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Short communication

Heterocyclic compounds based on 3-(4-bromophenyl) azo-5-phenyl-2(3*H*)-furanone: Anti-avian influenza virus (H5N1) activity

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3-[2-(4-Bromphenyl)hydrazono]-5-phenyl-furan-2(3*H*)-one (**1**) was used for preparation of some novel pyrazole, pyridazinone, oxadiazole, triazole, thiazolidine and thioxopyrimidine derivatives. Some of the prepared products were tested for anti-avian influenza virus activity and revealed promising antiviral activity against H5N1 virus [A/Chicken/Egypt/1/20 % (H5N1)] by determination of both EC_{50} and LD_{50} and confirmed by plaque reduction assay on Madin-Darby canine kidney cells. Compounds 3-[2-(4-bromophenyl)hydrazono]-5-phenylfuran--2(3*H*)-one (**1**), 1-(4-bromophenyl)-*N*-hydroxy-5-phenyl--1*H*-pyrazole-3-carboxamide (**5**) and 1-(4-bromophenyl)--*N*-{2,3-dihydro-4-hydroxy-3-phenyl-6-oxo-2-thioxopyrimidin-1(6*H*)-yl}-5-phenyl-1*H*-pyrazole-3-carboxamide (**12a**) showed the highest effects. Detailed synthesis, spectroscopic data, and antiviral activity of the synthesized compounds are reported.

Keywords: furanone, pyrazole, pyridazinone, oxadiazole, triazole, anti-avian influenza virus (H5N1)

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2(3*H*)-Furanones are a type of five-membered heterocyclic compounds of synthetic and biological importance. The importance of this type is due to the facile opening of the lactone ring to give acyclic products, which undergo ring closure to give other synthetically and biologically important heterocyclic compounds (1, 2). The chemistry of pyrazole-containing compounds is particularly interesting for antiviral (1), anti-angiogenic activity (3), and as apoptosis-inducing agents (4), potent peroxisome proliferator-activated receptor gamma [PPARg] partial agonists (5) and antibiotics (6). Moreover, pyridazinones have promising biological activities as antiplatelet (7), antihypertensive agents (8). Biochemical studies revealed that oxadiazole caused activation on glutamic oxalo-

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acetic transaminase (GOT) and glutamate pyruvate transaminase (GPT) enzymes, inhibition of gamma-glutamyl transpeptidase (γ-GT) enzyme activity (9) and potent inhibition of tyrosine (10). On the other hand, substituted 1,3,4-oxadiazoles exhibit fungicidal (11), antimicrobial and antitubercular activity (12). Simple 1,2,4-triazoles also display some biological activities as antioxidant, urease inhibitors (13) and antiviral (14) agents. These diverse pharmacological activities prompted us to convert furanone into other heterocycles bearing pyrazolyl moiety, and evaluate them for anti-viral activity. On the other hand, influenza viruses are respiratory pathogens that affect humans and are responsible for substantial morbidity, mortality and decreased productivity worldwide (15). Several synthetic compounds have already been utilized as potential inhibitors of the avian virus (H5N1) and some of them demonstrated the ability to inhibit viral replication at a level comparable to the approved anti-influenza drugs zanamivir and oseltamivir (16).

EXPERIMENTAL

Melting points were measured using an Electrothermal 9100 digital melting point apparatus (Büchi, Switzerland) and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1600 FTIR (Perkin-Elmer, USA) in KBr discs. ^{1}H NMR spectra were measured on a Jeol 270 MHz spectrometer (Jeol, Japan) and a Bruker Avance spectrometer (300 MHz) (Bruker, Germany) in DMSO-d_6, and chemical shifts were recorded in δ ppm relative to the internal standard TMS. Mass spectra were run at 70 eV with a Finnigan SSQ 7000 spectrometer (Thermo electron corporation, USA) using EI; *m/z* values are indicated in Dalton. Elemental analyses were performed on a Perkin-Elmer 2400 analyzer (Perkin-Elmer) and were found within ±0.4 % of the theoretical values (Table I). Follow-up of the reactions and checking of the purity of compounds were done by TLC on silica gel-precoted aluminum sheets (type 60 F_{254}, Merck, Germany). All solvents and reagents were purchased from Aldrich (Germany).

Physicochemical and spectral data for the synthesized compounds are given in Tables I and II, respectively.

Syntheses

*3-[2-(4-Bromophenyl)hydrazono]-5-phenylfuran-2(3*H*)-one (**1**).* – To a solution of bromoaniline (6.88 g, 0.04 mol) in glacial acetic acid (15 mL), conc. HCl (8 mL) was added, then while the mixture was being cooled in ice under stirring, sodium nitrite (4.96 g, 0.068 mol) was added dropwise. To the diazonium mixture, freshly fused sodium acetate (6.0 g) was added. Aroyl propionic acid (6.0 g, 0.04 mol) was added to acetic anhydride (40 mL), the mixture was refluxed for 3 h, then allowed to cool at room temperature. This solution was added dropwise, with stirring and cooling in ice, to the diazo- nium mixture. The reaction mixture was allowed to stand overnight and the precipitate was collected, washed with water several times and recrystallized from acetic acid to give high yield (2).

