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A Series of Biguanide Ligands and Their Cu(II) Complexes: Cholinesterase Inhibitory and Antimicrobial Properties

Julide Nacaroglu Ballı,[a] Ozge Gungor,[a] and Muhammet Kose*[a]

In this work, a series of biguanide hydrochloride salts and their Cu(II) complexes were synthesized and screened for their acetyl/butyryl choline esterase inhibitory and antimicrobial properties. The structures of the synthesized compounds were characterised by common spectroscopic and analytical methods. Biguanide compounds showed considerably lower inhibitory activity compared to the reference drugs donepezil and galantamine. On the other hand, complexation of the biguanide compounds with Cu(II) resulted in dramatic increase in the inhibitory activity. The Cu(II) complexes showed AChE inhibitory activity with the IC_{50} values of 21.29 ± 0.95–82.53 ± 0.20 μM and those values are comparable to that of donepezil (IC_{50}: 18.54 ± 1.03 μM). The synthesised compounds were also screened for their antimicrobial activity towards gram positive (+) and gram negative (−) bacteria. Compounds (12.50 mg/mL) showed important antibacterial properties with inhibition zones of 8–28 mm diameter against gram-positive and gram-negative microorganisms. Compounds A03 and A08 exhibited more antimicrobial properties towards *E. coli* than standard antibiotics amikacin and gentamicin.

1. Introduction

Biguanidines are organic compounds and biguanidine derivatives such as metformin, buformin and phenformin are pharmaceutically active compounds.[1,2] Biguanidines carry electrons with the functional N–C(N)=N–C(N)=N moiety in an electron-rich π-conjugated system. They are quite basic due to their electron richness, so they are mostly used in their monoprotonated form in medicinal chemistry.[3] Additionally, biguanidines are N,N′-bidentate ligands and therefore have the ability to form stable metal complexes.[4] Derivatives of biguanidines have proven useful in the treatment of hyperglycemia, malaria, filaria and influenza.[5–8] Biguanidines such as metformin, phenformin, and buformin have been widely used in the treatment of non-insulin-dependent diabetes as antihyperglycemic drugs.[9] In addition to common use of biguanides as antihyperglycemic agents, several biguanides have been shown to exhibit wide range of biological activity such as antibacterial, antimalarial, anticancer and so on.[2,10–12] Some biological active biguanides are shown in Figure 1.

Based on the cholinergic hypothesis that postulates an increase in acetylcholine (ACh) levels, carbamate-like drugs inhibit cholinesterases (ChEs) and are considered one of the mainstays of contemporary pharmacotherapy for Alzheimer's disease (AD).[13–17] Although the etiology of AD has not yet been fully elucidated, several factors are assumed to play a role in the pathogenesis of this disease, including accumulation of amyloid-β peptide deposits in the brain and oxidative stress. Basically, there are two types of ChE enzymes that belong to the serine hydrolases group in the human nervous system: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). In fact, both enzymes can catalyze ACh hydrolysis at cholinergic synapses. However, BChE can also hydrolyze other esters; therefore, its concentration in the brain appears to be important for Alzheimer's patients. Inhibition of both enzymes produces an increase in ACh concentration at cholinergic synapses, showing symptomatic efficacy in the treatment of AD; therefore, drugs such as cholinesterase inhibitors galantamine, rivastigmine, tacrine and donepezil are clinically applied to alleviate the neuromuscular symptoms of AD.[18,19]

In this work, we prepared a series biguanide ligands and their Cu(II) complexes. The structures of the compounds were determined by FTIR, NMR (for biguanide ligands), elemental analysis and mass spectra. Crystal structures of compounds A06 and A07 were determined by single crystal X-ray diffraction studies. Finally, the synthesized biguanide compounds were screened for their acetyl/butyryl choline esterase inhibitory and antimicrobial properties.

Experimental

Materials and physical measurements

The reagents used in the study were purchased from commercial sources and used as received. For the UV-Vis spectra, a Perkin-Elmer Lamda 25 spectrometer was used. FT-IR spectra of the compounds were measured on a Perkin Elmer Spectrum 100 FT-IR with ATR (attenuated total reflectance) attachment. FT-IR spectra (Figs. S1–S16), NMR spectra (Figure S17–S24) and MALDI TOF (Figure S25–S32) spectra were given in the supplementary documents.

Synthesis of biguanide hydrochloride salts (A01-A09)

2-cyanoguanidine (1.1 mmol) and substituted aniline (1 mmol) derivatives were added to a 100 mL glass flask, 0.5 mL of 37% HCl

[a] J. N. Ballı, O. Gungor, Prof. Dr. M. Kose
Science Faculty, Chemistry Department, Kahramanmaras Sutcu Imam University, Avsar Campus, Kahramanmaras, TURKEY
E-mail: muhammetkose@ksu.edu.tr

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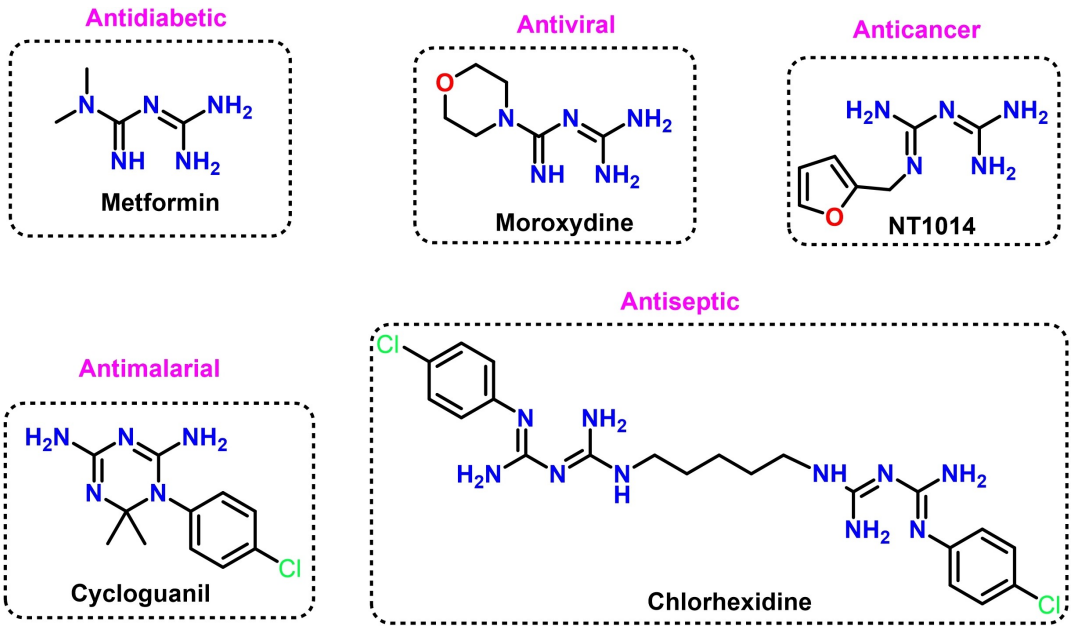


Figure 1. Some examples of pharmaceutically active biguanide compounds.

and 10 mL of ^{n}BuOH were added, and the mixture was refluxed at 90–100 °C for approximately 18 hours. The precipitated product was vacuum filtered. The filtered product was washed with cold water and 2-propanol.

