Quorum Quenching and Antimicrobial Activity of Goldenseal (*Hydrastis canadensis*) against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Abstract

The popular herbal remedy goldenseal (*Hydrastis canadensis* L.) is traditionally used to treat skin infections. With this study, we show activity of *H. canadensis* extracts *in vitro* against methicillin-resistant *Staphylococcus aureus* (MRSA). An extract from *H. canadensis* leaves demonstrated more potent antimicrobial activity than the alkaloid berberine alone (MICs of 75 μg/mL and 150 μg/mL, respectively). LC-MS detected alkaloids and efflux-pump inhibitory flavonoids in the extract, and the latter may explain the enhanced efficacy of the extract compared to berberine alone. We also show evidence of anti-virulence activity as a second mechanism by which *H. canadensis* acts against *S. aureus*. The *H. canadensis* leaf extract (but not the isolated alkaloids berberine, hydrastine, and canadine) demonstrated quorum quenching activity against several clinically relevant MRSA isolates (USA300 strains). Our data suggest that this occurs by attenuation of signal transduction through the AgrCA two-component system. Consistent with this observation, the extract inhibited toxin production by MRSA, and prevented damage by MRSA to keratinocyte cells *in vitro*. Collectively, our results show that *H. canadensis* leaf extracts possess a mixture of constituents that act against MRSA via several different mechanisms. These findings lend support for the traditional application of crude *H. canadensis* extracts in the treatment of prevention of infection.

Keywords

Introduction

The European Antimicrobial Surveillance Systems estimates that as many as 52 million people worldwide are colonized with multi-drug resistant bacteria. Among the most

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prevalent and lethal of these is methicillin-resistant *Staphylococcus aureus* (MRSA), which now causes more annual deaths in the US than HIV/AIDS [1]. With the growing rates of MRSA levels in community and healthcare settings, and the increased disease burden and higher treatment costs associated with drug-resistant bacterial infections [2], there is a pressing need to devise new strategies to combat problematic pathogens such as MRSA.

Anti-virulence based approaches to disarm drug-resistant bacterial pathogens are increasingly being developed [3]. These agents will be more pathogen-specific than current antibiotics, but the enormity of disease burden caused by *S. aureus* and MRSA, including an estimated 76% of all skin and soft tissue infections [4], highlights the value of developing MRSA targeted therapeutics. The specificity and the anti-virulence nature of such agents may limit the evolution of widespread drug resistance, facilitate the development of immune responses that will prevent subsequent infection, and avoid negative impacts on the normal microbial flora [3]. One appealing target as a MRSA anti-virulence strategy is the quorum-sensing system [5, 6].

S. aureus quorum-sensing, also called the accessory gene regulator or "*agr*" system (Figure 1), is a cell density dependent regulatory system that controls the production of many host-damaging agents or "virulence factors". This system responds to a secreted peptide signal, called an autoinducing peptide (AIP), which is biosynthesized and secreted by *S. aureus*. Depending on the *S. aureus* strain, the structure of the secreted AIP can be any one of four types (AIP-1, AIP-2, AIP-3, or AIP-4). Once the extracellular concentration of the AIP signal is high enough, it binds to a surface receptor, called AgrC, activating a signal transduction cascade. The key components of this cascade are the AgrCA two-component regulatory pair, and when activated, they induce the production of extracellular virulence factors. Mutations in the *agr* system are known to reduce pathogenesis in animal models of infection [5], and numerous studies have shown that small-molecule inhibition of the *agr* system attenuates skin and soft tissue infections in animal models [5, 7, 8].

It was our objective to investigate the *in vitro* effectiveness of the popular [9] herbal medicine goldenseal *(Hydrastis canadensis* L.), against several clinical isolates of MRSA. *H. canadensis* possesses an array of alkaloids, the most abundant of which is berberine [10]. Previously, *H. canadensis* has demonstrated antimicrobial activity against various Gram positive bacteria [10–13], and berberine has been shown to inhibit the growth of MRSA [14]. However, the antimicrobial activity of crude *H. canadensis* extracts against MRSA has never been reported, nor has their quorum quenching activity been investigated. With these studies, we tested the central hypothesis that antimicrobial effects of *H. canadensis* leaf extracts are due to the combined action of multiple constituents acting via different mechanisms. This hypothesis was based in part on seminal work by Stermitz et. al, in which the antimicrobial activity of berberine has been shown to be potentiated by other plant constituents [15, 16]. We focused specifically on *H. canadensis* leaf extracts in these experiments, because our previous studies have shown leaf extracts to be more effective than root extracts as synergists to the antimicrobial activity of berberine [17]. Although *H. canadensis* root extracts are most commonly used in traditional medicine applications [18], there is precedent for topical use of goldenseal leaf extracts against infection [19]. Our goals with this study were to evaluate an *H. canadensis* leaf extract as an antimicrobial agent and

quorum quencher for MRSA, and to compare the activity of the crude extract to that of its major alkaloid, berberine. In particular, we investigated activity against USA300 MRSA isolates, which have emerged as the most common strain of MRSA observed in community-acquired soft-tissue infections [4, 20].

