Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus*

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A R T I C L E   I N F O

A B S T R A C T

Aim of study: One-third of botanical remedies from southern Italy are used to treat skin and soft tissue infection (SSTI). *Staphylococcus aureus*, a common cause of SSTI, has generated increasing concern due to drug resistance. Many plants possess antimicrobial agents and provide effective remedies for SSTI. Our aim was to investigate plants from different ethnobotanical usage groups for inhibition of growth and biofilms in methicillin-resistant *Staphylococcus aureus* (MRSA).
Materials and methods: Three groups were assessed: plant remedies for SSTI, plant remedies not involving the skin, and plants with no ethnomedical application. We screened 168 extracts, representing 104 botanical species, for activity against MRSA (ATCC 33593). We employed broth dilution methods to determine the MIC after 18 h growth using an optical density (OD_{600nm}) reading. Anti-biofilm effects were assessed by growing biofilms for 40 h, then fixing and staining with crystal violet. After washing, 10% Tween 80 was added and OD_{570nm} readings were taken.
Results: Extracts from 10 plants exhibited an $IC_{50} \leq 32\,\mu g/ml$ for biofilm inhibition: *Lonicera alpigena*, *Castanea sativa*, *Juglans regia*, *Ballota nigra*, *Rosmarinus officinalis*, *Leopoldia comosa*, *Malva sylvestris*, *Cyclamen hederifolium*, *Rosa canina*, and *Rubus ulmifolius*. Limited bacteriostatic activity was evident.
Conclusion: This study has demonstrated that the anti-biofilm activity of medicinal plants used for SSTI is significantly greater than plants without any ethnomedical applications.

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1. Introduction

The increase of microbial resistance to antibiotics threatens public health on a global scale as it reduces the effectiveness of treatments and increases morbidity, mortality, and health care costs (Coast et al., 1996). Evolution of highly resistant bacterial strains has compromised the use of newer generations of antibiotics (Levy, 2002). Although the active constituents may occur in lower concentrations, plant extracts may be a better source of antimicrobial compounds than synthetic drugs (Cox and Balick, 1994). The phenomenon of additive or synergistic effects is often crucial to bioactivity (Aqil et al., 2006; Kamatou et al., 2006) in plant extracts and in some cases, the activity is lost in purified fractions (Cos et al., 2006). Development of bacterial resistance to synergistic drug combinations, such as those found in plants, may be slower than for single drug therapies.

Only a small percent of plants have been investigated for their bioactivity (Cox and Balick, 1994). Furthermore, most studies on the antimicrobial activity of plant extracts have been restricted to analysis of their bacteriostatic and bactericidal properties. New assays investigating other potential roles, such as the mediation of pathogenicity via quorum sensing inhibition, have recently emerged (Rasmussen and Givskov, 2006). Investigations into the anti-pathogenic potential of natural products may open new avenues for drug development in the control of antibiotic resistant pathogens.

The use of plants against skin disease is a common practice in the popular medicine of most cultures, although the precise causation of disease and mechanism of cure is not always understood (Grierson and Afolayan, 1999; Srinivasan et al., 2001). While 1–3% of pharmaceutical drugs are used to treat skin disorders and wounds, 33% of botanical therapies in non-Western societies are used to this end (Mantle and Gok, 2001). The topical application of many plant remedies for the treatment of various skin conditions has proven effective due to the antimicrobial and anti-inflammatory properties of plant compounds.

In the Vulture-Alto Bradano area of Basilicata Province, southern Italy (Fig. 1), wild plants are regularly gathered as sources of

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Fig. 1. Map of the study area: Vulture-Alto Bradano, Basilicata Province, Italy.

food and medicine, making up an integral facet of home health-care (Pieroni et al., 2002b,c; Pieroni and Quave, 2005; Quave et al., 2008). At least one-third of all medicinal plants utilized in the folk-pharmacopoeia of this area are applied towards the treatment of skin and soft tissue infection (SSTI) (Quave et al., 2008). Vulture-Alto Bradano is characterized by small scale agriculture and its inhabitants maintain strong ties with the land. The demographic and socioeconomic aspects of this area are described in previous works (Pieroni et al., 2002c; Quave and Pieroni, 2005; Quave et al., 2008).

Staphylococcus aureus, a Gram-positive species, is a ubiquitous colonizer of the skin and mucous membranes of humans and animals (Fauci et al., 1998). It is the most prominent etiological agent for SSTI and is a common target for natural product drug screenings. Many strains of *Staphylococcus aureus* carry resistance genes for penicillin antibiotics, tetracyclines, methicillin and now, vancomycin. Methicillin-resistant *Staphylococcus aureus* (MRSA) presents a significant threat to public health in the USA. A surveillance study on MRSA found that an estimated 94,360 patients in the USA had invasive MRSA infections in 2005, resulting in an estimated 18,650 deaths (Klevens et al., 2007), surpassing the mortality estimates for AIDS in the USA.

Biofilm-related infections caused by staphylococci are among the leading causes of nosocomial infection in the USA (Kong et al., 2006). Biofilms comprise sessile microbial communities that embed themselves within a self-made extracellular polymeric matrix. This phenotype confers some protection to bacteria against host defenses and impedes the delivery of certain large molecule antimicrobials (Lewis, 2001). Moreover, bacteria associated with biofilms grow slowly, and this restricts the efficacy of many antibiotic classes, including the β-lactams (Lewis, 2005). The first stages of staphylococcal biofilm formation involve adherence of planktonic bacterial cells to cellular or prosthetic surfaces,

such as surgically implanted medical devices, including catheters, plates, screws, artificial joints and cardiac valves (Costerton et al., 1999). Biofilms present significant therapeutic barriers for many antibiotics and the discovery of agents which could prevent their formation or adherence would be of great use.