A01: Molecular Formula: $C_{10}H_{15}N_5$·HCl; MW: 241.77 g/mol; Yield: 76.7 %; Color: Colorless Elementel Analysis Calculated ($C_{10}H_{15}N_5$·HCl): C% 49.69; H% 6.67; N% 28.97; Found: %C 49.55; H% 6.53; N% 28.74; FT-IR (ATR, cm^{-1}): 3296, 3178, 2972, 1634, 1555, 1525, 1481, 1380, 1256, 1185, 1050, 869, 736, 613 cm^{-1}. ^{1}H (d_6-DMSO, ppm): δ 9.20 (1H, s, NH), 7.29 (1H, s, Ar–H), 7.21 (3H, d, Ar–H), 7.16 (1H, d, NH), 7.13 (4H, d, NH_2), 2.62 (2H, dd, CH_2), 1.13 (3H, t, CH_3);^{13}C (d_6-DMSO, ppm) :δ 161.16 (N–C-N), 157.63 (N–C-N), 138.62 (N–Ar-C), 135.92 (Ar–C), 129.02 (Ar–C), 127.05 (Ar–C), 126.45 (Ar–C), 126.20 (Ar–C), 24.33 (–CH_2–), 14.95 (–CH_3).

A02: Molecular Formula: $C_{10}H_{15}N_5$·HCl; MW: 241.77 g/mol; Yield: 68 % ; Color: Colorless; Elementel Analysis Calculated ($C_{10}H_{15}N_5$·HCl): % C 49.69; H% 6.67; N% 28.97 Found: %C 49.58; H% 6.56; N% 28.84; FT-IR (ATR, cm^{-1}):3301, 3183, 2973, 1626, 1599, 1557, 1528, 1505, 1487, 1378, 1258, 1193, 1055, 862, 824, 737, 613, 538, 488 cm^{-1}; ^{1}H (d_6-DMSO, ppm): δ 9.70 (1H, s, NH), 7.26 (4H, d, NH_2), 7.23 (1H, s, NH), 7.12 (2H, d,, Ar–H), 7.07 (2H, s,, Ar–H), 2.58 – 2.52 (2H, m,-CH_2–), 1.14 (3H, t,-CH_3); ^{13}C (d_6-DMSO, ppm) δ 161.03 (N–C-N), 155.57 (N–C-N), 138.98 (Ar–C), 136.18 (Ar–C), 127.90 (Ar–C), 121.31(Ar–C), 27.60(–CH_2–), 15.80 (–CH_3).

A03: Molecular Formula: $C_9H_{13}N_5O$·HCl; MW: 243.74 g/mol; Yield: 59 %; Color: Colorless; Elementel Analysis Calculated ($C_9H_{13}N_5O$·HCl): C% 44.36; H% 5.79; N% 28.74; Found: C% 44.18; H% 5.62; N% 28.49; FT-IR (ATR,cm^{-1}): 3340, 3299, 3184, 2961, 1634, 1562, 1481, 1382, 1269, 1226, 1114, 1051, 1022, 932, 871, 751, 734, 604, 501 cm^{-1}; ^{1}H (d_6-DMSO, ppm): δ 8.60 (1H, s, NH), 7.68 (1H, d, NH), 7.30 (4H, s, NH_2), 7.14 (2H, s, Ar–H), 7.05 (2H, d, Ar–H), 6.89 (1H, s, NH), 3.83 (3H, s, -OCH_3); ^{13}C (d_6-DMSO, ppm) δ 161.65 (N–C-N), 155.93 (N–C-N), 150.72 (Ar–C-O–), 127.36 (Ar–C), 124.99 (Ar–C), 123.58 (Ar–C), 120.57 (Ar–C), 111.64 (Ar–C), 56.17 (O-CH3).

A04: Molecular Formula: $C_9H_{13}N_5O$·HCl; MW:243.74 g/mol; Yield: 60.8 %; Color: Colorless; Elementel Analysis Calculated ($C_9H_{13}N_5O$·HCl): C% 44.36; H% 5.79; N% 28.74; Found: C% 44.23; H% 5.63; N% 28.68; FT-IR (ATR, cm^{-1}): 3302, 3133, 2972, 1607, 1530, 1487, 1418, 1377, 1295, 1247, 1193, 1168, 1053, 1033, 826, 610 cm^{-1}; 1H (d6-DMSO, ppm): δ 9.55 (1H, s, NH), 7.23 (3H, d, NH_2 ve NH), 7.19 (3H, s, NH_2 ve NH), 7.02 (2H, s, Ar–H), 6.88 (2H, s, Ar–H), 3.71 (3H, s, -OCH_3); ^{13}C (d_6-DMSO, ppm) δ 161.33 (N–C-N), 156.43 (N–C-N), 156.27 (Ar–C-O–), 131.72 (Ar–C), 123.77 (Ar–C), 114.33 (Ar–C), 55.93 (O-CH3)

A05: Molecular Formula: $C_{10}H_{15}N_5O_2$·HCl; MW: 273.76 g/mol; Yield: 69.1 %; Color: Pink; Elementel Analysis Calculated ($C_{10}H_{15}N_5O_2$·HCl): C% 43.88; H% 5.89; N% 25.59; Found: C% 43.72; H% 5.69; N% 25.37; FT-IR (ATR, cm^{-1}): 3298, 3193, 2958, 1627, 1531, 1483, 1377, 1242, 1201, 1155, 1021, 857, 830, 754, 726, 499 cm^{-1}; ^{1}H (d6-DMSO, ppm): δ 9.64 (1H, s, NH), 7.22 (4H, s, NH_2), 7.08 (2H, s, Ar–H), 7.03 (d, 1H, Ar–H), 6.89 (1H, d, NH), 6.81 (1H, dd, NH), 3.72 (6H, d, -OCH_3); ^{13}C (d_6-DMSO, ppm): δ 161.11 (N–C-N), 156.46 (N–C-N), 148.97 (Ar–C-O–), 145.91 (Ar–C-O–) 132.04 (Ar–C), 114.27 (Ar–C), 112.34 (Ar–C), 107.40 (Ar–C), 56.18 (O-CH_3), 55.91(O-CH_3).

A06: Molecular Formula: $C_8H_{10}N_5Cl$·HCl; MW: 248.15 g/mol; Yield: 44.6 %; Color: Colorless; Elementel Analysis Calculated ($C_8H_{10}N_5Cl$·HCl): C% 38.73; H% 4.47; N% 28.23; Found: C% 38.65; H% 4.24; N% 28.15; FT-IR (ATR, cm^{-1}):3340, 3307, 3191, 3127, 2967, 1632, 1598, 1562, 1525, 1477, 1387, 1286, 1248, 1191, 1067, 874, 741, 724, 624, 513 cm^{-1}; ^{1}H (d_6-DMSO, ppm): δ 9.01 (1H, s, NH), 7.64 (1H, d, NH), 7.46 (1H, d, Ar–H), 7.36 (2H, s, NH_2), 7.29 (3H, Ar–H), 7.15 (1H, t, NH); ^{13}C (d_6-DMSO, ppm) δ161.57 (N–C-N), 155.60 (N–C-N), 135.06 (N–Ar-C–C), 129.38 (Ar–C), 127.29 (Ar–C), 126.76 (Ar–C), 126.62 (Ar–C), 125.97 (Ar–C).