Materials and Methods

Test Bacteria, Chemicals, and Biochemicals

Wild type *S. aureus* strains used in this study were CA-MRSA USA300 TCH1516 [21], LAC [22], AH1263 (also called LAC*) [23], and SA502A [24]. *S. aureus* quorum-sensing reporter strains containing plasmid pDB59 were also employed, and included representatives of *agr* group I (CA-MRSA USA300 LAC), *agr* group II (SA502A), and *agr* group III (CA-MRSA MW2) [25]. Additional studies to investigate quenching made use of *S. aureus lux* reporter strain ROJ143 [26, 27]. For the epithelia toxicity tests, alpha-toxin (*hla::*Erm) [28] or *agr* (*agr::*Tet) [29] mutants of USA300 strain AH1263 (LAC*) were used. Tryptic soy broth (TSB), Mueller Hinton broth, carbonyl cyanide m-chloro-phenylhydrazone (CCCP, purity >98% by TLC), berberine (purity >98% by HPLC), (1R,9S)-(-)-β-hydrastine (purity >98% by HPLC) and DMSO were purchased from Sigma Aldrich (Saint Louis, MO, USA) and canadine (tetrahydroberberine, purity >98% by HPLC, stereochemistry unconfirmed) from Chromadex (Irvine, CA, USA). Sideroxylin, 8-desmethyl-sideroxylin, and 6-desmethyl-sideroxylin were isolated previously from *H. canadensis* leaves [30] with >98% purity. Acetic acid was purchased from Fisher Chemical (Pittsburgh, PA, USA). Ethanol (95%), HPLC grade acetonitrile, and HPLC grade methanol were obtained from Pharmaco-AAPER (Shelbyville, KY, USA). Water was purified with a nanodiamond system (Barnstead, Dubuque, IA, USA).

Plant material

Cultivated *Hydrastis canadensis* L. (Ranunculaceae) was harvested in September 2008 from: N 35° 24.277', W 082° 20.993', 702.4 m elevation. A voucher specimen is retained at the University of North Carolina Herbarium (NCU583414) and identity was confirmed by Dr. Alan S. Weakly.

Extraction and LC-MS

Extracts were prepared according to standard procedures employed in the manufacture of hydroethanolic *Hydrastis canadensis* supplements [31], as described elsewhere [17]. A solvent of 50% ethanol:50% water was used, in a ratio of 1:5 (g plant material:mL solvent). The solvent (500 mL) was blended with the entire aerial (above ground) portion of *H. canadensis* plants (100 g) and macerated for 24 hr, then filtered under vacuum. The resulting extract, which will be referred to as *H. canadensis* leaf extract throughout this paper, was stored in an amber bottle at room temperature. Biological activity and HPLC profile were consistent for this extract over a three year time period.

The extract was profiled with liquid chromatography – mass spectrometry (LC-MS) to determine alkaloid and flavonoid quantity and identity. Quantitative analysis of alkaloids and flavonoids (Table 1, Supplemental Information) was accomplished using an established

method [17, 30]. Constituent identities were confirmed on an LTQ-Orbitrap high resolution mass spectrometer (Thermo, San Jose, CA, USA) coupled to an ultra-performance liquid chromatograph (UPLC) (Waters, Alliance) by comparing accurate mass, fragmentation, and retention time with those of authenticated standards, as reported previously [30].

The following gradient was employed, where A = HPLC grade acetonitrile and B = 0.1% aqueous formic acid: 90%‑70% B from 0 to 5.0 min; 70% to 50% B from 5 to 7.0 min; 50% to 45% from 7.0 to 7.5 min, 45% to 0% B from 7.5 to 8.0 min, isocractic at 0% B from 8.0 to 8.5 min and 0 to 90% from 8.5 to 9.0 min. MS analysis was conducted in the positive ion mode for alkaloids and the negative ion mode for flavonoids, with mass range of 100–1000 m/z. Capillary temperature was 300°C, sheath gas pressure was 28 (arbitrary units), and source, capillary, and tube lens voltages were 4.30 kV, 29 V, and 110 V, respectively.

Broth Microdilution MIC Assays

Minimum inhibitory concentration (MIC) was measured according to Clinical Laboratory Standards Institute (CLSI) standard procedures [32]. Briefly, extracts or purified berberine (+ control) were added to 96 well plates in triplicate at concentrations ranging from 4.7 to 300 μg/mL in Mueller Hinton Broth. Vehicle (2% DMSO) was the negative control, and DMSO content was fixed at 2% in all wells. A 24 hr culture of a single colony isolate of *S. aureus* AH1263, TCH1516, or LAC was grown to log-phase in Mueller Hinton broth and added at a final density of 1.0×10^5 CFU/mL. Absorbance at 600 nm was measured after 24 hr using a POLARstar Optima microplate reader (BMG Labtech, Inc). MIC was defined as the concentration at which there was no statistically significant difference between the treatment and vehicle control. Absorbance for replicate wells containing all assay components except bacteria was subtracted from the absorbance of assay wells.

Quorum quenching assays with fluorescent reporters

S. aureus quorum-sensing reporter strains containing pDB59 were grown overnight in TSB supplemented with chloramphenicol at 10 μg/mL at 37°C with shaking. For testing extracts or samples, cultures were diluted 500-fold into TSB (approximately $4–10 \times 10^6$ CFU/mL after dilution) and 180 μL was dispensed into a microtiter plate. Sample or vehicle control was added (20 μL) to reach the appropriate concentration, and the plates were incubated with shaking (200 rpm) at 37°C. At designated time points, absorbance (600 nM) and fluorescence (excitation 485 nm, emission 530 nm) was measured in a Tecan Infinite M200 plate reader. Reported values are for 30 hr of growth.