The objectives of this study were to evaluate extracts of south Italian plants for effects on growth, biofilm formation and adherence in MRSA. Our hypothesis was that plants used specifically for the treatment of SSTI would demonstrate higher mean activity for growth and biofilm inhibition than plant remedies not involving the skin or plants with no ethnomedical application.

2. Materials and methods

2.1. Plant material and extraction

Plants from three ethnobotanical usage categories were selected for analysis: Group 1: medicinal plants for skin and soft tissue infection (SSTI) ($n = 25$), Group 2: medicinal plants not involved in skin therapies ($n = 28$), and Group 3: plants with no ethnomedical application ($n = 51$). Plants were categorized based on previous ethnobotanical surveys conducted in the Vulture-Alto Bradano area of southern Italy (Pieroni et al., 2002b,c; Pieroni and Quave, 2005, 2006; Quave et al., 2008). Parameters for collecting a sample of non-medicinal species of Vulture-Alto Bradano were that they grew in abundance and were flowering at the time of collection. Plants were field-collected and identified using the standard botanic work *Flora d'Italia* (Pignatti, 2002). Familial nomenclature follows the current Angiosperm Phylogeny Group (Stevens, 2001 onwards). Bulk samples were separated by part (e.g., leaves, stems, flowers, and roots), cut into small pieces and dried in a plant drier using a heat and air flow source (35 °C) for 48–72 h. In the case of herbaceous species, efforts were made to collect all parts of the plant, including both above-ground and below-ground parts. Only the above-ground parts were typically collected from trees and large shrubs. Bulk specimens were composed only of the targeted plant species—parts from other plants and soil were removed prior to drying and vacuum storage. Cross-contamination was avoided by using clean drying nets and storage containers. After drying, plant materials were packed into plastic bags with several silica packets and vacuum sealed to prevent mold growth. Voucher specimens were deposited at the *Herbarium Lucanum* (HLUC) in Potenza, Italy and Fairchild Tropical Botanic Gardens (FTG) in Miami, FL, USA. Bulk specimens were shipped to the US under USDA permit #DP63438 for bioassays.

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$_2$O) for 30 min. 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$_2$O) were prepared, sterile filtered (0.2 μm) and stored in the dark at 4 °C. The excipient (DMSO or dH$_2$O) made up less than 5.1% of the final test solution for MIC assays and less than 2.5% for biofilm assays.

2.2. Bacteria and culture conditions

Methicillin-resistant *Staphylococcus aureus* (ATCC 33593) was grown in Tryptic Soy Broth (TSB) or on agar plates for 18 h at 37 °C. A 0.5 McFarland Standard was used to create inoculum densities of 1.5×10^8 cfu/ml in PBS using the direct suspension method (Isenberg, 2004) for MIC and biofilm assays.

2.3. Determination of minimum inhibitory concentrations (MICs)

MICs were determined by the microtiter broth method (Amsterdam, 1996) in sterile flat-bottom 96-well polystyrene plates. Serial dilution techniques were used to determine the MIC_{50} and MIC_{90} of extracts at concentrations of 8–512 μg/ml after 18 h growth. Negative controls (cells + TSB), positive controls (cells + TSB + antibiotics – vancomycin, ampicillin, and trimethroprim-sulfamethoxazole), vehicle controls (cells + TSB + DMSO), and media controls (TSB) were included. Positive controls for antibiotics were prepared at 8–512 μg/ml via serial dilution techniques. All tests were performed in triplicate. Optical density readings were taken using a KC4 microplate reader at 600 nm at 0 and 18 h post-inoculation. Results are reported as the MIC for growth at 18 h post-inoculation. To account for the effect of extract color on the OD_{600nm} reading, a formula for calculating percent inhibition was used. The mean % inhibition of replicate tests was used to determine the final MIC values.

$$\% \, \text{inhibition} = \left(1 - \left(\frac{\text{ODt18} - \text{ODt0}}{\text{ODgc18} - \text{ODgc0}} \right) \right) \times 100$$

ODt18 = optical density (600 nm) of the test well at 18 h post-inoculation; ODt0 = optical density (600 nm) of the test well at 0 h post-inoculation; ODgc18 = optical density (600 nm) of the growth control well at 18 h post-inoculation; ODgc0 = optical density (600 nm) of the growth control well at 0 h post-inoculation.

2.4. Biofilm formation and adherence

A modified microtiter dish system (Christensen et al., 1985; Schadow et al., 1988; Yarwood et al., 2004) was employed to test the effect of plant extracts on biofilm formation and adherence. Assays were performed in sterile flat-bottom 96-well polystyrene plates. Serial dilution techniques were employed to determine the IC_{50} of extracts at sub-inhibitory (for growth) concentrations of 4–128 μg/ml after 40 h growth. Negative controls (cells + TSB), positive controls (cells + TSB + antibiotics – vancomycin, ampicillin, and trimethroprim-sulfamethoxazole), vehicle controls (cells + TSB + DMSO), and media controls (TSB) were included. Positive controls for antibiotics were prepared at 4–128 μg/ml via serial dilution techniques. All tests were performed in triplicate.

To test for the effects of extracts on biofilm formation, the appropriate concentration of extract was added to the test wells prior to inoculation. Plates were placed in a 37 °C incubator and bacteria were shaken for 40 h. The contents of the wells were then aspirated, rinsed 3 times with PBS, and fixed by drying for 1 h in the 37 °C incubator. Once the wells were fully dry, we added 200 μl of 0.1% crystal violet stain to wells to stain for 15 m. The excess stain was rinsed off with tap water and 200 μl of 10% Tween 80 was added to wells. We pulled off the stain adhering to the biofilm biomass with the Tween 80 and transferred to new 96-well plates for spectrophotometric analysis (OD_{570nm}).

