

# *In vitro* activity of *Inula helenium* against clinical *Staphylococcus aureus* strains including MRSA

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## Introduction

*Staphylococcus aureus* is an extraordinarily versatile pathogen that causes a wide spectrum of infections due to its extensive range of virulence factors.<sup>1</sup> It can cause infections to skin and soft tissue such as abscesses, impetigo and cellulitis and can also cause systemic and life-threatening conditions such as bacteraemia, endocarditis and pneumonia.<sup>2</sup> Of particular significance to healthcare settings is methicillin-resistant *S. aureus* (MRSA), which has had a significant impact on patient morbidity and mortality.<sup>3</sup>

This study aims to investigate the antibacterial properties of the herbal compound *Inula helenium* as an alternative to antibiotics, in which the extract is directed specifically against *S. aureus* isolates. Some of the isolates comprising the collection were selected for this study as they were resistant to mupirocin (Bactroban), the conventional agent of choice used to control MRSA carriage and infection.<sup>4,5</sup>

*I. helenium* is a large perennial herb indigenous to south-east Europe and western Asia, but is found as a naturalised escapee in Ireland, Britain and north mid-west USA.<sup>6</sup> Cytotoxic and antiproliferative activities have been attributed to *I. helenium* and it has been established that the oil of *I. helenium* possesses strong antibacterial properties.<sup>7-11</sup> The efficacy of *I. helenium* with regard to antistaphylococcal properties is examined against a large population of clinical strains ( $n=200$ ) including 100 each of methicillin-sensitive and -resistant isolates. To date, there has been no published study regarding the efficacy of *I. helenium* against a large collection of clinical staphylococcal isolates. The herb used in this study is sourced locally and tested against a locally derived population of clinical staphylococci.

## Materials and methods

### Preparation of extracts

Dried roots and rhizomes of *I. helenium* in powder form were provided by Bandon Medicinal Herbs. The powder preparation (100 mg/mL) was extracted in 50% ethanol (v/v)

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## ABSTRACT

The present study aims to investigate the bactericidal activity (specifically antistaphylococcal) of *Inula helenium*. The antimicrobial activity of the extract is tested against 200 clinically significant Irish *Staphylococcus aureus* isolates consisting of methicillin-resistant (MRSA) and -sensitive (MSSA) *S. aureus* using a drop test method and a microbroth dilution method. The antibacterial effect is evaluated by measuring the area of the inhibition zone against the isolates. Results proved *I. helenium* to be 100% effective against the 200 staphylococci tested, with 93% of isolates falling within the ++ and +++ groups. The minimum bactericidal concentration of *I. helenium* was examined on a subset of isolates and values ranged from 0.9 mg/mL to 9.0 mg/mL. The extract was equally effective against antibiotic-resistant and -sensitive strains. This plant therefore possesses compounds with potent antistaphylococcal properties, which in the future could be used to complement infection control policies and prevent staphylococcal infection and carriage. This research supports other studies wherein herbal plants exhibiting medicinal properties are being examined to overcome the problems of antibiotic resistance and to offer alternatives in the treatment and control of infectious diseases.

KEY WORDS: Antimicrobial.

*Inula helenium*.

Methicillin-resistant *Staphylococcus aureus*.

Plants, medicinal.

*Staphylococcus aureus*.

for 28 days at room temperature. All extracts were filter sterilised through cellulose nitrate filters with a pore size of 0.45  $\mu\text{m}$  (Sartorius, Germany).

### Bacterial cultures

A total of 200 random, anonymised *S. aureus* isolates comprising 100 each of MRSA and MSSA were collected in the Department of Microbiology at Cork University Hospital (CUH) between January 2006 and March 2007. These strains were isolated from a variety of clinical specimens (blood, respiratory and urine samples). Antimicrobial susceptibility testing using CLSI guidelines<sup>12</sup> demonstrated that these isolates showed different sensitivities to a spectrum of antibiotics tested (erythromycin, fusidic acid, gentamicin, linezolid, rifampicin, teicoplanin, tetracycline and vancomycin [data not shown]). The strains were also examined for high- and low-level mupirocin resistance.<sup>13</sup>

### Determination of antimicrobial activity

Susceptibility to *I. helenium* extract was determined on Mueller-Hinton agar (Oxoid) using CLSI guidelines for

susceptibility testing of bacteria that grow aerobically.<sup>12</sup> The surface of a Mueller-Hinton agar plate was inoculated with a  $1.5 \times 10^8$  colony-forming unit (cfu)/mL suspension of the test bacterial isolate. In place of a disc impregnated with an antimicrobial agent, an inoculum of 200  $\mu$ L *I. helenium* extract was applied to the surface of the plate and allowed to dry for 1 h. A control plate was prepared by applying 200  $\mu$ L 50% ethanol (EtOH; v/v) to another inoculated plate. The plates were then incubated overnight at 37°C. Following incubation, antibacterial activity was qualitatively assessed by the presence or absence of inhibition zones, and the area of the inhibition zone was measured using a Mitutoyo calliper (Giles Scientific, Santa Barbara CA).

