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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and biological activities of new Mannich bases of chlorokojic acid derivatives

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Abstract A novel series of 6-chloromethyl-3-hydroxy-2-substituted 4*H*-pyran-4-one derivatives were synthesized and tested for their antibacterial, antifungal and antiviral in vitro properties. In the view of activity results, compounds **8–11** (MIC: 8 μg/ml) were more remarkably active against *Staphylococcus aureus* and *Enterococcus faecalis*. Compounds **1–7** were highly active against *Candida albicans* and *C. parapsilosis* with MIC value of 8 μg/ml. Compound **9** bearing 3-chlorophenyl moiety was determined to be the most active compound against RNA virus *PI*-3.

Keywords Chlorokojic acid · Kojic acid · Mannich bases · Antimicrobial · Antiviral

Introduction

The development of antimicrobial agents to treat infections has been one of the most notable medical achievements of the past century. However, these advances in medical care are threatened by a natural phenomenon known as "antimicrobial resistance". Antimicrobial resistance, whereby microbes mutate and become less susceptible to these "miracle drugs" over time, creates problems to physicians for infectious diseases, as they must work quickly to determine what drug will work on a patient's infection. *Candida* species, especially *C. albicans* which is the most frequent pathogen, are the most widespread and threatening fungal pathogens today, and responsible for the majority of invasive and non-invasive fungal infections. The emergence of organisms resistant to nearly all the class of antimicrobial agents has become a serious public health concern. To overcome the drawbacks of the current antifungal drugs and obtain more efficacious drugs, an antifungal drug having a novel mode of action should be developed (Thomas and Patterson, 2002). On the other hand, *Herpes simplex* virus Type-1 (*HSV*-1) is known as a very common human pathogen that causes a variety of illnesses. Influenza virus infection is also common and it may cause life-threatening events in high-risk patients. Eventually, there is a need for safe and effective antiviral agents in the management of the signs and symptoms of *Herpes simplex* and influenza virus infections (Esquenazi *et al.*, 2002; Özçelik *et al.*, 2009).

Some natural antibiotics contain a siderophore structure, e.g., albomycin. Siderophores are low-molecular weight compounds manufactured by microorganisms to facilitate the uptake of iron(III). They contain either hydroxamate or catechol groups, which are able to sequester iron cations (Hider *et al.*, 1992). Chelating ability of kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one), which has a catechol-like function, has been used for analytical purposes, also plays a significant role in its antibacterial and antifungal activities (Weinberg, 1957; Synytsya *et al.*, 2008; Fassihi *et al.*, 2009). Also, metal-binding compounds which were formed by the reaction of hydroxypyranone derivatives with metallic ions have proven activities in fungal infectious (Brtko *et al.*, 2004).

Previous antimicrobial activity studies showed that kojic acid and its derivatives have antibacterial activity (Kotani *et al.*, 1975; Pirselova *et al.*, 1996; Aytemir *et al.*, 2003; Bentley, 2006; Fassihi *et al.*, 2009). Moreover, 2-chloromethyl-5-

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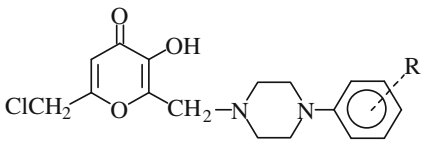
hydroxy-4*H*-pyran-4-one (chlorokojic acid) inhibited *Aeromonas aerogenes*, *Micrococcus pyogenes* var. *aureus*, *Samonellal typhosa*, *Penicilium digitalum*, *Russula nigricans* and *Saccharomyces cerevisiae* (Wolf and Westveer, 1950). Brtko and co-workers reported that chlorokojic acid and other halogen derivatives have significant antifungal activity. Also, their copper(II) salts' complex derivatives were prepared and found to be more active than chlorokojic acid (Brtko *et al.*, 2004). It was shown that kojic acid derivatives were determined as having significant antifungal effects against different *Candida species* and *Pythium graminicola* by the different researchers (Kayahara *et al.*, 1990; Aytemir *et al.*, 2003, 2004; Fassihi *et al.*, 2009). According to its antibacterial and fungicidal properties, kojic acid is used as a food additive to prevent browning (Burdock *et al.*, 2001; Brtko *et al.*, 2004; Bentley, 2006). Moreover, kojic acid derivatives exhibit a number of interesting bioactivities including herbicidal (Veverka and Kralovicova, 1990), anti-speck (Uchino *et al.*, 1988), pesticide and insecticide (Alverson, 2003), antitumor activity (Higa *et al.*, 2007), anti-diabetic (Xiong and Pirrung, 2008), antiepileptic (Aytemir *et al.*, 2004, 2010), modest anti-inflammatory effect (Brtko *et al.*, 2004), and as a skin-whitening product in cosmetic (Burdock *et al.*, 2001; Bentley, 2006).

Literature survey reveals that piperazine ring has an important factor for antimicrobial activity. Eperezolid, posaconazole, ketoconazole and itraconazole, which are currently important antimicrobial drugs used for the treatment of microbial infections, contain a piperazine ring in their structures (Upadhayaya *et al.*, 2004; Foroumadi *et al.*, 2006). In the framework of this study, we aim to synthesize new Mannich bases of chlorokojic acid derivatives having substituted piperazine ring in order to develop new antimicrobial and antiviral compounds (Table 1).

