Planta Medica

Journal of Medicinal Plant and Natural Product Research

Publishers

Georg Thieme Verlag KG
Stuttgart · New York
Rüdigerstraße 14
D-70469 Stuttgart
Postfach 30 11 20
D-70451 Stuttgart

Thieme Publishers
333 Seventh Avenue
New York, NY 10001, USA
www.thieme.com

Quorum Sensing Inhibitors of *Staphylococcus aureus* from Italian Medicinal Plants

Authors **Cassandra L. Quave**[1], **Lisa R. W. Plano**[2], **Bradley C. Bennett**[1]

Affiliations
[1] Department of Biological Sciences, Florida International University, Miami, FL, USA
[2] Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL, USA

Key words
- quorum sensing
- MRSA
- medicinal plants
- δ-toxin
- δ-hemolysin
- *agr*

Abstract

Morbidity and mortality estimates due to methicillin-resistant *Staphylococcus aureus* (MRSA) infections continue to rise. Therapeutic options are limited by antibiotic resistance. Anti-pathogenic compounds, which inhibit quorum sensing (QS) pathways, may be a useful alternative to antibiotics. Staphylococcal QS is encoded by the *agr* locus and is responsible for the production of δ-hemolysin. Quantification of δ-hemolysin found in culture supernatants permits the analysis of *agr* activity at the translational rather than transcriptional level. We employed reversed phase high performance chromatographic (RP-HPLC) techniques to investigate the anti-QS activity of 168 extracts from 104 Italian plants through quantification of δ-hemolysin. Extracts from three medicinal plants (*Ballota nigra*, *Castanea sativa*, and *Sambucus ebulus*) exhibited a dose-dependent response in the production of δ-hemolysin, indicating anti-QS activity in a pathogenic MRSA isolate.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Emerging infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) pose a significant threat to hospital patients as the rates of nosocomial infection steadily rise [1]. Healthcare-associated MRSA (HA-MRSA) is often multidrug-resistant (MDR), and therapeutic options are rapidly becoming more limited as new resistant phenotypes arise. Even more alarming, perhaps, is the recent emergence of highly virulent community-acquired MRSA (CA-MRSA) [2,3]. Many of these CA-MRSA strains, most notably those of the USA300 clonal lineage, have the capacity to cause a serious, life-threatening infection even in otherwise healthy individuals [4]. This accounts in large part for the observation that, in the United States alone in 2005, an estimated 94360 patients suffered from invasive infection caused by MRSA, with approximately 18650 resulting in a fatal outcome [5].

One approach to drug discovery for the treatment of staphylococcal infections is through the study of natural products from traditional remedies, such as those made from plants. Plants are engaged in a struggle for survival that involves the production and release of secondary metabolites into their immediate environment both as a means of defense against potential pathogens and offense against competing species. If viewed in the light of chemical ecology, it is logical then that certain plants would be geared towards the production of antimicrobial compounds that limit the ability of microbes to produce the factors required for virulence and successful colonization.

Most research on the activity of botanical natural products towards *S. aureus* is focused on growth inhibition, while some have focused on inhibition of the MDR mechanisms, such as efflux pumps [6–9]. No studies on the *agr*-inhibiting or quorum sensing inhibiting (QSI) activity of botanical natural products on *S. aureus* have been conducted thus far. Inhibition of staphylococcal QS pathways could potentially limit the degree of pathogenicity posed by some *S. aureus* strains by blocking the production of certain virulence factors. Moreover, the inhibition of staphylococcal pathogenesis could be accomplished without growth inhibition, thus potentially avoiding selective pressures for drug resistance.

The staphylococcal QS system is a cell-density-dependent mechanism for controlling protein expression, including the production of staphylococcal virulence factors such as α-, β-, and δ-he-

received January 24, 2010
revised June 23, 2010
accepted June 26, 2010

Bibliography
DOI http://dx.doi.org/10.1055/s-0030-1250145
Published online July 19, 2010
Planta Med 2010; 76: 1–8

ISSN 0032-0943

Correspondence
Dr. Cassandra L. Quave
Department of Microbiology and Immunology
College of Medicine
University of Arkansas for Medical Sciences
4301 W. Markham St.
Mail Slot 511
Little Rock, AR 72205-7199
USA
Phone: + 17 8 62 39 80 46
Fax: + 15 0 16 86 53 59
cassy.quave@gmail.com

This is a copy of the author's personal reprint

molysins. It is encoded by the *agr* locus, which is a quorum-sensing gene cluster of five genes (*hld, agrA, agrB, agrC*, and *agrD*) [10].

Staphylococcal δ-hemolysin, or δ-toxin, is a translational protein product of RNAIII. It is a 26-amino-acid polypeptide with surfactant-like properties [11]. Translation of *hld*, the gene for δ-hemolysin, occurs about one hour after transcription of RNAIII. There are two forms of δ-toxin that can be found in the culture supernatant: formylated (with an N-terminal methionine) and deformylated. These forms are represented by two distinct peaks in the reversed phase high-performance chromatography (RP-HPLC) chromatogram (**Fig. 1S**). δ-Toxin accumulates in the culture medium in both forms and is approximately 90% formylated and 10% deformylated. This ratio is due to the arrest of deformylated δ-toxin production during the post-exponential growth phase, whereas formylated δ-toxin continues to accumulate. Somerville et al. [12] suggest that this change may be linked to iron availability in the culture medium.