To test for the effects of extracts on biofilm adherence, biofilms were established in the 96-well plates by growing shaking for 20 h at 37 °C. At 20 h post-inoculation, planktonic cells and TSB were aspirated and fresh TSB was added with the appropriate concentration of test extract. Plates were then placed back into the 37 °C incubator on a shaker for 20 h. The staining methods were the same as those described above.

Replicate absorbance readings for each concentration tested were averaged and the average of the media control (TSB + extract) was subtracted. This value was then divided by the mean absorbance of the vehicle control and multiplied by 100. The con-

centration at which the extract depleted the biofilm biomass by at least 50% was labeled as the IC_{50}.

2.5. Statistical analyses

The mean values and standard deviations of all replicates described in the above tests were calculated using Excel software. Differences between means were analyzed with a One-Way ANOVA, followed by multiple comparison analysis using the Bonferroni method on SPLUS software. Differences were considered significant with p-values < 0.05.

3. Results

3.1. Effects on planktonic growth of MRSA

Plant extracts demonstrated limited bacteriostatic activity (Table 1). Roughly 18% of the extracts tested demonstrated an IC_{50} of 256 or 512 μg/ml. Of those demonstrating activity, the majority were not associated with any particular ethnobotanical application. The mean percent inhibition of growth for each ethnobotanical usage group when screened at 512 μg/ml ranged from 15.93 to 19.46% (Group 1 = 19.46%, Group 2 = 15.93%, and Group 3 = 18.43%). Based on results from a One-Way ANOVA ($p > 0.05$), we found that there was no significant difference in the mean bacteriostatic activity of extracts from plants in each ethnobotanical usage group (Table 2a).

3.2. Effects on MRSA biofilm formation

Extracts from 10 plants exhibited an $IC_{50} \leq 32$ μg/ml for inhibition of biofilm formation: *Lonicera alpigena*, *Castanea sativa*, *Juglans regia*, *Ballota nigra*, *Rosmarinus officinalis*, *Leopoldia comosa*, *Malva sylvestris*, *Cyclamen hederifolium*, *Rosa canina* var. *canina*, and *Rubus ulmifolius* (Table 1). The mean percent inhibition of biofilm formation for each ethnobotanical usage group when screened at 64 μg/ml ranged from 0.58 to 8.73% (Group 1 = 8.73%, Group 2 = 6.6%, and Group 3 = 0.58%). Plants used in folk remedies for SSTI made up 38% of plants exhibiting an IC_{50} of 8–128 μg/ml for MRSA biofilm formation.

Based on the results from a One-Way ANOVA test ($p < 0.05$), the mean anti-biofilm activity of extracts from plants in each ethnobotanical usage group was not the same (Table 2a). Multiple comparison analysis using the Bonferroni method revealed a significant difference between groups 1 and 3. The anti-biofilm activity of plants categorized for medicinal applications to the skin was significantly greater than activity from plants without any ethnomedical applications (Table 2b).

4. Discussion

A significant dose-dependent response in biofilm inhibition was evident in 5 of the 168 extracts tested: *Arundo donax*, *Ballota nigra*, *Juglans regia*, *Leopoldia comosa*, *Marrubium vulgare*, and *Rubus ulmifolius* (Fig. 2). Here, we offer a discussion of the ethnomedicinal applications of these plants in southern Italy and review the literature for other reports of anti-staphylococcal activity.

4.1. Arundo donax (Poaceae)—canna

The giant reed is commonly used in the construction of musical instruments and the production of industrial cellulose. It is also used for several other purposes in southern Italy, especially to support grape vines. This use was so important to local agriculture

Table 1
Effect of plant extracts on growth and biofilm formation in MRSA (ATCC 33593)