2-[2-(4-Bromophenyl)hydrazono]-4-oxo-4-phenylbutane hydrazide (**2**). – A mixture of furanone **1** (3.42 g, 0.01 mol) and hydrazine hydrate (0.64 g, 0.02 mol, 98 %) in absolute ethanol (20 mL), was stirred at room temperature for 0.5 h. The solvent was evaporated under vacuum and crystals were obtained.

*4-[2-(4-Bromophenyl)hydrazinyl]-6-phenylpyridazin-3(*2H*) one* (**3**). – A mixture of furanone **1** (3.42 g, 0.01 mol) and hydrazine hydrate 98 % (0.64 g, 0.02 mol) in absolute ethanol (25 mL) was refluxed for 2 h. The solvent was evaporated under vacuum and the solid residue was recrystallized from benzene.

*4-[2-(4-Bromophenyl)hydrazono]-1,2-dihydro-1,6-diphenylpyridazin-3(4*H*)-one* (**4**). – A mixture of furanone **1** (3.42 g, 0.01 mol) and phenylhydrazine (1.3 g, 0.012 mol) in absolute ethanol (25 mL) was refluxed for 3 h. The solvent was evaporated under vacuum and the solid residue was recrystallized from benzene/petroleum ether (60–80 °C, 1:1).

1-(4-Bromophenyl)-N-*hydroxy-5-phenyl-1*H-*pyrazole-3-carboxamide* (**5**). – A mixture of furanone **1** (3.42 g, 0.01 mol), hydroxylamine hydrochloride (0.7 g, 0.01 mol) and sodium acetate (1 g) in ethanol (25 mL) was refluxed for 4 h. The solvent was evaporated under vacuum and the solid residue was recrystallized from benzene/petroleum ether (60–80 °C, 1:1).

*5-[1-(4-Bromophenyl)-5-phenyl-1*H*-pyrazole-3-yl]-1,3,4-oxadiazole-2(3*H*)-thione* (**6**). – A mixture of hydrazide **2** (1.13 g, 0.003 mol), carbon disulfide (5 mL) and sodium hydroxide (4 %, 3 mL) in ethanol (30 mL) was stirred at room temperature for 6 h. The solvent was evaporated under vacuum, dissolved in hot water, and then the filtrate was neutralized with diluted hydrochloric acid. The yellow precipitate was collected after washing with water several times. The product obtained was recrystallized from benzene/petroleum ether (60–80 °C, 1:1).

*2-{[1-(4-Bromophenyl)-5-aryl-1*H*-pyrazol-3-yl]carbonyl}*-N-*phenyl-hydrazinecarbo-thioamides* (**7a,b**). – A mixture of hydrazides **2** (3.75 g, 0.01 mol) and phenyl or *p*-methoxyphenyl isothiocyanate (0.01 mol) in ethanol (30 mL), was refluxed for 2 h. The mixture was cooled, and then filtered off. The solid so obtained was recrystallized from benzene/ethanol (1:2.5).

*1-(4-Bromophenyl)-5-phenyl-*N′*-(phenylcarbonyl)-1*H*-pyrazole-3-carbohydrazide* (**8**). – To a suspension of hydrazide **2** (1.13 g, 0.003 mol) in dry benzene (15 mL), benzoyl chloride (0.42 g, 0.003 mol) was added at 25 °C and the mixture was heated under reflux at 75 °C for 3 h. The solvent was evaporated under vacuum, and the solid residue was recrystallized from benzene.

*2-{1-(4-Bromophenyl)-5-phenyl-1*H*-pyrazole-3-yl}-5-phenyl-1,3,4-oxadiazole* (**9**). – A mixture of diaroylhydrazine **8** (0.92 g, 0.002 mol) and phosphorus oxychloride (10 mL) was refluxed for 1 h. The mixture was cooled, poured onto crushed ice and then neutralized with 4 % NaOH. The white precipitate was collected by filtration, washed with water and recrystallized from benzene.

*4-(4-Bromophenyl)-5-(1-aryl-5-phenyl-1*H*-pyrazol-3-yl)-*2H*-1,2,4-triazole-3(4*H*)-thiones* (**10a,b**). – A mixture of thiosemicarbazides **7a,b** (0.001 mol) and NaOH (4 %, 20 mL) was

refluxed for 2 h. The mixture was cooled, poured onto crushed ice and then neutralized with diluted hydrochloric acid. The white precipitate was collected by filtration and washed with water. The solid obtained was recrystallized from ethanol/water (5:1).