Quantification of δ-toxin produced by *S. aureus* and found in the culture supernatants allows for the analysis of *agr* activity at the translational rather than transcriptional level. The identification of *agr*-inhibiting drugs or staphylococcal QS-inhibitors has been proposed by several research groups as a potential anti-staphylococcal therapy [13–17]. In 2000, Otto and Götz [11] provided a fast method for δ-toxin quantification using RP-HPLC techniques for the analysis of staphylococcal culture filtrates. We apply this method for the first time as a screening tool for identifying plant extracts with QSI activity concerning a strain of HA-MRSA known as pulsed-field type (PFT) USA500.

USA500 isolates are SCCmecIV and MLST ST8. USA500 demonstrates high virulence in animal infection models and has a high capacity to evade host defense mechanisms, including the lysis of neutrophils [18]. USA500 isolates are highly multidrug-resistant and tend to be associated with nosocomial transmission [19,20]. They are also associated with the production of many virulence factors, including δ-hemolysin cytolytic toxins and phenol-soluble modulins (including α-toxin), among others. A recent genetic study has demonstrated that the emerging highly virulent CA-MRSA USA300 lineage actually evolved from USA500 [18]. There is a critical need for novel therapeutic options in the treatment of highly virulent MDR staphylococcal infection, such as those caused by USA500.

We have quantified the amount of δ-hemolysin found in the supernatant of *S. aureus* cultures treated with plant extracts as a means of measuring the impact of plant products on the staphylococcal quorum sensing (QS) system. We examined 168 crude extracts made from 104 Italian plants, representing 44 plant families.

Materials and Methods

▼

Plant material and extraction

Ethnobotanical surveys of plants used in the traditional pharmacopoeia of the Vulture-Alto Bradano region of Basilicata, southern Italy were conducted, and the results are described in previous works [21–24]. Bulk and voucher specimens were collected and identified in 2006 by C.L. Quave. Voucher specimens of plants were deposited at the Herbarium Lucanum (HLUC) in Potenza, Italy and Fairchild Tropical Botanic Gardens (FTG) in Miami, FL, USA.

Dry plant materials were ground into a fine powder using a homogenizer. Ethanolic extracts of all plant samples were made by soaking in 95% denatured EtOH using a ratio of 1 g (plant material):10 mL (EtOH) for 72 h. Flasks were agitated daily. Water extracts were made by boiling 1 g (plant material): 50 mL (dH_2O) for 30 minutes. Extracts were vacuum filtered and rotary evaporated, then frozen and lyophilized. Stock concentrations of 10 mg/mL of dry extract in the excipient (DMSO or dH_2O) were prepared, sterile filtered (0.2 µm) and stored in the dark at 4 °C. The excipient (DMSO or dH_2O) made up less than 5.1% of the final test solution for MIC assays and less than 2.5% for δ-toxin assays.

Bacteria and culture conditions

HA-MRSA PFT USA500 (NRS385) was obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) repository [19]. We screened 10 different *S. aureus* isolates from our lab collection for δ-hemolysin production and selected this particular strain based on the high levels of δ-hemolysin detected in the RP-HPLC method detailed below. Bacteria were grown on tryptic soy broth (TSB) or agar plates (TSA) for 18 h at 37 °C. A 1 : 20 dilution of a standardized inoculum (0.5 McFarland Standard) was used to create final inoculum densities of $5–8 \times 10^5$ CFU/mL from overnight cultures using the direct suspension method [25] for MIC and δ-toxin assays. Inoculum densities were confirmed by taking colony counts using the spread plate method at the time of inoculation.

Determination of minimum inhibitory concentrations (MIC)

MIC's were determined following the NCCLS standard broth microdilution methods [26] in sterile flat-bottom 96-well polystyrene plates. Briefly, test strains were inoculated into 0.1 mL tryptic soy broth (TSB) containing the extract at concentrations ranging from 8–512 µg/mL. The optical density (OD_{600}) was assessed immediately after inoculation and again after 18 hrs using a KC4 microplate reader. Corrections for extract color, which can alter the OD output, were done as previously described [27]. We included negative controls (cells + TSB), positive controls (cells + TSB + antibiotics – vancomycin, ampicillin, and trimethroprim-sulfamethoxazole; MP Biomedicals, > 98% purity for all antibiotics), vehicle controls (cells + TSB + DMSO), and media controls (TSB). All tests were performed in triplicate. Results are reported as the MIC for growth at 18 hours post-inoculation.

Quantification of δ-toxin production

Polystyrene 24-well culture plates were prepared with a total volume of 1 mL per well of TSB, an initial sub-MIC test concentration of 64 µg of extract suspended in DMSO (< 1% DMSO in total well volume) and bacteria. We chose this concentration as it was well below the MIC for all of the extracts examined. By testing at concentrations too low to significantly impact growth, we could gain a better assessment of how δ-toxin production was affected. Screening at concentrations high enough to inhibit growth will yield more false-positives as δ-toxin production is lower in cultures that do not grow well.

Extracts demonstrating activity, as exhibited by lower δ-toxin levels, were also investigated at a range of test concentrations from 8–256 µg/mL. Controls for media, growth, and growth in the carrier solvent (DMSO) were also performed. Liquid test cultures were grown for 15 hours at 37 °C and aerated by shaking at 150 rpm. All tests were performed in triplicate.

Aliquots of bacteria (2 mL) were centrifuged for 5 min at 14 000 × g with a microcentrifuge. Supernatants were removed and stored at − 20 °C until HPLC analysis. The concentration of δ-toxin was measured by RP-HPLC with a 1-mL Resource PHE column (GE Healthcare) as previously described [11], except that 200 µL of supernatant (as opposed to 500 µL) was injected onto the column using a Thermo Spectra-System HPLC apparatus, equipped with a Diode Array Detector and autosampler (Thermo Electron Corporation) as well as ChromQuest 4.1 software.

