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The Sensitization of *Legionella pneumophila* to Some Antibiotics by Reserpine and Anti-*Legionella* Effects of Different Benzofuranone Derivatives

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ABSTRACT

Background: *Legionella pneumophila* is a dangerous pathogenic bacterium can cause serious infectious diseases especially in hospitalized immuno-compromised patients. This bacterium is shown to be resistant against different antibiotics. Resistance against a wide range of antibiotics is usually mediated by efflux pump in bacteria. Efflux pumps are proteinaceous transporters localized in the cytoplasmic membrane of all kinds of cells which excreted antibiotics outside the cells. However, synthesis of new anti-*Legionella* compounds or selection of resistant modulating agents are useful strategy to combat with *L. pneumophila*in the future.

Methods: In this study the antibacterial activity of some benzofuranone derivatives have been investigated by disk diffusion method against *L. pneumophila*. Also the sensitivity of this test strain was evaluated against 19 antibiotics and the combination effect of reserpine at a sub-inhibitory concentration was further studied with these antibiotics using disk diffusion method with some modifications.

Conclusion: Among the different synthetic compounds which were tested against *L. pneumophila*, the most antibacterial activity was observed for compounds 1j and 1m which contain hydroxyl and methoxy groups on the C-6 and C-7 positionsagainst *L. pneumophila*. To evaluate whether efflux pumps are active in *L. pneumophila* or not an efflux inhibitor (reserpine) was tested in combination of different antibiotics against this test strain. Reserpine significantly enhanced the antibacterial activities of kanamycin, nitrofurantoin, co-trimoxazole, erythromycin, ofloxacillin, gentamycin, rifampin, ciprofloxacin, nalidixic acid, minocycline, tobramycin, and amikacin against *L. pneumophila* which shows the resistances to these antibiotics are mediated by efflux system in this bacterium.

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Introduction

Legionnaires disease, one of the most common cause of hospital and community acquired pneumonia is because of *Legionella pneumophila* infection. *L. pneumophila* and all of other species of this genus are Gram negative facultative intracellular bacteria (1, 2). There is some limitation on the selection of an appropriate antibiotic for treatment of infections caused by this bacterium. Intrinsic resistance of *L. pneumophila* against conventional antibiotics modulated by efflux pumps as well as intracellular nature of this microorganism during their life cycle. There are two important factors to limit the usage of conventional antibiotics against *L. pneumophila* (3). Before erythromycin was considered as the first line drug to treat *L. pneumophila* infection, but because bacterial resistance to this antibiotic, it was replaced by more active agents from macrolides and quinolones groups (i.e. azithromycin, ciprofloxacin, levofloxacin). Rifampin has been also recently added to this category (1, 3). However this antibiotic replacement could not completely reduced the mortality of patient suffering from hospital acquired pneumnonia caused by *L. pneumophila* especially in immunocompromised cases (3). Because of the reasons cited, synthesis of new anti-*Legionella* compounds and selection of resistant modulating agentswhich decrease the resistance of *L. pneumophila* towards conventional antibiotics are useful strategy to overcome the above limitation in the near future.

Recently, novel (z)-2-(nitroimidazolulmethylen)-3 (2H)-benzofuranone derivatives have been synthesized and their antimicrobial effects against wide variety of test strains have been reported (4). The main goal of this work is testing these novel compounds on the *L. pneumophila*. Also we investigated the effect of reserpine, since it is famous for efflux pump inhibitor, on the antibacterial activity of conventional antibiotics against *L. pneumophila*.

Materials and Methods

Bacterial test strain and other materials

A previously isolated *L. pneumophila* strain (GenBank HQ840733), which has been identified by 16S rRNA gene amplification method, was used in this research as a test strain. Five mg of each benzofuranone derivatives which listed in Table 1 were precisely weighted and dissolved in *1 ml dimethyl sulfoxide* (DMSO). Second, sterile blank paper disks were loaded by aliquots 8µl of the above stock solutions to obtain 40 µg of each compound per each paper disks. All impregnated disks were dried and reserved in dark at 4°C for further antibacterial assay. Standard antibiogram disks listed in Table 2 were obtained from Mast Co., UK and used for reserpine-antibiotic combination assay.

Susceptibility testing

Antimicrobial activity of different benzofuranone derivatives were determined by conventional disk diffusion method. For this purpose, a fresh culture of the *L. pneumophila* (48 hours) was harvested and a 0.5 MacFarland microbial suspension was prepared as an inoculum with viable counts of approximately 1×10^8 CFU/ml.

