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Original article

A study of cytotoxicity of novel chlorokojic acid derivatives with their antimicrobial and antiviral activities☆

Mutlu Dilsiz Aytemir [a,*], Berrin Özçelik [b]

[a] Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 06100 Sıhhiye – Ankara, Turkey
[b] Gazi University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 06330 Ankara, Turkey

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ABSTRACT

A series of 6-chloromethyl-3-hydroxy-2-substituted 4*H*-pyran-4-one derivatives were synthesized and tested for their antimicrobial and antiviral activities. Mannich base derivatives were prepared through the reaction of substituted piperazine or piperidine derivatives on chlorokojic acid and formaline. The structures of the synthesized compounds were confirmed by IR, ^{1}H and ^{13}C NMR, ESI-MS, and elemental analysis. According to the activity studies, compounds **2**–**7** (MIC: 1–2 μg/mL) were found to be highly active against *Bacillus subtilis* and *Staphylococcus aureus,* while compounds **3**, **5** and **6** showed significant activity against *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Acinetobacter baumannii.* Also, compounds **2**–**7** were more remarkably active against *Candida albicans* and *Candida parapsilosis* (MIC: 4–8 μg/mL). Additionally, compound **2** was the most active one against RNA virus *PI*-3.
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1. Introduction

In the last few decades, the frequency and spectrum of antimicrobial-resistant infections have increased both in the hospitals and community. It is reported that certain infections that are essentially untreatable have begun to occur as epidemics both in the developing and other developed regions as a result of antimicrobial resistance. The most serious concern with antibiotic resistance is that some bacteria have become resistant to almost all of the readily available antibiotics and these bacteria can cause serious diseases. Examples include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) and multidrug-resistant *Streptococcus pyogenes* (Group A Streptococcus: GAS) [1]. This is a major public health issue. On the other hand, the development of effective antiviral drugs is an important biomedical scientific achievement of the late 20th century. These are highly potent drugs against herpes viruses, human immunodeficiency virus (HIV), hepatitis B virus, influenza virus and with extension of the list to papilloma viruses, respiratory viruses, enteroviruses, and hepatitis C virus over the next 5–10 years. But this exciting background comes with the problem of drug resistance. Virally encoded drug resistance has been documented against nearly all compounds with antiviral activity, and the genetic basis of resistance is known now [2]. Many screening efforts have been made to find new antimicrobial or antiviral agents from natural or synthetic compounds that can specifically act on different molecular targets to control infections caused by various microorganisms [3–7].

Wolf and Westveer showed that 2-chloromethyl-5-hydroxy-4*H*-pyran-4-one (chlorokojic acid) contains catechol group-inhibited *Aeromonas aeruginosa, Micrococcus pyogenes* var. *aureus, Salmonella typhosa, Penicillium digitatum, Russula nigricans* and *Saccharomyces cerevisiae* [8]. Kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one) and its derivatives also have an inhibitory effect on the growth of *E. coli* and *S. aureus* [9,10]. Previous antimicrobial activity studies had shown that kojic acid was more active against Gram-negative bacteria than against Gram-positive ones [11]. However, some of its derivatives have shown adverse effects different from kojic acid's antibacterial activity results [12–15]. Also, chlorokojic acid and other halogen derivatives have significant antifungal activity. Moreover, their copper(II) salts' complex derivatives were prepared and found to be more active than chlorokojic acid [16]. Kojic acid and its derivatives exhibit a number of interesting bioactivities and are used in food additives [11,16,17], herbicidals [12,18], anti-speck agents [19], pesticides and insecticides [20–22], and also as a skin-whitening product in cosmetic products [11,17].

☆ The authors have declared no conflict of interest.

* Corresponding author. Tel.: +90 312 324 03 33x114; fax: + 90 312 311 4777.
E-mail address: mutlud@hacettepe.edu.tr (M.D. Aytemir).

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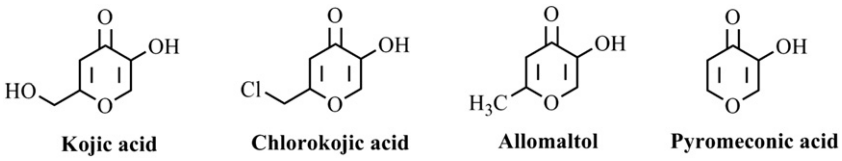


Fig. 1. Hydroxypyranone derivatives.

Previously, some of the Mannich bases of allomaltol (5-hydroxy-2-methyl-4*H*-pyran-4-one) derivatives were prepared in our laboratory for their anticonvulsant and antimicrobial activities. All these derivatives have a lower antifungal activity than chlorokojic acid [23]. Therefore, in order to continue our research program, seven novel Mannich bases of chlorokojic acid derivatives were synthesized and screened for their antimicrobial and antiviral activities.

