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Source / Izvornik: **Acta Pharmaceutica, 2019, 69, 277 - 285**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.2478/acph-2019-0020

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:163:045890

Acta Pharm. **69** (2019) 277–285 Short communication

https://doi.org/10.2478/acph-2019-0020

Antimicrobial assesment of aroylhydrazone derivatives *in vitro*

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Aroylhydrazones **1–13** were screened for antimicrobial and antibiofilm activities *in vitro*. *N*′-(2-hydroxy-phenylmethylidene)-3-pyridinecarbohydrazide (**2**), *N*′-(5-chloro-2-hydroxyphenyl-methylidene)-3-pyridinecarbohydrazide (**10**), *N*′-(3,5-chloro-2-hydroxyphenylmethylidene)-3-pyridinecarbohydrazide (**11**), and *N*′-(2-hydroxy-5-nitrophenylmethylidene)-3-pyridinecarbohydrazide (**12**) showed antibacterial activity against *Escherichia coli*, with *MIC* values (in μmol mL^{-1}) of 0.18–0.23, 0.11–0.20, 0.16–0.17 and 0.35–0.37, resp. Compounds **11** and **12**, as well as *N*′-(2-hydroxy-3-methoxyphenylmethylidene)-3-pyridinecarbohydrazide (**6**) and *N*′-(2-hydroxy-5-methoxyphenylmethylidene)-3-pyridinecarbohydrazide (**8**) showed antibacterial activity against *Staphylococcus aureus*, with the lowest *MIC* values of 0.005–0.2, 0.05–0.12, 0.06–0.48 and 0.17–0.99 μmol mL^{-1}. *N*′-(2-hydroxy-5-methoxyphenylmethylidene)-3-pyridinecarbohydrazide (**7**) showed antifungal activity against both fluconazole resistant and susceptible *C. albicans* strains with IC_{90} range of 0.18–0.1 μmol mL^{-1}. Only compound **11** showed activity against *C. albicans* ATCC 10231 comparable to the activity of nystatin (the lowest *MIC* 4.0 ×10^{-2} *vs*. 1.7 × 10^{-2} μmol mL^{-1}). Good activity regarding multi-resistant clinical strains was observed for compound **12** against MRSA strain (*MIC* 0.02 μmol mL^{-1}) and compounds **2**, **6** and **12** against ESBL+ *E. coli* MFBF 12794, with the lowest *MIC* for compound **12** (IC_{50} 0.16 μmol mL^{-1}). Anti-biofilm activity was found for compounds **2** (*MBFIC* 0.015–0.02 μmol mL^{-1} against MRSA) and **12** (*MBFIC* 0.013 μmol mL^{-1} against EBSL+ *E. coli*). In the case of compound **2** against MRSA biofilm formation, *MBFIC* values were comparable to those of gentamicin sulphate, whereas in the case of compound **12** and EBSL+ *E. coli* even more favourable activity compared to gentamicin was observed.

Keywords: aroylhydrazones, antimicrobial, antibiofilm, MDR strains

Accepted December 14, 2018
Published online February 11, 2019

Aroylhydrazones contain an azomethine group –NHN=CH– connected with carbonyl group, which is responsible for their different pharmaceutical applications. Therefore, aroylhydrazones have attracted considerable attention for their wide range of biological

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activities, such as antimicrobial, anticonvulsant, antitumor, analgesic, antiplatelet, antitubercular and anti-inflammatory activities (1, 2). Further, hydrazones as chelating agents were investigated as potential drugs for the treatment of a disease called "iron overload disease" (3). Salicylaldehyde derivatives were shown to be selective iron chelators with promising pharmacological properties (4). However, pharmacokinetic studies have stressed the relatively short biological half-life of certain hydrazones due to hydrolysis of the hydrazone bond, which is typical for this class of compounds (4, 5). Molecular structural changes of aroylhydrazones derived from salicylaldehyde, *o*-vanilin and nicotinic acid hydrazide in dimethylsulphoxide/water (DMSO/water) mixtures were studied by NMR, UV–Vis, ATR and Raman spectroscopy (6). Results obtained by Galić *et al.* (6) revealed that addition of water to the system did not induce tautomeric conversion of the ketoamino hydrazide part and the enolimino aldehyde part. Furthermore, addition of water showed formation of hydrated molecules and compounds were quite stable in DMSO/water mixtures (V(DMSO)/V(H_2O), of 9/1, 8/2, 7/3 and 6/4 volume ratios).

