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Isolation of praeruptorins A and B from *Peucedanum praeruptorum* Dunn. and their general pharmacological evaluation in comparison with extracts of the drug☆

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Abstract

The root of *Peucedanum praeruptorum* Dunn. was extracted with solvents at different polarity obtaining three chemical fractions: aqueous (H_2O), *n*-butanol (BuOH) and ethyl acetate (AcOEt). From AcOEt praeruptorins A and B were isolated by column chromatography on silica gel, using toluene/ethyl acetate as eluent, and identified by ^1H and ^{13}C NMR analysis. The extracts and the praeruptorins were tested for gross behavioural effects and acute toxicity in mice; the cytotoxicity on *Artemia salina* Leach and the antimicrobial activity were also evaluated. None of the tested substances evoked behavioural effects or acute toxicity after oral administration in mice; delayed mortality was observed with AcOEt and praeruptorin A only after intraperitoneal administration of high doses (1 g/kg). In *Artemia salina* test AcOEt, and praeruptorins A and B had LC_{50} values of 40.2, 121.2 and 34.5 μg/ml, respectively. AcOEt and praeruptorin A showed antimicrobial activity on *Streptococcus agalactiae*; their MIC values were 250 and 100 μg/ml, respectively. © 2001 Éditions scientifiques et médicales Elsevier SAS

Keywords: *Peucedanum praeruptorum*; Praeruptorins; Cytotoxicity; *Artemia salina*; Gross behaviour

1. Introduction

The root of *Peucedanum praeruptorum* Dunn. (Apiaceae) is widely employed in traditional Chinese medicine as antitussive, anti-asthma and as a remedy for angina [1]. At present the drug is also sold in Italy. The main chemical components of *P. praeruptorum* are angular-type pyranocoumarins named praeruptorins (A,B,C,E) and their glucoside derivatives; moreover, the plant also contains a group of linear-type furanocoumarin glucosides and simple coumarin glucosides [2–5].

Pharmacological studies in vitro, aimed to confirm the anti-asthma and anti-angina effects, showed that *P. praeruptorum* relaxes the musculature of gut, trachea, vessels and uterus; the miorelaxing activity is referred to praeruptorins that are supposed to have calcium channels blocking activity [6–9]. Nevertheless, at present *P. praeruptorum* and its chemical components have not been characterised from a general pharmacological point of view and no data exist in current literature regarding their general pharmacological effects and toxicity.

In this work commercial drug consisting of *P. praeruptorum* root was submitted to a phytochemical study and pure praeruptorins A and B were obtained. Pure compounds and total extracts were studied in order to evaluate their gross behavioural effects and acute toxicity. Moreover, the cytotoxic activity on *Artemia salina* was determined considering that other pyranocoumarins such as decursin showed cytotoxicity in in vitro experiments [10]. Finally, the antimicrobial activity was evaluated.

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2. Phytochemical study

Dried roots (500 g) of *P. praeruptorum* cut in pieces were obtained from Tong Ren Tang Pharmacy of Beijing (China) and identified by one of us; a sample is stored at our Department. The drug was powdered and extracted with methanol at room temperature by maceration and subsequent percolation. The extract was evaporated *under vacuum* at 45°C; the residue was dissolved in a mixture of H_2O/ethyl acetate 1:1 and partitioned; the resulting aqueous phase was again partitioned with ethyl acetate (1:1). The ethyl acetate fractions were combined and the residual aqueous fraction extracted twice with an equal volume of *n*-butanol. The resulting butanolic fractions were combined to give an extract containing compounds with medium polarity. The yield of the three fractions, determined after evaporation of the solvent, was 36 g (7.2%), 10 g (2.0%) and 43.5 g (8.7%) for the aqueous (H_2O), butanolic (BuOH) and ethyl acetate (AcOEt) fraction, respectively.

