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In vitro study of antiyersinious effects of Oxadin®

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Yersinia pseudotuberculosis and *Yersinia enterocolitica* are the etiologic agents of yersiniosis – a zoonosis with a widespread distribution in the world [1, 2]. Both microorganisms may cause various syndromes in humans and animals ranging from enterocolitis, mesenteric lymphadenitis, pseudoappendicitis and arthritis to severe septicemia and systemic infections with abscesses in spleen and liver [3–5]. For yersiniosis, antibiotic treatment recommendations from the World Health Organization include tetracycline, chloramphenicol, gentamycin, and cotrimoxazol [6].

Despite the diversity of antibacterial agents available, *Yersinia* infections are still a serious problem with unsolved pathogenesis [7]. Moreover, many antibiotics are overused in veterinary practice and are considered as the primary cause of multiple resistant strains in humans. With regard to this, intensive investigations on synthesis and characterization of new antibacterial agents have been performed [8, 9].

Aim of the present work was to study *in vitro* the antibacterial effect of the Bulgarian antimicrobial chemotherapeuticum Oxadin® towards the growth of clinical strains of *Y. pseudotuberculosis* and *Y. enterocolitica*.

Oxadin® is the dinatrium salt of 2-(4-uracilmethylene)-5-(4-bromphenyl)-6-hydroxy-2,3-dihydro-(6 *H*)-1,3,4-oxadiazin synthesized in Troya-Farm Company (Bulgaria). It has successfully been administered as an antiviral agent against rota-, corona- and herpes virus infections in animals. *In vitro* Oxadin® has shown growth inhibition and bactericidal effects against clinical pathogenic strains of *Staphylococcus aureus* [10, 11].

In the course of the present examination 10 strains of *Y. pseudotuberculosis* and 10 strains of *Y. enterocolitica* were used (Table 1). The antiyersinious effect was tested by a routine agar-diffusion method. In each *Yersinia* inoculated Petri dish 4 wells (9 mm in diameter) were prepared and filled up with 1 ml of 10% solution of Oxadin®. The zones of bacterial growth inhibition were measured after 24–48 h incubation at 37 °C. The spectrum of resistence was also determined against the WHO recomended drugs – tetracycline (4 μg/ml), chloramphenicol (8 μg/ml), gentamycin (4 μg/ml), and cotrimoxazol (0.2 μg/ml) (Difco Laboratories, USA). The minimal inhibiting concentration (MIC) towards one of *Y. pseudotuberculosis* and *Y. enterocolitica* strains was determined in dynamics. MIC was defined as the lowest concentration of Oxadin® at which no visible growth was observed at 24, 48, 72 and 96 h. The minimal bactericidal concenration (MBC) was determined after subculturing of 0.1 ml from all clear MIC tubes onto MPA. All experiments were performed in five replicates. Statistical analysis was carried out by Student's t-test.

Results summarizing the effect of Oxadin® on *Y. pseudotuberculosis* and *Y. enterocolitica* strains are presented in Table 1. The growth of all examined strains was inhibited by Oxadin® used in 10% concentration. The diameter of inhibiting zones varied from 15 to 20 mm. All strains of both *Yersinia* species were susceptible to tetracycline (16.3–21.3 mm inhibiting zones), chloramphenicol (16.2–19.2 mm), gentamycin (18.9–22.4 mm), and cotrimoxazol (18.6–22.5 mm). No secondary stocks have been observed in these areas, which indicates an expressed bactericidal effect of the chemotherapeuticum and the others antimicrobial agents. Analysis of data shows that the differences observed with Oxadin® are probably related with the individual strain peculiarities. Moreover, no correlation was found between the presence of virulence plasmid, serotype and the sensitivity to Oxadin®.

Table 2 shows the results from determination of MIC and MBC of Oxadin towards *Y. pseudotuberculosis* IP 2969 and *Y. enterocolitica* IP 8896. Both strains showed a middle inhibiting zone in agar, i.e. they possess a good sensitivity to this agent. Obviously, after augmentation of the time for direct contact of test-microorganisms and Oxadin® in MPB, a better inhibiting effect was observed. For example, 24 h after inoculation of the tube with *Y. pseudo-*

Table 1: Inhibiting effect of Oxadin® on the growth of *Y. pseudotuberculosis* and *Y. enterocolitica* strains

