Accepted Manuscript

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Please cite this article as: Aytemir, M.D., Özçelik, B., Karakaya, G., Evaluation of bioactivities of chlorokojic acid derivatives against dermatophytes couplet with cytotoxicity, *Bioorganic & Medicinal Chemistry Letters* (2013), doi: http://dx.doi.org/10.1016/j.bmcl.2013.03.098

EVALUATION OF BIOACTIVITIES OF CHLOROKOJIC ACID DERIVATIVES AGAINST DERMATOPHYTES COUPLET WITH CYTOTOXICITY

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Abstract

In an attempt to find novel antifungal agents with improved activity, a series of compounds bearing 6-chloromethyl-3-hydroxy-2-substituted 4*H*-pyran-4-one moiety were synthesized and examined for their cytotoxic evaluation and antifungal activities against both standard and isolated dermatophytic fungal species *M. gypseum*, *T. mentagrophytes var. erinacei* and *E. floccosum*.

Keratin is a protein found in only some of the eukaryotic cells which is also the basic component of the structures like hair, nail and skin. Although the structure of keratin is resistant to many external factors, some of microorganisms can invade this tissue. These keratinized tissues are damaged by superficial fungal infection, particularly dermatophyte infections. Those are among the most common forms of skin diseases all around the world which are mainly caused by three genera such as *Microsporum*, *Epidermophyton* and *Tricophyton*. In the recent years, we witnessed an increasing trend in the incidence of fungal infections because of the increased number of immunocomprimised hosts. In addition to the side effects regarding the use of the drugs given against dermatophytosis (e.g. ketoconazole, fluconazole), drug resistance to these drugs may also be seen. Therefore, there is an emerging necessity for the discovery of new, safer and more effective antifungal agents.[1-4]

In our previous studies, Mannich bases of hydroxypyrone derivatives such as kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one), chlorokojic acid (2-chloromethyl-5-hydroxy-4*H*-pyran-4-one) and allomaltol (5-hydroxy-2-methyl-4*H*-pyran-4-one) were synthesized and their extensive bioactivities were determined including anticonvulsant, antibacterial, antifungal and antiviral activities.[5-12] In a previous study, we also showed that allomaltol derivatives of Mannich compound bearing phenylpiperazine moieties with halogen atoms exhibited significant antifungal activities against *Candida* species. Their minimal inhibitory concentration (MIC) values were found to be the same as the reference compound Fluconazole against *Candida krusei*.[7] These data led us to focus on halogen derivatives to develop more effective compounds. So in a recent study performed in our laboratory, chlorokojic acid derivatives containing piperazine and piperidine structure were synthesized and tested for their antimicrobial and antiviral activities. The results of this study clearly indicated that the series of Mannich bases were active towards growth inhibition of pathogenic fungi *C. albicans* and *C. parapsilosis*.[10-11] The synthesis studies continued concerning this group of compounds followed with further bioactivity studies. Finally, novel derivatives of chlorokojic acid derivatives were synthesized as our latest paper and evaluated for their anticonvulsant, antifungal, antibacterial including antitubercular activites against *M. tuberculosis* (main cause of tuberculosis) and *M. avium* (nontuberculous mycobacteria which causes pulmonery diseases resembling tuberculosis) by using Resazurin microplate assay procedure (REMA). Compounds generally showed greater inhibition activity over *M. avium* growth than *M. tuberculosis*.[12]

Concerning these results, in the present study it is aimed to examine the antifungal activities of compounds against dermatophytes, which have 6-chloromethyl-3-hydroxy-2-substituted 4*H*-pyran-4-one structure. The seven derivatives of title compounds namely, compound **5** and compounds **20-25**, were newly synthesized as Mannich base derivatives. The other eighteen compounds that were reported to be synthesized before as presented in our latest paper were evaluated for their antidermatophytic activities for the first time.[12]

