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Original article

Synthesis and antimicrobial activities of hexahydroimidazo[1,5-*a*]pyridinium bromides with varying benzyl substituents

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ABSTRACT

Variously substituted benzyl bromides were employed to quaternize hexahydrobenzylimidazo[1,5-*a*]pyridine (**A**) and the resulting bromides (**1**–**11**) were evaluated for their *in vitro* antimicrobial activity against 10 pathogenic microorganisms: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Micrococcus luteus*, *Proteus vulgaris*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumonia*, *Candida albicans* and *Candida krusei*. Antimicrobial activities were surprisingly high (MIC: 0.78–400 μg/mL) and the sensitivity of the salts tested has been found to depend strongly both on the benzyl substituents and the microorganisms used. However, the correlation observed between antimicrobial activity and calculated partition coefficient (Clog*P*) was poor. Acute toxicity assessment of these salts showed LD_{50} of 757–2000 mg/kg, after oral administration in mice in 24 h.

1. Introduction

The chemistry of imidazole derivatives has attracted more attention during recent years due to their reactivity and biological activities. They have been shown to possess antihelmintic, antifungal, antibacterial and anticancer activities [1]. Besides their biological actions, diazol(in)ium salts find wide applications in the preparation of *N*-heterocyclic carbenes (NHCs) and their metal complexes [2–10], some of which have attracted important attention as potential pharmaceuticals [7]. Additionally, diazol(in)ium salts, as potential antiseptics/disinfectants, have been the research subject of several groups [11–30]. Our previous work in this area has concerned the antimicrobial activity of substituted imidazolinium, benzimidazolium and tetrahydropyrimidinium salts and their metal complexes [31–35]. Studies have shown that annulations at various positions of the imidazole scaffolds significantly influence the stability of the carbene species [36]. Therefore, it can be envisioned that such fusion(s) may be used as a tool for tuning their biological properties. For example, 1-*p*-chlorophenylhexahydroimidazo[1,5-*a*]pyridines, containing fused piperidine moiety on the N^1–C^5 atoms of the imidazoline (ring **A**) are hypotensive agents [37].

The most commonly used antibacterial reagents, benzalkonium chloride and cetyl chloride, contain benzyl and pyridine moieties, respectively. On the other hand, more efficacious compounds can be designed by joining two or more biologically active systems together in a single molecular framework. In view of the above mentioned facts and in continuation of our interest in the synthesis of imidazoline moiety [31–35], we synthesized and evaluated the antimicrobial activity of benzyl substituted hexahydroimidazo[1,5-*a*]pyridinium bromides (**1**–**11**). It has been hoped that combination of these active groups in the new molecular design would lead to better antimicrobial agents with less toxic properties.

2. Results and discussion

2.1. Chemistry

The target salts (**1**–**11**) shown in Scheme 1 have been obtained by quaternization of 1,5,6,7,8,8a-hexahydroimidazo[1,5-*a*]pyridine (**A**), by variously substituted benzyl bromides. 2-Benzyl-1,5,6,7,8,8a-hexahydroimidazo[1,5-*a*]pyridin-2-ium bromide (**1**), 2-(2,4,6-tetramethylbenzyl)-1,5,6,7,8,8a-hexahydroimidazo[1,5-*a*]pyridin-2-ium bromide (**4**), 2-(2,3,5,6-tetramethylbenzyl)-1,5,6,7,8,8a-hexahydroimidazo[1,5-*a*]pyridin-2-ium bromide (**5**), 2-(pentamethylbenzyl)-1,5,6,7,8,8a-hexahydroimidazo[1,5-*a*]pyridin-2-ium bromide (**6**) are known in the literature [38,39]. The other salts

