 3 and 4 (p-value ≤ 0.001).

Mycobacterium phlei. Optical density assays of *M. phlei* treated with 4.8 μL of alkaloid (Figure 4A) showed that alkaloids from Site 1 and Site 3 inhibited the growth of the bacteria significantly more than the methanol control (p ≤ 0.001 in both cases). There was no statistically significant difference between the inhibition caused by alkaloids from Site 1 and Site 3 (p-value = 0.910). The samples treated with alkaloids from Site 5 grew significantly more than those treated with methanol (p-value ≤ 0.001). However, colony-forming unit assays of *M. phlei* treated with 4.8 μL of alkaloid (Figure 4B) indicated no significant differences in lethality between any population and the methanol control (p-value > 0.05 in all comparisons).

For optical density assays of *M. phlei* treated with 9.6 μL of alkaloid (Figure 4C), samples treated alkaloids from Site 4 grew significantly more than the methanol control (p-value = 0.019). Colony-forming unit assays of *M. phlei* treated with 9.6 μL of alkaloid (Figure 4D) indicated that alkaloids from Site 1 (p-value = 0.025) and Site 3 (p-value = 0.031) were significantly more lethal to the bacteria than the methanol control. The mean colony-forming units per milliliter for Site 6 was much lower than that of methanol; however, this difference was not statistically significant (p-value = 0.093), likely because Site 6 had a relatively smaller sample size than the rest of the populations due to some of the assays being unreadable or too numerous to count. There were no significant differences in lethality between Site 1 and Site 3 (p-value = 0.133).

Discussion

This study investigated the hypothesis that alkaloid-based skin secretions protect the poison frog *M. simplex* from infection by bacteria in the genus *Mycobacterium*, some of which are known to be pathogenic in frogs. Two methods, optical density assays and colony-forming unit assays, were used to measure the antimycobacterial properties of the alkaloids. The data for *M. smegmatis* showed that alkaloids from several populations inhibited growth and were lethal to the bacteria at 4.8 μL. Nearly all of the populations inhibited growth at 9.6 μL in both optical density and colony-forming unit assays, demonstrating a dose-response relationship. Further, there were significant differences in inhibition among populations. The data from *M. phlei* was inconsistent and inconclusive, and control trials suggested that the methanol in which the alkaloids were suspended had a significant effect on bacterial growth, which acted as a confounding factor. The data from *M. smegmatis* lends further support to the hypothesis that certain alkaloid compounds inhibit microbial growth, with variation in inhibition based on geographic location.

The first method of measuring microbial inhibition, optical density, indicated that alkaloids inhibit the growth of *M. smegmatis*. This inhibition varies among populations and exhibits a dose-response relationship. When *M. smegmatis* was treated with 4.8 μL of alkaloid, the optical density indicated that three populations (Sites 1, 3, and 6) inhibited microbial growth significantly more than the methanol control. When the volume of alkaloid was increased to 9.6 μL, five of the six populations inhibited bacterial growth significantly more than the methanol

control. Though not statistically significant, there was some degree of inhibition in Site 1 (though this specific test was likely confounded by an outlier frog with less alkaloid diversity and richness).[1] The fact that more populations were inhibitory at 9.6 μL than at 4.8 μL suggests a dose-response relationship, which is consistent with microbial inhibition. Further, there were significant differences in inhibition among several populations, supporting the idea that differences in alkaloid composition as a result of geographic isolation results in differential microbial inhibition.

On the other hand, the optical density results for *M. phlei* were inconsistent, with alkaloids from some populations (Site 1 and Site 3) inhibiting growth at 4.8 μL, but without significant inhibition at 9.6 μL. Further, cultures treated with alkaloids from two populations (Site 5 at 4.8 μL and Site 4 at 9.6 μL) grew significantly more than the methanol control. Clearly, there are some discrepancies in these results. However, the growth curve of *M. phlei* treated with methanol alone suggests that the methanol in which the alkaloids were suspended had a significant effect on *M. phlei*, which was not seen in *M. smegmatis.* As a result, it is impossible to determine the effect caused by the alkaloids alone based on this study. For studies in which alkaloids are suspended in methanol, it is likely that *M. phlei* is not a good model for mycobacterial growth inhibition due to the methanol acting as a confounding factor. Further research should fully investigate the effect of methanol on this particular bacterial species.