A07: Molecular Formula: $C_8H_{10}N_5Cl$·HCl; MW: 248.15 g/mol; Yield: 66.3 %; Color: Colorless; Elementel Analysis Calculated ($C_8H_{10}N_5Cl$·HCl): C% 38.73; H% 4.47; N% 28.23; Found: C% 38.59; H% 4.32; N% 28.17; FT-IR (ATR, cm^{-1}): 3302, 3130, 2975, 1655, 1636, 1606, 1562, 1525, 1486, 1408, 1377, 1274, 1258, 1194, 1089, 1057, 1013, 859, 819, 727, 619, 531, 464 cm^{-1}; ^{1}H (d_6-DMSO, ppm): δ 9.96 (1H, s, NH), 7.40 (6H, NH_2 ve Ar–H), 7.34 (2H, d, NH_2), 7.11 (2H, s, NH_2);^{13}C (d_6-DMSO, ppm) δ161.85 (N–C-N), 155.30 (N–C-N), 138.28 (N–Ar-C), 128,94 (Ar–C), 127.29 (Ar–C), 122,57 (Ar–C).

A08: Molecular Formula: $C_8H_{10}FN_5$·HCl; MW: 294.7 g/mol; Yield: 65%; Color: Light Pink; Elementel Analysis Calculated ($C_8H_{10}FN_5$·HCl): C% 41.48; H% 4.79; N% 30.23 Found: C% 41.17; H% 4.61; N% 30.08; FT-IR (ATR, cm^{-1}):3696, 3296, 3161, 1640, 1562, 1525, 1506, 1481, 1417, 1376, 1218, 1157, 1100, 1012, 923, 865, 830, 772, 746, 709, 606, 577, 527, 484 cm^{-1}; ^1H (d_6-DMSO, ppm): δ 9.79 (1H, s, NH), 7.39–7.30 (5H, m, NH_2), 7.14 (2H, t, Ar–H), 7.08 (2H, s, Ar–H), 6.69 (1H, s, NH); ^{13}C (d_6-DMSO, ppm) δ 163.39 (N–C-N), 161.74 (N–C-N), 159.96 (Ar–C), 157.57 (Ar–C), 155.81 (Ar–C), 135.45 (Ar–C), 135.43 (Ar–C), 123.40 (Ar–C), 123.32.

Synthesis of biguanide Cu(II) complexes

Dicyanamide (0.5 g, 6 mmol) and $Cu(ClO_4)_2.6H_2O$ (1.1 g, 3 mmol) were dissolved in acetonitrile (15 mL). The green-colored solution was stirred for 30 minutes and the amine (6 mmol) was dissolved in acetonitrile (10 mL) and added dropwise. The color of the solution changed with the addition of the amine compound. The reaction was cooled to room temperature after reflux for 24 hours, washed with water and allowed to dry.

$[Cu(A01)_2]\cdot(ClO_4)_2$; MW:668.93 g/mol; Yield: 44%; Color: Purple; Elementel Analysis Calculated ($[Cu(A01)_2]\cdot(ClO_4)_2$): C% 35.80; H% 4.21; N% 20.88 Found: C% 35.64; H% 4.07; N% 20.63; FT-IR (ATR, cm^{-1}): 3439, 3345, 3230, 2967, 2928, 2873, 1659, 1487, 1248, 1190, 1056, 927, 755, 619 cm^{-1}; MALDI-TOF (m/z) Calculated: 471.18, Found: 474.855 $\{[Cu(A01)_2]\}^+$.

$[Cu(A02)_2]\cdot(ClO_4)_2$; MW: 668.93 g/mol; Yield: 68%; Color: Purple; Elementel Analysis Calculated ($[Cu(A02)_2]\cdot(ClO_4)_2$): C% 35.80; H% 4.21 N%, 20.88; Found: C% 35.57; H% 4.13; N% 20.53; FT-IR (ATR, cm^{-1}): 3461, 3362, 3339, 3247, 2969, 2934, 2873, 1645, 1583, 1531, 1503, 1454, 1415, 1366, 1252, 1223, 1090, 1048, 984, 932, 840, 751, 723, 662, 623, 568, 503 cm^{-1}; MALDI-TOF (m/z) Calculated: 470.04, Found: 472.114 $[Cu(A02)_2+2H]^+$.

$[Cu(A03)_2]\cdot(ClO_4)_2$; MW:672.88 g/mol; Yield: 60.8%; Color: Purple; Elementel Analysis Calculated ($[Cu(A03)_2]\cdot(ClO_4)_2$): C% 46.47; H% 5.54; N% 28.53; Found: C% 46.19; H% 5.35; N% 28.42; FT-IR (ATR,cm^{-1}): 3456, 3335, 1667, 1596, 1496, 1462, 1243, 1163, 1085, 1042, 1015, 930, 748, 621, 471 cm^{-1}; MALDI-TOF (m/z) Calculated: 769.10, Found: 769.405 $\{[Cu(A03)_2]\cdot(ClO_4)_2\ 2\ C_2H_5OH\}^+$.

$[Cu(A04)_2]\cdot(ClO_4)_2$; MW:672.88 g/mol; Yield: 56%; Color: Purple; Elementel Analysis Calculated ($[Cu(A04)_2]\cdot(ClO_4)_2$): C% 46.47; H% 5.54; N% 28.53 Found: C% 46.32; H% 5.38; N% 28.42; FT-IR (ATR,cm^{-1}): 3460, 3362, 3336, 2961, 2923, 2901, 2835, 1646, 1588, 1552, 1539, 1506, 1462, 1366, 1300, 1242, 1166, 1083, 1056, 1026, 982, 930, 861, 831, 757, 730, 653, 524 cm^{-1}; MALDI-TOF (m/z) Calculated: 477.15, Found: 476.357 $\{[Cu(A04)_2]–H\}^+$.

$[Cu(A05)_2]\cdot(ClO_4)_2$; MW: 732.93 g/mol; Yield: 48%; Color: Purple; Elementel Analysis Calculated ($[Cu(A05)_2]\cdot(ClO_4)_2$): C% 44.81; H% 5.27; N% 26.13; Found: C% 44.75; H% 5.14; N% 26.02; FT-IR (ATR, cm^{-1}): 3334, 3208, 2934, 2835, 1591, 1495, 1231, 1076, 1015, 617 cm^{-1}. MALDI-TOF (m/z) Calculated: 553.16, Found: 557.319 $\{[Cu(A05)_2]+H_2O]\}^+$.