δ-Hemolysin eluted at a retention time of about 6.4 minutes (deformylated) and 6.8 minutes (formylated) after sample injection as two distinct peaks. Integration of the δ-toxin peak area was performed at 280 nm. We confirmed the identity of δ-toxin peaks by peak fractionation and LC-mass spectrometry (**Fig. 1S**) using a Thermo Finnigan Deca XP max ion trap mass spectrometer and surveyor LC with autosampler and diode array detector (Thermo Electron Corporation) using conditions previously described [12]. The peak areas for both the formylated and deformylated forms of δ-hemolysin were calculated using ChromQuest software. The sum of both peaks was calculated and used to calculate the mean percent inhibition of δ-toxin production for the replicate tests in relation to the mean peak area of the excipient (DMSO) growth controls.

Regarding the issue of plant color in the protocol for δ-toxin detection, we ran controls with extract and media alone using the same RP-HPLC conditions described above, and no peaks were observed, indicating that extract does not interfere with the results of this particular test.

Statistical analysis

All experiments were carried out in triplicate. Pairwise testing was performed based on t-tests as formatted in Microsoft Excel and SPLUS software with p values < 0.05 considered significant.

Supporting information

A HPLC chromatogram of the *S. aureus* supernatant showing both δ-hemolysin peaks (formylated and deformylated) is provided with accompanying LC-MS spectrograms, confirming the peak identities (**Fig. 1S**). In addition, HPLC chromatograms of supernatants collected after treatment with a range of 8–256 µg/mL of extract (*Ballota nigra* stems, *Castanea sativa* leaves, and *Sambucus ebulus* leaves) are provided and demonstrate a dose-dependent response in δ-hemolysin production (**Fig. 2S**).

Results

There was a broad low level response of δ-toxin inhibition to the screening test concentration of 64 µg/mL. QSI activity was apparent in 90% of the extracts tested (**Table 1**). No QSI activity was apparent in aqueous extracts. This suggests that the active QSI components are predominantly low to medium in polarity. Extracts were not effective at inhibiting growth of this multidrug-resistant strain of HA-MRSA (USA500/NRS385). Only 6% of extracts demonstrated a MIC_{50} at concentrations of 256–512 µg/mL. None demonstrated a MIC_{90} at concentrations ≤ 512 µg/mL. Cytotoxicity of the extracts against a CCRF-CEM human T lymphoblastoid cell line (ATCC CCL-119) was assessed using an MTT cell proliferation assay and has been previously reported [28].

A total of 15 ethanolic extracts inhibited the production of δ-toxin by ≥ 50% in comparison to controls at the preliminary screen concentration of 64 µg/mL: *Sambucus ebulus* (leaves), *Achillea ageratum* (aerial parts), *Borago officinalis* (whole plant), *Cerinthe major* (whole plant), *Knautia arvensis* (aerial parts), *Acacia dealbata* (inflorescence), *Castanea sativa* (leaves), *Ballota nigra* (stems), *Rosmarinus officinalis* (aerial parts), *Salvia pratensis* (inflorescence), *Malva sylvestris* (flowers), *Aceras anthropophora* (whole plant), *Orchis purpurea* (whole plant), *Orchis italica* (whole plant), and *Crataegus monogyna* (aerial parts). However, upon further testing of these extracts at a concentration range of 8–256 µg/mL, we found that only three of these demonstrated significant dose-dependent δ-toxin inhibition independent of any growth-inhibitory effects: *B. nigra, C. sativa*, and *S. ebulus* (**Fig. 1** and **2S**).

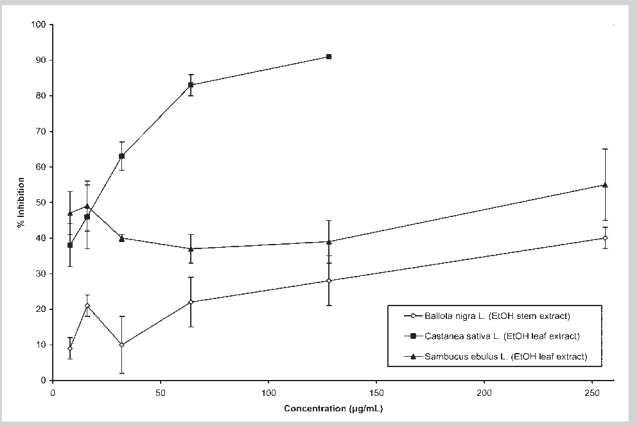

Fig. 1 Percent inhibition of δ-toxin peak area after treatment with extracts of *Ballota nigra, Castanea sativa*, and *Sambucus ebulus*.

Discussion

Quantification of δ-hemolysin in the supernatant of staphylococcal cultures can be used as a measure of *agr* system or QS activity [11–13]. The *agr* system controls approximately 150 genes and is critical to *S. aureus* virulence [29]. Although blocking certain virulence pathways (such as *agr*-regulated toxin production) would not necessarily prevent *S. aureus* from entering and growing in a host, it could impede processes related to colonization and delay the establishment of infection, affording the host immune response more time to combat the pathogen.

While the staphylococcal QS system is a useful target for the discovery and development of new anti-pathogenic drugs, the dynamic nature of the *agr* system must not be overlooked. There is the risk that downregulation or manipulation of certain gene promoters could result in the unintended consequence of upregulating the production of another virulence factor. In particular, a better understanding of the effect that *agr* manipulation can have on the development of infection *in vivo* is necessary. For example, inhibiting *agr* activity during certain times in the infection process can lead to deleterious effects, such as increased biofilm formation [30].