Family	Botanic name	Voucher ID	Plant part	Ethnobotanical use[a]	Extract solvent	[b]MIC_{50} (growth)	[b]IC_{50} (biofilm formation)
Adoxaceae	*Sambucus ebulus* L.	CQ-168	Inflorescence	N	EtOH	–	–
			Leaves	S	EtOH	–	–
			Stems	N	EtOH	–	–
	Sambucus nigra L.	CQ-151	Woody parts	R	EtOH	–	–
			Leaves	S	EtOH	512	–
					dH$_2$O	–	–
			Inflorescence	S; R	EtOH	–	–
					dH$_2$O	–	–
			Infructescence	F	EtOH	–	–
Alliaceae	*Allium cepa* L.	CQ-206	Leaves; bulbs; roots	S; M; F	EtOH	–	–
Apiaceae	*Daucus carota* L.	CQ-215	Leaves; stems	N	EtOH	–	–
			Inflorescence; infructescence	N	EtOH	–	–
	Foeniculum vulgare ssp. *piperitum* (Ucria) Coutinho	CQ-192	Leaves; stems	M; F	EtOH	–	–
	Foeniculum vulgare ssp. *vulgare* Mill.	CQ-196	Leaves; stems	M	EtOH	–	–
	Tordylium apulum L.	CQ-101	Flowers; leaves; roots; stems	N	EtOH	–	–
Apocynaceae	*Vinca major* L.	CQ-117	Flowers; leaves; roots; stems	M	EtOH	–	–
Aracaeae	*Arum italicum* Mill.	CQ-175	Stems	N	EtOH	–	–
			Fruits	N	EtOH	–	–
			Stalks	N	EtOH	–	–
			Leaves	S	EtOH	–	–
Asphodelaceae	*Asphodelus microcarpus* Salzm. & Viv.	CQ-109	Inflorescence	N	EtOH	–	–
			Leaves	N	EtOH	512	–
Asteraceae	*Achillea ageratum* L.	CQ-219	Leaves; stems; flowers	M	EtOH	256	–
	Achillea millefolium L.	CQ-176	Inflorescence	M	EtOH	–	–
			Leaves; stems	M	EtOH	512	–
			Leaves; stems; flowers	M	EtOH	–	–
	Anacyclus tomentosus DC.	CQ-167	Leaves; stems; flowers	N	EtOH	–	–
	Cichorium intybus L.	CQ-106	Basal leaves; roots	F	EtOH	512	–
					dH$_2$O	–	–
			Leaves; stems; flowers	F	EtOH	–	–
	Matricaria recutita L.	CQ-118	Flowers; leaves; roots; stems	S; M	EtOH	512	–
					dH$_2$O	–	–
	Scolymus hispanicus L.	CQ-199	Leaves; stems; flowers	N	EtOH	–	–
	Tussilago farfara L.	CQ-202	Leaves; stems; roots	S	EtOH	–	–
	Urospermum dalechampii (L.) Scop.	CQ-134	Flowers; leaves; roots; stems	N	EtOH	–	–
Boraginaceae	*Anchusa officinalis* L.	CQ-128	Leaves; stems; flowers	N	EtOH	–	–
	Borago officinalis L.	CQ-100	Flowers; leaves; roots; stems	M	EtOH	–	–
					dH$_2$O	–	–
	Cerinthe major L.	CQ-110	Flowers; leaves; roots; stems	N	EtOH	–	–
	Echium italicum L.	CQ-162	Leaves; stems; flowers	N	EtOH	–	–
Brassicaceae	*Brassica rapa* subsp. rapa	CQ-104	Flowers; leaves; roots; stems	F	EtOH	–	–
	Cardaria draba (L.) Desv.	CQ-140	Flowers; leaves; roots; stems	N	EtOH	512	–
	Eruca sativa Mill.	CQ-102	Flowers; leaves; roots; stems	N	EtOH	–	–
	Sisymbrium officinale (L.) Scop.	CQ-131	Flowers; leaves; roots; stems	N	EtOH	–	–

Table 1 (*Continued*)

Family	Botanic name	Voucher ID	Plant part	Ethno-botanical use[a]	Extract solvent	[b]MIC_{50} (growth)	[b]IC_{50} (biofilm formation)
Caprifoliaceae	*Lonicera alpigena* L.	CQ-213	Woody parts	N	EtOH	–	32
			Leaves	N	EtOH	–	–
Caryophyllaceae	*Saponaria officinalis* L.	CQ-210	Leaves; stems; flowers	N	EtOH	–	–
	Silene alba (Mill.) E.H.L. Krause	CQ-123	Leaves; stems; flowers	N	EtOH	–	–
	Silene nutans L.	CQ-125	Leaves; stems; flowers	N	EtOH	–	–
Cucurbitaceae	*Ecballium elaterium* (L.) A. Richard	CQ-169	Leaves; stems; flowers	S	EtOH	512	–
Dennstaedtia-ceae	*Pteridium aquilinium* (L.) Kuhn	CQ-211	Leaves	N	EtOH	–	–
			Stems	N	EtOH	–	–
Dipsacaceae	*Dipsacus fullonum* L.	CQ-201	Leaves; stems	N	EtOH	–	–
			Flowers	N	EtOH	–	–
	Knautia arvensis Coult.	CQ-190	Leaves; stems; flowers	N	EtOH	–	–
	Knautia lucana Lacaita & Szabo	CQ-166	Leaves; stems; flowers	N	EtOH	–	–
Equisetaceae	*Equisetum arvense* L.	CQ-226	Stems; leaves	N	EtOH	–	–
Fabaceae	*Acacia dealbata* Link	CQ-115	Inflorescence	O	EtOH	–	–
			Stems	O	EtOH	–	–
			Leaves; stems	O	EtOH	–	–
	Anthyllis vulneraria L.	CQ-147	Leaves; stems; flowers	N	EtOH	–	–
	Astragalus monspessulanus L.	CQ-112	Leaves; stems; flowers; roots	N	EtOH	–	–
	Coronilla emerus L.	CQ-137	Leaves; flowers	N	EtOH	–	–
			Woody stems	N	EtOH	–	–
	Melilotus alba Medik.	CQ-193	Leaves; stems; flowers	N	EtOH	512	–
	Robinia pseudoacacia L.	CQ-155	Woody parts	N	EtOH	–	–
			Leaves	N	EtOH	512	–
			Inflorescence	N	EtOH	–	–
	Spartium junceum L.	CQ-144	Leaves; stems; flowers	A	EtOH	–	–
	Trifolium repens L.	CQ-138	Leaves; stems; flowers; roots	N	EtOH	–	–
	Vicia craca L.	CQ-149	Leaves; stems; flowers; roots	N	EtOH	512	–
	Vicia faba L.	CQ-103	Leaves; stems; flowers; roots	F	EtOH	–	–
	Vicia sativa subsp. *angustifolio*	CQ-124	Leaves; stems; flowers	N	EtOH	512	–
	Vicia sativa subsp. *sativa*	CQ-119	Leaves; stems; flowers	N	EtOH	–	–
	Wisteria sinensis (Sims) Sweet	CQ-126	Inflorescence	O	EtOH	512	–
			Stems	O	EtOH	–	–
			Leaves	O	EtOH	512	–
Fagaceae	*Castanea sativa* Mill.	CQ-191	Inflorescence	N	EtOH	256	16
			Leaves	N	EtOH	256	–
			Woody parts	A	EtOH	512	–
	Quercus cerris L.	CQ-228	Leaves	N	EtOH	–	–
			Stems; fruits	N	EtOH	–	–
Gentianaceae	*Centaurium pulchellum* (Sw.) Druce	CQ-217	Leaves; stems; flowers; roots	N	EtOH	–	–
Geraniaceae	*Erodium ciconium* (L.) L'Hér.	CQ-142	Leaves; stems; flowers; roots	N	EtOH	–	–
	Erodium malacoides (L.) L'Hér. ex Aiton	CQ-121	Leaves; stems; flowers	N	EtOH	128	–
	Geranium columbinum L.	CQ-129	Leaves; stems; flowers	N	EtOH	512	–
Hyacinthaceae	*Leopoldia comosa* (L.) Parl.	CQ-105	Bulbs	M; F	EtOH	–	16
					dH_2O	–	8
			Leaves; inflorescence	N	EtOH	–	–
Hypericaceae	*Hypericum perforatum* L.	CQ-183	Leaves; stems; flowers	S	EtOH	256	128