$[Cu(A06)_2]\cdot(ClO_4)_2$; MW: 681.71 g/mol; Yield: 45%; Color: Purple; Elementel Analysis Calculated ($[Cu(A07)_2]\cdot(ClO_4)_2$): C% 39.64; H% 3.74; N% 28.89 Found: C% 39.52; H% 3.65; N% 28.73; FT-IR (ATR, cm^{-1}): 3582, 3544, 3450, 3363, 3230, 1651, 1577, 1550, 1531, 1464, 1347, 1034, 926, 616 cm^{-1}; MALDI-TOF (m/z) Calculated: 528.07, Found: 528.37 $\{[Cu(L^6)_2\ C_2H_5OH]\}^+$; Calculated: 567.04, Found: 567,248 $\{[Cu(A07)_2]+(C_2H_5OH)+K]\}^+$

$[Cu(A07)_2]\cdot(ClO_4)_2$; MW: 681.71 g/mol; Yield: 66.3%; Color: Purple; Elementel Analysis Calculated ($[Cu(A06)_2]\cdot(ClO_4)_2$): C% 39.64; H% 3.74; N% 28.89 Found: C% 39.52; H% 3.52; N% 28.53; FT-IR (ATR, cm^{-1}): 3364, 3362, 3335, 2989, 2906, 1646, 1589, 1550, 1538, 1487, 1403, 1366, 1273, 1252, 1220, 1088, 1042, 1012, 982, 930, 861, 824, 764, 721, 623, 501 cm^{-1}; MALDI-TOF (m/z) Calculated: 727.97, Found: 727.596 $\{Cu(A06)_2\cdot(ClO_4)_2]_+C_2H_5OH\}^+$; Calculated: 485.04, Found: 485.886 $\{[Cu(L^6)_2]\}^+$; Calculated:211.06, Found: 211.806 $\{[L^6+H]\}^+$

$[Cu(A08)_2]\cdot(ClO_4)_2$; MW: 648.81 g/mol; Yield: 54%; Color: Purple; Elementel Analysis 1646, 1589, 1550, Calculated ($[Cu(A08)_2]\cdot(ClO_4)_2$): C% 29.53; H% 2.79; N 21.52 Found: C% 29.45; H% 2.47; N 21.41; FT-IR (ATR, cm^{-1}): 3462, 3364, 3329, 3115, 1659, 1645, 1555, 1530, 1505, 1412, 1366, 1286, 1210, 1149, 1090, 1050, 1012, 984, 930, 869, 836, 801, 779, 721, 624, 502, 474 cm^{-1}; MALDI-TOF (m/z) Calculated: 611.06, Found: 612,796 $\{[Cu(A08)_2]\cdot(ClO_4)(C_2H_5OH)(H_2O)+H]\}^+$.

Caution: Perchlorate salts and their Cu(II) complexes are potentially explosive and handled carefully in small quantities with no hazards.

Single crystal X-ray diffraction study

Crystal structures of compounds A06 and A07 were determined by single crystal XRD studies. X-ray crystallographic reflection data were collected at ambient temperature (293 K) using the Agilent Supernova diffractometer (Mo–Kα irradiation λ = 0.71073 Å). By using Olex2, the structures were solved by SHELXT and refined with SHELXL.[20,21] The crystal structures were solved using the direct method and by collecting all reflections over the refinement F^2 process. The hydrogen atoms attached to the carbon atoms were placed in the calculated positions using the binding model and refined by the temperature factor.

AChE and BChE inhibition studies

The inhibitory activities of synthesized biguanide compounds on cholinergic enzymes (AChE and BuChE) were determined using the Ellman Method.[22] Enzyme solution 0.22 units/mL, donepezil, galantamine and synthesized compounds were studied at 1×10^{-4}–1×10^{-7} M concentrations. Solutions of donepezil and galantamine as standard inhibitors, synthesized compounds in DMSO: water mixture (2:8) were prepared. 140 μL of phosphate buffer (pH: 7.4), different concentrations of the compounds (20 μL) and AChE or BChE (20 μL/well) were added to each well (96 well-late) and incubated at 25 °C for 10 minutes. The chromatographic reagent DTNB [5,5-dithio-bis(2-nitrobenzoic acid)] (3 mM, 20 μL/well) and the substrates acetylthiocholine iodine (ATCI) (3 mM, 20 μL/well) or butrylthiocholine iodine (BTCI) (3 mM, 20 μL/well) were added to the enzyme-inhibitor mixture after the formation of 3 mM, 20 μL/well, in the enzyme-inhibitor mixture and measured in 412 in the enzyme-inhibitor mixture. It was determined by preparing an identical solution of the enzyme without compounds as a control. Control and inhibitor readings were corrected by blank reading. All operations were repeated three times. The concentrations of the samples that inhibited the degradation of the substrate (acetylcholine or butyrylcholine) by 50% (IC_{50}) were determined using the excel program by linear regression analysis between percent inhibition and sample concentration.

Antimicrobial activity studies

Antimicrobial properties of the compounds were screened against microorganisms (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium* and *Candida albican* strains). Muller Hilton Broth (MHB) by inoculation into Maller Hilton Broth (MHB) was achieved by incubating at °C for 18 hours. Müller Hilton Agar (MHA) was used as the medium for antimicrobial activity and Malt Extract Agar (MEA) was used for yeast strain.

Strains were inoculated into sterile petri dishes, standardized with 0.5 McFarland standard, and incubated for 1 hour at 37±1 °C.[23,24] Disc containing DMSO and Amikacin (AK: 30 μg) and Gentamicin (CN: 10 μg) were used for control. The antimicrobial activity of the synthesized compounds was determined using the Kirby-Bauer Disk Diffusion Method. 12.50 mg/mL of the synthesized compounds were dissolved with 10% DMSO and absorbed at a concentration of 25 μL on discs made of 6 mm diameter empty sterile Whatman papers. Discs prepared on the cultures of bacteria on MHA and yeast strains on MEA were placed. In order to determine the inhibition zones, it was incubated at 37±1 °C for 18–24± 2 hours.[23–25] The study was performed in three repetitions and the mean values were given.

Minimum inhibition concentration

In order to determine the MIC values of the synthesized compounds, they were dissolved with 10% DMSO as 12.5 mg/mL, 1.25 mg/mL and 0.125 mg/mL, and their minimum effect values (MIC) on gram positive (+) and gram negative (−) bacteria strains were determined.

2. Results and Discussion

In this work, a series of biguanide hydrochloride salts (**A01**–**A08**) and their Cu(II) complexes ($[Cu(A^{01-08})_2]\cdot(ClO_4)_2$) were synthesised. Synthesis reactions of the compounds is shown in Figure 2. Biguanide hydrochloride salts were obtained as a result of the reaction of dicyandiamide and substituted aniline derivatives in the acidic conditions. The structure of biguanide salts were characterized by $^1H/^{13}C$ NMR, FTIR spectroscopies and elemental analysis. The Cu(II) complexes were synthesized in one pot reaction of dicyandiamide, substituted aniline and $Cu(ClO_4)_2.6H_2O$ in acetonitrile. The compounds were obtained in good yields and purities. The structures of Cu(II) complexes were determined by FTIR, elemental analysis and MALDI-TOFF mass spectra.

