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Quaternary salts derived from 3-substituted quinuclidine as potential antioxidative and antimicrobial agents

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Abstract: Two series of novel ammonium salts containing the quinuclidine moiety were prepared in order to evaluate their antioxidative, antibacterial and antifungal potential. The synthesized homologues of 3-hydroxy (QOH) and 3-chloroquinuclidine (QCl) with the different *N*-benzyl substituents at the *para*-position (bromo, chloro or nitro group) were obtained in very good yields and characterized by IR and NMR spectroscopies and elemental analysis. All compounds were tested for antioxidative activity using the oxygen radical absorbance capacity (ORAC) assay and among tested samples, *N-p*-nitrobenzyl-3-hydroxyquinuclidinium bromide (QOH-4) exhibited the highest antioxidative potential (293.80 nmol (TE) mL^{-1}), which was further investigated by the DNA nicking assay. The biological activity of selected compounds was evaluated by measuring the zone of inhibition and by determining the minimal inhibitory concentration (MIC) against three Gram-positive bacteria (*B. cereus, E. faecalis* and *S. aureus*), three Gram-negative bacteria (*E. coli, P. aeruginosa* and *C. sakazakii*) and three fungi species (*C. albicans*, *A. niger* and *P. notatum*). The bioactivity assay showed that some newly synthetized quaternary quinuclidinium compounds display a comparable or even better antibacterial and antifungal activity than the reference drugs such as gentamicin (GEN), cefotaxime (CTX) and amphotericin B (AMPHB). Among the tested compounds, *N-p*-chlorobenzyl-3-hydroxyquinuclidinium bromide (QOH-3) exhibited a considerable antibacterial efficiency against *P. aeruginosa* (MIC=0.39 μg mL^{-1}) and QOH-4 displayed a potent antifungal activity against *C. albicans* (MIC=1.56 μg mL^{-1}).

Keywords: quaternary ammonium salts, ORAC assay, DNA damage protection activity, antioxidative and antimicrobial activity

1 Introduction

Quinuclidine (1-azabicyclo[2.2.2]octane) is found to be a part of the structure of numerous naturally occurring bioactive compounds, such as cinchona, indole alkaloids and synthetic drugs [1-3]. Many clinically useful chemotherapeutic agents contain quinuclidine nucleus as an essential part of their structure and several scientific papers regarding its synthesis and/or biological activity have been published [4]. Recently some synthetic quinuclidines have been identified as a specific muscarinic agonists with potential in Alzheimer's dementia therapy [1-5] and some 3-substitued quinuclidine derivatives have been shown to be potential antidotes against poisoning by organophosphorus compounds such as pesticides, insecticides and chemical warfare agents [1-3], [5]. Today, few esters of quinuclidine-3-ol are even commercially available as therapeutic agents [6], such as zacopride and RG 12915, which are classical 5-hydroxytryptamine 3 ($5\text{-}HT_3$) receptor antagonists [7-10]. In addition, an antimicrobial activity of a quinuclidine-based FtsZ inhibitor and its synergistic potential with β-lactam antibiotics has been reported. FtsZ is an essential GTP-ase in bacterial cell division which is highly conserved among Gram-positive and Gram-negative bacteria. Therefore, it represents an interesting molecular target that could be manipulated in order to develop new antibacterial agents [11-12].

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It is well known that during the process of cell respiration, reactive oxygen species (ROS) are generated as byproducts of normal metabolism. The accumulation of ROS results in the damage of essential cellular compartments and biomolecules (proteins, DNA, fatty acids) which can ultimately lead to the development of serious pathological conditions. Cells have evolutionarily developed several ROS defending mechanisms such as enzymes superoxide dismutase, glutathione reductase, and catalase. However, the ability to effectively neutralize ROS by acquired mechanisms gradually decreases with cell aging; therefore an external consumption of antioxidants able to scavenge ROS is principally recommended [13]. The most popular natural source of antioxidants are plants and their extracts, but recently some synthetic forms have also gained much attention such as stobadine and derivatives of dihydropyridine [14].

A variety of quaternary ammonium salts (-N^+RR'R'', QAS) containing different heterocyclic moieties and synthesized via the Menschutin reaction have been reported for an increased pharmacological potential [15-19]. As such, chitin and chitosan have lately become extensively popular because of their broad application possibilities in medicine and several other industries. Chitosan is a nitrogeneous polysaccharide derived from chitin by a process of *N*-deacetylation. In the work reported by Liu et al. the antioxidative potential of different chitosan derivatives was explored, among which QAS have shown the highest activity [20]. This increased activity was attributed to the positive charge density of the nitrogen atom in QAS which was later explored by the introduction of the different substituting groups, namely highly electronegative $-CBr_3$ and $-CCl_3$ [21]. These studies have shown that QAS have strong free radical scavenging activity and that this property could be modulated by the introduction of the different electronegative substituents that would further strengthen the positive charge on the QAS nitrogen atom.

Given the persistent incidence of a bacterial multidrug resistance and the antimicrobial potency of QAS that we have previously shown with imidazole and quinuclidine moieties [22-23], in this paper we have designed and explored new quinuclidine compounds, 3-hydroxyquinuclidine and 3-chloroquinuclidine (QOH and QCl) quaternized by benzyl derivatives. In order to explore the effect of a positive charge density of the nitrogen atom on antioxidative and antimicrobial activities, three powerful electronegative groups -Br, -Cl and $-NO_2$ were introduced into the *para* position of *N*-benzyl-3-substituted quinuclidinium salts. The compound with $-NO_2$ group at *para* position on the benzyl ring and -OH group on the quinuclidine ring have shown the best antioxidative and DNA protection properties with a promising potential in the development of quinuclidine QAS with new applications in medicine, pharmacy and the food industry.