1-(4-Bromophenyl)-N-*(4-oxo-3-arylthiazolidine-2-ylidene)-5-phenyl-1*H*-pyrazole-3-carbohydrazides (**11a,b**).* – A mixture of thiosemicarbazide **7a** or **b** (0.5 g, 0.001 mol), ethylchloroacetate (0.12 g, 0.001 mol) and NaOH pellets (0.4 g) in absolute ethanol (30 mL) was re-

Table I. Physical and analytical data of new compounds

Compd.	Formula (M_r)	M.p. (°C)	Yield (%)	Analysis (%) (calcd./found)				
				C	H	Br	N	S
1	$C_{16}H_{11}BrN_2O_2$ (342.80)	295–296	65	56.00 56.20	3.23 3.20	23.28 23.21	8.16 8.10	
2	$C_{16}H_{15}BrN_4O_2$ (375.22)	89–90	80	51.22 51.32	4.03 4.38	21.30 21.16	14.93 14.73	
3	$C_{16}H_{13}BrN_4O$ (357.22)	160–161	86	53.80 53.72	3.67 3.68	22.37 22.10	15.68 15.00	
4	$C_{22}H_{17}BrN_4O$ (433.30)	84–85	81	60.98 60.79	3.95 3.78	18.44 18.10	12.93 12.60	
5	$C_{16}H_{12}BrN_3O_2$ (358.19)	103–104	78	53.65 53.69	3.38 3.28	22.31 22.06	11.73 11.60	
6	$C_{17}H_{11}BrN_4OS$ (399.26)	179–180	79	51.14 51.23	2.78 2.64	20.01 19.89	14.03 13.88	8.03 8.00
7a	$C_{23}H_{18}BrN_5OS$ (492.39)	199–200	76	56.10 56.00	3.68 3.61	16.23 16.13	14.22 14.00	6.51 6.58
7b	$C_{24}H_{20}BrN_5O_2S$ (522.42)	147–148	74	55.18 55.20	3.86 3.66	15.30 15.09	13.41 13.21	6.12 6.00
8	$C_{23}H_{17}BrN_4O_2$ (461.31)	120–121	77	59.88 59.64	3.71 3.50	17.32 17.12	12.15 12.00	
9	$C_{23}H_{15}BrN_4O$ (443.30)	167–168	80	62.32 62.12	3.41 3.21	18.03 17.87	12.64 12.48	
10a	$C_{23}H_{16}BrN_5S$ (474.38)	189–190	77	58.23 58.00	3.40 3.19	16.84 16.62	14.76 14.63	6.76 6.49
10b	$C_{24}H_{18}BrN_5OS$ (504.40)	225–226	81	57.15 56.98	3.60 3.40	15.84 15.62	13.88 13.48	6.36 6.34
11a	$C_{25}H_{18}BrN_5O_2S$ (532.41)	191–192	72	56.40 56.38	3.41 3.43	15.01 14.84	13.15 13.00	6.02 5.91
11b	$C_{26}H_{20}BrN_5O_3S$ (562.44)	139–140	76	55.52 55.49	3.52 3.50	14.21 14.11	12.45 12.39	5.70 5.68
12a	$C_{26}H_{18}BrN_5O_3S$ (560.42)	186–187	70	55.72 55.61	3.24 3.13	14.26 14.00	12.50 12.22	5.72 5.70
12b	$C_{27}H_{20}BrN_5O_4S$ (590.45)	124–125	72	54.92 54.69	3.41 3.20	13.53 13.19	11.86 11.66	5.43 5.39

fluxed for 2 h. The solvent was evaporated under reduced pressure; the reaction mixture was poured onto water. The obtained precipitate was filtered off, washed with water, dried and recrystallized from ethanol.

1-(4-Bromophenyl)-N-*{2,3-dihydro-4-hydroxy-3-aryl-6-oxo-2-thioxopyrimidin-1(6*H*)-yl}--5-phenyl-1*H*-pyrazole-3-carboxamide (**12a,b**).* – A solution of **7a** or **b** (0.001 mol) in sodium ethoxide (0.023 g sodium metal in 30 mL ethanol) was heated for 30 min at 80 °C. The reaction mixture was cooled, and then diethylmalonate (0.016 g, 0.001 mol) was poured into water, with stirring at 70 °C for 3 h. The precipitated solid was filtered off, washed with water, dried and recrystallized from methanol to give compounds **12a,b**.