According to the work done by Da Costa *et al.* (7), *in vitro* evaluated *N*-acylhydrazone derivatives of different amino acids such as L-phenylalanine, L-leucine and L-alanine against *M. tuberculosis* showed a *MIC* between 12.5–50 μg mL^{-1}. In another study, done by Mandewale *et al.* (8), zinc(II) complexes with quinoline hydrazone ligands were synthesized and screened for activity against *M. tuberculosis* (H37 RV strain) ATCC 27294. Results showed that two of the tested complexes were very active, with *MIC* value ranging from 8.00–7.42 μmol L^{-1}, which is comparable to the drugs ciprofloxacin (*MIC* value 9.41 μmol L^{-1}), pyrazinamide (*MIC* value 25.34 μmol L^{-1}) and streptomycin (*MIC* 10.74 μmol L^{-1}) used to treat tuberculosis.

Benzimidazole derivatives bearing hydrazone moiety revealed that some of the compounds had a bactericidal effect on the growth of *Salmonella typhimurium*, two times better or equal to the activity of chloramphenicol, which was used as a positive control (9). Activities of these compounds against other Gram-negative bacterial strains such as *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* were good, with the *MIC* value of 25–100 μg mL^{-1}. Activities against Gram-positive bacteria were determined on four strains: *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtilis*. The best activity, equal to the activity of chloramphenicol, was found against an *E. faecalis* strain with the *MIC* value of 12.5 μg mL^{-1}.

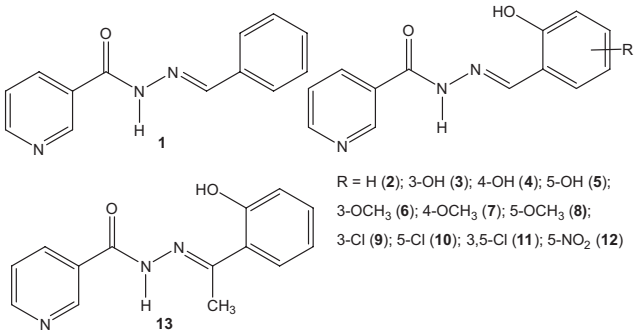

Fig. 1. Structures of aroylhydrazones **1–13**.

As a part of our investigations on aromatic hydrazones derived from nicotinic acid hydrazide (10), a group of 13 derivatives (Fig. 1) was studied for antimicrobial activity in the present work. Our research was focused on antibacterial, antifungal and antibiofilm studies *in vitro*.

EXPERIMENTAL

Chemicals

Chemicals used in this study, such as acetic acid glacial (Panreac, Spain), dimethyl sulfoxide pure (Lach-Ner, Czech Republic), methanol (Merck KgaA, Germany), are commercially available and were used without further purification, while aroylhydrazone derivatives were synthesized by previously described procedures (11) and analyzed using standard analytical methods. Drugs used as positive controls were gentamicin sulphate and colistin purchased from Sigma-Aldrich, nystatin from PLIVA Hrvatska (Croatia), norfloxacin from Krka-Farma (Slovenia) and voriconazole from Pfizer (USA).

