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Synthesis and biological evaluation of new *N*-(2-hydroxy-4(or 5)-nitro/aminophenyl)benzamides and phenylacetamides as antimicrobial agents

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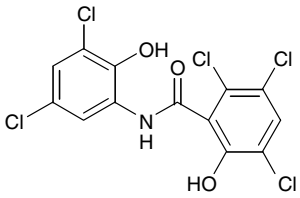
Abstract—A new series of *N*-(2-hydroxy-4(or 5)-nitro/aminophenyl)benzamide and phenylacetamide derivatives (**1a**–**1n**, **2a**–**2n**) were synthesized and evaluated for antibacterial and antifungal activities against *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, and their drug-resistant isolates. Microbiological results indicated that the compounds possessed a broad spectrum of activity against the tested microorganisms at MIC values between 500 and 1.95 μg/ml. Benzamide derivative **1d** exhibited the greatest activity with MIC values of 1.95, 3.9, and 7.8 μg/ml against drug-resistant *B. subtilis*, *B. subtilis*, and *S. aureus*, respectively.

1. Introduction

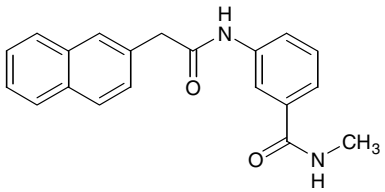
The dramatically rising prevalence of multidrug-resistant microbial infections in the past few decades has become a serious health care problem. In particular, the emergence of multi-drug resistant strains of Gram-positive bacterial pathogens such as methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermis* and vancomycin-resistant *Enterococcus* is a problem of ever-increasing significance.[1–5] In order to prevent this serious medical problem, the elaboration of the new types of the previously known drugs is a very actual task.

Benzamide derivatives exhibit various types of biological properties such as anthelmentic, antihistaminic, antifungal, and antibacterial.[6–14] 6-*N*-(2-hydroxy-3,5-dichlorophenyl)-2-hydroxy-3,5,6-trichlorobenzamide (oxyclozanide), which has a benzamide structure, was discovered in 1969 as an anthelmentic agent effective against *Fasciola hepatica* for the treatment of liver fluke infection.[6] 3,4-Dihydroxy-6-(*N*-ethylamino)benzamide is a natural product that has been found in green pepper (*Piper nigrum* L.) as an antibacterial by Pradhan et al.[11]



Oxyclozanide

Additionally, a benzamide derivative, BAS-118, has been found to be a novel anti-*Helicobacter pylori* agent with a potent and selective antibacterial activity, which includes clarithromycin (CAM)- and metronidazole (MNDZ)-resistant isolates.[15]



BAS-118

Keywords: Antibacterial activity; Antifungal activity; Benzamide; Phenylacetamide.
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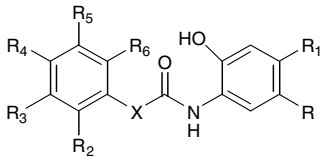
In the last years, we reported some novel microbiologically active *N*-(2-hydroxy-5-substitutedphenyl)benzamide/phenylacetamide/phenoxyacetamide/thiophenoxyacetamide derivatives as seen in Formula 1.[10,12–14] According to our previous study, synthesized compounds showed significant antimicrobial effects at MIC values between 12.5 and 200 μg/ml. It was noticeable that the compounds having a nitro group on position 5 of *N*-(2-hydroxyphenyl) moiety of benzamide or phenylacetamide were found to be more active than the other analogues for either antibacterial activities against some Gram-positive and Gram-negative bacteria or antifungal activity against *Candida albicans* among the tested structures.[14] Herein, we have described the synthesis of a series of benzamide and phenylacetamide derivatives which have a nitro group attached on position 4 or 5 of *N*-(2-hydroxyphenyl) binding them as a new class of synthetic antimicrobial agents along with their in vitro antimicrobial activity. Additionally, we also put an electron donating group such as amine instead of nitro which is an electron withdrawing group for the same position in order to be able to discuss the effect of substituent for biological activity.



X = –, CH_2, CH_2O, CH_2S
R = H, Cl, CH_3, NO_2; $\mathbf{R_1}$ = H, CH_3, NO_2; $\mathbf{R_2}$ = H, CH_3, OCH_3; $\mathbf{R_3}$ = H, OCH_3;
$\mathbf{R_4}$ = H, Cl, Br, F, CH_3, NO_2, OCH_3, $C(CH_3)_3$; $\mathbf{R_5}$ = H, OCH_3; $\mathbf{R_6}$ = H, OCH_3

Formula 1

2. Results and discussion

2.1. Chemistry

The *N*-(2-hydroxy-4(or 5)-nitro/aminophenyl)benzamides and phenylacetamides described in this paper were prepared following a general synthetic route represented in Schemes 1 and 2.

The synthesis of compounds **1a**–**1n** was performed by reacting suitable 2-aminophenols with appropriate carboxylic acid chlorides, obtained in turn by treating carboxylic acids with thionyl chloride (Scheme 1). Reduction of the nitro group of **1a**–**1n** afforded **2a**-**2n** as seen in Scheme 2. The compounds **2c**, **2d**, **2h**–**2j**, **2l**, and **2n** were obtained from **1c**, **1d**, **1h**–**1j**, **1l**, and **1n**, respectively, by using $NiCl_2{\cdot}6H_2O$ and Zn in methanol for reduction. Ten percent Pd–C was used to synthesize the other amines (**2a**, **2b**, **2e**–**2g**, **2k**, **2m**).

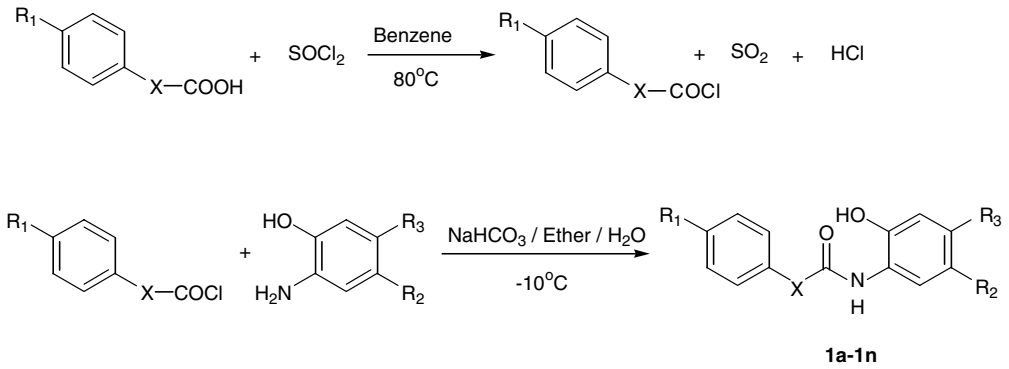
All compounds are new products except **1a**,[16] **1b**,[16,17] **1f**,[14] **1i**,[13] **1j**,[18] **2a**,[16] **2b**,[16] **2c**,[19] and **2j**.[18] The purity of the compounds was checked by TLC (Merck TLC plates Silica gel 60 F_{254}) using two types of developing solvents S1 ($CHCl_3$/MeOH 15:1 for **1a**–**1n**) and S2 ($CHCl_3$/isopropanol 8:1 for **2a**–**2n**). The plates were visualized using UV light. Melting points (uncorrected) were determined. All of the structures were supported by spectral data. The IR, ^{1}H NMR, Mass spectra, and elemental analyses are in agreement with the proposed structures. The chemical, physical, and spectral data of all the synthesized compounds **1a**–**1n** and **2a**–**2n** are reported in Tables 1 and 2, respectively.