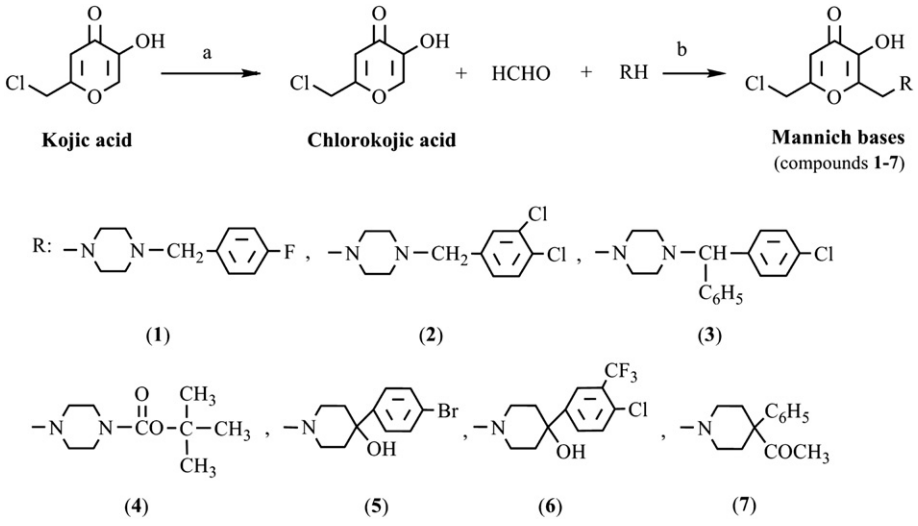
2. Results and discussion

2.1. Chemistry

Kojic acid provides a promising skeleton for development of new and more potent derivatives such as chlorokojic acid, allomaltol and pyromeconic acid (3-hydroxy-4*H*-pyran-4-one) (Fig. 1). They are good ligands for the nucleophilic and electrophilic substitution reactions [24–26]. Therefore, many researchers used them as a starting or intermediate compound for the preparation of new compounds in medicinal chemistry [13,20,23,27–31].

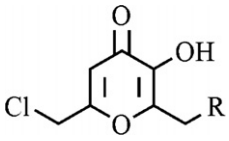
Chlorokojic acid was synthesized from commercially available kojic acid in a one-step reaction as suggested in previous studies [13,23,27,28]. Chlorination of the 2-hydroxymethyl moiety of kojic acid using thionyl chloride at room temperature produced chlorokojic acid, with the ring hydroxyl being unaffected. Mannich-type reactions are three-component condensation reactions involving carbonyl compounds, existing as keto–enol tautomeric forms, formaline, and a primary or secondary amine. Because of its phenol-like properties, kojic acid readily undergoes aminomethylation at room temperature during the Mannich reaction *ortho* to the enolic hydroxyl group. Woods has reported di-Mannich derivatives obtained in an acidic medium from kojic acid, formaline and aromatic amine [30]. Using dimethylamine, diethylamine, pyrrolidine, morpholine, piperidine or 4-methylpiperazine, and chlorokojic acid, only 6th-position of kojic acid was substituted by a Mannich group [27]. Additionally, 6-morpholino or piperidinomethyl chlorokojic acid was prepared via Mannich reaction [30]. In a later study, Ichimoto et al. synthesized Mannich bases of kojic acid and pyromeconic acid in either an acidic or a basic medium using aliphatic or heterocyclic secondary amines such as dimethylamine, diethylamine or morpholine, respectively [24].

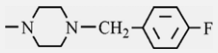
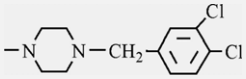
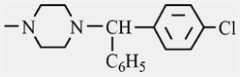
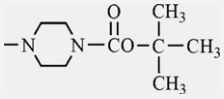
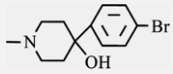
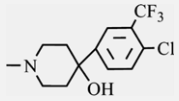
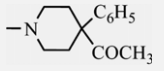
Within the scope of the present study, using the methodology shown in Scheme 1, seven new 6-chloromethyl-3-hydroxy-2-substituted 4*H*-pyran-4-one derivatives were synthesized as Mannich base derivatives. The basic substituent was introduced in the 6th-position of chlorokojic acid via a Mannich-type reaction, using formaline and an appropriate substituted piperidine or piperazine derivatives in methanol at room temperature. The reaction proceeded very rapidly. Formation of the desired new Mannich base derivatives was confirmed on the basis of elemental analysis, and the structures of the compounds were supported by spectral data. The IR, ^{1}H and ^{13}C NMR, and ESI-MS were in agreement with the proposed structures. Yields and the melting points of the synthesized compounds are presented in Table 1. In the IR spectra of all compounds, the stretching (st) bands associated with C=O (pyranone), C=C and C–O were observed at 1657–1622, 1597–1456, and 1227–1198 cm^{-1}, respectively. With ^{1}H NMR spectra, characteristic singlet peaks of the 4*H*-pyran-4-one (H^5) ring proton were found in the region 6.53–6.56 ppm in accordance with literatures [13,23,28,31]. The methylene group (ClCH_2–) proton signals were displayed as singlet peaks at 4.63–4.66 ppm. The other –CH_2– group proton signals of **1**–**7** appeared also as singlet peaks at 3.43–3.62 ppm. ^{13}C NMR spectra of compounds **1**–**7** had typical peaks for 6-chloromethyl and carbonyl of pyranone ring were observed in the range of 41.33–42.10 and 173.36–174.2 ppm, respectively. Compounds **1** and **6** bearing fluoro atoms showed suitable spin–spin decoupling. All these results were found acceptable with literature [23]. The ESI-MS of all compounds showed (M^+ + H), (M^+ + H+2) and (M^+ + Na) peaks.



Scheme 1. Preparation of Mannich bases. Reagents and conditions a: $SOCl_2$; b: MeOH.

Table 1
Yields and melting points of the synthesized compounds (**1**–**7**).



