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Antibacterial and Cytotoxic Activities of the Sesquiterpene Lactones Cnicin and Onopordopicrin

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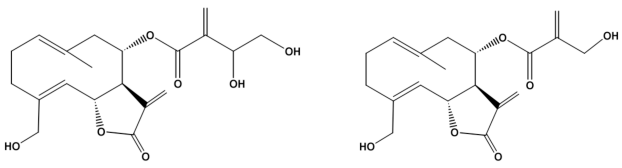
The antimicrobial and cytotoxic activities of chloroform extracts from the weeds *Centaurea tweediei* and *C. diffusa*, and the main sesquiterpene lactones isolated from these species, onopordopicrin and cnicin, respectively, were assayed. Results show that the chloroform extracts from both *Centaurea* species possess antibacterial activities against a panel of Gram-positive and Gram-negative bacteria. Remarkable antibacterial activity against methicillin-resistant *Staphylococcus aureus* was also measured. Both the extracts and the purified sesquiterpene lactones show high cytotoxicity against human-derived macrophages. Despite this cytotoxicity, *C. diffusa* chloroform extract and cnicin are attractive candidates for evaluation as antibiotics in topical preparations against skin-associated pathogens.

Keywords: cnicin, onopordopicrin, cytotoxicity, antibacterial activity, *Centaurea*, sesquiterpene lactones.

The genus *Centaurea* (Asteraceae) has close to 600 species ubiquitously distributed around the world. Some members of this genus are edible vegetables, whereas others have been incorporated into popular medicine [1,2]. In contrast, several species are weeds with significant economical importance in cattle farming [3]. Most of the compounds isolated from the genus *Centaurea* are terpenoids [4] and mainly sesquiterpene lactones [5-11].

Probably one of the most studied compounds isolated from several species of *Centaurea* is cnicin (**1**), a germacranolide, exhibiting hypoglycemic, antifungal, cytostatic, phytotoxic, alellopathic, and insecticidal activities [1,9,12-18]. Another sesquiterpene lactone isolated from *Centaurea* species is onopordopicrin (**2**) [6], a germacranolide similar to cnicin that differs in the nature of the ester residue at C-8 (4-hydroxymethacrylate *vs*. 4-hydroxymethyl-4-hydroxymethacrylate) [5,8]. Only partial screening for antibacterial and alellopathic activities has been reported for onopordopicrin [8,16]. Cnicin has also been evaluated as an antibacterial agent [9,19]. Recent study into cnicin's mechanism of action suggests that it mediates the inactivation of MurA, an enzyme involved in the first step of synthesis of peptidoglycans, major



1 **2**

Figure 1: Structures of cnicin (**1**) and onopordopicrin (**2**).

constituents of the bacterial cell wall [20,21]. In continuation of our on-going development of new medicinal formulations using cheap natural sources, we evaluated the antibacterial activity of chloroform extracts of *C. diffusa* and *C. tweediei,* and compared them with the main sesquiterpene lactone isolated from each species; i.e. cnicin and onopordopicrin, respectively [6,7]. We focused our antibacterial screen study on a panel of *Staphylococcus* strains, and also evaluated the cytotoxic effect on a human-derived monocyte THP-1 cell line.

The antibacterial activity of chloroform extracts, cnicin and onopordopicrin were assayed against a panel of opportunistic and pathogenic bacteria using the disk diffusion agar assay. The *C. diffusa* extract was slightly more active than that of *C. tweediei*, with diameters of the

Table 1. Antibacterial activities of *C. tweediei* and *C. diffusa* extracts, cnicin and onopordopicrin, expressed as diameter of the inhibition zone (in mm).

Strain	CTC [µg]		CDC [µg]		Onopordopicrin [µg]		Cnicin [µg]		PC
	250	500	250	500	25	50	25	50	
Gram-positive									
B. subtilis	11±0.8	14±0.9	10±1.1	13±1.1	NA	NA	11±1.0	16±1.2	22±1.1 (G_{10})
R. aurantiacum	NA	NA	NA	NA	NA	NA	NA	15±1.0	21±0.9 (G_{10})
S. aureus 25923	11±0.9	13±1.0	10±0.9	14±1.2	NA	NA	12±1.1	15±1.1	20±0.9 (G_{10})
S. aureus 25178	NA	NA	NA	12±1.0	NA	NA	11±1.0	14±1.0	20±1.2 (G_{10})
S. aureus 27217	10±0.5	12±1.0	10±0.8	13±1.1	NA	NA	11±1.1	15±1.1	19±1.1 (G_{10})
S. aureus 6538	12±1.2	13±0.9	10±1.0	13±1.0	NA	NA	11±1.2	14±1.2	20±1.3 (G_{10})
MRSA	NA	14±1.3	10±1.1	15±1.1	NA	NA	13±1.3	17±1.4	18±1.5 (G_{50})
Gram-negative									
E. coli	NA	NA	NA	9±0.9	NA	NA	12±0.9	20±1.3	23±1.6 (G_{20})
K. pneumoniae	ND	ND	ND	ND	NA	NA	NA	10±0.9	ND
P. aeruginosa	NA	NA	NA	11±1.2	NA	NA	NA	14±0.9	24±1.0 (G_{50})

CTC= Chloroform extract of *C. tweediei*, CDC= Chloroform extract of *C. diffusa*. MRSA= methicillin-resistant *S. aureus*.
PC= Positive control, ND= Not determined, NA= No activity detected. G_{10}, G_{20}, G_{50}= Gentamicin 10, 20 and 50 µg, respectively.

inhibition zone ranging between 9-15 mm and 12-14 mm, respectively when 500 µg/mL of each extract was assayed (Table 1). Both extracts showed higher activity against Gram-positive than Gram-negative bacteria.