Table 1 Yields, melting points and elemental analysis results of the synthesized compounds **1–11**



Comp. no.	R	M.p. (°C)	Yield (%)	Empirical formula	MW	Elemental Anal. Calc./Found (%)		
						C	H	N
1	3-CF_3	153-4	82	$C_{18}H_{18}ClF_3N_2O_3$	402.10	53.67	4.50	6.95
						53.90	4.01	6.88
2	4-CF_3	167-8	88	$C_{18}H_{18}ClF_3N_2O_3$	402.10	53.67	4.50	6.95
						53.51	4.47	6.92
3	2-F	148-9	71	$C_{17}H_{18}ClFN_2O_3$	352.09	57.88	5.14	7.94
						57.85	5.11	7.84
4	4-F	154-5	65	$C_{17}H_{18}ClFN_2O_3$	352.09	57.88	5.14	7.94
						57.53	5.10	7.80
5	2-OCH_3	125-6	54	$C_{18}H_{21}ClN_2O_4$	364.11	59.26	5.80	7.68
						59.54	5.83	7.62
6	3-OCH_3	136-7	79	$C_{18}H_{21}ClN_2O_4$	364.11	59.26	5.80	7.68
						59.04	5.72	7.57
7	4-OCH_3	165-6	92	$C_{18}H_{21}ClN_2O_4$	364.11	59.26	5.80	7.68
						59.45	5.77	7.65
8	2-Cl	137-8	50	$C_{17}H_{18}Cl_2N_2O_3$	368.06	55.30	4.91	7.59
						54.73	4.80	7.41
9	3-Cl	151-2	74	$C_{17}H_{18}Cl_2N_2O_3$	368.06	55.30	4.91	7.59
						54.95	4.85	7.47
10	4-Cl	174-5	86	$C_{17}H_{18}Cl_2N_2O_3$	368.06	55.30	4.91	7.59
						54.55	4.61	7.58
11	3,4-di-Cl	160-1	83	$C_{17}H_{18}Cl_3N_2O_3$	402.03	50.58	4.24	6.94
						50.12	4.27	6.80

Results and discussion

Chemistry

Kojic acid provides a promising skeleton for development of new more potent derivatives such as chlorokojic acid, allomaltol and pyromeconic acid (Fig. 1). They are good ligands for the nucleophilic and electrophilic substitution reactions (Ichimoto and Tatsumi, 1965; Uher *et al.*, 2000). Therefore, many researchers used them for starting or intermediate material for preparation of new compounds (O'brien *et al.*, 1960; Ichimoto and Tatsumi, 1965; Ellis *et al.*, 1996; Aytemir *et al.*, 2003, 2004; Dehkordi *et al.*, 2008).

In this study, chlorokojic acid was used as the starting material. The enolic structure of neutral chlorokojic acid is expected to be the most stable one (Zborowski *et al.*, 2004). It was synthesized from commercially available kojic acid in one-step reaction according to literature. Chlorination of 2-hydroxymethyl moiety of kojic acid using clean thionyl chloride at room temperature afforded chlorokojic acid (Ellis *et al.*, 1996; Aytemir *et al.*, 2003, 2004).

Because of phenol-like property of kojic acid, it readily undergoes aminomethylation in the Mannich reaction *ortho* to enolic hydroxyl group. It was reported that di-Mannich derivatives which were formed at 3- and 6-positions, obtained in an acidic medium by the reaction of kojic acid, formaline and aromatic amine derivatives. However, O'brien *et al.* (1960) showed that derivatives of Mannich bases occured at only 6-position of kojic acid, which were synthesized using dimethylamine, diethylamine, pyrrolidine, morpholine, piperidine or 4-methylpiperazine and chlorokojic acid. Additionally, 6-morpholino or piperidinomethyl chlorokojic acid were prepared via Mannich reaction (O'brien *et al.*, 1960). At latter study, Ichimoto and Tatsumi (1965) synthesized Mannich bases of kojic acid and pyromeconic acid in either acidic or basic medium, giving details of mechanism, using aliphatic or heterocyclic secondary amines such as dimethylamine, diethylamine or morpholine, respectively.

The 6-chloromethyl-3-hydroxy-2-(substituted phenylpiperazin-1-yl)methyl-4*H*-pyran-4-one derivatives (**1–11**) were prepared by the reaction of appropriate substituted phenyl piperazine derivatives with chlorokojic acid and formaline at room temperature as shown in Scheme 1. The derivatives of chlorokojic acid were obtained in good yields. The structures of the synthesized compounds **1–11** were identified by using IR, ^{1}H NMR, ESI-MS data and elemental analysis. Yields, the melting points and elemental analysis of the synthesized compounds are presented in Table 1. IR spectra of **1–11** showed stretching bands associated with C=O, C=C and C–O stretching at 1642–1622, 1597–1429 and 1218–1162 cm^{-1}, respectively. When the ^{1}H NMR spectra was investigated for the all synthesized compounds, characteristic singlet peaks of the 4*H*-pyran-4-one (H^5) ring proton were found in the region 6.45–6.63 ppm in accordance with literatures (Ellis *et al.*, 1996; Aytemir *et al.*, 2003, 2004). Substituted piperazine ring protons were found at 2.50–2.71 and 2.61–3.29 ppm as two triplet peaks. Also, signals of ClCH_2– group protons were displayed as singlet peaks at 4.55–4.73 ppm. The methylene group protons of **1–11** appeared as singlet peaks at 3.51–3.72 ppm. The ESI mass spectra of all compounds showed suitable peaks in accordance with previously study (Aytemir *et al.*, 2010).