Yersinia strains*	Country of isolation	pYV	Serotype	Inhibitory zones				
				Oxadin®	Tetracycline	Chloramphenicol	Gentamycin	Cotrimoxazol
Yp IP 32981	France	+	I	16.9 ± 1.2	18.9 ± 2.1	16.8 ± 1.2	19.6 ± 1.7	20.8 ± 2.4
Yp IP 32979	France	+	I	17.4 ± 1.5	16.3 ± 1.5	18.2 ± 1.6	21.4 ± 2.2	18.6 ± 1.9
Yp IP 2911	Argentina	–	I	18.4 ± 1.4	16.8 ± 1.7	16.4 ± 1.4	19.8 ± 1.8	21.4 ± 2.8
Yp IP 2969	France	–	II	18.3 ± 1.6	18.2 ± 1.4	17.3 ± 1.6	20.6 ± 1.7	22.1 ± 3.0
Yp IP 2951	France	+	II	20.5 ± 1.9	17.4 ± 1.8	19.1 ± 1.8	19.4 ± 1.8	19.7 ± 1.9
Yp IP 2926	France	+	II	15.6 ± 1.3	16.9 ± 1.7	18.4 ± 2.1	21.3 ± 2.2	19.9 ± 2.0
Yp IP 32984	Spain	+	III	19.2 ± 1.4	17.3 ± 1.5	16.7 ± 1.3	20.6 ± 1.9	21.2 ± 2.1
Yp IP 2861	France	+	III	19.7 ± 1.4	18.8 ± 1.9	16.2 ± 1.4	19.8 ± 2.1	21.3 ± 2.0
Yp IP 2637	Switzerland	+	III	19.5 ± 1.6	19.1 ± 2.0	17.1 ± 1.6	21.6 ± 2.3	20.7 ± 2.2
Yp IP 2952	Argentina	+	V	16.9 ± 1.5	17.5 ± 1.5	18.4 ± 1.5	22.4 ± 2.5	21.3 ± 2.3
Ye IP 8896	Italy	+	0:3	17.4 ± 1.5	19.4 ± 1.6	16.8 ± 1.4	20.7 ± 1.8	22.5 ± 2.7
Ye IP 7841	Africa	–	0:3	15.8 ± 1.2	17.6 ± 1.5	16.3 ± 1.2	19.2 ± 2.3	21.0 ± 2.2
Ye IP 23111	France	+	0:3	16.4 ± 1.3	18.1 ± 1.4	18.5 ± 1.7	19.8 ± 2.5	20.5 ± 2.1
Ye IP 21100	France	–	0:5	19.1 ± 1.4	18.7 ± 1.8	16.7 ± 1.6	20.3 ± 2.4	19.7 ± 1.8
Ye IP 22981	France	+	0:5	17.6 ± 1.2	16.9 ± 1.3	17.4 ± 1.5	21.5 ± 2.2	20.9 ± 2.1
Ye IP 22464	Australia	–	0:5	19.8 ± 2.1	19.2 ± 1.6	18.3 ± 1.7	18.9 ± 1.8	19.4 ± 1.8
Ye ATCC 8081	USA	–	0:8	18.7 ± 2.0	17.8 ± 1.4	19.2 ± 1.8	19.6 ± 2.1	21.2 ± 2.1
Ye IP 383	Belgium	+	0:9	19.3 ± 1.4	18.2 ± 1.8	16.6 ± 1.4	20.8 ± 1.8	20.4 ± 1.9
Ye IP 21447	UK	+	0:9	19.0 ± 1.6	21.3 ± 2.4	16.8 ± 1.2	22.4 ± 2.6	21.3 ± 2.0
Ye IP 23067	France	–	0:9	20.2 ± 1.7	19.7 ± 1.8	17.4 ± 1.7	21.8 ± 2.0	21.6 ± 2.1

Legend: IP - Institute Pasteur Collection; ATCC - American Collection; Yp - Yersinia pseudotuberculosis; Ye - Yersinia enterocolitica
* All the strains were kindly provided by Dr. Carniel (Yersinia Reference Center, Institute Pasteur, Paris, France).