Kojic acid, which is an important and bioactive fungal metabolite, produced by many species of *Aspergillus*, *Acetobacter* and *Penicillium*, 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). Therefore, it has been used by many researchers as a beginning material for the preparation of new compounds in medicinal chemistry.[6-8,13-16] The enolic structure of neutral chlorokojic acid is expected to be the most stable one.[17] Because of their phenol-like properties, chlorokojic acid and other kojic acid derivatives readily undergo aminomethylation at room temperature during the Mannich reaction *ortho* to the enolic hydroxyl group.[18] The mechanisms of this Mannich reaction both in an acidic and a basic medium were investigated in an earlier study and

found that, enhanced reactivity in basic medium is due to increase in the electronegativity at 6-position. [19]

In the present study, chlorokojic acid was synthesized from commercially available kojic acid in a one-step reaction as suggested in previous studies. [6,7,10-12,15] Chlorination of the 2-hydroxymethyl moiety of kojic acid using thionyl chloride at room temperature produced chlorokojic acid, with the ring hydroxyl is being unaffected. The scope of the present study, using the methodology is shown in **Scheme**. 6-Chloromethyl-3-hydroxy-2-substituted 4*H*-pyran-4-one derivatives were synthesized as Mannich base derivatives.

Scheme.

Table.

The yields of the reactions and melting points of the compounds were determined, their structures were demonstrated by using spectroscopic techniques such as IR, ^{1}H-NMR, ^{13}C-NMR, ESI-MS and proved by elemental analysis results.

The selected diagnostic bands of IR spectra of compounds provided useful information for determining structures. Because of hydroxymethyl moiety showing two hydrogen bondings both intra- and intermolecular, (C=O) streching gave signals at lower frequency. IR spectra of all compounds showed ν (C−O) at about 1200 cm^{-1} and ν (C = C) at 1450 cm^{-1}. The formation of the Mannich bases of chlorokojic acid was further confirmed by using the ^{1}H-NMR spectra. Assignments of the signals were based on the chemical shifts and intensity pattern. Nitrogen atoms of piperidine ring exhibited broad singlet peaks. Aromatic hydrogens of phenyl rings were at 7.3-7.5 ppm with suitable integral values. ^{13}C-NMR spectra analysis was supported by DEPT and APT spectra to establish the carbon skeleton of the compounds. The results confirmed the structures. The distinctive signals of compounds were observed in the mass spectra which followed the similar fragmentation pattern. The entire spectrums showed molecular ion peaks, M$^+$+23 (Na) peaks and isotope peaks owing to chlorine atom.

Nowadays, due to the significant increase in the incidence of fungal infections on the growing population of immunocompromised patients and the emerging resistance to existing drugs, many authors emphasize the importance of newly synthesized active compounds, which may have valuable new targets with improved effect beyond the existing antifungals. [25] In the present study, the determined antidermatophytic activity of compounds by using eukaryotic cells (He-La, MRC-5) in lower toxicity (MIC: 250-500µg mL^{-1}) to evaluate cytotoxicity of chlorokojic acid derivatives is found in a range of 4-32 µg mL^{-1} against *Microsporum gypseum*, *Trichophyton mentagrophytes var erinacei* and *Epidermophyton floccosum.* Antidermatophytic activities were determined under non-toxic concentrations (MNTCs: ≥512 - ≥64 µg mL^{-1}) in He-La and MRC-5 cell line. As shown in Table, compounds **1**, **3**, **16-22** and **25** showed the highest activity against *E. floccosum* (MIC: 4 µg mL^{-1}). The existing activity of starting material chlorokojic acid (MIC: 8 µg mL^{-1}) was increased by these Mannich bases. Compounds **3**, **4**, **16-22** and **25** exhibited the same activity with chlorokojic acid against both standard and isolated strains of *T. mentagrophytes var. erinacei*. Compounds **1, 9, 16-18, 20-22, 27** and **28** were found as active as chlorokojic acid but safer (MNTCs: ≥ 512 µg mL^{-1} and ≥ 256 µg mL^{-1}) than it (MNTCs: ≥ 256 µg mL^{-1}) when compared with cytotoxicty results. Against both isolated and standard strains of *E. floccosum*, compounds **16** and **17** showed higher activity (MIC: 4-8 µg mL^{-1}) than chlorokojic acid (MIC: 8-16 µg mL^{-1}). As for antidermatofitic activity against *M. gypseum* compounds **3, 16, 17-22, 25** were bearing remarkable activity at MICs

values of 4 µg mL^{-1}. In addition, the rest of the compounds show moderate activity with MIC values of 4-32 µg mL^{-1}. Besides this, during the investigation of maximum non-toxic concentrations (MNTCs), they were less toxic than chlorokojic acid on the MRC-5 and He-La cell line suggesting that these compounds have promising properties which can lead to the discovery of novel antifungal agents.