2.1. Characterization of the compounds

The FT-IR spectra of the synthesized compounds were obtained in the range of 4000–450 cm^{-1} to investigate the characteristic bond stretching's. The FT-IR spectra of the all compounds are given in the supplementary documents (**Figure S1**–**S16**). The

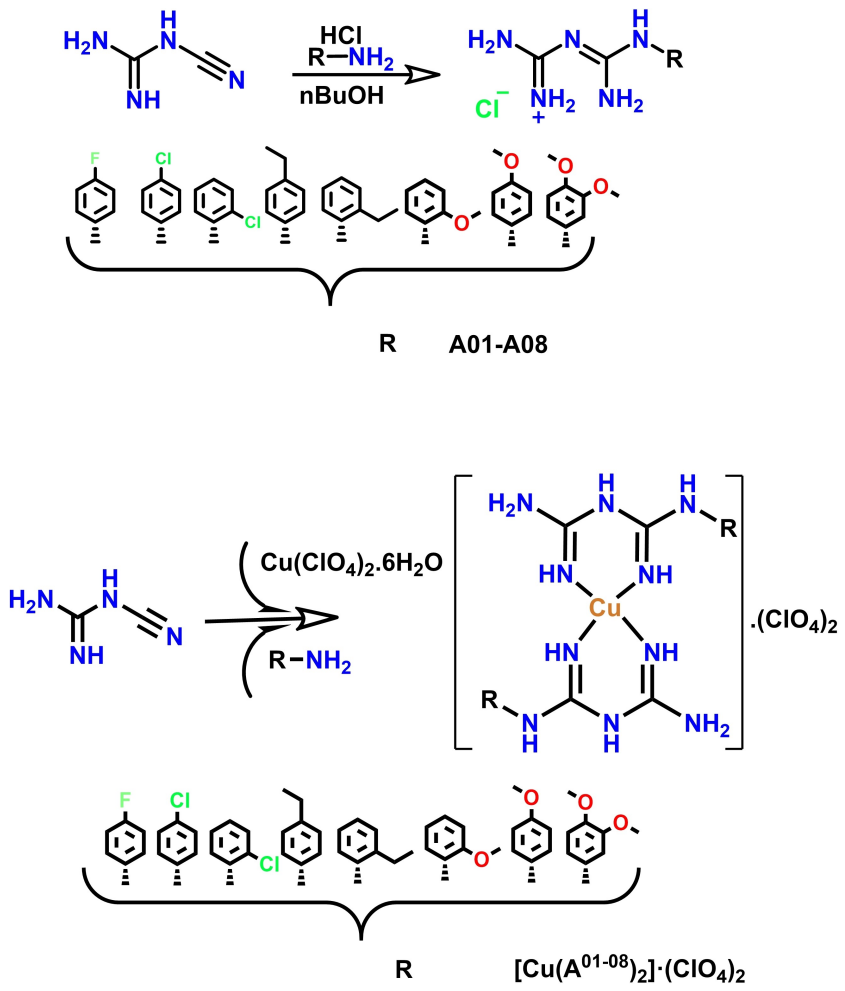


Figure 2. Synthesis scheme of synthesized metal complexes.

characteristic bond stretching's in the FT-IR spectra of the synthesized compounds were examined and tabulated in Table 1. Stretching peaks seen at 3100–3400 cm^{-1} range in the FT-IR spectra of the compounds are due to the υ(N–H). The υ(C≡N) vibrational band of the nitrile group observed at approximately 2160 cm^{-1} in the starting dicyandiamide, was not absent in the compounds showing the nucleophilic addition of the anile nitrogen atom to the nitrile group of the dicyandiamide. Vibration bands at around 2920 and 2870 cm^{-1} can be assigned to the υ(C–H) group stretching's. In the spectra of **A01**–**A08**, υ(C=N) stretching's were observed at 1630–1640 cm^{-1}. The absence of vibrations of the nitrile group in the starting compound in the spectra of the A01-A08 compounds, instead the observation of C=N stretching vibrations, indicates that the compounds in question were synthesized.

The FT-IR spectra of the Cu(II) complexes showed peaks in the range of 2900–3400 cm^{-1} which were interpreted as asymmetric and symmetric υ(N–H) vibrations. The relatively sharp peaks in the range of 1660–1610 cm^{-1} are assigned to the υ(C=N) stretching's. In the spectra of the complexes, characteristic perchlorate stretching's υ(ClO_4^-) were seen at around 624 and 1056 cm^{-1}, confirming the presence of perchlorate anions as counter ions in the structures. Moreover, perchlorate stretching peaks do not show any splitting which suggest no-coordination bond occur between the perchlorate anion and Cu(II). When the FT-IR spectral data of all compounds are examined, the characteristic peaks support the proposed structures of the compounds.

$^1H/^{13}C$ NMR spectra of the compounds **A01**–**A08** were taken and spectra of the compounds are provided in the supplementary documents (**Figure S17**–**S24**). ^1H-NMR spectra of the compounds are differing only in the signals due to the substitutes in the phenyl ring. In the spectra of the compounds, the aromatic phenyl group protons were observed at 7.12–7.00 ppm range. Singlet signals at around 7.20-7.30 ppm and 9.64 ppm due to the –NH_2 and –NH–Ph group protons, respectively. The ^1H-NMR signals of the compounds A01-A03 are in well agreement with the previous reports. When ^{13}C NMR of the compounds are examined, the two biguanide carbon atom signals were seen at around 160–165 ppm range. Rest of the carbon atoms were observed at 120–160 ppm range. The number of carbon atom signals are in well agreement with their proposed structures. Both ^1H and ^{13}C NMR spectra of the compounds revealed that there are significant impurities in the samples.

MALDI TOF mass spectra of Cu(II) complexes were performed and all spectra are given in Supplementary documents (Figs. S25–S32). In the spectra of the complexes, complex ion $[Cu(L)_2]^+$ peaks were observed for each complex. Finally, in order to verify the elementel composition of all compounds were investigated by microanalysis (C, H and N). The experimental data showed that there was a good correlation between the calculated and experimental values.

2.2. Crystal structures of A06 and A07

Single crystals of compounds A06 and A07 were grown from recrystallisation in ethanol. Crystal structures of the compounds were solved in triclinic unit cell with $P2_1/n$ space group. Crystal structures of A06 and A07 are similar differing in the substitüe groups in the phenyl rings. In the structures of both compounds, the asymmetric unit contains a biguanidinium cation with +1 charge and a chloride as a counter ion. Crystal structures of the compounds are shown in Figure 3a and 3c. For both compounds, the C–N bond distances in the biguanidine unit are in the range of 1.316(3) and 1.344(3) Å, this indicates π-electron delocalization through the biguanide unit. In the crystal structure, two cationic guanidinium cations forms a dimeric structure with two concordant N1–H····N3 hydrogen bonds as shown in Figure 3b and 3d. The -NH_2 and -NH groups in biguanidinium cations are involved in hydrogen bonds with chloride ions. These hydrogen bonds observed in the crystal structure form a supramolecular hydrogen bond network. The crystal packing diagrams of the compounds emphasising the hydrogen bonds are provided in the supplementary documents (Figure S33–S34).