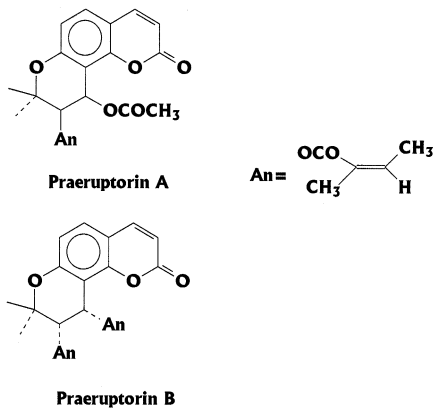

Fig. 1.

Table 1
^1H NMR spectrum of praeruptorin A and praeruptorin B (ppm from TMS in $CDCl_3$)

^1H	Praeruptorin A	Praeruptorin B
H-3	6.20, d, $J = 9.6$ Hz	6.13, d, $J = 9.6$ Hz
H-4	7.56, d, $J = 9.6$ Hz	7.57, d, $J = 9.6$ Hz
H-5	7.32, d, $J = 8.0$ Hz	7.32, d, $J = 8.0$ Hz
H-6	6.87, d, $J = 8.0$ Hz	6.72, d, $J = 8.0$ Hz
H-3′	6.55, d, $J = 6.0$ Hz	6.46, d, $J = 6.0$ Hz
H-4′	5.37, d, $J = 6.0$ Hz	5.40, d, $J = 6.0$ Hz
2′-Me	1.45, s	1.45, s
2′-Me	1.42, s	1.49, s
Angeloyl		
H-3″	6.10, d, $J = 6.0$ Hz	6.08, d, $J = 6.0$ Hz; 5.95, d, $J = 6.0$ Hz
Me-2″	1.93, s	1.80, s; 1.82, s
Me-3″	1.90, d, $J = 6.0$ Hz	1.94, d, $J = 6.0$ Hz; 1.93, d, $J = 6.0$ Hz
$COCH_3$	2.01, s	

Table 2
^{13}C NMR spectrum of praeruptorin A and praeruptorin B (ppm from TMS in $CDCl_3$)

Carbon	Praeruptorin A	Praeruptorin B
C-2	159.8	159.6
C-3	112.9	113.0
C-4	143.3	143.2
C-4a	107.3	113.0
C-5	129.1	129.2
C-6	114.3	114.2
C-7	156.7	156.5
C-8	107.8	107.8
C-8a	153.0	153.0
C-4′	60.8	60.6
C-3′	69.6	70.1
C-2′	76.7	77.2
Me-2′	22.2	22.2
Me-2′	25.2	25.2
Angeloyl		
C-2″	126.8	126.8, 127.2
C-3″	134.6	134.6, 139.7
Me-2″	20.3	20.3, 20.2
Me-3″	15.6	15.6, 15.4
Acetyl		
CO	179.0	
Me	20.5	

The AcOEt was separated by column chomatography (CC) on silica gel using toluene/ethyl acetate 7:3 as eluent. The two main constituents, praeruptorin A and B (Fig. 1), were obtained and crystallised from AcOEt/*n*-hexane. Compounds were identified by comparison of their physico-chemical and spectroscopic (^1H and ^{13}C NMR, MS) data (Tables 1 and 2). NMR analyses were performed using a Bruker AM-500 spectrometer. The yields were 17.5 g for praeruptorin A and 2.5 g for praeruptorin B.

3. Biological assays

3.1. Materials and methods

For biological tests aqueous fraction was diluted in distilled water, BuOH, AcOEt and praeruptorins were dissolved in DMSO at 5%: DMSO in preliminary tests did not interfere with biological responses.

Gross behavioural effects. Male mice (Harlan, S. Pietro al Natisone-UD, Italy) weighing 18–20 g were used. Animals were administered orally or intraperitoneally with the test substances and after 60 min (oral route) or 20 min (intraperitoneal route) were observed according to the Irwin [11] protocol. The acute mortality (number of animals died within 4 h) and the delayed mortality (number of animals died within 7 days) were

also determined. The substances were tested at the doses of 0.125–0.250–0.500 and 1 g/kg. Controls were treated with solvent, administered orally or intraperitoneally.