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20. 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. Mannich bases were prepared by the reaction of substituted piperazine or piperidine derivatives and chlorokojic acid in MeOH with 37% formaline. The mixture was stirred vigorously for 15-25 min at room temperature. The resulting precipitate was collected by filtration and washed with cold MeOH. Formation of the desired new Mannich base derivatives were confirmed on the basis of elemental analysis, and the structures of the compounds were supported by spectral data. *Chlorokojic acid* was synthesized as described before.[10-12,15] Yield 60%, m.p. 166-167°C.6-(Chloromethyl)-3-hydroxy-2-((4-(4-iodophenyl)piperazin-1-yl)methyl)-4*H*-pyran-4-one **(Compound 5)** $C_{17}H_{18}ClIN_2O_3$, Yield: 56%; mp: 182-3°C; %CHN Found (Calculated): C 44.65 (44.32), H 3.91 (3.94), N 6.61 (6.08); IR ν (cm-1): 1630 (C=O), 1456 (C=C), 1202 (C-O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm: 2.60 (4H; t; *J*= 4.8; piperazine-$H^{2'}$, $H^{6'}$), 3.13 (4H; t; *J*= 4.8; piperazine-$H^{3'}$,$H^{5'}$), 3.62 (2H; s; -CH_2-), 4.66 (2H; s; ClCH_2-), 6.55 (1H; s; H^5), 6.76 (2H; d; *J*= 9.2; Ar-$H^{2''}$, $H^{6''}$), 7.46 (2H; d; *J*= 9.2; Ar-$H^{3''}$, $H^{5''}$), 9.22 (1H; s; -OH); ^{13}C-NMR (DMSO, 400 MHz) δ ppm: 42.11, 48.35, 52.77, 54.02, 81.32, 113.12, 118.49, 137.93, 144.82, 148.14, 151.19, 161.93, 174.15; ESI-MS (m/z): 399 (100%), 461 (M[+]+H), 463 (M[+]+H+2), 483 (M[+]+Na). Ethyl 4-((6-(chloromethyl)-3-hydroxy-4-oxo-4*H*-pyran-2-yl)methyl)piperazine-1-carboxylate **(Compound 20)** $C_{14}H_{19}ClN_2O_5$, Yield: 63%; mp: 136-7°C; %CHN Found (Calculated): C 50.59 (50.84), H 5.63 (5.79), N 8.38 (8.47); IR ν (cm-1): 1679 (C=O), 1424 (C=C), 1220 (C-O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm: 1.67 (3H; t; *J*= 7.0; -CH_3), 2.44 (4H; t; *J*= 5.2; piperazine-$H^{2'}$, $H^{6'}$), 3.36 (4H; t; *J*= 5.2; piperazine-$H^{3'}$,$H^{5'}$), 3.60 (2H; s; -CH_2-), 4.02 (2H; q; *J*= 6.8; -CH_2CH$_3$), 4.65 (2H; s; ClCH_2-), 6.55 (1H; s; H^5), 9.19 (1H; brs; -OH); ^{13}C-NMR (DMSO, 400 MHz) δ ppm: 15.22, 42.10, 43.93, 