2.3. AChE and BuChE enzyme inhibition properties

Biguanidine-based compounds A01–A08 with different functional groups were synthesized and the inhibitory activities of AChE and BuChE were evaluated using the Ellman Colorimetric

Table 1. Characteristic FT-IR peak assignments for compounds A01-A08.

	υ(C≡N)	υ(N–H)	$υ(C–H)_{Ar}$	$υ(C–H)_{alph}$	υ(C=N)	υ(C=C)
Dicyandiamide	2197	3155	–	–	1663	–
A01	–	3296	3178	2972	1634	1481
A02	–	3301	3183	2973	1626	1487
A03	–	3340	3184	2961	1634	1481
A04	–	3302	3133	2972	1607	1487
A05	–	3298	3193	2958	1627	1483
A06	–	3307-3340	3191-3127	–	1632	1477
A07	–	3302	3130	–	1655	1486
A08	–	3296	3161	–	1640	1481

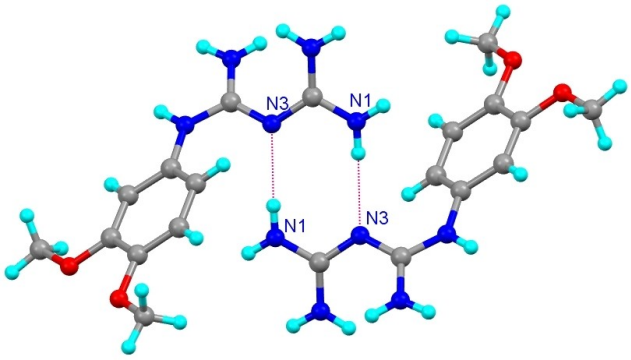


Figure 3. a) Crystal structure of A07 (thermal ellipsoid plotted with a probability of 50%). b) Hydrogen bond dimer in the structure of A07 c) Crystal structure of A06 (thermal ellipsoid drawn with a probability of 50%). d) Hydrogen bond dimer in the structure of A06.

test. According to the results obtained, all synthesized compounds showed inhibitory activity against both AChE and BuChE enzymes. Enzyme inhibition IC_{50} values for compounds A01–A08 are given in Table 2. The highest inhibition activity against AChE enzyme was obtained for compound A02 with an IC_{50} value of 94.18 ± 3.36 μM. All the other compounds exhibited inhibitory activity, with IC_{50} values between 223.93–356.22 μM. The highest inhibition activity against BChE enzyme were seen for compound A03 (IC_{50}: 87.80 ± 0.18 μM). The lowest inhibitory activity against the BChE enzyme is A01 compound with a value of 1819.18 ± 1.52 μM. As compared with donepezil and galantamine, all biguanide compounds showed considerably lower inhibitory activity. When the substituent groups in the compounds are evaluated within themselves; when A01–A02 compounds with an ethyl group attached in the different positions of the phenyl ring are compared, the A02 compound having an ethyl group in the para position exhibited the higher inhibitory activity towards AChE. Although A01 and A02 compounds exhibit different inhibition against AChE enzyme, their selectivity (0.165–0.168, respectively) is almost the same, which is the lowest selectivity value among the synthesized compounds.

Comparing the compounds A03–A05 having methoxy ($-OCH_3$) group(s) in their structure, the compound A03 with a methoxy group at the *o*-position exhibited the higher inhibition activity (IC_{50}: 236.25 ± 0.80 μM) against the AChE enzyme. Almost equivalent results were observed for the compounds containing *p*- and 3,4-methoxy groups against the AChE enzyme (356.22 ± 5.05 and 273.95 ± 3.09 μM, respectively). The highest inhibition activity against BChE enzyme (IC_{50}: 87.80 ± 0.18 μM) were observed for compound A03. When A06-A08 compounds containing halogen groups in their structure show similar inhibition activity against AChE enzyme (317.14 ± 1.41, 268.28 ± 3.19 and 223.93 ± 3.15 μM, respectively), the highest selectivity belongs to A06 compound with a value of 0.191. The highest inhibition activity against BuChE enzyme belongs to compound A07 with IC_{50} value of 494.32 ± 2.80 μM.

Enzyme inhibition IC_{50} values for compounds $[Cu(A^{01-08})_2]\cdot(ClO_4)_2$ are given in Table 3. Cu(II) complexes of biguanide ligands showed dramatically higher AChE and BuChE enzyme inhibitory activity than their ligands. AChE enzyme inhibitory activity of compounds $[Cu(A01)_2]\cdot(ClO_4)_2$ (IC_{50} 21.29 ± 0.95 μM), $[Cu(A07)_2]\cdot(ClO_4)_2$ (IC_{50}: 23.19 ± 0.37 μM), $[Cu(A06)_2]\cdot(ClO_4)_2$ (IC_{50}: 21.99 ± 2.63 μM) and $[Cu(A08)_2]\cdot(ClO_4)_2$ compound (IC_{50}: 25.66 ± 0.12) are comparable to that of standard drug donepezil (IC_{50}: 18.54 ± 1.03 μM). The highest

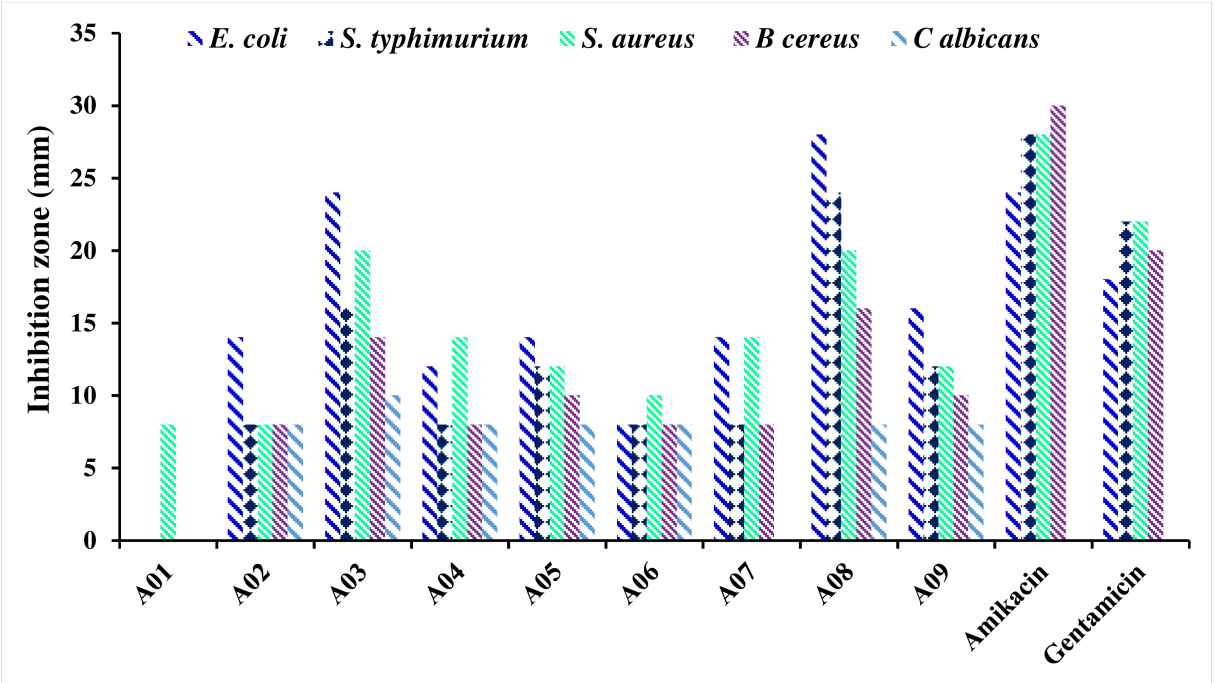
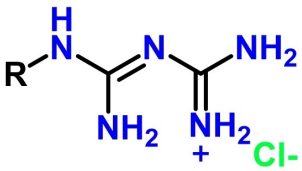
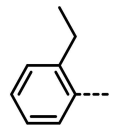
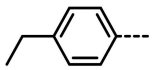
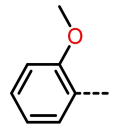
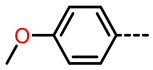
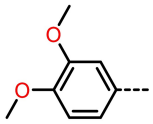
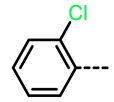
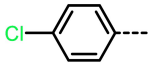
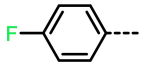


