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Synthesis and characterization of azobenzene derivatives and azobenzene-imidazolium conjugates with selective antimicrobial potential

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ABSTRACT

Five new non-fluorinated and fluorinated azo derivatives, as well as a new series containing five azo-imidazolium conjugates of varying alkyl chain length in the imidazolium head group were prepared and characterized using FTIR and NMR spectroscopy, and elemental microanalysis. Antimicrobial activities of these compounds against pathogenic Gram positive bacterium *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli, Salmonella enterica* serovar Typhimurium, as well as yeasts *Candida* albicans and *Saccharomyces cerevisiae* were evaluated using the disc diffusion method. The azo derivatives and their imidazolium conjugates were selectively active against the tested microorganisms with zone of inhibition diameters ranging from 10mm-21mm. Their biological efficacy was dependent on the chain length and level of fluorine substitution. The presence of long alkyl chains is essential for the azo derivatives to exhibit microbial efficacy. Active azo-imidazolium conjugates on *Staphylococcus aureus* contained 16 and 18 carbons in the alkyl chains while Gram negative strains exhibited a cut-off effect at chain length C16. Difference in antimicrobial behavior of the azo derivatives could be due to the different cell wall between the Gram positive and negative bacteria. Fluorination had no effect on the activity against *E. coli* and *Salmonella*. However, a clear correlation between the level of fluorine substitution and the antimicrobial activity against *Staphylococcus aureus, Candida albicans* and *Saccharomyces cerevisiae* was observed.

1. Introduction

The emergence of multi-drug resistant microbial strains is one of the major public health threats and hence it is essential to design and develop new, efficient and tolerant alternative antimicrobial drugs [1]. According to literature, the development of new antimicrobial agents against Gram-negative bacteria is difficult as their cell membrane forms a strong barrier that prevents the permeation of antibacterial agents [2]. Hence, the majority of the existing antibacterial agents for Gram positive bacteria are ineffective against their Gram negative counterparts. Yeasts from the *Candida* genus also pose serious health risks to humans due to their opportunistic nature [3]. Azo compounds are used in a wide range of technological applications [4,5]. This class of compounds also exhibit various biological activities such as antiseptic [6], antimicrobial [6–14], anti-tumor [15], antineoplastic [16], anti-androgenic [17] and antidiabetic [18] activities. Azo compounds are known to interact with the bacterial cell membranes. Their ability to photoswitch enable them to modulate membrane interaction of peptides [19], and hence they are used as photocontrollers in biological systems [20–24]. Recently, azo-amphiphiles, in particular those containing a cationic ammonium head group were reported to show a high antimicrobial potential [25]. On the other hand, heterocyclic imidazole derivatives, a class of azole compounds play a critical role in many biological systems and functions [26]. They constitute a major class of compounds with medicinal and pharmacological properties [35–38]. The biological efficacy of imidazolium derivatives depends on the counter anions, the head groups and the *N*-substituents on the imidazolium moiety [27]. In both azo and imidazolium compounds, hydrophobicity/lipophilicity was found to increase their biological efficacy [25,28]. Due to the unique chemical and physiological properties of the fluorine atom, fluorination

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could modulate the chemical and biological properties, pharmacodynamics and pharmacokinetics of organo-compounds [29]. Over the years, research and development of fluorine-containing drugs have progressed significantly. The presence of fluorine atom(s) could increase drugs' selectivity and lipophilicity, thus resulted in increased antimicrobial activities [30]. Hence, we are interested to investigate the antimicrobial potency of compounds comprising azo, imidazolium and fluorine components. To the best of our knowledge, there is no reports on the antimicrobial activities of fluorinated azo compounds and azo-imidazolium conjugates. Therefore, in the present work, our aim is to synthesize new azo derivatives of different fluorine substitution, and azo-imidazolium conjugates with varying chain length on the ionic imidazolium head group. These compounds were assayed against pathogenic Gram positive bacterium *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli, Salmonella enterica* serovar Typhimurium, as well as yeasts *Candida* albicans and *Saccharomyces cerevisiae* to evaluate their antimicrobial potential.

2. Experimentals

2.1. Materials and methods

Reagents and chemicals used in this research were of analytical grade and used as procured. A Stuart SMP10 melting point apparatus was used to measure the melting points of the compounds. FTIR spectra were recorded on a Perkin Elmer 2000 spectrophotometer and NMR spectra were recorded on a Bruker ADVANCE 500MHz spectrophotometer. Elemental analyses were carried out by using a Perkin Elmer 2400 LS series CHNS/O analyser.

2.2. Synthesis

All azo derivatives were synthesised following the steps as depicted in Scheme 1 [4]

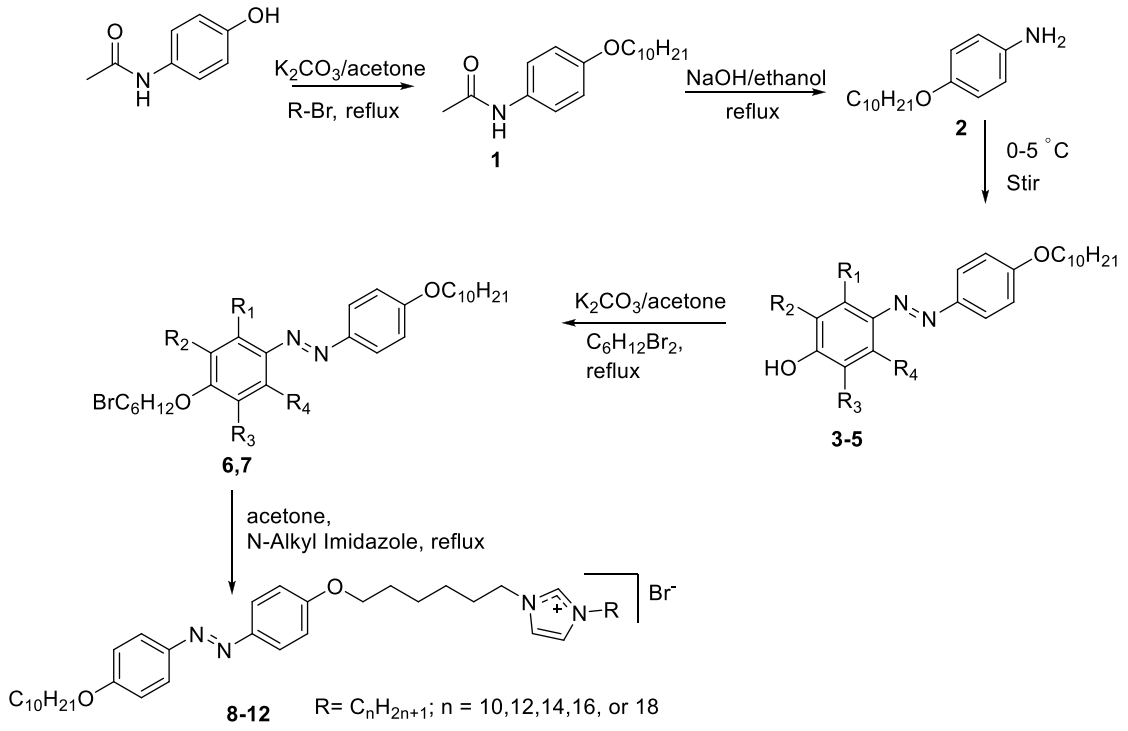
2.2.1. Synthesis of N-*[4-(decyloxy)phenyl]acetamide,* ***1***