52.62, 53.10, 61.39, 113.12, 144.86, 147.90, 155.23, 161.94, 174.16; ESI-MS (m/z): 331 (M[+]+H), 333 (M[+]+H+2), 353 (M[+]+Na, 100%). Benzyl 4-((6-(chloromethyl)-3-hydroxy-4-oxo-4*H*-pyran-2-yl)methyl)piperazine-1-carboxylate **(Compound 21)** $C_{19}H_{21}ClN_2O_5$, Yield: 64%; mp: 141-2°C; %CHN Found (Calculated): C 57.86 (58.09), H 5.25 (5.39), N 7.07 (7.13); IR ν (cm-1): 1679 (C=O), 1446 (C=C), 1225 (C-O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm: 2.45 (4H; t; *J*= 4.8; piperazine-$H^{2'}$, $H^{6'}$), 3.40 (4H; m; piperazine-$H^{3'}$,$H^{5'}$), 3.60 (2H; s; -CH_2-), 4.65 (2H; s; ClCH_2-), 5.07 (2H; s; -CH_2COO-), 6.55 (1H; s; H^5), 7.28-7.38 (5H; m; Ar-H), 9.20 (1H; brs; -OH); ^{13}C-NMR (DMSO, 400 MHz) δ ppm: 42.10, 44.15, 52.64, 54.01, 66.89, 113.12, 128.20, 128.50, 129.09, 137.57, 144.82, 148.05, 155.05, 161.92, 174.17; ESI-MS (m/z): 393 (M[+]+H), 395 (M[+]+H+2), 415 (M[+]+Na, 100%). 1-((6-(Chloromethyl)-3-hydroxy-4-oxo-4*H*-pyran-2-yl)methyl)-4-phenylpiperidine-4-carbonitrile **(Compound 22)** $C_{19}H_{19}ClN_2O_3$, Yield: 71%; mp: 156-7°C; %CHN Found (Calculated): C 64.35 (63.60), H 5.39 (5.34), N 7.91 (7.81); IR ν (cm-1): 1660 (C=O), 1455 (C=C), 1200 (C-O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm: 1.99-2.56 (8H; m; piperidine); 3.68 (2H; s; -CH_2-), 4.66 (2H; s; ClCH_2-), 6.56 (1H; s; H^5), 7.34-7.54 (5H; m; Ar-H); 9.24 (1H; brs; -OH); ^{13}C-NMR (DMSO, 400 MHz) δ ppm: 36.15, 42.09, 42.29, 50.69, 54.03, 113.19, 122.67, 126.33, 128.75, 129.71, 140.82, 144.87, 148.19, 161.95, 174.19; ESI-MS (m/z): 381 (100%, M[+]+Na), 359 (M[+]+H), 361 (M[+]+H+2). 6-(Chloromethyl)-3-hydroxy-2-((4-phenyl-5,6-dihydropyridin-1(2H)-yl)methyl)-4*H*-pyran-4-one **(Compound 23)** $C_{18}H_{18}ClNO_3$, Yield: 76%; mp: 165-6°C; %CHN Found (Calculated): C 64.67 (65.16), H 5.33 (5.47), N 4.16 (4.22); IR ν (cm-1): 1622 (C=O), 1456 (C=C), 1198 (C-O); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ ppm: 2.49-3.19 (6H; m; pyridine), 3.69 (2H; s; -CH_2-), 4.67 (2H; s; ClCH_2-), 6.14 (1H; t; *J*= 3.6; pyridine), 6.55 (1H; s; H^5), 7.22-7.42 (5H; m; Ar-H); ^{13}C-NMR (DMSO, 400 MHz) δ ppm: 27.99, 42.13,