Subsequently, the *surface* of BCYE-agar plateswere *inoculated with* a lawn of 0.5

McFarland suspension of the bacterial suspension.

Table 1. The antibacterial effect of different of benzofuranone derivatives against *Legionella pneumophila* at content of 40 µg/disk

Compounds	X-Residue	Zone of inhibition (mm)
1a	H	10
1b	5-Cl	8.5
1c	5-Br	-
1d	5-CH_3	-
1e	5-OCH_3	25
1f	5-I	-
1g	6-Cl	-
1h	6-CH_3	-
1i	6-OCH_3	-
1j	6-OH	-
1k	7-CH_3	10
1l	7-OCH_3	7
1m	6,7-$(OCH_3)_2$	25

In the next step disks which contain different benzofuranone derivatives compounds were applied on the surface of the inoculated plates. All plates were incubated for 48 h in 37°C. They were protected in the candle jar with 5% CO_2 and high humidity condition. After this step the inhibition zone diameters were measured and reported.

To validate the combination effect of reserpine with antibiotics, different sub-inhibitory amounts of reserpine were over-loaded onto the standard antibiotic disks (20 and 40 µg/disks). For this purpose, a stock solution of reserpine (1mg/ml) was prepared and two aliquots (20 and 40 µl) of this stock solution were separately loaded onto different standard antibiotic disks which listed in Table 2. The reserpine supplemented antibiogram disks as well as a new series of the standard antibiotic disks were placed onto the inoculated BCYE-agar media. All plates were incubated for 48 h in 37°C. After this step the diameter of inhibition zone around the paper disks were precisely measured by a ruler and the mean surface area of each inhibition zone (mm^2) were calculated from the mean diameter of each tested antibiotic. The fold of increase in the inhibition zone areas for different antibiotics against *L. pneumophila* were calculated by the following equation; $(b^2-a^2)/a^2$

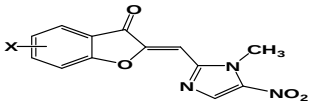
In the above formula, *a* is the inhibition zone diameter at the presence of antibiotic only and *b* represents the inhibition zone in the presence of antibiotic plus reserpine. All experiments were performed in three different time frames.

Results and Discussion

Antibacterial effect of benzofuranones derivatives

The compounds depicted in Figure 1, (*Z*)-2-(1-methyl-5-nitroimidazole-2-ylmethylene)-3(2*H*)-benzofuranones (1a-n), were prepared by the condensation of appropriate 3(2*H*)-benzofuranones with 1-methyl-5-nitroimidazole-2-carbaldehyde in acetic acid in the presence of catalytic amount of sulfuric acid (5). The antibacterial properties of these compounds were assayed in vitro against *L. pneumophila*by a disk diffusion method *(Table 1).* Considering the activities of compounds, generally it appears that changing the position of substituents on benzofuran have significant influence on antibacterial activity. Among the 13 different substituents, compounds 1j and 1m having hydroxyl and methoxy groups on the C-6 and C-7 positions, respectively, revealed to be the most active compounds. In addition, compounds having small lipophilic

residues such as chloro and methyl substituents on the C-5 and C-7 positions (1b and 1l), respectively, as well as unsubstituted derivative (1a) were shown to be moderately active against *L. pneumophila*. The rest were inactive against selected bacteria.



1a-n
X= 1a, H; 1b, 5-Cl; 1c, 5-Br; 1d, 5-CH_3; 1e, 5-OCH_3; 1f, 5-I; 1g, 6-Cl; 1h, 6-CH_3; 1i, 6-OCH_3; 1j, 6-OH; 1k, 7-CH_3; 1l, 7-CH_3; 1m, 7-OCH_3; 1n, 6,7-$(OCH_3)_2$.

Figure 1. The structure of synthesized compounds **1a-n**

Table 2. Zone of inhibition (mm) and increase in inhibition zone area of different antibiotics against *Legionella pneumophila* (in absence and in presence of reserpine at contents of 20 and 40 µg/disk)