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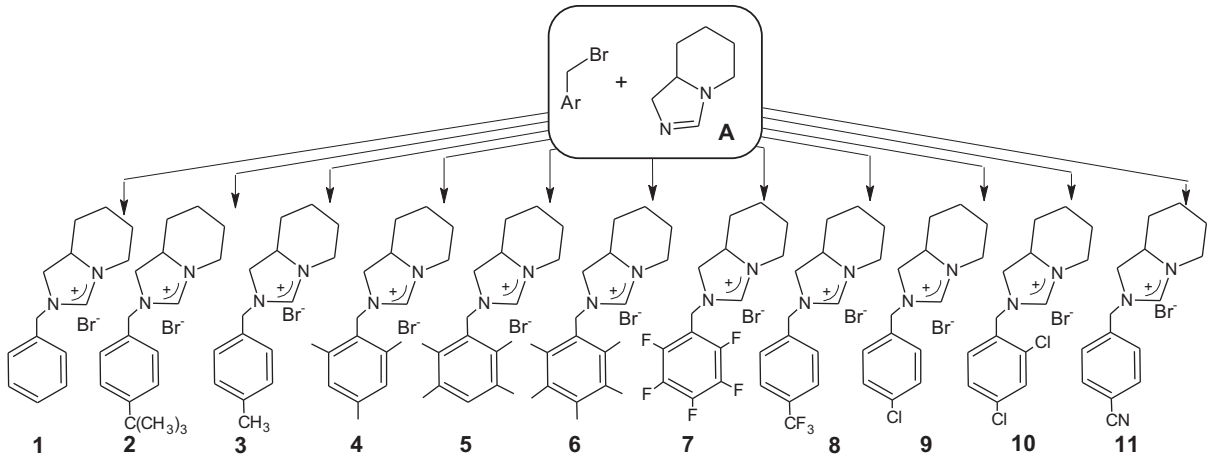


Scheme 1. Synthesis and formula of the salts used for antimicrobial and acute toxicity tests.

were prepared according to the published procedure. The purity of the salts was checked by TLC with Merck Kieselgel GF 254 Plates, elemental analyses and ^{1}H and ^{13}C NMR (Varian Mercury AS 400).

2.2. Calculated logP

For theoretically calculated logarithms of 1-octanol/water partition coefficient parameter (Clog*P*) MarvinSketch v 5.3.5 – Calculator Plugin was used [40,41].

2.3. Pharmacology

2.3.1. Antimicrobial activity

The *in vitro* antibacterial screening of the compounds (**1**–**11**) against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Micrococcus luteus*, *Proteus vulgaris*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and the antifungal screening against *Candida albicans* and *Candida krusei* was carried out by the MIC method. The MIC values together with Clog*P* and LD_{50} values are given in Table 1. 1-Octanol/water partition coefficient (log*P*) is indicative of the lipophilicity of the compounds and it is accepted that more lipophilic molecules cross the cell membrane more easily. Gentamycin and nystatin were used as reference compounds as a comparison and a check on the reliability of the method used.

All of these salts showed remarkable selectivities and effective activities against tested microorganisms (>400–0.78 μg/mL). However, among these compounds tested, unsubstituted benzyl derivative (**1**) affords the lowest inhibitory activity (MIC: >400 μg/mL) against Gram-positive *B. cereus* (CCM 99), Gram-negative bacteria and two yeasts. The antimicrobial mechanism of cationic biocides has been believed to involve their destructive interaction with cell wall and/or cytoplasmic membranes leading to the death of the microorganism and this interaction is known to be enhanced by hydrophobic moieties bonded to the nitrogen of the imidazole nitrogen. It is also known that the chain length of the alkyl substituent on the N atom enhances the antibacterial activity [14]. CH_3 groups therefore, have been added incrementally to the benzyl ring with the intention of increasing lipophilicity and as a consequence improving the activity. The bromide **2**, bearing one CH_3 at C-4 of the benzyl substituent displayed a very good activity against all microorganism (except *S. typhimurium*). Despite their increasing Clog*P* values, this change was accompanied by significant reductions in the antimicrobial activity of multi methylated benzyl substituted salts, such as **3**–**6**. This observation suggests poor correlation between lipophilicity and MIC values. Thus, other factors, such as steric and electronic might be influential. For example, inhibitory activity increases in going from electron-rich to electron-poor benzyls. This may indicate that the negatively charged walls of the microorganism prefer a more positive cation to bind.

In agreement with this the electron-withdrawing substituents (Cl, CN, CF_3) at the *p*-position of the phenyl ring also increases the positive charge density on the imidazolinium cation. The salt **10**, bearing two Cl atoms on the 2,4-position of the benzyl ring, shows a generally improved activity when compared to the mono chlorobenzyl bromide, **9**. However, acute toxicity of **10** is slightly higher than **9**. However, pentafluorobenzyl derivative (**7**) was less active and less toxic than **8**.