2.2. In vitro antibacterial and antifungal activity

All the synthesized *N*-(2-hydroxy-4(or 5)-nitro/aminophenyl)benzamide and phenylacetamide derivatives (**1a**–**1n**, **2a**–**2n**) were assayed in vitro for antibacterial activity against *Klebsiella pneumoniae* RSHM 574, *Pseudomonas aeruginosa* ATCC 25853, *Escherichia coli* ATCC 25922, *K. pneumoniae* isolate (resistant to trimethoprim sulfamethoxazole, amoxicilin clavulonat, ceftriaxon, cephepim, aztreonam), *P. aeruginosa* isolate (resistant to amoxicilin clavulonat), *E. coli* isolate (resistant to trimethoprim sulfamethoxazole, cephepim, tazobactam) as Gram-negative bacteria, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *B. subtilis* isolate (resistant to ceftriaxon), *S. aureus* isolate (resistant to oxacilin, gentamycin, aztreonam, trimethoprim sulfamethoxazole) as Gram-positive bacteria, and the antifungal activity was evaluated against *C. albicans* ATCC 10231, *C. albicans* isolate. The MIC values were determined by the 2-fold serial dilution technique in Mueller–Hinton broth and Sabouraud dextrose agar for the antibacterial and antifungal assay, respectively. For comparison of the antimicrobial activity, rifampicin, ampicillin trihydrate, gentamycin sulfate, and ofloxacine were used as the reference antibacterial agents and fluconazole, amphotericin B were employed as the reference antifungal agents. All the biological results of the tested compounds are given in Table 3. The combined data reported that the synthesized compounds (**1a**–**1n**, **2a**–**2n**) showing MIC values between 500 and 1.95 μg/ml were able to inhibit the in vitro growth of the microorganisms screened.

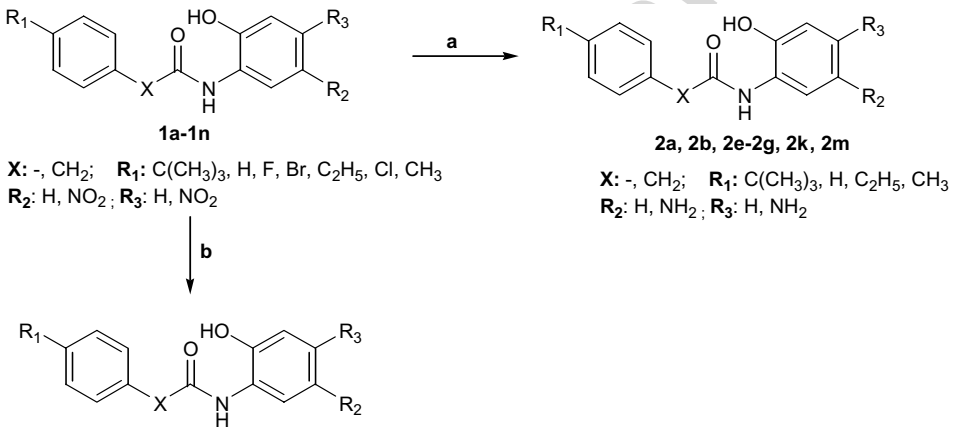
As shown in Table 3, all tested compounds exhibited moderate inhibitory effect with MIC values between 250 and 125 μg/ml against drug-resistant *K. pneumoniae* except **2g**. Derivative **2g**, 4-ethyl-*N*-(2-hydroxy-5-aminophenyl)benzamide, showed only a significant activity with a MIC value of 62.5 μg/ml even more active than tested standard drugs, rifampicin, ampicillin, and gentamycin. Changing the position of amine from 4 to 5 (see compound **2e**) caused 2-fold less potency against drug-resistant *K. pneumoniae*. Most of the derivatives indicated better activity against *K. pneumoniae* RSHM 574 than its isolate. In particular, **1h** came out with very significant activity at a MIC value of 31.25 μg/ml. Derivatives **2g** and **2h** had a good inhibitory effect as well. Additionally, all of these three compounds showed better activity than the standard drugs ampicillin and gentamycin.

Only compound **2a**, 4-*t*-butyl-*N*-(2-hydroxy-4-aminophenyl)benzamide, showed more activity than other



1a-1e; **X :** –; **R_1 :** $C(CH_3)_3$, H, F, Br, C_2H_5; **R_2 :** H; **R_3 :** NO_2
1f-1h; **X :** –; **R_1 :** H, C_2H_5, F; **R_2 :** NO_2; **R_3 :** H
1i-1l; **X :** CH_2; **R_1 :** Br, Cl, CH_3, F; **R_2 :** H; **R_3 :** NO_2
1m, 1n; **X :** CH_2; **R_1 :** CH_3, F; **R_2 :** NO_2; **R_3 :** H

Scheme 1. Synthesis of the target *N*-(2-hydroxy-4(or 5)-nitrophenyl)benzamides/phenylacetamides (**1a**–**1n**).



X: -, CH_2; **R_1:** $C(CH_3)_3$, H, F, Br, C_2H_5, Cl, CH_3
R_2: H, NO_2; **R_3**: H, NO_2

X: -, CH_2; **R_1:** $C(CH_3)_3$, H, C_2H_5, CH_3
R_2: H, NH_2; **R_3**: H, NH_2

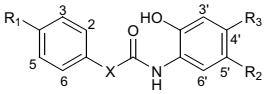
X: -, CH_2; **R_1:** F, Br, Cl
R_2: H, NH_2; **R_3**: H, NH_2

2a-2e; **X :** –; **R_1 :** $C(CH_3)_3$, H, F, Br, C_2H_5; **R_2 :** H; **R_3 :** NH_2
2f-2h; **X :** –; **R_1 :** H, C_2H_5, F; **R_2 :** NH_2; **R_3 :** H
2i-2l; **X :** CH_2; **R_1 :** Br, Cl, CH_3, F; **R_2 :** H; **R_3 :** NH_2
2m, 2n; **X :** CH_2; **R_1 :** CH_3, F; **R_2 :** NH_2; **R_3 :** H