Artemia salina test. The cytotoxicity was evaluated on *Artemia salina* Leach according to the method of Mongelli et al. [12], slightly modified by Renzini et al. [13]. Brine shrimps eggs (Euroaquarium S.p.A., Bologna, Italy) were hatched in artificial sea water; after 48 h the phototrophic nauplii were collected and a suspension of 10–15 nauplii (100 μl) was placed into each well of a 48-well microplate (Kartell, Milan, Italy) containing 900 μl of extract in artificial sea water; control wells containing artificial sea water were also prepared. After 24 h of incubation the dead nauplii were counted using a Zeiss binocular microscope (10X), then 200 μl of methanol were added to each well and after 60 min the total number of nauplii was counted. Podophyllotoxin, dissolved in artificial sea water, was used as the reference substance.

The cytotoxicity, expressed as LC_{50} with 95% confidence limits, was calculated with the test of Lichtfield and Wilcoxon [14].

Antimicrobial activity. Antimicrobial activity was evaluated on Gram-positive (*Staphylococcus aureus*, *Staphylococcus capitis*, *Streptococcus agalactiae*, *Streptococcus pyogenes* ATCC 12345, *Bacillus subtilis* ATCC 6333, *Listeria monocytogenes* ATCC 19111, *Listeria seligeri* IAL 1820), and Gram-negative (*Escherichia coli*, *Klebsiella ozanae*, *Pseudomonas aeruginosa*, *Shigella flexneri* IAL 1517, *Shigella sonnei* IAL 1580, *Salmonella enteritidis* IAL 1132, *Salmonella thyphymurium* CDC 9767 IAL 1472) bacteria and on yeasts (*Candida albicans*, *Candida krusei*), obtained from collection or clinically isolated, cultured on Mueller–Hinton broth (Becton–Dickinson, Milan, Italy) at pH 7.4.

The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The MIC was determined on 96-well culture plates by a microdilution method. Eight two-fold dilutions of the extracts were carried out starting from the concentration of 2000 μg/ml for the extracts or 100 μg/ml for praeruptorins. All test solutions were sterilised with a 0.22-μm filter and placed in the wells that were inoculated with a microorganism suspension at a density of 10^5 cells/ml. The plates were incubated at 37°C for 24 h (bacteria) or 48 h (yeasts). After incubation the plates were observed in order to determine the MICs. The cultures that did not present growth were used to inoculate plates of solid medium in order to determine the MBC. Tetracycline and miconazole were used as reference substances on bacteria and yeasts, respectively. Proper blanks were tested in parallel; samples were tested in triplicate.

3.2. Results

Gross behavioural effects. After oral administration neither the fractions (H_2O, BuOH and AcOEt) nor praeruptorins (A and B) evoked changes in behavioural parameters, within the range of doses used; mortality did not occur. After intraperitoneal administration neither the fractions nor praeruptorins showed behavioural effects during the observation period, however, delayed mortality was observed with AcOEt and praeruptorin A that caused, at the dose of 1 g/kg, the death of 100 and 40%, respectively, of the animals (Table 3).

Artemia salina test. AcOEt showed cytotoxic activity: its LC_{50} value was 40.2 μg/ml (C.L. 25.3–63.8) (Table 4). BuOH and H_2O showed low or none cytoxicity: their LC_{50} values were 585.0 μg/ml (C.L. 462.3–740.3) and >1000 μg/ml, respectively. Praeruptorins A and B were both cytotoxic being their LC_{50} values of 121.2 μg/ml (C.L. 90.0–163.5) and 34.5 μg/ml (C.L. 21.4–55.8), respectively. Podophyllotoxin showed an LC_{50} value corresponding to 9.5 μg/ml (C.L. 6.4–14.0).