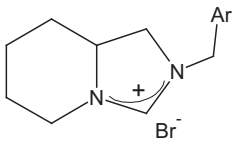
The position of the annulation seemed to be important. Therefore, related literature data [31] and data from this work were compiled in Table 2. Comparison of the antimicrobial activity of 1, 5- and 4,5-annulated imidazolinium salts (**12**, **13**) suggests that 1,5-annulation enhances the antimicrobial activity against SA, SE, EC and CE.

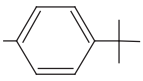
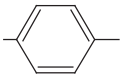
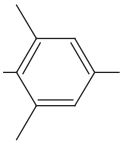
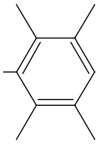
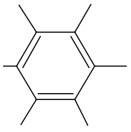
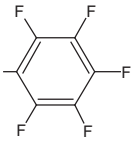
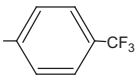
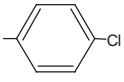
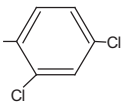
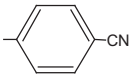
It is worth noting that among the tested compounds, **8**–**11** and **2** showed a very good antifungal activity against *C. albicans* (ATCC 10231) and *C. krusei* (ATCC 6258) with MICs 0.78 μg/mL, which were better than the values obtained for standard drug nystatin (6.25 and 3.12 μg/mL, respectively). In this study, compounds **8**–**11** are generally more effective on the bacteria than the other compounds, which were comparable with that for gentamycin. *E. coli* (ATCC 8739), *S. typhimurium* (CCM 5445) and *K. pneumoniae* (CCM 2318) are highly pathogenic to humans. Indeed, the bacterium *K. pneumoniae* showed a lower microbial susceptibility compared to the other tested microorganisms. This is probably due to its capsular material surrounding the microorganism. The compounds tested here generally showed lower antimicrobial activities against Gram-negative bacteria. The antimicrobial activities against Gram-positive bacteria and fungus may depend on the differences between the cell structures of these microorganisms. Whether differences in antimicrobial activities are due to differential uptake or different intramolecular interactions requires further investigation.

2.3.2. Acute toxicity

Despite their promising properties, less attention has been paid to the possible use of ionic liquids in pharmaceutical formulation and processing, presumably due to lack of information on their

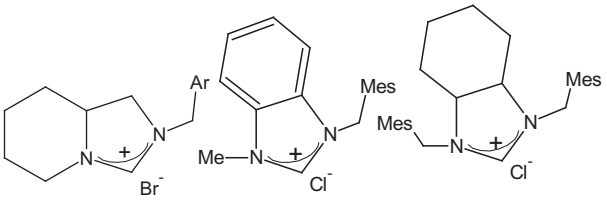
Table 1
Clog*P*, LD_{50} and *in vitro* MIC values for hexahydroimidazo[1,5-*a*]pyridinium bromides.



Salt	Ar	Clog*P*	LD_{50} (mg/kg)	MIC (μg/mL)									
				Gram-positive bacteria				Gram-negative bacteria				Yeasts	
				SA	SE	BC	ML	PV	EC	ST	KP	CA	CK
1		−1.35	907	100	50	>400	3.12	>400	>400	>400	>400	>400	>400
2		0.14	838	100	100	200	12.5	50	200	100	200	25	50
3		−0.85	>2000	3.12	1.56	<0.78	6.25	6.25	0.78	50	0.78	3.12	0.78
4		0.17	758	50	25	200	6.25	50	200	100	200	50	12.5
5		0.68	1946	50	6.25	100	12.5	6.25	50	50	200	50	6.25
6		1.19	1106	25	3.12	12.5	12.5	6.25	12.5	12.5	50	50	3.12
7		−0.72	978	3.12	0.78	6.25	0.78	12.5	25	25	12.5	12.5	3.12
8		−0.47	824	1.56	0.78	3.12	0.78	6.25	0.78	0.78	12.5	0.78	0.78
9		−0.72	>2000	1.56	0.78	3.12	0.78	6.25	3.12	0.78	12.5	0.78	0.78
10		−0.08	1891	0.78	0.78	3.12	0.78	1.56	3.12	0.78	12.5	0.78	0.78
11		−0.53	>2000	1.56	1.56	3.12	0.78	6.25	0.78	0.78	12.5	0.78	0.78
Gentamycin				3.12	1.56	6.25	1.56	0.78	3.12	6.25	25	–	–
Nystatin				–	–	–	–	–	–	–	–	6.25	3.12