Microorganisms and media

Several microorganisms, standard laboratory strains from the American Type Culture Collection (ATCC) and resistant clinical strains, were obtained from stock cultures of the Collection of Microorganisms (MFBF), Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. Experiments included *Staphylococcus aureus* ATCC 6538 (methilicin susceptible strain), methicillin resistant *S. aureus* MFBF 10679 (MRSA), *Escherichia coli* ATCC 10536, extended-spectrum beta-lactamase-positive *E. coli* (ESBL+ *E. coli*) MFBF 12794, *Candida albicans* ATCC 10231 (fluconasole-susceptible), fluconasole-resistant *C. albicans* MFBF 11103 (Ca^{FLR+}), and *Aspergillus brasiliensis* ATCC 16404. Microbiological media Mueller-Hinton broth, Mueller-Hinton+2 % glucose (*m*/*V*) broth and RPMI 1640+2 % (*m*/*V*) glucose broth were purchased from Merck (Germany).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (*MIC*s) were measured by the serial two-fold microdilution broth assay in Mueller-Hinton broth for bacterial strains, and RPMI with 2 % of glucose broth for fungi according to the recommendations of EUCAST protocols (12, 13).

Compounds **1**–**13** were first dissolved in DMSO/water (1:1, *V*/*V*), followed by dilution with sterile physiological saline to a stock concentration of 800 μg mL^{-1}. The solvent mixture was tested as a negative control in all assays.

Inoculum suspensions of bacteria and fungi were prepared from fresh cultures of microbial strains cultured on the surface of tryptic-soy agar for 18 h at 35 °C for bacterial species, and on Sabouraud 2 % (*m*/*V*) glucose agar for 48 h at 35 °C for yeast and fungi. Inocula were prepared with physiological saline and cell density was adjusted to 0.5 McFarland units using a nephelometer (Kisker, Germany). Working microbial suspensions were prepared as 1:10 dilutions in Mueller-Hinton broth for bacteria, and RPMI-2 % glucose broth for yeast and fungi. Final bacterial suspensions contained approximately 10^7 CFU mL^{-1} and the yeast/mold suspensions 5×10^6 CFU mL^{-1}. Serial two-fold microdilution

Table I. MIC *values* (IC_{90} *and* IC_{50}) *of* in vitro *antimicrobially active aroylhydrazone derivatives*