2 Experimental procedure

2.1 Synthesis

Quaternization reagents were obtained from Sigma-Aldrich. Dry solvents were used throughout the synthesis. Reactions were monitored by thin layer chromatography using DC-Alufolien Aluminiumoxide 60 F_{254} plates (Merck) with 9:1 chloroform/methanol as the eluent. The detection of spots was achieved by UV light and by the reversible absorption of iodine. Melting points were determined in open capillaries using a Büchi B-540 apparatus and are uncorrected. Elemental analyses were performed with a PerkinElmer PE 2400 Series II CHNS/O Analyser. FTIR spectra were recorded on a PerkinElmer FTIR 1725 X spectrometer. All samples were prepared by mixing FTIR-grade KBr (Sigma-Aldrich) with 1% (w/w) salt and grinding to a fine powder. Spectra were recorded over the 400-4000 cm^{-1} range without baseline corrections. ^1H and ^{13}C 1D NMR spectra were recorded in DMSO-d_6 solutions with a Bruker Avance III HD 400 MHz/54 mm Ascend spectrometer (400 MHz) at room temperature. Chemical shifts are reported as δ values in ppm using TMS as an internal standard. Abbreviations for data quoted are: s, singlet; d, doublet; dd, doublet of doublets and m, multiplet. Coupling constants (J) are given in Hz. Atomic numbering of quinuclidine heterocyclic ring is in accordance with the IUPAC recommendations. ^1H and ^{13}C NMR spectra are given in Supporting information. NMR spectroscopic signals were assigned after reference to the literature [24,25]

2.2 General procedure for synthesized compounds

To the solution of 3-hydroxyquinuclidine (1.00 mmol) in dry acetone, an equimolar amount of the appropriate quaternization reagent (benzyl bromide, *para*-chlorobenzyl bromide, *para*-bromobenzyl bromide and *para*-nitrobenzyl bromide, respectively) was added at room temperature. The reaction mixture was kept in the dark for 5 days to obtain a solid product. The excess of the solvent was then removed under reduced pressure and the solid was washed

several times with dry diethyl ether to give quaternary compounds as white crystals. The QCl-1-4 crystals were synthesized following the same procedure as for QOH-1-4 by using 3-chloroquinuclidine with prior removal of HCl. All crystals were obtained in very good yields.

N-benzyl-3-hydroxyquinuclidinium bromide (**QOH-1**). Yield: 89.5%; mp: 178.4-180.3°C; IR (KBr) ν/cm^{-1}: 3500-3100, 3002, 2940, 1466, 1120, 1105, 1079, 1040, 1011, 951, 941, 595. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 1.67 – 2.06 (m, 5 H, H4, H5, H8), 3.03 – 3.08 (m, 1 H, H2b), 3.26 - 3.50 (m, 4H, H6 and H7), 3.62 - 3.70 (m, 1 H, 2a), 4.06 (m, 1 H, H3), 4.44 - 4.54 (m, 2 H, CH_2-Bn), 5.54 (d, J=3.67 Hz, 1 H, OH), 7.49 - 7.56 (m, 5 H, Ar); ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 17.80 (C5) 21.37 (C8), 27.24 (C4), 53.23 (C6), 54.24 (C7), 63.02 (C2),63.75 (C3), 66.44 (CH_2-Bn), 128.09 (C1'), 129.41 (C3' and C5'), 130.62 (C4'), 133.53 (C2' and C6'). Anal. calcd. for $C_{14}H_{20}BrNO$: C 56.38; H 6.76; Br 26.79; N 4.70; O 5.36. Found: C 56.36, H 6.76, N 4.71.

N-*p*-bromobenzyl-3-hydroxyquinuclidinium bromide (**QOH-2**). Yield: 98.0%; mp: 243.6-245.0°C; IR (KBr) ν/cm^{-1}: 3526-3158, 3012, 2942, 1533, 1463, 1072, 658. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 1.65 – 2.16 (m, 5 H, H4, H5 and H8), 3.03 - 3.08 (m, 1 H, H2b), 3.20 - 3.45 (m, 4 H, H6 and H7), 3.60 – 3.68 (m, 1 H, H2a), 4.04 - 4.08 (m, 1 H, H3), 4.40 - 4.52 (m, 2 H, CH_2-Bn), 5.55 (d, J=3.18 Hz, 1 H, OH), 7.44 - 7.51 (m, 2 H, H2' and H6'), 7.69 - 7.77 (m, 2 H, H3' and H5'); ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 17.79 (C5), 21.34 (C8), 27.22 (C4), 53.06 (C6), 54.33 (C7), 63.07 (C2), 63.75 (C3), 65.53 (CH_2-Bn), 124.50 (C1'), 127.42 (C4'), 132.41 (C2' and C6'), 135.60 (C3' and C5'). Anal. calcd. for $C_{14}H_{19}Br_2NO$: C 44.59; H 5.08; Br 42.38; N 3.71; O 4.24. Found: C 44.58, H 5.08, N 3.71.

N-*p*-chlorobenzyl-3-hydroxyquinuclidinium bromide (**QOH-3**). Yield: 84.0%; mp: 220.8-222.3°C; IR (KBr) ν/cm^{-1}: 3512-3132, 3002, 2941, 1536, 1466, 1131, 1094, 1041, 951, 652.^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 1.69 – 2.16 (m, 5 H, H4, H5 and H8), 3.02 – 3.10 (m, 1 H, H2b), 3.21 - 3.44 (m, 4 H, H6 and H7), 3.62 – 3.69 (m, 1 H, H2a), 4.02 – 4.09 (m, 1 H, H3), 4.44 - 4.55 (m, 2 H, CH_2), 5.55 (d, J=3.67 Hz, 1 H, OH) 7.53 - 7.64 (m, 4 H, Ar); ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 17.79 (C5), 21.34 (C8), 27.23 (C4), 53.05 (C6), 54.29 (C7), 63.05 (CH_2-Bn), 63.74 (C3), 65.39 (C2), 127.07 (C1'), 129.46 (C2' and C6'), 135.35 (C3' and C5'), 135.65 (C4'). Anal. calcd. for $C_{14}H_{19}ClBrNO$: C 50.55; H 5.76; Br 24.02; Cl 10.66; N 4.21; O 4.81. Found: C 50.54, H 5.76, N 4.22.