Table 1 (*Continued*)

Family	Botanic name	Voucher ID	Plant part	Ethno-botanical use[a]	Extract solvent	[b]MIC$_{50}$ (growth)	[b]IC$_{50}$ (biofilm formation)
Juglandaceae	*Juglans regia* L.	CQ-181	Immature fruits	S; C	EtOH	–	16
			Leaves	R	EtOH	–	–
			Woody parts	N	EtOH	512	–
Juncaceae	*Juncus articulatus* L.	CQ-216	Leaves; fruits	N	EtOH	–	–
Lamiaceae	*Ballota nigra* L.	CQ-160	Stems	S; M	EtOH	–	–
			Roots	N	EtOH	–	–
			Leaves	S; M	EtOH	–	–
			Leaves; stems; flowers	S; M	EtOH	–	–
					dH$_2$O	–	8
	Clinopodium vulgare L.	CQ-182	Leaves; stems; flowers	N	EtOH	–	–
	Marrubium vulgare L.	CQ-170	Leaves; stems; flowers	S; M	EtOH	–	–
					dH$_2$O	–	–
			Roots	N	EtOH	–	–
	Mentha pulegium L.	CQ-200	Leaves; stems; flowers; roots	F	EtOH	–	–
	Mentha spicata L.	CQ-224	Leaves; stems; flowers	F	EtOH	–	–
	Origanum heracleoticum L.	CQ-207	Leaves; stems; flowers	F	EtOH	–	–
	Phlomis herba-venti L.	CQ-168	Leaves; stems; flowers	N	EtOH	–	–
	Rosmarinus officinalis L.	CQ-113	Leaves; stems; flowers	F; S	EtOH	512	16
	Salvia pratensis L.	CQ-165	Leaves; stems	N	EtOH	512	–
	Salvia virgata Jacq.	CQ-127	Leaves; stems; flowers	N	EtOH	–	–
	Stachys tymphaea Hausskn.	CQ-189	Leaves; stems; flowers	N	EtOH	–	–
Liliaceae	*Lilium candidum* L.	CQ-174	Leaves; stems	N	EtOH	–	–
			Inflorescence	N	EtOH	–	–
Malvaceae	*Alcea rosea* L.	CQ-205	Leaves; stems; flowers; roots	O	EtOH	–	–
	Malva sylvestris L.	CQ-156	Stems	S; M	EtOH	–	–
					dH$_2$O	–	32
			Flowers	S; M	EtOH	–	–
			Leaves	S; M	EtOH	256	–
					dH$_2$O	–	32
Moraceae	*Ficus carica* L.	CQ-173	Leaves	N	EtOH	–	–
			Woody parts	N	EtOH	–	–
			Immature fruits	S; F	EtOH	–	–
Myrsinaceae	*Cyclamen hederifolium* Aiton	CQ-186	Tubers	M	EtOH	–	8
Nyctaginaceae	*Mirabilis jalapa* L.	CQ-222	Leaves; flowers; fruits	N	EtOH	–	–
Oleaceae	*Olea europaea* L.	CQ-197	Leaves	N	EtOH	–	–
			Woody parts	A	EtOH	–	–
Orchidaceae	*Aceras anthropophora* R. Br.	CQ-153	Leaves; stems; flowers; roots	N	EtOH	–	–
	Orchis italica Poir.	CQ-133	Inflorescence; leaves; stems	N	EtOH	–	–
	Orchis purpurea Huds.	CQ-132	Inflorescence; leaves; stems	N	EtOH	–	–
Papaveraceae	*Fumaria officinalis* L.	CQ-107	Leaves; stems; flowers; roots	N	EtOH	–	–
	Papaver rhoeas subsp. *rhoeas*	CQ-145	Leaves; stems; flowers; roots	F	EtOH	–	–
	Papaver somniferum L.	CQ-178	Leaves; stems; flowers; roots	M; R	EtOH	–	–

Table 1 (*Continued*)