Compounds	R	M.p. (°C)	Yield (%)
1		157–8	68
2		147–8	86
3		98–9	78
4		143–4	80
5		138–9	43
6		122–3	50
7		158–9	82

2.2. In vitro antibacterial and antifungal activities

The antibacterial and antifungal activity profiles of the newly synthesized compounds were assessed for antimicrobial activity against both the standard and the isolated strains of bacteria. For antibacterial activity assessment, standard strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *S. aureus*, *Enterococcus faecalis* and *Bacillus subtilis*) and their drug-resistant isolates were tested; and for antifungal activity *Candida albicans* and *C. parapsilosis* were used. Ampicillin, vancomycin, gentamicin, ofloxacin, levofloxacin, ketoconazole, and fluconazole were also tested under identical conditions for comparison in antibacterial and antifungal assays, respectively. Tables 2 and 3 describe the *in vitro* antimicrobial activity with MIC values of compounds **1**–**7**.

According to our data (Tables 2 and 3), the synthesized compounds showed a broad spectrum of activity against Gram-positive and Gram-negative standard strains with MIC values between 1 and 64 μg/mL. In the meantime, the synthesized compounds showed activity against drug-resistant isolates of both Gram-positive and -negative strains with MIC values of 2 to ≥128 μg/mL.

As given in Table 2, the antibacterial activity against Gram-negative bacteria of the synthesized compounds **3**, **5**, and **6** bearing (4-chlorophenyl)benzylpiperazine, 4-bromophenyl-4-hydroxypiperidine and 4-chloro-3-(trifluoromethyl)phenyl-4-hydroxypiperidine moiety at the 2nd-position of pyran-4-one ring, was found to have significantly high antibacterial potential against standard strains of *E. coli, P. aeruginosa, K. pneumoniae, A. baumannii* with a bacterial inhibition between 2 and 4 μg/mL. Also, these compounds showed more activity (MIC: 2 μg/mL) against *E. coli* and *P. aeruginosa* than the other Gram-negative bacteria compared with control drugs ampicillin (MIC: 2 μg/mL), ofloxacin (1 μg/mL) and levofloxacin (1 μg/mL). The least efficiency of the compounds among Gram-negative bacteria was seen on *P. mirabilis* with a concentration range of 8–16 μg/mL in comparison with ofloxacin and levofloxacin (MICs: <0.12–1 μg/mL).

As for compounds **2** and **4**, it was found that they had the same effect against all Gram-negative bacteria. The MIC values of both compounds were 4 μg/mL against *P. aeruginosa*; 8 μg/mL against *E. coli*, *K. pneumoniae*, and *A. baumannii*; and 16 μg/mL against *P. mirabilis.* Furthermore, on comparison, compound **7** had similar antibacterial activity with MIC values between 4 and 16 μg/mL as compounds **2** and **4**.

In the entire series, compound **1** possessing 4-fluorobenzyl piperazine moiety was less effective (MIC: 16–32 μg/mL) towards standard strains of all Gram-negative bacteria besides compound **7** (16 μg/mL), which showed the same activity on *P. mirabilis.*

Multiple drug-resistant organisms used in the current study are becoming common causes of infections in the acute and long-term care units of hospitals. The emergence of resistance to the beta-lactam antibiotics has become more serious in recent decades. Broad use of antibiotics is followed by emergence of resistant strains such as extended-spectrum beta-lactamases (ESβLs). *E. coli* and also *A. baumannii* are the opportunistic nosocomial pathogens associated with significant morbidity and mortality. Additionally, among drug-resistant microorganisms from highly prevalent opportunistic pathogens, *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 12% of hospital-acquired urinary tract infections, 8% of surgical wound infections, and 10% of bloodstream infections [1,3].

As shown in Table 2, the synthesized compounds revealed a broad spectrum of activity with MIC values of 4–64 μg/mL against tested drug-resistant isolated strains. Especially, compound **2** which has 3,4-dichlorobenzylpiperazine moiety showed remarkable activity against ESβL(+) strains of *E. coli* and *K. pneumoniae* with MIC values of 4 μg/mL, compared with the reference drugs, ampicillin (MIC: ≥128 μg/mL), ofloxacin (MIC: 0.5 μg/mL) and levofloxacin (MIC: 0.5–1 μg/mL, respectively). The other compounds (**1**, **3**–**7**) were found to manifest a moderate effect (MICs: 16–64 μg/mL) for all isolated drug-resistant Gram-negative strains.

As for structure–activity relationship (SAR), fluoro substitution in *para* position of the benzyl ring (compound **1**) has the worst activity with MIC values between 16 and 32 μg/mL in this series, whereas the 3,4-dichloro substitution (compound **2**) of benzyl ring increases antibacterial activity with four-folds (MIC: 4–8 μg/mL) towards isolated strains of *A. baumannii* and *P. aeruginosa* and two-folds (MIC: 8 μg/mL) against *E. coli* and *K. pneumoniae.* Moreover, antibacterial activity of the compounds **1** and **2** against *P. mirabilis* was determined to be the same. Furthermore, compounds **2** and **4** containing ester moiety had shown same activity against all bacteria except from *P. aeruginosa.* In the Mannich base derivatives bearing piperazine ring, compound **3** was the most remarkable and active one (MIC: 2–8 μg/mL). It has two phenyl rings in its structure, of which one of them was a p-chlorophenyl ring and the other a nonsubstituted phenyl ring. When compared with compounds **2**

Table 2
Antibacterial activity of the synthesized compounds (**1**–**7**) and the control drugs (MIC in μg/mL).