Quorum quenching assay with *lux* reporter

AIP-1 and AIP-2 were obtained by filter sterilizing overnight TSB cultures of *S. aureus* strains AH1263 and SA502A, respectively [24]. An overnight culture of the *lux* reporter strain ROJ143 was subcultured 1:50 in TSB containing chloramphenicol (10 μg/mL) and grown at 37°C with shaking to OD_{600} of 0.8. Aliquots (164 μL) of the *lux* reporter strain were added to quadruplicate microtiter plate wells that contained 20 μL of AH1263 conditioned medium (i.e. AIP-1) and *H. canadensis* extract at various sub-inhibitory concentrations, such that all wells contained a final DMSO concentration of 0.8%. Following 90 min of incubation at 37°C with shaking (200 rpm), bioluminescence was

measured in a Tecan Infinite M20 plate reader. For comparison, the *lux* reporter was inhibited with AIP-2 by replacing *H. canadensis* with SA502A conditioned medium (also in 0.8% DMSO).

Human skin epithelia toxicity assay

USA300 strain AH1263 (LAC*) and derivatives with an alpha-toxin mutation or an *agr* deletion were grown overnight in TSB. Culture flasks (50 mL) containing 10 mL of TSB, and a range of sub-inhibitory concentrations of *H. canadensis* (with constant 0.8% DMSO), were inoculated 1:500 with AH1263 overnight culture. TSB aliquots containing DMSO vehicle only were inoculated with the *agr* and *hla* mutants. Following 11 hr of growth at 37°C with shaking (225 rpm), cultures were filtered through SpinX filters (0.22 μm, Corning) and α-toxin content of the medium was measured by immunoblotting, as described previously [28]. Human keratinocytes (HaCaT) were cultured in RPMI with 10% fetal calf serum with penicillin/streptomycin [33]. The spent medium was filter sterilized, and diluted 5-fold in HaCaT medium. Aliquots of this spent medium (0.5 mL) were added to confluent HaCaT in 24 well plates and incubated for 24 hr. To assess toxicity, the release lactate dehydrogenase was measured with a commercially available kit (Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay).

Statistics

Significance of means as compared to controls was calculated with two-sample equal variance, 2-tailed student's t-tests.

Results and Discussion

One of the objectives of this study was to compare the antimicrobial activity of an *H. canadensis* extract against MRSA to that of its major alkaloid berberine. An *H. canadensis* leaf extract (see chromatogram in Figure 1S, Supporting Information) was standardized to berberine content, and its antimicrobial activity was evaluated against MRSA strain USA300 AH1263 (LAC*) [22] side by side with the same concentrations of berberine alone (Figure 2). The extract displayed a lower MIC value (75 μg/mL, reported as μg berberine/mL assay volume) as compared to that of the pure berberine (150 μg/mL), indicating that constituents other than berberine play a role in its activity against MRSA. Note that berberine serves at the positive control in this assay, and our observed MIC is consistent with numerous previous reports of the activity of this alkaloid against *S. aureus* [10, 34, 35]. It is very likely that the enhanced activity of the extracts as compared to berberine alone is due to flavonoids such as sideroxylin, 8-desmethyl-sideroxylin, and 6-desmethyl-sideroxylin (Figure 3), which have previously been reported at constituents of *H. canadensis* [30, 36]. *H. canadensis* flavonoids have been shown to enhance antimicrobial activity of berberine via efflux pump inhibition [30], and were detected in the *H. canadensis* extracts under investigation here (Table 1S, Supporting Information).

Our results show that *H. canadensis* leaf extracts possess antimicrobial activity. This activity is likely due to the action of the alkaloid berberine , which demonstrated MIC values ranging from 150 μg/mL to 300 μg/mL against three different MRSA strains (Table 2S,

Supporting Information). Notably, two other goldenseal alkaloids [10], canadine and
hydrastine (Figure 3), did not demonstrate any antimicrobial activity against the three
MRSA strains evaluated (Table 2S).

The current study demonstrates that relatively high concentrations of *H. canadensis* leaf
extracts or purified berberine are required to achieve antimicrobial activity; however, there
are many anecdotal reports of this botanical being a highly effective treatment for various
infections [18]. The *S. aureus agr* quorum sensing system plays an important role in skin
infections. Thus, we hypothesized that quorum quenching activity might partially explain
the activity of *H. canadensis* extracts against MRSA. A reporter assay was employed that
has previously been used to characterize other quorum quenching agents [37]. When extracts
at sub-inhibitory concentrations were incubated with the reporter strains, we observed a
consistent and robust dose-dependent quenching effect with *agr* type I, II, and III reporters
(Figure 4). These three *agr* classes represent the vast majority of all *S. aureus* strains [38],
indicating that *H. canadensis* leaf extracts contain a general quorum quenching agent. This
quorum quenching effect appeared not to be attributable to the major *H. canadensis* alkaloid
berberine (Figure 4D), which did not inhibit the *agr* system even at 75 μg/mL. We also
failed to observe quenching activity with the other alkaloids hydrastine or canadine (data not
shown).

Further experiments were conducted to gain insight into the *H. canadensis* leaf extract
mechanism of quorum quenching using a *S. aureus* reporter strain that contains a quorum
sensing controlled *lux* promoter [27]. This *lux* reporter strain does not make its own AIP,
due a deletion in the *agrBD* genes. When AIP-1 is added to the medium, the *lux* reporter
turns on, generating bioluminescence. This reporter strain was employed to test whether the
AgrCA two-component system (Figure 1) could be a target of quenching action. We
generated samples containing a constant level of AIP-1, combined them with various sub-
inhibitory concentrations of *H. canadensis* (0–75 μg/mL, standardized to berberine),
exposed these mixtures to the reporter strains, and measured bioluminescence (Figure 5).
We observed dose dependent inhibition, suggesting that AgrCA is a probable target of some
constituent of the extract. In support of this hypothesis, the extract inhibited to the same
level as an AgrC competitive antagonist AIP-2 (Figure 5). The most obvious mechanism of
inhibition is the AIP receptor on AgrC, which is exposed to the extracellular environment
and vulnerable to exogenous agents [5]. Binding to this receptor would cause the observed
suppression of bioluminescence by the *lux* reporter. However, an alternative explanation for
the observed effect is that the active compound sequestered or inactivated AIP through a
novel interaction, or possibly repressed the *agr* P3 promoter through a different regulatory
mechanism.