Scheme 2. Synthesis of the target *N*-(2-hydroxy-4(or 5)-aminophenyl)benzamides/phenylacetamides (**2a**–**2n**). Reagents: (a) 10% Pd–C, H_2, EtOH; (b) $NiCl_2 \cdot 6H_2O$, Zn, MeOH.

tested compounds and gentamycin with a MIC value of 62.5 μg/ml against drug-resistant *P. aeruginosa*. Besides, five compounds **1g**, **1h**, **2a**, **2g**, **2h** were found to have inhibitory effect with the same MIC value against *P. aeruginosa.* It could be considered that attaching H-acceptor functional groups for position R_2 and hydrophobic groups such as $C(CH_3)_3$, C_2H_5 or F for position R_1 played very important role for enhancing activity against the Gram-negative enterobacter *P. aeruginosa*, which is effective in nosocomial infections and often resistant to antibiotic therapy. None of compounds indicated more activity than ampicillin neither against *P. aeruginosa* nor its isolate. Interestingly, all the tested benzamides and phenylacetamides showed very significant activity in comparison to gentamycin. While **1d**, **1n**, and **2l** showed a good inhibitory effect with a MIC value of 62.5 μg/ml against the other Gram-negative bacteria *E. coli*, none of the compounds was found to have an important activity against drug-resistant *E. coli* isolate. Structure–activity relationships revealed that compounds possessing *p*-fluoro-phenylacetamide instead of *p*-fluoro-benzamide improved the potency as

Table 1. Yields, physicochemical and spectral properties of the compounds (**1a**–**1n**)



Compound	R_1	R_2	R_3	X	Empirical formulas	Mp (°C)	Yield (%)	Elemental analyses: calculated, found	IR (cm^{-1})	^1H NMR (DMSO-d_6) δ ppm J = Hz
1a	$C(CH_3)_3$	H	NO_2	—	$C_{17}H_{18}N_2O_4$	283–284 (Ref. 15[a])	32	C, 64.96; H, 5.77; N, 8.91 C, 64.85; H, 5.63; N, 8.65	3421, 2966, 1645, 1499–1610, 1525, 1338, 610–952	1.18 (s, 9H, $C(CH_3)_3$), 6.96 (dd, 1H, J_o = 9.2 and J_m = 1.6, 6′ − *H*), 7.42 (d, 2H, J = 8.4, 3-*H*, 5-*H*), 7.77 (d, 2H, J_o = 8.0, 2-*H*, 6-*H*), 7.85 (dd, 1H, J_o = 9.2 and J_m = 2.8, 5′ − *H*), 8.66 (d, 1H, J_o = 2.8, 3′ − *H*), 9.39 (s, 1H, O*H*)
1b	H	H	NO_2	—	$C_{13}H_{10}N_2O_4$	269–270 260 (Ref. 15) 266–267 (Ref. 16)	38	C, 60.47; H, 3.90; N, 10.85 C, 60.57; H, 4.057; N, 10.88	3396, 2963, 1650, 1501–1590, 1539, 1345, 615–946	7.39–7.44 (m, 2H, 3-*H*, 5-*H*), 7.47–7.49 (m, 1H, 4-*H*), 7.59 (d, 1H, J_o = 2.8, 3′ − *H*), 7.65 (dd, 1H, J_o = 8.8 and J_m = 2.8, 5′ − *H*), 7.83 (m, 2H, 2-*H*, 6-*H*), 8.09 (d, 1H, J_o = 9.2, 6′ − *H*), 9.45 (s, 1H, O*H*)
1c	F	H	NO_2	—	$C_{13}H_9N_2O_4F$	244–245	33	C, 56.53; H, 3.28; N, 10.14 C, 56.97; H, 3.268; N, 10.08	3402, 3092, 1652, 1502–1603, 1547, 1345, 1159, 618–945	7.34–7.38 (m, 2H, 3-*H*, 5-*H*); 7.70 (d, 1H, J_m = 2.8, 3′ − *H*); 7.76 (dd, 1H, J_o = 8.8 and J_m = 2.8, 5′ − *H*); 8.01–8.05 (m, 2H, 2-*H*, 6-*H*); 8.14 (d, 1H, J_o = 8.8, 6′ − *H*); 9.65 (s, 1H, O*H*)
1d	Br	H	NO_2	—	$C_{13}H_9N_2O_4Br$	243–244	29	C, 46.31; H, 2.69; N, 8.31 C, 46.15; H, 2.706; N, 8.042	3388, 1650, 1504-1590, 1542, 1336, 651–947	7.71–7.92 (m, 6H, Ar-*H*); 8.14 (dd, 1H, J_o = 8.4 and J_m = 1.2, 5′ − *H*); 9.19 (s, 1H, O*H*)
1e	C_2H_5	H	NO_2	—	$C_{15}H_{14}N_2O_4$	246–247	44	C, 62.93; H, 4.93; N, 9.79 C, 62.40; H, 4.822; N, 9.681	3398, 2971, 1649, 1505–1591, 1539, 1347, 630–946	1.19 (t, 3H, CH_3); 2.67 (q, 2H, CH_2); 7.37 (d, 2H, J_o = 8.4, 3-*H*, 5-*H*); 7.71 (d, 1H, J_m = 2.8, 3′ − *H*); 7.77 (dd, 1H, J_o = 8.4 and J_m = 2.8, 5′ − *H*); 7.87 (d, 2H, J_o = 8.0, 2-*H*, 6-*H*); 8.22 (d, 1H, J_o = 8.4, 6′ − *H*); 9.48 (s, 1H, O*H*)
1f	H	NO_2	H	—	$C_{13}H_{10}N_2O_4$	284–286 297 (Ref. 13)	47	C, 60.47; H, 3.90; N, 10.85 C, 60.34; H, 4.237; N, 10.84	3407, 2960, 1644, 1530–1591, 1339, 614–952	7.06 (d, 1H, J_o = 8.8, 3′ − *H*), 7.51–7.62 (m, 3H, 4′ − *H*, 3-*H*, 5 -*H*), 7.94–7.97 (m, 3H, 2-*H*, 4-*H*, 6-*H*), 8.75 (d, 1H, J_o = 3.2, 6′ − *H*), 9.63 (s, 1H, O*H*)

(*continued on next page*)

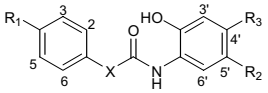
Table 1 (*continued*)