N-*p*-nitrobenzyl-3-hydroxyquinuclidinium bromide (**QOH-4**). Yield: 95.1%; mp: 210.4-210.3°C; IR (KBr) ν/cm^{-1}: 3510-3140, 3003, 2940, 1530, 1465, 1120, 1042, 1010, 952. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 1.68 – 2.17 (m, 5 H, H4, H5 and H8), 3.07 – 3.14 (m, 1 H, H2b), 3.25 - 3.49 (m, 4 H, H6 and H7), 3.66 - 3.71 (m, 1 H, H2a), 4.04 - 4.08 (m, 1 H, H3), 4.60 - 4.72 (m, 2 H, CH_2-Bn), 5.57 (d, J=3.18 Hz, 1 H, OH), 7.78 - 7.89 (m, 2 H, H2' and H6') 8.31 - 8.40 (m, 2 H, H3' and H5'); ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 17.81 (C5), 21.35 (C8), 27.13 (C4), 53.30 (C6), 54.61 (C7), 63.32 (C2), 63.74 (C3), 64.92 (CH_2-Bn), 124.27 (C3' and C5'), 135.11 (C2' and C6'), 149.02 (C1'). Anal. calcd. for $C_{14}H_{19}BrN_2O_3$: C 48.99; H 5.58; Br 23.28; N 8.16; O 13.98. Found: C 49.01, H 5.58, N 8.15.

N-benzyl-3-chloroquinuclidinium bromide (**QCl-1**). Yield: 80.0%; mp: 197.9-199.3°C; IR (KBr) ν/cm^{-1}: 3002, 2955-2851, 1530, 1460, 1322, 952, 844; 700, 620. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 1.84 – 2.35 (m, 5 H, H4, H5 and H8), 3.27 - 3.67 (m, 5 H, H2a, H6 and H7), 3.97 - 4.08 (m, 1 H, H2b), 4.55 (s, 2 H, CH_2-Bn), 4.69 - 4.77 (m, 1 H, H3), 7.47 - 7.59 (m, 5 H, Ar); ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 18.71 (C5), 22.77 (C8), 28.35 (C4), 52.45 (C6), 54.09 (C3), 54.23 (C7), 62.03 (C2), 66.34 (CH_2-Bn), 127.72 (C1'), 129.51 (C5' and C3'), 130.78 (C4'), 133.57 (C2' and C6'). Anal. calcd. for $C_{14}H_{19}BrClN$: C 53.10; H 6.05; Br 25.23; Cl 11.20; N 4.42. Found: C 53.11, H 6.04, N 4.43.

N-*p*-bromobenzyl-3-chloroquinuclidinium bromide (**QCl-2**). Yield: 88.6%; mp: 219.7-221.3°C; IR (KBr) ν/cm^{-1}: 3004, 2959-2851, 1531, 1462, 1320, 1074, 953, 841; 700, 619. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 1.84 – 2.34 (m, 5 H, H4, H5 and H8), 3.27 - 3.67 (m, 5 H, H2a, H6 and H7), 3.96 - 4.04 (m, 1 H, H2b), 4.54 (s, 2 H, CH_2-Bn), 4.67 - 4.75 (m, 1 H, H3), 7.47 - 7.52 (m, 2 H, H3' and H5'), 7.71 - 7.76 (m, 2 H, H2' and H6'); ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 18.71 (C5), 22.76 (C8), 28.35 (C3), 52.35 (C6), 54.05 (C3), 54.29 (C7), 61.99 (C2), 65.41 (CH_2-Bn), 124.67 (C1'), 127.06 (C4'), 132.49 (C2' and C6'), 135.65 (C3' and C5'). Anal. calcd. for $C_{14}H_{18}Br_2ClN$: C 42.51; H 4.59; Br 40.40; Cl 8.96; N 3.54. Found: C 42.52, H 4.60, N 3.54.

N-*p*-chlorobenzyl-3-chloroquinuclidinium bromide (**QCl-3**). Yield: 64.5%; mp: 186.7-188.3°C; IR (KBr) ν/cm^{-1}: 3003, 2958-2852, 1530, 1460, 1322, 1092, 951, 844; 700, 618. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 1.82 – 2.36 (m, 5 H, H4, H5 and H8), 3.23 - 3.67 (m, 5 H, H2a, H6 and H7), 3.96 – 4.05 (m, 1 H, H2b), 4.56 (s, 2 H, CH_2-Bn), 4.67 - 4.75 (m, 1 H, H3), 7.53 - 7.64 (m, 4 H, Ar); ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 18.71 (C5), 22.75 (C8), 28.34 (C4), 52.36 (C6), 54.05 (C3), 54.27 (C7), 61.99 (CH_2-Bn), 65.35 (C2), 126.67 (C1'), 129.55 (C2' and C6'), 135.40 (C3' and C5'), 135.81 (C4'). Anal. calcd. for $C_{14}H_{18}BrCl_2N$: C 47.89; H 5.17; Br 22.76; Cl 20.19; N 3.99. Found: C 47.88, H 5.17, N 3.99.

N-*p*-nitrobenzyl-3-chloroquinuclidinium bromide (**QCl-4**). Yield: 99.0%; mp: 177.4-179.0°C; IR (KBr) ν/cm^{-1}: 3012, 2955-2847, 1559, 1473, 1348, 1092, 950, 870; 701, 622. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 1.82 – 2.35 (m, 5 H, H4, H5 and H8), 3.32 - 3.69 (m, 5 H, H2a, H6 and H7), 4.00 - 4.09 (m, 1 H, H2b), 4.68 - 4.76 (m, 3 H, CH_2-Bn and H3), 7.83 - 7.87 (m, 2 H, H2' and H6'), 8.32 - 8.41 (m, 2 H, H3' and H5'); ^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 18.73 (C5), 22.77(C8),

28.25 (C4), 52.67 (C6), 54.00 (C3), 54.56 (C7), 62.20 (C2), 64.86 (CH_2-Bn), 124.33 (C2' and C6'), 134.80 (C1'), 135.17 (C3' and C5'), 149.11 (C4'). Anal. calcd. for $C_{14}H_{18}BrClN_2O_2$: C 46.49; H 5.02; Br 22.09; Cl 9.80; N 7.75; O 8.85. Found: C 46.50, H 5.02, N 7.74.