As further evidence of the relevance of the observed quorum quenching activity of the *H.
canadensis* leaf extract, we investigated its effect on alpha-toxin production by *S. aureus*.
Alpha-toxin is the primary cause of skin abscess dermonecrosis [26] and is, thus, potentially
relevant for *H. canadensis* bioactivity. This toxin is encoded by the *hla* gene and is under
direct control of the *agr* quorum-sensing system [5]. When the USA300 strain AH1263 was
incubated with increasing concentrations of extract, alpha-toxin levels decreased in concert
as assessed by immunoblot (Figure 6A). These findings verify the transcriptional reporter

assay results (Figures 4 and 5). As expected, the USA300 hla mutant, which was included as a control for the immunoblot, did not produce alpha-toxin.

To relate the *H. canadensis* leaf extract quorum quenching observations to skin infections, we employed an assay to evaluate MRSA interactions with cultured human keratinocyte cells (HaCaT). Based on the precedent that inhibition of the *agr* system, or mutations in alpha-toxin, reduced skin abscess formation [5, 28], we tested MRSA wildtype and mutant strains as controls. Spent medium from USA300 strain AH1263, or derivatives with agr or hla mutation, were exposed to the HaCaT cells, and cell viability was assessed using a lactate dehydrogenase (LDH) release assay (Figure 6B). As anticipated, the wildtype USA300 caused significant damage (83% killing), while the agr and hla mutants had almost no effect on HaCaT cell integrity. After exposure of the USA300 strain to an *H. canadensis* extract (80 μg/mL), the USA300 damage to HaCaT cells dropped markedly to 38%. These results demonstrate that *H. canadensis* inhibition of the *agr* system results in reduced alpha toxin production, which in turn attenuates damage to human skin keratinocytes.