Compound	MIC ($\mu mol\ mL^{-1}$)	Microorganism					
		S. a. ATCC 6538	*E. coli* ATCC 10536	*C. a.* ATCC 10231	MRSA MFBF 10679	ESBL+ *E. coli* MFBF 12794	*C. a.*$^{FLR+}$ MFBF 11103
2	IC_{90}	> 0.83	0.23	> 0.83	> 0.83	0.45	0.49
	IC_{50}	> 0.83	0.18	> 0.83	> 0.83	0.29	0.36
6	IC_{90}	0.48	> 0.74	> 0.74	> 0.74	0.52	> 0.74
	IC_{50}	6.00×10^{-2}	> 0.74	> 0.74	> 0.74	0.37	> 0.74
7	IC_{90}	> 0.74	> 0.74	0.18	> 0.74	> 0.74	0.10
	IC_{50}	> 0.74	> 0.74	0.16	> 0.74	> 0.74	9.00×10^{-2}
8	IC_{90}	0.99	> 0.73	> 0.73	> 0.73	> 0.73	> 0.73
	IC_{50}	0.17	> 0.73	> 0.73	> 0.73	> 0.73	> 0.73
10	IC_{90}	> 0.73	0.20	> 0.73	> 0.73	> 0.73	> 0.73
	IC_{50}	> 0.73	0.11	> 0.73	> 0.73	> 0.73	> 0.73
11	IC_{90}	0.2	0.17	5.0×10^{-2}	> 0.65	> 0.65	0.4
	IC_{50}	5.16×10^{-3}	0.16	4.0×10^{-2}	> 0.65	> 0.65	0.36
12	IC_{90}	0.12	0.37	> 0.70	2.30×10^{-2}	0.36	> 0.70
	IC_{50}	4.70×10^{-2}	0.35	> 0.70	2.10×10^{-2}	0.16	> 0.70
13	IC_{90}	> 0.78	> 0.78	0.44	> 0.78	> 0.78	> 0.78
	IC_{50}	> 0.78	> 0.78	0.37	> 0.78	> 0.78	> 0.78
Gentamicin sulphate	IC_{90}	1.35×10^{-4}	$> 3.35 \times 10^{-2}$	$> 3.35 \times 10^{-2}$	6.97×10^{-4}	$> 3.35 \times 10^{-2}$	$> 3.35 \times 10^{-2}$
	IC_{50}	5.03×10^{-5}	$> 3.35 \times 10^{-2}$	$> 3.35 \times 10^{-2}$	2.97×10^{-4}	$> 3.35 \times 10^{-2}$	$> 3.35 \times 10^{-2}$
Norfloxacin	IC_{90}	$> 6.26 \times 10^{-3}$	4.95×10^{-5}	$> 6.26 \times 10^{-3}$	$> 6.26 \times 10^{-3}$	$> 6.26 \times 10^{-3}$	$> 6.26 \times 10^{-3}$
	IC_{50}	$> 6.26 \times 10^{-3}$	4.68×10^{-5}	$> 6.26 \times 10^{-3}$	$> 6.26 \times 10^{-3}$	$> 6.26 \times 10^{-3}$	$> 6.26 \times 10^{-3}$
Colistin	IC_{90}	$> 2.16 \times 10^{-2}$	$> 2.16 \times 10^{-2}$	$> 2.16 \times 10^{-2}$	$> 2.16 \times 10^{-2}$	5.07×10^{-5}	$> 2.16 \times 10^{-2}$
	IC_{50}	$> 2.16 \times 10^{-2}$	$> 2.16 \times 10^{-2}$	$> 2.16 \times 10^{-2}$	$> 2.16 \times 10^{-2}$	4.63×10^{-5}	$> 2.16 \times 10^{-2}$
Nystatin	IC_{90}	> 0.27	> 0.27	2.95×10^{-2}	> 0.27	> 0.27	2.28×10^{-2}
	IC_{50}	> 0.27	> 0.27	1.70×10^{-2}	> 0.27	> 0.27	1.68×10^{-2}

S. a. – *Staphylococcus aureus, E. coli* – *Escherichia coli, C. a.* – *Candida albicans, C. a.*$^{FLR+}$ – fluconazole-resistant *Candida albicans*

testing was performed for all compounds in concentrations from 200 to 0.1 μg mL^{-1}. After inoculation and aerobic incubation for 18 h at 35 °C in the dark, *MIC*s of bacterial strains were determined by adding the redox-indicator 2,3,5-triphenyl-2*H*-tetrazolium chloride [TTC, 1.0 % (*m*/*V*), in sterile physiological saline]. After 3 hours of incubation at 35 °C in the dark, 0.04 mol L^{-1} HCl in isopropanol was added to all wells and absorbance was recorded at 540 nm using a microtiter plate reader (Labsystems iEMS Reader MF, Finland). *MIC* was defined as the lowest concentration of investigated compounds that inhibited 90 % of the growth compared to the negative control; values for 50 % growth inhibition are shown as well. *MIC*s for *C. albicans* strains were obtained by determing absorbance at 540 nm using the above mentioned microtiter plate reader. *MIC* values for mold *A. brasiliensis* ATCC 16404 were determined visually after 48 h of incubation, aerobically at 35 °C in the dark. *MIC*s were calculated using the Gompertz non-linear regression model, evaluating the % microbial viability *vs*. $\log_{conc}$ (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) to get the final IC_{90} and IC_{50} values expressed in μg mL^{-1}. All tests were performed in triplicate and results are expressed as the mean value. Minimal inhibitory concentrations are shown in Table I.