Abbreviations: Clog*P*: calculated logarithm of 1-octanol/water partition coefficient: LD_{50}: the dose that kills half of the mice tested; MIC: minimum inhibitory concentration; SA: *Staphylococcus aureus*; SE: *Staphylococcus epidermidis*; BC: *Bacillus cereus*; ML: *Micrococcus luteus*; PV: *Proteus vulgaris*; EC: *Escherichia coli*; ST: *Salmonella typhimurium*; KP: *Klebsiella pneumoniae*; CA:*Candida albicans*; and CK: *Candida krusei*.

Table 2
Comparison of hexahydroimidazo[1,5-*a*]pyridinium bromides (**1** and **4**) with 4,5-annulated salts (**12** and **13**).



Ar = Ph(**1**); Mes(**4**) **12** **13**
Mes = C_6H_2-2,4,6-Me

	MIC (μg/mL)			
	SA	SE	EC	CA
1	100	50	>400	>400
4	50	25	200	50
12	1600	1600	1600	1600
13	100	1600	1600	1600

toxicity [42]. Some of the salts tested here for their antimicrobial activity displayed a very efficient performance. Hence, they were all assayed for acute toxicity which is one of the basic requirements in drug development. Acute toxicity results, LD_{50} values, were shown in Table 1. No lethality was observed among mice treated with 2000 mg/kg oral doses of imidazole derivates **2**, **10** and **11** on the limit test. LD_{50} values of other salts after oral administration in mice in 24 h showed low toxicity according to EPA and WHO.

3. Conclusions

As a fused, reduced pyridine at the N^1–C^5 positions of the imidazolinium ring resulted in compounds which in general were more active than simple imidazol(in)ium salts. For example, the least active compounds of this group, unsubstituted benzyl bromide (**1**) exhibited a higher activity than the analogous 1,3-dibenzylbenzimidazolinium chloride [31]. However, the data indicate that hydrophobic properties of the bromides, characterized by Clog*P*, are of minor importance for the *in vitro* antimicrobial activity. Therefore, factors other than lipophilicity such as electronic and/or steric might be in operation.

The MICs of the most active derivatives (**2**, **8**, **9**, **10** and **11**) were shown to be as low as 0.78 μg/mL against Gram-negative, Gram-positive bacteria and fungi. A simple inspection of Table 1 indicates that the presence of electron-withdrawing groups (Cl, CF_3 and CN, and in certain cases CH_3) at C-4 of the benzyl ring is mandatory for the broad-spectrum antimicrobial activity and with the exception of *P. vulgaris*, the resulting activity is superior to the standards gentamycin and nystatin. It is worth mentioning that the salts **2**, **10** and **11** with $LD_{50} > 2000$ mg/kg offer great potential for further development of new antimicrobials and our efforts are continuing along these lines.

4. Experimental

4.1. General procedure for the preparation of salts

All chemicals used in this study were purchased from E. Merck, Aldrich, Fluka or Alfa Aesar.

Variously substituted benzyl bromides (15 mmol) and compound **A** (1.86 g; 15 mmol) were refluxed in toluene (5 mL) for 4 h. The mixture was cooled to room temperature, diethyl ether (15 mL) was added and vigorously shaken and then decanted. The solid residue was washed with Et_2O (3 × 20 mL) to obtain an organic solid which was recrystallized from methanol/diethyl ether (3 mL/20 mL).