The second method of analysis, colony-forming unit assays, also demonstrated inhibition of *M. smegmatis*, which was consistent with the optical density results for this microbe. The alkaloids collected from Sites 1, 2, and 3 were lethal to *M. smegmatis* at 4.8 μL. At this volume, only alkaloids from Sites 1 and 3 inhibited growth of the bacteria in the optical density assay, but

[1] Although the mean endpoint optical density was approximately equal to that of the other populations and lower than that of methanol (Figure 3C), the inhibition was not statistically significant when compared to the methanol control (p-value = 0.07). However, the cultures treated with alkaloids from one particular frog from Site 1 had an unusually high optical density at both 4.8 μL and 9.6 μL. Because there is variation in the alkaloids of individuals from the same population (Saporito et al. 2012), it is suspected that this individual had a less diverse alkaloid composition or lower quantities of alkaloids, which may have resulted in lower inhibition. The presence of an outlier increases the standard error and reduces the ability to detect a statistical significant difference. The fact that the alkaloids collected from Site 1 were inhibitory or lethal to *M. smegmatis* in all other analyses (optical density at 4.8 μL, colony-forming units at 4.8 μL and 9.6 μL) supports the hypothesis that some frogs from Site 1 are, in fact, inhibitory, but as an entire site, are confounded by an outlier in this specific test.

the alkaloids from Site 2 did not. However, this result is not unusual because optical density may "[measure] live as well as dead bacterial cell debris" (Pan et al. 2014); therefore, it is possible that a culture with a high optical density may have low viability as determined by the colony-forming unit assay. At 9.6 μL, alkaloids from Sites 1, 2, 3, and 4 were found to be lethal to *M. smegmatis*, which is consistent with the optical density results that showed that alkaloids from all sites were inhibitory (although the inhibition of Site 1 was not statistically significant; see above for further discussion on Site 1). These results also indicate that alkaloids from Sites 5 and 6 inhibited the growth of the bacteria, but were not lethal to the bacteria (i.e., they did not reduce of the viability of the bacteria). Moreover, at 9.6 μL, there were significant differences in lethality among populations, lending further support to the hypothesis that difference in alkaloid composition results in difference in microbial inhibition.

In *M. phlei*, colony-forming unit assays showed that none of the alkaloids were significantly more lethal to the methanol at 4.8 μL, and only the alkaloids from Site 1 and Site 3 were more lethal to the bacteria at 9.6 μL. The lack of significant difference in inhibition between the methanol control and each population, as well as the lack of consistency with the optical density results for *M. phlei*, lend further support to the hypothesis that the methanol vehicle was a confounding factor for the results with this species of mycobacteria. Thus, no meaningful conclusions can be drawn regarding the alkaloids' ability to affect the growth or viability of *M. phlei*.

The relationship between microbial inhibition and alkaloids cannot be fully described without an analysis of the specific alkaloid compounds that are most and least effective in terms of microbial growth inhibition and lethality. Unfortunately, the alkaloids present in the frog skins of the present study have not yet been characterized. However, previous research has demonstrated that there are significant differences in alkaloid quantity and diversity between geographically isolated populations of *M. simplex* (Grant et al. 2012). Grant et al. (2012) also reported that pumiliotoxins, 5,8-disubstituted indolizidines, 5,6,8-trisubstituted indolizidines, 3,5-disubstituted pyrrolizidines, and some other izidines are well-distributed among *M. simplex* populations in the region represented by the present study. Some of these alkaloid classes, such as pumiliotoxins and indolizidines, were shown by Macfoy et al. (2005) to inhibit the growth of *Escherischia coli*, *Bacillus subtilis*, and *Candida albicans*. Based on the results of the present study, it is likely that the alkaloids collected from frogs in Site 1 and Site 3 have the strongest

antimicrobial effects because they were active against *M. smegmatis* in both assays even at a lower volume (4.8 μL), compared to Site 5, which showed no inhibitory activity at 4.8 μL. A future study will need to characterize the specific alkaloid compounds present in the samples used in the present study to determine which alkaloids serve as the best defense against the mycobacterial species *M. smegmatis*.