Compound	R_1	R_2	R_3	X	Empirical formulas	Mp (°C)	Yield (%)	Elemental analyses: calculated, found	IR (cm^{-1})	^1H NMR (DMSO-d_6) δ ppm J = Hz
1g	C_2H_5	NO_2	H	—	$C_{15}H_{14}N_2O_4$	254–256	46	C, 62.93; H, 4.93; N, 9.79 C, 62.58; H, 5.218; N, 9.788	3407, 2970, 1646, 1597, 1530, 1345, 631–948	1.21 (t, 3H, CH_3); 2.69 (q, 2H, CH_2); 7.08 (d, 1H, J_o = 9.2, $3' - H$); 7.38 (d, 2H, J_o = 8.4, 3-*H*, 5-*H*); 7.91 (d, 2H, J_o = 8.8, 2-*H*, 6-*H*); 7.99 (dd, 1H, J_o = 9.2 and J_m = 2.8, $4' - H$); 8.78 (d, 1H, J_m = 3.2, $6' - H$); 9.55 (s, 1H, O*H*)
1h	F	NO_2	H	—	$C_{13}H_9N_2O_4F$	263–265	35	C, 56.53; H, 3.28; N, 10.14 C, 56.97; H, 3.268; N, 10.08	3422, 1650, 1586, 1547, 1339, 1162, 615–955	7.08 (d, 1H, J_o = 8.8, $3' - H$); 7.35–7.40 (m, 2H, $3 - H$, $5 - H$); 7.99–8.07 (m, 3H, $4' - H$, $2 - H$, $6 - H$); 8.71 (d, 1H, J_m = 2.8, $6' - H$); 9.72 (s, 1H, O*H*); 11.61 (s, 1H, N*H*)
1i	Br	H	NO_2	CH_2	$C_{14}H_{11}N_2O_4Br$	234–235 220 (Ref 17)	49	C, 47.89; H, 3.16; N, 7.98 C, 47.81; H, 3.413; N, 7.987	3383, 3090, 1651, 1509–1591, 1542, 1336, 620–942	3.70 (s, 2H, CH_2), 7.16 (d, 2H, J_o = 8.4, $3 - H$, $5 - H$), 7.38 (d, 2H, J_o = 8.4, $2 - H$, $6 - H$), 7.53 (d, 1H, J_m = 2.8, $3' - H$), 7.57 (dd, 1H, J_o = 8.8 and J_m = 2.8, $5' - H$), 8.13 (d, 1H, J_o = 9.2, $6' - H$), 9.57 (s, 1H, O*H*), 10.94 (s, 1H, N*H*)
1j	Cl	H	NO_2	CH_2	$C_{14}H_{11}N_2O_4Cl$	221–223 220–221 (Ref. 18)	39	C, 54.83; H, 3.61; N, 9.13 C, 54.57; H, 3.864; N, 9.163	3343, 2922, 1663, 1504–1555, 1339, 620–939	3.72 (s, 2H, CH_2), 7.21–7.60 (m, 6H, Ar-*H*), 8.14 (d, 1H, J_o = 8.8, $6' - H$), 9.57 (s, 1H, O*H*), 10.95 (s, 1H, N*H*)

1k	CH_3	H	NO_2	CH_2	$C_{15}H_{14}N_2O_4$	232–234	60	C, 62.93; H, 4.93; N, 9.79 C, 62.78; H, 5.066; N, 9.763	3333, 1663, 1504, 1554, 1339, 620–939	2.25 (s, 3H, C*H*$_3$); 3.77 (s, 2H, C*H*$_2$); 7.1 (d, 2H, J_o = 8.0, 3 − *H*, 5 − *H*); 7.2 (d, 2H, J_o = 8.4, 2 − *H*, 6 − *H*); 7.36 (d, 1H, J_m = 2.0, 3′ − *H*); 7.68 (dd, 1H, J_o = 8.8 and J_m = 2.8, 5′ − *H*); 8.26 (d, 1H, J_o = 8.8, 6′ − *H*); 9.56 (s, 1H, O*H*); 11.06 (s, 1H, N*H*)
1l	F	H	NO_2	CH_2	$C_{14}H_{11}N_2O_4F$	226–228	33	C, 57.93; H, 3.82; N, 9.65 C, 57.68; H, 3.787; N, 9.649	3343, 3046, 2711, 1662, 1588–1625, 1555, 1339, 1224, 620–940	3.83 (s, 2H, C*H*$_2$); 7.08–7.18 (m, 2H, 3 − *H*, 5 − *H*); 7.35–7.40 (m, 2H, 2 − *H*, 6 − *H*); 7.65–7.71 (m, 2H, 3′ − *H*, 5′ − *H*); 8.26 (d, 1H, J_o = 8.8, 6′ − *H*); 9.67 (s, 1H, O*H*); 11.09 (s, 1H, N*H*)
1m	CH_3	NO_2	H	CH_2	$C_{15}H_{14}N_2O_4$	271–273	59	C, 62.93; H, 4.93; N, 9.79 C, 62.44; H, 4.759; N, 9.708	3464, 3332, 2709, 1662, 1506–1590, 1555, 1339, 630–946	2.26 (s, 3H, C*H*$_3$); 3.74 (s, 2H, CH_2); 7.05 (d, 1H, J_o = 9.2, 3′ − *H*); 7.17 (d, 2H, J_o = 8.0, 3 − *H*, 5 − *H*); 7.22 (d, 2H, J_o = 7.6, 2 − *H*, 6 − *H*); 7.86 (d, 1H, J_o = 9.2 and J_m = 2.8, 4′ − *H*); 8.93 (d, 1H, J_m = 2.4, 6′ − *H*); 9.54 (s, 1H, O*H*); 11.65 (s, 1H, N*H*)
1n	F	NO_2	H	CH_2	$C_{14}H_{11}N_2O_4F$	253–255	49	C, 57.93; H, 3.82; N, 9.65 C, 57.69; H, 3.692; N, 9.628	3359, 2958, 1655, 1509–1590, 1549, 1332, 1223, 638–957	3.79 (s, 2H, C*H*$_2$); 7.01 (d, 1H, J_o = 8.8, 3′ − *H*); 7.12–7.17 (m, 2H, 3 − *H*, 5 − *H*); 7.35–7.39 (m, 2H, 2 − *H*, 6 − *H*); 7.88 (dd, 1H, J_o = 8.8 and J_m = 2.8, 4′ − *H*); 8.92 (d, 1H, J_m = 2.8, 6′ − *H*); 9.65 (s, 1H, O*H*); 11.68 (s, 1H, N*H*)

[a] Melting point was not shown in the literature.