Determination of anti-biofilm activity

Compounds that showed antimicrobial activity against the tested clinical microbial strains MRSA MFBF 10679, ESBL+ *E. coli* MFBF 12794 and Ca^{FLR+} MFBF 11103 were investigated for anti-biofilm activity using the crystal violet assay according to Vlainić *et al.* (14) with slight modifications. The effect on biofilm formation was evaluated by determining the minimum biofilm formation inhibition concentration (*MBFIC*). Inoculum suspensions of fresh cultures of microbial strains were prepared with sterile physiological saline and cell density was adjusted to 0.5 McFarland units using a nephelometer (Kisker). Working suspensions of bacteria and yeast used in experiments were prepared as 1:10 dilutions in RPMI 1640+2 % (*m*/*V*) glucose broth for yeast, and Mueller-Hinton+2 % glucose (*m*/*V*) broth for bacteria. Final bacterial suspensions contained 10^7 CFU mL^{-1} and yeast suspensions 5 × 10^6 CFU mL^{-1}. Experiments were performed in sterile 96-well flat-bottom plastic tissue plates (TPP, Switzerland). Investigated compounds were tested in the concentration range from 500 to 3.91 μg mL^{-1}. Untreated controls contained inoculum in broth, and standard antimicrobial drugs were used for positive controls – gentamicin sulphate (stock concentration used for ESBL+ *E. coli* MFBF 12794 was 1 mg mL^{-1}, while stock concentration for MRSA MFBF 10679 was 600 μg mL^{-1}), norfloxacin (stock concentration of 1 mg mL^{-1}) for bacteria, nystatin and voriconazole (stock concentration of 400 μg mL^{-1} each) for fungi. Only broth was used for negative controls. After a 24-h incubation period for bacteria and 48 h of incubation for yeast (37 °C, aerobic, in the dark), wells were aspirated and washed four times with 250 μL of PBS and shaken vigorously. Adherent microbial cells were treated with methanol for 20 min and left to dry overnight. The formed biofilm was stained with crystal violet (0.5 %, *m*/*V*, in methanol) for 10 min. Residual crystal violet was aspirated and plates were rinsed with tap water and left to dry overnight. Adherent biofilm was resolubilized with glacial acetic acid. Results were obtained by measuring absorbance at 540 nm using a microtiter plate reader (Labsystems iEMS Reader MF). The $MBFIC_{50}$ and $MBFIC_{90}$ values represent the lowest compound dilutions at which microbial growth during biofilm formation was inhibited by 50 and 90 %, resp., compared to the untreated control (inoculum with broth). *MBFIC*s were calculated from linear regression of the log

of concentration of compounds *vs*. % reduction, using the GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. All tests were performed in triplicate and the results are expressed as the mean value.

RESULTS AND DISCUSSION

All the compounds **1–13** were screened for their *in vitro* antibacterial activity against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) strains, *C. albicans* (yeast) and *A. brasiliensis* (filamentous fungus).