4.1.1. 2-(4-tert-Butylbenzyl)-1,5,6,7,8,8a-hexahydroimidazolo[1,5-a] pyridin-2-ium bromide, ***2***

Yield: 4.6 g, 96%, m.p.: 112–115 °C. ^{1}H NMR ($CDCl_3$, δ, ppm): 9.98 (s, 1 H, NCH), 7.39 (d, $J = 2.1$ Hz, 2 H, $CH_2C_6H_4C(CH_3)_3$), 7.34 (d,, $J = 2.1$ Hz, 2 H, $CH_2C_6H_4C(CH_3)_3$), 4.82 (s, 2 H, $CH_2C_6H_4C(CH_3)_3$), 4.33–4.18 (m, 2 H, piperidin-*H*), 3.96 (m, 1 H, piperidin-*H*), 3.34 (m, 2 H, NCH_2CH), 2.00–1.53 (m, 6 H, piperidin-*H*), 1.29 (s, 9 H, $CH_2C_6H_4C(CH_3)_3$). ^{13}C NMR ($CDCl_3$, δ, ppm): 155.9 (NCH), 152.3, 129.8, 128.8, 126.3 ($CH_2C_6H_4C(CH_3)_3$), 59.6 ($NHCH_2CH$), 53.7 ($CH_2C_6H_4C(CH_3)_3$), 51.9, 46.1, 34.8, 31.7, 31.4, 25.6, 22.4 (piperidin-C, $CH_2C_6H_4C(CH_3)_3$). Anal. Calc. for $C_{15}H_{18}N_3Br$ (M: 320.23): C, 61.54; H, 7.75; N, 7.97. Found: C, 61.60; H, 7.65; N, 8.01%.

4.1.2. 2-(4-Methylbenzyl)-1,5,6,7,8,8a-hexahydroimidazolo[1,5-a] pyridin-2-ium bromide, ***3***

Yield: 4.2 g, 90%, m.p.: 101–103 °C. ^{1}H NMR ($CDCl_3$, δ, ppm): 9.97 (s, 1 H, NCH), 7.30 (d, $J = 2.0$ Hz, 2 H, $CH_2C_6H_4CH_3$), 7.17 (d, $J = 1.9$ Hz, 2 H, $CH_2C_6H_4CH_3$), 4.81 (s, 2 H, $CH_2C_6H_4CH_3$), 4.28 (m, 2 H, piperidin-*H*), 3.97 (m, 1 H, piperidin-*H*), 3.52 (m, 2 H, NCH_2CH), 2.33 (s, 3 H, $CH_2C_6H_4CH_3$), 1.98–1.49 (m, 6 H, piperidin-*H*). ^{13}C NMR ($CDCl_3$, δ, ppm): 155.8 (NCH), 139.0, 130.0, 129.8, 128.9 ($CH_2C_6H_4CH_3$), 59.6 ($NHCH_2CH$), 53.6 ($CH_2C_6H_4CH_3$), 51.9, 46.1, 31.8, 25.6, 22.3, 21.4 (piperidin-C, $CH_2C_6H_4CH_3$). Anal. Calc. for $C_{15}H_{21}N_2Br$ (M: 309.24): C, 58.26; H, 6.84; N, 9.06. Found: C, 58.31; H, 6.85; N, 9.08%.

4.1.3. 2-(Pentafluorobenzyl)-1,5,6,7,8,8a-hexahydroimidazo[1,5-a] pyridin-2-ium bromide, ***7***

Yield: 5.19 g, 90%, m.p.: 142–144 °C. ^{1}H NMR ($CDCl_3$, δ, ppm): 9.75 (s, 1 H, NCH), 5.10 (s, 2 H, $CH_2C_6F_5$), 4.32 (m, 2 H, piperidin-*H*), 4.11 (m, 1 H, piperidin-*H*), 3.37 (m, 2 H, NCH_2CH), 2.03–1.49 (m, 6 H, piperidin-*H*). ^{13}C NMR ($CDCl_3$, δ, ppm): 156.3 (NCH), 139.9, 138.7, 129.0, 124.9 ($CH_2C_6F_5$), 59.5 ($NHCH_2CH$), 53.7 ($CH_2C_6F_5$), 46.8, 45.8, 32.1, 26.1, 22 (piperidin-C). Anal.Calc. for $C_{14}H_{14}N_2F_5Br$ (M: 385.17): C, 43.66; H,3.66; N,7.27. Found: C, 43.60; H, 3.59; N, 7.34%.