Compounds	Gram-negative standard and clinical isolated strains									
	E. coli		*P. aeruginosa*		*P. mirabilis*		*K. pneumoniae*		*A. baumannii*	
	ATCC 35218	Isolated strain	ATCC 10145	Isolated strain	ATCC 7002	Isolated strain	RSKK 574	Isolated strain	RSKK 02026	Isolated strain
1	16	64	16	64	16	64	16	64	32	64
2	8	4	4	16	16	64	8	4	8	16
3	4	64	2	16	8	64	4	64	4	16
4	8	64	4	16	16	64	8	64	8	16
5	4	64	2	16	8	64	4	64	4	16
6	4	64	2	16	8	64	4	64	4	16
7	8	64	4	16	16	64	8	64	4	16
AMP	2	>128	–	–	2	>128	2	>128	2	>128
GM	–	–	0.5	2	–	–	–	–	–	–
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: ampicillin; GM: gentamicin; OFX: ofloxacin; LVX: levofloxacin, *E. coli* isolates (+ESβL enzyme), *P. aeruginosa* isolates (resist to Trimethoprim-Sulfamethoxazole, tazobactam), *P. mirabilis* isolates (+ESβL enzyme), *K. pneumoniae;* isolates (+ESβL enzyme), *A. baumannii* isolates (resist to cephalosporin) -: No activity observed.

and **4**, the addition of phenyl ring on the structure of compound **3** increased the activity two-folds against all Gram-negative bacteria and *S. aureus.* When antibacterial activity of compounds **5**–**7** possessing piperidine ring was investigated, it was observed that compounds **5** and **6** were found to have the same activity and higher effect than compound **7** without halogen substitution at its structure. Hence, when hydroxy substitution at the 4th-position of piperidine ring was changed with acetyl, antibacterial activity was decreased. In addition, there was no difference in the antimicrobial activity with the location and type of the halogen substituted on phenyl ring. Also, the compounds (**5** and **6**) had exactly the same activity as compound **3** possessing piperazine ring against all Gram-negative bacteria.

Among Gram-positive bacteria, *S. aureus* has been recognized for long as one of the major resistant pathogens that can cause diseases in humans. Likewise, multi-drug resistant *Enterococci* have become a serious threat for public health. High level resistance for penicillin and aminoglycosides are being reported of this bacterium [32,33].

According to the obtained data (Table 3), antibacterial activity results of compounds **2**–**7** (MIC: 1–2 μg/mL) against standard Gram-positive bacteria were encouraging, although compound **1** was found to manifest moderate (MIC: 32 μg/mL) activity against standard strains of *S. aureus.* All the synthesized substituted piperazine or piperidine derivatives, except compound **1**, were found to be highly active against *B. subtilis* showing a bacterial inhibition value at 1 μg/mL but all synthesized compounds were less effective against *E. faecalis* than the other Gram-positive bacteria.

Candida species are the most widespread and threatening fungal pathogens today, and are responsible for most of the invasive and non-invasive fungal infections. Among all *Candida* species, *C. albicans* is the most frequent pathogen [3,4].

The results obtained clearly indicate that the series of Mannich bases discussed here are active towards growth inhibition of pathogenic fungi. In general, all compounds, except compound **1**, exhibited excellent antifungal activity (MIC: 4–8 μg/mL) against *Candida* spp when compared to the reference drugs, ketoconazole (MIC: 1 μg/mL) and fluconazole (MIC: 2–4 μg/mL). The compounds **2**–**7** may be promoted as fungicides. In general, the compounds showed an improved antibacterial activity when compared to their antifungal activity.

2.3. Antiviral activity

All compounds were assayed against both herpes simplex virus-1 (*HSV*-1) and human parainfluenza virus type 3 (*PI*-3) by using Madin Darby Bovine Kidney and Vero cell lines with the aim to capture structure relationship in each of the compounds. Acyclovir and oseltamivir were used as control agents. Correlation between toxicity on uninfected cells (Vero, MDBK) and antiviral activity of the synthesis compounds were determined in the same microtiter plate. The results of the antiviral study are presented in Table 4.

HSV-1 is a DNA virus that causes cold sore, a recurrent labial infection, as well as genital infections less frequently. Parainfluenza viruses are enveloped RNA viruses which are the major

Table 3
Antibacterial and antifungal activities of the synthesized compounds (**1**–**7**) and the control drugs (MIC in μg/mL).

Compounds	Gram-positive standard and clinical isolated strains						Fungi	
	S. aureus		*E. faecalis*		*B. subtilis*		*C. albicans*	*C. parapsilosis*
	ATCC 25923	Isolated strain	ATCC 29212	Isolated strain	ATCC 6633	Isolated strain	ATCC 10231	ATCC 22019
1	32	64	16	32	32	64	16	16
2	2	128	16	128	1	2	4	8
3	1	64	16	32	1	2	4	8
4	2	128	8	128	1	2	4	8
5	1	64	16	32	1	2	4	8
6	1	64	16	32	1	2	4	8
7	2	128	64	128	1	2	4	8
AMP	<0.12	>128	0.5	>128	0.12	0.5	–	–
VAN	0.12	2	–	–	–	–	–	–
OFX	0.25	64	1	32	–	–	–	–
LVX	0.25	128	0.5	32	–	–	–	–
KET	–	–	–	–	–	–	1	1
FLU	–	–	–	–	–	–	2	4

AMP: ampicillin; VAN: vancomycin; OFX: ofloxacin; LVX: levofloxacin, KET; ketoconazole; FLU: fluconazole, *S. aureus* isolates (meticillin resist; MRSA), *E. faecalis* isolates (cephalosporin resist), *B. subtilis* isolates (ceftriaxon resist) -: No activity observed.