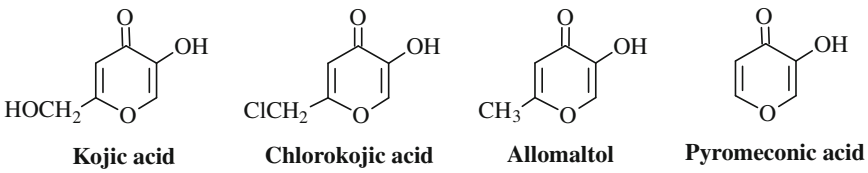
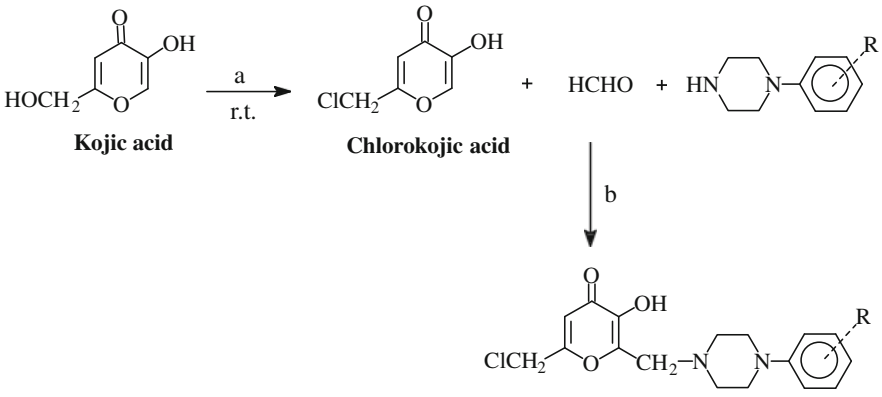


Fig. 1 Hydroxypyranone derivatives



Scheme 1 Schematic representation of the synthesized compounds. R = 3-Trifluoromethyl-, 4-trifluoromethyl-, 2-fluoro-, 4-fluoro-, 2-methoxy-, 3-methoxy-, 4-methoxy-, 2-chloro-, 3-chloro-, 4-chloro-, 3,4-dichloro-, (*a*) $SOCl_2$, (*b*) MeOH, *r.t.* room temperature

In vitro antibacterial and antifungal activity

The antibacterial and antifungal activity profiles of the newly synthesized compounds were assessed for antimicrobial activity against both standards and the isolated strains of microorganism. Tables 2 and 3 describe the in vitro antimicrobial activity with the MIC values of compounds **1–11**. According to our data (Tables 2 and 3), the synthesized compounds showed a broad spectrum of activity with MIC values 8–32 μg/ml against Gram-positive and Gram-negative standard strains. In the meantime, the synthesized compounds showed moderate effect with MIC values 32 to ≥128 μg/ml against drug-resistant isolated both of strains.

As given in the Table 2, antibacterial activity of compounds **1–4**, showed better activity against standards strains of Gram-negative bacteria like *E. coli*, *P. aeruginosa*, *P. mirabilis* and *K. pneumoniae*. As for Gram-positive bacteria, MIC values 8–64 μg/ml and 64 to ≥128 μg/ml were determined for standard and isolated drug-resistant strains, respectively. Compounds **1–4** which bearing fluoro and trifluoromethyl-substituents on phenyl rings have more potent activity (MIC: 16 μg/ml) among this Mannich base derivatives series towards *E. coli*. The other synthesized compounds have similar activity against to *E. coli*. Furthermore, compounds **1**, **2**, **7** and **11** (MIC: 16 μg/ml) showed high activity against *P. aeruginosa* in the entire series. Investigation among other Gram-negative bacteria, compounds **1**, **3**, **4**, and **7** and compounds **2** and **7** were more active derivatives against *K. pneumoniae* and *A. baumannii*, respectively, than the others (MIC: 16 μg/ml). Also, compounds **1**, **2** and **4** (MIC: 16 μg/ml) were the most effective compounds towards *P. mirabilis*.

According to the obtained data in Table 3, it can be considered that structural differences on the substitution at 2-position of hydroxypyranone nucleus changed their activity against *S. aureus* but not its isolated strains of methicillin-resistant *S. aureus* (MRSA). The antibacterial activity of the synthesized compounds **8–11** possessing a chloro-substituent on phenyl ring were found to have significantly high activity against Gram-positive bacteria such as *S. aureus* and *E. faecalis* showing a bacterial inhibition 8 μg/ml. On the other hand, the other synthesized compounds caused slight activity against isolated strains of *S. aureus.* As structure–activity relationship (SAR), while, methoxy substitution in the *ortho*, *meta* and *para* position of the phenyl rings (**5–7**) have less activity but the chloro, fluoro and trifluoromethyl substitution (**2–4** and **8–11**) of

Table 2 Antibacterial activity of the synthesized compounds **1–11** and the control drugs (MIC in μg/ml)