Family	Botanic name	Voucher ID	Plant part	Ethno-botanical use[a]	Extract solvent	[b]MIC$_{50}$ (growth)	[b]IC$_{50}$ (biofilm formation)
Plantaginaceae	*Digitalis ferruginea* L.	CQ-227	Leaves; stems; flowers	N	EtOH	–	–
	Linaria vulgaris Hill	CQ-223	Leaves; stems; flowers; roots	N	EtOH	–	–
	Plantago major L.	CQ-225	Leaves; stems; flowers; roots	S; M	EtOH	–	–
Poaceae	*Agropyron repens* (L.) P. Beauv.	CQ-208	Leaves; stems; roots	M	EtOH	–	–
	Arundo donax L.	CQ-146	Stem internodes	A; R	EtOH	–	–
			Stem nodes	S	EtOH	–	–
					dH$_2$O	–	128
			Leaves; stems	A; R	EtOH	–	–
Polygonaceae	*Rumex crispus* L.	CQ-171	Leaves; stems; fruits	S	EtOH	512	–
Pottiaceae	*Syntrichia ruralis* (Hedw.) Web. & Mohr	CQ-229	Whole plant	N	EtOH	–	–
Ranunculaceae	*Delphinium fissum* Waldst. & Kit.	CQ-187	Leaves; stems; flowers; fruits	N	EtOH	–	–
	Ranunculus acris L.	CQ-135	Leaves; stems; flowers	N	EtOH	–	–
Rosaceae	*Crataegus monogyna* Jacq.	CQ-116	Leaves; stems; flowers	M	EtOH	–	–
	Prunus spinosa L.	CQ-163	Woody parts; leaves	M	EtOH	–	–
			Fruits	N	EtOH	512	–
	Rosa canina var. *canina*	CQ-152	Fruits	N	EtOH	–	32
			Woody parts	N	EtOH	–	–
			Leaves; stems	N	EtOH	512	–
	Rubus ulmifolius Schott	CQ-164	Leaves; stems; flowers	S	EtOH	–	–
			Leaves	S	EtOH	–	–
			Roots	M	EtOH	512	8
			Woody stems	N	EtOH	512	–
Rubiaceae	*Galium verum* L.	CQ-177	Leaves; stems; flowers	N	EtOH	–	–
Scrophulariaceae	*Verbascum sinuatum* L.	CQ-218	Leaves; stems; flowers	N	EtOH	–	–
	Verbascum thapsus L.	CQ-172	Stems	M	EtOH	–	–
			Leaves	M	EtOH	–	–
			Inflorescence	M	EtOH	–	–
Ulmaceae	*Ulmus minor* L.	CQ-195	Leaves	N	EtOH	–	–
			Woody parts	M	EtOH	–	–
Urticaceae	*Parietaria diffusa* Mert. & Koch	CQ-212	Leaves; stems; fruits; roots	M	EtOH	–	–
	Urtica dioica L.	CQ-179	Leaves; stems; flowers	S; M; F	EtOH	–	–
Valerianaceae	*Centranthus ruber* (L.) DC.	CQ-143	Leaves; stems; inflorescence	M	EtOH	256	–
Vitaceae	*Vitis vinifera* var. *aglianico*	CQ-209	Wine	S; F		–	64
			Stems	N	EtOH	–	–
			Fruits	F	EtOH	–	–
			Leaves	N	EtOH	–	–

The MIC$_{50}$ for growth was tested in the range of 8–512 μg/ml, whereas the IC$_{50}$ for biofilm formation was tested from 4 to 128 μg/ml due to issues with the percent excipient (DMSO) in the test solution. A dash (–) represents that no IC$_{50}$ was identified within the concentration range tested.

[a] 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.

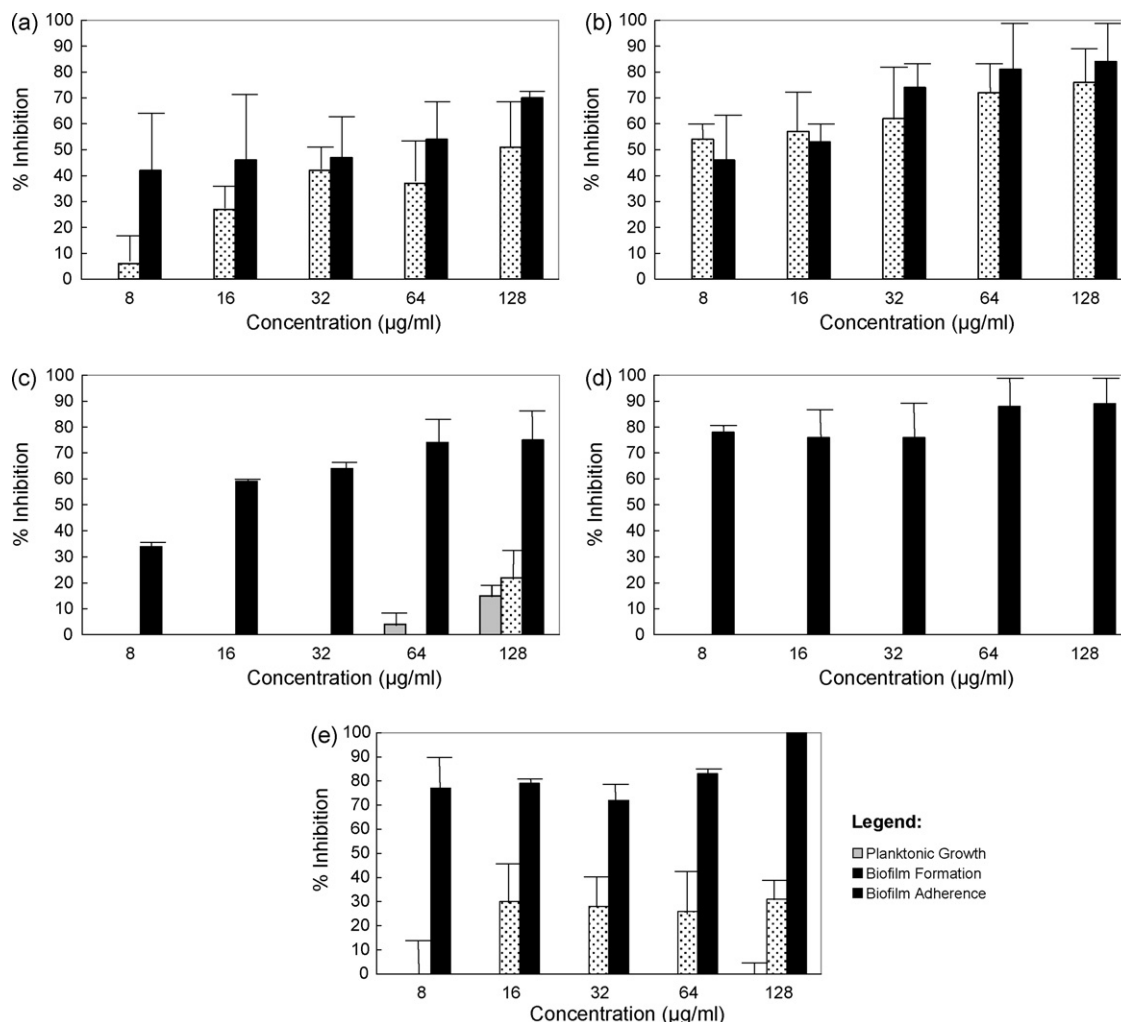[b] MIC$_{50}$ and IC$_{50}$ values expressed as μg/ml.

