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 prevention of infection.

Keywords: Hydrastis canadensis | Ranunculaceae | quorum quenching | berberine | methicillin-resistant Staphylococcus aureus | anti-virulence

Article:

Introduction
The European Antimicrobial Surveillance System estimates that as many as 52 million people worldwide are colonized with multidrug resistant bacteria. Among the most 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 drugresistant 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 anMRSA anti-virulence strategy is the quorum sensing system [5, 6].

S. aureus quorum sensing system, also called the accessory gene regulator or “agr” system (Fig. 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 sig-nal 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].
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.

It was our objective to investigate the in vitro effectiveness of the popular [9] herbal medicine goldenseal (Hydrastis canadensis L., Ranunculaceae) 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, alphatoxin (Δhla::Erm) [28] or agr (Δagr::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 and canadine (tetrahydroberberine, purity > 98% by HPLC, stereochemistry unconfirmed) from Chromadex. 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. Ethanol (95%), HPLC grade acetonitrile, and HPLC grade methanol were obtained from Pharmaco-AAPER. Water was purified with a nanodiamond system (Barnstead).

Plant material

Cultivated Hydrastis canadensis L. was harvested in September 2008 from: N 35°24.277′, W 082°20.993′, 702.4m 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 1S, Supporting Information) was accomplished using an established method [17,30]. Constituent identities were confirmed on an LTQ-Orbitrap high-resolution mass spectrometer (Thermo) 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 5.0min; 70% to 50% B from 5 to 7.0min; 50% to 45% from 7.0 to 7.5min, 45% to 0% B from 7.5 to 8.0 min, isocratic at 0% B from 8.0 to 8.5min and 0 to 90% from 8.5 to 9.0min. MS analysis was conducted in the positive ion mode for alkaloids and the negative ion mode for flavonoids, with a 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 600nm 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 × 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 OD600 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 subinhibitory 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 M200 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 (50mL) containing 10mL of TSB and a range of subinhibitory 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.

Supporting information

Tables summarizing the concentrations of flavonoids and alkaloids in the Hydrastis canadensis leaf extract and MIC values of H. canadensis alkaloids against several strains of MRSA as well as a comparison of the base peak LC-MS chromatograms for an equimolar mixture of the standard alkaloids berberine, hydrastine, and canadine are available as Supporting Information.

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 Fig. 1S) 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 (Fig. 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 as 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-desmethyisideroxylin (Fig. 3), which have previously been reported as 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).
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 twofold 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 OD600 of triplicate assay wells.

Fig. 3 Structures of major alkaloids, berberine (1), (1R,9S)-(-)-β-hydrastine (2), and canadine (tetrahydroberberine) (3), as well as flavonoids, sideroxylin (4), 8-desmethyl-sideroxylin (5), and 6-desmethyl-sideroxylin (6), found in Hydrastis canadensis.
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). Notably, two other goldenseal alkaloids [10], canadine and hydrastine (Fig. 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 subinhibitory 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 (Fig. 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 (Fig. 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 to 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 (Fig. 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 (Fig. 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 (Fig. 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.
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 the extract (standardized to berberine) was tested at 25 and 50 μg/mL. These concentrations were chosen because they were subinhibitory 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 a 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 (OD600 = 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.

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. Alphatoxin 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, alphatoxin levels decreased in concert as assessed by immunoblot (Fig. 6A). These findings verify the transcriptional reporter assay results (Figs. 4 and 5). As expected, the USA300 Δhla 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 wild-type and mutant strains as controls. Spent media from USA300 strain AH1263 or derivatives with a Δagr or Δhla mutation were exposed to the HaCaT cells, and cell viability was assessed using a lactate dehydrogenase (LDH) release assay (Fig. 6B). As anticipated, the wild-type USA300 caused significant damage (83% killing), while the Δagr and Δhla 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 hydastine. 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 subinhibitory concentrations quench the agr quorum sensing system, and that this activity is not due to the major alkaloids berberine, hydastine, or canadine. The most likely mechanism by which this quorum quenching effect occurs is attenuation of signal transduction through the AgrCA twocomponent 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.

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Conflict of Interest

The authors report no conflict of interest.

References


6 Antunes LCM, Ferreira RBR, Buckner MMC, Finlay BB. Quorumsensing in bacterial virulence. Microbiology 2010; 156: 2271-2282


9 Blumenthal M. Herb market levels after five years of boom. Herbalgram 1999; 47: 64–65


13 Villinski JR, Dumas ER, Chai HB, Pezzuto JM, Angerhofer CK, Gafner S. Antibacterial activity and alkaloid content of Berberis thunbergii, Berberis vulgaris and Hydrastis canadensis. Pharm Biol 2003; 41: 551–557


19 Moore M. Herb formulas for clinic and home. Albuquerque, NM: Southwest School of Botanical Medicine; 1995


31 Cech RA. Making plant medicine. Williams: Horizon Herbs; 2000: 276

32 Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd edition. Villanova, PA: National Committee for Clinical Laboratory Standards; 1993