Comp. no.	Gram-negative standard and clinic isolated strains									
	E. coli		*P. aeruginosa*		*P. mirabilis*		*K. pneumoniae*		*A. baumannii*	
	ATCC 35218	Isolated strain ESβL+	ATCC 10145	Isolated strain	ATCC 7002	Isolated strain ESβL+	RSKK 574	Isolated strain ESβL+	RSKK 02026	Isolated strain
1	16	64	16	64	16	64	16	64	32	64
2	16	64	16	64	16	64	32	64	16	64
3	16	64	32	64	32	64	16	64	32	64
4	16	64	32	64	16	64	16	64	32	64
5	32	64	32	64	32	64	32	64	32	64
6	32	64	32	64	32	64	32	64	32	64
7	32	64	16	64	32	64	16	32	16	32
8	64	>128	64	>128	64	>128	64	>128	64	>128
9	32	64	32	64	32	64	32	64	32	64
10	64	>128	64	>128	64	>128	64	>128	64	>128
11	64	64	16	64	32	64	32	64	64	32
AMP	2	>128	–	–	2	>128	2	>128	2	>128
VAN	–	–	–	–	–	–	–	–	–	–
GM	–	–	–	–	–	–	–	–	–	–
OFX	0.12	0.5	1	64	<0.12	1	<0.12	0.5	0.12	64
LVX	<0.12	0.5	1	64	<0.12	1	<0.12	1	0.12	64

AMP ampicilline, *VAN* vancomisine, *GM* gentamicin, *OFX* ofloxacin, *LVX* levofloxacine

– No activity observed, *E. coli* isolates; (resist to trimethoprim–sulfamethoxazole, cefepime, tazobactam), *P. aeruginosa* isolates (resist to trimethoprim–sulfamethoxazole, tazobactam), *P.mirabilis* isolates (resist to trimethoprim–sulfamethoxazole, cefepime, tazobactam), *K. pneumoniae* isolates (resist to trimethoprim–sulfamethoxazole, amoxicillin clavulonat, cefriaxon, cephepim, aztreonam) *A. baumannii* isolates (resist to trimethoprim–sulfamethoxazole, cefepime)

Table 3 Antibacterial and antifungal activities of the synthesized compounds **1–11** and the control drugs (MIC in μg/ml)

Comp. no.	Gram-positive standard and clinic isolated strain bacteria							
	Bacteria						Fungi	
	S. aureus		*E. faecalis*		*B. subtilis*		*C. albicans*	*C. parapsilosis*
	ATCC 25923	Isolated strain MRSA	ATCC 29212	Isolated strain	ATCC 6633	Isolated strain	ATCC 10231	ATCC 22019
1	32	64	16	32	32	64	8	8
2	16	64	8	32	8	64	8	8
3	16	64	16	32	8	16	8	8
4	16	64	16	32	16	32	8	8
5	64	128	64	128	16	32	8	8
6	64	128	64	128	16	32	8	8
7	32	64	32	64	32	64	8	8
8	8	>128	8	>128	16	>128	16	16
9	8	128	8	128	16	32	16	16
10	8	>128	8	>128	16	>128	16	16
11	8	64	8	64	16	64	16	16
AMP	<0.12	>128	0.5	>128	0.12	0.5	–	–
VAN	0.12	2	–	–	–	–	–	–
GM	–	–	–	–	–	–	–	–
OFX	0.25	64	1	32	–	–	–	–
LVX	0.25	128	0.5	32	–	–	–	–
KET	–	–	–	–	–	–	1	1
FLU	–	–	–	–	–	–	2	4

AMP ampicilline, *VAN* vancomisine, *GM* gentamicin, *OFX* ofloxacin, *LVX* levofloxacine, *KET* ketoconazole, *FLU* fluconazole

– No activity observed, *S. aureus* isolates (resist to oxacillin, gentamicin, aztreonam, trimethoprim–sulfamethoxazole), *E. faecalis* isolates (resist to cephalosporins and beta-lactam), *B. subtilis* isolates (resist to ceftriaxon)

phenyl leads to increase in antibacterial activity towards *S. aureus* (MIC: 8–16 μg/ml). In addition, there is no difference in antimicrobial activity with the location of the chloro-substituent on phenyl ring. Their antibacterial activity results were encouraging, although some compounds (**1**, **5**, **6** and **7**) manifested moderate antibacterial activity against standard strains of *S. aureus*. The antibacterial potential against *E. faecalis* was exhibited by compounds **2** and **8–11** at concentration 8 μg/ml among the synthesized compounds. Beside, compounds **8–11** were found to be active against Gram-positive while they were less effective towards Gram-negative bacteria. The other compounds had manifested moderate inhibitory activity against *E. faecalis*. Moreover, compounds **2** and **3**, which bears trifluoromethyl- and fluorophenylpiperazinemethyl moiety at the 2-position of pyran-4-one ring, were the most active derivatives against standard strains of *B. subtilis* with a MIC value of 8 μg/ml.

As seen in the Table 3, all Mannich base derivatives showed similar activity profile in fungi (MIC: 8–16 μg/ml). The screening data indicates that especially compounds **1–7** (MIC: 8 μg/ml), which have trifluoromethyl, fluoro and methoxy phenyl derivatives, were determined to posses high antifungal potential against *C. albicans* and *C. parapsilosis* when compared to other tested compounds. These compounds had comparable results with fluconazole (MIC: 4 μg/ml) against *C. parapsilosis*.