To a 250-ml round bottom flask, 4-acetaminophenol (5.0 g, 33.1 mmol) was dissolved in acetone (100mL). 1-Bromodecane (36.4 mmol, 8.05g), and K_2CO_3 (59.4mmol, 13.72g) were then added. The reaction mixture was refluxed for 24 hours. After the reaction was complete, excess solvent was removed using a rotatory evaporator. Excess K_2CO_3 was removed by washing the crude product with dichloromethane (DCM). The solid was filtered and the residue was dried. The sticky, whitish solid precipitate was then purified with n-hexane.

Yield: 56.37 %. FTIR (KBr) υ/cm^{-1}: 3320, 3140 (N-H), 2923, 2850 (C_{sp3}-H), 1661 (C=O), 1607 (C=C), 1236 (C-O). ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.88 (t, 3H, J = 7 Hz, CH_3), 1.35 (m, 12H, CH_2), 1.46 (m, 2H, CH_2CH_3), 1.69 (m, 2H, OCH_2CH_2), 1.87 (t, 3H, J = 7.3 Hz, $OCCH_3$), 3.91 (t, 2H, J = 7 Hz, OCH_2), 6.84 (d, 2H, J = 8.9 Hz, Ar-H), 7.09 (s, 1H, NH), 7.36 (d, 2H, J = 10 Hz, Ar-H).

2.2.2. Synthesis of 4-*(decyloxy)aniline,* ***2***

To a 250-ml round bottom flask containing compound **1** in ethanol (80ml)**,** an aqueous solution of sodium hydroxide (22.4 equiv.) was added. The reaction mixture was refluxed for 24 h. Subsequently, excess solvent was removed using a rotatory evaporator. The crude product was washed with distilled water and recrystallized from ethanol. Yield: 91.10 %. FTIR (KBr) υ/cm^{-1}: 3381, 3310 (N-H), 2920, 2852 (C_{sp3}-H), 1515 (C=C), 1247 (C-O). ^{1}H NMR



3,6: R1= R2 = R3= R4= H
4,7: R1= R4= H; R2= R3= F
5: R1= R2 = R3= R4= F
8-12: R1= R2 = R3 = R4= H

Scheme 1. Synthesis routes toward the formation of azo derivatives, **3-12**.

(500 MHz, $CDCl_3$) δ/ppm: 0.88 (t, 3H, J = 7 Hz, CH_3), 1.33 (m, 12H, CH_2), 1.44 (m, 2H, CH_2CH_3), 1.73 (m, 2H, OCH_2CH_2), 3.88 (t, 2H, J = 7 Hz, OCH_2), 6.24 (s, 2H, NH_2), 6.79 (d, 4H, Ar-H).

2.2.3. Synthesis of (E)-4-((4′-(decyloxy)phenyl) diazenyl)phenols/fluorinatedphenols, **3-5**

A 250-mL round bottom flask containing a mixture of compound **2** (3.33g, 13.4 mmol) and hydrochloric acid (3ml) in acetone (100ml) was placed in an ice-water bath between 0-5 °C and stirred for 1 h. A cold aqueous solution of phenol (1.0 equiv.), sodium carbonate (1.0 equiv.) and sodium hydroxide (1.0 equiv.) was then added dropwise. The reaction mixture was stirred for 1-2 h while maintaining the temperature between 0-5 °C. Subsequently, the ice-water bath was removed and the reaction mixture was stirred overnight at room temperature. The reaction mixture was then neutralized with an aqueous solution of sodium hydroxide upon which a reddish brown precipitate was formed. The crude product was filtered, washed with distilled water, and purified using column chromatography in DCM/Petroleum Ether (80:20). Compounds **4** and **5**, were synthsised following the same procedure, except that phenol was replaced with 2,6-difluorophenol or 2,3,5,6-tetrafluorophenol.

3: Yield: 52.9 %. M.p: 103-106 °C. FTIR (KBr) υ/cm^{-1}: 3166 (O-H), 2919, 2850 (C_{sp3}-H),1600 (C=C), 1497 (N=N), 1250 (C-O), 1150 (C-N). ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.88 (t, 3H, J = 6.9 Hz, CH_3), 1.27 (m, 12H, CH_2), 1.46 (m, 2H, $OCH_2CH_2CH_2$), 1.81 (m, 2H, OCH_2CH_2), 4.01 (t, 2H, J = 7 Hz, OCH_2), 6.91 (d, 2H, J = 8.9 Hz, Ar-H), 6.98 (d, 2H, J = 9 Hz, Ar-H), 7.81 (d, 2H, J = 8.9 Hz, Ar-H), 7.83 (d, 2H, J = 9 Hz, Ar-H)**.** **4**: Yield: 80.3%. M.p: 87-90 °C. FTIR (KBr) υ/cm^{-1}: 3374(O-H), 2920, 2852 (C_{sp3}-H), 1604 (C=C), 1501 (N=N), 1249 (C-O), 1147 (C-N), 1021 (C-F). ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.88 (t, 3H, J = 7 Hz, CH_3), 1.29 (m, 12H, CH_2), 1.46 (m, 2H, OCH_2CH_2 CH_2), 1.81 (m, 2H, OCH_2CH_2), 4.03 (t, 2H, J = 6.6 Hz, OCH_2), 6.99 (d, 2H, J = 9 Hz, Ar-H), 7.53 (d, J = 8.6 Hz, 2H, Ar-H)**,** 7.86 (d, J = 9 Hz, 2H, Ar-H). **5**: Yield: 73%. M.p.: 97-100°C. FTIR (KBr) υ/cm^{-1}: 3383(O-H), 2920, 2853(C_{sp3}-H), 1603(C=C), 1509 (N=N), 1252 (C-O), 1155 (C-N), 1019 (C-F). ^{1}H NMR (500 MHz, CD_3OD) δ/ppm: 0.91 (t, 3H, J = 7 Hz, CH_3), 1.32 (m, 12H, CH_2), 1.49 (m, 2H, CH_2CH_3),1.79 (m, 2H, OCH_2CH_2), 4.07 (t, 2H, J = 8 Hz, OCH_2), 7.03 (d, 2H, J = 9.1 Hz, Ar-H), 7.77 (d, 2H, J = 9 Hz, Ar-H).