4.1.4. 2-[4-(Trifluoromethyl)benzyl]-1,5,6,7,8,8a-hexahydroimidazolo [1,5-a]pyridin-2-ium bromide, ***8***

Yield: 5.0 g, 92%. ^{1}H NMR ($CDCl_3$, δ, ppm): 9.94 (s, 1 H, NCH), 7.56 (q, $J = 2.1$ Hz 4 H, $CH_2C_6H_4CF_3$), 4.95 (s, 2 H, $CH_2C_6H_4CF_3$), 4.15 (m, 2 H, piperidin-*H*), 3.95 (m, 1 H, piperidin-*H*), 3.10 (m, 2 H, NCH_2CH), 1.93–1.47 (m, 6 H, piperidin-*H*). ^{13}C NMR ($CDCl_3$, δ, ppm): 156.3 (NCH), 137.2, 131.4, 129.6, 126.3, 126.2, ($CH_2C_6H_4CF_3$), 59.8 ($NHCH_2CH$), 53.8 ($CH_2C_6H_4CF_3$), 51.5, 46.2, 31.6, 25.5, 22.3 (piperidin-C). Anal. Calc. for $C_{15}H_{18}N_2F_3Br$ (M: 363.22): C, 46.60; H, 5.00; N, 7.71. Found: C, 46.63; H, 5.05; N, 7.77%.

4.1.5. 2-(4-Chlorobenzyl)-1,5,6,7,8,8a-hexahydroimidazolo[1,5-a] pyridin-2-ium bromide, ***9***

Yield: 4.7 g, 96%, m.p.: 89–91 °C. ^{1}H NMR ($CDCl_3$, δ, ppm): 10.08 (s, 1 H, NCH), 7.27 (dd, $J = 0.6$ Hz 2 H, $CH_2C_6H_4Cl$), 7.18 (dd, $J = 0.6$ Hz, 2H, $CH_2C_6H_4Cl$), 4.75 (s, 2 H, $CH_2C_6H_4Cl$), 4.05 (m, 2 H, piperidin-*H*), 3.84 (m, 1 H, piperidin-*H*), 3.18 (m, 2 H, NCH_2CH), 1.86–1.33 (m, 6 H, piperidin-*H*). ^{13}C NMR ($CDCl_3$, δ, ppm): 156.5 (NCH), 134.9, 131.7, 130.5, 129.4, ($CH_2C_6H_4Cl$), 59.6 ($NHCH_2CH$), 53.5 ($CH_2C_6H_3Cl$), 51.2, 46.0, 31.8, 25.6, 22.3 (piperidin-C). Anal. Calc. for $C_{14}H_{18}N_2ClBr$ (M: 329.66): C, 51.01; H, 5.50; N, 8.50. Found: C, 51.10; H, 5.60; N, 8.47%.

4.1.6. 2-(2,4-Dichlorobenzyl)-1,5,6,7,8,8a-hexahydroimidazolo[1,5-a] pyridin-2-ium bromide, ***10***

Yield: 5.0 g, 92%, m.p.: 156–158 °C. ^{1}H NMR ($CDCl_3$, δ, ppm): 10.16 (s, 1 H, NCH), 7.28 (m, 1 H, $CH_2C_6H_3Cl_2$), 7.18 (m, 2 H,

$CH_2C_6H_4Cl_2$), 7.06 (m, 1 H, $CH_2C_6H_3Cl_2$), 4.94 (s, 2 H, $CH_2C_6H_3Cl_2$), 4.19 (m, 2 H, piperidin-*H*), 3.94 (m, 1 H, piperidin-*H*), 3.25 (m, 2 H, NCH_2CH), 1.91–1.44 (m, 6 H, piperidin-*H*). ^{13}C NMR ($CDCl_3$, δ, ppm): 157.0 (NCH), 135.9, 135.0, 129.8, 129.5, 128.4, 125.4 ($CH_2C_6H_3Cl_2$), 59.7 ($NHCH_2CH$), 53.9 ($CH_2C_6H_3Cl_2$), 48.7, 46.2, 31.9, 25.7, 22.3 (piperidin-C). Anal.Calc. for $C_{14}H_{17}N_2Cl_2Br$ (M: 364.11): C, 46.18; H, 4.71; N, 7.69. Found: C, 46.19; H, 4.68; N, 7.77%.

