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Susceptibility of some clinical isolates of *Staphylococcus aureus* to fractions from the aerial parts of *Leuzea carthamoides*

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Abstract: The antimicrobial activity of the dichloromethane extract from aerial parts of *Leuzea carthamoides* DC. was tested *in vitro* against 19 *Staphylococcus aureus* strains (ATCC 25923, CNCTC Mau 43/60, clinical isolates). The extract was fractionated by column chromatography on silica gel into six fractions (petroleum ether, toluene, dichloromethane, ethyl acetate, methanol and water). The minimum inhibitory concentrations (MICs) of the fractions ranged from 64 to 1024 μg/mL. An ethyl acetate fraction (EA 1) with the widest range of activity inhibited all of the strains with MIC in the range 128–512 μg/mL. This fraction exhibited potent activity against strains which showed associated resistance to oxacillin, ciprofloxacin and erythromycin.

Key words: *Leuzea carthamoides*; aerial parts; antimicrobial activity; fractionation; *Staphylococcus aureus*; MRSA

Introduction

Staphylococcal infections are important causes of morbidity, mortality, and increased economic costs. *Staphylococcus aureus* has overcome all the therapeutic agents that have been developed in the past 50 years. The most notable example of this phenomenon is methicillin-resistant *S. aureus* (MRSA), which causes serious hospital and community infections worldwide (Hiramatsu 2001). *Leuzea carthamoides* DC. (Asteraceae), syn. *Rhaponticum carthamoides* (Willd.) Iljin, is a medicinal plant of southern Siberia, where it has been used in cases of overstrain and common weakness after illness. Within previous phytochemical investigations of the aerial parts of *L. carthamoides*, the content of 20-hydroxyecdysone, sesquiterpene lactones, flavonoids (Opletal et al. 1997) and the composition of essential oil (Geszprych & Weglarz 2002) were investigated. Pharmacological studies indicated immunomodulatory (Lamer-Zarawska et al. 1996) and radical scavenging (Miliauskas et al. 2004) activities. In our previous study aerial part of *L. carthamoides* was the most active among four *Leuzea* species against ten bacterial pathogens including *S. aureus* (Kokoska et al. 2005). The aim of this study was to evaluate the effect of the dichloromethane extract of *L. carthamoides* aerial parts and its fractions on growth of some *Staphylococcus aureus* clinical isolates.

Material and methods

Plant material
The dried aerial parts of *L. carthamoides* were purchased from Adavo (Velký Osek, Czech Republic). The voucher specimen (No. Kok 025), authenticated by Dr Kokoska, was deposited in the herbarium of the Institute of Tropics and Subtropics, Czech University of Life Sciences Prague.

Bacterial strains
17 clinical isolates of *S. aureus* were obtained from different hospitals in the Czech Republic (Faculty Hospital Motol, Prague; Regional Hospital Liberec; Česká Lípa Hospital; Masaryk's Hospital, Ústí nad Labem). Prior to determination of minimum inhibitory concentrations (MICs) they

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Table 1. MICs of antibiotics and bioactive fractions (μg/mL) against standard strains and clinical isolates of *Staphylococcus aureus*.

Strains	Antibiotics				Fractions				
	vancomycin	oxacillin	ciprofloxacin	erythromycin	DM 1	EA 1	EA 2	EA 3	M 3
ATCC 25923	0.5	1	0.5	1	>1024	128	512	1024	1024
CNCTC Mau 43/60	0.5	0.5	0.5	0.5	1024	128	1024	1024	512
428	0.5	0.25	0.5	0.5	1024	128	1024	>1024	1024
429	0.5	4	64	>128	>1024	128	1024	>1024	1024
430M	0.25	0.5	0.5	0.5	>1024	128	1024	1024	1024
430V	0.5	2	128	>128	1024	128	256	512	64
431	0.5	0.25	0.5	0.5	>1024	512	1024	>1024	1024
579	0.5	0.5	0.25	1	>1024	256	1024	1024	512
584	0.5	1	0.25	0.5	>1024	256	1024	>1024	512
624	0.5	2	0.25	0.5	>1024	512	1024	>1024	512
625	0.5	1	0.25	0.5	>1024	512	1024	>1024	512
685	1	2	0.5	1	>1024	512	1024	>1024	512
2858	0.5	32	128	>128	>1024	256	1024	>1024	1024
2882	0.5	32	>128	>128	>1024	256	1024	>1024	1024
2884	0.5	16	>128	>128	>1024	256	1024	>1024	1024
2885	0.5	32	>128	>128	>1024	128	1024	>1024	1024
3014	0.5	32	>128	>128	>1024	256	1024	>1024	1024
42924	0.5	64	>128	>128	>1024	256	1024	>1024	1024
44301	0.5	16	>128	>128	>1024	256	1024	>1024	1024

oxacillin (resistant $\geq$ 4 μg/mL); ciprofloxacin (resistant $\geq$ 4 μg/mL); vancomycin (resistant $\geq$ 32 μg/mL); erythromycin (resistant $\geq$ 8 μg/mL) (Jorgensen et al. 1999); DM-dichlormethane, EA-ethyl acetate, M-methanol

were stored on Columbia Blood Agar Base at 4°C and subcultured monthly. *S. aureus* standard strain ATCC 25923 was obtained from Oxoid. CNCTC Mau 43/60 standard strain was obtained from the Czech National Collection of Type Cultures, the National Institute of Public Health, Prague, Czech Republic. All microorganisms were tested in Mueller-Hinton broth. Cultivation media were purchased from Oxoid.