Antiviral activity

In this study, our goal was to investigate the role of efficient substitution on different position of phenylpiperazine ring. All compounds were assayed against both *HSV*-1 and *PI*-3 by using Madin–Darby Bovine Kidney and Vero cell lines with the aim of to capture structure relationship in each of the compounds. The results of the antiviral study are presented in Table 4.

As given in CPE inhibitory concentration ranging, compound **7** bearing 4-methoxy substituent showed significant activity against *HSV*-1 as potent as the reference compound acyclovir, but limited activity at maximum and minimum concentration ranges of 1.6 to <0.1 μg/ml with the maximum non-toxic concentration (MNTC) value of 1.6 μg/ml. Additionally, compound **9** (0.8–0.1 μg/ml) was

Table 4 Cytotoxicity on MDBK and Vero cells as well as antiviral activity against *HSV*-1 and *PI*-3 results of the compounds **1–11**

Comp. no.	MDBK cells			Vero cells		
	MNTCs (μg/ml)	CPE inhibitory concentration		MNTCs (μg/ml)	CPE inhibitory concentration	
		HSV-1			*PI-3*	
		Max.	Min.		Max.	Min.
1	0.8	0.8	0.05	0.8	0.4	0.025
2	0.8	0.8	0.05	0.4	0.4	0.025
3	0.8	0.8	0.05	0.8	0.2	0.025
4	0.8	0.8	0.05	1.6	0.2	0.025
5	0.8	0.8	0.2	1.6	0.2	0.05
6	0.8	0.8	0.2	1.6	0.4	0.1
7	1.6	1.6	0.1	1.6	0.8	0.05
8	1.6	0.4	0.1	0.4	0.4	0.2
9	1.6	0.8	0.1	0.8	0.8	0.025
10	1.6	0.4	0.1	0.4	0.4	0.025
11	1.6	0.4	0.1	0.4	0.4	0.05
Asiklovir	1.6	1.6	<0.012	–	–	–
Oseltamivir	–	–	–	1.6	1.6	<0.012

MNTCs maximum non-toxic concentrations, *CPE* cytopathogenic effect, *HSV-1 Herpes simplex* virus Type-1, *PI-3 Parainfluenza*-3 virus, *Max.* Maximum, *Min.* Minimum

– Not done, activity observed

shown anti-*Herpes simplex* activity but less potent. On the other hand, compounds **1–4** were shown as same activity as compound **7** but on higher non-toxic concentrations (MNTC: 0.8 μg/ml). Among the tested Mannich bases derivatives, compounds **5**, **6**, **8**, **10** and **11** were less active against DNA virus. Taken into account CPE inhibitory concentration ranging against the RNA viruses *PI*-3, compound **9** (0.8–0.025 μg/ml) had remarkable antiviral activity in Mannich base derivatives. Thereto, compounds **7** and **1** were less active than compound **9**. While the activities of compounds **2** and **10** (0.4–0.025 μg/ml) against *PI*-3 were in similar CPE inhibitory concentration range, compounds **3**, **4** and **11** had lower activity than those had. Also, compounds **5** and **6** were negligible values as seen in Table 4.

Concerning SAR, when the effects of mono or dichloro substitution in the *ortho*, *meta* and *para* position of the phenyl ring (**8–11**) were investigated, compound **9** containing 3-chloro-substituent was more active than 2-chloro, 4-chloro and 3,4-dichlorophenyl derivatives which showed similar activity against *HSV*-1. Additionally, when the effects of methoxy substituent were searched on activity against both viruses, 4-methoxy derivative showed higher activity than 2- and 3-methoxy derivatives. Also, changing substitution of any position of trifluoromethyl (**1**, **2**) and fluoro (**3**, **4**) phenyl derivatives were not effective on activity towards both DNA and RNA viruses. Finally, evaluation of effect on chloro-substitution against *PI-3* infected Vero cell, compound **9** was shown to display good antiviral activity. While 4-chloro (**10**) and 3,4-dichloro (**11**) were less active, 2-chloro derivative was found to be inactive.

Investigation among MNTCs of synthesized compounds **1–11** on the Vero and MDBK cell line was found 0.4–1.6 μg/ml. As this result, compounds **1–6** (0.8 μg/ml) were more toxic than compounds **7–11** (1.6 μg/ml) on MDBK cell culture line. At this concentration, the cells did not exhibit altered morphology or growth characteristics indicative of cytotoxic effects. Thus, toxicity prevented the evaluation of their potential antiviral effect at higher concentration.

Conclusion

Further modification of the above structures may provide compounds with better antibacterial and antiviral activities among synthesized derivatives. In this study, the impact of halogen substituent at phenylpiperazine moiety was investigated. Different substituents on phenyl ring and chelating effect were related with their antimicrobial activity. The results of SAR studies indicated that the presence of electron withdrawing groups is necessary for the antimicrobial activity. Looking at the SAR between hydroxypyranones, it can be concluded that the presence of the halogen at the phenyl ring compounds **2** and **8–11** bearing chlorophenyl-moiety, have been found to enhance

the antibacterial activity towards *S. aureus* and *E. faecalis*. Compounds **1** and **2** will have proved to be promising antibacterial agents against Gram-negative bacteria in the trifluoromethyl-moiety series. All of synthesized compounds have better antibacterial effect against Gram-positive than Gram-negative bacteria. According to antifungal potential, compounds **1–7** determined to be the most remarkable active Mannich base derivatives against *C. albicans* and *C. parapsilosis*. The synthesis studies should be continued concerning this group of compounds followed with further antimicrobial activity studies.