50.20, 52.76, 53.62, 113.13, 122.47, 125.24, 127.69, 129.04, 134.55, 140.75, 144.76, 148.52, 161.94, 174.18; ESI-MS (m/z): 332 (100%, M^{+}+H), 334 (M^{+}+H+2), 354 (M++Na). 6-(Chloromethyl)-2-((4-(4-chlorophenyl)-5,6-dihydropyridin-1(2H)-yl)methyl)-3-hydroxy-4*H*-pyran-4-one **(Compound 24)** $C_{18}H_{17}Cl_2NO_3$, Yield: 97%; mp: 166-7°C; %CHN Found (Calculated): C 59.15 (59.03), H 4.61 (4.68), N 4.01 (3.82); IR ν (cm-1): 1631 (C=O), 1458 (C=C), 1194 (C-O); ^{1}H-NMR (DMSO-*d*$_6$, 400 MHz) δ ppm: 2.47-3.27 (6H; m; pyridine), 3.69 (2H; s; -C*H$_2$*-), 4.66 (2H; s; ClC*H$_2$*-), 6.18 (1H; brs; pyridine), 6.55 (1H; s; *H^5*), 7.36 (2H; d; *J*= 8.4; Ar-*H$^{2''}$*, *H$^{6''}$*), 7.44 (2H; d; *J*= 8.4; Ar-*H$^{3''}$*, *H$^{5''}$*); ^{13}C-NMR (DMSO, 400 MHz) δ ppm: 27.86, 42.13, 50.08, 52.70, 53.55, 113.13, 123.38, 127.02, 128.92, 132.19, 133.46, 139.54, 144.76, 148.47, 161.94, 174.17; ESI-MS (m/z): 218 (100%), 366 (M^{+}+H), 368 (M^{+}+H+2), 388 (M^{+}+Na). 6-(Chloromethyl)-2-((4-(4-fluorophenyl)-5,6-dihydropyridin-1(2*H*)-yl)methyl)-3-hydroxy-4*H*-pyran-4-one **(Compound 25)** $C_{18}H_{17}ClFNO_3$, Yield: 80%; mp: 164-5°C; %CHN Found (Calculated): C 61.20 (61.81), H 4.77 (4.90), N 4.01 (4.00); IR ν (cm-1): 1626 (C=O), 1456 (C=C), 1200 (C-O); ^{1}H-NMR (DMSO-*d*$_6$, 400 MHz) δ ppm: 2.52-3.20 (6H; m; pyridine), 3.70 (2H; s; -C*H$_2$*-), 4.69 (2H; s; ClC*H$_2$*-), 6.12 (1H; brs; pyridine), 6.58 (1H; s; *H^5*), 7.14-7.19 (2H; m; Ar-*H$^{2''}$*, *H$^{6''}$*), 7.45-7.49 (2H; m; Ar-*H$^{3''}$*, *H$^{5''}$*) ^{13}C-NMR (DMSO, 400 MHz) δ ppm: 28.05, 42.11, 50.12, 52.67, 53.56, 113.12, 115.62, 115.83, 122.46, 127.15, 133.58, 137.22, 144.76, 148.47, 160.83, 161.92, 163.25, 174.16; ESI-MS (m/z): 350 (100%, M^{+}+H), 352 (M^{+}+H+2), 372 (M^{+}+Na).
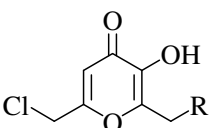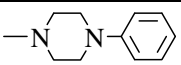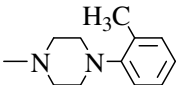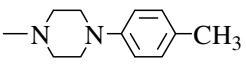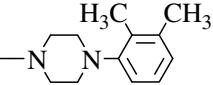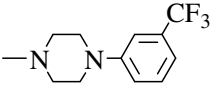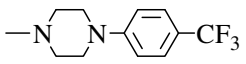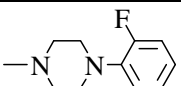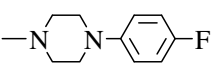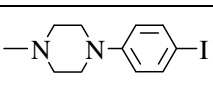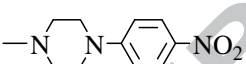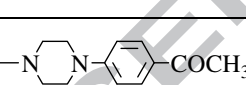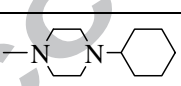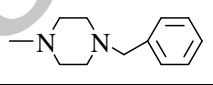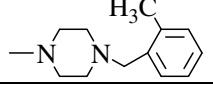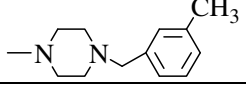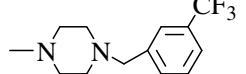
21. *In vitro* antidermatophytic activity of the compounds against *Microsporum gypseum*, *Trichophyton mentagrophytes var. erinacei* and *Epidermophyton floccosum* were screened by broth microdilution method. Terbinafine, griseofulvin and itraconazole were used as the control agents. The minimal inhibition concentration (MIC) of each extract was determined by using broth microdilution techniques according to Clinical and Laboratory Standards Institute. The medium used was RPMI 1640 broth with l-glutamine and buffered to pH 7.0 with 0.165 M 3-(N-morpholino) propanesulfonic acid but without sodium bicarbonate, sterilized by filtration. A sterile 96 well microdilution plates were prepared, as series of test materials dilutions (128-0.0625 µg mL^{-1}). For each test plate, two drug-free controls were included, one with the medium alone (sterility control) and the other with medium plus inoculum suspension which served as the growth control. Tests were performed in triplicate. Each well was inoculated with 100 µL of the corresponding inoculums and incubated at 28°C for 7 days. The microplates were read visually with the aid of an inverted reading mirror after 7 days for dermatophytes.[22-23] Cytotoxicity was evaluated by the maximum non-toxic concentrations (MNTCs) of each samples, which were determined by the method described previously by Özçelik et al.[24] based on cellular morphologic alteration in the table.

