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Synthesis and Pharmacokinetic studies of Effective and Safe New Antimicrobial Derivatives of Substitited Hydroquinoxaline-2,3-diones

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ABSTRACT

A series of 1,4-disubstituted octahydroquinoxaline-2,3-dione derivatives was prepared through two steps reaction. The latter involves the formation of N,N-disubstituted cyclohexane-1,2-diamine derivatives (**la-g**) through reductive alkylation of 1,2-cyclohexanediamine with different acetophenones in presence of sodium cyanoborohydride. Fusion of compounds (**1a-g**) with diethyl oxalate affording the target compounds (**2a-g**). Elucidation of structures of compounds (**2a-g**) was based upon different spectral data as well as the elemental methods of analyses. In addition, mass spectrometry and X-ray diffraction analyses were carried out. Moreover, the lipophilicity of the target compounds as expressed from the Clog P. Most of the test compounds (**2a-g**) showed weak to moderate antibacterial and antifungal activities against most of the used bacterial and fungal strains in comparison to norfloxacin and clotrimazole as reference drugs respectively. The pharmacokinetics of the most active hydroquinoxaline-2,3-diones derivative (**2e**) was determined in Rabbit plasma after intravenous and oral administration by a **s**imple, sensitive and selective high-performance liquid chromatographic (HPLC) assay with ultraviolet detection (HPLC-UV). Norfloxacin (NFL) was used as internal standard. The mean C_{max}, t_{max} and AUC_{0-8h} were 16.50 ± 2.10 µg/ml, 2 ± 0.11h and 74.84 ± 5.11 µg h/ml respectively for compound (**2e**). The mean elimination half-life ($t_{0.5e}$), absorption half-life ($t_{0.5a}$), elimination rate constant k_e and absorption rate constant k_a values were 3.11 ± 0.22h, 0.60 ± 0. 1h, 0.231± 0.03 h^{-1} and 0.1.155 ± 0.13 h^{-1}, respectively. The absolute bioavailability is 80.96% indicating good absorption after oral administration.

Keywords: antimicrobial, activity, octahydroquinoxaline-2,3-dione, diffraction, synthesis, structure elucidation, pharmacokinetic study

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INTRODUCTION

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The emergence of microbial resistance is an evolutionary process based on selection for organisms that have enhanced ability to survive doses of antibiotics that would have previously been lethal. Survival of bacteria often results from an inheritable resistance.[1,2] Many studies were relied on highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* a causative agent of human respiratory tract and skin infections, also hospital-acquired infections and resistant fungi such as *Candida albicans* a causative agent of oral and genital infections.[1,2] Moreover, antibiotic resistance may impose a biological cost and consequently, spread of antibiotic resistant bacteria may be hampered by reduced fitness associated with the resistance. However, additional mutations may compensate for this fitness cost and aids the survival of this bacteria.[3-6] Hence, the search for new and potent antimicrobial agents is gaining interest. On the other hand, nitrogen containing heterocycles are indispensable structural units for medicinal chemists. Among the various heterocyclic compounds, quinoxalines form an attractive biologically active molecules as those form part of various antibiotics such as echinomycin, levomycin and actinoleutin[7] that are known to possess other biological potentials such as adenosine receptor antagonist, anticancer, anthelimintic, antidepressant, anti-inflammatory, and antituberular activities.[8-13] Moreover, quinoxaline and its analogues have been investigated as the catalyst's ligands.[14] In view of the literature regarding antimicrobial potency of quinoxalines and their mode of action that prevent DNA-directed RNA synthesis by virtue of binding to CpG site on DNA, the quinoxaline nucleus is focused on synthesizing newer derivatives to explore potent antimicrobial activities.[4,8]

The continued interest in designing new flexible quinoxaline and quinoxalinedione molecules stems mainly because of the outstanding biological activities exhibited by several derivatives incorporating such heterocyclic moieties.[15-17] Accordingly, the present work aims to the design and synthesis of new substituted octahydroquinoxalinedione derivatives and elucidation of their structures, in addition to, testing the target compounds for their expected antimicrobial effects, if any.

Moreover, many studies are carried out for the quantitative determination[18-24], bioavailability[23-25], mechanism of action and pharmacokinetics of quinolone derivatives[25-30]. This study aims to optimize a rapid, sensitive and selective high performance liquid chromatography (HPLC) method for the simultaneous quantification of octahydroquinozaline-2,3-dione derivatives in rabbit plasma. The validation of the method will demonstrate its specificity (selectivity), limit of detection (LOD), precision, repeatability or reproducibility, linearity and working range. In addition, accuracy (bias), absolute and relative recoveries and stability of octahydroquinozaline-

2,3-dione derivatives in plasma and working solutions will be carried out and norfloxacin will be used as an internal standard. The method will be applied for determination of the pharmacokinetic parameters and the absolute bioavailability of the prepared octahydroquinozaline-2,3-dione derivatives in rabbits after both Intravenous and oral administration. The pharmacokinetic parameters will include C_{max}, t_{max}, $t_{0.5}$, K_e, V_d, Cl_t, AUC $_{0-t}$, AUC $_{0-\infty}$ and the absolute bioavailability. Statistical analysis of data will be performed using ANOVA computerized system[23-24].

MATERIALS AND METHOD

Chemistry

Melting points were determined in an electrothermal melting point apparatus (Stuart Scientific, Staffordshine, STIS, SMP3, UK) and were uncorrected. Monitoring of the chemical reactions was carried out by TLC using pre-coated silica gel plates (kieselgel 0.25 mm, 60G F254, Merck, Germany) and $CHCl_3/CH_3OH$ as the mobile phase. Visualization of the spots was effected by ultraviolet lamp (model CM-10, USA) at wavelengths 254 and 365 nm and/or iodine stain.

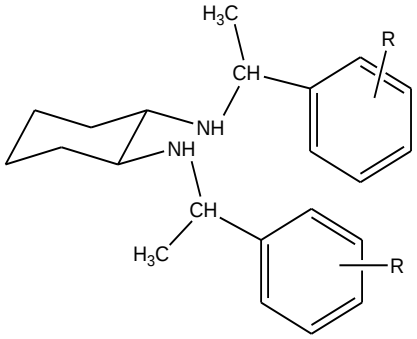
IR spectra were carried out as KBr discs on a Shimadzu IR-470 Spectrometer (Shimadzu, Japan). ^{1}H-NMR spectra were scanned on a Varian EM-360L NMR spectrometer (60 MHz, Varian, USA). Chemical shifts are expressed in δ-values (ppm) relative to TMS as an internal standard; using $CDCl_3$ as a solvent and D_2O was used for the detection of the exchangeable protons.