Table II. Spectral data of the new compounds

Compd.	IR (ν_{max}, cm^{-1})	^1H and ^{13}C NMR (δ, ppm)	MS (*m/z*, %)
1	1735 (C=O), 3335 (NH)	^1H NMR: 6.32 (s, 1H, =CH), 3.42 (s, 1H, NH, D_2O exchangeable), 7.80 (s, 1H, NH, D_2O exchangeable), 7.30–7.45 (m, 9H, Ar-H)	342 (M^+, 100)
2	1652, 1690 (2 C=O), 3270–3335 (NH, NH_2)	^1H NMR: 2.60–2.85 (m, 2H, $-CH_2$), 3.42 (s, 2H, NH_2, D_2O exchangeable), 7.00 (s, 1H, NHCO–, D_2O exchangeable), 7.30–7.45 (m, 9H, Ar–H), 8.65 (s, 1H, NH, D_2O exchangeable)	374 (M^+, 100)
3	1648 (C=O), 3275–3315 (NH)	^1H NMR: 5.60 (s, 1H, H pyridazinone), 6.55 (d, 2H, *J* = 8Hz, Ar-H), 7.25 (d, 2H, *J* = 8Hz, Ar-H), 7.40 (m, 3H, Ph-H), 7.65 (m, 2H, Ph-H), 7.90 (s, 1H, NH, pyridizinone, D_2O exchangeable), 8.70 (s, 1H, NH, D_2O exchangeable), 11.80 (s, 1H, NH-Ar, D_2O exchangeable)	357 (M^+, 100)
4	1655 (C=O), 3235 (NH)	^1H NMR: 5.9 (s, 1H, H pyridazinone), 6.35–7.4 (m, 14H, Ar-H), 8.2 (br., 1H, NHCO–, D_2O exchangeable), 11.1 (s, 1H, NHPh exchangeable)	332 (M^+, 100)
5	1645 (C=O), 3130 (NH), 3340 (OH)	^1H NMR: 7.00 (s, 1H, pyrazole H), 7.20–7.48 (m, 9H, Ph-H + Ar-H), 9.20 (s, 1H, OH, D_2O exchangeable), 11.10 (s, 1H, NH, D_2O exchangeable)	357 (M^+, 100)
6	1250 (C=S), 3100 (NH)	^1H NMR: 7.08 (s, 1H, pyrazole H), 7.20–7.40 (m, 9H, Ph-H + Ar-H), 14.35 (br, 1H, NH, D_2O exchangeable)	398 (M^+, 100)
7a	1252 (C=S), 1646 (C=O), 3200–3290 (NH)	^1H NMR: 7.14 (s, 1H, pyrazole H), 7.30–7.66 (m, 14H, 3 Ph-H), 9.70 (br, 2H, NH, D_2O exchangeable), 10.30 (s, 1H, NH, D_2O exchangeable) ^{13}C NMR: 108.31, 120.56–138.70, 143.30 (Ar-20C), 146.38 (C=N), 161.05 (C=O), 198.40 (C=S)	491 (M^+, 100)
7b	1252 (C=S), 1650 (C=O), 3196–3292 (NH)	^1H NMR: 3.7 (s, 3H, OCH_3), 7.11 (s, 1H, pyrazole H), 7.31–7.62 (m, 13H, 3 Ph-H), 9.90 (br, 2H, NH, D_2O exchangeable), 10.60 (s, 1H, NH, D_2O exchangeable)	523 (M^+, 100)

Table II. cont.