In conclusion, this is the first study to demonstrate that frog alkaloids from *M. simplex* effectively inhibit the growth of bacteria in a genus known to be pathogenic in anurans. Therefore, if *M. smegmatis* is a good model for other species of mycobacteria, it is possible that alkaloids defend this species of poison frog (and possibly others) from infection as well as predators. As suggested by the variation in inhibition and lethality among populations, the level of defense from infection likely varies among populations, which is likely based on differences in the alkaloid profiles of geographically isolated locations. Future research should investigate microbial growth inhibition and lethality in microbes that are known to be pathogenic to anurans and that are known to naturally occur in poison frog habitats.

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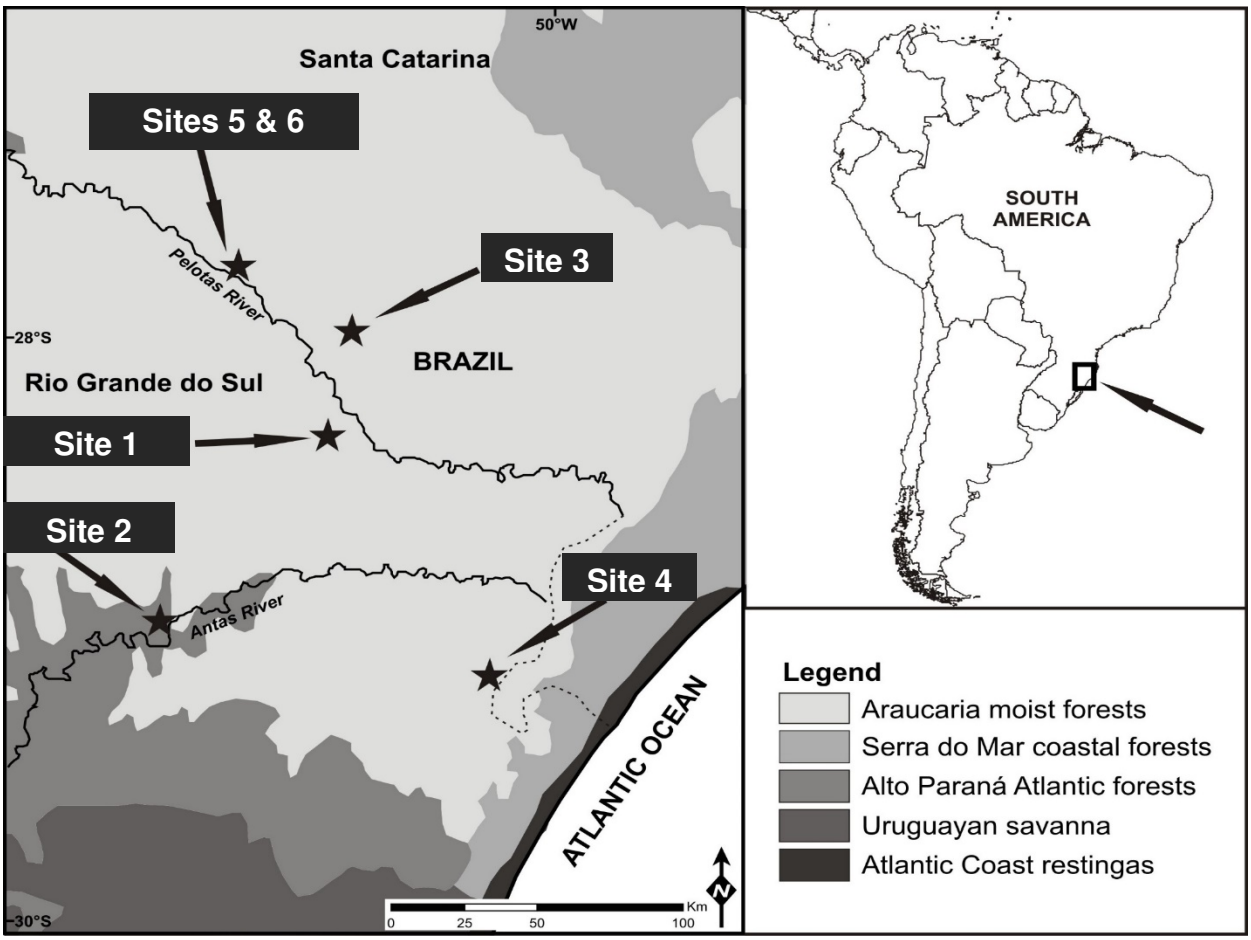