In a previous study of these same extracts, we examined the anti-biofilm and bacteriostatic activity against another strain of *S. aureus* (ATCC 33593) [27]. We found that none of the most promising QS inhibitors reported here (*B. nigra, C. sativa*, and *S. ebulus*) exhibited any anti-biofilm activity, and they did not appear to promote biofilm formation in an *in vitro* context. However, in order

This is a copy of the author's personal reprint

Table 1 Inhibition of δ-toxin and minimal inhibitory concentrations of plant extracts against MRSA (strain I. D. NRS385/PFT USA500).

Family	Botanic Name	Voucher ID	Plant Part	Ethnobotanical Use*	Extract Solvent	Percent Inhibition of δ-toxin Production**	MIC_{50}***
Adoxaceae	*Sambucus ebulus* L.	CQ-168	inflorescence	N	EtOH	45	–
			leaves	S	EtOH	48	–
			stems	N	EtOH	28	–
	Sambucus nigra L.	CQ-151	woody parts	R	EtOH	29	–
			leaves	S	EtOH	36	–
					dH_2O	–	–
			inflorescence	S; R	EtOH	38	–
					dH_2O	–	–
			infructescence	F	EtOH	34	–
Alliaceae	*Allium cepa* L.	CQ-206	leaves; bulbs; roots	S; M; F	EtOH	22	–
Apiaceae	*Daucus carota* L.	CQ-215	leaves; stems	N	EtOH	2	–
			inflorescence; infructescence	N	EtOH	39	–
	Foeniculum vulgare ssp. *piperitum* (Ucria) *Coutinho*	CQ-192	leaves; stems	M; F	EtOH	–	–
	Foeniculum vulgare ssp. *vulgare* Mill.	CQ-196	leaves; stems	M	EtOH	8	–
	Tordylium apulum L.	CQ-101	flowers; leaves; roots; stems	N	EtOH	25	–
Apocynaceae	*Vinca major* L.	CQ-117	flowers; leaves; roots; stems	M	EtOH	26	–
Aracaeae	*Arum italicum* Mill.	CQ-175	stems	N	EtOH	28	–
			fruits	N	EtOH	15	–
			stalks	N	EtOH	2	–
			leaves	S	EtOH	22	–
Asphodelaceae	*Asphodelus microcarpus* Salzm. & Viv.	CQ-109	inflorescence	N	EtOH	19	–
			leaves	N	EtOH	17	–
Asteraceae	*Achillea ageratum* L.	CQ-219	leaves; stems; flowers	M	EtOH	66	512
	Achillea millefolium L.	CQ-176	inflorescence	M	EtOH	41	–
			leaves; stems	M	EtOH	23	–
			leaves; stems; flowers	M	EtOH	38	–
	Anacyclus tomentosus DC.	CQ-167	leaves; stems; flowers	N	EtOH	36	–
	Cichorium intybus L.	CQ-106	basal leaves; roots	F	EtOH	23	–
					dH_2O	–	–
			leaves; stems; flowers	F	EtOH	8	–
	Matricaria recutita L.	CQ-118	flowers; leaves; roots; stems	S; M	EtOH	29	512
					dH_2O	–	–
	Scolymus hispanicus L.	CQ-199	leaves; stems; flowers	N	EtOH	23	–
	Tussilago farfara L.	CQ-202	leaves; stems; roots	S	EtOH	16	–
	Urospermum dalechampii (L.) Scop.	CQ-134	flowers; leaves; roots; stems	N	EtOH	14	–
Boraginaceae	*Anchusa officinalis* L.	CQ-128	leaves; stems; flowers	N	EtOH	34	–
	Borago officinalis L.	CQ-100	flowers; leaves; roots; stems	M	EtOH	54	–
					dH_2O	–	–
	Cerinthe major L.	CQ-110	flowers; leaves; roots; stems	N	EtOH	48	–
	Echium italicum L.	CQ-162	leaves; stems; flowers	N	EtOH	32	–
Brassicaceae	*Brassica rapa* subsp. *rapa*	CQ-104	flowers; leaves; roots; stems	F	EtOH	27	–
	Cardaria draba (L.) Desv.	CQ-140	flowers; leaves; roots; stems	N	EtOH	12	–
	Eruca sativa Mill.	CQ-102	flowers; leaves; roots; stems	N	EtOH	13	–
	Sisymbrium officinale (L.) Scop.	CQ-131	flowers; leaves; roots; stems	N	EtOH	20	–
Caprifoliaceae	*Lonicera alpigena* L.	CQ-213	woody parts	N	EtOH	28	–
			leaves	N	EtOH	25	–
Caryophyllaceae	*Saponaria officinalis* L.	CQ-210	leaves; stems; flowers	N	EtOH	4	–
	Silene alba (Mill.) E. H. L. Krause	CQ-123	leaves; stems; flowers	N	EtOH	43	–
	Silene nutans L.	CQ-125	leaves; stems; flowers	N	EtOH	43	–
Cucurbitaceae	*Ecballium elaterium* (L.) A. Richard	CQ-169	leaves; stems; flowers	S	EtOH	21	–
Dennstaedtiaceae	*Pteridium aquilinium* (L.) Kuhn	CQ-211	leaves	N	EtOH	–	–
			stems	N	EtOH	24	–
Dipsacaceae	*Dipsacus fullonum* L.	CQ-201	leaves; stems	N	EtOH	28	–
			flowers	N	EtOH	28	–
	Knautia arvensis Coult.	CQ-190	leaves; stems; flowers	N	EtOH	48	–
	Knautia lucana Lacaita & Szabo	CQ-166	leaves; stems; flowers	N	EtOH	6	–

continued next page

This is a copy of the author's personal reprint

Table 1 Inhibition of δ-toxin and minimal inhibitory concentrations of plant extracts against MRSA (strain I. D. NRS385/PFT USA500). *continued*