Table 4
Cytotoxicity on MDBK and Vero cells as well as antiviral activity against *HSV*-1 and *PI*-3 results.

MDBK cells				Vero cells		
Compounds	MNTCs (μg/mL)	CPE inhibitory concentration		MNTCs (μg/mL)	CPE inhibitory concentration	
		HSV-1			*PI*-3	
		Max.	Min.		Max.	Min.
1	1.6	0.4	0.1	0.4	0.4	0.05
2	0.4	–	–	0.8	0.8	0.05
3	0.4	–	–	0.4	0.2	0.1
4	0.8	0.8	0.4	0.4	0.4	0.2
5	0.4	–	–	0.8	0.2	0.05
6	0.8	0.8	0.2	0.4	0.2	0.05
7	0.4	–	–	0.4	0.4	0.05
Asiklovir	1.6	1.6	<0.012	–	–	–
Oseltamivir	–	–	–	1.6	1.6	<0.012

$MNTC_s$, 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.

causative agents of respiratory viral infections, and carry significant high rates of morbidity and mortality in infants and young children [34].

As given in CPE inhibitory concentration ranging, compounds **1** and **6** showed anti-*Herpes simplex* activity with less potency. Among the tested Mannich base derivatives, compounds **2**, **3**, **5** and **6** were not active against DNA virus. Taking into account the CPE inhibitory concentration ranging against the RNA viruses *PI*-3, compound **2** containing 3,4-dichlorobenzyl moiety (0.8–0.05 μg/mL) had remarkable antiviral activity in Mannich base derivatives. Thereto, compounds **1** and **7** were less active than compound **2**. While the activities of compounds **1** and **7** (0.4–0.05 μg/mL) against *PI*-3 were in similar CPE inhibitory concentration range, compounds **5** and **6** had lower activity than that of compounds **1** and **7**. Also, the activities of compounds **3** and **4** were of negligible values as seen in Table 4.

Investigation among maximum non-toxic concentrations (MNTCs) of synthesized compounds **1**–**7** on the Vero and MDBK cell line was found to be 0.4–1.6 μg/mL. As per this result, compounds **2**–**7** (0.4–0.8 μg/mL) were more toxic than compound **1** (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 concentrations.

3. Conclusion

In this study, the impact of halogen substituent at piperazine or piperidine moiety was investigated. Among these Mannich base derivatives, compounds **3**, **5**, and **6** were the most active compounds towards *S. aureus* and *B. subtilis* with MIC values of 1 μg/mL and *P. aeruginosa* with MIC values of 2 μg/mL, respectively. Antibacterial activities of compounds **2**–**7** with MIC values of 2 μg/mL were determined for isolated drug-resistant strains of *B. subtilis*. All the synthesized compounds had better antibacterial effect against Gram-positive bacteria than Gram-negative ones. According to antifungal potential, compounds **2**–**7** were determined to be the most remarkably active Mannich base derivatives against *C. albicans* and *C. parapsilosis*. When the synthesized compounds were compared within CPE inhibitory concentration ranges, compound **2** appeared the most active derivative against *PI*-3.

4. Experimental procedure

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 as KBr disc (γ, cm^{-1}). ^1H NMR spectra were obtained with a Varian 400 MHz spectrophotometer in d_6-dimethylsulfoxide (DMSO-d_6) and tetramethylsilane (TMS) was used as an internal standard (chemical shift in δ, ppm). ^{13}C NMR spectra were recorded on a Varian Mercury 400 spectrophotometer. 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 Elementar Analysensysteme GmbH varioMICRO CHNS at The Scientific & Technological Research Council of Turkey-Ankara Testing and Analyses Laboratory (TÜBİTAK-ATAL). The purity of the compounds was assessed by thin layer chromatography (TLC) on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) chromatoplates.

Chlorokojic acid was synthesized as described by Ellis et al. [28]. Yield 60%, m.p. 166–167 °C.

4.1. General synthesis of Mannich bases (compounds **1**–**7**)

Mannich bases were prepared by the reaction of substituted piperazine or piperidine 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 methanol. All crude products recrystallized from MeOH except compound **6** which used chloroform as a solvent.

Seven compounds were synthesized according to the procedures as shown below.