Figure 1. Map of *Melanophryniscus simplex* collection locations in Brazil. **Note**: Sites 5 and 6 are geographically close, but represent distinct collection locations.

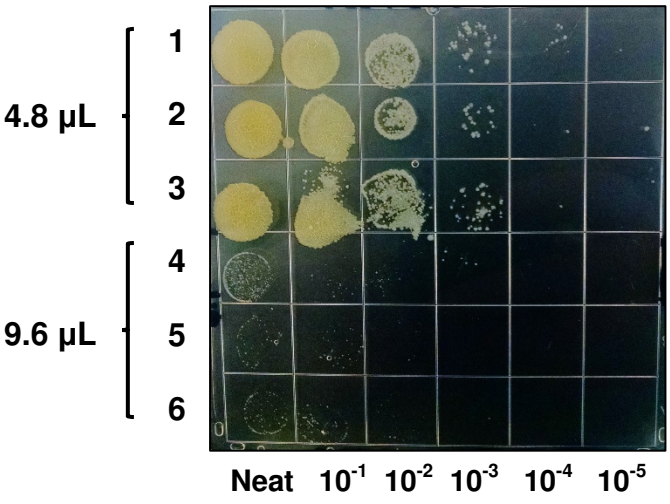

Figure 2. Colony-forming unit (CFU) assay for frog alkaloids tested against *Mycobacterium phlei*. Rows 1-3 were treated with 4.8 microliters of alkaloid; rows 4-6 were treated with 9.6 microliters of alkaloid. Column A is a pure culture of *M. phlei*, followed by a tenfold dilution in each successive column.