Family	Botanic Name	Voucher ID	Plant Part	Ethnobotanical Use*	Extract Solvent	Percent Inhibition of δ-toxin Production**	MIC_{50}***
Equisetaceae	*Equisetum arvense* L.	CQ-226	stems; leaves	N	EtOH	22	–
Fabaceae	*Acacia dealbata* Link	CQ-115	inflorescence	O	EtOH	56	–
			stems	O	EtOH	38	–
			leaves; stems	O	EtOH	21	–
	Anthyllis vulneraria L.	CQ-147	leaves; stems; flowers	N	EtOH	28	–
	Astragalus monspessulanus L.	CQ-112	leaves; stems; flowers; roots	N	EtOH	36	–
	Coronilla emerus L.	CQ-137	leaves; flowers	N	EtOH	33	–
			woody stems	N	EtOH	14	–
	Melilotus alba Medik.	CQ-193	leaves; stems; flowers	N	EtOH	43	–
	Robinia pseudoacacia L.	CQ-155	woody parts	N	EtOH	32	–
			leaves	N	EtOH	–	–
			inflorescence	N	EtOH	21	–
	Spartium junceum L.	CQ-144	leaves; stems; flowers	A	EtOH	22	–
	Trifolium repens L.	CQ-138	leaves; stems; flowers; roots	N	EtOH	4	–
	Vicia craca L.	CQ-149	leaves; stems; flowers; roots	N	EtOH	19	–
	Vicia faba L.	CQ-103	leaves; stems; flowers; roots	F	EtOH	14	–
	Vicia sativa subsp. *angustifolio*	CQ-124	leaves; stems; flowers	N	EtOH	22	512
	Vicia sativa subsp. *sativa*	CQ-119	leaves; stems; flowers	N	EtOH	29	–
	Wisteria sinensis (Sims) Sweet	CQ-126	inflorescence	O	EtOH	36	–
			stems	O	EtOH	39	–
			leaves	O	EtOH	41	–
Fagaceae	*Castanea sativa* Mill.	CQ-191	inflorescence	N	EtOH	20	–
			leaves	N	EtOH	70	512
			woody parts	A	EtOH	32	512
	Quercus cerris L.	CQ-228	leaves	N	EtOH	27	–
			stems; fruits	N	EtOH	37	–
Gentianaceae	*Centaurium pulchellum* (Sw.) Druce	CQ-217	leaves; stems; flowers; roots	N	EtOH	21	–
Geraniaceae	*Erodium ciconium* (L.) L'Hér.	CQ-142	leaves; stems; flowers; roots	N	EtOH	34	–
	Erodium malacoides (L.) L'Hér. ex Aiton	CQ-121	leaves; stems; flowers	N	EtOH	7	512
	Geranium columbinum L.	CQ-129	leaves; stems; flowers	N	EtOH	–	–
Hyacinthaceae	*Leopoldia comosa* (L.) Parl.	CQ-105	bulbs	M; F	EtOH	21	–
					dH_2O	–	–
			leaves; inflorescence	N	EtOH	31	–
Hypericaceae	*Hypericum perforatum* L.	CQ-183	leaves; stems; flowers	S	EtOH	36	–
Juglandaceae	*Juglans regia* L.	CQ-181	immature fruits	S; C	EtOH	–	–
			leaves	R	EtOH	39	–
			woody parts	N	EtOH	17	–
Juncaceae	*Juncus articulatus* L.	CQ-216	leaves; fruits	N	EtOH	32	–
Lamiaceae	*Ballota nigra* L.	CQ-160	stems	S; M	EtOH	76	–
			roots	N	EtOH	37	–
			leaves	S; M	EtOH	47	–
			leaves; stems; flowers	S; M	EtOH	47	–
					dH_2O	–	–
	Clinopodium vulgare L.	CQ-182	leaves; stems; flowers	N	EtOH	40	–
	Marrubium vulgare L.	CQ-170	leaves; stems; flowers	S; M	EtOH	40	–
					dH_2O	6	–
			roots	N	EtOH	40	–
	Mentha pulegium L.	CQ-200	leaves; stems; flowers; roots	F	EtOH	36	–
	Mentha spicata L.	CQ-224	leaves; stems; flowers	F	EtOH	28	–
	Origanum heracleoticum L.	CQ-207	leaves; stems; flowers	F	EtOH	30	–
	Phlomis herba-venti L.	CQ-168	leaves; stems; flowers	N	EtOH	16	–
	Rosmarinus officinalis L.	CQ-113	leaves; stems; flowers	F; S	EtOH	58	256
	Salvia pratensis L.	CQ-165	leaves; stems	N	EtOH	23	–
			inflorescence	N	EtOH	58	–
	Salvia virgata Jacq.	CQ-127	leaves; stems; flowers	N	EtOH	42	256
	Stachys tymphaea Hausskn.	CQ-189	leaves; stems; flowers	N	EtOH	41	–
Liliaceae	*Lilium candidum* L.	CQ-174	leaves; stems	N	EtOH	37	–
			inflorescence	N	EtOH	30	–

continued next page

This is a copy of the author's personal reprint

Table 1 Inhibition of δ-toxin and minimal inhibitory concentrations of plant extracts against MRSA (strain I. D. NRS385/PFT USA500). *continued*