8	1632 (C=N), 1675 (C=O), 3202 (NH)	^{1}H NMR: 7.10 (s, 1H, pyrazole H), 7.20–7.90 (m, 14H Ar-H), 10.20 (s, 1H, NH, D_2O exchangeable), 10.60 (s, 1H, NH exchangeable) ^{13}C NMR: 108.86, 121.91, 128.05–132.70, 139.02, 144.87 (Ar-20C), 146.41 (C=N), 161.05, 166.19 (2C=O)	460 (M^+, 100)
9	1600 (C=N)	^{1}H NMR: 7.00 (s, 1H, pyrazole), 7.2–7.78 (m, 14H, Ar-H)	443 (M^+,100)
10a	1251 (C=S), 3080 (NH)	^{1}H NMR: 6.70 (s, 1H, pyrazole H), 6.9–7.56 (m, 14H, 3Ph-H), 14.16 (br, 1H, NH, D_2O exchangeable)	475 (M^+,100)
10b	1252 (C=S), 3085 (NH)	^{1}H NMR: 3.40 (s, 3H, OCH_3), 6.70 (s, 1H, pyrazole H), 6.90–7.66 (m, 13H, 3Ph-H), 14.20 (br, 1H, NH, D_2O exchangeable)	504 (M^+,100)
11a	3085 (NH), 1680 (C=O)	^{1}H NMR: 4.86 (s, 2H, CH_2), 6.70 (s, 1H, pyrazole H), 6.90–7.66 (m, 14H, 3Ph-H), 10.60 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR: δ 48.1 (CH_2), 106.3 (pyrazole C-4), 122.12–159.90 (18 Ar-C, pyrazole C-3,5 and thiazole C-2,5), 170.36, 172.52 (2C=O) ^{13}C NMR: 40.1 (CH_2), 106.3, 116.12–142.90 (Ar-20C), 152.10, 155.25 (2 C=N), 170.36, 172.52 (2 C=O)	531 (M^+, 100)
11b	3088 (NH), 1681 (C=O)	^{1}H NMR: 3.60 (s, 1H, OCH_3), 4.82 (s, 2H, CH_2), 6.70 (s, 1H, pyrazole H), 6.92–7.62 (m, 13H, 3Ph-H), 10.2 (s, 1H, NH, D_2O exchangeable) ^{13}C NMR: 39.70 (CH_2), 55.32 (CH_3), 107.10, 114.42, 118.65, 121.37–132.29, 138.27, 144.81 (Ar-20C), 153.08, 154.57 (2C=N), 160.10, 168.64 (2C=O)	563 (M^+, 100)
12a	1252 (C=S), 1682 (C=O), 1660 (C=O), 3085 (NH)	^{1}H NMR: 6.70 (s, 1H, pyrazole H), 6.9–7.66 (m, 14H, 3Ph-H), 14 (br,1H, NH, D_2O exchangeable) ^{13}C NMR: 39.84 (CH_2), 108.69, 121.46, 127.47–132.29, 138.50, 139.34, 144.26 (Ar-20C), 145.96 (C=N), 166.65, 168.17, 168.49 (3C=O), 181.16 (C=S)	559 (M^+, 100)
12b	1252 (C=S), 1690 (C=O), 1661 (C=O), 3085 (NH)	^{1}H NMR: 3.4 (s, 3H, OCH_3),5.4 (s, 1H, ethylene), 6.7 (s, 1H, pyrazole H), 7.1–7.66 (m, 13H, 3Ph-H), 9.2 (s, 1H, NH, D_2O exchangeable), 15.2 (br, 1H, OH, D_2O exchangeable)	589 (M^+, 100)

Virus and cells. – Avian influenza-A virus (H5N1) [isolated from cloacal swabs from chicken, in Qalubiya governorate, Egypt, 2006 (A/chicken/Egypt/1/2006 (H5N1), accession no. FJ472343], was used to prepare low pathogenic rH5N1 by plasmid-based reverse genetics. The prepared H5N1 vaccine strain was used in this study to evaluate antiviral activity of some synthetized compounds. Madin-Darby canine kidney (MDCK) cells kindly provided by Dr. Richard Webby (St. Jude Children's Research Hospital, Memphis, TN, USA) were used for virus propagation. The MDCK cells were routinely

passaged in Dulbecco's modified Eagle medium (DMEM) containing 10 % fetal bovine serum (FBS) and 1 % antibiotic-antimycotic mixture (penicillin-streptomycin-amphotericin B).

Compound preparation for biological assays. – Prepared compounds were dissolved in 10 % dimethyl sulphoxide (DMSO) in doubly destilled water to a concentration of 10 mg mL^{-1} stock solutions used for further dilutions according to the assay applied.

Cytotoxicity assay (MTT assay). – Cytotoxic activity of the extracts was tested in MDCK cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (17) with minor modification. Briefly, the cells were seeded in 96 well--plates (100 µL per well containing 3×10^5 cells mL^{-1}) and treated with 5, 10, 20, 40, 80 and 120 µg of the sample per well. At 24 h, cells were washed with sterile phosphate buffer (PBS) three times and the supernatant was discarded. MTT solution (20 µL of 5 mg mL^{-1}) was added to each well and incubated at 37 °C for 4 h. Then the medium was aspirated. In each well, the formed formazan crystals were dissolved with 200 µL of acidified isopropanol (0.04 mol L^{-1} HCl in absolute isopropanol). Absorbance of formazan was detected with a dual wavelength UV spectrometer at 540 nm with 620 nm reference wavelength. The percentage of cytotoxicity compared to the untreated cells was determined. The 50 %-cytotoxicity (LD_{50}) for each compound was calculated from standard curve of percentage of cytotoxicity *vs.* sample concentration.