Figure 4. Antimicrobial activities of the biguanide compounds A01–A09 (mm).

Table 2. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme inhibition parameters of biguanide compounds.



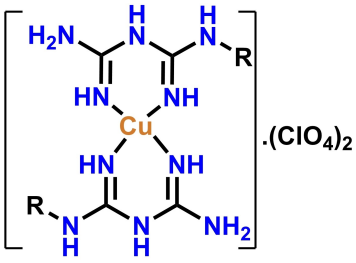
Compounds	R	IC_{50} (μM) EeAChE (μM)	eqBuChE (μM)	Selectivity EeAChE/EqBuChE
A01		281.45 ± 3.24	1819.18 ± 1.52	0.165
A02		94.18 ± 3.36	560.91 ± 0.63	0.168
A03		236.25 ± 0.80	87.80 ± 0.18	2.691
A04		356.22 ± 5.05	966.27 ± 6.76	0.369
A05		273.95 ± 3.09	180.77 ± 1.14	1.516
A06		317.14 ± 1.41	1659.77 ± 2.8	0.191
A07		268.28 ± 3.19	494.32 ± 2.80	0.543
A08		223.93 ± 3.15	895.70 ± 7.28	0.25
Donepezil		18.54 ± 1.03	15.29 ± 0.85	1.212
Galantamin		1.90 ± 0.75	6.42 ± 0.94	0.295

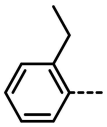
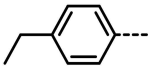
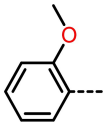
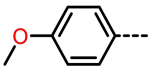
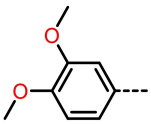
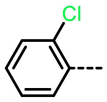
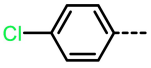
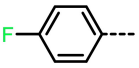
inhibition activity against BuChE enzyme was obtained for compound $[Cu(A03)_2]\cdot(ClO_4)_2$ (IC_{50} 32.61 ± 1.14 μM), while the lowest inhibition activity belongs to $[Cu(A05)_2]\cdot(ClO_4)_2$ (IC_{50} 269.29 ± 0.77 μM).

2.4. Antimicrobial properties

Antimicrobial activities of the synthesized biguanide compounds and their Cu(II) complexes were investigated against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli, Salmonella typhimurium* and *Candida albicans* strains. Microorganism used in this study were exposed to the synthesised compounds at 12.50 mg/mL concentrations. The inhibition zones are shown in Figures 4 and 5. In the study, it was determined that the compounds showed inhibition zones of 8–28 mm diameter against gram-positive and gram-negative microorganisms. The DMSO solution used as a control had no effect against the strains. It was found that compound A08 showed the highest inhibition zone (28 mm) against the microorganisms used. It was determined that the best inhibition effect for gram-negative strains was shown against E coli strain, with compound A08 showing the best effect with 28 mm inhibition zone. No effect was observed against C. albicans strain in the study. It is thought that these different findings may be due to the unique cell wall structure of each strain and the different resistance characteristics of microorganisms. In addition, a slightly weaker effect of the compounds was found compared to the antibiotics used. Antimicrobial activity of the complexes at 12.50 mg/mL concentration showed 8–14 mm diameter inhibition zones against gram-positive and gram-negative microorganisms. The complexation of the ligands with Cu(II) did

Table 3. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme inhibition parameters for Cu(II) complexes.



Complexes	R	IC_{50} (mM) EeAChE (μM)	eqBuChE (μM)	Selectivity EeAChE/EqBuChE
$[Cu(A01)_2]\cdot(ClO_4)_2$		21.29 ± 0.95	34.48 ± 1.00	0.635
$[Cu(A02)_2]\cdot(ClO_4)_2$		44.04 ± 0.10	69.00 ± 0.29	0.638
$[Cu(A03)_2]\cdot(ClO_4)_2$		28.41 ± 0.85	32.61 ± 1.14	0.871
$[Cu(A04)_2]\cdot(ClO_4)_2$		30.47 ± 2.01	50.40 ± 1.15	0.604
$[Cu(A05)_2]\cdot(ClO_4)_2$		82.53 ± 0.20	269.29 ± 0.77	0.590
$[Cu(A06)_2]\cdot(ClO_4)_2$		21.99 ± 2.63	258.19 ± 0.56	0.085
$[Cu(A07)_2]\cdot(ClO_4)_2$		23.19 ± 0.37	67.35 ± 5.35	0.344
$[Cu(A08)_2]\cdot(ClO_4)_2$		25.66 ± 0.12	51.23 ± 0.05	0.501
Donepezil		18.54 ± 1.03	15.29 ± 0.85	1.212
Galantamin		1.90 ± 0.75	6.42 ± 0.94	0.295

not increase the antimicrobial activity. Among the complexes, complex $[Cu(A08)_2]\cdot(ClO_4)_2$ exhibited the highest antimicrobial activity against E. coli strain with 28 mm inhibition zone.

2.5. Minimum inhibition concentration (MIC)

The minimum inhibition concentrations (MIC) of all compounds on microorganism strains are given in Tables 4 and 5. It was determined that the compounds showed predominantly 12.5–0.125 mg/mL antimicrobial activity on microorganism strains. The determined MIC concentration was determined by considering the mechanism of action of antibiotics. It was determined that the best result against microorganisms was the MIC value of 0.125 mg/mL. It was determined that all complexes, except A01, A02 and A06 compounds at 12.5 mg/mL had an effect against C. albicans strains. It was determined that the best results were obtained from the compounds A02, A07 and A08 with MIC value of 0.125 mg/mL. Complex $[Cu(A05)_2]\cdot(ClO_4)_2$ showed antimicrobial activity against *E. coli* with MIC value of 0.125 mg/mL. While compound A01 had a MIC concentration of 12.5 mg/mL against sarius, it was determined that the compound [Cu(A01)2]·(ClO4)2 had an MIC almost 10 times lower. Compound A02 1.25 mg/mL against *C. albicans* strain it was observed that this concentration increased 10-fold in the metal complex when exhibiting MIC.