Family	Botanic Name	Voucher ID	Plant Part	Ethnobotanical Use*	Extract Solvent	Percent Inhibition of δ-toxin Production**	MIC_{50}***
Malvaceae	*Alcea rosea* L.	CQ-205	leaves; stems; flowers; roots	O	EtOH	18	–
	Malva sylvestris L.	CQ-156	stems	S; M	EtOH	22	–
					dH_2O	–	–
			flowers	S; M	EtOH	53	–
			leaves	S; M	EtOH	34	–
					dH_2O	–	–
Moraceae	*Ficus carica* L.	CQ-173	leaves	N	EtOH	24	–
			woody parts	N	EtOH	21	–
			immature fruits	S; F	EtOH	41	–
Myrsinaceae	*Cyclamen hederifolium* Aiton	CQ-186	tubers	M	EtOH	10	–
Nyctaginaceae	*Mirabilis jalapa* L.	CQ-222	leaves; flowers; fruits	N	EtOH	16	–
Oleaceae	*Olea europaea* L.	CQ-197	leaves	N	EtOH	40	–
			woody parts	A	EtOH	24	–
Orchidaceae	*Aceras anthropophora* R. Br.	CQ-153	leaves; stems; flowers; roots	N	EtOH	51	–
	Orchis italica Poir.	CQ-133	inflorescence; leaves; stems	N	EtOH	49	–
	Orchis purpurea Huds.	CQ-132	inflorescence; leaves; stems	N	EtOH	57	–
Papaveraceae	*Fumaria officinalis* L.	CQ-107	leaves; stems; flowers; roots	N	EtOH	44	–
	Papaver rhoeas subsp. *rhoeas*	CQ-145	leaves; stems; flowers; roots	F	EtOH	25	–
	Papaver somniferum L.	CQ-178	leaves; stems; flowers; roots	M; R	EtOH	36	–
Plantaginaceae	*Digitalis ferruginea* L.	CQ-227	leaves; stems; flowers	N	EtOH	32	–
	Linaria vulgaris Hill	CQ-223	leaves; stems; flowers; roots	N	EtOH	25	–
	Plantago major L.	CQ-225	leaves; stems; flowers; roots	S; M	EtOH	12	–
Poaceae	*Agropyron repens* (L.) P. Beauv.	CQ-208	leaves; stems; roots	M	EtOH	19	–
	Arundo donax L.	CQ-146	stem internodes	A; R	EtOH	22	–
			stem nodes	S	EtOH	35	–
					dH_2O	–	–
			leaves; stems	A; R	EtOH	10	–
Polygonaceae	*Rumex crispus* L.	CQ-171	leaves; stems; fruits	S	EtOH	25	–
Pottiaceae	*Syntrichia ruralis* (Hedw.) Web. & Mohr	CQ-229	whole plant	N	EtOH	45	–
Ranunculaceae	*Delphinium fissum* Waldst. & Kit.	CQ-187	leaves; stems; flowers; fruits	N	EtOH	29	–
	Ranunculus acris L.	CQ-135	leaves; stems; flowers	N	EtOH	34	–
Rosaceae	*Crataegus monogyna* Jacq.	CQ-116	leaves; stems; flowers	M	EtOH	57	–
	Prunus spinosa L.	CQ-163	woody parts; leaves	M	EtOH	33	–
			fruits	N	EtOH	29	512
	Rosa canina var. *canina*	CQ-152	fruits	N	EtOH	16	–
			woody parts	N	EtOH	44	–
			leaves; stems	N	EtOH	14	512
	Rubus ulmifolius Schott	CQ-164	leaves; stems; flowers	S	EtOH	10	–
			leaves	S	EtOH	17	–
			roots	M	EtOH	21	–
			woody stems	N	EtOH	16	512
Rubiaceae	*Galium verum* L.	CQ-177	leaves; stems; flowers	N	EtOH	27	–
Scrophulariaceae	*Verbascum sinuatum* L.	CQ-218	leaves; stems; flowers	N	EtOH	34	–
	Verbascum thapsus L.	CQ-172	stems	M	EtOH	31	–
			leaves	M	EtOH	40	–
			inflorescence	M	EtOH	38	–
Ulmaceae	*Ulmus minor* L.	CQ-195	leaves	N	EtOH	23	–
			woody parts	M	EtOH	–	–
Urticaceae	*Parietaria diffusa* Mert. & Koch	CQ-212	leaves; stems; fruits; roots	M	EtOH	19	–
	Urtica dioica L.	CQ-179	leaves; stems; flowers	S; M; F	EtOH	36	–
Valerianaceae	*Centranthus ruber* (L.) DC.	CQ-143	leaves; stems; inflorescence	M	EtOH	31	–
Vitaceae	*Vitis vinifera var. aglianico*	CQ-209	wine	S; F		–	–
			stems	N	EtOH	–	–
			fruits	F	EtOH	21	–
			leaves	N	EtOH	22	–

* Ethnobotanical use of specific plant part(s) in the study region: S = medicinal application to skin; M = medicinal application not involving the skin; C = cosmetic applications; A = agricultural tool; O = ornamental; R = ritual or spiritual use; F = food; N = no reported use; ** Percent inhibition for δ-toxin based on an initial screening concentration of 64 µg/mL for MRSA PFT USA500; *** MIC values are reported as µg/mL for MRSA PFT USA500; – No inhibitory activity

This is a copy of the author's personal reprint

to account for variations in staphylococcal genotypes and phenotypes, we strongly caution that additional testing against all of the major PFT strains (especially CA-MRSA isolates from the USA-300 lineage) should be undertaken with these active extracts and fractions thereof in regards to QSI, anti-biofilm, bacteriostatic, and bactericidal activity prior to the initiation of *in vivo* studies.