Antimicrobial activity. *P. praeruptorum* fractions did not inhibit the growth of most of the microorganisms tested; only AcOEt was active on *Streptococcus agalactiae* (MIC 250 μg/ml and MBC 1000 μg/ml). Praeruptorin A inhibited *S. agalactiae* too (MIC and MBC 100 μg/ml) while praeruptorin B resulted inactive. MIC values of reference substances were between 0.4 and 6.2 μg/ml for tetracycline and between 0.5 and 32.0 μg/ml for miconazole.

3.3. Discussion

The phytochemical study of *Peucedanum praeruptorum* root allowed to separate the total extract in three chemical fractions with different polarity and to obtain

Table 3
Toxicity of chemical fractions of *Peucedanum praeruptorum* and of praeruptorins in mice

Dose (g/kg i.p.)	Delayed mortality (%)				
	H_2O fraction	BuOH fraction	AcOEt fraction	Praeruptorin A	Praeruptorin B
1.0	0	0	100	40	0
0.5	0	0	0	0	0

Table 4
Cytotoxicity of *Peucedanum praeruptorum* fractions and praeruptorins on *Artemia salina* [a]

Concentration (μg/ml)	Dead nauplii (%)					
	Podophyllotoxin	H_2O fraction	BuOH fraction	AcOEt fraction	Praeruptorin A	Praeruptorin B
1000	n.t.		83.0 ± 1.1	n.t.	n.t.	n.t.
750	n.t.		62.0 ± 2.9	n.t.	n.t.	n.t.
500	n.t.		25.0 ± 1.4	n.t.	n.t.	n.t.
200	n.t.		9.0 ± 1.1	100.0 ± 0.0	63.7 ± 11.9	82.0 ± 2.5
100	n.t.		3.0 ± 0	86.4 ± 2.8	43.0 ± 5.5	75.0 ± 5.5
50	70.0 ± 3.6	n.t.	n.t.	51.2 ± 3.3	29.8 ± 4.2	66.7 ± 4.4
25	60.0 ± 2.2	n.t.	n.t.	21.3 ± 1.4	22.7 ± 5.7	49.0 ± 6.2
12.5	55.0 ± 3.6	n.t.	n.t.	11.7 ± 1.9	17.3 ± 3.2	30.7 ± 8.2
6.2	49.0 ± 4.2	n.t.	n.t.	n.t.	n.t.	n.t.
3.1	32.0 ± 8.2	n.t.	n.t.	n.t.	n.t.	n.t.
	0.3 ± 0.1	2.0 ± 0.5	1.0 ± 0.2	3.0 ± 1.5	2.0 ± 0.3	1.0 ± 0.3
LC_{50}	9.5 (6.4–14)	n.d.	585.0 (462.3–740.3)	40.2 (25.3–63.8)	121.2 (90–163.5)	34.5 (21.4–55.8)

[a] Blank cells denote no effect; n.t. not tested; n.d. not detectable.

two pure compounds, praeruptorins A and B. The Irwin test showed that the three extracts and praeruptorins A and B after oral administration do not provoke behavioural effects in mice and are devoid of acute toxicity. After intraperitoneal administration only AcOEt and praeruptorin A resulted toxic at the high dose of 1 g/kg i.p. In *Artemia salina* test AcOEt resulted cytotoxic as well as the two praeruptorins, among which praeruptorin B was more potent.

On the whole, these results show that *P. praeruptorum* extracts are devoid of behavioural effects and acute toxicity when administered by mouth. Regarding the pure components only praeruptorin A appears toxic, although only after injection of high doses.

Taking into account the good correlation between the cytotoxicity on *Artemia salina* with that on tumour cell lines such as KB, P-388, L5178Y and L1210 [15], praeruptorins, and particularly praeruptorin B, that show an LC_{50} value not too different from that of podophyllotoxin, is worthy of interest. Further studies on *P. praeruptorum* will be carried out to better define the spectrum and the selectivity of the cytotoxic activity.

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