Electron Impact mass spectra (EI-MS) were run with JEOL JMS600 mass spectrometer at 70 eV (Thermo Electron Corporation, Japan) at the Micro Analytical Central Lab, Assiut University. Elemental microanalyses were performed on a Perkin-Elmer, 240 Elemental Analyzer, at the Unit of Microanalysis, Assiut University. X-ray diffraction (XRD) was carried out using XRD unit, at Dept., of physics, Faculty of Science, Assiut University.

N,N-Disubstituted cyclohexane-1,2-diamine derivatives (1a-g)

Sodium cyanoborohydride (48.0 mmol) was added portion wise to a solution of the appropriate acetophenone derivative (60 mmol) and 1,2-cyclohexylamine (30 mmol) in methanol (70 ml) at 0^{o}C. The reaction mixture was stirred for 3 h at ambient temperature, an additional amount of sodium cyanoborohydride (8.1 mmol) was added portion wise and stirring was continued for further 3-4 h. The reaction mixture was quenched with distilled water, concentrated and extracted with chloroform; the combined organic extract was washed with distilled water, and dried. Concentration and column chromatography ($CHCl_3/CH_3OH$) afforded compounds (**1a-g**) as pale yellow oils, Scheme 1. Yields and ^{1}HNMR data are given in Table 1.

Table 1: Yields and ^{1}HNMR data of compounds (1a-g)

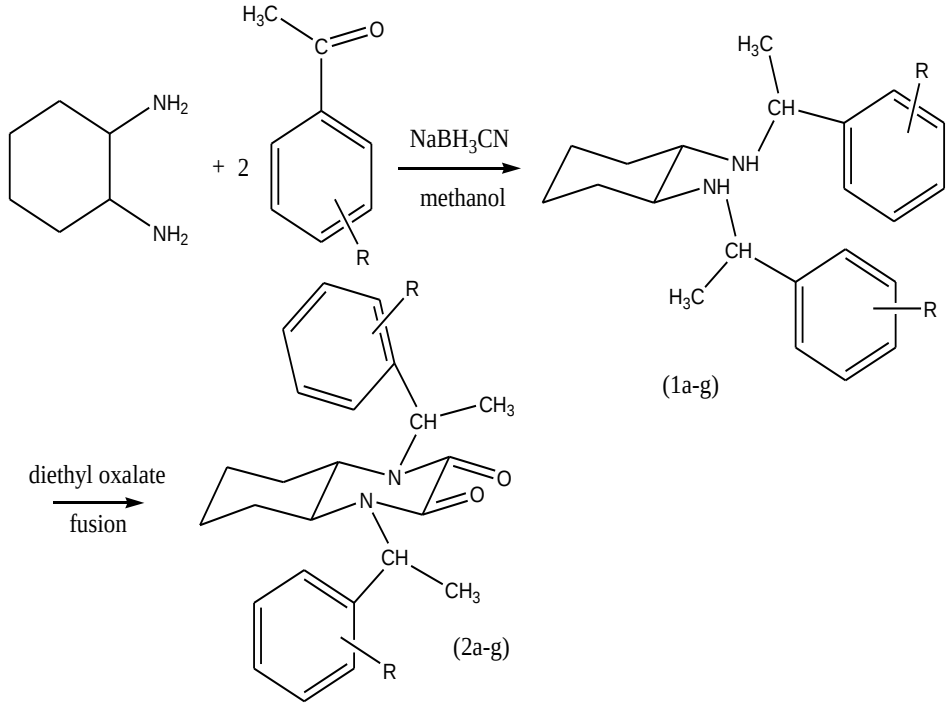


Compd. No.	R	Yield%	^{1}HNMR
1a	H	75	1.10-1.30 (m, 8H, *c*-hexyl) 1.38 (s, 3H, 2CH_3) 2.00-2.15 (m, 4H, 2CH of *c*-hexyl and 2NH exchangeable), 4.08- (q, 4H, 2CH), and 7.08-7.20 (m, 10H, 2 Ph).
1b	*p*-Br	80	1.13-1.25 (m, 8H, *c*-hexyl) 1.38 (s, 3H, 2CH_3) 2.00-2.20 (m, 4H, 2CH of *c*-hexyl and 2NH exchangeable), 4.08 (q, 4H, 2CH), and 7.00-7.38 (dd, 8H, 2 Ph).
1c	*p*-Cl	78	1.10-1.23 (m, 8H, *c*-hexyl) 1.36 (s, 3H, 2CH_3) 1.95-2.10 (m, 4H, 2CH of *c*-hexyl and 2NH exchangeable), 4.06 (q, 4H, 2CH), and 7.00-7.36 (dd, 8H, 2 Ph).
1d	*o*-Cl	62	1.14-1.23 (m, 8H, *c*-hexyl) 1.36 (s, 3H, 2CH_3) 1.96-2.13 (m, 4H, 2CH of *c*-hexyl and 2NH exchangeable), 4.07 (q, 4H, 2CH), and 7.00-7.36 (dd, 8H, 2 Ph).
1e	*p*-CH_3	78	1.12-1.63 (m, 8H, *c*-hexyl) 1.36 (s, 3H, 2CH_3) 1.96-2.13 (m, 4H, 2CH of *c*-hexyl and 2NH exchangeable), 2.35 (s, 6H, 2CH_3), 4.08 (q, 4H, 2CH), and 7.01 (s, 8H, 2Ph).
1f	*p*-OCH_3	83	1.16-1.66 (m, 8H, *c*-hexyl) 1.38 (s, 3H, 2CH_3) 1.98-2.15 (m, 4H, 2CH of *c*-hexyl and 2NH exchangeable), 3.37 (s, 6H, 2OCH_3), 4.08 (q, 4H, 2CH), and 6.70-6.78 and 6.90-7.01 (two m, 8H, 2Ph).
1g	*p*-F	80	1.12-1.62 (m, 8H, *c*-hexyl) 1.38 (s, 3H, 2CH_3) 1.96-2.10 (m, 4H, 2CH of *c*-hexyl and 2NH exchangeable), 4.10 (q, 4H, 2CH), and 6.92-7.10 (m, 8H, 2Ph).