Susceptibility of the tested microbial species to aroylhydrazone compounds showed species-dependent and compound-dependent activity. Pronounced activities were detected for compounds **2**, **6**, **7**, **8**, **10**, **11**, **12** and **13**. Compounds **2**, **6**, **7**, **11** and **12** showed good activity against multi-resistant clinical strains MRSA, ESBL+ *E. coli* and *C. a.*$^{FLR+}$, still lower than that of reference drugs. The same applies to compounds **11** and **12**, which were highly active against *S. aureus* ATCC 6538 (both **11** and **12**), *C. albicans* ATCC 10231 (**11**) and *E. coli* ATCC 10536 (**11**) but still not as active as the standard drugs. Compound **6** showed activity against the *S. aureus* ATCC 6538 strain with IC_{90} range 6.0×10^{-2}–0.48 μmol mL^{-1}, while compounds **11** and **12** showed even better antibacterial activity against *S. aureus*, with the lowest *MIC* value of 5.16×10^{-3} μmol mL^{-1} (*MIC* range 5.16×10^{-3}–0.2 μmol mL^{-1} for compound **11**) as well as against the *E. coli* ATCC 10536 strain (IC_{90} range 0.17–0.37 μmol mL^{-1}). Compound **2** showed antibacterial activity against *E. coli* with the *MIC* range 0.18–0.23 μmol mL^{-1}, whereas compound **10** showed enhanced activity against *E. coli* with the *MIC* range 0.11–0.20 μmol mL^{-1}. In all of these cases, *MIC* values were markedly higher than for standard drugs. Good activity against both investigated *C. albicans* strains was shown by compound **11**. The lowest *MIC* value of 4.0×10^{-2} μmol mL^{-1} against *C. albicans* ATCC 10231 was fairly comparable with the lowest *MIC* value of nystatin of 1.7×10^{-2} μmol mL^{-1}. Compound **7** showed somewhat lower activity against both *C. albicans* strains with higher *MIC* values (IC_{90} range 0.18–0.10 μmol mL^{-1}). Only compound **12** showed good activity against the MRSA strain, but still markedly lower than gentamicin sulphate. Compounds **2**, **6** and **12** showed activity against ESBL+ *E. coli* MFBF 12794, with the lowest *MIC* in the case of compound **12** (IC_{50} 0.16 μmol mL^{-1}); however, still markedly higher than that of colistin (IC_{50} 4.63×10^{-5} μmol mL^{-1}).

None of the tested compounds showed a wide spectrum of antibacterial activity against multi-resistant clinical strains and none of the compounds showed activity against *A. brasiliensis* in the concentration range tested.

Antibacterial tests performed in this study showed that compounds **10** and **11**, bearing one and two chloro atoms, resp., as well as compound **12** with a nitro group, showed good inhibition of bacterial growth whereas the compounds without an electron withdrawing group (chloro- and nitro-) showed weak antimicrobial activity. This may be due to the fact that electron withdrawing substituents (chloro-, nitro-) increase the lipophilicity of the compounds, which leads to higher partitioning of such compounds into the lipophilic phase of a microbial membrane. This causes higher local concentration of the compound near a biological target site (1). Furthermore, hydrazones with a strong electron withdrawing substituent (fluorine) showed high bioactivity and were commonly used in medicine to improve metabolic stability, bioavailability and protein ligand interactions (15).

Table II. $MBFIC_{50}$ *and* $MBFIC_{90}$ *values of* in vitro *antimicrobially active aroylhydrazone derivatives*

Compound	*MBFIC* (μmol mL^{-1})	Microorganism	
		MRSA MFBF 10679	ESBL+ *E. coli* MFBF 12794
2	$MBFIC_{50}$	1.54×10^{-2}	$> 2.07 \times 10^{-2}$
	$MBFIC_{90}$	2.03×10^{-2}	$> 2.07 \times 10^{-2}$
12	$MBFIC_{50}$	$> 1.75 \times 10^{-2}$	1.32×10^{-2}
	$MBFIC_{90}$	$> 1.75 \times 10^{-2}$	1.34×10^{-2}
Gentamicin sulphate	$MBFIC_{50}$	1.17×10^{-2}	> 0.17
	$MBFIC_{90}$	1.36×10^{-2}	> 0.17
Norfloxacin	$MBFIC_{50}$	> 0.78	> 0.78
	$MBFIC_{90}$	> 0.78	> 0.78