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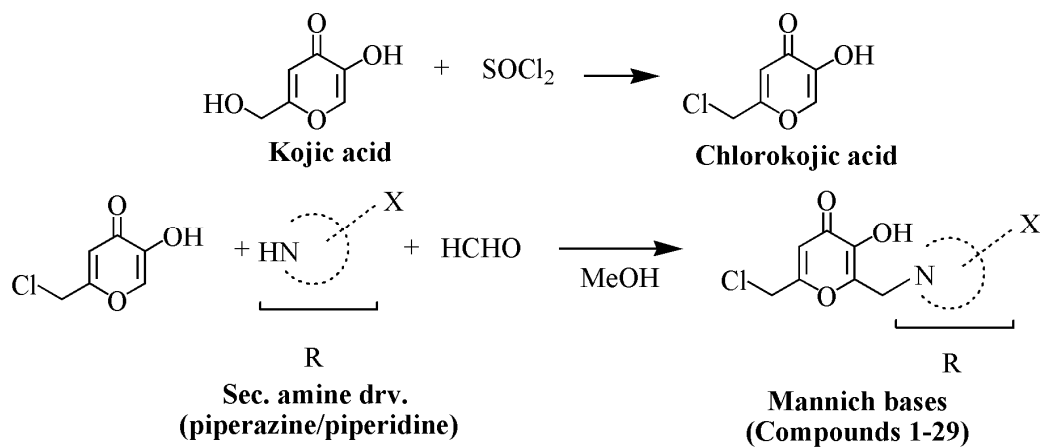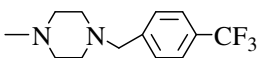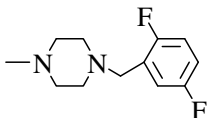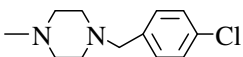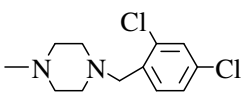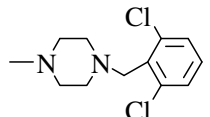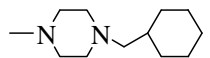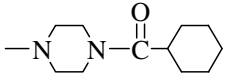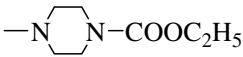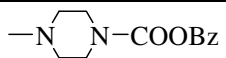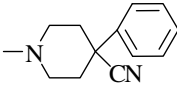
Scheme. General synthesis of Mannich bases of chlorokojic acid (Compounds 1-29)