Table 2. Yields, physicochemical and spectral properties of the compounds (**2a**–**2n**)



Compound	R_1	R_2	R_3	X	Empirical formulas	Mp (°C)	Yield (%)	MS (ESI+) *m/z* (%X)	IR (cm^{-1})	^1H NMR (DMSO-d_6) δ ppm J = Hz
2a	$C(CH_3)_3$	H	NH_2	—	$C_{17}H_{20}N_2O_2$	162–164 (Ref. 15[a])	57	285.21 (100)	3413, 3257, 2953, 1650, 1512–1541, 603–946	1.31 (s, 9H, $C(CH_3)_3$), 4.59 (s, 2H, NH_2), 6.33 (dd, 1H, J_o = 8.8 and J_m = 2.4, 5′ − *H*), 6.64 (dd, 1H, J_o = 8.8 and J_m = 1.6, 6′ − *H*), 7.04 (d, 1H, J_m = 2.8, 3′ − *H*), 7.53 (d, 2H, J_o = 8.4, 3 − *H*, 5 − *H*), 7.83 (d, 2H, J_o = 8.4, 2 − *H*, 6 − *H*), 8.63 (s, 1H, O*H*), 9.39 (s, 1H, N*H*)
2b	H	H	NH_2	—	$C_{13}H_{12}N_2O_2$	129–131 130 (Ref. 15)	80	229.18 (100)	3364, 3301, 1649, 1516, 1092, 623-969	5.14 (s, 2H, NH_2), 5.95 (dd, 1H, J_o = 8.4 and J_m = 2.0, 5′ − *H*), 6.06 (d, 1H, J_m = 2.0, 3′ − *H*), 6.98 (d, 1H, J_o = 8.4, 6′ − *H*), 7.34–7.42 (m, 3H, 2 − *H*, 4 − *H*, 6 − *H*), 7.81 (d, 2H, J_o = 7.2, 3 − *H*, 5 − *H*), 9.14 (s, 1H, O*H*), 9.29 (s, 1H, N*H*)
2c	F	H	NH_2	—	$C_{13}H_{11}N_2O_2F$	218–220 (Ref. 19[a])	91	247.14 (100)	3565, 3252, 1647, 1547, 1160, 616–962	4.96 (s, 2H, NH_2), 6.02 (d, 1H, J_o = 7.2, 5′ − *H*), 6.13 (s, 1H, 3′ − *H*); 7.01 (d, 1H, J_o = 8.0, 6′ − *H*), 7.27–7.98 (m, 4H, 2 − *H*, 3 − *H*, 5 − *H*, 6 − *H*), 9.15 (s, 1H, O*H*), 9.42 (s, 1H, N*H*)
2d	Br	H	NH_2	—	$C_{13}H_{11}N_2O_2Br$	218–220	33	307.05 (100), 309.05 (95)	3408, 3253–3208, 1652, 1542–1607, 1092, 618–963	4.98 (s, 2H, NH_2), 6.05 (d, 1H, J_o = 8.4, 5′ − *H*), 6.16 (s, 1H, 3′ − *H*), 7.06 (d, 1H, J_o = 8.4, 6′ − *H*), 7.71 (d, 2H, J_o = 8.0, 3 − *H*, 5 − *H*), 7.89 (d, 2H, J_o = 8.4, 2 − *H*, 6 − *H*), 9.18 (s, 1H, O*H*), 9.48 (s, 1H, N*H*)
2e	C_2H_5	H	NH_2	—	$C_{15}H_{16}N_2O_2$	159–160	28	257.2 (100)	3366, 3303, 1646, 1503–1646, 623–968	1.17–1.22 (m, 3H, CH_3), 2.67 (q, 2H, CH_2), 6.05 (dd, 1H, J_o = 8.8 and J_m = 2.0, 5′ − *H*), 6.16 (d, 1H, J_m = 2.0, 3′ − *H*), 7.19 (d, 1H, J_o = 8.4, 6′ − *H*), 7.3 (d, 2H, J_o = 8.0, 3 − *H*, 5 − *H*), 7.9 (d, 2H, J_o = 8.0, 2 − *H*, 6 − *H*), 9.25 (s, 1H, N*H*); 9.40 (s, 1H, O*H*)

2f	H	NH_2	H	—	$C_{13}H_{12}N_2O_2$	228–230	45	229 (100)	3245, 3185–3126, 1653, 1513–1608, 620–868	4.73 (s, 2H, NH_2), 6.32 (dd, 1H, J_o = 8.4 and J_m = 2.8, 4′ − H), 6.64 (d, 1H, J_o = 8.0, 3′ − H), 7.05 (d, 1H, J_m = 2.8, 6′ − H), 7.52–7.98 (m, 5H, Ar-H), 8.67 (s, 1H, OH) 9.47 (s, 1H, NH)
2g	C_2H_5	NH_2	H	—	$C_{15}H_{16}N_2O_2$	177–179	47	257.21 (100)	3431, 3387–3348, 2958, 1645, 1513–1609, 611–940	1.20 (t, 3H, CH_3), 2.68 (q, 2H, CH_2), 4.59 (s, 2H, NH_2), 6.29 (dd, 1H, J_o = 8.4 and J_m = 2.4, 4′ − H), 6.63 (d, 1H, J_o = 8.4, 3′ − H), 7.03 (d, 1H, J_m = 2.8, 6′ − H), 7.36 (d, 2H, J_o = 8.0, 3 − H, 5 − H), 7.87 (d, 2H, J_o = 8.4, 2 − H, 6 − H), 8.63 (s, 1H, OH), 9.40 (s, 1H, NH)
2h	F	NH_2	H	—	$C_{13}H_{11}N_2O_2F$	173–175	81	247.16 (100)	3378, 3249, 1634, 1531–1594, 1181, 612–935	4.86 (s, 2H, NH_2), 6.33 (dd, 1H, J_o = 8,4 and J_m = 2.4, 4′ − H), 6.63 (d, 1H, J_o = 8.4, 3′ − H), 6.99 (d, 1H, J_m = 2.4, 6′ − H), 7.34 (m, 2H, 3 − H, 5 − H), 8.01 (d, 2H, 2 − H, 6 − H), 8.63 (s, 1H, OH), 9.47 (s, 1H, NH)
2i	Br	H	NH_2	CH_2	$C_{14}H_{13}N_2O_2Br$	243–245	44	321.03 (100), 323.03 (98)	3243, 3186–3126, 1657, 1532–1610, 1072, 667–841	3.62 (s, 2H, CH_2), 4.88 (s, 2H, NH_2), 5.98 (d, 1H, J_o = 8.0, 5′ − H), 6.09 (s, 1H, 3′ − H), 7.10 (d, 1H, J_o = 8.4, 6′ − H), 7.28 (dd, 2H, J_o = 7,2 and J_m = 2.0, 3 − H, 5 − H), 7.51 (dd, 2H, J_o = 8.0 and J_m = 2.0, 2 − H, 6 − H), 9.24 (s, 1H, OH), 9.30 (s, 1H, NH)
2j	Cl	H	NH_2	CH_2	$C_{14}H_{13}N_2O_2Cl$	238–240 155–157 (Ref. 18)	60	277.10 (100)	3242, 3185–3126, 1658, 1532–1610, 1094, 682–865	3.49 (s, 2H, CH_2), 4.72 (s, 2H, NH_2), 5.83 (dd, 1H, J_o = 8.4 and J_m = 2.0, 5′ − H), 5.95 (d, 1H, J_m = 1.6, 3′ − H), 6.96 (d, 1H, J_o = 8.8, 6′ − H), 7.21 (m, 4H, Ar-H), 9.08 (s, 1H, OH), 9.13 (s, 1H, NH)
2k	CH_3	H	NH_2	CH_2	$C_{15}H_{16}N_2O_2$	143–145	22	257.20 (100)	3409, 3249, 1651, 1507–1607, 619–962	2.25 (s, 3H, CH_3), 3.55 (s, 2H, CH_2), 4.83 (s, 2H, NH_2), 5.95 (dd, 1H, J_o = 8.8 and J_m = 2.4, 5′ − H), 6.05 (d, 1H, J_m = 2.4, 3′ − H), 7.06–7.19 (m, 4H, Ar-H), 9.17 (s, 1H, OH), 9.28 (s, 1H, NH)