2.3 *In vitro* antioxidant activity

2.3.1 Oxygen radical absorbance capacity (ORAC) assay

The antioxidant activities were determined by ORAC method [26], which measures the decay in fluorescence intensity (FI) of a probe in time. Prior to the experiment, all chemicals were dissolved in phosphate buffer (0.075 mM, pH 7.0). The FI decay of the reaction mixture containing 190 µL fluorescein (160 µM), 60 µL AAPH (150 mM) and 30 µL of the tested sample or a reference standard Trolox (6.25-50 µM) was measured in a microtiter plate (*Porvair Sciences*) at 37°C in a Perkin-Elmer LS55 spectrofluorimeter using the excitation wavelength of 485 nm. The fluorescence decay was monitored at 530 nm during the period of 60 min. The data were analyzed using FW WinLab software and the obtained results represent an average of at least three independent experiments.

2.3.2 DNA protection assay

The DNA protection assay was performed in a reaction mixture containing supercoiled pQE60 plasmid (200 ng) and different concentrations of the **QOH-4** compound (0-100 mM) or catalase (0,2 U and 0,5 U) as a positive control. After a 10 min incubation period at room temperature, in the reaction mixture 10 µL Fenton's reagent (30 mM H_2O_2, 50 µM ascorbic acid and 80 µM of $FeCl_3$) was added. The reaction mixture was incubated for another 30 min at 37°C following detection using the ethidium bromide (0.5 µg mL^{-1}) in a 1% agarose gel. The DNA bands were separated by applying the electric current of 75 mA for 30 min in the standard TAE buffer (40 mM Tris, pH 7.6; 20 mM acetic acid and 1 mM EDTA) and visualized using the ChemiDock XRS imaging system (*Bio-Rad*).

2.4 Biological assays

2.4.1 Assay for in vitro antimicrobial activity

The tested microorganisms were obtained from the culture collection at the American Type Culture Collection (ATCC) (Rockville. MD. USA) and at the Microbiology laboratory. Department of Biology, Faculty of Science, University of Split, Croatia (FNSST). The assayed collection included three Gram-positive bacteria *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923) and three Gram-negative ampicillin-resistant bacterial strains *Escherichia coli* (FNSST 111), *Pseudomonas aeruginosa* (FNSST 982) and *Cronobacter* (*Enterobacter*) *sakazakii* (FNSST 014). Bacterial strains were cultured overnight at 37°C in tryptic soy broth (TSB) and fungi were cultured overnight at 30°C in Sabouraud dextrose broth (SDB) to achieve optical densities corresponding to 10^6 colony forming units (cfu/mL) for bacteria and 10^4 cfu/mL for fungal strains. Antifungal activity was assessed on the yeast *Candida albicans* (ATCC 10231) and fungal strains *Penicillium notatum* (FNSST 3729) and *Aspergillus niger* (FNSST 3843).

2.4.2 Disc diffusion assay

In order to investigate the antimicrobial activities of the novel synthesized ammonium salts containing quinuclidine moiety a disc diffusion assay was employed according to the CLSI guidelines [CLSI. 2008]. Briefly, 100 µL of suspension containing 10^6 colony-forming units (cfu/mL) of bacterial cells were spread on a Mueller Hinton agar (Becton Dickinson, Sparks, MD). The stock solutions of quaternary salts were prepared by dissolving in 96% ethanol to a final concentration of 10 mg mL^{-1}. The sterile filter discs (6 mm) were individually loaded with 40 µL of the stock solution equivalent to a final concentration of 400 µg/disc of synthesized compounds and then placed on the nutrient agar that had been previously inoculated with the target microbial strains. Additionally, 96% ethanol was used as a negative control cefotaxime (30 µg) gentamicin (15 µg) and commercial fungicide amphotericin B (10 µg) were used as positive controls. The plates were incubated for 24 h at 37°C for bacterial strains and 48 h or 72 h at 30°C for yeast and mold isolates respectively. Antibacterial activity was assessed by measuring the diameter of the inhibition zone in millimeters including disc diameter for the test isolates compared to the controls. Samples were assayed in triplicate for each condition and the diameter of inhibition zones were presented as mean ± SE values.

2.4.3 Minimal inhibitory concentration assay

In addition antimicrobial activities of the novel synthesized ammonium salts containing quinuclidine moiety were

tested by a broth microdilution assay in 96 well microtitre plates. The standard two fold serial microdilution assay described by the Clinical and Laboratory Standards Institute [CLSI. 2008] was performed for the assessment of the minimal inhibitory concentrations (MICs). Bacteria and fungi were grown overnight in Mueller-Hilton broth (MHB) at 37°C and Sabouraud dextrose broth (SDB) at 30°C to the stationary phase. The microbial cultures were diluted in fresh MHB and SDB to a final concentration of 10^6 cfu/mL for bacteria. The compounds in the aqueous nutrient medium were previously dissolved in 96 % ethanol to the highest concentration of 10 mg mL^{-1}. The stock solution was then serially two fold diluted to obtain concentrations ranging from 0.06-1024 μg mL^{-1} in sterile plates containing Mueller Hinton broth (MHB) for bacteria. Serial dilutions of the investigated compounds were added to the microtiter plates in a volume of 100 μL. Each well was additionally inoculated with 100 μL of inoculums of the target microorganism and incubated at 37°C for 18-24 h. The MIC value was determined as the lowest concentration of the sample at which the tested microorganisms did not demonstrate any visible growth after incubation. As an indicator of bacterial growth, 50 μL of 0.2 mg mL^{-1} *p*-iodonitrotetrazoliumchloride (INT; Sigma-Aldrich Co. Ltd, Poole, UK) was added to the wells and incubated at 37°C for 30 min. Following addition of INT and incubation the MIC was determined as the lowest sample concentration at which no pink colour appeared. Cefotaxime, gentamicin and amphotericin B were used as positive controls. Minimal inhibitory concentrations of the commercial antibiotics were determined by the E-test (AB Biodisk, Solna, Sweden). The MIC was interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge. In this study no bioactivity was defined as a MIC >1000 μg/mL, mild bioactivity as a MIC in the range 512-1000 μg mL^{-1}, moderate bioactivity as a MIC in the range 128-512 μg mL^{-1}, good bioactivity as a MIC in the range 32-128 μg mL^{-1}, strong bioactivity as a MIC in the range 10-32 μg mL^{-1}, and very strong bioactivity as a MIC<10 μg mL^{-1}.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and discussion