2.2.4. Synthesis of (E)-N-(4-bromohexyl)oxy)phenyl)-N′-decyloxy) phenyl/substitutedphenyl) diazenes, **6,7**

In a 250mL round bottom flask, compound **3** (3g, 8.5mmol) and 1,6-dibromohexane (7 equiv.) were dissolved in acetone (80ml). Potassium carbonate (3.0 equiv.) was then added and the reaction mixture was refluxed for 7 h. Subsequently, the solvent was removed using a rotary evaporator and the crude product was recrystalized from methanol to yield compound **6**. Compound **7** was synthsised following the same procedure, except compound **3** was replaced with compound **4**.

6 Yield: 53.42%. M.p.: 96-99 °C. FTIR (KBr) υ/cm^{-1}: 2919, 2850(C_{sp3}-H), 1603 (C=C), 1497 (N=N), 1246(C-O), 553 (C-Br). ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.88 (t, 3H, J = 6.92 Hz, CH_3), 1.33 (m,12H, CH_2), 1.47 (m, 2H, $CH_2CH_2CH_2Br$), 1.53 (t, 4H, $OCH_2CH_2CH_2$), 1.82 (m, 4H, OCH_2CH_2), 1.91 (m, 2H, CH_2CH_2Br), 3.43 (t, 2H, J = 6.8 Hz, CH_2Br), 4.03 (m, 4H, OCH_2), 6.97 (d, 4H, Ar-H), 7.87 (d, 4H,Ar-H). **7** Yield: 50.5%. M.p.: 60- 62 °C. FTIR (KBr) υ/cm^{-1}: 2919, 2850 (C_{sp3}-H), 1604 (C=C), 1470 (N=N), 1259 (C-O), 1031 (C-F), 569 (C-Br).. ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.88 (t, 3H, J = 6.9 Hz, CH_3), 1.31 (m,12H, CH_2), 1.47 (m, 2H, $CH_2CH_2CH_2Br$), 1.53 (m, 4H, $OCH_2CH_2CH_2$), 1.81 (m, 4H, OCH_2CH_2), 1.90 (m, 2H, CH_2CH_2Br), 3.43 (t, 2H, J = 6.8 Hz, CH_2Br), 4.03 (t, 2H, J = 6.6 Hz, OCH_2), 4.21 (t, 2H, J = 6.4 Hz, OCH_2) 6.98 (d, 2H, J = 9 Hz, Ar-H), 7.47 (d, 2H, J = 9.05 Hz, Ar-H), 7.86 (d, 2H, J = 9 Hz, Ar-H).

2.2.5. Synthesis of (E)-N-alkyl-N′-6-(4-(4′-decyloxyphenyl) diazenyl)phenoxy)hexan-1-imidazolium bromides, **8-12**

To a 100 mL round bottom flask containing compound **6** (0.3g, 0.6 mmol) in acetone (40ml), the corresponding *N*-alkyl-imidazole (1.0 equiv.) was added and the reaction mixture was refluxed for 48hrs. Subsequently, the reaction mixture was cooled to room temperature and excess solvent was removed using a rotary evaporator. The crude products were recrystalised from diethyl ether to yield compounds **8**-**12** in pure form.