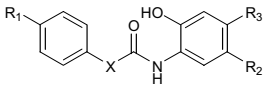
(*continued on next page*)

Table 2 (*continued*)

Compound	R_1	R_2	R_3	X	Empirical formulas	Mp (°C)	Yield (%)	MS (ESI+) *m/z* (%X)	IR (cm^{-1})	^1H NMR (DMSO-d_6) δ ppm J = Hz
2l	F	H	NH_2	CH_2	$C_{14}H_{13}N_2O_2F$	214–216	40	261.18 (100)	3244, 3185–3126, 1653, 1513–1608, 1233, 619–958	3.62 (s, 2H, CH_2), 4.88 (s, 2H, NH_2), 5.98 (dd, 1H, J_o = 8.4 and J_m = 2.4, 5′ − *H*), 6.01 (d, 1H, J_m = 2.4, 3′ − *H*), 7.10–7.17 (m, 3H, 3 − *H*, 5 − *H*, 6′ − *H*), 7.33–7.38 (m, 2H, 2 − *H*, 6 − *H*), 9.23 (s, 1H, O*H*), 9.31 (s, 1H, N*H*)
2m	CH_3	NH_2	H	CH_2	$C_{15}H_{16}N_2O_2$	284–285	89	257.19 (100)	3376, 1651, 1530–1595, 636–936	2.26 (s, 3H, CH_3), 3.63 (s, 2H, CH_2), 4.51 (s, 2H, NH_2), 6.18 (dd, 1H, 4′ − *H*), 6.38 (d, 1H, J_o = 8.0, 3′ − *H*), 7.01 (d, 1H, 6′ − *H*), 7.11 (d, 2H, J_o = 7.6, 3 − *H*, 5 − *H*), 7.21 (d, 2H, J_o = 8.0, 2 − *H*, 6 − *H*), 8.62 (s, 1H, O*H*), 9.19 (s, 1H, N*H*)
2n	F	NH_2	H	CH_2	$C_{14}H_{13}N_2O_2F$	234–235	46	261.16 (100)	3302, 3250–3201, 1629, 1509–1539, 1237	3.71 (s, 2H, CH_2), 4.53 (s, 2H, NH_2), 6.20 (d, 1H, J_o = 7.6, 4′ − *H*), 6.55 (d, 1H, J_o = 8.0, 3′ − *H*), 7.01 (s, 1H, 6′ − *H*), 7.17 (m, 2H, 3 − *H*, 5 − *H*), 7.38 (m, 2H, 2 − *H*, 6 − *H*), 8.67 (s, 1H, O*H*), 9.26 (s, 1H, N*H*)

[a] Melting point was not shown in the literature.

Table 3. The antimicrobial and antimicotic activity of the synthesized compounds (**1a**–**1n**, **2a**–**2n**) and the control drugs (MIC in μg/ml)



Compound	R_1	R_2	R_3	X	A	B	C	G	H	I	E	F	K	L	M	N
1a	$C(CH_3)_3$	H	NO_2	—	250	250	250	125	125	125	15.6	1.95	7.8	7.8	250	125
1b	H	H	NO_2	—	250	250	250	125	125	125	31.25	125	31.25	62.5	62.5	125
1c	F	H	NO_2	—	250	250	250	125	125	125	15.6	15.6	3.9	7.8	250	250
1d	Br	H	NO_2	—	125	250	250	125	250	62.5	1.95	500	3.9	7.8	250	125
1e	C_2H_5	H	NO_2	—	125	250	125	500	250	125	15.6	125	250	62.5	125	125
1f	H	NO_2	H	—	250	250	250	125	125	125	62.5	15.6	7.8	31.25	125	250
1g	C_2H_5	NO_2	H	—	125	250	250	250	62.5	125	31.25	7.8	3.9	7.8	125	62.5
1h	F	NO_2	H	—	125	125	125	31.25	62.5	125	62.5	15.6	3.9	7.8	62.5	62.5
1i	Br	H	NO_2	CH_2	125	250	250	250	250	125	125	125	31.25	250	250	250
1j	Cl	H	NO_2	CH_2	125	250	125	125	125	125	31.25	125	250	125	125	125
1k	CH_3	H	NO_2	CH_2	250	250	250	125	250	125	250	500	125	250	62.5	250
1l	F	H	NO_2	CH_2	125	250	250	250	125	125	31.25	62.5	250	125	125	125
1m	CH_3	NO_2	H	CH_2	250	250	250	125	125	125	62.5	125	15.6	250	250	250
1n	F	NO_2	H	CH_2	125	250	250	125	250	62.5	31.25	31.25	15.6	31.25	250	125
2a	$C(CH_3)_3$	H	NH_2	—	125	62.5	125	125	62.5	125	7.8	7.8	15.6	15.6	62.5	62.5
2b	H	H	NH_2	—	250	250	125	125	125	125	62.5	15.6	62.5	125	62.5	62.5
2c	F	H	NH_2	—	250	250	250	125	125	250	125	15.6	125	62.5	125	125
2d	Br	H	NH_2	—	250	250	250	250	250	125	31.25	7.8	62.5	125	250	125
2e	C_2H_5	H	NH_2	—	250	250	250	125	250	125	31.25	7.8	250	125	125	125
2f	H	NH_2	H	—	250	250	250	125	125	125	62.5	31.25	15.6	31.25	125	125
2g	C_2H_5	NH_2	H	—	62.5	125	125	62.5	62.5	125	31.25	15.6	62.5	15.6	62.5	62.5
2h	F	NH_2	H	—	125	125	125	62.5	62.5	125	62.5	15.6	62.5	15.6	62.5	62.5
2i	Br	H	NH_2	CH_2	125	250	125	125	250	125	125	31.25	125	125	125	250
2j	Cl	H	NH_2	CH_2	250	125	125	125	125	125	62.5	31.25	250	62.5	125	125
2k	CH_3	H	NH_2	CH_2	250	250	250	125	125	125	62.5	62.5	62.5	250	125	250
2l	F	H	NH_2	CH_2	125	125	125	125	125	62.5	62.5	62.5	250	125	125	125
2m	CH_3	NH_2	H	CH_2	250	250	250	125	125	125	62.5	62.5	15.6	31.25	125	125
2n	F	NH_2	H	CH_2	250	250	250	125	250	125	62.5	31.25	15.6	62.5	250	125
Rifampicin					256	32	62.5	16	32	>4096	256	512	256	>4096	—	—
Ampicillin trihydrate					256	2	500	1024	4	>4096	8	>4096	16	64	—	—
Gentamycin sulfate					1024	1024	0.97	>4096	512	1024	2048	1024	512	>4096	—	—
Ofloxacin					64	64	32	64	8	2048	32	>4096	>4096	>4096	—	—
Fluconazol					—	—	—	—	—	—	—	—	—	—	4	512
Amphotericin B					—		—	—	—	—	—	—	—	—	512	128