Table. Antidermatophytic activities couplet with cytotoxicity of the compounds and reference agents as MICs (µg mL^{-1}) values.

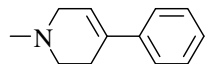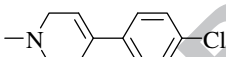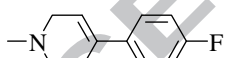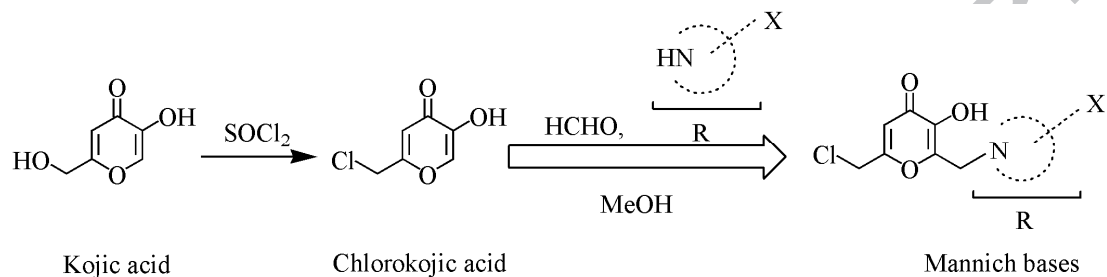|	-R	Trichophyton mentagrophytes var. erinacei		Epidermophyton floccosum		Microsporum gypseum		Cell Line	
		NCPF 375	Isolated strain	RSKK 3027	Isolated strain	NCPF 580	Isolated strain	MRC-5	He-La
		MIC		MIC		MIC		MNTCs	
1	—N N— (phenyl)	8	16	4	8	8	8	≥256	≥256
2	—N N— (2-CH$_3$ phenyl, H$_3$C)	8	16	16	32	16	32	≥128	≥128
3	—N N—◯—CH$_3$	4	8	4	8	4	8	≥64	≥64
4	—N N— (2,3-diCH$_3$ phenyl, H$_3$C CH$_3$)	4	8	8	16	8	16	≥256	≥256
5	—N N— (CF$_3$ phenyl)	32	64	32	64	32	64	≥128	-
6	—N N—◯—CF$_3$	32	64	32	64	32	64	≥128	-
7	—N N— (F phenyl)	8	16	16	32	8	16	≥128	-
8	—N N—◯—F	8	8	8	16	8	16	≥128	-
9	—N N—◯—I	8	16	8	16	8	16	≥512	≥512
10	—N N—◯—NO$_2$	16	32	16	32	16	32	≥512	≥512
11	—N N—◯—COCH$_3$	16	16	16	16	16	16	≥512	≥512
12	—N N— (cyclohexyl)	16	16	16	32	16	32	≥512	≥512
13	—N N—CH$_2$— (benzyl)	16	32	16	32	16	32	≥512	≥512
14	—N N—CH$_2$— (2-CH$_3$ benzyl, H$_3$C)	16	32	16	32	16	32	≥512	≥512
15	—N N—CH$_2$— (3-CH$_3$ benzyl, CH$_3$)	16	16	16	32	16	32	≥512	≥512
16	—N N—CH$_2$— (CF$_3$ benzyl)	4	8	4	8	4	8	≥512	≥512

No.	Structure								
17	piperazine–N–CH$_2$–C$_6$H$_4$–CF$_3$	4	8	4	8	4	8	≥512	≥512
18	piperazine–N–CH$_2$–C$_6$H$_3$(F)$_2$ (2,5-diF)	4	8	4	8	4	8	≥256	≥256
19	piperazine–N–CH$_2$–C$_6$H$_4$–Cl	4	8	4	8	4	8	≥128	≥128
20	piperazine–N–CH$_2$–C$_6$H$_3$(Cl)$_2$ (2,4-diCl)	4	8	4	8	4	8	≥512	≥256
21	piperazine–N–CH$_2$–C$_6$H$_3$(Cl)$_2$ (2,6-diCl)	4	8	4	8	4	8	≥512	256
22	piperazine–N–CH$_2$–cyclohexyl	4	8	4	8	4	8	≥512	≥256
23	piperazine–N–C(=O)–cyclohexyl	8	16	16	32	16	32	≥128	≥128
24	piperazine–N–COOC$_2$H$_5$	16	32	16	32	16	32	≥128	≥256
25	piperazine–N–COOBz	4	8	4	8	4	8	≥128	≥256
26	4-phenyl-4-cyano-piperidine	16	32	16	32	16	32	≥256	≥256
27	4-phenyl-1,2,3,6-tetrahydropyridine	8	16	8	16	8	16	≥256	≥256
28	4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine	8	8	8	8	8	8	≥256	≥256
29	4-(4-fluorophenyl)-1,2,3,6-tetrahydropyridine	16	16	16	16	16	16	≥256	≥256
30	**Chlorokojic Acid**	4	8	8	16	2	4	≥256	≥256
	Terbinafine	0.125	0.25	0.25	0.5	0.25	0.5		
	Griseofulvin	0.5	1	0.5	1	0.5	1		
	Itraconazole	0.25	0.5	0.125	0.25	0.125	0.25		

9

Graphical Abstract

Mannich bases of chlorokojic acid were synthesized and examined for their cytotoxic evaluation and antifungal activities against both standard and isolated dermatophytic fungi species *M. gypseum*, *T. mentagrophytes var. erinacei* and *E. floccosum*.