*Compounds (1a-g) exhibited IR bands attributed to 2NH stretching functions at 3455-3525 cm^{-1}.

1,4-Disubstituted octahydroquinoxaline-2,3-dione (2a-g):

A mixture of the *N,N*-disubstituted cyclohexane-1,2-diamine derivatives (**1a-g**) (7.5 mmol), and freshly distilled diethyl oxalate (7.5 mmol) was fussed for 3-5 h, treated with ether (30 ml) and left 2-3 h. The product was filtered, dried and crystallized from absolute ethanol, Scheme 1, Tables 2, and 3.



R = (a) H, (b) *p*-Br, (c) *p*-Cl, (d) *o*-Cl, (e) *p*-CH_3, (f) *p*-OCH_3, (g) *p*-F, and

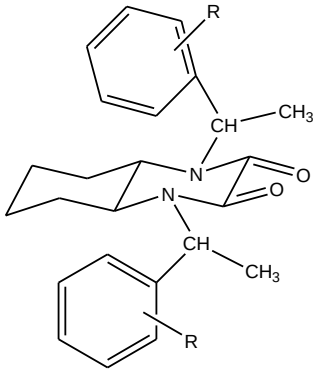
Scheme 1: Synthesis of compounds 1a-g and 2a-g.

Scheme 1: Synthesis of compounds (1a-g) and (2a-g).

Calculation of the log P values:

The log P values of the target compounds (**2a-g**), were computed with a routine method called calculated log P (Clog P) contained in a PC-software package (McLogP 2.0, BioByte Corp., CA, USA). A representation of the molecular structure where hydrogens were omitted or 'suppressed' (SMILES notation) was entered into the program, which computes the log P based on Crippen's fragmentation[31], and the results are given in Table 2.

Table 2: The physicochemical data and elemental microanalyses of compounds (2a-g).



Compd No.	R_1	M. formula (M.Wt)	Yield* %	Mp** °C	R_f***	ClogP	CHN Found/Cacd

2a	H	$C_{24}H_{28}N_2O_2$ (376.49)	73	110-1	0.38	4.08	75.57 7.26 7.86 76.56 7.50 7.44
2b	*p*-Br	$C_{24}H_{26}Br_2N_2O_2$ (534.28)	80	183-5	0.40	5.74	52.09 4.02 5.86 53.95 4.90 5.24
2c	*p*-Cl	$C_{24}H_{26}Cl_2N_2O_2$ (445.38)	78	186-7	0.46	5.20	63.77 5.36 6.61 64.72 5.88 6.29
2d	*o*-Cl	$C_{24}H_{26}Cl_2N_2O_2$ (445.38)	75	179	0.50	5.20	63.00 5.54 6.51 64.72 5.88 6.29
2e	*p*-CH_3	$C_{26}H_{32}N_2O_2$ (404.54)	75	183-4	0.31	5.06	76.03 7.77 7.19 77.19 7.97 6.92
2f	*p*-OCH_3	$C_{26}H_{32}N_2O_4$ (436.54)	80	198-9	0.33	3.83	70.36 7.19 6.63 71.53 7.39 6.42
2g	*p*-F	$C_{24}H_{26}F_2N_2O_2$ (412.47)	70	200-2	0.36	4.40	68.47 5.45 6.94 69.89 6.35 6.53

*Developing solvent system is $CHCl_3/CH_3OH$ (6:3)

**The crystallization solvent for the solid compounds is ethanol.

X-ray diffraction

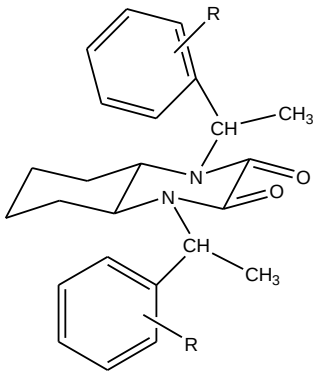
X-ray diffraction (XRD) was carried out using XRD unit, model PW1710 control unit, PW1710 generator and PW1050 Goniometer, Anode material Cu, Optics automatic divergence slit 0.1 and beta filtering graphite monochromator and software visualx, traces and PDF2 1999[32] at Dept., of physics, Faculty of Science, Assiut University.

Table 1: Yields and ^{1}HNMR data of compounds (1a-g)

Table 2: The physicochemical data and microanalyses of compounds (2a-g)

Table 3: ^{1}HNMR and MS data of compounds (2a-g)

Table 3: ^{1}HNMR and mass fragmentation data of compounds (2a-g)



Compd. No.	R	^{1}HNMR	MS
2a*	H	1.00-1.90 (m, 8 H, *c*-hexyl), 1.57 (d, 6H, $2CH_3$), 4.12-4.30 (m, 2H, 2CH of *c*-hexyl), 5.10-5.30 (q, 2H, 2CH), and 7. 08-7.21 (m, 10 H, 2 Ph).	M^+ (376.22, 4.01%) and M^++1 (377.20, 0.08%) and C_6H_{10}+2 (86, 100%).