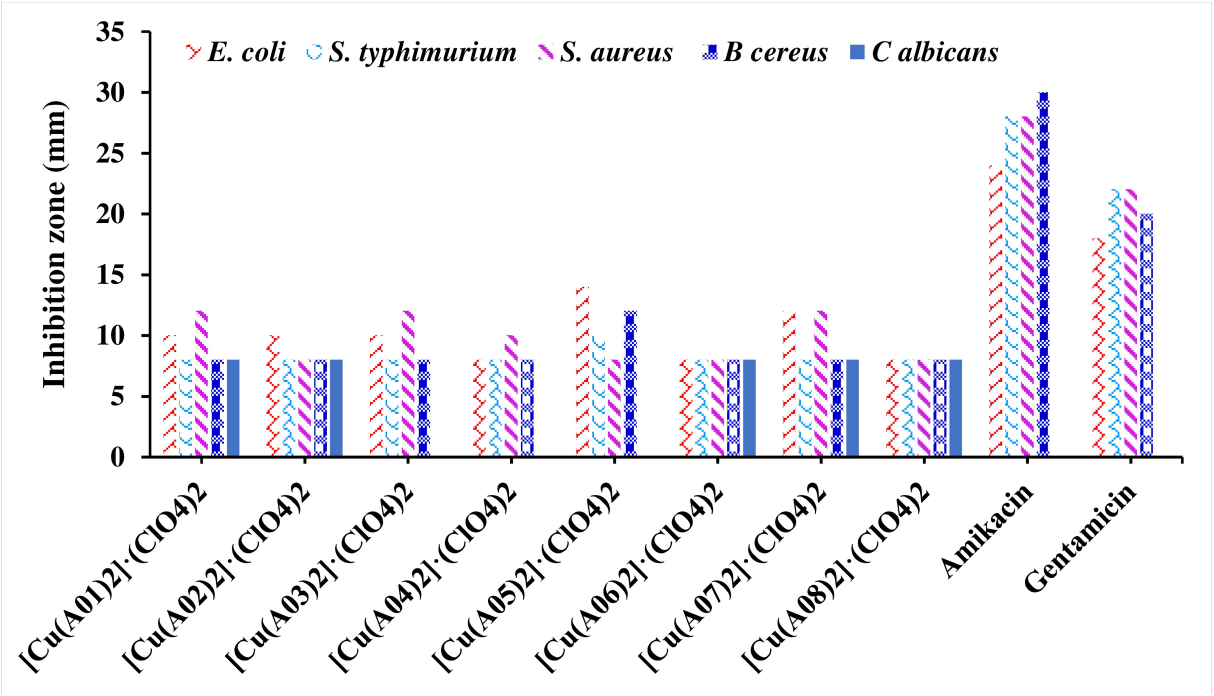


Figure 5. Antimicrobial properties of complexes $[Cu(A^{01-08})_2]\cdot(ClO_4)_2$

Table 4. MIC values of A01-A09 compounds in gram-negative strains.

MIC (mg/mL) Compound	*E. coli*	*S. typhimurium*	*S. aureus*	*B. cereus*	*C.albicans*
A01	0	0	12.5	0	0
A02	0.125	1.25	0.125	1.25	1.25
A03	1.25	12.5	1.25	12.5	12.5
A04	1.25	12.5	12.5	12.5	12.5
A05	12.5	12.5	12.5	12.5	12.5
A06	1.25	12,5	1.25	12.5	0
A07	0.125	1.25	0.125	1.25	12.5
A08	0.125	1.25	1.25	1.25	12.5
Amikacin[26,27]	0.002	0.008	0.004	4	ND
Gentamicin[26,27]	0.016	0.032	0.008	0,5	ND

ND: Not Determined.

Table 5. MIC values of $[Cu(A^{01-08})_2]\cdot(ClO_4)_2$ compounds in gram-negative strains.

MIC (mg/mL) Complexes	*E. coli*	*S. typhimurium*	*S. aureus*	*B. cereus*	*C. albicans*
$[Cu(A01)_2]\cdot(ClO_4)_2$	12.5	12.5	1.25	12.5	12.5
$[Cu(A02)_2]\cdot(ClO_4)_2$	1.25	12.5	12.5	12.5	12.5
$[Cu(A03)_2]\cdot(ClO_4)_2$	12.5	12.5	1.25	12.5	0
$[Cu(A04)_2]\cdot(ClO_4)_2$	12.5	12.5	12.5	12.5	0
$[Cu(A05)_2]\cdot(ClO_4)_2$	0.125	1.25	12.5	1.25	0
$[Cu(A06)_2]\cdot(ClO_4)_2$	12.5	12.5	12.5	12.5	12.5
$[Cu(A07)_2]\cdot(ClO_4)_2$	1.25	12.5	12.5	12.5	12.5
$[Cu(A08)_2]\cdot(ClO_4)_2$	12.5	12.5	12.5	12.5	12.5
Amikacin[26,27]	0.002	0.008	0.004	4	ND
Gentamicin[26,27]	0.016	0.032	0.008	0.5	ND

ND: Not determined.

Most of the complexes were found to have low MIC values. In the light of the data obtained in the study, it was determined that the ligands and their Cu(II) complexes have antibacterial and antifungal activities.

Compared to the Gentamicin and Amikacin antibiotics used in the study, it is possible that some complexes have higher effects than the inhibition zones of these antibiotics, and that results can be obtained by using higher concentrations of complexes with no results. According to the findings, these complex structures are likely to replace antibiotics in intensively used antibiotic treatments against gram-negative and positive strains.

3. Conclusions

In this work, we reported the synthesis, characterization, AChE/BuChE inhibitory and antimicrobial activities of biguanide compounds and their Cu(II) complexes. The structures of the synthesized compounds were characterized by FTIR, elemental analysis, NMR (for biguanide compounds) and mass spectra. Crystal structures of two biguanide derivatives (A06 and A07) were determined by XRD studies. Biguanide compounds showed AChE inhibitory activity and their inhibitory activities are dramatically lower than donepezil and galantamine. Yet, Cu(II) complexes of biguanide ligands showed comparable AChE inhibitory properties. Almost all compounds showed higher AChE inhibitory properties than BuChE. The compounds

were also investigated for their antimicrobial activity towards gram positive (+) and gram negative (−) bacteria. Compounds (12.50 mg/mL) showed important antibacterial properties with inhibition zones of 8–28 mm diameter against gram-positive and gram-negative microorganisms.

Acknowledgements

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Biguanide compounds · Cu(II) complexes · acetyl/butyryl choline esterase inhibitory · Antibacterial properties

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*J. N. Ballı, O. Gungor, Prof. Dr. M. Kose**

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A Series of Biguanide Ligands and Their Cu(II) Complexes: Cholinesterase Inhibitory and Antimicrobial Properties

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Ozge Gungor
Prof. Dr. Muhammet Kose
Julide Nacaroglu Ballı