According to the experimental results, none of the compounds under investigation showed anti-biofilm activity of *C. a.*$^{FLR+}$ at the tested concentrations. As shown in Table II, anti-biofilm activity of compounds **2** and **12** was detected in the case of MRSA and EBSL + *E. coli*. Compound **2** showed good activity only against the formation of biofilm formed by MRSA, while compound **12** showed good activity only against the formation of biofilm formed by EBSL + *E. coli*. In the case of compound **2** against MRSA biofilm formation, *MBFIC* values (1.5×10^{-2} μmol mL^{-1}–2.0×10^{-2} μmol mL^{-1}) were comparable to those of gentamicin sulphate (1.2×10^{-2} μmol mL^{-1}–1.4×10^{-2} μmol mL^{-1}), whereas in the case of compound **12** and EBSL+ *E. coli* even more favourable activity *versus* gentamicin was recorded. Results reveal that the type of substituent in the molecule that determines hydrophobicity/hydrophilicity of the hydrazone could be affecting quorum sensing systems (QS) involved in anti-biofilm activity and, therefore, in future, further investigation of this issue should be performed.

Compounds containing electron withdrawing groups generally exert better antimicrobial activity against Gram-negative bacteria. These findings can be explained by the differences between Gram-negative and Gram-positive bacteria. Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide. Gram-positive bacteria lack an outer membrane but are surrounded by layers of peptidoglycan many times thicker than those found in Gram-negative bacteria. Nitro group is a strong electron-withdrawing group, situated in *para* position to a hydroxyl group in compound **12**, which leads to increased lipophilicity. Increased lipophilicity promotes entrance of substances into the cell membrane and causes structural defects leading to cell death. As already mentioned, compound **12** (nitro substituted) showed high activity against the formation of a biofilm formed by the Gram-negative bacterium (EBSL+ *E. coli*).

CONCLUSIONS

All the title compounds were screened for their *in vitro* antimicrobial activity against *S. aureus* (Gram-positive bacterium), *E. coli* (Gram-negative bacterium), *C. albicans* and *A.*

brasiliensis. The activity was detected for the compounds *N'*-(2-hydroxy-phenylmethylidene)-3-pyridinecarbohydrazide (**2**), *N'*-(2-hydroxy-3-methoxyphenylmethylidene)-3-pyridinecarbohydrazide (**6**), *N'*-(2-hydroxy-5-methoxyphenylmethylidene)-3-pyridinecarbohydrazide (**7**), *N'*-(2-hydroxy-5-methoxyphenylmethylidene)-3-pyridinecarbohydrazide (**8**), *N'*-(5-chloro-2-hydroxyphenylmethylidene)-3-pyridinecarbohydrazide (**10**), *N'*-(3,5-chloro-2-hydroxyphenylmethylidene)-3-pyridinecarbohydrazide (**11**), and *N'*-(2-hydroxy-5-nitrophenylmethylidene)-3-pyridinecarbohydrazide (**12**). Compounds **11** and **12** showed antibacterial activity against *S. aureus* and *E. coli* strains, while compounds **6** and **8** showed activity only against *S. aureus*. Compounds **2** and **10** were active against *E. coli*. Experimental results reveal that compounds **6**, **8**, **11** and **12** exhibited strong antibacterial activity against *S. aureus*. As the obtained results show, only compound **12** showed strong activity against MRSA. Furthermore, only compound **11** showed activity against *C. albicans* ATCC 10231 comparable to the activity of nystatin.

Compounds **2**, **6**, **10**, **11** and **12**, which showed good antimicrobial activity, were also investigated on multi-resistant clinical strains MRSA, ESBL+ *E. coli* and *C. a.*$^{FLR+}$. Experimental results showed that only compounds **2** and **12** showed good activity against biofilm formation. Compound **2** showed good activity against formation of the biofilm formed by MRSA, while compound **12** showed only good activity against formation of the biofilm formed by EBSL + *E. coli*. In the case of compound **2** against MRSA biofilm formation, *MBFIC* values were comparable to those of gentamicin sulphate, whereas in the case of compound **12** and EBSL+ *E. coli* even more favourable activity compared to gentamicin was observed.

Acknowledgements. – The research was supported by the Croatian Science Foundation (Project IP-2014-09-4841).

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