Interestingly, each of the three most promising extracts comes from species that are topically applied in south Italian folk remedies for skin and soft tissue infection and inflammation [24]. Specifically, the leaves of *S. ebulus* are used to wrap the feet before putting on shoes in order to prevent the formation of foot blisters and sores. The aerial parts (leaves, flowers, and stems) of *B. nigra* are prepared in an aqueous decoction and then used to wash the skin as an anti-inflammatory and hemostatic. The leaves of *C. sativa* are topically applied to decrease swelling from varicose veins. Other ethnobotanical studies in the Mediterranean have also documented the application of these species in traditional wound healing practices [31, 32].

Regarding the chemistry of these species, *B. nigra* is rich in phenolic compounds [33], including phenylpropanoid glycosides [34], phenylpropanoid derivatives [35, 36], and flavonoids [37]. *C. sativa* [38] and *S. ebulus* [39] are also rich in phenolics, specifically regarding flavonoid content. At this point, we have not conducted bioassay-guided fractionation tests for the isolation of the active constituent(s), but it is reasonable to hazard a guess that the activity may be related to phenolic compound(s) with a low to medium level of polarity. Based on analyses of δ-hemolysin production, we have offered the first reports of plant extracts interfering with QS pathways in *S. aureus*. These results indicate that some small degree of QSI activity is evident in 90% of the ethanolic plant extracts screened, including those extracts with no growth inhibitory activity. Future changes to the protocol that may afford a more selective hit rate could be to conduct the preliminary screen at a lower test concentration of 32 µg/mL.

The validity of plant-based therapies for infection that do not exhibit activity in the standard *in vitro* bacteriostatic or bactericidal assays is oftentimes questioned. These data, however, support the concept that other mechanisms of action may be in play, which do not necessarily impact bacterial growth, but virulence mechanisms instead. These data offer a level of validity to the use of south Italian folk remedies incorporating *B. nigra*, *C. sativa*, and *S. ebulus* for the treatment of skin and soft tissue infection when *S. aureus* is implicated. Further investigation, including the bioassay-guided fractionation and isolation of active components from these three species, is recommended.

Acknowledgements

This work was funded by NIH, NCCAM (Grant # F31AT004288, PI: C. L. Quave). The content is solely the responsibility of the authors and does not necessarily reflect the official views of the National Center for Complementary and Alternative Medicine or the National Institutes of Health. Additional support for the project was provided by Botany in Action (C. L. Quave) and Anne Chatham Fellowship in Medicinal Botany (C. L. Quave). We thank Dr. Carmine Colacino (Università della Basilicata, Potenza, Italy) and Dr. Andrea Pieroni (University of Gastronomic Sciences, Pollenzo/Bra, Italy) for assisting in the taxonomic identification of plants collected. Special thanks to Dr. Michael Otto (National Institutes of Health/National Institute of Allergy and Infectious Diseases, Bethesda, USA) for assisting in the setup of the experiment. We also thank Dr. Horacio Preistap and Myron Georgiadis (Florida International University, Miami, USA) for technical support in the use of HPLC and MS equipment, respectively.

References

1 *Klevens RM, Edwards JR, Tenover FC, McDonald LC, Horan T, Gaynes R.* Changes in the epidemiology of methicillin-resistant *Staphylococcus aureus* in intensive care units in US hospitals, 1992–2003. Clin Infect Dis 2006; 43: 387–388
2 *Gould IM.* Community-acquired MRSA: can we control it? Lancet 2006; 368: 824–826
3 *Yamamoto T, Nishiyama A, Takano T, Yabe S, Higuchi W, Razvina O, Shi D.* Community-acquired methicillin-resistant *Staphylococcus aureus*: community transmission, pathogenesis, and drug resistance. J Infect Chemother, in press
4 *Hildron AI, Low CE, Honig EG, Blumberg HM.* Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* strain USA300 as a cause of necrotising community-onset pneumonia. Lancet Infect Dis 2009; 9: 384–392
5 *Klevens RM, Morrison M, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim G, McDougal LK, Carey RB, Fridkin SK.* Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA 2007; 298: 1763–1771
6 *Gibbons S, Oluwatuyi M, Kaatz GW.* A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*. J Antimicrob Chemother 2003; 51: 13–17
7 *Gibbons S, Oluwatuyi M, Veitch NC, Gray AI.* Bacterial resistance modifying agents from *Lycopus europaeus*. Phytochemistry 2003; 62: 83–87
8 *Gibbons S, Udo EE.* The effect of reserpine, a modulator of multidrug efflux pumps, on the *in vitro* activity of tetracycline against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) possessing the *tet*(K) determinant. Phytother Res 2000; 14: 139–140
9 *Pereda-Miranda R, Kaatz GW, Gibbons S.* Polyacylated oligosaccharides from medicinal Mexican morning glory species as antibacterials and inhibitors of multidrug resistamce in *Staphylococcus aureus*. J Nat Prod 2006; 69: 406–409
10 *Sakoulas G, Eliopoulos GM, Moellering RC, Wennersten C, Venkataraman L, Novick RP, Gold HS.* Accessory gene regulator *(agr)* locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. Antimicrob Agents Chemother 2002; 46: 1492–1502
11 *Otto M, Götz F.* Analysis of quorum sensing activity in staphylococci by RP-HPLC of staphylococcal delta-toxin. BioTechniques 2000; 28: 1088–1096
12 *Somerville GA, Cockayne A, Durr M, Peschel A, Otto M, Musser JM.* Synthesis and deformylation of *Staphylococcus aureus* δ-toxin are linked to tricarboxylic acid cycle activity. J Bacteriol 2003; 185: 6686–6694
13 *Otto M.* Quorum-sensing control in Staphylococci – a target for antimicrobial drug therapy? FEMS Microbiol Lett 2004; 241: 135–141
14 *Dell'Acqua G, Giacometti A, Cirioni O, Ghiselli R, Saba V, Scalise G, Gov Y, Balaban N.* Suppression of drug-resistant staphylococcal infections by the quorum-sensing inhibitor RNAIII-inhibiting peptide. J Infect Dis 2004; 190: 318–320
15 *Balaban N, Goldkorn T, Nhan RT, Dang LB, Scott S, Ridgley RM, Rasooly A, Wright SC, Carlson JR.* Autoinducer of virulence as a target for vaccine and therapy against *Staphylococcus aureus*. Science 1998; 280: 438–440
16 *Otto M, Submuth R, Vuong C, Jung G, Gotz F.* Inhibition of virulence factor expression in *Staphylococcus aureus* by the *Staphylococcus epidermidis agr* pheromone and derivatives. FEBS Lett 1999; 450: 257–262
17 *Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick RP, Muir TW.* Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc Natl Acad Sci USA 1999; 96: 1218–1223
18 *Li M, Diep BA, Villaruz AE, Braughton KR, Jiang X, DeLeo FR, Chambers HF, Lu Y, Otto M.* Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci USA 2009; 106: 5883–5888