4.1.1. 6-Chloromethyl-2-[4-(4-fluorobenzyl)piperazin-1-ylmethyl]-3-hydroxy-4H-pyran-4-one (**1**)

IR (KBr disc) 1629 (C=O st), 1508, 1458 (C=C st) and 1205 cm^{-1} (C–O st). ^1H NMR (400 MHz, DMSO-d_6, in ppm): 2.36 (4H; br s; piperazine), 2.50 (4H; br s; piperazine), 3.43 (2H; s; $-CH_2-$), 3.56 (2H; s;=N–CH_2-), 4.65 (2H; s; ClCH_2-), 6.54 (1H; s; H^5), 7.11 (2H; t; $J = 9.0$; Ph-$H^{3'}$, $H^{5'}$), 7.30 (2H; t; $J = 7.0$; Ph-$H^{2'}$, $H^{6'}$), 9.00–9.20 (1H; br; $-OH$). ^{13}C NMR (100 MHz, DMSO-d_6, in ppm): 42.09, 52.98, 53.04, 54.09, 61.66, 113.11, 115.50 (d; $J = 20.6$), 131.26 (d; $J = 7.6$), 134.93, 144.73, 148.34, 161.82, 161.89 (d; $J = 240.8$; C–F), 174.12. ESI-MS m/z 195 (100%), 367 (M^++H), 369 (M^+ + H+2), 389 (M^+ + Na). Anal. Cal. for $C_{18}H_{21}ClN_2O_4$ MW: 366.11 Cal. C: 58.94 H: 5.50 N: 7.64. Found C: 58.83 H: 5.39 N: 7.56.

4.1.2. 6-Chloromethyl-2-[(4-(3,4-dichlorobenzyl)piperazin-1-yl) methyl]-3-hydroxy-4H-pyran-4-one (**2**)

IR (KBr disc) 1625 (C=O st), 1597, 1456 (C=C st) and 1227 cm^{-1} (C–O st). ^1H NMR (400 MHz, DMSO-d_6, in ppm):2.37 (4H; br s; piperazine), 2.50 (4H; br s; piperazine), 3.45 (2H; s; $-CH_2-$), 3.56 (2H; s;=N–CH_2-), 4.65 (2H; s; ClCH_2-), 6.54 (1H; s; H^5), 7.28 (1H; dd; $J = 8.0$; $J = 1.6$; Ph-$H^{5'}$), 7.51 (1H; d; $J = 1.6$; Ph-$H^{2'}$), 7.55 (1H; d; $J = 8.0$; Ph-$H^{6'}$), 9.00–9.20 (1H; br; $-OH$). ^{13}C NMR (100 MHz, $CDCl_3$, in ppm): 41.43, 52.96, 53.07, 55.59, 61.73, 112.55, 128.51, 130.42, 131.96, 131.22, 132.56, 138.66, 144.51, 146.23, 161.64, 173.89. ESI-MS m/z 101 (100%), 417 (M^+ + H), 419 (M^+ + H+2), 439 (M^+ + Na). $C_{18}H_{19}Cl_3N_2O_3$ for MW: 416.04 Anal. Cal. for $C_{18}H_{19}Cl_3N_2O_3.1/2H_2O$ MW: 426.04 Cal. C: 50.66 H: 4.72 N: 6.56. Found C: 50.88 H: 4.59 N: 6.54.

4.1.3. 6-Chloromethyl-2-{[4-((4-chlorophenyl)(phenyl)methyl) piperazin-1-yl]methyl}-3-hydroxy-4H-pyran-4-one (**3**)

IR (KBr disc) 1633 (C=O st), 1586, 1479 (C=C st) and 1200 cm^{-1} (C–O st). ^1H NMR (400 MHz, DMSO-d_6, in ppm): 2.30 (4H; br s; piperazine), 2.50 (4H; br s; piperazine), 3.56 (2H; s; $-CH_2-$), 4.32 (1H; s; $-CH=$), 4.64 (2H; s; $ClCH_2-$), 6.54 (1H; s; H^5), 7.16–7.43 (9H; m; Ph). ^{13}C NMR (100 MHz, $CDCl_3$, in ppm): 41.43, 51.72, 53.41, 55.61, 75.42, 112.60, 127.51, 128.02, 128.89, 128.94, 129.37, 132.88, 141.26, 142.03, 144.53, 146.28, 161.64, 173.93. ESI-MS *m/z* 101 (100%), 459 ($M^+ + H$), 461 ($M^+ + H+2$), 481 ($M^+ + Na$). Anal. Cal. for $C_{24}H_{24}Cl_2N_2O_3$ MW: 458.11 Cal. C: 61.54 H: 5.38 N: 5.98. Found C: 61.65 H: 5.33 N: 5.90.

4.1.4. tert-Butyl 4-[(6-(chloromethyl)-3-hydroxy-4-oxo-4H-pyran-2-yl)methyl]piperazine-1-carboxylate (**4**)

IR (KBr disc) 1698 (C=O st, ester) and 1622 (C=O st, pyranone), 1457 cm^{-1} (C=C st) and 1207 cm^{-1} (C–O st). ^1H NMR (400 MHz, DMSO-d_6, in ppm): 1.38 (9H; s; $-CH_3$), 2.41 (4H; t; $J = 4.80$; piperazine), 3.31 (4H; t; $J = 4.80$; piperazine), 3.59 (2H; s; $-CH_2-$), 4.65 (2H; s; $ClCH_2-$), 6.54 (1H; s; H^5), 9.10–9.35 (1H; br; $-OH$). ^{13}C NMR (100 MHz, $CDCl_3$, in ppm): 28.62, 41.43, 52.80, 52.90, 55.25, 80.12, 112.11, 144.28, 146.04, 154.81, 162.07, 173.79. ESI-MS *m/z* 101 (100%), 359 ($M^+ + H$), 361 ($M^+ + H+2$), 381 ($M^+ + Na$). Anal. Cal. for $C_{16}H_{23}ClN_2O_5$ MW: 358.12 Cal. C: 53.56 H: 6.46 N: 7.81. Found C: 53.47 H: 6.42 N: 7.72.