3.1 Synthesis

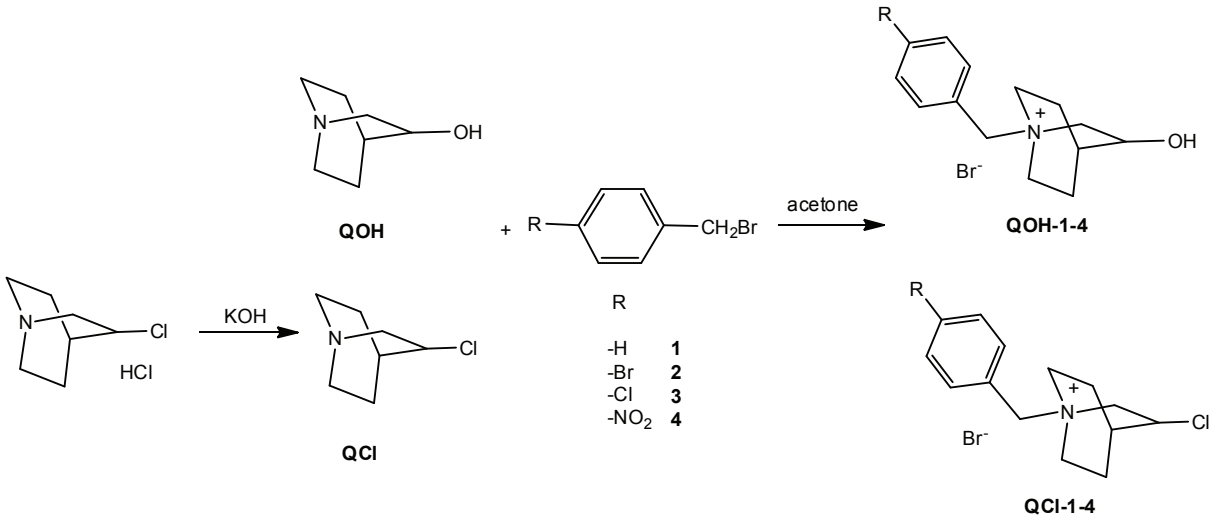
Two series of quaternary ammonium salts (QAS) have been synthesized by the reaction of 3-hydroxy or 3-chloroquinuclidine quaternized with benzyl or *para*-bromo, *para*-chloro and *para*-nitrobenzyl bromides (Scheme 1). All synthesized products were of a satisfactory purity and therefore no further purification by recrystallization was necessary. The white crystals of quaternary salts were obtained in very good yields. The acquired ^{1}H- and ^{13}C-NMR signals of the quinuclidine moiety were in accordance with the previously published data [24, 25]. The assignment of the aromatic benzyl rings was based on the NMR signals of chemical shifts and multiplicity of ^{1}H-NMR. The attributions of the aromatic ^{1}H-NMR chemical shifts are in full agreement with those previously reported for the *N*-benzyl-3-benzamidoquinuclidines except the signal of H-4, which is missing due to the *para*-substitution. On the other hand, the aromatic ^{13}C–signals are not in a correlation with the previously obtained *N*-benzyl-3-benzamidoquinuclidine spectral data, instead somewhat higher ^{13}C-chemical shifts of C-3, C-4 and C-5 atoms in the benzyl rings are observed and it is most likely due to the effect of a substituent.

3.2 In vitro *Antioxidant activity*

3.2.1 ORAC assay

The observed biological activity of some quinuclidinium salts [23] could be, among others, associated with the antioxidative potential that has not been investigated so far. In order to determine the antioxidative potential of the newly synthetized compounds, a standard ORAC method was used [26,27]. This method measures the decay in fluorescence intensity (FI) of a probe upon the reaction with the free peroxyl radicals generated by AAPH. In the presence of the standard (Trolox), the FI decay is postponed until the antioxidative agent is fully expended. From the resultant spectra the area under the curve (AUC) is obtained after the blank (Blk) AUC subtraction, and the standard curve is generated by plotting the AUC with nmol of the Trolox standard. The obtained antioxidative potential expressed in nmol of trolox eqvivalents (TE) is presented in Table 1.

The observed data show that all quaternary quinuclidinium compounds exhibit better antioxidative activity than the precursor compounds (QOH and QCl). Among the tested quaternary quinuclidinium salts, compounds **QOH-2** and **QOH-4** exhibit the highest antioxidative potential. Therefore, it can be concluded that the hydroxy group at the position 3 on quinuclidine ring has a significant influence on the antioxidative activity. This finding might be explained by the polarity



Scheme 1: Synthesis of quaternary ammonium salts.

Table 1: Antioxidative activity of the tested compounds, determined by ORAC method.

Compound	nmol (TE) mL^{-1}
QOH	22.85 ± 0.45
QCl	21.36 ± 0.64
QOH-1	40.09 ± 0.24
QCl-1	51.24 ± 0.32
QOH-2	111.6 ± 0.38
QCl-2	57.43 ± 0.29
QOH-3	27.21 ± 0.19
QCl-3	63.72 ± 0.25
QOH-4	293.80 ± 0.85
QCl-4	88.40 ± 0.12

of the hydroxyl group which could act as a proton donor in the redox reaction and thereby diminish the influence of the peroxyl radicals. Moreover, it appears that in the series of the QOH compounds, the NO_2 substituent at *para* position on the benzyl ring strongly influences antioxidative activity. This was also observed with the QCl series, which albeit of generally lower antioxidative potential, detain similar influence of the NO_2 substituent on the antioxidative activity.

3.2.2 DNA protection assay

The assay is based on the ability of the tested sample to neutralize free radicals and prevent breaking the DNA strands. The DNA damage is induced by Fenton's reagent, which serves as a generator of the hydroxyl ($^{\bullet}OH$) radicals that react with the nitrogen bases of the DNA. The produced base radicals further interact with sugar moiety, causing the breakage of the sugar-phosphate backbone and linearization of the DNA molecule. The resultant DNA molecule is visualized by gel electrophoresis whereby linear and circular DNA forms are easily distinguished.

The results of ORAC assay have shown that among tested compounds, **QOH-4** have the strongest antioxidative potential. In order to further characterize this finding we have performed the DNA protection assay aiming to evaluate the capacity of **QOH-4** to prevent linearization of pQE60 plasmid upon the addition of the Fenton's reagent. Figure 1. shows the protective effect of **QOH-4** and its ability to shield DNA from the Fe^{3+}-dependent nicking.