2b	*p*-Br	1.10-1.93 (m, 8 H, *c*-hexyl), 1.57 (d, 6H, $2CH_3$), 4.10-3.25 (m, 2H, 2CH of *c*-hexyl), 5.00-5.15 (q, 2H, 2CH), and 6.85-7.50 (m, 8 H, 2 Ph).	M^+ (534.03, 0.2%), M^++2 (536.03, 0.2%), M^++4 (538.13, 0.2%), and C_6H_{10}+2 (86, 100%).
2c	*p*-Cl	0.75-2.00 (m, 8 H, *c*-hexyl), 1.56 (d, 6H, $2CH_3$), 4.11-3.26 (m, 2H, 2CH of *c*-hexyl), 5.10-5.17 (q, 2H, 2CH), and 6.90-7.50 (m, 8 H, 2 Ph).	M^+ (444.14, 2.2%), M^++2 (446.09, 0.8%), M^+-2Cl (373.05, 0.2%), and $C_7H_5Cl^+$ (124, 100%).
2d	*o*-Cl	0.92-2.00 (m, 8 H, *c*-hexyl), 1.58 (d, 6H, $2CH_3$), 4.10-4.30 (m, 2H, 2CH of *c*-hexyl), 5.10-5.18 (q, 2H, 2CH), and 6.85-7.40 (m, 8 H, 2 Ph).	M^+ (444.14, 4.0%), M^++2 (446.10, 1.4%), and $C_7H_5Cl^+$ (124, 100%).
2e	*p*-CH_3	0.92-2.15 (m, 8 H, *c*-hexyl), 1.50 (d, 6H, $2CH_3$), 2.3 (s, 6H, 2 CH_3), 3.15-3.60 (m, 2H, 2CH of *c*-hexyl), 4.50-5.15 (m, 2H, 2CH), and 7.2 (s, 8 H, 2 Ph).	M^+ (404.30, 3.8%), M^++1-$2CH_3$, (375.09, 16.3%), C_6H_{10}+2 (86, 100%).
2f	*p*-OCH_3	0.75-2.10 (m, 8 H, *c*-hexyl), 1.50 (d, 6H, $2CH_3$), 3.00-3.20 (m, 2H, 2CH of *c*-hexyl), 3.70 (s, 6H, 2 OCH_3), 4.00-5.30 (q, 2H, 2CH), and 6.50-7.30 (m, 8 H, 2 Ph).	M^++1 (437.24, 7.0%), M^+-1 (435.33, 0.3%), *p*-methoxybenzyl (121.08, 100%).
2g	*p*-F	0.75-2.00 (m, 8 H, *c*-hexyl), 1.45 (d, 6H, $2CH_3$), 3.15-3.70 (m, 2H, 2CH of *c*-hexyl), 4.20-5.20 (q, 4H, 2CH), and 6.85-7.15 (m, 8 H, 2 Ph-*p*-F).	M^+ (412.05, 8.0%), M^+-2 (410.85, 45.6%), $C_7H_4F^+$ (105.83, 100%).

*Compounds (2a-g) exhibited IR bands attributed to 2C=O stretching functions at 1671-1628 cm^{-1}.

Antimicrobial activity

1- Antibacterial activity

The new compounds (**2a- g**) were tested for their antibacterial activity *in vitro*, in comparison with norfloxacin as a reference drug using the standard agar cup diffusion method[33] using six bacterial species representing both Gram-positive and Gram-negative strains at the Assiut University Mycological Center (AUMC). The strains are common contaminants of the environment in KSA and some of which are involved in human and animal diseases. The used bacterial strains are *Serratia marscens* (AUMC B55), *Pseudomonas aeruginosa* (AUMC B73), and *Escherichia coli* (AUMC B53) as representatives for the Gram negative strains, while the Gram positive strains were represented by *Staphylococcus aureus* (AUMC B54), *Bacillus cereus* (AUMC B52), and *Micrococcus luteus* (AUMC B112).[34,35]

Bacterial strains were individually cultured for 48 h in 100 mL conical flasks containing 30 ml Nutrient Agar (NA) medium. Bioassay was done in 10 cm sterile plastic Petri dishes in which

One mL suspension and 15 mL of NA were poured. Plates were shaken gently to homogenize the inocula.

After solidification of the media, 5 mm cavities were cut in the solidified agar (4 cavities/plate) using sterile cork borer. The test compounds (2a-g) and chloramphenicol were dissolved in dimethyl sulphoxide (10 µmol/mL) were loaded in the cavities. In addition, other cavities were loaded with the solvent (DMSO) and served as a negative control. The seeded plates were incubated at 28±2°C for 48 h. The radii of inhibition zones (in mm) of triplicate sets were measured and the results are cited in Table 4.

Table 4: Antibacterial activity of compounds 2a-g and norfloxacin (inhibition zone in mm).

No.	Conc. µmol/mL	(G -ve)			(G +ve)		
		S. marcescens	***P. aeruginosa***	***E. coli***	***Staph. aureus***	***Bacillus cereus***	***Micrococcus luteus***
2a	10	14	10	21	18	22	24
	5	3	8	15	15	18	19
	2.5	0	2	8	10	11	15
	1.25	-	0	-	8	10	7
	0.6	-	-	-	0	2	0
2b	10	12	4	14	15	22	16
	5	8	0	4	6	12	9
	2.5	2	-	0	2	3	0
	1.25	0	-	-	0	0	-
	0.6	-	-	-	-	-	-
2c	10	18	8	22	21	28	24
	5	12	5	15	18	17	18
	2.5	5	0	4	15	10	11
	1.25	0	-	0	6	0	0
	0.6	-	-	-	0	-	-
2d	10	12	8	12	19	17	20
	5	11	6	10	15	15	18
	2.5	8	0	8	13	13	15
	1.25	0	-	0	10	12	13
	0.6	-	-	-	0	10	0
	0.3	-	-	-	-	0	-
2e	10	16	17	24	20	26	24
	5	10	13	14	14	15	18
	2.5	4	5	`10	10	10	13
	1.25	0	0	8	8	0	5
	0.6	-	-	0	0	-	0
2f	10	10	12	12	17	24	20
	5	6	6	12	15	20	18
	2.5	0	2	10	11	20	16
	1.25	-	0	6	10	16	13
	0.6	-	-	0	0	10	0

	0.3	-	-	-	-	0	-
2g	10	14	18	18	10	18	17
	5	6	12	13	10	18	13
	2.5	0	10	10	6	10	5
	1.25	-	0	0	0	10	0
	0.6	-	-	-	-	6	-
	0.3	-	-	-	-	0	-
Ref.	10	40	18	34	20	36	22
	5	40	14	30	20	34	18
	2.5	39	11	26	15	30	10
	1.25	36	10	20	15	30	6
	0.6	30	8	16	10	25	0
	0.3	26	8	14	10	20	-

Serratia marcescens (-ve) AUMC No. B-55, *Pseudomonas aeruginosa* (-ve) AUMC No. B-73, *Escherichia coli* (-ve) AUMC No. B-53, *Staphylococcus aureus* (+ve) AUMC No. B-54, *Bacillus cereus* (+ve) AUMC No B-52, *Micrococcus luteus* (+ve) AUMC No B-112, Refer. = Norfloxacin as antibacterial standard and AUMC = Assiut University Mycological Center