In conclusion, our data lend support for the traditional use of *H. canadensis* to treat skin infections. *H. canadensis* leaf extracts possess direct antimicrobial activity that is due in part to the alkaloid berberine, but not canadine or hydrastine. Importantly, antimicrobial activity is just one of several mechanisms by which *H. canadensis* appears to act against MRSA. We show that *H. canadensis* leaf extracts at sub-inhibitory concentrations quench the *agr* quorum sensing system, and that this activity is not due to the major alkaloids berberine, hydrastine, or canadine. The most likely mechanism by which this quorum quenching effect occurs is attenuation of signal transduction through the AgrCA two-component system. Such attenuation would cause the observed reduction in toxin production by *H. canadensis* exposed MRSA. Collectively, our results demonstrate that *H. canadensis* leaf extracts contain several classes of constituents that act against MRSA with different mechanisms. Such a mixture would be expected to demonstrate better efficacy than its components alone in the treatment or prevention of MRSA infections, by virtue of its ability to target the pathogen via multiple pathways. This prediction could be evaluated with future *in vivo* investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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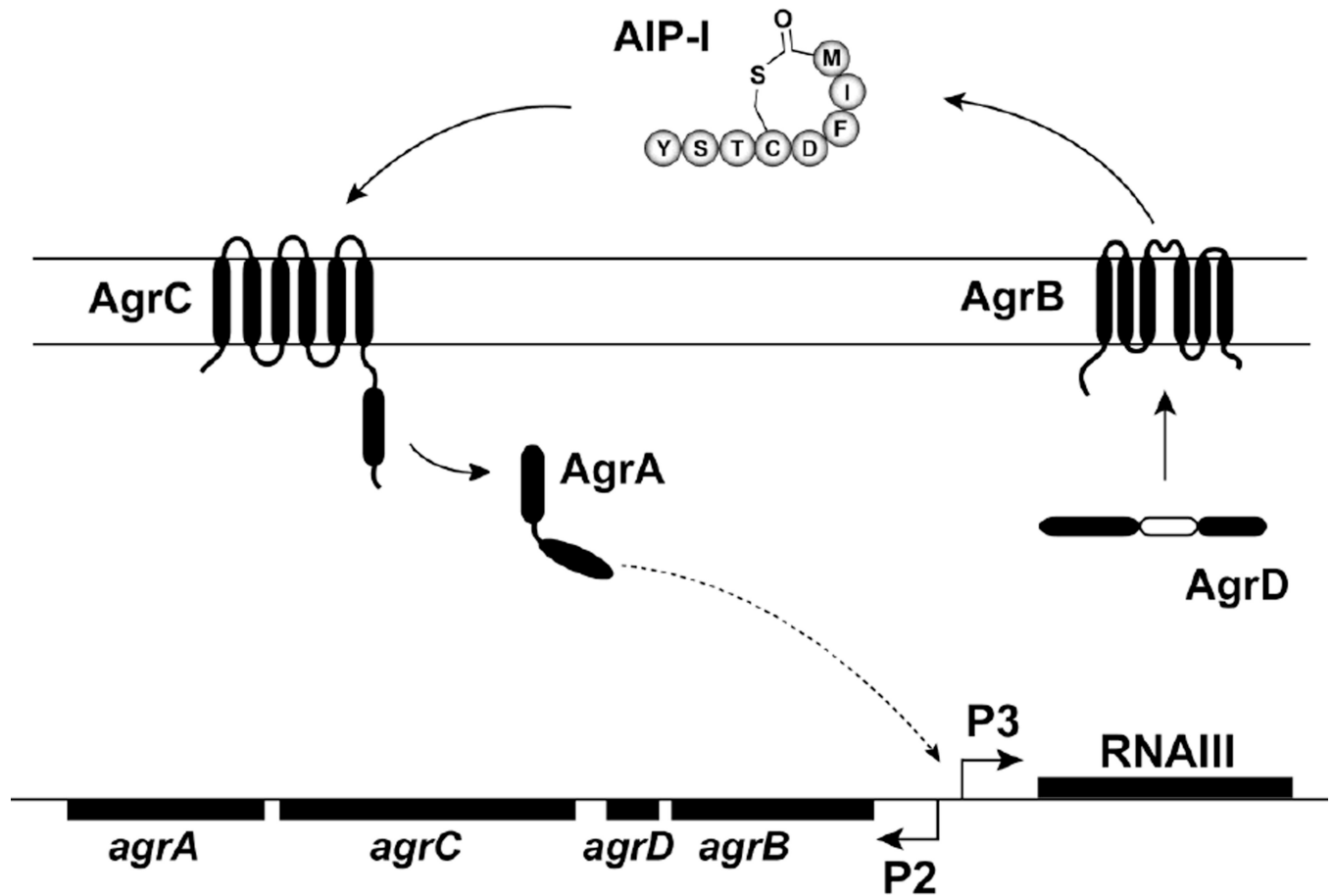
Fig. 1.
Schematic of the *S. aureus agr* quorum sensing system. AgrD is the peptide precursor of the autoinducing peptide (AIP) signal. AgrB is a membrane bound endopeptidase that generates the AIP (AIP-1 sequence is shown). AgrC is a membrane histidine kinase that binds AIP and phosphorylates the response regulator AgrA, which in turn induces transcription from the P2 and P3 promoters in the *agr* chromosomal locus. This induction autoinduces the system and leads to the production of RNAIII transcript. RNAIII is the primary effector of the system and turns on the secretion of virulence factors.
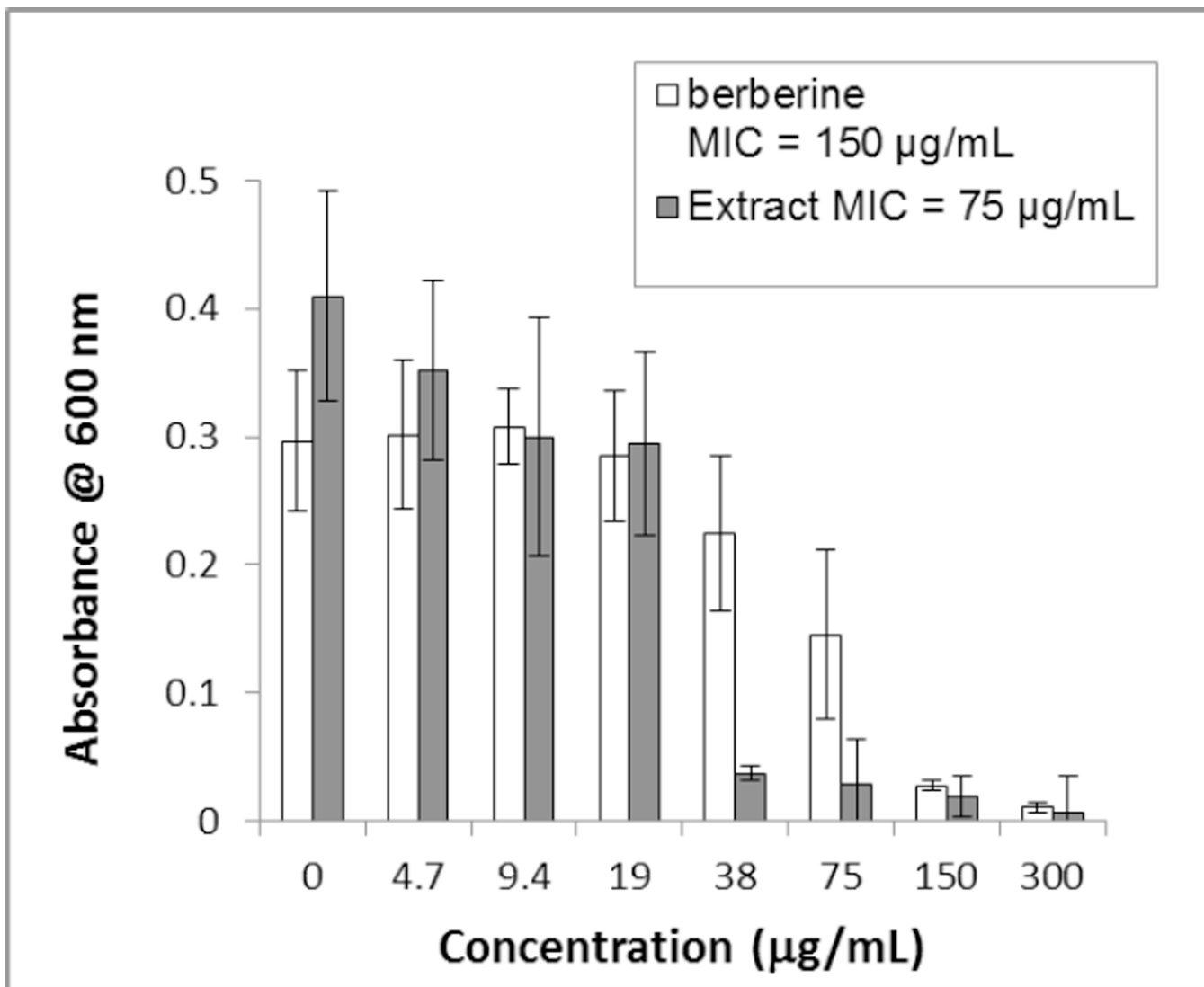
Fig. 2.