A, *Klebsiella pneumoniae* isolate; B, *Pseudomonas aeruginosa* isolate; C, *E. coli* isolate; E, *Bacillus subtilis* isolate; F, *Staphylococcus aureus* isolate; G, *K. pneumoniae* RSHM 574; H, *P. aeruginosa* ATCC 25853; I, *E. coli* ATCC 25922; K, *B. subtilis* ATCC 6633; L, *S. aureus* ATCC 25923; M, *Candida albicans* ATCC 10231; N, *C. albicans* isolate.

onefold (compounds **1n** and **2l**). Besides, all of the compounds showed more activity than standard drug, ofloxacin.

The newly synthesized compounds showed more potent antibacterial activity against Gram-positive bacteria than Gram-negative ones. Among the tested series, 4-bromo-*N*-(2-hydroxy-4-nitrophenyl)benzamide **1d** was found to be the most potent derivative with a MIC value of 1.95 μg/ml against drug-resistant *B. subtilis* providing higher potencies than the compared standard drugs. When we generally glanced at all of the value for this bacterium it should be pointed out that benzamide structure played a noticeable role for increasing the activity. When compared to the effect of nitro and amine group for this activity, it can be concluded that compounds including a nitro group on the phenolic ring slightly enhanced the activity. Furthermore, it was noticeable that the number of active derivatives against *B. subtilis* ATCC 6633 had increased. All the tested compounds were found to be more active than the standard drugs, rifampicin and gentamycin, against either *B. subtilis* or its isolate. While **1d** was the most potent derivative for drug-resistant *B. subtilis*, it showed very weak activity against drug-resistant *S. aureus*. For this time, compound **1a** indicated a very good inhibitory effect with a MIC value of 1.95 μg/ml. Besides, the derivatives **1g**, **2a**, **2d**, and **2e** exhibited significant antibacterial activity with MIC values of 7.8 μg/ml. Additionally, all of the compounds except **1d** and **1k** had more inhibitory effect than all the tested standard drugs. Most of the derivatives having benzamide groups were found to be significantly active with MIC value between 62.5 and 7.8 μg/ml against *S. aureus* ATCC 25923. We could point out that benzamide structure instead of phenylacetamide in these series improved the potency. Moreover, all of the compounds displayed more potent antibacterial activity against the same bacterium than standard drugs, rifampicin, gentamycin, and ofloxacin. While compounds **1a**, **1c**, **1d**, **1f**, **1g**, **1h**, **1n**, **2a**, **2f**, **2g**, **2h**, and **2m** were found to be more active than all standard drugs, **1b**, **1e**, **2c**, **2j**, and **2n** displayed an antibacterial activity against *S. aureus* comparable to that of ampicillin.

On the other hand, the SAR results against *C. albicans* and its isolate revealed that the benzamide moiety exhibited slightly better activity than the phenylacetamide. As seen in Table 3, even if all tested compounds had a moderate activity against *C. albicans* ATCC 10231, they showed more potent activity than standard drug, amphotericin B. Besides, all compounds exhibited more antifungal activity against *C. albicans* isolate than fluconazole.

3. Conclusion

In conclusion, we have discovered a novel series of benzamide and phenylacetamide antimicrobial agents. According to this study, we could point out that the benzamide structure played a very important role for increasing in vitro antibacterial activity against Gram-positive bacteria. Although the alternates of the substituents such as nitro or amine attached at phenolic moiety made no important difference for the antimicrobial activity, placing of them at position R_2 and R_3 was found to be important for enhancing the potency against *P. aeruginosa*, and drug-resistant *B. subtilis*, respectively. In particular, compound **1d** having benzamide group exhibited the greatest activity with MIC values of 1.95, 3.9, and 7.8 μg/ml against drug-resistant *B. subtilis*, *B. subtilis*, and *S. aureus*, respectively. Additionally, the result against *B. subtilis* for **1a** also is quite encouraging. These observations provide some predictions in order to design further antimicrobial active compounds prior to their synthesis followed by QSAR and molecular modeling studies.

4. Experimental

The chemicals were purchased from the commercial venders and were used without purification. The reactions were monitored and the purity of the products was checked by thin layer chromatography (TLC). Silica gel 60 F_{254} chromatoplates were used for TLC. The solvent systems were chloroform/methanol (15:1) for **1a**–**1n**, chloroform/isopropanol (8:1) for **2a**–**2n**. Final compounds were purified by recrystallization using appropriate solvents as given in Sections 4.1 and 4.1. All the melting points were measured with a capillary melting point apparatus (Buchi SMP 20 and Electrothermal 9100) and are uncorrected. Yields were calculated after recrystallization. The IR spectra were recorded on a Jasco FT/IR-420 spectrometer with KBr disks. Compounds **1a**–**1i**, **2a**–**2h**, **2k**–**2n**: 1629–1653 cm^{-1} (C=O amide I); **1j**–**1n**, **2i**, **2j**: 1655–1663 cm^{-1} (C=O amide I). The ^1H NMR spectra were recorded employing a VARIAN Mercury 400 MHz FT spectrometer, chemical shifts (δ) are in ppm relative to TMS, and coupling constants (J) are reported in Hertz. Mass spectra for compounds **2a**–**2n** were taken on a Waters Micromass ZQ by using ESI (+) method. Elemental analyses of compounds **1a**–**1n**, most of which were not ionized on Waters Micromass ZQ, were taken on a Leco 932 CHNS-O analyzer. The results of the elemental analyses (C, H, N) were within ±0.4% of the calculated amounts.

4.1. General procedure for synthesis of *N*-(2-hydroxy-4(or 5)-nitrophenyl)benzamides/phenylacetamides (1a–1n)

Thionyl chloride (1.5 ml) and appropriate carboxylic acid (0.5 mmol) were refluxed in benzene (5 ml) at 80 °C for 3 h, and then excess thionyl chloride was removed in vacuo. The residue was dissolved in ether (10 ml) and the solution added during 1 h to a stirred, ice-cold mixture of appropriate *o*-aminophenol (0.5 mmol), sodiumbicarbonate (0.5 mmol), diethyl ether (10 ml), and water (10 ml). The mixture was stirred overnight at room temperature and filtered. After the precipitate was washed with water, 2 N HCl and water, respectively, and finally with ether, **1a**–**1n** were obtained (Scheme 1). The crude product was purified by recrystallization from ethanol. The crystals were dried in vacuo.