Fig. 2. The percent inhibition of extracts for planktonic growth, biofilm formation and adherence in MRSA. (a) *Arundo donax*, aqueous extract of nodes; (b) *Ballota nigra*, aqueous extract of aerial parts; (c) *Juglans regia*, ethanolic extract of immature fruits; (d) *Leopoldia comosa*, ethanolic extract of bulbs; (e) *Marrubium vulgare*, ethanolic extract of roots.

in the past that land was often divided along the border lines of reed colonies. The white hemicellulose membrane located at the nodes of the reed is inserted into fresh lacerations as a haemostatic agent (Passalacqua et al., 2007; Pieroni et al., 2002c) (Fig. 3).

Small tooth-sized pieces of the reed are also cut up and used in the ritual treatment of toothache (Quave and Pieroni, 2005). Antiproliferative activity against several human cancer cell lines has been reported for a lectin isolated from the rhizomes of this plant (Kaur

Table 2
Statistical tests for differences in the inhibitory activity of the three ethnobotanical usage groups: Group 1 includes plants used for SSTI ($n = 25$); Group 2 includes plants used medicinally, but not on the skin ($n = 28$); and Group includes plants with no ethnomedical application ($n = 51$)

	d.f.	Sum of Sq	Mean Sq	F Value	p-Value
(a)					
Planktonic growth	2	960.1	480.0347	0.7320253	0.48121
Biofilm formation	2	2073.34	1036.669	7.159144	0.00104
(b)					
Groups compared	Estimate	Standard error	Lower bound	Upper bound	
1–2	2.13	3.25	−5.730	9.98	
1–3[****]	8.15	2.36	2.460	13.90	
2–3	6.03	2.75	−0.634	12.70	

(a) One-way ANOVA tests were performed. These results indicate that there was no significant difference in the inhibition of planktonic growth ($p > 0.05$) between ethnobotanical usage groups. There was, however, a significant difference in the inhibition of biofilm formation ($p < 0.05$) between groups. (b) Multiple comparison analysis using the Bonferroni method was performed on the data for anti-biofilm activity. [****] represents a significant difference between groups.

Fig. 3. The white hemicellulose membrane found at the node of the giant reed (*Arundo donax* L. [Poaceae]) is used as a haemostatic agent for minor lacerations.

et al., 2005). *Arundo donax* is rich in alkaloids, including bufoteni-dine and gramine. Small amounts of DMT have also been isolated from the flowers (Duke, 2008).

We found that aqueous extracts of the reed nodes (which contain the white hemicellulose membrane) demonstrated a marked dose-dependent response for anti-biofilm activity, both in preventing MRSA biofilm formation and disrupting established biofilms (Fig. 2a). These results may suggest that the traditional application of the reed membrane to fresh lacerations may be useful as a prophylactic for biofilm-related infection. The effect of giant reed extract on biofilm formation and adherence in MRSA has not been previously reported.

4.2. Ballota nigra (Lamiaceae)—erba cane

Infusions of the aerial parts of black horehound are used in the south Italian pharmacopoeia as a rinse for skin rashes and are also drunk to promote circulation. Extensive phytochemical studies (Bertrand et al., 2000; Bruno et al., 1986; Seidel et al., 1997, 2000) have been performed on black horehound, and several phenylpropanoid glycosides have been identified and linked to moderate growth inhibition in *Staphylococcus aureus* (Didry et al., 1999). Black horehound also contains diterpenes, such as marubiin, as well as caffeic and ferulic acid derivatives (Gruenwald et al., 1998). Potent anti-oxidant activity has been reported in infusions of the aerial parts (Citoglu et al., 2004; Vrchovska et al., 2007). We prepared several types of extractions of the different parts of the plant, and found that an aqueous extraction of the aerial parts, similar to that prepared in the folk medical tradition, was the most effective at inhibiting both biofilm growth and adherence. Although this extract actually promoted planktonic growth of this MRSA strain by almost twofold, significant dose-dependent responses for the inhibition of both biofilm formation and adherence were evident (Fig. 2b). The effect of black horehound extract on biofilm formation and adherence in MRSA has not been previously reported.