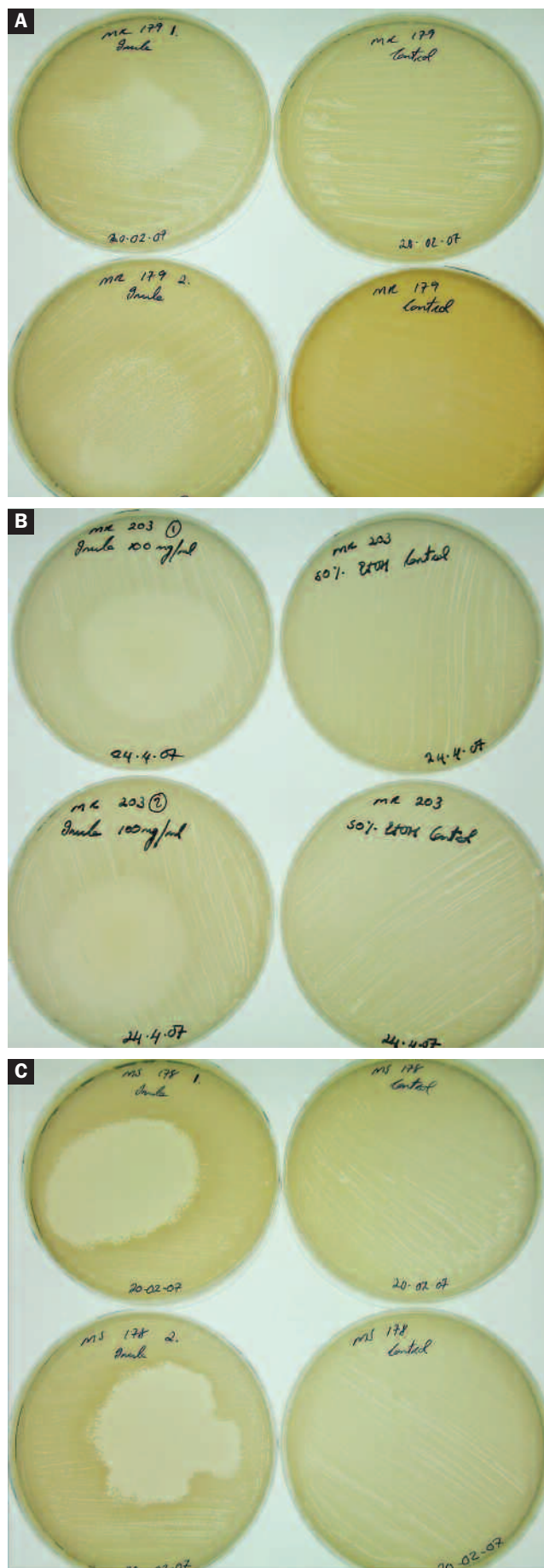
The experiment was performed in duplicate and the mean of the area of the inhibition zones was calculated. The isolates were classified into three groups based on the area of inhibition that resulted from the herbal extract. The groups were as follows: + (partial inhibition, which was less growth than the control plate to which no antibacterial suspension had been added, and which varied from discrete small colonies to an extremely light haze), ++ (strong inhibition of bacterial growth causing a distinct clearing effect up to 24 mm) and +++ (strong inhibition of bacterial growth causing a distinct clearing effect greater than 25 mm).

#### Minimum inhibitory concentration/ minimum bactericidal concentration

The method used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was a microbroth dilution method based on those described by Karaman<sup>14</sup> and Şahin.<sup>15</sup> This test was performed on a subset of 43 isolates which comprised MRSA ( $n=22$ ) and MSSA ( $n=21$ ) isolates, and included four high-level mupirocin-resistant isolates detected within these groups. Control strain ATCC MSSA 25922 was also included. Each 96-well microtitre plate was prepared by dispensing 95  $\mu$ L nutrient broth (Lab M, Bury, Lancashire, UK) and 5  $\mu$ L inoculum for each isolate corresponding to a 0.5 McFarland turbidity standard (prepared according to CLSI guideline M2-A912) into each well. A volume of 100  $\mu$ L *I. helenium* extract of varying concentration was added to each well.