8 (n = 10) yield: 68.6%. M.p.: 122-124 °C. FTIR (KBr) υ/cm^{-1}: 3069 (C_{sp2}-H), 2922, 2852 (C_{sp3}-H), 1601 (C=C),1499, 1473 (N=N), 1243 (C-O), 1147 (C-N). Anal. calcd. $[C_{41}H_{65}BrN_4O_2]^+$: C = 67.84, H = 9.03, N = 7.72; Found: C = 66.99, H = 9.05, N = 7.74. ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.88 (m, 6H, CH_3), 1.28 (m, 26H, CH_2), 1.45 (m, 4H, $OCH_2CH_2CH_2$), 1.57 (m, 2H, $NCH_2CH_2CH_2$), 1.82 (m, 4H,OCH_2CH_2), 1.90 (m, 2H, NCH_2CH_2), 1.98 (m, 2H, NCH_2CH_2), 4.03 (m, 4H, OCH_2), 4.31 (t, 2H, J = 7.5 Hz, NCH_2), 4.40 (t, 2H, J = 7.4 Hz, NCH_2), 6.97 (d, 4H, Ar-H), 7.18 (s, 1H, NCH), 7.22 (s,1H, NCH), 7.87 (d, 4H, Ar-H), 10.76 (s,1H, NCH). ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm: 162.27, 161.66, 146.72, 146.01, 137.19, 125.08, 121.37, 121.27, 114.83, (C_{arom}), 68.46, 67.74, 50.36, 50.16, (OCH_2,NCH_2), 31.90, 31.85, 30.31, 30.19, 29.58-29.17, 28.99, 26.29, 26.01, 25.80, 25.17, 22.70, 22.67 (CH_2), 14.14, (CH_3). **9** (n = 12) yield: 80.6%. M.p.: 127-129 °C. FTIR (KBr) υ/cm^{-1}: 3071 (C_{sp2}-H), 2922, 2851 (C_{sp3}-H), 1601 (C=C), 1499, 1468 (N=N), 1243(C-O), 1147 (C-N). Anal. calcd. $[C_{43}H_{69}BrN_4O_2]^+$: C = 68.50, H = 9.22, N = 7.43; Found: C = 68.35, H = 9.56, N = 7.32. ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.87 (m, 6H, CH_3), 1.28 (m, 30H, CH_2), 1.46 (m, 4H, $OCH_2CH_2CH_2$), 1.58 (m, 2H, $NCH_2CH_2CH_2$), 1.82 (m, 4H, OCH_2CH_2), 1.89 (m, 2H, NCH_2CH_2), 1.90 (m, 2H, NCH_2CH_2), 4.03 (m, 4H, OCH_2), 4.31 (t, 2H, J = 7.5 Hz, NCH_2), 4.40 (t, 2H, J = 7.4 Hz, NCH_2), 6.98 (d, 4H, Ar-H), 7.17 (s, 1H, NCH), 7.21 (s,1H, NCH), 7.89 (d, 4H, Ar-H), 10.76 (s,1H, NCH). ^{13}C NMR(125 MHz, $CDCl_3$) δ/ppm: 161.28, 160.98, 146.93, 146.80, 137.89, 124.35, 121.51, 121.39, 114.69, (C_{arom}), 68.37, 67.85, 50.24, 50.03, (OCH_2,NCH_2), 31.90, 30.29, 30.23, 29.59-29.22, 28.99, 28.87, 26.28, 26.03, 25.88, 25.46, 22.68, (CH_2),14.13 (CH_3). **10** (n = 14) yield: 50.6%. M.p: 129-132 °C. FTIR (KBr) υ/cm^{-1}: 3073 (C_{sp2}-H), 2920, 2850 (C_{sp3}-H), 1601(C=C), 1499, 1467 (N=N), 1243 (C-O), 1148 (C-N). Anal. calcd. $[C_{45}H_{73}BrN_4O_2]$+: C = 69.12, H = 9.41, N = 7.16; Found: C = 69.31, H = 9.72, N = 7.18. ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.87 (m, 6H, CH_3), 1.30 (m, 34H, CH_2), 1.46 (m, 4H, $OCH_2CH_2CH_2$), 1.56 (m, 2H, $NCH_2CH_2CH_2$), 1.82 (m, 4H, OCH_2CH_2), 1.87 (m, 2H, NCH_2CH_2), 1.98 (m, 2H, NCH_2CH_2), 4.03 (m, 4H, OCH_2), 4.31 (t, 2H, J = 7.5 Hz, NCH_2), 4.40 (t, 2H, J = 7.4 Hz, NCH_2), 6.98 (d, 4H, Ar-H), 7.17 (s, 1H, NCH), 7.21 (s,1H, NCH), 7.93 (d, 4H, Ar-H), 10.74 (s,1H, NCH). ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm: 161.24, 160.94, 146.99, 146.87, 137.85, 124.32, 121.52, 121.41, 114.67, (C_{arom}), 68.35, 67.83, 50.29, 50.09, (OCH_2,NCH_2), 31.93, 31.90, 30.31, 30.29, 30.23, 29.69-29.00, 28.87, 26.28, 26.03, 25.90, 25.47, 22.70, (CH_2), 14.14 (CH_3). **11** (n = 16) yield: 48.7%. M.p: 122-124 °C. FTIR (KBr) υ/cm^{-1}: 3073 (C_{sp2}-H), 2921, 2851 (C_{sp3}-H), 1601(C=C), 1499, 1473 (N=N), 1250 (C-O), 1144 (C-N). Anal. calcd. $[C_{47}H_{77}BrN_4O_2]^+$: C = 69.69, H = 9.58, N = 6.92; Found: C = 69.78, H = 10.00, N = 7.08. ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.88 (m, 6H, CH_3), 1.24 (m, 38H, CH_2), 1.47 (m, 4H, $OCH_2CH_2CH_2$), 1.57 (m, 2H, $NCH_2CH_2CH_2$), 1.81 (m, 4H, OCH_2CH_2), 1.91 (m, 2H, NCH_2CH_2), 1.97 (m, 2H, NCH_2CH_2), 4.04 (m, 4H, OCH_2), 4.32 (t, 2H, J = 7.5 Hz, NCH_2), 4.40 (t, 2H, J = 7.4 Hz, NCH_2), 6.99 (d, 4H, Ar-H), 7.17 (s, 1H, NCH), 7.21 (s,1H, NCH), 7.91 (d, 4H, Ar-H), 10.74 (s,1H, NCH). ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm: 161.61, 161.32, 147.01, 146.31, 138.14, 124.67, 121.38, 121.32, 114.81, (C_{arom}), 68.43, 67.91, 50.34, 50.13, (OCH_2,NCH_2), 31.94, 31.91, 30.30, 30.24, 29.71-29.20, 28.99, 28.84,

26.29, 26.02, 25.89, 25.46, 22.70, (CH_2) 14.14 (CH_3). **12** (n = 18) yield:46.25%. M.p: 104-106 °C. FTIR (KBr) υ/cm^{-1}: 3070 (C_{sp2}-H), 2919, 2850 (C_{sp3}-H), 1601 (C=C),1499, 1467 (N=N), 1244 (C-O), 1148 (C-N). Anal. calcd. $[C_{49}H_{81}BrN_4O_2]^+$: C = 70.22, H = 9.74, N = 6.68; Found: C = 70.02, H = 10.02, N = 6.46. ^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm: 0.87 (m, 6H, CH_3), 1.27 (m, 42H, CH_2), 1.45 (m, 4H, $OCH_2CH_2CH_2$), 1.56 (m, 2H, $NCH_2CH_2CH_2$), 1.80 (m, 4H, OCH_2CH_2), 1.91 (m, 2H, NCH_2CH_2), 1.96 (m, 2H, NCH_2CH_2), 4.03 (m, 4H, NCH_2), 4.31 (t, 2H, J = 7.5 Hz, OCH_2), 4.41 (t, 2H, J = 7.4 Hz, OCH_2), 6.97 (d, 4H, Ar-H), 7.18 (s, 1H, NCH), 7.23 (s,1H, NCH), 7.87 (d, 4H, Ar-H), 10.65 (s,1H, NCH). ^{13}C NMR (125 MHz, $CDCl_3$) δ/ppm: 162.09, 161.38, 147.86, 147.29, 138.61, 124.80, 121.29, 121.23, 114.86, (C_{arom}), 68.47, 67.96, 50.37, 50.16, (OCH_2,NCH_2), 31.93, 31.90, 30.32, 30.26, 29.71-29.00, 28.90, 28.83, 26.30, 26.01, 25.90, 25.46, 22.70, (CH_2), 14.12 (CH_3).