Antifungal activity

Compounds (**2a-g**) were tested for their antifungal activity *in vitro*, in comparison with clotrimazole as a reference drug using the standard agar cup diffusion method[33] at the Assiut University Mycological Center (AUMC), Faculty of Science, Assiut University. Six pathogenic [*T. rubrum* (Castellani) Sabouraud AUMC 1145, and *C. albicans* (Robin) Berkhout AUMC 421], phytopathogenic (*F. oxysporum* Schlechtendal AUMC 208) and food deteriorating fungal species [*A. flavus* Link AUMC 3372, *G. candidum* Link AUMC 228, and *S. brevicaulis* (Saccardo) Bainier AUMC 363] were used in the present study.[35-41]

Spore suspension in sterile distilled water was prepared from 7 days old culture of the test fungi growing on Sabouraud' dextrose broth (30 mL) media in 100 mL conical flasks. The final spore concentration was 5×10^4 spores/mL. About 15 mL of growth medium was introduced on sterilized Petri dishes of 10 cm diameter and inoculated with 1 mL of spore suspension. Plates were shaken gently to homogenize the inocula. Antifungal activity of the test compounds (**2a-g**) was performed by the standard agar cup diffusion method as follow:

After solidification of the media, 5 mm cavities were cut in the solidified agar (4 cavities/plate) using sterile cork borer and was filled with the solutions of the test compounds and clotrimazole (10 µmol/mL in DMSO). In addition, other cavities were impregnated with the solvent (DMSO) and served as a negative control. The seeded plates were incubated at 28±2°C for 7 days. The radii of inhibition zones (in mm) of triplicate sets were measured at successive intervals during the incubation period and the results are cited in Table 5.

Table 5: Antifungal activity of compounds 2a-g and clotrizmazole (inhibition zone in mm)

No.	Conc. µmol/mL	*Candida albicans*	*Geotrichum candidum*	*Fusarium oxysporum*	*Aspergillus flavus*	*Scopulariopsis brevicaulis*	*Trichophyt-on rubrum*
2a	10	14	14	15	10	14	12
	5	10	12	13	10	10	8
	2.5	8	10	10	0	4	6
	1.25	0	10	0	-	0	0
	0.6	-	0	-	-	-	-
2b	10	8	20	10	12	14	18
	5	5	14	6	5	6	12
	2.5	0	8	0	0	0	5
	1.25	-	0	-	-	-	0
2c	10	18	19	19	17	18	25
	5	12	16	10	8	10	16
	2.5	7	10	10	0	6	10
	1.25	2	0	3	-	0	0
	0.6	0	-	0	-	-	-
2d	10	16	18	10	14	8	18
	5	14	18	4	14	6	18
	2.5	10	16	0	10	0	16
	1.25	0	14	-	0	-	10
	0.6	-	10	-	-	-	0
	0.3	-	0	-	-	-	-
2e	10	18	16	10	18	12	20
	5	14	12	6	10	10	18
	2.5	10	10	2	7	3	6
	1.25	5	0	0	3	0	5
	0.6	0	-	-	0	-	0
2f	10	20	26	20	18	22	24
	5	14	22	20	15	18	22
	2.5	13	18	14	10	12	14
	1.25	10	14	12	5	10	10
	0.6	0	10	10	0	7	0
	0.3	-	0	0	-	0	-
2g	10	10	14	8	10	8	8
	5	4	10	5	4	3	3
	2.5	0	6	2	0	0	0
	1.25	-	2	0	-	-	-
	0.6	-	0	-	-	-	-
Ref.	10	30	26	24	27	28	33
	5	30	24	24	27	24	32
	2.5	28	24	24	27	23	32
	1.25	26	24	24	23	23	32
	0.6	26	24	20	23	20	32
	0.3	20	24	20	23	20	32
	0.15	20	22	17	23	17	30

Candida albicans AUMC No. 418, *Geotrichum candidum* AUMC No. 226, *Fusarium oxysporum*

AUMC No. 5119, *Aspergillus flavus* AUMC No. 1276, *Scopulariopsis brevicaulis* AUMC No. 729, *Trichophyton rubrum* AUMC No. 1804, Refer, = clotrimazole as antifungal standard and AUMC = Assiut University Mycological Center

The minimum inhibitory concentrations (MICs):

The test compounds giving positive results were diluted with DMSO to prepare a series of descending concentration down to 0.15 µmol/mL. Diluted solutions were similarly assayed as mentioned before and the least concentration (below which no activity) was recorded. The squares of inhibition zone diameters were plotted against log concentrations of the tested compounds, extrapolation of the resulting straight line to intersect with log concentration scale in the curve corresponded to log MIC, and MIC was obtained as antilog, and the results are cited in Tables 6 and 7.

Pharmacokinetics

Chemicals and reagents

Hydroquinoxaline-2,3-diones derivative (**2e**) was newly synthesized. Norfloxacin (used as an IS) was obtained from Shionogi Pharmaceutical.

Apparatus and chromatographic system

The HPLC apparatus used was composed of a Gilson pump, and a Synchroback RP (250 x 4.6 mm) column. The mobile phase composed of acetonitrile: water in the ratio of 65:35 adjusted to pH 4 with glacial acetic acid. The mobile phase was pumped at a flow rate of 1.0 mL/min. Detection was conducted using a UV-Vis Gilson detector (United State) set at 237 nm. Integration was achieved using a Hewlett Pachard detector (France) set at 10 mm/min[18]. Stock solutions of compound (**2e**) (100 µg/ml) and norfloxacin, internal standard (100 µg/ml) were prepared in the mobile phase. Additional dilutions were done in the mobile phase to produce norfloxacin working solutions (20 µg/ml).