This is a copy of the author's personal reprint

19 *NARSA*. The Network on Antimicrobial Resistance in *Staphylococcus aureus*. Available at http://www.narsa.net. Accessed March 20, 2010
20 *McDougal LK, Steward CD, Killgore GE, Chaitram J, McAllister SK, Tenover FC.* Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol 2003; 41: 5113–5120
21 *Pieroni A, Nebel S, Quave CL, Munz H, Heinrich M.* Ethnopharmacology of *liakra*: traditional weedy vegetables of the Arbëreshë of the Vulture area in southern Italy. J Ethnopharmacol 2002; 81: 165–185
22 *Pieroni A, Quave CL, Nebel S, Heinrich M.* Ethnopharmacy of the ethnic Albanians (Arbëreshë) of northern Basilicata, Italy. Fitoterapia 2002; 73: 217–241
23 *Pieroni A, Quave CL.* Traditional pharmacopoeias and medicines among Albanians and Italians in southern Italy: a comparison. J Ethnopharmacol 2005; 101: 258–270
24 *Quave CL, Pieroni A, Bennett BC.* Dermatological remedies in the traditional pharmacopoeia of Vulture-Alto Bradano, inland southern Italy. J Ethnobio Ethnomed 2008; 4: 5
25 *Isenberg HD.* Clinical microbiology procedures handbook, 2nd edition. Washington D.C.: ASM Press; 2004
26 *NCCLS.* Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 5th edition. Wayne, PA: National Center for Clinical Laboratory Standards; 2001
27 *Quave CL, Plano LWR, Pantuso T, Bennett BC.* Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus*. J Ethnopharmacol 2008; 118: 418–428
28 *Quave CL.* An ethnopharmacological approach to multi-drug resistant *Staphylococcus aureus*: Evaluation of Italian plants used in the traditional healing of skin disease [dissertation]. Miami, FL: Florida International University; 2008: 169
29 *Dunman PM, Murphy E, Haney S, Palacios D, Tucker-Kellogg G, Wu S, Brown EL, Zagursky RJ, Shlaes D, Projan SJ.* Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J Bacteriol 2001; 183: 7341–7353
30 *Harraghy N, Kerdudou S, Herrmann M.* Quorum-sensing systems in staphylococci as therapeutic targets. Anal Bioanal Chem 2007; 387: 437–444
31 *Pieroni A, Quave CL, Santoro RF.* Folk pharmaceutical knowledge in the territory of the Dolomiti Lucane, inland southern Italy. J Ethnopharmacol 2004; 95: 373–384
32 *Kültür Ş.* Medicinal plants used in Kirklareli Province (Turkey). J Ethnopharmacol 2007; 111: 341–364
33 *Vrchovska V, Spilkova J, Valentao P, Sousa C, Andrade PB, Seabra RM.* Antioxidative properties and phytochemical composition of *Ballota nigra* infusion. Food Chem 2007; 105: 1396–1403
34 *Seidel V, Bailleul F, Libot F, Tillequin F.* A phenylpropanoid glycoside from *Ballota nigra*. Phytochemistry 1997; 44: 691–693
35 *Daels-Rakotoarison DA, Seidel V, Gressier B, Brunet C, Tillequin F, Bailleul F, Luyckx M, Dine T, Cazin M, Cazin J-C.* Neurosedative and antioxidant activities of phenylpropanoids from *Ballota nigra*. Arzneimittelforschung 2000; 50: 16–23
36 *Didry N, Seidel V, Dubreuil L, Tillequin F, Bailleul F.* Isolation and antibacterial activity of phenylpropanoid derivatives from *Ballota nigra*. J Ethnopharmacol 1999; 67: 197–202
37 *Bertrand M-C, Tillequin F, Bailleul F.* Two major flavonoids from *Ballota nigra*. Biochem Syst Ecol 2000; 28: 1031–1033
38 *Basile A, Sorbo S, Giordano S, Ricciardi L, Ferrara S, Montesano D, Cobianchi RC, Vuotto ML, Ferrara L.* Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. Fitoterapia 2000; 71: S110–S116
39 *Süntar IP, Akkol EK, Yalçin FN, Koca U, Keleş H, Yesilada E.* Wound healing potential of *Sambucus ebulus* L. leaves and isolation of an active component, quercetin 3-*O*-glucoside. J Ethnopharmacol 2010; 129: 106–114

This is a copy of the author's personal reprint