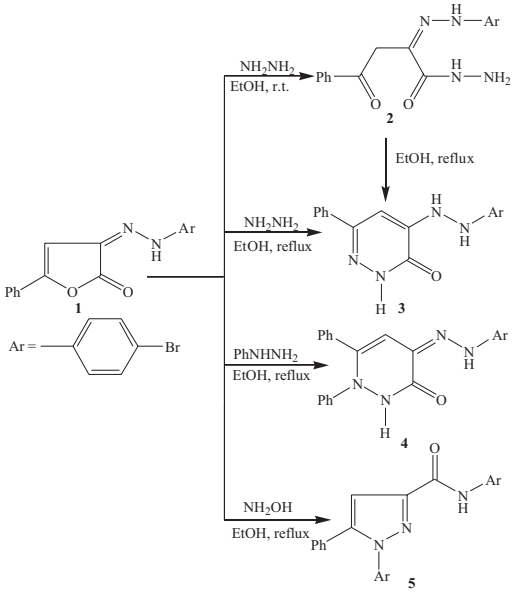
Antiviral activity and therapeutic index. – Antiviral activity of the compounds was determined using the cytopathogenicity (CPE) assay against low pathogenic reassortant avian influenza virus (rH5N1). Cells were seeded in 96-well cell culture plates (100 µL per well at a density of 3×10^5 cells mL^{-1}) and grown to confluency. Cells were then infected with 100 µL of stock virus. After the virus adsorption period on cells of 2 hours at 37 °C, the virus was removed and serial dilutions (10, 20, 40 µg μL^{-1}) of the tested compounds were added, then incubated with infected cells using maintenance DMEM with 2 % FBS (100 µl per well) at 37 °C for 3 days of monitoring until complete CPE was observed in the infected and untreated virus control.

Plaque reduction assay. – In a six-well cell culture plate, confluent MDCK cells were infected with a pre-incubated mixture of 100 µL of avian influenza H5N1 virus (80–100 plaques per well) and 100 µL of DMEM [containing 2 % antibiotics and 1 mg mL^{-1} of L-1-tosyl-amido-2-phenylethyl chloromethyl ketene and a different concentration of each compound (5, 10, 20, and 40 µg mL^{-1})]. The plates were incubated for 1 h at 37 °C in 5 % CO_2 to allow virus adsorption. After adsorption, 2 mL of agarose overlayer (2 % agarose in DMEM 2 x containing 1 % FBS) was added to each well and mixed. The cultures were incubated at 37 °C in 5 % CO_2 for 3–4 days of plaque formation monitoring. Plaques were fixed with 10 % formalin in phosphate-buffered saline for 2 h followed by removal of the agar overlayer and staining with 0.1 % crystal violet in distilled water. Plaques were counted manually from triplicate wells based on the plaque number but not plaque size. Viral counts and percentage of virus reduction were calculated according to Hayden *et al.* (18).

RESULTS AND DISCUSSION

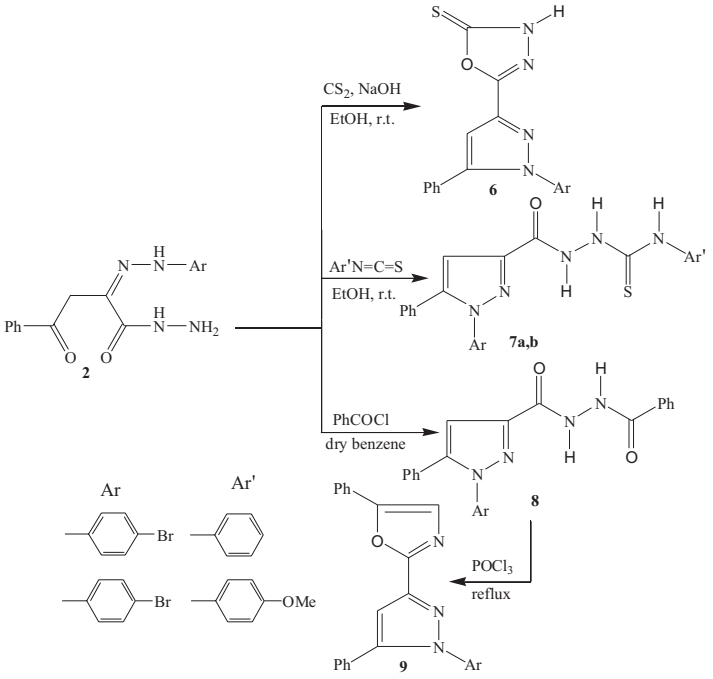
Chemistry

3-[2-(4-Bromophenyl)hydrazono]-5-phenylfuran-2(3*H*)-one (**1**) was prepared by coupling the diazotized aniline with 5-phenyl-2(3*H*)-furanone according to the reported procedures (2). Furanone **1** was reacted with hydrazine hydrate in absolute ethanol at room temperature to give the corresponding acid hydrazide **2**, which was refluxed in ethanol to give pyridazinone derivative **3**. Also, compound **3** was prepared by refluxing compound **1** with hydrazine hydrate in absolute ethanol. The reaction of furanone **1** with phenylhydrazine was also investigated. When the reaction was carried out under reflux conditions, pyridazinone derivative **4** was formed. Moreover, furanone **1** was reacted with hydroxylamine hydrochloride in ethanol under reflux to give pyrazole derivative **5** (Scheme 1).