Dilutions of the extract were prepared outside the wells and concentrations ranging from 100 mg/mL to 0.1 mg/mL were tested. For each sample, the concentrations tested were between 100 mg/mL and 10 mg/mL in 10-fold dilutions, and between 20 mg/mL and 1 mg/mL in single-fold dilutions, and, finally, between 0.9 mg/mL and 0.1 mg/mL in intervals of 0.1 mg/mL. The second to last well of each row contained 100  $\mu$ L EtOH without *I. helenium* extract, and the last well contained 195  $\mu$ L nutrient broth and 5  $\mu$ L inoculum only – these acted as experimental controls. The final volume in each well was 200  $\mu$ L.

**Fig. 1.** *In vitro* activity of *I. helenium* against MRSA. All are Mueller Hinton agar plates inoculated with MRSA isolates to which *I. helenium* has been applied. Plates on left (top and bottom) are the *I. helenium* test plates, and plates on right (top and bottom) are the 50% EtOH control plates. Results are shown in duplicate. Panel A shows MRSA 179 demonstrating + inhibition. Panel B shows MRSA 203 demonstrating ++ inhibition. Panel C shows MRSA 178 demonstrating +++ inhibition.



The microtitre plate was left uncovered under sterile laboratory conditions for 4 h to allow evaporation of EtOH and was followed by incubation at 37°C for 24 h. The MIC was defined as the lowest concentration of the compound to inhibit growth. The MBC was determined by plating 5 µL sample from all wells on nutrient agar plates (Oxoid), which were then incubated at 37°C for 24 h. The MBC was the concentration at which there was no microbial growth.<sup>14</sup> Each organism was tested in duplicate.

#### Stability studies

The stability of the active extracts in soluble form (stored at room temperature in the dark) was measured by testing the same extract for antibacterial activity against three each of MRSA and MSSA isolates periodically over 26 weeks.

## Results

#### Preliminary evaluation of antibacterial activity

Extensive optimisation experiments on *I. helenium* extract determined that a concentration of 100 mg/mL in 50% EtOH was appropriate to test the extract against all 200 *S. aureus* isolates using the method described. The ethanolic extract exhibited different degrees of inhibitory effect against the tested microorganisms. All inhibition and clearing caused by *I. helenium* was measured (mm) and results were classified into three groups as outlined previously. Figure 1 shows a representative MRSA isolate from each of the three categories of inhibition described for the *I. helenium* extract antibacterial effect (namely MRSA 179 +, MRSA 203 ++ and MRSA 178 +++). For each isolate tested, a 50% solution of EtOH was also applied to inoculated Mueller-Hinton plates treated identically to exclude the possibility of EtOH-related inhibition of the organisms. All 200 staphylococcal isolates were inhibited by the *I. helenium* extract; 93% of isolates were within the ++ and +++ groups (55.5% and 37.5%, respectively).

#### Minimum inhibitory concentration/ minimum bactericidal concentration

Following incubation of the microtitre plates, the MIC of the *I. helenium* extract could not be determined visually or spectrophotometrically as the combination of extract and nutrient broth alone resulted in a turbid solution. Therefore, a change in turbidity or absorbance could not be attributed to the *I. helenium* extract exerting an antibacterial effect on the test organisms. Consequently, samples from all wells of the 96-well microtitre plate were plated on nutrient agar to determine the MBC. The MBC of the *I. helenium* extract was determined for a selection of the MRSA ( $n=22$ ) and MSSA ( $n=21$ ) isolates using a modification of the microbroth assay of Karaman<sup>14</sup> and Şahin.<sup>15</sup> The isolates were selected to represent each of the three categories of inhibition, specifically 11 from the + category, 15 from the ++ category and 18 from the +++ category. *I. helenium* extract MBC for each isolate tested was within the range 0.9 mg/mL to 9.0 mg/mL.

#### Determination of stability of the active extracts

It was observed from the stability study conducted that the antistaphylococcal effect of the *I. helenium* crude extract remained as effective six months after the initial extraction as it was at the beginning of the study (data not shown).

## Discussion

The use of herbal medicine and medicinal plants has been examined as an alternative to conventional treatment for many diseases. In recent times it has been considered particularly worthy of investigation because of the emergence of antibiotic resistance. There are many reports of the antimicrobial activity of crude plant extracts, and the use of plants and their preparations to treat infections is traditional practice in many parts of the world.<sup>16-18</sup> The herbal plant selected for this study was *I. helenium*. This was chosen in conjunction with Bandon Medicinal Herbs as one of its traditional uses cited by the British Herbal Medicine Association (1983) was as an antibacterial agent.<sup>19</sup> This study focused on the assessment of the susceptibility of clinical isolates of MRSA and MSSA to *I. helenium* extracts because of their acknowledged status as significant human pathogens.