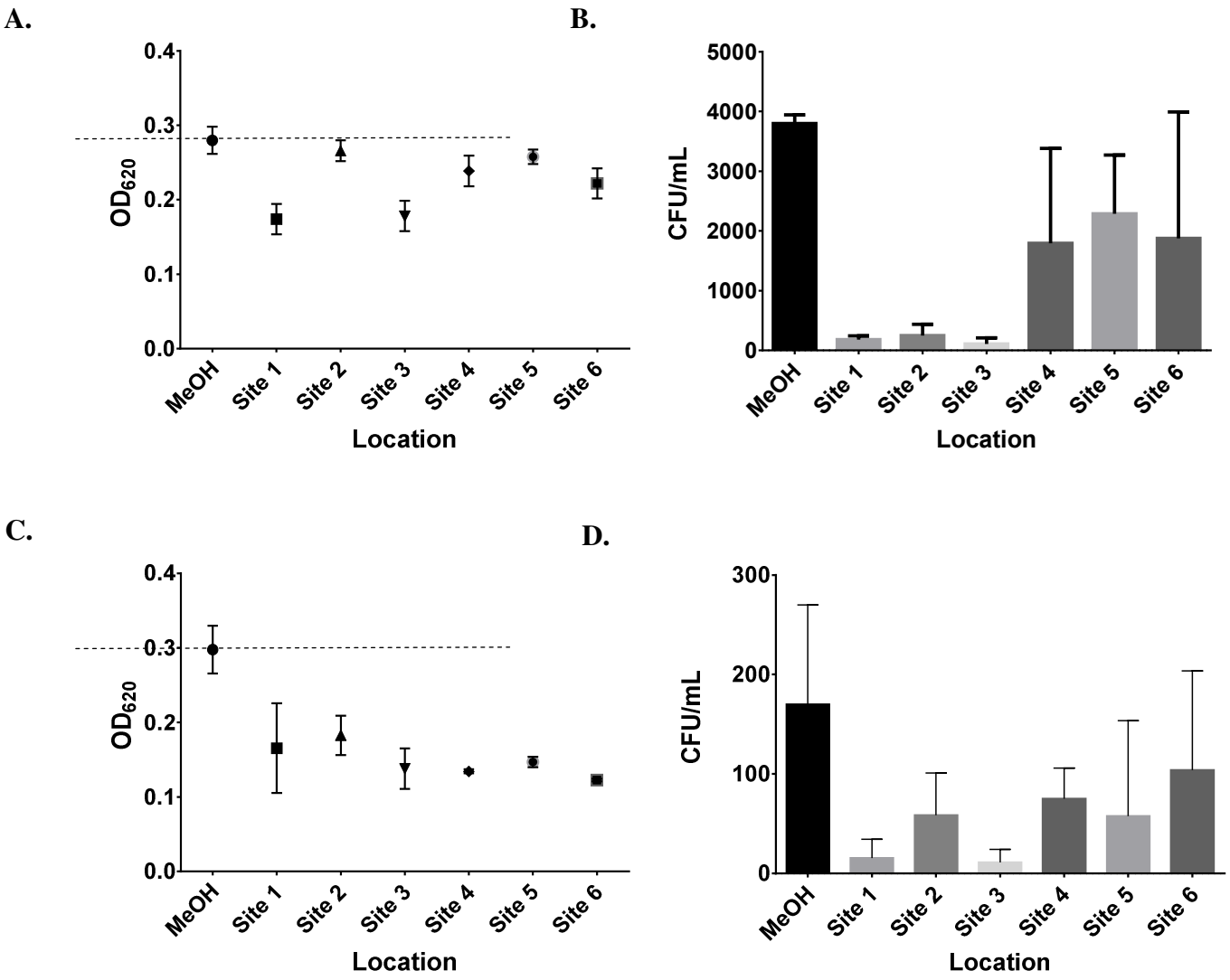

Figure 3. Optical density and colony-forming unit analyses for *Mycobacterium smegmatis*. (A) Mean endpoint optical density measurements for *Mycobacterium smegmatis* treated with 4.8 microliters of alkaloid. The dotted line represents the level of inhibition by the methanol control. (B) Mean colony-forming units per mL for *Mycobacterium smegmatis* treated with 4.8 microliters of alkaloid. (C) Mean endpoint optical density measurements for *Mycobacterium smegmatis* treated with 9.6 microliters of alkaloid. The dotted line represents the level of inhibition by the methanol control. (D) Mean colony-forming units per mL for *Mycobacterium smegmatis* treated with 9.6 microliters of alkaloid.