When DNA was exposed to Fenton's reagent it resulted in the formation of the linear (nicked) DNA form. Addition of **QOH-4** resulted in a partial preservation of the circular DNA, which was shown to be concentration dependent. These results suggest that **QOH-4** have the ability to shield DNA in an oxidative stress condition which might be explained either by the **QOH-4**-iron complex formation or by a proton donor ability of the hydroxyl-group. Hydroxyl-groups are known to have a reducing potential and are able to reduce highly oxidizing free radicals such as superoxide, peroxyl, aloxyl and hydroxyl radicals [28].

3.2.3 Biological evaluation

3.2.3.4 Disc diffusion assay

In this study the preliminary antimicrobial potential of the *N*-quaternary salts was tested against a diverse panel of selected antibiotic susceptible Gram-positive bacteria including *Bacillus cereus* (ATCC 11778), *Enterococcus*

faecalis (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), Gram-negative ampicillin-resistant bacteria namely *Escherichia coli* (FNSST 111), *Pseudomonas aeruginosa* (FNSST 982) and *Cronobacter* (*Enterobacter*) *sakazakii* (FNSST 014) and fungi such as *Candida albicans* (ATCC 10231) *Penicillium notatum* (FNSST 3729) and *Aspergillus niger* (FNSST 3843) by the disc diffusion assay.

As a comparison, we tested the inhibition zones of conventional antimicrobial agents that are used in clinical settings to treat infections caused by pathogenic bacteria, such as gentamicin (GEN) and cefotaxime (CTX). The antifungal agent amphotericin B (AMPHB), was used as a referent antimicotic.

As shown in Table 2 the results of the disc diffusion assay indicate that all compounds exhibit a potent and broad spectrum activity against tested bacterial and fungal strains.

The mean zones of inhibition were found to be in the range of 9.3 ± 0.3 to 24.3 ± 1.3 mm. It is noteworthy that the tested samples displayed not only antibacterial activity against the susceptible Gram-positive bacteria but also against Gram-negative bacteria with various antibiotic resistance profiles. Among the tested Gram-negative bacteria, *Pseudomonas aeruginosa* displayed promising sensitivity towards all compounds with considerable zones of growth inhibition which were larger than the ones with the reference positive controls, GEN and CTX respectively (Figure 2 A).

This is important, because *P. aeruginosa* is a major opportunistic pathogen that causes a wide range of nosocomial infections and accumulated enough resistance mechanisms to render them virtually untreatable by modern antibacterial chemotherapy [29].

Promising antimicrobial potential of all new compounds was also observed against *E. coli* but only in comparison to GEN (Figure 2 B). CTX was still more efficient for that bacterium, displaying a larger zone of growth inhibition than either of the compounds. It is worth noting that among the tested quinuclidinium salts **QCl-4** displayed a potential antimicrobial activity against both, bacteria and fungi (Figure 3). However, it should be noted that the amount of tested quinuclidines was 20 folds higher than the reference antibiotics, thus more detailed evaluation of antimicrobial potential was further needed.

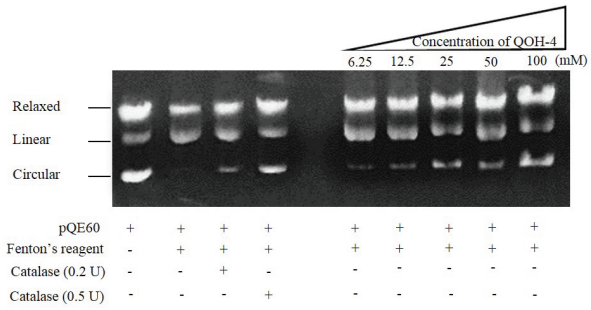


Figure 1: DNA protection assay of **QOH-4**. The pQE60 plasmid in the presence and absence of the DNA nicking reagent (Fenton's reagent). Catalase was used as a positive control.

3.2.4 Minimal inhibitory concentrations (MICs)

The antimicrobial activity was investigated by a broth microdilution method to determine the minimal inhibitory concentrations (MICs) for two series of quaternary quinuclidinium salts. MIC is the minimal concentration of the tested compound at which inhibition of the bacterial growth is observed. As a control, we have also tested MICs of the relevant conventional antimicrobial agents such as gentamicin and cefotaxime which are currently used in clinical treatments. The results presented in Table 3 reveal that all compounds display a remarkable selectivity and

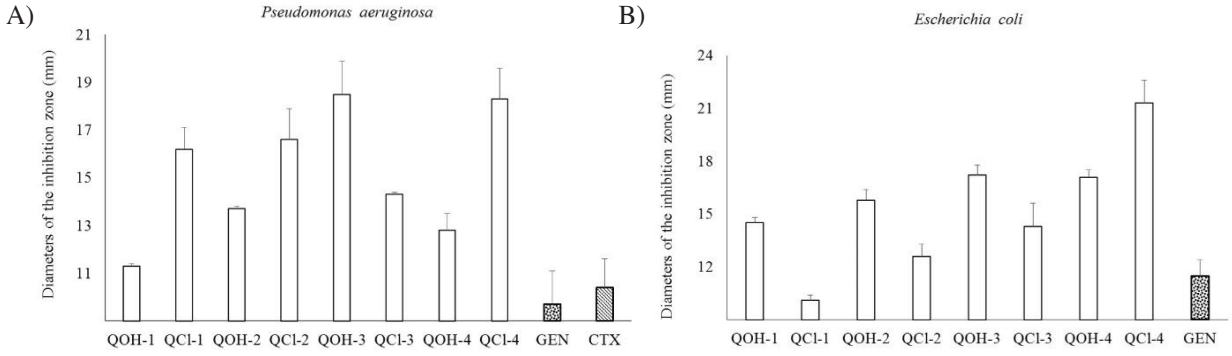


Figure 2: Diameters of the inhibition zones of all tested quinuclidinium salts against *P. aeruginosa* (A) *and E. coli* (B). Reference antibiotics GEN and CTX (A) and GEN (B) served as positive controls.

Table 2: Diameters of the inhibition zones of 3-substituted quinuclidines (**QOH** and **QCl**) and their quaternary salts (**QOH-1-4** and **QCl-1-4**) evaluated by the disc diffusion assay.