A previous study examined the antibacterial activity of the essential oil of *I. helenium*, using a single *S. aureus* test strain, and a second study investigated an isolated constituent of the plant for its antibacterial properties against two *S. aureus* strains; however, neither study included MRSA strains.<sup>9,20</sup>

Deriu *et al.*, in 2008, demonstrated the antimicrobial activity of extracts prepared from *I. helenium* using supercritical fluid extraction and hydrodistillation against a panel of organisms.<sup>11</sup> In the current study, crude *I. helenium* plant extracts were examined for antimicrobial activity specifically against a collection of 200 clinically significant *S. aureus* (100 MSSA and 100 MRSA). Four strains were also resistant to the antibiotic mupirocin, which is used as a topical agent both prophylactically and to treat staphylococcal infection.

After an extensive study of the efficacy of the extract (results not shown), the optimal concentration for extraction was 100 mg/mL in 50% EtOH for the *I. helenium*. A drop test method was selected as an appropriate method by which to test the antimicrobial effect, as any possible inhibition due to the EtOH could be eliminated by an evaporation step. Optimisation of this method was performed assessing different evaporation times and the effect of using different volumes of extract in the assay (results not shown). It was observed that the length of evaporation time did not affect or contribute to the inhibitory effect, and, as expected, a dosage effect was observed whereby an increase in the volume of the applied inoculum caused greater inhibition of the bacterial isolate.

The antimicrobial activity of the *I. helenium* extract against the microorganisms examined in the present study was qualitatively assessed by the presence and absence of inhibition zones. Zone diameters were classified into three groups (+, ++, +++), which was a modified system developed from those described by Chariandy<sup>21</sup> and Bonjar.<sup>16</sup>

On preliminary examination of the antibacterial effect of the herbal compound in the current study, it was clear that all isolates were inhibited to varying degrees, indicating the need to subdivide levels of inhibition into three categories as shown for representative isolates in Figure 1. All isolates were inhibited by *I. helenium*. Of the entire collection, 7% of isolates showed partial inhibition that was less-confluent growth than that shown by the control plate in each case, and varied from discrete small colonies to an extremely light haze ( $n=14$ ); a further 55.5% ( $n=111$ ) showed zones of inhibition of 1–24 mm, and the remaining 37.5% of isolates ( $n=75$ ) demonstrated zones of inhibition of greater than 25 mm. Neither methicillin nor mupirocin susceptibility status appeared to affect the inhibition of staphylococcal strains by *I. helenium*.

Based on the initial antimicrobial screening test, the inhibitory effect of the *I. helenium* was quantitatively assessed for a subset of 43 isolates by MBC determination. The MBC values were between 0.9 mg/mL and 9.0 mg/mL, with the majority of isolates (75%) having an MBC of 2 mg/mL or less. These results are comparable to MICs reported by Deriu *et al.* for the single strain of *S. aureus* tested.<sup>11</sup> No deterioration in antimicrobial activity was observed on the isolates tested over the six months' duration of these stability assays. It is therefore postulated that the stability could extend for considerably longer, which could be significant when developing new antimicrobial agents.

The results highlight that MRSA isolates were as sensitive to the tested plant extracts as were the MSSA strains. Ahmand and Beg,<sup>22</sup> who examined 45 Indian medicinal plants (*I. helenium* was not included), observed similar results with their isolates and compounds, whereby antibiotic-resistant strains were inhibited in addition to non-antibiotic-resistant isolates. This indicates that antibiotic resistance does not interfere with antibacterial action of the plant extracts and that different plants have different modes of action on the test organisms.

Scientific publications have suggested that Gram-positive bacteria, including *Staphylococcus* species, are more susceptible to the antibacterial compounds of herbal medicinal plants than are Gram-negative organisms, due to their morphological constitutions.<sup>18</sup> It has been reported that the sesquiterpene lactones of *I. helenium* are potent and irreversible inhibitors of the bacterial enzyme MurA, which is responsible for the first step in the cytoplasmic biosynthesis of peptidoglycan precursor molecules, and that this accounts for the significant effect on this Gram-positive pathogen.<sup>23</sup>

A further advantage of using *I. helenium* is that this plant is naturalised to the locality in which it was tested. From the available literature, this study appears to be the first of its kind to demonstrate that herbal plants grown in southern Ireland and tested against Irish clinical staphylococcal isolates can prove effective in their control.

In conclusion, this *in vitro* investigation demonstrated that *I. helenium* possesses significant antistaphylococcal activity. This study is beneficial at a time when conventional chemotherapeutic treatment options are diminishing and new alternatives are crucial. □

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