Standard calibration curves

In a screw-capped 10ml centrifuge tubes, a 500 µl of Rabbit plasma was mixed with 100 µl of compound (**2e**) reference solution to produce concentrations of range of 0.01 to 20 µg of compound (**2e**) in 1ml of plasma and the tubes were vortex mixed for 10 s. A 100 µl of the norfloxacin stock solution (IS) was added to each tube to achieve a final norfloxacin concentration of 5 µg/ml and the tubes were vortex mixed for 10 s. Extraction of both the drug

and the internal standard was done with 5 ml chloroform by vortex mixing for 30s and centrifugation for 10 min at 5000 rpm. The lower organic layer was carefully separated by automatic pipette into clean glass tubes, evaporated to dryness under nitrogen at 45^0C in a block bath. The residue was reconstituted in 200 µl of mobile phase, filtered with Millipore filter and a 25 µl was injected into the HPLC loop. The calibration curves were obtained by plotting the peak height ratios of **2e**/NFL versus their respective concentrations of compound (**2e**)[23-24].

Validation of bio-analytical Method

The described method was validated in terms of linearity, lower limit of quantification (LLOQ), recovery, selectivity, accuracy, precision, and stability according to international guidelines regarding bio-analytical methods validation[23].

Selectivity

The selectivity of the method was demonstrated by lacking the interferences at the retention times of compound (**2e**) and norfloxacin (internal standard) due to endogenous compounds in the plasma or direct injected drugs in the mobile phase.

Lower Limit of Quantification and Linearity

The lower limit of quantification (LLOQ) was the smallest analytical concentration that could be measured with accuracy between 80 to 120% and precision lower than 20%. In the proposed method, the linearity was tested by the calibration curve ranging from 0.01 to 20 µg/mL in three replicates at each concentration.

Extraction efficiency (absolute recovery)

Absolute recoveries of compound (**2e**) and norfloxacin from plasma were determined by comparing chromatographic peak ratios of **2e**/NFL from extracted plasma samples containing norfloxacin (0.01 to 20 µg/mL) to those ratios (ciprofloxacin/ norfloxacin) obtained by direct injection of standard samples (un-extracted) prepared in the mobile phase at the entire calibration range of compound (**2e**) (0.01 to 20 µg/mL) and norfloxacin (5 µg/mL) in four replicates at each concentration.

Accuracy and Precision

Intra assay accuracy and precision evaluations were performed by repeated analysis of compound (**2e**) in Rabbit plasma at the entire calibration range of compound (**2e**) (5 µg/mL) in three replicates at each concentration. The overall precision of the method was expressed as relative standard deviation (RSD%) and the accuracy was expressed as the percentage ratio between the experimental concentration and the nominal concentration for each sample .

Stability

Long-term stability of compound (**2e**) in frozen human plasma was tested after storage at -20^{0}C for up to five months. The stability was evaluated by analyzing compound (**2e**) in spiked rabbit plasma samples over the entire calibration concentration range (0.01-20 µg/ml). Ten sets of standard curves were prepared and immediately wrapped in aluminum foil to protect them from incident light and frozen at -20°C. Compound (**2e**) concentrations of two sets of the frozen standard curves were determined monthly over a period of five months. Stability was determined by comparison with ciprofloxacin concentration in freshly prepared samples (zero h). Each value represents the mean %compound (**2e**) of three determinations.

Quantitation of compound (2e) in Rabbit:

The same extraction and chromatographic procedures described for the preparation of the calibration plots were used for the quantitation of compound (**2e**) in Rabbit samples after administration of compound **2e** (5.7 mg/kg).

Blood sampling

Venous blood samples (1 ml) for determination of compound (**2e**) were collected pre-dose (0 hours) and at 0.5, 1, 2, 3, 6 and 8 hours after drug administration. Plasma was separated immediately by centrifugation and stored at 20ºC until analysis.

Pharmacokinetic parameters

The method will be applied for determination of the pharmacokinetic parameters and the absolute bioavailability of the prepared octahydroquinozaline-2,3-dione derivative (**2e**) in rabbits after both Intravenous and oral administration. The pharmacokinetic parameters will include C_{max}, t_{max}, $t_{0.5}$, K_e, V_d, Cl_t, AUC $_{0-t}$, AUC $_{0-\infty}$ and the absolute bioavailability. In this study, norfloxacin a strong antibacterial will be utilized as a reference drug for comparing all pharmacokinetic parameters with that of the prepared octahydroquinozaline-2, 3-dione derivatives. Statistical analysis of data will be performed using ANOVA computerized system[23-24].

RESULTS AND DISCUSSION

Chemistry

Compounds (**la-g**) were prepared according to reported procedures.[16] Target compounds, 1,4-disubstituted octahydroquinoxaline-2,3-dione derivatives (**2a-g**) were prepared by fussing compounds (**1a-g**) and freshly distilled diethyl oxalate. Structures of compounds, (**2a-g**) were confirmed by IR, ^{1}H-NMR, and MS, in addition to elemental method of analyses. IR spectra of

compounds (**2a- g**) were characterized by lack of the characteristic bands due to NH functions of the intermediates (**1a-g**) and exhibited bands attributed to C=O stretching function at 1678-1622 cm^{-1}. ^{1}H-NMR spectra of compounds (**1a-g**), Table 1 exhibited a general pattern for the cyclohexyl moiety and 2NH (exchangeable with D_2O) groups which is characterized as a multiplet signal between 0.75 and 2.47 ppm. The two methine C<u>H</u>CH_3 protons appeared as a quartet equivalent to two proton around 4.06 and 4.10 ppm. The methyl protons $CHC\underline{H_3}$ appeared as doublets equivalent to 6 protons around 1.36-1.38 ppm. In addition, the protons of the specific function groups appeared in positions that are in accordance with their structures. Moreover, the aromatic protons showed patterns at positions which are in accordance to the structures of the target compounds (1a-g). ^{1}H-NMR spectra of compounds (**2a-g**), Table 3 revealed absence of signals corresponding to the exchangeable protons of the 2NH groups proved the process of cyclization. In addition, the spectra showed a general pattern for the cyclohexyl moiety as a broad multiplet signal at 0.75 and 2.15 ppm. The spectra of the target compounds (**2a-g**) showed a characteristic quartet equivalent to 2 protons of the methine protons of the C<u>H</u>CH_3 moiety at 4.20 and 5.18 ppm. Also, they showed doublets at 1.45-1.58 ppm equivalent to 6 protons corresponding to the two methyl group of $CHC\underline{H_3}$ groups. MS of compound (**2a-g**) revealed the molecular ion peaks M^+ corresponding to the molecular weight for compounds **2a, 2b**, **2c**, **2d**, **2e**, and **2g** and M^++1 for compounds **2f**.