4.3. Juglans regia (Juglandaceae)—noce

The common walnut has been reported in the ethnobotanical literature for its use as an aromatic for cheese and to protect it from parasites (Guarrera et al., 2005a) in Italy, and as a medicine for rheumatism, fever, fungal infection, hemorrhoid, cough and eczema in Turkey (Erdemoglu et al., 2003; Kultur, 2007). The bark is prepared in a decoction and gargled for toothache and fresh leaves are applied topically to reduce the swelling associated with varicose veins (Quave et al., 2008). The immature fruits are used for cosmetic (hair dye) applications (Pieroni et al., 2004) and the flowers are employed in ritual healing ceremonies for *mal d'arco* (rainbow illness) (Quave and Pieroni, 2005).

An ethanolic extract of the immature fruits reduced biofilm adherence and formation. Limited growth inhibition was noted at higher doses 128–512 μg/ml (Fig. 2c). Similar activity in staphylococcal growth inhibition by aqueous extracts of walnut leaves has also been reported (Pereira et al., 2007). The walnut tree is rich in phenolic compounds, including naphtoquinones and flavonoids that are likely responsible for the anti-bacterial activity of extracts derived from this plant (Pereira et al., 2007; Stampar et al., 2006). The use of walnut leaves for mild skin inflammations has been approved by the German Commission E (Blumenthal et al., 2000). This is the first report of anti-biofilm activity for MRSA by walnut extract.

4.4. Leopoldia comosa (Hyacinthaceae)—cipuldjin

In southern Italy, the tassel grape hyacinth is used in folk remedies for toothache (bulb is grated and applied topically) (Guarrera et al., 2005b) and for food (Casoria et al., 1999; Pieroni et al., 2002b). Extracts from the bulbs have demonstrated potent *in vitro* anti-oxidant activity (Pieroni et al., 2002a). Both the aqueous and ethanolic extracts of the bulb demonstrated potent anti-biofilm activity for this strain of MRSA. The IC_{50} for preventing biofilm formation and adherence was 16 and 8 μg/ml, respectively. The IC_{90} for biofilm adherence was 128 μg/ml (Fig. 2d). This is the first report of anti-biofilm activity for this plant.

4.5. Marrubium vulgare (Lamiaceae)—maruggē

White horehound is one of the most frequently quoted medicinal species applied in south Italian traditional medicine. A local rhyme is associated with this plant: "*U' marrugxhə, ogni mal' destruggə*" (the white horehound destroys every disease). It is used in the treatment of furuncle, boil, athlete's foot, skin infection, dermatitis, foot and mouth disease (in animals), gastritis, and malaria. It is thought to be particularly good at cleansing the liver and is used as a hepatoprotectant and general panacea (Pieroni et al., 2002c; Pieroni and Quave, 2005). Aqueous decoctions of the aerial parts are either used to rinse the skin or are drunk, depending upon the illness being treated. An ethanolic extract of the roots was effective in reducing adherence of established biofilms at very low concentrations (IC_{50} = 8 μg/ml and IC_{90} = 128 μg/ml). This extract was not, however, very effective at preventing the formation of biofilms on plastic surfaces. The maximum percent inhibition for biofilm formation was 31% at a concentration of 128 μg/ml (Fig. 2e).

Previous studies have demonstrated that white horehound extracts exhibit antispasmodic (Schlemper et al., 1996), anti-oxidant (Berrougui et al., 2006; Matkowski and Piotrowska, 2006; Weel et al., 1999), hypotensive (El Bardai et al., 2001), insecticidal (Pavela, 2004), and analgesic (Meyre-Silva et al., 2005) properties. *Marrubium* species are rich in diterpenes, caffeic acid derivatives, sterols, and flavonoids (Khanavi et al., 2005; Lazari et al., 1999). One of the principle medicinal components of horehound extract is marubiin, a furanic labdane diterpene (Blumenthal et al., 2000). It has been found to exhibit non-selective anti-inflammatory properties in mouse models, which may support its use as a folk remedy for dermatitis (Stulzer et al., 2006). The German Commission E approved the use of this herb for the treatment of loss of appetite and dyspepsia (Blumenthal et al., 2000). The effect of white horehound extract on biofilm formation and adherence in MRSA has not been previously reported.

5. Conclusion

Plant extracts demonstrated limited bacteriostatic activity, and in fact, many actually promoted planktonic growth. More significantly, extracts from five plants exhibited dose-dependent inhibition of biofilm formation and adherence. We identified a significant correlation in anti-biofilm activity with medicinal plants used for SSTI when compared with plants with no apparent ethnomedical use. These results validate the efficacy of the topical application of *Arundo donax*, *Ballota nigra*, *Juglans regia*, *Leopoldia comosa*, and *Marrubium vulgare* in Italian folk remedies for SSTI.

While these results are based on the analysis of crude, unfractionated extracts, they also represent the first of many steps towards the development of new anti-biofilm drugs. Inhibition of biofilms provides a method of controlling the effects of pathogenic bacteria without strong selection for drug resistance. The emer-

gence of infections due to highly resistant MRSA capable of forming and many times involving biofilms presents a significant dilemma to medicine today. The healthcare community is ill-prepared to effectively treat such infections as the old classes of antibiotics currently in use rapidly lose their efficacy. Effective treatments for the disruption of established biofilms could save many lives and decrease healthcare costs related to the treatment and potential replacement of infected implanted prosthetic devices. Prophylactic agents for biofilms embedded in surgically implanted devices and catheters could significantly diminish morbidity and subsequently reduce healthcare costs associated with nosocomial infection.

We recommend further investigation of natural products from the following plants for anti-biofilm activity in MRSA: *Lonicera alpigena*, *Castanea sativa*, *Juglans regia*, *Ballota nigra*, *Rosmarinus officinalis*, *Leopoldia comosa*, *Malva sylvestris*, *Cyclamen hederifolium*, *Rosa canina*, and *Rubus ulmifolius*.

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