Preparation and fractionation of extracts

First, dry powdered plant material (100 g) was subsequently extracted for 48 h with dichloromethane, chloroform, ethyl acetate and methanol using a Soxhlet apparatus and tested against standard strain of *S. aureus* ATCC 25923. Dichloromethane extract was selected for further fractionation as it was the most active and gave the highest yield. The extract was fractionated by column chromatography (44 × 572 mm, 600 mL) on silica gel (63–200 μm, Merck) using a set of eluents (petroleum ether, toluene, dichloromethane, ethyl acetate, methanol, water), which were selected to cover whole range of polarity. After evaporation of the solvents *in vacuo*, the petroleum ether (PE 1–2, yields 7.9% and 0.4%), toluene (TO 1–3, yields 11.2%, 1.7% and 1.2%), dichloromethane (DM 1–3, yields 8.3%, 6.2% and 1.2%), ethyl acetate (EA 1–3, yields 17.5%, 4.6% and 7.9%), methanol (M 1–3, yields 23.7%, 1.6% and 2.6%), and water (H 1–2, yields 2.1% and 1.7%) fractions were obtained. Each fraction consisted of 600 mL (column volume) of the corresponding solvent.

Determination of minimum inhibitory concentrations (MICs) of the fractions and standard antibiotics

Susceptibility assays were performed by a micro-dilution technique using 96-well microplates (Jorgensen et al. 1999). First, 2048 μg/mL stock solution of each tested fraction were prepared in DMSO (10% v/v) in sterile Tris buffer saline (TBS) of pH 7.6 (Sigma). The reconstituted fractions were serially diluted (two-fold) to give a concentration range of 1024 to 1.00 μg/mL using Rotatiter (Dynex, Prague, CR). Each well was inoculated with 5 μL of bacterial suspension at a density of 10^7 CFU/mL. The plates were incubated at 37°C for 24 h and the solution of DMSO (5% v/v) in TBS served as the negative control. The growth of microorganisms was observed as turbidity determined by the UV-VIS spectrophotometer Helios ε (Spectronic Unicam, Cambridge, UK) at 600 nm. MICs were calculated based on the density of the growth control and they were the lowest concentrations that resulted in 80% reduction in growth compared with that of the control. Ciprofloxacin, erythromycin, oxacillin and vancomycin (Sigma) were used as positive controls and were tested in the same manner in concentration range of 128 to 0.25 μg/mL. All determinations were carried out in triplicate. The isolates were classified as resistant or susceptible according to NCCLS interpretive standards (Jorgensen et al. 1999). The significance of the differences in MICs was determined by the Student's t-test (unpaired, unequal variance). $P < 0.01$ was considered significant.

Results and discussion

MICs (yields) of the Soxhlet extracts were as follows: dichloromethane 1 mg/mL (2.26 g), chloroform 2 mg/mL (0.54 g), ethyl acetate 2 mg/mL (0.26 g), methanol 8 mg/mL (6.5 g). Even though all extracts exhibited some activity, the dichloromethane one was selected for further fractionation because of combination of high yield and activity. The MICs of antibiotics and bioactive column fractions are shown in Table 1. It reveals that 8 *S. aureus* isolates (47%) were resistant to oxacillin and 9 (53%) to ciprofloxacin and erythromycin, while all were sensitive to vancomycin. All 8 isolates resistant to oxacillin showed associated resistance to ciprofloxacin and erythromycin. Fractions PE 1,2; TO 1–3; DM 2,3; M 1,2 and H 1,2 exhibited no activity against tested microorganisms. The most susceptible strain was 430V, which was inhibited by all fractions tested with MICs in the range of 64–1024

μg/mL. The fraction with the widest range of activity was EA 1, which inhibited all of the strains tested with MIC range 128–512 μg/mL. When compared to erythromycin and ciprofloxacin, 9 (all) and 7 resistant strains respectively, showed the same or higher susceptibility to the EA 1 fraction than to these antibiotics. The difference in MICs of all antibiotics except vancomycin between groups of susceptible and resistant strains were statistically significant ($P < 0.01$), while there was no significant difference in any of the fractions.

Chemical composition and biological activities of *L. carthamoides* were reviewed by Opletal et al. (1997). Until now, researchers' interest has been focused mainly on the roots and rhizomes, and as a result we have good knowledge about their phytochemistry and biological action. On the contrary, there are only few reports concerning chemical or biological properties of the aerial parts. However, some compounds found in the aerial parts (e.g. the flavonoid apigenin and the sesquiterpene lactone cynaropicrin) have already shown *in vitro* antibacterial activity, but with contradictory results. In a study of antibacterial activity of *Scutellaria barbata*, apigenin was identified as the most active principle (MIC range 3.9–15.6 μg/mL) in diethyl ether extract when tested against several *S. aureus* strains including MRSA (Sato 2000). In contrast, antimicrobial evaluation of apigenin and cynaropicrin isolated from *Moquinia kingii* revealed only moderate antibacterial activity of these compounds (MIC range 500–2500 μg/mL) against *S. aureus* ATCC 25923 (Schinor et al. 2004). In addition, the main biologically active constituent of *L. carthamoides*, 20-hydroxyecdysone, also showed moderate activity against some bacterial pathogens, including *S. aureus* (Ahmad 1996).

In conclusion, the bioactive column fractions (mainly EA 1) from *L. carthamoides* aerial parts dichloromethane extract possess potent antibacterial activity against strains of *S. aureus* with associated resistance to oxacillin, ciprofloxacin and erythromycin. In light of previous phytochemical reports discussed above we suppose that some of the previously identified compounds may participate in the antibacterial action reported in this study. Purifying the active fractions, and subsequent isolation and chemical characterization of the active substances, which is currently underway in our laboratory, could contribute to elucidation of the biological action of *L. carthamoides* and provide some additional information about its phytochemistry.

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