Table 2: Number of colonies (CFU) of the tested *Yersinia* strains after treatment with Oxadin®

Test strain	Time (h)	Dilutions						Control
		1 : 2 (50)[1]	1 : 4 (25)	1 : 8 (12.5)	1 : 16 (6.25)	1 : 32 (3.125)	1 : 64 (1.56)	
	24	–	–	–	9 ± 2	236 ± 40	SC[2]	CG[3]
YP	48	–	–	–	–	164 ± 25	SC	CG
IP 2969	72	–	–	–	–	39 ± 14	SC	CG
	96	–	–	–	–	–	348 ± 74	CG
	24	–	–	–	2	24 ± 6	SC	CG
YE	48	–	–	–	–	8 ± 2	540 ± 60	CG
IP 8896	72	–	–	–	–	1	110 ± 21	CG
	96	–	–	–	–	–	32 ± 6	CG

Legend: YE – Yersinia enterocolitica
YP – Yersinia pseudotuberculosis;
[1] Quantity of Oxadin® (mg/ml) in 10% concentration
[2] Semi–confluent bacterial growth
[3] Confluent bacterial growth

tuberculosis IP 2969 containing 6.25 mg/ml Oxadin® (dilution 1 : 16), only nine colonies were detected. Later, after 48 h contact at a dilution of 1 : 32, 164 colonies were counted. The total inhibiting effect was proved after 96 h at the same dilution. When *Y. enterocolitica* IP 8896 was used as test strain, similar dynamics of the inhibitory effect was observed. The number of bacterial cells reported periodically at every 24 h at a dilution of 1 : 16 and 96 h at a dilution of 1 : 32 is equal to zero. The MIC determinations for tetracycline (4 μg/ml), chloramphenicol (8 μg/ml), gentamycin (4 μg/ml), and cotrimoxazol (0.2 μg/ml) are in agreement with those reported by other authors [12–14]. Serotype or strain specific patterns of susceptibility were not found, irrespective of the geographic and host origin of the strains used.

The data received allow the conclusion that Oxadin® has a well expressed inhibiting and bactericidal *in vitro* effect on both *Yersinia* pathogens, which are in accordance with the factors concentration and time of contact. Further experiments aiming to establish an antibacterial effect of Oxadin® *in vivo* are in progress in our laboratory.

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A new steroidal saponin from the bulbs of *Lilium candidum* L.

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In preceding communications [1, 2], we reported the isolation of three steroidal saponins from *Lilium candidum* L. These glycosylated compounds contain two molecules of

Table: ^{13}C and ^{1}H NMR data of the new steroidal glycoside 1

Position	Carbon-13 chemical shifts (CD_3OD)	Proton chemical shifts (coupling constants) (CD_3OD) Aglycone
1	41.44	1.88; 1.07
2	33.18	1.90; 1.60
3	78.64	3.59 m
4	38.55	2.45 ddd; 2.30 bt
5	141.90	–
6	122.63	5.39 m
7	30.75	2.01; 1.58
8	32.80	1.57
9	51.70	0.97
10	38.04	–
11	21.97	c
12	39.55	c
13	40.90	–
14	57.84	c
15	32.77	2.00; 1.28
16	80.97	4.51 ddd (*6.7; 7.6; 8.4*)
17	63.90	1.80 dd *(6.5; 8.5)*
18	16.74	0.81 s
19	19.82	1.05 s
20	42.94	1.94 p
21	14.99	1.00 d *(6.8)*
22	113.16	–
23	31.94	c
24	28.98	c
25	36.28	1.41 m
26	103.23	4.36 d *(8.6)*
27	16.83	0.90 d *(6.6)*
26-OR	65.35	3.49 dq and 3.82 dq (*9.6; 7.1*)
	15.68	1.20 t (*7.1*)
		Saccharide part
Glc: 1′	100.41	4.40 d *(7.8)*
2′	77.92	3.20 dd *(7.8; 9.3)*
3′	76.25	3.36 t *(9.3)*
4′	82.54	c
5′	77.82	c
6′	62.48	c
Rha: 1″	102.06	5.24 d *(1.6)*
2″	72.24	3.89 dd *(1.6; 3.3)*
3″	72.39	3.66 dd *(3.3; 9.5)*
4″	73.93	3.39 t *(9.5)*
5″	69.74	4.13 dq *(9.5; 6.3)*
6″	17.94	1.24 d *(6.3)*
Glc: 1‴	104.64	4.52 d *(7.8)*
2‴	75.08	3.42 dd *(7.8; 9.0)*
3‴	79.38	3.65 t *(9.0)*
4‴	71.41	c
5‴	78.13	c
6‴	61.91	c

[c] the value of parameter could not be determined