Comparison of antimicrobial activity of goldenseal (*Hydrastis canadensis*) leaf extract and berberine against MRSA strain USA300 AH1263 [22]. The extract was standardized to berberine content, which is displayed on the x-axis. Different concentrations were prepared via two-fold serial dilution for both test samples. The leaf extract showed an MIC value at 38 μg/mL (expressed as μg berberine/mL assay volume), which is three times lower than the MIC of isolated berberine at 150 μg/mL. Error bars represent standard deviation for OD_{600} of triplicate assay wells.
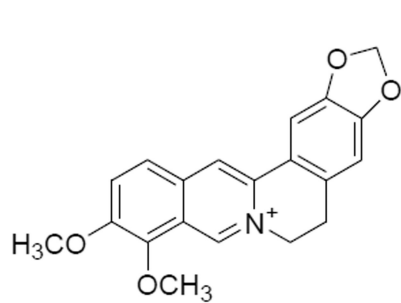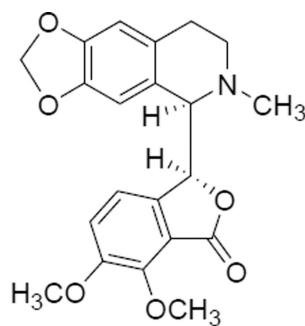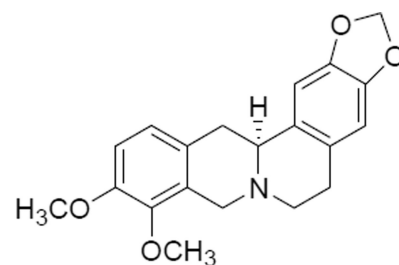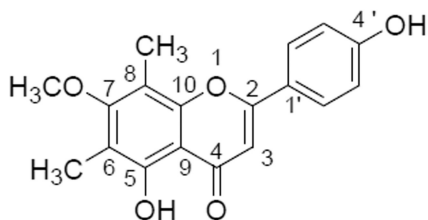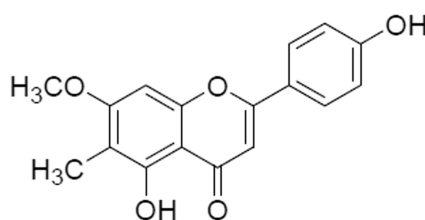
Fig. 3.
Structures of major alkaloids, berberine (**1**), (1R,9S)-(-)-β-hydrastine (**2**) and canadine (tetrahydroberberine) (**3**), and flavonoids, sideroxylin (**4**), 8-desmethyl-sideroxylin (**5**), and 6-desmethyl-sideroxylin (**6**), found in *Hydrastis canadensis*.
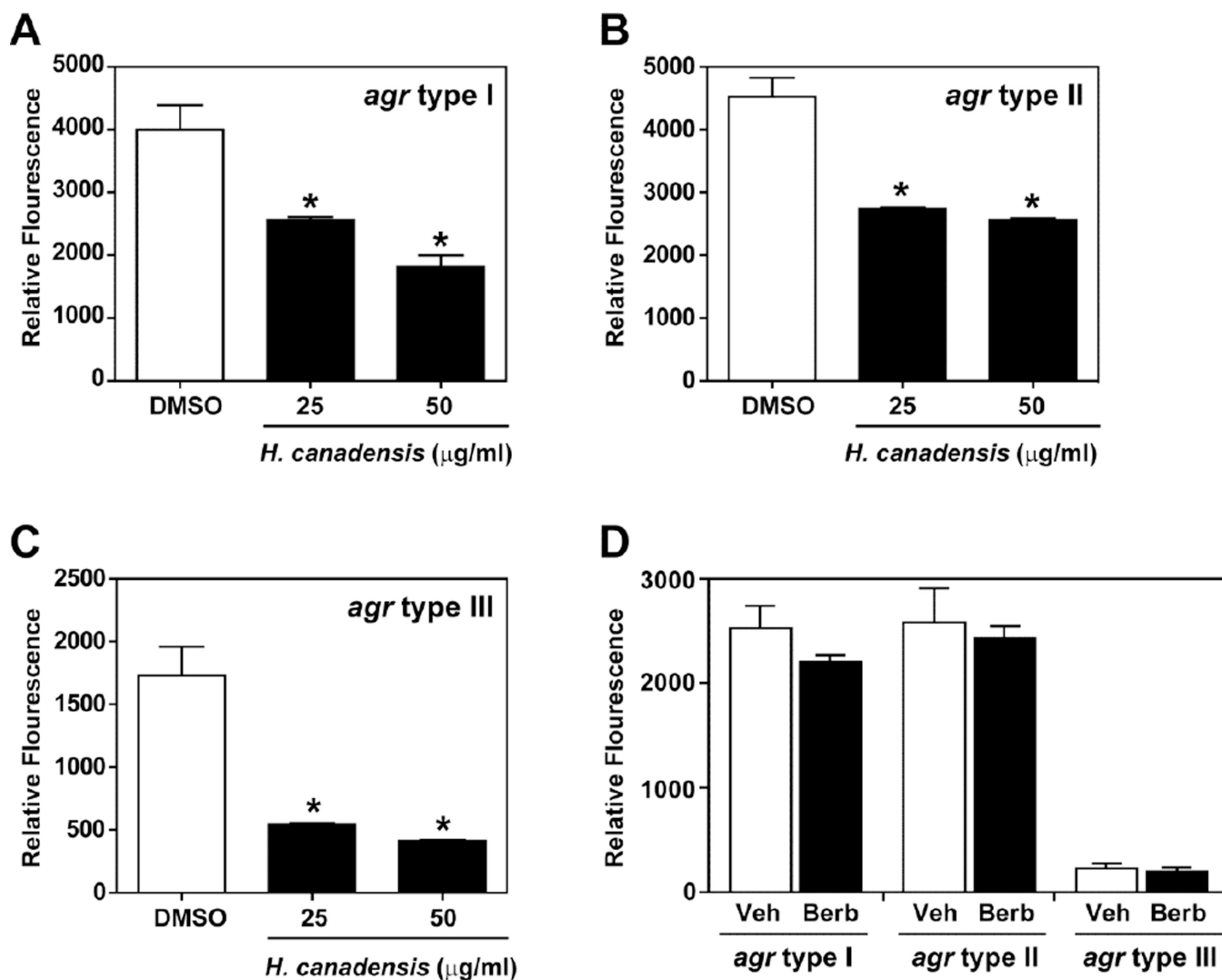
Fig. 4.