2.3. Disc Diffusion Method

The non-fluorinated and fluorinated azo derivatives, **3-7** and the azo-imidazolium conjugates, **8-12** were examined for their antimicrobial activities against five pathogenic microorganisms using the agar well diffusion method. All the pathogenic bacteria were inoculated in nutrient broth and incubated at 37°C for 12-14 hours. The inoculated microbes were spread, and wells were produced on the nutrient agar. The wells were filled with 100 μL of each sample. Agar well diffusion agar was used to assess the antibacterial and antifungal potentials of the synthesized compounds. The tested pathogenic microorganisms *Staphylococcus aureus, Escherichia coli, Salmonella enterica* serovar Typhimurium, *Candida albicans* and *Saccharomyces cerevisiae* were inoculated into the Brain Heart Infusion (BHI) broth and incubated at 37°C for 16 h. The cultures were then diluted with sterile 0.9 % saline solution to achieve turbidity of 0.5 McFarland standard. The Mueller Hinton agar was then inoculated by streaking the test microorganisms using a sterile swab over the entire agar surface. 20 µl of each sample as well as the positive and negative controls were pipetted respectively onto each sterile paper discs (7 mm) which were placed on the agar and the plates were incubated at 37°C for for 24 hours. The zone of inhibition was measured and recorded after the incubation period. Samples at concentrations ranging from at 0.5-2.5mg/mL were prepared by dissolving 0.5-2.5mg of each sample in 1 mL of methanol, except for compounds **7** and **8** which were dissolved in chloroform. The positive controls Vancomycin, ampicillin, streptomycin sulphate and chloramphenicol were prepared at 300 µg/mL. As positive controls, 300 µg/mL vancomycin was used for *Staphylococcus aureus*, 300 µg/mL ampicillin for *Escherichia coli* and *Salmonella enterica* serovar Typhimurium and 300 µg/mL streptomycin sulphate as well as chloramphenicol was used for *Candida albicans* and *Saccharomyces cerevisiae*, respectively.

3. Results and Discussion

3.1. FTIR Spectroscopy

Diagnostic peaks in compounds **3-12** were observed within the expected regions: ν (C_{sp3}-H) at 2919 and 2850 cm^{-1}, ν (C=C) aromatic within 1583-1604 cm^{-1}, ν (N=N) within 1497-1523 cm^{-1} and ν (C-O) ether within 1243-1259 cm^{-1}. The disappearance of the N-H peak at 3381 cm^{-1} and the appearance of O-H peak as well as the N=N peak at 3166 cm^{-1} and 1497 cm^{-1}, respectively indicated the successful formation of the azo derivatives, compounds **3, 4** and **5** (see Fig.1(a)). The formation of compounds **4** and **5** was substantiated by the C-F peak at 1021 cm^{-1}, similar to that reported by Pilati et al. and Gurumurthy et al. [31,32]. On the other hand, upon alkylation of compounds **3** and **4,** the O-H peak at 3166 cm^{-1} disappeared [33] and the presence of the C-Br peak at 553 cm^{-1} confirmed the formation of compounds **6** and **7** as shown in Fig. 1(b). The successful incorporation of the imidazolium moiety into the latter was confirmed by the appearance of the characteristic C-N peak at 1144-1155 cm^{-1} in the FTIR spectra of the azo-imidazolium conjugates, compounds **8-12**, which conformed to the reported values [24]. Stretching vibrations of the *para*-substituted phenyl rings was observed around 845 cm^{-1}. Fig. 1(c) shows the diagnostic peaks in compound **8**.

3.2. NMR Spectroscopy

In order to further elucidate the molecular structures of the synthesised compounds, the ^{1}H-and ^{13}C NMR spectroscopy were employed. Similar spectral characteristics were observed in the ^{1}H NMR spectra of compounds **3, 4** and **5**. The following discussion was based on compound **3**. The NMR spectrum with peak integrations (in red) which reveal the ratio of the numbers of chemically different hydrogen atoms in compound **3** is shown in Fig. 2. A triplet at 0.88 ppm (J = 7.0 Hz) could be assigned to the methyl protons. Signals corresponded to the methylene protons were observed as three mutiplets within 1.27 ppm-1.82 ppm. The oxymethylene protons in these compounds were observed as one triplet at 4.02 ppm (J = 7 Hz). Due to electron withdrawing effect of the oxygen atoms, these protons are more deshielded and hence, their signals are shifted downfield as compared to that of other methylene protons in the terminal chains. In compound **3**, signals attributable to the aromatic protons were observed as four doublets, each integrated to two protons, at 6.90 ppm (J = 8.9 Hz), 6.98 ppm (J = 9 Hz), 7.80 ppm (J = 8.9 Hz) and 7.84 ppm (J = 9 Hz). However, in the difluorinated and tetrafluorinated analogues, compounds **4** and **5**, respectively, the same type of protons gave rise to three doublets at 6.98 ppm (J =9 Hz), 7.52 ppm (J = 8.6 Hz) and 7.85 ppm (J = 9 Hz) in the former, and two doublets at 7.03 ppm (J = 9 Hz), and 7.79 ppm (J = 9 Hz) in the latter. Similar spectral characteristics in terms of chemical shift values and splitting patterns as those discussed for compounds **3** and **4** were observed in the ^{1}H NMR spectra of the alkylated analogues compounds **6** and **7**, except for an additional triplet at 3.43 ppm (J = 6.8 Hz) which could be assigned to the protons attached to the brominated carbon, (-CH_2Br).

Due to close structural resemblance, similar splitting patterns and chemical shift values were observed in the ^{1}H-NMR spectra of compounds **8-12**. The following discussion was based on the spectral data of compound **8** as a representative homologue. Compound **8** showed similar spectral characteristics as those discussed for compound **6**. However, a singlet corresponded to the acidic proton at C2 at 10.76 ppm, two additional triplets at 4.30 (J = 7.5 Hz), and 4.38 ppm (J = 7.4 Hz) ascribable to the methylene protons adjacent to the N1 and N3 as well as two singlets at 7.18 ppm and 7.22 ppm attributable to the N-CH of the imidazolium moiety were observed in compound **8** (see Fig. 3 with peak integration in red).

The formation of the azo-imidazolium conjugates, compounds **8-12** was further confirmed using the ^{13}C NMR spectroscopy. Compound **8** contained 43 carbons but only 29 signals were observed in its ^{13}C NMR spectrum as some of the peaks were overlapped (Fig. 4). The aromatic carbons of the azobenzene moiety were observed within 162.27 ppm – 114.83 ppm. The acidic carbon, C2 of the imidazolium ring was seen at 137.19 ppm while that of the alkenyl carbons were seen at 121.37 ppm and 121.27 ppm. Peaks at 68.46 ppm and 67.74 ppm could be assigned to the oxymethylene carbons, while the two nitrogenated carbons were observed at 50.36 ppm and 50.16 ppm. Other methylene carbons in the alkyl chains and spacer were observed within 31.90 ppm-22.67 ppm. The methyl carbons were observed at 14.14 ppm which was in good agreement with that reported in the literature [34–36].