Lipophilicity

The lipophilicity of the target compounds **2a-g** is expressed in the term of Clog P values (Table 2). The values were computed with a routine method called calculated log P (Clog P) contained in a PC-software package as mentioned under the experimental section[31] The target compounds exhibited high values for Clog P, Table 2. This work devoted to study the effect of the lipophilicity on the antimicrobial activity.

X-ray diffraction (XRD)

The X-ray diffraction (XRD) is based on observing the scattered intensity of an X-ray beam hitting a sample as a function of incident and scattered angle, polarization and wave length or energy. On the other hand, to identify unknown substance by comparing diffraction data against a data base maintained by the international center for diffraction data.[32] The latter contains no XRD data about the new compounds (**2a- g**). The X-ray diffraction spectra for compounds **2b** and **2e** are rich in bands corresponding to C, H, Br, and N.

Antimicrobial activity

Antibacterial activity

The test compounds (**2a-g**) were assayed using the standard agar cup diffusion method[33] at a concentration of 10 µmol/mL and those giving positive results were diluted with DMSO to prepare a series of descending concentrations down to 0.15 µmol/mL and were similarly assayed and the least concentration (below which no activity) was recorded as the MIC.

Results of the antibacterial activity, Table 4, indicated that at a concentration of 10 µmol/mL most of the test compounds were active against most of the used bacterial strains. The test compounds (**2a-g**) showed 25.0-45.0% of the antibacterial activity of norflonoxacin against *S. Marcescens*, 44.5-100.0% against *P. Aeruginosa*, 35.3-70.6% against *E. coli*, 50.0-100.0% against *S. aureus*, 47.3-72.3% against *B. Cereus*, and 90.9-120.0% against *M. Luteus*. Moreover the variation of the antibacterial activity with concentrations was indicated in Table 4. It was noted that the most sensitive organisms to the test compounds were *S. aureus, B. cereus, E. coli* and *M. Luteus*. It is noteworthy to mention that, compound 2c, 2e, 2f, and 2g bearing *p*-Cl, *p*-CH_3, *p*-OCH_3, and *p*-F moieties respectively were the most active ones and gave antibacterial activities up to concentration of 1.25 µmo/mL. The most active derivative of the target compounds was compound 2e bearing in its structure *p*-CH_3, moiety.

Antifungal activity

Results of the antifungal activity, Table 5 revealed that the test compounds (**2a-g**) showed variable activities against the used fungal strains in comparison to clotrimazole as a reference drug. In addition the test compounds (**2a-g**) showed 26.7-66.7% of the antifungal activity of clotrimazole against *C. albicans*, 53.9-100.0% against *G. Candidum*, 41.7-83.4% against *F. oxysporum*, 37.1-66.7% against *A. flavus*, 28.6-78.6% against *S. bervicularis*, and 36.4-75.8% against *T. rubrum*. Moreover the variation of the antifungal activity with concentrations was indicated in table 6. It was noted that, most of the tested compounds showed effective antifungal activity against the used fungi. Again, It is noteworthy to mention that, the least active antifungal compound was 2c (R = *p*-Cl) giving activity at 2.5 µmo/mL, while compounds 2d and 2f bearing *o*-Cl, *p*-OCH_3 moieties respectively gave antifungal activity at 0.6 µmo/mL. On the other hand, compound 2e (R = *p*-OCH_3) showed antifungal activity at 0.3 µmo/mL.

Table 6: Antibacterial activity (inhibition zone in mm and MICs given in brackets at 10 µmol/mL) of compounds (2a-g) and chloramphenicol.

Sample No.	***Serratia marcescens***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Staphylococcus aureus***	***Bacillus cereus***	***Micrococcus luteus***
2a	14 (2.3)	10(2.6)	21(5.0)	18(0.98)	22(0.97)	24(1.8)
2b	12(2.4)	4 (6.0)	14(5.0)	15(4.0)	22(3.22)	16(3.7)

2c	18(3.5)	8 (4.5)	22(2.4)	21(0.7)	28(2.1)	21(1.24)
2d	12(1.23)	8 (4.5)	12(1.23)	19(0.97)	17(0.98)	20(0.6)
2e	16(2.8)	17(2.9)	24(1.9)	20(0.96)	26(2.2)	24(1.22)
2f	10 (2.6)	12 (2.4)	14(0.6)	17(0.55)	24(0.29)	20(0.5)
2g	14 (2.3)	18 (3.5)	18 (5.0)	10(2.4)	18(0.29)	17(2.5)
Refer.	40 (0.04)	18(0.07)	34(0.16)	20(0.04)	36(0.006)	22(1.51)

Table 7: Antifungal activity (inhibition zone in mm and MICs given in brackets at 10 µmol/mL) of test compounds (2a-g) and clotrizmazole

Sample No.	*Candida albicans*	*Geotrichum candidum*	*Fusarium oxysporum*	*Aspergillus flavus*	*Scopulariopsis brevicaulis*	*Trichophyton rubrum*
2a	14(1.52)	14(0.6)	15(1.25)	10(2.5)	14(2.87)	12(1.25)
2b	8(5.0)	20(1.9)	10 (2.5)	12 (2.3)	14(4.0)	18(4.21)
2c	18(2.2)	19(1.5)	19(1.5)	17(2.57)	18(3.9)	25(3.86)
2d	16(1.22)	18(2.3)	10 (2.5)	14(1.25)	8 (4.5)	18(0.65)
2e	18(2.4)	16(0.15)	10 (2.5)	18(2.3)	12(2.5)	20(3.96)
2f	20(1.0)	26(0.36)	20(0.3)	18(2.5)	22(1.79)	24(0.90)
2g	10 (2.5)	14(2.5)	8 (4.5)	10 (2.5)	8 (4.5)	8 (-)
Refer.	30(0.005)	26(0.012)	24(0.005)	27(0.17)	28(0.005)	33(0.003)

Pharmacokinetics

Limit of quantitation (LOQ):

The minimum concentration of compound (**2e**) in plasma that could be detected (LOD) with a statistically acceptable RSD (%) in the peak height ratio was 0.02 compound (**2e**) per ml of plasma[18].