Quorum quenching in *S. aureus agr* P3-GFP reporter strains by *H. canadensis* leaf extract and berberine. Reporter strains (*agr* type I, panel **A**; *agr* type II, panel **B**; *agr* type III, panel **C**) were grown in tryptic soy broth and extract (standardized to berberine) was tested at 25 and 50 μg/mL. These concentrations were chosen because they were sub-inhibitory under the conditions of this assay. The reporter strains were incubated for 30 hr with the extract and GFP fluorescence was measured. For comparison, a competitive inhibitor of the AIP receptor quenches to ~95% (not shown). In panel **D**, each *agr* reporter strain was grown and measured in the same manner and berberine (Berb) was added to 75 μg/mL and compared to sterile water as vehicle control (Veh). * indicates $p < 0.01$, compared to vehicle (DMSO) in panels **A**, **B**. and **C**. p- values were calculated with a two-tailed student's t-test, and error bars represent standard deviation of the mean for triplicate measurements. Differences in the results between vehicle and berberine in panel **D** were not statistically significant.
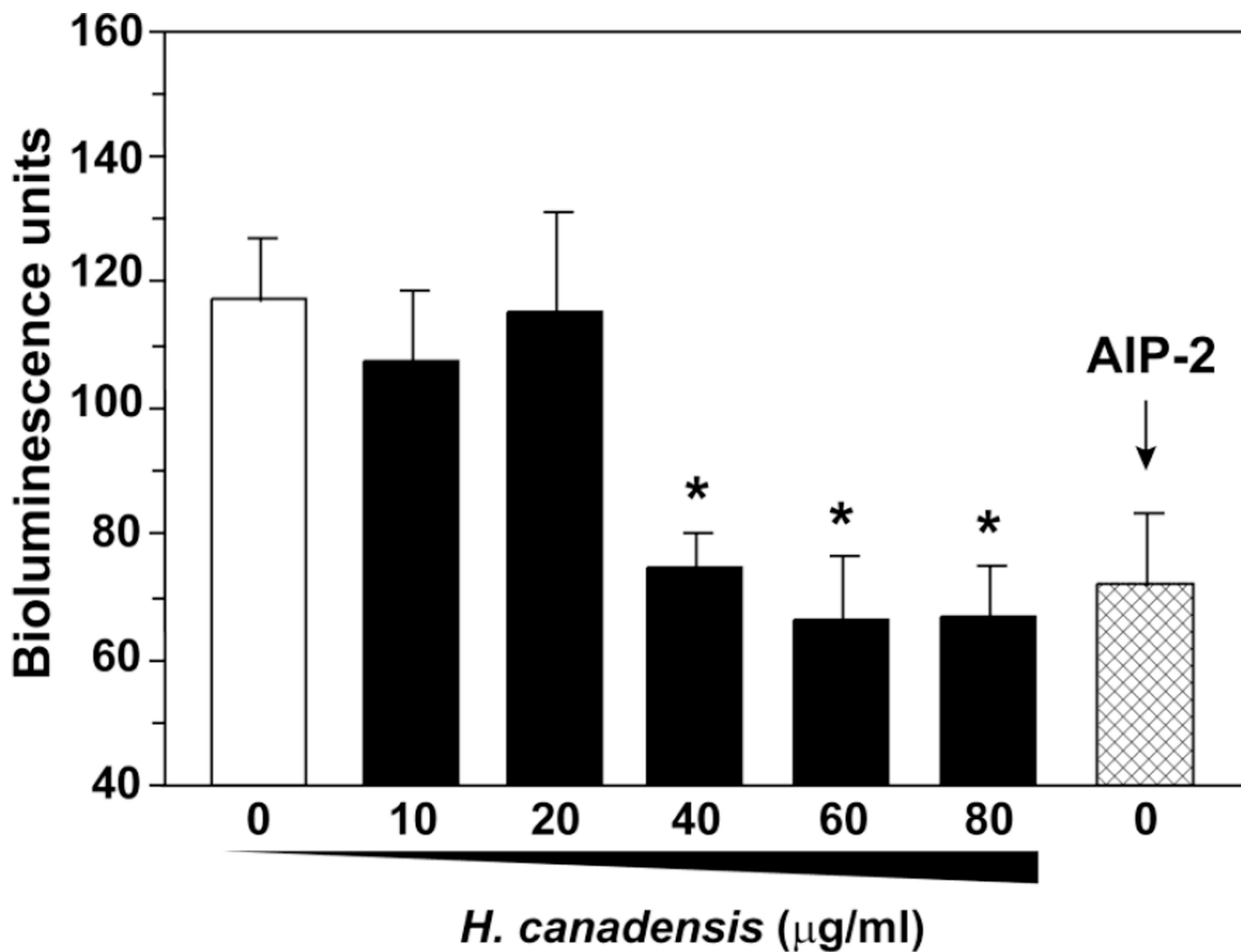
Fig. 5.