Microorganisms	Diameters of the inhibition zone[a,c] (mm)											
	Compound										Standard antibiotic[b,c]	
	QOH	QCl	QOH-1	QCl-1	QOH-2	QCl-2	QOH-3	QCl-3	QOH-4	QCl-4	CTX	GEN
Gram-positive bacteria												
B. cereus	10.8 ± 0.6	21.4 ± 1.3	11.5 ± 1.3	21.4 ± 1.3	13.6 ± 0.7	21.3 ± 0.7	19.3 ± 1.3	19.3 ± 0.9	13.7 ± 1.1	24.3 ± 1.3	26.8 ± 0.4	18.2 ± 0.7
E. faecalis	11.7± 0.6	21.4 ± 1.3	12.3 ± 0.9	15.3 ± 0.9	12.7 ± 0.1	11.6 ± 0.5	17.9 ± 0.6	16.3 ± 1.1	16.6 ± 0.9	15.3 ± 0.8	23.5 ± 0.1	14.6 ± 1.4
S. aureus	10.7± 0.9	21.4 ± 1.3	15.6 ± 1.4	20.6 ± 1.4	14.7 ± 0.3	12.6 ± 0.8	17.5 ± 0.1	22.3 ± 1.0	17.8 ± 0.3	19.3 ± 1.1	21.7 ± 0.4	23.9 ± 0.9
Gram-negative bacteria												
E. coli	10.5± 0.7	21.4 ± 1.3	14.5 ± 0.3	10.1 ± 0.3	15.8 ± 0.6	12.6 ± 0.7	17.2 ± 0.6	14.3 ± 0.8	17.1 ± 0.4	21.3 ± 1.0	22.8 ± 0.7	11.5 ± 0.9
P. aeruginosa	10.9± 0.9	21.4 ± 1.3	11.2 ± 0.1	16.2 ± 0.1	13.7 ± 1.4	16.6 ± 0.7	18.5 ± 0.9	14.3 ± 1.3	12.8 ± 0.1	18.3 ± 0.7	10.4 ± 1.2	9.7 ± 1.4
C. sakazakii	10.2± 0.3	21.4 ± 1.3	13.3 ± 1.2	13.2 ± 0.1	16.8 ± 1.3	17.6 ± 0.9	19.4 ± 1.3	17.3 ± 1.2	17.8 ± 0.3	22.3 ± 0.8	24.7 ± 0.6	21.6 ± 0.9
Fungi									**AMPHB**			
C. albicans	12.0± 0.3	21.4 ± 1.3	18.2 ± 0.6	17.2 ± 0.6	15.4 ± 0.7	19.4 ± 0.7	13.4 ± 0.1	23.4 ± 0.1	17.9 ± 0.7	24.0 ± 1.2	21.9 ± 0.7	
A. niger	9.3± 0.3	21.4 ± 1.3	12.6 ± 0.7	19.6 ± 1.7	13.7 ± 0.9	19.7 ± 1.5	14.8 ± 0.6	14.8 ± 1.9	19.3 ± 1.1	22.0 ± 1.0	19.3 ± 1.1	
P. notatum	11.2± 0.6	21.4 ± 1.3	17.9 ± 0.3	21.9 ± 0.8	12.3 ± 0.3	22.3 ± 1.4	15.6 ± 0.4	17.2 ± 0.7	21.5 ± 0.3	24.0 ± 0.8	23.5 ± 0.3	

[a] Diameter of inhibition zone (values in mm) around the disc: 400 µg/disc.

[b] Standard antibiotics disc: CTX, cefotaxime (30 µg/disc), GEN, gentamicin (15 µg/disc), AMPHB, amphotericin B (10 µg/disc).

[c] Values are expressed as mean ± SE.

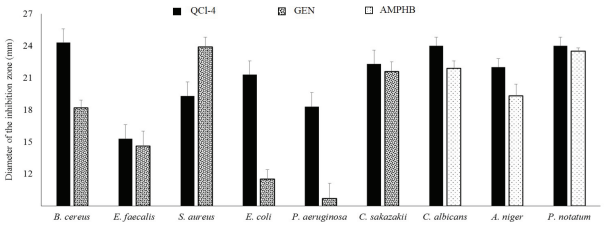


Figure 3: Antibacterial and antifungal activities of **QCl-4** against all tested microorganism in comparison with GEN and AMPHB as positive controls.

effective activity against the tested Gram-positive and Gram-negative bacteria with MIC values ranging from 0.39 to 100.00 μg mL^{-1}. Detailed analysis of the obtained MIC values show that quaternary salts with substituents at *para* position such as Br, Cl and NO_2, display generally better activity than the compounds without substituents on the benzyl ring regardless of the substituent type. However, the exception to this observation are data for *P. aeruginosa* which for all tested compounds had lower MICs then the standard antibiotic GEN and for most compounds displayed lower MICs than the reference CTX.

Among the eight newly synthetized quinuclidinium salts, two compounds namely **QOH-3** and **QCl-3** displayed better antibacterial potential against the Gram-positive (*B. cereus* and *E. faecalis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) than the reference antibiotics with MIC values ranging from 0.39-3.12 μg mL^{-1}. **QCl-3** had 2.5-fold better antibacterial activity than GEN against *B. cereus* and **QOH-3** had similar MIC value against *E. faecalis* as GEN. For *E. coli* MIC value observed in the presence of the **QCl-3** is 10-fold lower than the reference GEN. These values for **QOH-3** are even 164-fold lower then GEN and 41-fold lower than CTX.

Strong and potent antimicrobial activity for other quinuclidinium compounds have also been observed. For example, **QCl-4** showed identical MIC values (6.25 μg mL^{-1}) for all tested Gram-positive bacteria which is comparable to the GEN. Furthermore, **QCl-2** and **QOH-4** display MIC values for *P. aeruginosa* (3.12 μg mL^{-1}) which are 20-fold lower than the MIC observed for GEN and 5-fold lower than the one observed for CTX. Lower MIC values ranging from 3.12-6.25 μg mL^{-1} have been accounted for all tested Gram negative bacteria in the presence of **QCl-2** relative to GEN. For *C. sakazakii* MIC for **QCl-2** was 2.5-fold lower than for GEN and CTX.