Linearity of the calibration plots:

The peak-height ratio, (PHR) of compound (**2e**)/NFL versus the spiked of compound (**2e**) in the range of 0.01-20 µg/ml gave excellent linear responses using simple computer program with iterative technique[23].

A typical standard plot of ciprofloxacin in plasma can be described by the equation:

$PHR = 0.032\ (Conc.)^{0.793}$ Eq.1

, $r^2>0.999$

By which compound (**2e**) plasma concentrations of dosed samples is given by:

$$Measuresd\ Conc. = \ ^{0.032}\sqrt[1]{\frac{PHR}{0.793}} \qquad \text{Eq. 2}$$

The correlation coefficient (r^2) of greater than 0.999, indicating a good fit to the least square linear regression analysis[23].

Precision and reproducibility of the assay

The precision of the assay was assessed by the acceptable variability in the peak height ratio at each concentration of inter-day and intra-day reproducibility of the calibration curves of compound (**2e**) in plasma[23].

Extraction efficiency (absolute recovery):

Absolute recovery of compound (**2e**) by the utilized method[23] was more than 95%.

The overall mean absolute recovery of the assay was shown to be 99.20 % with a standard deviation of 0.782 and overall RSD (%) of 0.788 indicating excellent extraction efficiency and reproducibility of the extraction procedure[23].

Accuracy

In the blind study, accuracy and precision were remarkably good as measured by the overall mean recovery of 99.35% with a RSD (%) of 1.39% in the concentration range of 0.01 to 20 µg/ml of ciprofloxacin in plasma[23].

Stability of compound (2e) in frozen Rabbit plasma

The % compound (**2e**) recovered always higher than 95% over the entire concentration range of ciprofloxacin indicating good stability of ciprofloxacin in plasma[23].

In vivo application of the assay to pharmacokinetics of compound (2e).

The proposed method was successfully applied for accurate measurement of compound (**2e**) in dosed Rabbit plasma samples over a period of 8 hours following single oral administration Tables (8-11) summarizes the plasma concentrations Tables (8-9) and pharmacokinetic parameters Tables (10-11)of compound (**2e**) after oral administration and IV administration of compound (**2e**) to 6 RabbitsThe mean C_{max}, t_{max} and AUC_{0-8h} were 16.50 ± 2.10 µg/ml, 2 ± 0.11h and 74.84 ± 5.11 µg h/ml respectively for compound (**2e**). The mean elimination half-life $t_{0.5e}$, absorption half-life $t_{0.5a}$, elimination rate constant k_e and absorption rate constant k_a values were 3.11 ± 0.22h, 0.60 ± 0. 1h, 0.231± 0.03 h^{-1} and 0.1.155 ± 0.13 h^{-1}, respectively (tables 8-11). The absolute bioavailability is 80.96% indicating good absorption after oral administration. The results presented here demonstrate that the method is suitable for determination the pharmacokinetics, bioavailability and bioequivalence of compound (**2e**) in plasma[23-24].

Table 8: Plasma concentrations of compound (2e) after Intravenous (*iv*) administration to rabbits.

Time (h)	Concentration (µg/ml)	
	Mean ± SD	CV%
0.5	20.19 ± 1.50	7.40
1	17.20 ± 1.11	6.45
2	13.11 ± 0.70	6.80

3	10.05 ± 0.89	8.86
6	5.13 ± 0.13	7.99
8	2.10 ± 0.11	5.23

Table 9: Plasma concentrations of compound (2e) after oral administration to rabbits.

Time (h)	Concentration (µg/ml)	
	Mean ± SD	CV%
0.5	3.11 ± 0.22	7.07
1	7.10 ± 0.59	8.30
2	16.50 ± 2.10	12.72
3	13.10 ± 1.01	7.71
6	8.12 ± 0.90	11.08
8	5.01 ± 0.23	4.59

Table 10: Pharmacokinetic parameters of compound (2e) after intravenous (*iv*) administration to rabbits.

Pharmacokinetic Parameters	Mean ± SD	CV%
C_o (µg /ml)	22.12 ± 2.10	9.49
$t_{0.5}$	2.90 ± 0.20	6.89
K_e (h^{-1})	0.238 ± 0.03	12.60
V_d (L)	0.181 ± .02	11.04
Cl_T (L/h)	0.043 ± 0.005	11.62
AUC (µg.h/ml)	92.43 ± 6.10	6.59

Table 11: Pharmacokinetic parameters of compound (2e) after oral administration to rabbits.

Pharmacokinetic Parameters	Mean ± SD	CV%
Intercept (µg/ml)	25..23 ± 2.50	9.90
C_{max}	16.50	6.99
t_{max}	2.00	5.66
$t_{0.5\ elimin}$	3.11 ± 0.22	7.07
$t_{0.5abs}$	0.60 ± 0.10	16.67
K_e (h^{-1})	0.231 ± 0.03	12.98
K_a (h^{-1})	1.155 ± 0.13	11.25
V_d (L)	0.046 ± 0.01	21.73
Cl_T (L/h)	0.011 ± 0.002	18.18
AUC (µgh/ml)	74.84 ± 5.11	6.82
FA (%)	80.96	5.66

CONCLUSION

A number of 1,4-disubstituted octahydroquinoxaline-2,3-dione derivatives were prepared through two steps reaction. This protocol involves the formation of N,N-disubstituted cyclohexane-1,2-diamine derivatives (**1a-g**) followed by cyclization with diethyl oxalate. The structures of the target compounds, 1,4-disubstituted octahydroquinoxaline-2,3-dione derivatives (**2a-g**) were elucidated depending upon different spectral data as well as the elemental methods

of analyses. In addition, mass fragmentation and X-ray diffraction analyses were carried out. Moreover, the lipophilicity of the target compounds as expressed from the Clog P and the measured R_f values were cited. The antimicrobial and MIC activities of compounds (**2a-g**) were investigated. Most of the test compounds showed weak to moderate antibacterial activity in comparison to norfloxacin as a reference drug. Also some test compounds were equiactive as clotrimazole with regards to their antifugal activities. Moreover, The pharmacokinetics of the compound 2e was determined in Rabbit plasma by after Intravenous and oral administration by a simple, sensitive and selective high-performance liquid chromatographic assay The absolute bioavailability was 80.96% indicating good absorption of compound 2e after oral administration.

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