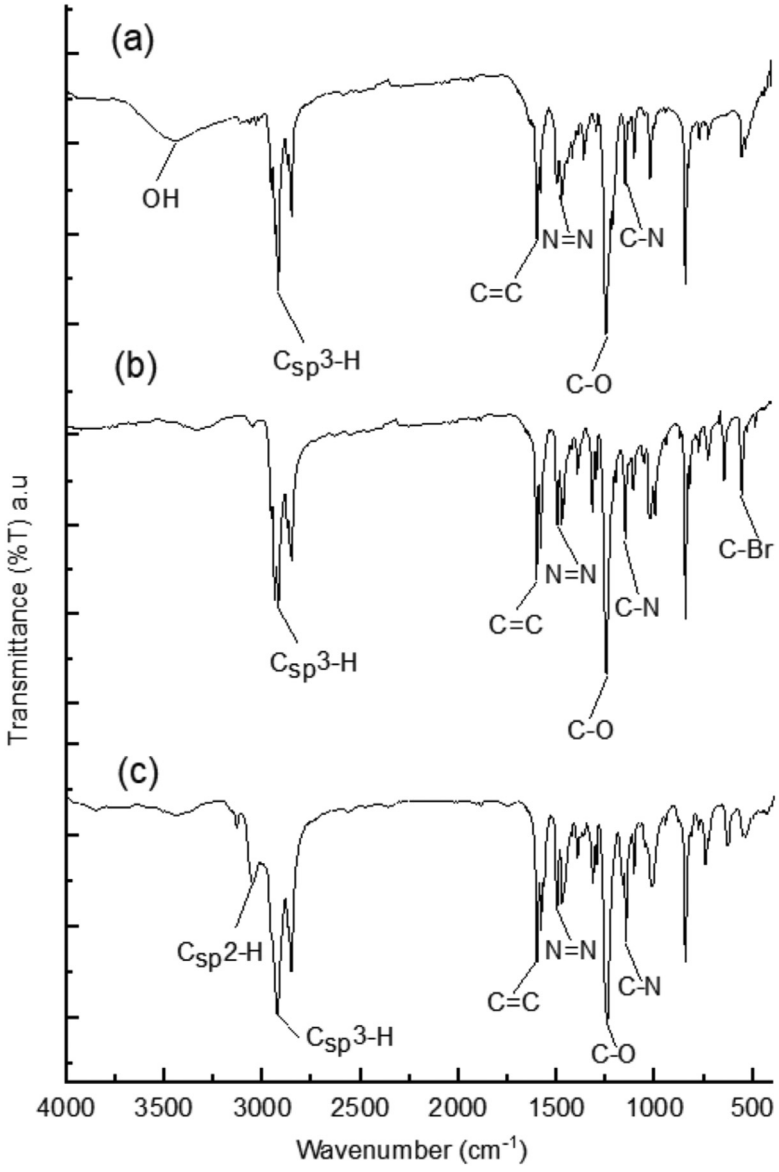


Fig. 1. FTIR spectra of compounds (a) **3**, (b) **6** and (c) **8**.

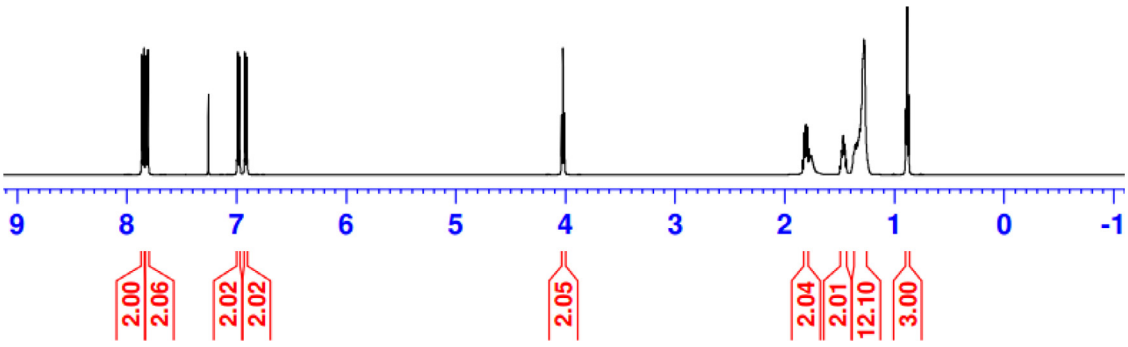


Fig. 2. ^{1}H NMR spectrum of compound **3** (in $CDCl_3$).

3.3. Antimicrobial Activity

The inhibitory effects of azo derivatives and the azo-imidazolium conjugates showed selective activities against the tested microorganisms at 1mg/mL are shown in Table 1. The results were expressed as zones of inhibition as illustrated in Fig. 5. In this study, the potency of these compounds was influenced by the hydrocarbon chain length in the cationic imidazolium head group and level of fluorine substitution on the azo moeity. Long alkyl chains and multiple fluorination are essential in inhibiting the growth of Gram positive bacterium *Staphylococcus aureus*. For azo derivatives, only those with two alkyl chains, compounds **6**