Scheme 1

Treatment of **2** with carbon disulphide in alcoholic sodium hydroxide gave oxadiazolthione derivative **6**. Hydrazide **2** was reacted with phenyl or *p*-methoxyphenyl isothiocyanate to give the corresponding thiosemicarbazide derivatives **7a,b**, respectively. Also, hydrazide **2** was reacted with benzoyl chloride in dry benzene to give the corresponding pyrazole derivative **8**, which was treated with phosphorus oxychloride to afford the corresponding 1,3,4-oxadiazole derivative **9** (Scheme 2).



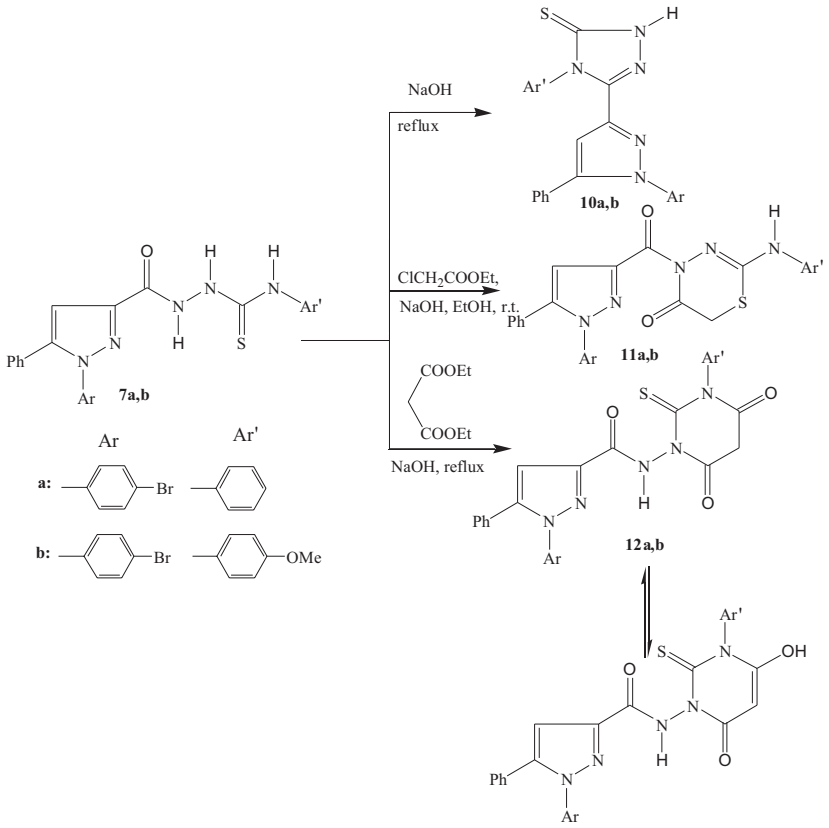
Scheme 2

Ring closure of thiosemicarbazides **7a,b** using sodium hydroxide solution, led to the formation of the corresponding triazolthiones **10a,b**. Also, the reaction of thiosemicarbazides **7a,b** with ethyl chloroacetate in the presence of alcoholic potassium hydroxide afforded thiazolidine derivatives **11a,b**, respectively. Moreover, when thiosemicarbazides **7a,b** were reacted with diethylmalonate in the presence of sodium ethoxide, the corresponding thioxopyrimidin derivatives **12a,b** were formed (Scheme 3).

Antiviral bioassays

Cytotoxicity assay (MTT) was carried out on all prepared compounds and according to the results obtained, only compounds **1**, **5**, **7a**, **8**, **9**, **10a**, **11a**, and **12a** showed notable toxic effects on the biology of cells used in assays. The LD_{50}, EC_{50}, and therapeutic index (*TI*) are presented in Fig. 1. Calculated LD_{50} value indicated that only **1**, **5**, **12a** passed the antiviral activity bioassay.

It was obvious that at concentrations of 10, 20 and 40 μg mL^{-1} compounds **1**, **5** and **12a** showed higher therapeutic indices than the other tested compounds compared to the anti-influenza drug zanamivir. Plaque reduction assay confirmed the antiviral activity of the three compounds as shown in Fig. 2.



Scheme 3

Structure-activity relationship

Structure modifications on the lead compounds **1** and **2** afforded derivatives with a variety of anti-avian influenza virus (H5N1) activity. For example, pyrazole-3-carboxyamide derivative **5** demonstrated remarkable activity due to the chromophor in the skeleton of this compound and displayed a significant EC_{50} value in regard to the parent compound **1** (Fig. 1). However, product **2** does not appear to be advantageous in terms of anti-avian influenza virus with respect to the parent **1** because of its open chain structure and higher hydrophilicity. Furthermore, chemical transformation/structure conversion of **2** into **7** and **8** influenced positively the activity profiles against influenza virus compared to their parent. On the contrary, a dramatic drop of activity was observed for compound **9** because of the building of the oxazole ring. This behavior confirmed that such functionalization hindered the interaction with the host cell receptor for virus entry and decreased the activity. Transformation of **7a,b** into **10a,b** and **12a,b** was accomplished with significant changes in activity. Increase of activity here can be attributed to the