When the antibacterial activities of the sesquiterpene lactones, onopordopicrin and cnicin, were assayed against the same panel of microorganisms, an increase in the antibacterial activity was observed for cnicin (50 µg), with diameters of the inhibition zone ranging between 14-17 mm for Gram-positive bacteria, and 10-20 mm for Gram-negative bacteria. Surprisingly, onopordopicrin showed no antibacterial activity suggesting that other compound/s in the *C. tweediei* extract were responsible for its antimicrobial activity.

As a result of the lack of onopordopicrin activity, we focused on determining the minimum inhibitory concentration (MIC) of the chloroform extract of *C. diffusa* and its isolated lactone cnicin. MICs between 42 to 250 µg/mL were measured when the chloroform extract was assayed against the same bacterial panel (Table 2). No activity was observed against *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Klebsiella pneumoniae*. When cnicin was tested, MICs between 42 to 124 µg/mL were recorded, except against *R. aurantiacum*, which was resistant to this compound (Table 2).

THP-1 human-derived monocytes were used to determine the cytotoxic effects of the compounds. The chloroform extracts of both *C. tweediei* and *C. diffusa* caused significant damage to macrophages, varying between 70-80%, similar to that produced by hydrogen peroxide used as a positive control. Onopordopicrin and cnicin, showed damage of about 80% and 65%, respectively (Figure 2).

Previous work has reported conflicting results on the activities of onopordopicrin and cnicin. It has been reported that onopordopicrin was active against *S. aureus* ATCC 25923 [8], but in our work such activity was not observed. We are not able to explain this discrepancy because the experimental details were not described completely in the mentioned work. On the other hand, MICs of cnicin have been reported against both Gram-positive and Gram-negative bacteria [9,19]. For instance,

Table 2. MICs of chloroform extract of *C. diffusa* (CDC) and cnicin (in µg/mL).

Strain	CDC	Cnicin
Gram-positive		
B. subtilis	NA	42
R. aurantiacum	83	NA
S. aureus 25923	250	124
S. aureus 25178	42	124
S. aureus 27217	250	42
S. aureus 6538	250	124
MRSA	83	124
S. epidermidis	NA	42
Gram-negative		
E. coli	250	124
K. pneumoniae	NA	124
P. aeruginosa	250	124

MRSA= methicillin-resistant *S. aureus*. NA= No activity detected

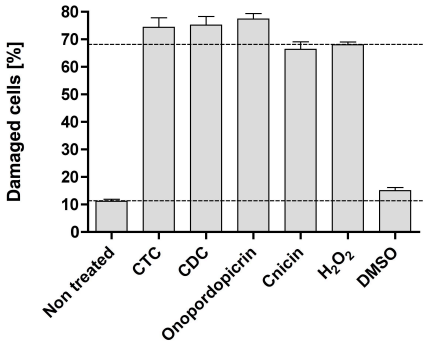


Figure 2: Cytotoxic effects of extracts, cnicin and onopordopicrin on THP-1 cells. Propidium iodide staining was used to determine the cytotoxic effects. Dashed lines represent treatment with 5% H_2O_2 (upper line) (positive control), and non treated cells (lower line) (negative control). Shown is the mean ± SD of three independent experiments. CDC= chloroform extract of *C. diffusa*, CTC= chloroform extract of *C. tweediei*.

Bruno *et al*. [19] reported MICs ranging between 12.5-50 µg/mL when *B. subtilis*, *S. aureus* ATCC 25923, *E. coli*, and *P. aeruginosa* were tested, but we measured slightly higher values ranging between 42-124 µg/mL for the same strains. Again, the differences in the values obtained might have originated from the application of different protocols. However, Skliar *et al*. [22] reported the antimicrobial activity of the methanol extract of *C. diffusa* on a panel of twelve microorganisms. Although they concluded that the extract was active against all the tested strains, MICs of 200 mg/mL were recorded. As noted before, cnicin exerts antibacterial effects by inhibiting the enzyme MurA [20].

Interestingly, the binding of cnicin to MurA, as elucidated by X-ray crystallography, is mediated by the binding of the vicinal diols at positions C18 and C19, which mimics the binding of a phosphate group [21], which irreversibly inhibits the enzyme activity. Therefore, the poor antibacterial activity of onopordopicrin can be attributed to the lack of a hydroxyl group, and, therefore, weak binding to the enzyme.