In conclusion, among the synthesized and tested quinuclidinium salts, **QCl-3** and **QOH-3** showed promising inhibitory profiles against clinically important pathogens such as *E. coli* and *P. aeruginosa*. It appears that different *para* substituents at benzyl rings of quaternary salts affects antibacterial activity as accounted for 3-chloroquinuclidines bearing Br substituent on the benzyl ring. For these compounds strong activity against Gram-negative bacteria was observed. On the other hand, quinuclidinium salts containing NO_2 group as a substituent on the benzyl group, show strong antibacterial activity against all tested Gram-positive bacteria. It should also be noted that Cl as a *para* substituent on the benzyl group positively affects antimicrobial activity against respective Gram-positive and Gram-negative bacteria, regardless if it is part of a 3-hydroxy or 3- chloroquinuclidinium heterocyclic core.

The obtained MIC values for all compounds against three fungal strains are presented in Table 4. Our results show better antifungal activities for *C. albicans* with the compounds from the QOH series. The most prominent MIC values were observed for **QOH-4** (MIC=1.56 μg mL^{-1}), which is in range with the AMPHB standard.

4 Conclusions

In this study, we have synthesized a novel series of *N*-quaternary salts of 3-hydroxy- and 3-chloroquinuclidines (**QOH-1-4** and **QCl-1-4)** to investigate their antimicrobial and antioxidative activities. All compounds were synthesized and characterized by spectral data (IR, 1D and 2D NMR). The ORAC test was used to evaluate the antioxidant properties of synthesized QAS. According to our ORAC results and the DNA protection assay the **QOH-4** compound was identified as the most potent antioxidants. In preliminary screening studies, the obtained antimicrobial results were very promising since many of the compounds demonstrated a potent *in vitro* activity against a broad range of clinically important Gram-negative bacteria, including multidrug resistant strains with MIC value from 0.39 to 3.12 μg mL^{-1} (**QCl-2**, **QOH-2**, **QCl-3** and **QOH-4**). Some of them, such as **QCl-4**, showed very strong bioactivity against all tested Gram-positive bacteria with MIC values of 6.25 μg mL^{-1} Furthermore, we discovered that among the present class of compounds, **QOH-3** and **QCl-3,** with Cl as the *para*-substituent on the benzyl group, were especially effective against Gram-positive and Gram-negative species with much lower MIC values than standard antibiotics tested in this study (for example, 164 fold lower than GEN) which could serve as a guidance to design new and promising antibacterial compounds.

Of special importance is the finding that **QOH-4** has a strong antioxidant activity and a high antifungal potential

Table 3: Minimal inhibitory concentration of 3-substituted quinuclidine (**QOH** and **QCl**) and their quaternary salts (**QOH-1-4** and **QCl-1-4**) against Gram-positive and Gram-negative bacteria. The MIC values lower than the reference antibiotic are indicated as bold numbers in dark grey squares and MIC values between the two referent antibiotics are colored in grey.

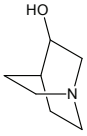
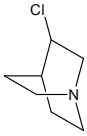
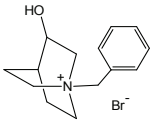
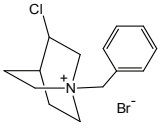
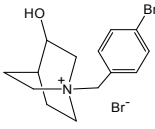
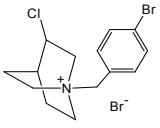
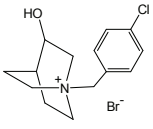
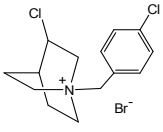
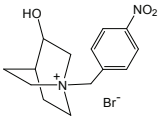
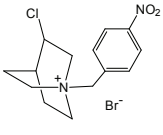
Comp/M_r	Structure	MIC (μg mL^{-1})					
		Gram-positive bacteria			Gram-negative bacteria		
		B. cereus	*E. faecalis*	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. sakazakii*
QOH 127.18		25.00	50.00	25.00	50.00	25.00	50.00
QCl 145.63		50.00	25.00	12.50	12.50	50.00	25.00
QOH-1 298.22		12.50	50.00	50.00	50.00	**6.25**	12.50
QCl-1 316.66		25.00	25.00	25.00	25.00	25.00	25.00
QOH-2 377.11		50.00	50.00	25.00	12.50	**12.50**	25.00
QCl-2 395.56		12.50	6.25	12.50	6.25	**3.12**	**3.12**
QOH-3 332.66		100.00	3.12	100.00	12.50	**0.39**	25.00
QCl-3 351.11		1.56	25.00	25.00	3.12	25.00	25.00
QOH-4 343.22		50.00	12.50	100.00	25.50	3.12	50.00
QCl-4 361.66		6.25	6.25	6.25	6.25	25.00	25.00
Gentamicin		4.00	4.00	1.00	32.00	64.00	8.00
Cefotaxime		0.25	0.50	0.50	0.50	16.00	8.00

Table 4: Minimal inhibitory concentration of 3-substituted quinuclidine (**QOH** and **QCl**) and their quaternary salts (**QOH-1-4** and **QCl-1-4**) against fungi. The MIC value lower than the reference antimycotic is indicated in grey.

Compound	MIC (μg mL^{-1}) Fungi		
	C. albicans	*A. niger*	*P. notatum*
QOH	12.50	25.00	25.00
QCl	25.00	25.00	12.50
QOH-1	12.50	3.12	3.12
QCl-1	100.00	50.00	50.00
QOH-2	6.25	25.00	100.00
QCl-2	50.00	50.00	25.00
QOH-3	50.00	25.00	25.00
QCl-3	50.00	50.00	25.00
QOH-4	1.56	25.00	50.00
QCl-4	6.25	12.50	12.50
Amphotericin B	2.00	1.00	1.00

against *C. albicans* when compared to other QAS derivatives (1.59 μg mL^{-1}). It is also worth mentioning that reference compounds **QOH** and **QCl** showed much lower values in all applied tests than the corresponding quaternary salts indicating that quaternization have improved biological potential of 3-substituted quinuclidine. The results of the present study are suggesting a promising starting point for ammonium salts structure optimization based on quinuclidine scaffold in order to design potent antimicrobial and antioxidative agents. Moreover, the mechanism of antibacterial activity and identification of the bacterial enzymes as potential targets of newly synthesized compounds is of a great interest in the future.

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