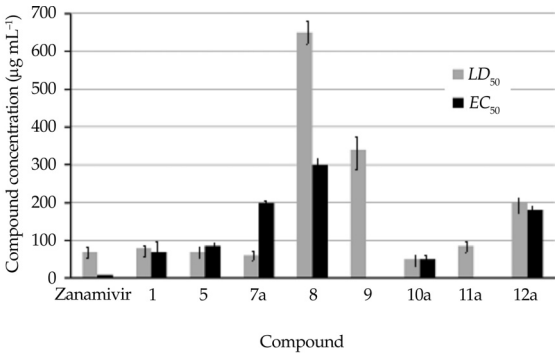


Fig. 1. The 50 % effective concentration (EC_{50}) and the concentration that exhibited 50 % cytotoxicity (LD_{50}) of the tested compounds, compared to the anti-influenza drug zanamivir. Data presented at mean ± SE (n = 5).

triazole and pyrimidine ring formation. In particular, **10a** and **12a** showed remarkable activity to the parent **7a** whereas a dramatic drop of activity was noted in case of **11a** compared to **7a** which was probably due to the thiazolidine ring formation. In conclusion, it follows that pyrazole nucleus is essential for anti-avian influenza virus (H5N1) activity; increased number of nitrogen atoms and amide functions sharply increases the activity; open chains containing amide C=O conjugated with pyrazole ring are more active than cyclized ones.

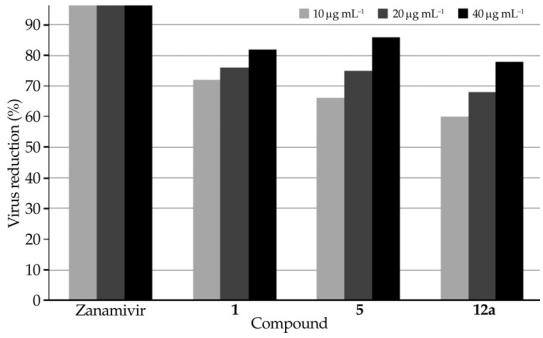


Fig. 2. Antiviral activity of compounds **1**, **5**, **12a** at different concentrations.

CONCLUSIONS

A series of novel pyrazole, pyridazinone, oxadiazole, triazole, thiazolidine and thioxopyrimidine derivatives were prepared and assayed in a variety of biological tests for antiviral activity. Also, some of the prepared products were tested for anti-avian influenza virus activity and revealed promising antiviral activity against H5N1 virus by determination of both EC_{50} and LD_{50} confirmed by the plaque reduction assay on MDCK cells. Compounds 3-[2-(4-bromophenyl)hydrazono]-5-phenylfuran-2(3*H*)-one (**1**), 1-(4--bromophenyl)-*N*-hydroxy-5-phenyl-1*H*-pyrazole-3-carboxamide (**5**) and 1-(4-bromo-

phenyl)-*N*-{2,3-dihydro-4-hydroxy-3-phenyl-6-oxo-2-thioxopyrimidin-1(6*H*)-yl}-5-phenyl--1*H*-pyrazole-3-carboxamide (**12a**) showed the highest anti-H5N1 activity of virus reduction varying from 75 % obtained by 10 μg per well to 84 % by 40 μg per well.

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S A Ž E T A K

Heterociklički derivati 3-(4-bromfenil) azo-5-fenil-2(3*H*)-furanona: Djelovanje na virus ptičje gripe (H5N1)

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3-[2-(4-Bromfenil)hidrazono]-5-fenil-furan-2(3*H*)-on (**1**) upotrjebljen je za pripravu novih derivata pirazola, piridazinona, oksadiazola, triazola, tiazolidina i tioksopirimidina. Neki od sintetiziranih spojeva imaju virustatski učinak na virus ptičje gripe H5N1. Farmakološki aktivnim spojevima određeni su EC_{50} i LD_{50} i dobiven je pozitivni test redukcije plaka na MDCK staničnoj liniji. Najjači učinak pokazali su 3-[2-(4-bromfenil)hidrazono]-5-fenilfuran-2(3*H*)-on (**1**), 1-(4-bromfenil)-*N*-hidroksi-5-fenil-1*H*-pirazol-3-karboksamid (**5**) i 1-(4-bromfenil)-*N*-{2,3-dihidro-4-hidroksi-3-fenil-6-okso-2-tioksopirimidin-1(6*H*)-il}-5-fenil-1*H*-pirazol-3-karboksamid (**12a**).

Ključne riječi: furanon, pirazol, piridazinon, oksadiazol, triazol**,** virus ptičje gripe (H5N1), virustatski učinak

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