When the synthesized compounds were compared within CPE inhibitory concentration ranges, compounds **1–4** and **7** against *HSV*-1 and compound **9** against *PI*-3 were appeared the most active derivatives as well as the controls. They could be selected as lead compounds for the development of novel antiviral agents.

Experimental

All chemicals used for the synthesis of the compounds were supplied by Merck (Darmstadt, Germany) and Aldrich Chemical Co. (Steinheim, Germany). Melting points were determined by a Thomas Hoover Capillary Melting Point Apparatus (Philadelphia, PA, USA) and were uncorrected. IR spectra were recorded on a Jasco FT/IR-420 spectrometer and Bruker Vector 22 IR (Opus Spectroscopic Software Version 2.0) as KBr disc (γ, cm^{-1}). ^1H NMR spectra were obtained with a Varian 400 MHz spectrophotometer in dimethylsulfoxide-d_6 (DMSO-d_6) and tetramethylsilane (TMS) was used as an internal standard (chemical shift in δ, ppm). Mass analysis was carried out with a Micromass ZQ LC–MS with Masslynx Software Version 4.1 by using ESI (+) method. The elemental analyses were performed with a Leco CHNS-932 (St. Joseph, MI, USA) at The Scientific & Technological Research Council of Turkey-Ankara Testing and Analyses Laboratory (TÜBİTAK-ATAL).

2-Chloromethyl-5-hydroxy-4H-pyran-4-one (chlorokojic acid) was synthesized as described before (Ellis *et al.*, 1996). Yield 60%, mp 166–167°C.

General synthesis of Mannich base derivatives (compounds **1–11**)

Mannich bases were prepared by the reaction of substituted phenyl piperazine derivatives (0.01 mol) and chlorokojic acid (0.01 mol) in MeOH (15 ml) with 37% formaline (1 ml). The mixture was stirred vigorously for 15–25 min at room temperature. The resulting precipitate was collected by filtration and washed with cold MeOH. The crude product was recrystallized from MeOH except compound **9** which was recrystallized from $CHCl_3$.

*6-(Chloromethyl)-3-hydroxy-2-({4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}methyl)-4H-pyran-4-one (**1**)*

IR (KBr disc) 1642 (C=O st), 1501 (C=C st) and 1162 cm^{-1} (C–O st). ^1H NMR δ (DMSO-d_6, 400 MHz) 2.71 (4H; t; piperazine), 3.29 (4H; t; piperazine), 3.72 (2H; s; –CH_2–), 4.73 (2H; s; ClCH_2–), 6.63 (1H; s; H^5), 7.11 (1H; d; $J = 7.51$; Ar–$H^{6'}$), 7.21(1H; s; Ar–$H^{2'}$), 7.26 (1H; d; $J = 8.43$; Ar–$H^{4'}$), 7.46 (1H; t; $J = 7.98$; Ar–$H^{5'}$), 9.41–9.48 ppm (1H; br; –O*H*). ESI *m/e* 195 (100%), 403 (M + 1, 18.16%), 405 (M + 3), 425 (M + 23).

*6-(Chloromethyl)-3-hydroxy-2-({4-[4-(trifluoromethyl)phenyl]piperazin-1-yl}methyl)-4H-pyran-4-one (**2**)*

IR (KBr disc) 1635 (C=O st), 1523 (C=C st) and 1202 cm^{-1} (C–O st). ^1H NMR δ (DMSO-d_6, 400 MHz) 2.50 (4H; t; $J = 4.00$; piperazine), 2.61 (4H; t; $J = 4.80$; piperazine), 3.63 (2H; s; –CH_2–), 4.66 (2H; s; ClCH_2–), 6.55 (1H; s; H^5), 7.04 (2H; d; $J = 9.20$; Ar–$H^{2'}$, $H^{6'}$), 7.48 (2H; d; $J = 8.40$; Ar–$H^{3'}$, $H^{5'}$), 9.20–9.30 ppm (1H; br; –O*H*). ESI *m/e* 101 (100%), 403 (M + 1, 23.65%), 405 (M + 3), 425 (M + 23).

*6-(Chloromethyl)-2-{[4-(2-fluorophenyl)piperazin-1-yl]methyl}-3-hydroxy-4H-pyran-4-one (**3**)*

IR (KBr disc) 1623 (C=O st), 1499, 1453 (C=C st) and 1200 cm^{-1} (C–O st). ^1H NMR δ (DMSO-d_6, 400 MHz) 2.64 (4H; t; $J = 4.40$; piperazine), 3.01 (4H; t; $J = 4.80$; piperazine), 3.63 (2H; s; –CH_2–), 4.66 (2H; s; ClCH_2–), 6.55 (1H; s; H^5), 6.92–7.12 (4H; m; Ar–H), 9.05–9.20 ppm (1H; br; –O*H*). ESI *m/e* 195 (100%), 353 (M + 1, 38.32%), 355 (M + 3), 375 (M + 23).