4.2. General procedure for the synthesis of *N*-(2-hydroxy-4(or 5)-aminophenyl)benzamides/phenylacetamides (2a–2n)

Compounds **2c**, **2d**, **2h**–**2j**, **2l**, and **2n** were synthesized from **1c**, **1d**, **1h**–**1j**, **1l**, and **1n**, respectively, which

(5 mmol) were treated with $NiCl_2 \cdot 6H_2O$ (15 mmol) and Zn (40 mmol) in methanol (25 ml) refluxing the mixture at 60 °C for 4 h. The precipitate was filtered. The crude product was purified by recrystallization from methanol. The crystals were dried in vacuo (Scheme 2).

Compounds **1a**, **1b**, **1e**–**1g**, **1k**, **1m** (5 mmol) in ethanol (50 ml) were reduced by hydrogenation using 40 psi of H_2 and 10% Pd–C (40 mg) until cessation of H_2 uptake to obtain compounds **2a**, **2b**, **2e**–**2g**, **2k**, **2m**, respectively. The catalyst was filtered on a bed of Celite, washed with ethanol, and the filtrate was concentrated in vacuo. The crude product was purified by recrystallization from ethanol. The crystals were dried in vacuo.

4.3. Microbiology

4.3.1. Materials. Mueller–Hinton Agar (MHA) (Merck), Mueller–Hinton Broth (MHB) (Merck), Sabouraud Dextrose Agar (SDA) (Merck), RPMI-1640 medium with L-glutamine (Sigma), 3-[*N*-morpholino]-propansulfonic acid (MOPS) (Sigma), 96-well microplates (Falcon®),Transfer pipette (Biohit) Rifampicin (Sifar Ilac Sanayii), Ampicillin trihydrate (Paninkret Chem. Pharm.), Gentamycin sulfate (Deva Ilac Sanayii), Ofloxacin (Zhejiang Huangyan East Asia Chemical CO.), Fluconazole (Nobel), Amphotericin B (Bristol Myers Squibb), Ethanol (Riedel de Haen®), Dimethylsulfoxide (DMSO) (Riedel de Haen®), Dimethylformamide (Riedel de Haen®).

Microorganisms. Klebsiella pneumoniae isolate (Resistant to Trimethoprim sulfamethoxazole, Amoxicilin clavulonat, Ceftriaxon, Cephepim, Aztreonam), *Pseudomonas aeruginosa* isolate (Resistant to Amoxicilin clavulonat), *E.coli* isolate (Resistant to Trimethoprim sulfamethoxazole, Cephepim, Tazobactam), *Bacillus subtilis* isolate (Resistant to Ceftriaxon), *Staphylococcus aureus* isolate (Resistant to Oxacilin, Gentamycin, Aztreonam, Trimethoprim sulfamethoxazole), *C. albicans* isolate (Biofilm positive), *K. pneumoniae* RSHM 574 (Refik Saydam Hıfzısıhha Merkezi Culture Collection), *P. aeruginosa* ATCC 25853 (American Type Culture Collection), *E. coli* ATCC 25922, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *C. albicans* ATCC 10231.

4.3.2. Method. Standard strains of *K. pneumoniae* RSHM 574, *P. aeruginosa* ATCC 25853, *E. coli* ATCC 25922, *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *C. albicans* ATCC 10231, and clinical isolates of these microorganisms that are known to be resistant to various antimicrobial agents were included in the study. Resistance was determined by Kirby Bauer Disk Diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI)[20] in the clinical isolates.

Standard powders of rifampicin, ampicillin trihydrate, gentamycin sulfate, ofloxacin, fluconazole, and amphotericin B were obtained from the manufacturers. Stock solutions were dissolved in dimethylsulfoxide (ofloxacin), methanol (rifampicin), pH 8 phosphate-buffered saline (PBS) (ampicillin trihydrate), and distilled water (gentamycin sulfate, fluconazole, and amphotericin B).

All bacterial isolates were subcultured in MHA plates and incubated overnight at 37 °C and all *Candida* isolates were subcultured in SDA plates at 35 °C for 24–48 h. The microorganisms were passaged at least twice to ensure purity and viability.

The solution of the newly synthesized compounds (**1a**–**1n**, **2a**–**2n**) and standard drugs was prepared at 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8, 3.9, 1.95, 0.98 μg/ml concentrations, at 4096, 2048, 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 μg/ml concentrations in the wells of microplates by diluting in MHB, respectively.

Bacterial susceptibility testing was performed according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) M100-S16.[21] The bacterial suspensions used for inoculation were prepared at 10^5 cfu/ml by diluting fresh cultures at MacFarland 0.5 density (10^7 cfu/ml). Suspensions of the bacteria at 10^5 cfu/ml concentration were inoculated to the 2-fold diluted solution of the compounds. There were 10^4 cfu/ml bacteria in the wells after inoculations. MHB was used for diluting the bacterial suspension and for 2-fold dilution of the compound. 80% DMSO, 20% EtOH, methanol, DMSO, PBS, pure microorganisms, and pure media were used as control wells. A 10 μl bacteria inoculum was added to each well of the microdilution trays. The trays were incubated at 37 °C in a humid chamber and MIC endpoints were read after 24 h of incubation. All organisms were tested in triplicate in each run of the experiments. The lowest concentration of the compound that completely inhibits macroscopic growth was determined and minimum inhibitory concentrations (MICs) were reported.

All *Candida* isolates were subcultured in SDA plates, incubated at 35 °C for 24–48 h prior to antifungal susceptibility testing, and passaged at least twice to ensure purity and viability. Susceptibility testing was performed in RPMI-1640 medium with L-glutamine buffered, pH 7, with MOPS and culture suspensions were prepared through the guideline of CLSI M27-A.[22] The yeast suspensions used for inoculation were prepared at 10^4 cfu/ml by diluting fresh cultures at MacFarland 0.5 density (10^6 cfu/ml). Suspensions of the yeast at 10^4 cfu/ml concentration were inoculated to the 2-fold diluted solution of the compounds. There were 10^3 cfu/ml bacteria in the wells after inoculations. A 10 μl yeast inoculum was added to each well of the microdilution trays. The trays were incubated at 35 °C in a humid chamber and MIC endpoints were read after 48 h of incubation. All organisms were tested in triplicate in each run of the experiments. The lowest concentration of the compound that completely inhibits macroscopic growth was determined and minimum inhibitory concentrations (MICs) was reported.

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