4.1.7. 2-(4-Cyanobenzyl)-1,5,6,7,8,8a-hexahydroimidazolo[1,5-a] pyridin-2-ium bromide, **11**

Yield: 4.5 g, 94%, m.p.: 82–84 °C. ^{1}H NMR ($CDCl_3$, δ, ppm): 10.03 (s, 1 H, NCH), 7.64 (s, 4 H, $CH_2C_6H_4CN$), 5.07 (s, 2 H, $CH_2C_6H_4CN$), 4.19 (m, 2 H, piperidin-*H*), 3.96 (m, 1 H, piperidin-*H*), 3.33 (m, 2 H, NCH_2CH), 1.98–1.49 (m, 6 H, piperidin-*H*). ^{13}C NMR ($CDCl_3$, δ, ppm): 156.6 (NCH), 138.5, 133.2, 129.9, 118.4, 113.1 ($CH_2C_6H_4CN$), 59.8 ($NHCH_2CH$), 53.9 ($CH_2C_6H_4CN$), 51.6, 46.3, 31.6, 25.4, 22.4 (piperidin-C). Anal. Calc. for $C_{15}H_{18}N_3Br$ (M: 320.23): C, 56.26; H, 5.67; N, 13.12. Found: C, 56.30; H, 5.65; N, 13.14%.

4.2. Antimicrobial activity tests

In vitro antimicrobial studies were carried out against 10 test microorganisms (four Gram-positive bacteria: *S. aureus* (6538/P), *S. epidermidis* (ATCC 12228), *B. cereus* (CCM 99) and *M. luteus* (ATCC 9341), four Gram-negative bacteria: *P. vulgaris* (ATCC 6897), *E. coli* (ATCC 8739), *S. typhimurium* (CCM 5445) and *K. pneumoniae* (CCM 2318)), and two yeasts: *C. albicans* (ATCC 10231) and *C. krusei* (ATCC 6258), which were obtained from the Microbiology Department Culture Collection of Ege University, Faculty of Science.

Stock cultures of bacteria were maintained on nutrient agar (NA) and yeasts were potato dextrose agar (PDA) at 4 °C. Determination of minimum inhibitory concentration (MIC) by microdilution method was performed according to the National Committee for Clinical Laboratory Standards [43,44]. The MIC was taken as the lowest concentration that inhibited growth after incubation. Dilution series using sterile distilled water were prepared at the required quantities of 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 μg/mL concentrations in test tubes, which were transferred to 96-well microtiter plates [45]. Overnight grown bacterial suspensions in double strength Mueller–Hinton broth, yeast suspensions in double strength yeast glucose broth were standardized to 10^8 cfu/mL using McFarland No. 0.5 standard solution. Microorganism suspension (100 μl) was then added into the wells. The last-well chain without microorganism was used as a negative control. Sterile distilled water and the medium served as a positive growth control. After incubation at 37 °C for 18 h for all strains, the first well without turbidity was determined as the MIC. Dimethyl sulfoxide (DMSO; Carlo-Erba, France) had no effect on the microorganisms in the concentrations studied. Gentamycin was used as standard antibacterial agent for positive control, whereas nystatin was used as antifungal. The final concentration of antimicrobial agents was between 0.78 and 400 μg/mL.

4.3. Acute toxicity testing

In this study, firstly the acute oral toxicity of imidazole derivates was assessed by the limit test in the mice. Limit dose (2000 mg/kg) for acute oral toxicity according to EPA/OECD was used ($n = 10$; 5 male and 5 female for each group) [46,47]. The limit test was used to determine if the toxicity of a test substance is above or below a specified dose. Toxic responses occurring within a given period were recorded. Based on the results, a regulatory action or additional testing was required. Therefore, in this study, the classical LD_{50} test was used to determine the lethal dose (LD_{50}) of a substance that will kill 50% of test animals. The test material is administered in increasing doses to groups of 10 male and 10 female animals. Mortalities were recorded within a given period, and the LD_{50} was determined with the aid of statistical calculations (Probit analyses in SPSS for Windows 10.0). In the tests, male and female Swiss albino mice weighing 20–30 g were used [48,49].

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