Inhibition of *S. aureus agr lux* reporter with *H. canadensis* leaf extract. An *S. aureus lux* reporter strain was used that responds to exogenous AIP-1. A constant level of AIP-1 was mixed with extract, incubated with the *lux* reporter, and bioluminescence was measured. The extract was standardized to berberine and added to a concentration range of 10–80 μg/mL. AIP-2, a known inhibitor, was used as positive control. Values reported represent means of quadruplicate assay wells, with error bars representing the standard deviation of the mean, and * indicates $p < 0.0001$ compared to vehicle (calculated with a two-tailed student's t-test).
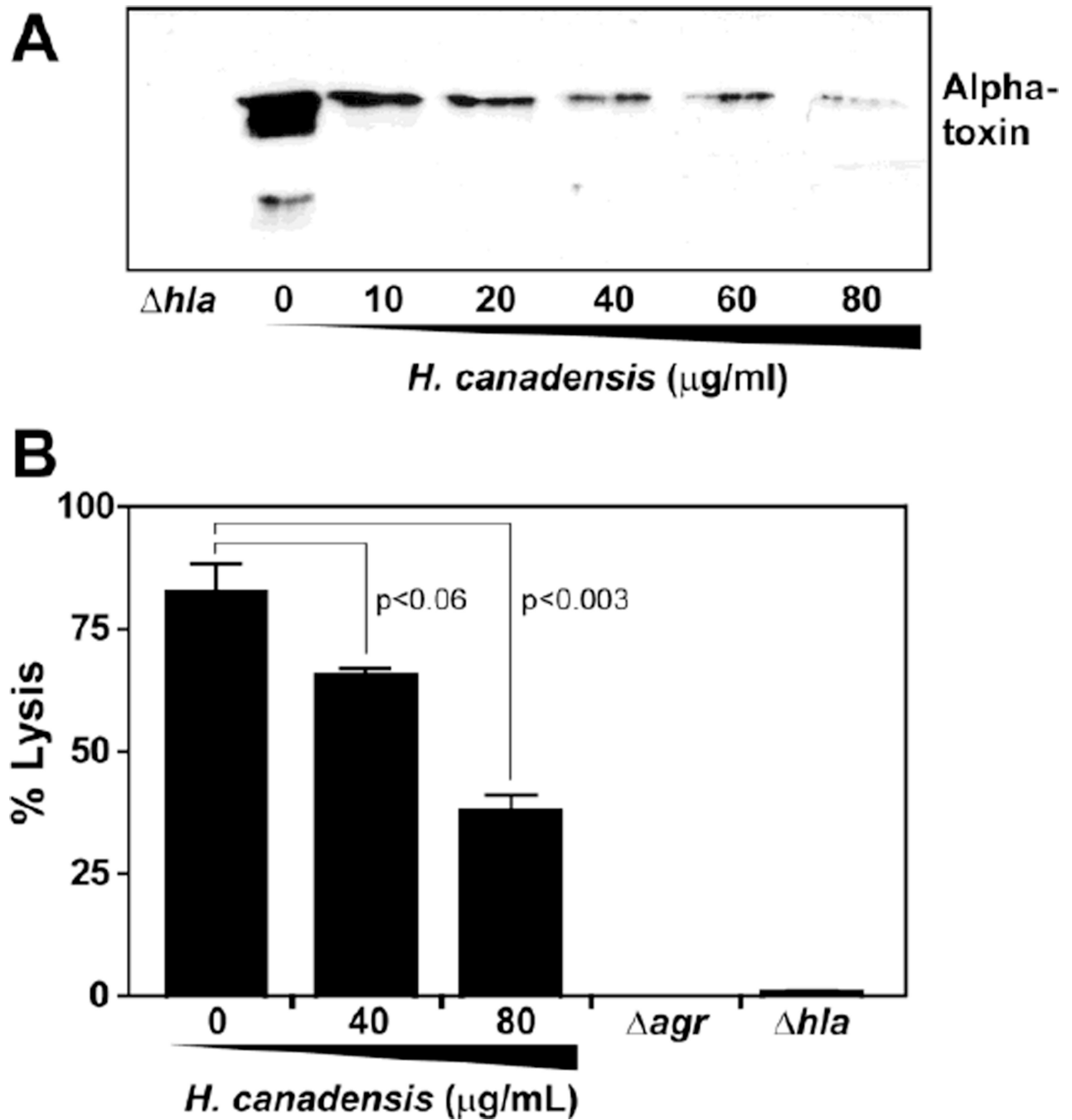
Fig. 6.
A *H. canadensis* extract inhibits MRSA toxin production and damage to human keratinocyte (HaCaT) cells. MRSA ($OD_{600}=0.1$) was exposed to extract at berberine-normalized concentrations (10, 20, 40, 60 and 80 μg/mL) of extract or vehicle control and incubated for 11 hr. Spent medium was collected and alpha-toxin immunoblots were performed (**A**). Confluent layers of HaCaT cells were generated and the same extract treated samples were incubated with the cells to assess damage with a lactate dehydrogenase assay (**B**). As controls, alpha-toxin (*hla*) and *agr* quorum sensing mutants were used. The values reported

for % lysis are averages of triplicate assay wells (with error bars representing standard deviation of the mean), and p- values were calculated with a two-tailed student's t-test.