*6-(Chloromethyl)-2-{[4-(4-fluorophenyl)piperazin-1-yl]methyl}-3-hydroxy-4H-pyran-4-one (**4**)*

IR (KBr disc) 1623 (C=O st), 1510, 1461 (C=C st) and 1202 cm^{-1} (C–O st). ^1H NMR δ (DMSO-d_6, 400 MHz) 2.61 (4H; t; $J = 4.80$; piperazine), 3.07 (4H; t; $J = 4.80$; piperazine), 3.63 (2H; s; –CH_2–), 4.66 (2H; s; ClCH_2–), 6.55 (1H; s; H^5), 6.90–7.04 (4H; m; Ar–H), 9.05–9.20 ppm (1H; br; –O*H*). ESI *m/e* 195 (100%), 353 (M + 1, 20.35%), 355 (M + 3), 375 (M + 23).

*6-(Chloromethyl)-3-hydroxy-2-{[4-(2-methoxyphenyl)piperazin-1-yl]methyl}-4H-pyran-4-one (**5**)*

IR (KBr disc) 1623 (C=O st), 1499, 1452 (C=C st) and 1199 cm^{-1} (C–O st). ^1H NMR δ (DMSO-d_6, 400 MHz)

2.62 (4H; t; $J = 4.40$; piperazine), 2.96 (4H; t; $J = 4.40$; piperazine), 3.62 (2H; s; –CH_2–), 3.76 (3H; s; –OCH_3), 4.67 (2H; s; ClCH_2–), 6.55 (1H; s; H^5), 6.87–6.94 ppm (4H; m; Ar–H). ESI *m/e* 365 (M + 1, 65.87%), 367 (M + 3), 387 (M + 23, 100%).

*6-(Chloromethyl)-3-hydroxy-2-{[4-(3-methoxyphenyl)piperazin-1-yl]methyl}-4H-pyran-4-one (**6**)*

IR (KBr disc) 1623 (C=O st), 1497, 1455 (C=C st) and 1202 cm^{-1} (C–O st). ^{1}H NMR δ (DMSO-d_6, 400 MHz) 2.61 (4H; t; $J = 4.80$; piperazine), 3.12 (4H; t; $J = 4.80$; piperazine), 3.62 (2H; s; –CH_2–), 3.70 (3H; s; –OCH_3), 4.67 (2H; s; ClCH_2–), 6.35 (1H; dd; $J = 7.60$, $J = 2.40$; Ar–$H^{2'}$), 6.43 (1H; t; $J = 2.40$; Ar–$H^{4'}$), 6.50 (1H; dd; $J = 8.40$, $J = 2.40$; Ar–$H^{6'}$), 6.55 (1H; s; H^5), 7.10 ppm (1H; t; $J = 8.20$; Ar–$H^{5'}$), 9.01–9.30 ppm (1H; br; –O*H*). ESI *m/e* 365 (M + 1, 34.13%), 367 (M + 3), 387 (M + 23, 100%).

*6-(Chloromethyl)-3-hydroxy-2-{[4-(4-methoxyphenyl)piperazin-1-yl]methyl}-4H-pyran-4-one (**7**)*

IR (KBr disc) 1625 (C=O st), 1514, 1458 (C=C st) and 1204 cm^{-1} (C–O st). ^{1}H NMR δ (DMSO-d_6, 400 MHz) 2.62 (4H; t; $J = 5.00$; piperazine), 3.00 (4H; t; $J = 4.60$; piperazine), 3.62 (2H; s; –CH_2–), 3.67 (3H; s; –OCH_3), 4.66 (2H; s; ClCH_2–), 6.55 (1H; s; H^5), 6.80 (2H; d; $J = 7.80$; Ar–$H^{2'}$, $H^{6'}$), 6.86 (2H; d; $J = 7.80$; Ar–$H^{3'}$, $H^{5'}$), 9.00–9.20 ppm (1H; br; –O*H*). ESI *m/e* 195 (100%), 365 (M + 1, 83.83%), 367 (M + 3), 387 (M + 23).

*6-(Chloromethyl)-2-{[4-(2-chlorophenyl)piperazin-1-yl]methyl}-3-hydroxy-4H-pyran-4-one (**8**)*

IR (KBr disc) 1622 (C=O st), 1479, 1454 (C=C st) and 1199 cm^{-1} (C–O st). ^{1}H NMR δ (DMSO-d_6, 400 MHz) 2.66 (4H; t; $J = 4.40$; piperazine), 2.98 (4H; t; $J = 4.40$; piperazine), 3.65 (2H; s; –CH_2–), 4.68 (2H; s; ClCH_2–), 6.57 (1H; s; H^5), 7.03 (1H; t; $J = 7.80$; Ar–$H^{5'}$), 7.14 (1H; dd; $J = 8.00$, $J = 1.20$; Ar–$H^{3'}$), 7.28 (1H; t; $J = 7.60$; Ar–$H^{4'}$), 7.39 (1H; dd; $J = 7.80$, $J = 1.40$; Ar–$H^{6'}$), 9.15–9.30 ppm (1H; br; –O*H*). ESI *m/e* 195 (100%), 369 (M + 1, 30.75%), 371 (M + 3), 391 (M + 23).