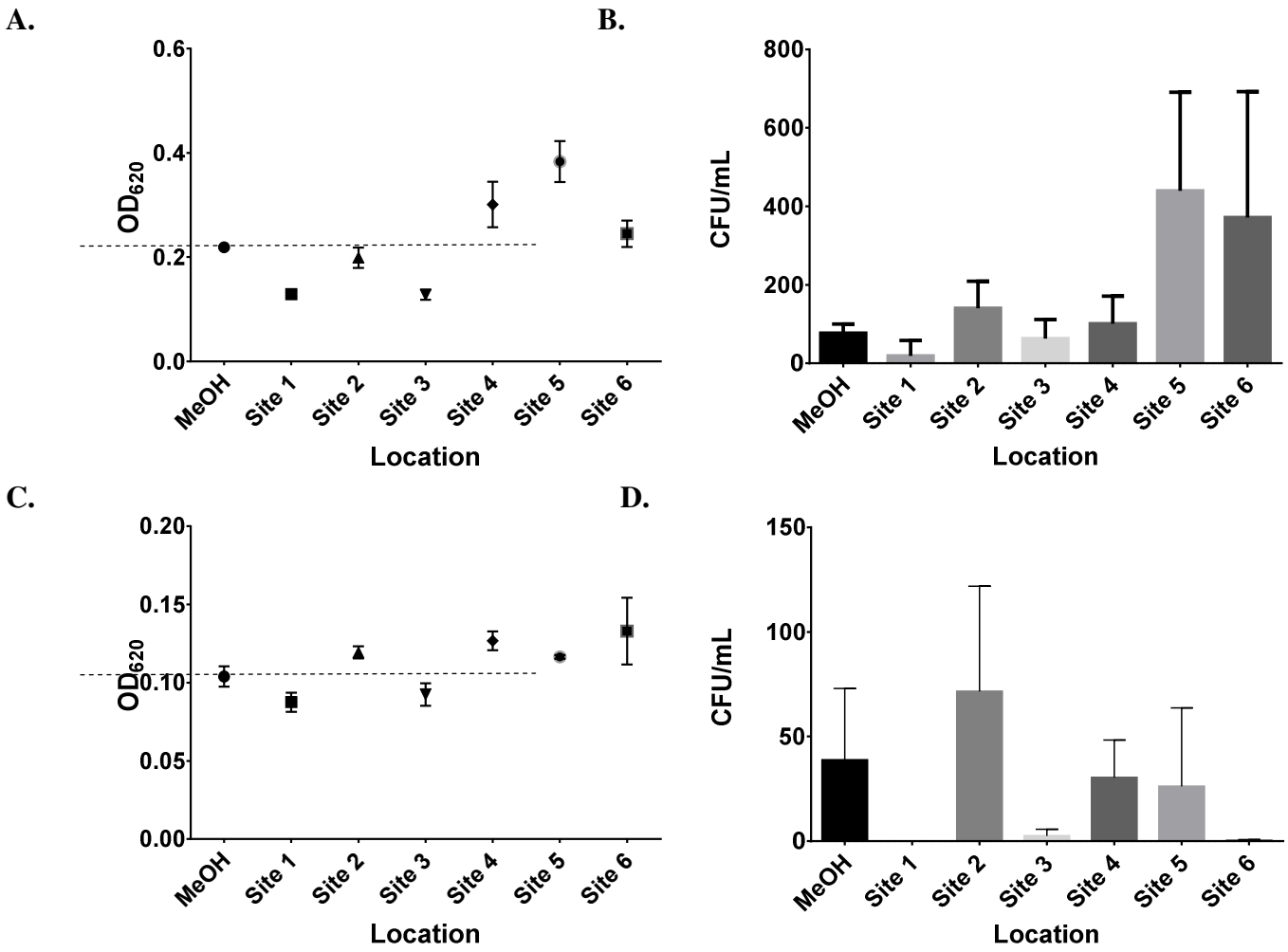

Figure 4. Optical density and colony-forming unit analyses for *Mycobacterium phlei*. (A) Mean endpoint optical density measurements for *Mycobacterium phlei* treated with 4.8 microliters of alkaloid. The dotted line represents the level of inhibition by the methanol control. (B) Mean colony-forming units per mL for *Mycobacterium phlei* treated with 4.8 microliters of alkaloid. (C) Mean endpoint optical density measurements for *Mycobacterium phlei* treated with 9.6 microliters of alkaloid. The dotted line represents the level of inhibition by the methanol control. (D) Mean colony-forming units per mL for *Mycobacterium phlei* treated with 9.6 microliters of alkaloid.