4.1.5. 2-{[4-(4-Bromophenyl)-4-hydroxypiperidin-1-yl]methyl}-6-chloromethyl-3-hydroxy-4H-pyran-4-one (**5**)

IR (KBr disc) 1655 (C=O st), 1585 (C=C st) and 1205 cm^{-1} (C–O st). ^1H NMR (400 MHz, DMSO-d_6, in ppm): 1.58 (2H; d; $J = 12.40$; piperidine), 1.91 (2H; t; $J = 12.60$; piperidine), 2.59 (2H; t; $J = 10.80$; piperidine), 2.66 (2H; d; $J = 10.80$; piperidine), 3.61 (2H; s; $-CH_2-$), 4.66 (2H; s; $ClCH_2-$), 6.55 (1H; s; H^5), 7.43 (2H; d; $J = 8.80$; Ph), 7.48 (2H; d; $J = 8.40$; Ph). ^{13}C NMR (100 MHz, DMSO-d_6, in ppm): 37.61, 41.33, 48.49, 48.65, 53.79, 69.08, 112.37, 119.23, 127.14, 130.56, 143.9, 147.90, 149.32, 161.05, 173.36. ESI-MS *m/z* 101 (100%), 428 ($M^+ + H$), 430 ($M^+ + H+2$), 450 ($M^+ + Na$). $C_{18}H_{20}BrClNO_4$ for MW: 427.02 Anal. Cal. for $C_{18}H_{19}BrClNO_4.1/2H_2O$ MW: 437.71 Cal. C: 49.39 H: 4.60 N: 3.19. Found C: 49.19 H: 4.95 N: 2.97.

4.1.6. 2-{[4-(4-Chloro-3-(trifluoromethyl)phenyl)-4-hydroxypiperidin-1-yl]methyl}-6-chloromethyl-3-hydroxy-4H-pyran-4-one (**6**)

IR (KBr disc) 1657 (C=O st), 1586, 1479 (C=C st) and 1198 cm^{-1} (C–O st). ^1H NMR (400 MHz, DMSO-d_6, in ppm): 1.58 (2H; d; $J = 12.00$; piperidine), 1.97 (2H; t; $J = 12.60$; piperidine), 2.59 (2H; t; $J = 10.8$; piperidine), 2.71 (2H; d; $J = 10.40$; piperidine), 3.62 (2H; s; $-CH_2-$), 4.66 (2H; s; $ClCH_2-$), 5.00–5.20 (1H; br; $-OH$), 6.56 (1H; s; H^5), 7.64 (1H; d; $J = 8.0$; Ph-$H^{3'}$), 7.76 (1H; dd; $J = 8.8$; $J = 2.0$; Ph-$H^{2'}$), 7.91 (1H; d; $J = 2.0$; Ph-$H^{6'}$). ^{13}C NMR (100 MHz, DMSO-d_6, in ppm): 38.20, 42.10, 49.30, 54.50, 69.98, 113.15, 122.36, 124.92, 126.73 (q; $J = 30.4$; $-CF_3$), 127.8, 129.2, 131.4, 131.9, 144.8, 148.6, 161.8, 174.2. ESI-MS *m/z* 101 (100%), 452 ($M^+ + H$), 454 ($M^+ + H+2$), 474 ($M^+ + Na$). $C_{19}H_{18}Cl_2F_3NO_4$ MW: 451.05 Anal. Cal. for $C_{19}H_{18}Cl_2F_3NO_4.H_2O$ MW: 469.06 Cal. C: 48.52 H: 4.28 N: 2.97. Found C: 48.04 H: 4.38 N: 2.80.

4.1.7. 2-[(4-Acetyl-4-phenylpiperidin-1-yl)methyl]-6-chloromethyl-3-hydroxy-4H-pyran-4-one (**7**)

IR (KBr disc) 1695 (C=O st, carbonyl), 1651 (C=O st, pyranone), 1586 (C=C st) and 1198 cm^{-1} (C–O st). ^1H NMR (400 MHz, DMSO-d_6, in ppm): 1.86 (3H; s; $-COCH_3$), 1.97 (2H; d; $J = 11.40$; piperidine), 2.03–2.65 (6H; m; piperidine), 3.51 (2H; s; $-CH_2-$), 4.63 (2H; s; $ClCH_2-$), 6.53 (1H; s; H^5), 7.25–7.99 (5H; m; Ph). ^{13}C NMR (100 MHz, DMSO-d_6, in ppm): 26.10, 32.84, 42.07, 50.75, 54.21, 54.47, 113.12, 127.11, 127.70, 129.52, 141.97, 144.70, 148.46, 161.82, 174.09, 209.49. ESI-MS *m/z* 101 (100%), 376 ($M^+ + H$), 378 ($M^+ + H+2$), 398 ($M^+ + Na$). Anal. Cal. for $C_{20}H_{22}ClNO_4$ MW: 375.12 Cal. C: 63.91 H: 5.90 N: 3.73. Found C: 63.52 H: 5.91 N: 3.61.