*6-(Chloromethyl)-2-{[4-(3-chlorophenyl)piperazin-1-yl]methyl}-3-hydroxy-4H-pyran-4-one (**9**)*

IR (KBr disc) 1630 (C=O st), 1454, 1429 (C=C st) and 1218 cm^{-1} (C–O st). ^{1}H NMR δ (DMSO-d_6, 400 MHz) 2.59 (4H; t; $J = 4.80$; piperazine), 3.16 (4H; t; $J = 4.40$; piperazine), 3.62 (2H; s; –CH_2–), 4.68 (2H; s; ClCH_2–), 6.57 (1H; s; H^5), 6.77 (1H; d; $J = 7.60$; Ar–$H^{6'}$), 6.88 (1H; dd; $J = 8.60$, $J = 2.20$; Ar–$H^{4'}$), 6.92 (1H; s; Ar–$H^{2'}$), 7.19 (1H; t; $J = 8.20$; Ar–$H^{5'}$), 9.22–9.32 ppm (1H; br; –O*H*). ESI *m/e* 195 (100%), 369 (M + 1, 33.43%), 371 (M + 3), 391 (M + 23).

*6-(Chloromethyl)-2-{[4-(4-chlorophenyl)piperazin-1-yl]methyl}-3-hydroxy-4H-pyran-4-one (**10**)*

IR (KBr disc) 1637 (C=O st), 1597, 1500 (C=C st) and 1205 cm^{-1} (C–O st). ^{1}H NMR δ (DMSO-d_6, 400 MHz) 2.50 (4H; t; $J = 4.40$; piperazine), 3.12 (4H; t; $J = 4.40$; piperazine), 3.51 (2H; s; –CH_2–), 4.55 (2H; s; ClCH_2–), 6.45 (1H; s; H^5), 6.84 (2H; d; $J = 8.95$; Ar–$H^{2'}$, $H^{6'}$), 7.10 (2H; d; $J = 8.95$; Ar–$H^{3'}$, $H^{5'}$), 9.05–9.17 ppm (1H; br; –O*H*). ESI *m/e* 101 (100%), 369 (M + 1, 23.58%), 371 (M + 3), 391 (M + 23).

*6-(Chloromethyl)-2-{[4-(3,4-dichlorophenyl)piperazin-1-yl]methyl}-3-hydroxy-4H-pyran-4-one (**11**)*

IR (KBr disc) 1630 (C=O st), 1596, 1487 (C=C st) and 1194 cm^{-1} (C–O st). ^{1}H NMR δ (DMSO-d_6, 400 MHz) 2.59 (4H; t; $J = 4.80$; piperazine), 3.18 (4H; t; $J = 4.40$; piperazine), 3.62 (2H; s; –CH_2–), 4.66 (2H; s; ClCH_2–), 6.56 (1H; s; H^5), 6.91 (1H; dd; $J = 8.80$, $J = 2.80$; Ar–$H^{6'}$), 7.10 (1H; s; Ar–$H^{2'}$), 7.36 (1H; d; $J = 8.80$; Ar–$H^{5'}$), 9.22–9.32 ppm (1H; br; –O*H*). ESI *m/e* 101 (100%), 403 (M + 1, 15.52%), 405 (M + 3), 425 (M + 23).

Microbiological studies

The compounds of **1**–**11** were dissolved in EtOH:hexane (1:1) by using 1% Tween 80 solution at a final concentration of 512 and 51.2 μg/ml and sterilized by filtration using 0.22 μm Millipore (MA 01730, USA) and used as the stock solutions. Reference antibacterial agents dissolved in phosphate buffer solution (ampicillin, pH 8.0; 0.1 mol/ml), dimethylsulphoxide (ketoconazole), or in water (gentamicin, ofloxacin, fluconazole) (NCCLS, 2006, 2008). Antibacterial activity test were carried out against standards; Gram-negative strains of *E. coli* ATCC 35218, *P. aeruginosa* ATCC 10145, *P. mirabilis* ATCC 7002, *K. pneumoniae* RSKK 574, *A. baumannii* RSKK 02026, and as Gram-positive strains of *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6633 and their drug resistant isolates were used for the determination of antibacterial activity. *C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 were used for the determination of antifungal activity. Mueller-Hinton Broth (MHB; Difco) and Mueller-Hinton Agar (MHA; Oxoid) were applied for growing and diluting of the bacteria suspensions (Özçelik *et al.*, 2006). The synthetic medium RPMI-1640 with

L-glutamine was buffered to pH 7 with 3-[*N*-morpholino]-propansulfonic acid and culture suspensions were prepared as described previously (Özçelik *et al.*, 2009). The microdilution method was employed for antibacterial and antifungal activity tests. Media were placed into each 96 wells of the microplates. Extract solutions at 512 μg/ml were added into first rows of microplates and twofold dilutions of the compounds (256–0.125 μg/ml) were made by dispensing the solutions to the remaining wells. The lowest concentration of the extracts that completely inhibit macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported as described previously study (Özçelik *et al.*, 2009).

Cytotoxicity and antiviral activity tests

Vero cell line (African green monkey kidney) used in this study was obtained from Department of Virology, Faculty of Veterinary, Ankara University (Ankara-Turkey). In order to determine the antiviral activity of the extracts, *Herpes simplex* virus Type-1 (*HSV*-1), as representative of DNA viruses and *Parainfluenza*-3 virus (*PI*-3), as representative of RNA viruses, were used. Media (EMEM) were placed into each 96 wells of the microplates (Greiner®; Essen, Germany). Stock solutions of the extracts were added into first raws of microplates and twofold dilutions of the extracts (51.2–0.012 μg/ml) were made by dispensing the solutions to the remaining wells. The MNTCs of each samples were determined by the method described previously study (Özçelik *et al.*, 2009) based on cellular morphologic alteration.

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