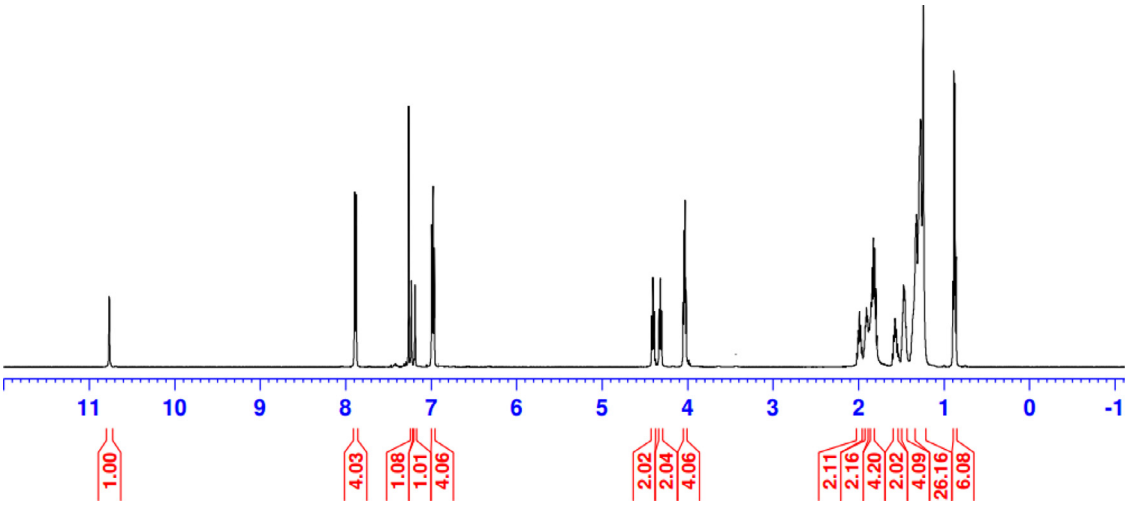


Fig. 3. ^{1}H NMR spectrum of compound **8** (in $CDCl_3$).

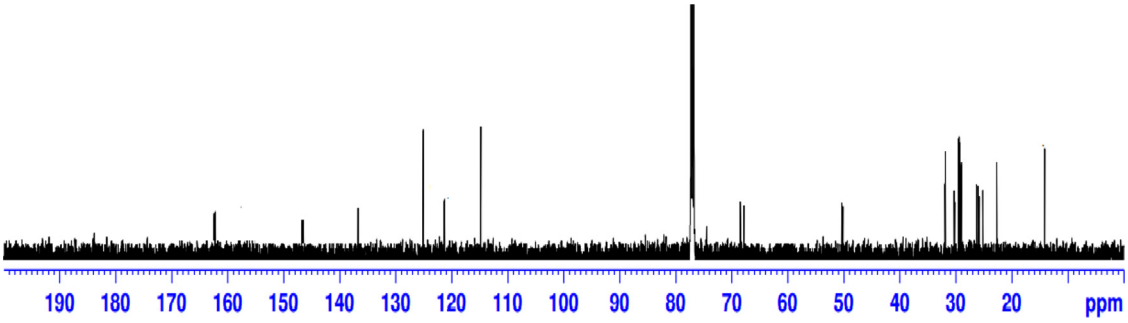


Fig. 4. ^{13}C NMR spectrum of compound **8** (in $CDCl_3$).

Table 1
Zones of inhibition diameters (mm) for compounds **3**-**12** on tested pathogenic Microorganisms.

	Zone of Inhibition diameter (mm)											
Sample	3	4	5	6	7	8	9	10	11	12	*Positive control	Negative control
Concentration (mg/mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.3	0.3
Gram-Positive bacterial												
Staphylococcus aureus	NA	NA	11	14	11	NA	NA	NA	10	11	16[a]	NA
Gram-Negative Bacteria												
Escherichia coli	10	NA	NA	13	14	10	11	10	NA	NA	22[b]	NA
Salmonella	10	NA	NA	12	12	10	10	10	NA	NA	25[b]	NA
Yeast												
Candida albicans	NA	20	21	NA	NA	NA	NA	NA	NA	16	17[c]	NA
Saccharomyces cerevisiae	14	16	21	14	13	17	14	15	15	13	24[d]	NA

Note: NA = Not activities; a = vancomycin; b = ampicillin; c = streptomycin sulphate;
d = chlorampenicol

and **7,** as well as the tetrafluorinated analogue, compound **5** were found to be active against this bacteria. On the other hand, the active azo-imidazolium conjugates, compounds **11** and **12** contained 16 and 18 carbons in the alkyl chain, respectively. It was reported that antibacterial activity is enhanced by hydrophobicity [37]. Molecules that contain hydrophobic alkyl chains are believed to disrupt the cell membranes of microorganisms. The extent of this membrane disruption is affected by chain length. Longer chains may be incorporated into the lipid bilayers of the plasma membrane of bacteria [38].

However, an opposite trend was observed for *E. coli* and *Salmonella*, the two tested Gram negative bacteria in this study. Fluorination had no effect on the activity against these bacteria. No activities were observed in compounds **4** and **5**. These organisms exhibited a cutoff effect at long alkyl chain length as observed for the azo-imidazolium conjugates, compounds **8**-**12**. Compounds **11** and **12** with long alkyl chains were inactive. Difference in antimicrobial behavior of the azo derivatives could be due to the different cell wall between the Gram positive and negative bacteria. The cell wall of Gram-positive bacteria is simple. It is composed of large fractions of negatively charged phospotidylglycerol. The attraction between the cationic head groups of the azo-imidazolium conjugates and the negatively charged bacterial membrane and the integration of the long alkyl chains into the lipid bilayer of the cell membrane could lead to perturbation of plasma membrane and induced cell death. On the other hand, the cell wall of Gram negative bacteria is more complex. It is made up of a cytoplasmic membrane and an outer membrane [39]. The latter contains lipopolysaccharides cross-bridge by divalent cations which stabilize the membrane, and creates a barrier that prevents the permeation