4.2. Microbiological studies

4.2.1. Test materials

The compounds **1**–**7** 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. Ampicillin, vancomycin, gentamicin, ofloxacin, levofloxacin, ketoconazole and fluconazole were used as the standard antibacterial and antifungal drugs. Reference antibacterial agents of ampicillin (AMP, Faco), gentamicin (GM; Faco), ofloxacin (OFX, Hoechst Marion Roussel) reference antifungal agents of ketoconazole (KET, Bilim) and fluconazole (FLU, Pfizer), were obtained from their respective manufacturers and dissolved in phosphate buffer solution (ampicillin, pH 8.0; 0.1 mol/mL), dimethylsulphoxide (ketoconazole), or in water (gentamicin, ofloxacin, fluconazole). The stock solutions of the agents were prepared in medium according to the Clinical and Laboratory Standards Institute [35,36].

4.2.2. Microorganisms and inoculums preparation

Antibacterial activity test was carried out against standard (ATCC; American type culture collection, RSKK; Culture collection of Refik Saydam Central Hygiene Institute) and isolated (clinical isolate and obtained from Department of Microbiology, Faculty of Medicine, Gazi University) strains.

As 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 Gram-positive strains of *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6633 were used for the determination of antibacterial activity. The isolated strains of *E. coli, P. mirabilis* and *K. pneumoniae* have extended-spectrum β-lactamase (ESβL) enzyme, *P. aeruginosa* isolate (resistant to Trimethoprim-Sulfamethoxazole, tazobactam), *A. baumannii* isolate (resistant to cephalosporin), Gram-positive isolated strains of *S. aureus* (resistant to methicillin; MRSA), *E. faecalis* (resistant to cephalosporin), *B. subtilis* (resistant to ceftriaxon) 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 growth and dilution of the bacterial suspensions. The synthetic medium RPMI-1640 with L-glutamine was buffered to pH 7 with 3-[N-morpholino]-propansulfonic acid and culture suspensions were prepared. The microorganism suspensions used for inoculation were prepared at 10^5 c.f.u. (colony forming unite/mL) by diluting fresh cultures at McFarland 0.5 density (10^8 c.f.u./mL). Suspensions of bacteria and fungi were added in each well of the diluted extracts with a density of 10^5 c.f.u./mL for fungi and bacteria. The bacterial suspensions used for inoculation were prepared at 10^5 c.f.u./mL by diluting fresh cultures at McFarland 0.5 density (10^8 c.f.u./mL). The fungi suspension was prepared by the spectrophotometric method of inoculums [36].

4.2.3. Antibacterial and antifungal tests

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 two-fold dilutions of the compounds

(256–0.125 μg/mL) were made by dispensing the solutions to the remaining wells. 10 μL culture suspensions were inoculated into all the wells. DMSO (80%) and EtOH (20%), pure microorganisms and pure media were used as control wells. All organisms were tested in triplicate in each run of the experiments. The sealed microplates were incubated at 35 °C for 24 h and 48 h in humid chamber. The lowest concentration of the compounds that completely inhibit macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported [3,35]. The MICs were evaluated on test samples that showed antimicrobial activity.

4.2.4. Cytotoxicity and antiviral activity tests

Vero cell line (African green monkey kidney), which was used in this study, was obtained from Department of Virology, Faculty of Veterinary, Ankara University (Ankara-Turkey). The culture of the cells was grown in EMEM (Eagle's Minimal Essential Medium; Seromed; Biochrom; Berlin; Germany) enriched with 10% fetal calf serum (Biochrom, Germany), 100 μg/mL of streptomycin and 100 IU/mL of penicillin in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were harvested using Trypsin solution (Bibco Life Technologies, UK).

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. The test viruses were obtained from Department of Virology, Faculty of Veterinary, and Ankara University.

Media (EMEM) were placed into each of the 96 wells of the microplates (Greiner^R; Essen, Germany). Stock solutions of the extracts were added into first rows of microplates and two-fold dilutions of the extracts (51.2–0.012 μg/mL) were made by dispensing the solutions to the remaining wells. Two-fold dilution of each material was obtained according to Log_2 on the microplates. Acyclovir (Biofarma Co.) and oseltamivir (Roche Co.) were used as the control agents. Strains of *HSV*-1 and *PI*-3 titers were calculated as tissue culture infecting dose ($TCID_{50}$) and inoculated into all the wells. The sealed microplates were incubated in 5% CO_2 at 37 °C for 2 h to detect the possible antiviral activities of the samples. Following incubation, 50 μL of the cell suspension of 300.000 cells/mL which were prepared in EMEM together with 5% fetal bovine serum were put in each well and the plates were incubated in 5% CO_2 at 37 °C for 48 h. After the end of this period, the cells were evaluated using cell culture microscope by comparison with treated-untreated control cultures and with acyclovir and oseltamivir. Consequently, maximum cytopathogen effect (CPE) concentrations as the indicator of antiviral activities of the extracts were determined.

The MNTCs of each sample were determined by the method described. Several concentrations of each sample were placed in contact with confluent cell monolayer and incubated in 5% CO_2 at 37 °C for 48 h. After the incubation period, drug concentrations that are not toxic to viable cells were evaluated as non-toxic and also compared with non-treated cells for confirmation. The rows that cause damage in all cells were evaluated as toxic in the present concentration. In addition, maximum drug concentrations that did not affect the cells were evaluated as non-toxic concentration. MNTCs were determined by comparing treated and controlling untreated cultures [5,36].

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