Examination of cytotoxic effects of the chloroform extracts show that both extracts, and the purified lactones cnicin and onopordopicrin, were significantly toxic to the human-derived macrophages used in this study. Our results are in agreement with previous work reporting cytotoxic effects in other human cell lines. For instance, cnicin has been shown to inhibit growth in breast and ovarian cell lines [9], while onopordopicrin was cytotoxic to epidermoid carcinoma cells [8]. Overall, we report that cnicin has remarkable antimicrobial activity against six *Staphylococcus* strains. Of special interest is the activity of cnicin against the multi-drug resistant pathogen MRSA, a prevalent worldwide strain infecting approximately 20,000 people annually in the United States, which is also associated with significant increases in the length of hospitalization [23,24].

The antibacterial activity observed in the *C. diffusa* chloroform extract suggests that this extract can be further developed without the need for cnicin purification. Since *C. diffusa* is an invasive weed in rangelands in countries such as Argentina and the US [25,26], its availability and exploitation as a cheap source for antibiotics represents an attractive source for its incorporation in topical formulations against human and animal skin infections. This application is especially significant bearing in mind that this extract is highly toxic to eukaryotic cells, limiting its use as a systemic treatment.

Experimental

Preparation of* C. diffusa *and* C. tweediei *extracts: Aerial parts of *C. diffusa* Lam. (Asteraceae, Cardueae, Centaureinae) were collected near Albahacas, Rio Cuarto, Cordoba, Argentina [6] and of *C. tweediei* Hook. et Arn. near Estacion Paul Groussac, Road M, km 856, Santa Fe, Argentina. Voucher specimens (#2487 and #2487, respectively) were deposited in the herbarium of the Instituto de Botanica del Nordeste, Corrientes, Argentina [6,7]. Both plants were processed and extrated with chloroform as published [6,7].

Isolation and preparation of cnicin and onopordopicrin: Cnicin and onopordopicrin were isolated from the chloroform extract of the aerial parts of *C. diffusa* (CDC) and *C. tweediei* (CTC), respectively [6,7].

Microbial strains and culture medium: Strains were grown in Mueller-Hinton broth (Becton & Dickinson) at 37^{o}C and stocks were maintained in the same medium solidified with 2% agar. The Gram-positive strains used in this study were *Bacillus subtilis* (ATCC 12432), *Rhodococcus aurantiacum* (ATCC 25938), and six strains of staphylococci including *Staphylococcus aureus* (ATCC 6538, ATCC 25178, ATCC 25923 and ATCC 27217), *S. aureus* methicillin-resistant (MRSA) (ATCC 700698), and *S. epidermidis* (ATCC 14990). *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumoniae* (ATCC 10031) were used as representatives of Gram-negative strains.

Antibacterial activity: The disk diffusion agar assay was used to determine the antibacterial activity of extracts and purified lactones. The inoculum density of bacteria was adjusted to be equivalent to 0.5 on the McFarland scale by diluting the cultures in fresh broth to an OD of 0.08-0.1 at 625 nm. Cultures were then spread uniformly on the surface of plates using a sterile swab. Disks were loaded with the chloroform extracts at final amounts of 6.25, 12.5, 25, 50, 100, 250 and 500 μg/disk, while final amounts of 6.25, 12.5, 25, 50 μg/disk were loaded for cnicin and onopordopicrin. Commercial disks embedded with 10, 20 or 50 μg gentamicin (Oxoid) were used as a positive control. DMSO was used as the negative control. All disks were distributed uniformly on the surface of the plate on which the culture was spread. Plates were incubated at 37°C for 16 h and the activity of the extracts and the compounds was measured according to the diameter of the inhibition zone expressed in mm. All the experiments were performed in triplicate.

Minimum inhibitory concentration (MIC) determination: The microdilution method for estimation of minimum inhibitory concentration (MIC) values was carried out to evaluate the antimicrobial activity. The MIC was determined in a final volume of 150 μL using 96-well microdilution plates according to established protocols [27]. Extracts and purified lactones were tested at a final concentration of 33, 42, 83, 124, and 250 μg/mL. All the experiments were carried out in triplicate.

Cytotoxicity assay: Monocytic cell line THP-1 (ATCC 202) was cultured in RPMI 1640 (Sigma) supplemented with 5% fetal calf serum (Hyclone, UT), and 2 mM L-glutamine (StemCell Technologies, Vancouver, Canada). Monocytes were activated with phorbol myristate acetate (Sigma) at a final concentration of 20 ng/mL and dispensed in 12-well plates at a density of $5x10^5$ cells/well. Cells were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2 for 24 h, and the cytotoxic effects of the compounds were assayed using the concentrations corresponding to the highest MIC obtained (250 μg/mL). Macrophages treated for 6 h with 5% hydrogen peroxide were used as a positive control, while untreated cells or those treated with DMSO were used as a negative control. The toxicity of the compounds was measured by staining the macrophages with propidium iodide (PI) (Sigma), according to published protocols [28]. All the experiments were performed in triplicate. PI emission was detected at 610-625 nm using the FL3 gate

of a BD FACS Vantage SE Turbo sort cell sorter (BD, ON, Canada).

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