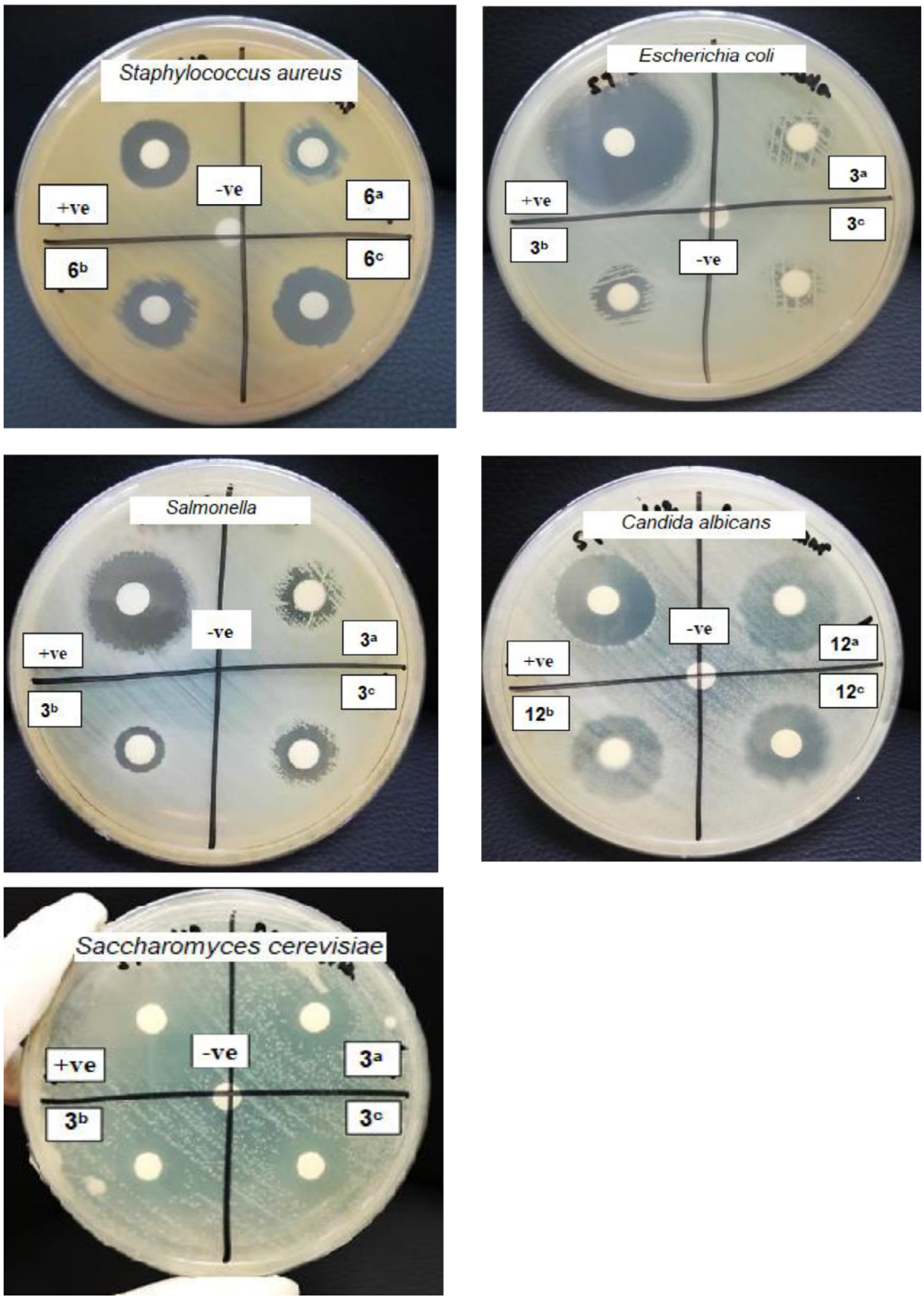


Fig. 5. Agar plates showing the growth inhibitory effect of compounds **3, 6**, and **12** on selected pathogenic microorganisms at different concentrations: a = 0.5 mg/mL; b = 1.0 mg/mL; c = 2.5mg/mL.

of lipophilic molecules [38]. Therefore, it could partially prevent the azo molecules from reaching and inducing the disruption of the inner plasma membrane, and thus resulting in lower or loss of antibacterial activities of the these compounds as observed in the present study.

Similar to *Staphylococcus aureus*, the growth inhibition of the tested fungus *Candida albicans* and *Saccharomyces cerevisiae* is influenced by long alkyl chains and fluorination. Non-fluorinated and short-chain azo derivatives and azo-imidazolium conjugates, showed no activities against *Candida albicans*. However, the fluorinated azo derivatives, compounds **4** and **5** were active against this fungus. At the same concentration, the antifungal activities of the fluorinated azo compounds were higher (inhibition zone diameters 20mm and 21mm, respectively) as compared to that of Streptomycin sulphate, the positive control (inhibition zone diameter 17mm). Among all the azo-imidazolium conjugates, only compound **12** with 18 carbons in the alkyl chain length on the imidazolium head group was active. On the other hand, all azo derivatives and azo-imidazolium conjugates were active against *Saccharomyces cerevisiae* with inhibition zone diameters ranging from

13mm to 21mm. The tetrafluorinated azobenzene derivative, **5** was the most potent against both fungi. The plasma membranes of fungal cells are similar to neutral eukaryotic membranes that contain zwitterionic phospholipids and ergosterol. According to literature, imidazolium derivatives (azole class) interact directly and damage the fungal cell membrane. They inhibit fungus growth by altering the cell membrane structure via inhibition of ergosterol biosynthesis [40,41].

Fluorination had no effect on the activity against Gram negative bacteria *E. coli* and *Salmonella* (Table 1). However, a clear correlation between the level of fluorine substitution and the antimicrobial activity against *Staphylococcus aureus*, a Gram positive bacterium and yeasts was observed. The presence of the highly electronegative and hydrophobic fluorine atoms enhanced lipophilicity of the azo compounds. Replacing the hydrogen with fluorine atoms resulted in little steric alterations as both elements are similar in size. It facilitated the interactions of fluorinated azo compounds with the receptor sites and the permeation into the fungal cell membrane [42], which resulted in enhanced antifungal activities as observed in the present study.

4. Conclusion

Five new non-fluorinated and fluorinated azo derivatives, as well as a new series containing five azo-imidazolium conjugates of varying alkyl chain length in the imidazolium moiety were prepared and characterized using FTIR and NMR spectroscopy, and elemental microanalysis. These compounds were assayed against pathogenic Gram positive bacterium *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli, Salmonella enterica* serovar Typhimurium, as well as yeasts *Candida* albicans and *Saccharomyces cerevisiae* using the disc diffusion method. The azo derivatives and their imidazolium conjugates were selectively active against the tested microorganisms with zone of inhibition diameters ranging from 10mm-21mm. Their biological efficacy was dependent on the chain length and level of fluorine substitution. It can be concluded that in the present study, Gram positive bacterium preferred the more hydrophobic molecules than their Gram negative counterparts. Fluorination had no effect on the activity against Gram negative bacteria. However, a clear correlation was observed between the level of fluorine substitution and the antimicrobial activity against Gram positive bacterium and yeasts. These azo derivatives and azo-imidazolium conjugates may represent new alternatives to fabricate selective and effective biocides.

Authors Contribution

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

Conception and design of study: W.S. Yam acquisition of data: H.F. Babamale, S. Thiagarajan analysis and/or interpretation of data: W.S. Yam, J.S. Tan

Drafting the manuscript: W.S. Yam, H.F. Babamale, J.S. Tan, S. Thiagarajan revising the manuscript critically for important intellectual content: W.S. Yam

Declaration of Competing Interest

None.

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