	Inhibition zone diameter (mm)			Increase in inhibition zone area	
Antibiotic[a]	**- reserpine**	**+ reserpine 20µg**	**+ reserpine 40µg**	**+ reserpine 20µg**	**+ reserpine 40µg**
Kanamycin(30)	15	16	37	0.14	5.1
Nitrofurantoin (300)	9	13	50	1.8	2.98
Cotrimoxazole (25)	17	18	35	0.12	3.23
Vancomycin (100)	15	15	15	·	·
Erythromycin (5)	24	24	34	·	1.006
Ofloxacin (5)	8	8	30	·	13.06
Clindamycin (2)	12	12	12	·	·
Gentamycin (10)	9	9	32	·	11.64
Cephalexin (30)	30	32	15	0.137	-0.75
Rifampin (5)	13	16	20	0.514	0.136
Ciprofloxacin (5)	10	10	22	·	3.84
Nalidixic acid (30)	9	9	38	·	16.82
Minocycline (30)	20	20	26	·	0.69
Ticarcillin (75)	22	22	10	·	-0.79
Coamoxiclave (30)	35	35	11	·	-0.9
Tobramycin (10)	7	8	30	0.3	17.36
Cefoporazone (75)	23	23	20	·	-0.24
Cephalotin (30)	22	35	17	1.53	-0.4
Amikacin (30)	10	13	20	0.69	3

[a] No inhibition zones were observed for following antibiotics in presence or absence of reserpine. Piperacillin (30), Carbenicilin (100), Bacitracin (0.04), Penicillin G (10 IU), Cefixime (5), Methicillin (5), Amoxicillin (25), Amikacin (30), Polymxin (100 IU), Aztreonam (30), Metronidazole (5), Furazolidone (50), Meropenem (10), Cefotaxime (30), Streptomycin (10), Ceftazidime (30), Oxacillin (1), Cefepime (30), Ampibactam (10), Colistin (10).

Combined effect of reserpine with conventional antibiotics

In this research effect of the indole alkaloid reserpine on the antibacterial of different antibiotics was investigated against *L. pneumophila*. The diameter of inhibition zones (mm) around the different antibiotic disks with or without reserpine against test strain are shown in Table 2. The antibacterial activities of kanamycin, nitrofurantoin, co-trimoxazole, erythromycin, ofloxacillin, gentamycin, rifampin, ciprofloxacin, nalidixic acid, minocycline, tobramycin and amikacin have been increased against *L. pneumophila*in the presence of reserpine (40 µg/disk). No enhancing effect on the antibacterial activities of others antibiotics was observed against *L. pneumophila* at tested concentration (40 µg/disk). The

highest fold increases in area were present for nitrofurantoin against *L. pneumophila* (a 29.86-fold increase). The fold increase in inhibition zone areas (%) for tobramycin, nalidixic acid, gentamycin and ofloxacillin were 17.36, 16.82, 11.64 and 13.06, respectively. Conversely, for cephalexin, cephalotin, cefoporazone, ticarcillin and coamoxiclave reserpine showed an antagonistic effect on the antibacterial activity of these antibiotics against the test strain. It should be pointed out that the sub-inhibitory contents of reserpine (20 or 40 µg/disc) were chosen to guarantee that the effect produced was due to the combination; not to the effect of the reserpine itself. So the effect observed in this condition could be due to the antibiotic-reserpine combination. At the concentration tested, reserpine significantly improved antibiotic efficacy against *L. pneumophila* when combined with kanamycin, nitrofurantoin, cotrimoxazole, erythromycin, ofloxacillin, gentamycin, rifampin, ciprofloxacin, nalidixic acid, minocycline, tobramycin, and amikacin (*Table 2*). As of now, the reason for the lack of enchantments and differences are not known. Efflux transporter mediated bacterial resistance to different antibiotics and reserpine may inhibit this efflux pump system (5). This is the first report of combination effect of reserpine with different antibiotics against *L. pneumophila*. Recently some natural products have been evaluated for increasing the antibacterial activities in different antibiotic (6-9). We showed that essential oils (*Menthalongifolia L.* and *Menthaspicata L.*) and different monoterpenes (piperitone, carvone and menthone) enhanced antibacterial activity of nitrofurantoin against Enterobacteria (6-8). Also the enhancement of cinnamon essential oil and its major component (Trans-cinnamaldehyde) on the antibacterial activity of clindamycin against a toxicogenic strain of *Clostridium difficile* has been previously reported (9). In this experiment using disk diffusion assay showed antibacterial activity of some antibiotics. They are currently used in treatment of Legionnaires's disease and they can be increased by the indole alkaloid reserpine.

Conclusion

The research focus was on examining the antibacterial activity of different benzofuranone derivatives against *L. pneumophila*. As a result, compounds bearing hydroxyl and methoxy groups on the C-6 and C-7 positions respectively, showed most antibacterial activity against this test strain. The effect of reserpine on the antibacterial activity of 19 antibiotics was tested against *L. pneumophila*. Results showed indole alkaloid can enhance the anti-*Legionella* activity of considerable numbers of antibiotics which examined during this investigation.

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Conflict of Interest

None declared conflicts of interest.

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