Synthesis and anti-microbial potencies of 1-(2-hydroxyethyl)-3-alkylimidazolium chloride ionic liquids: Microbial viabilities at different ionic liquids concentrations

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Abstract

Three 1-(2-hydroxyethyl)-3-alkylimidazolium chloride room temperature ionic liquids (ILs) [2OHimC_n][Cl]; (n = 0, 1, 4) have been synthesized from the appropriate imidazole precursors and characterized by IR and NMR spectroscopies and elemental analysis. Their anti-microbial activities were investigated using the well-diffusion method. The viabilities of *Escherichia coli*, *Aeromonas hydrophila, Listeria monocytogenes* and *Salmonella enterica* as a function of IL concentrations were studied. The minimal inhibitory concentrations (MICs) and EC_{50} values for the present ILs were within the concentration range from 60 to 125 mM and 23 to 73 mM, respectively. The anti-microbial potencies of the present ILs were compared to a standard antibiotic, gentamicin. The finding affords additional perspective on the level of ILs toxicity to aquatic lifeforms and yet, this characteristic can be readily harnessed to deter microbial growth and activity.

Keywords: Alkylimidazolium chloride ionic liquids, inhibition potential, human pathogens, microbial growth and viability.

1. Introduction

Room temperature ionic liquids (ILs) are generally salts that bear an organic cation, obtained by the extension of a valence of a nitrogen, phosphorus or sulphur atom, and an organic or inorganic anion with melting points below or not far above ambient temperatures. They are in demand as substitutes to conventional molecular solvents due to their high chemical and thermal

stabilities as well as their very low flammability and vapour pressures. ILs are generally excellent solvents for a wide range of inorganic and organic materials (Fuller et al., 1997; Suarez et al., 1998; Brennecke and Maginn, 2011; Gathergood et al., 2004; Couling et al., 2006; Hossain et al., 2011a). Despite their good industrial applicability, the high solubility of some ILs in water raises concerns as they may be potentially toxic to aquatic organisms. Correspondingly, IL researchers have focused their attention on determining IL toxicities on soil/aquatic-based organisms such as earthworms (Luo et al., 2009; Li et al., 2010), *Selenastrum capricornutum* (Cho et al., 2007) and *Vibrio fischeri* (Docherty and Kulpa, 2005) in order to afford a better understanding of their impact to the surrounding environment.

The notoriety of ILs as being toxic to aquatic organisms is, nonetheless, partially negated by the observation that their toxicities can be readily harnessed to deter microbial growth and activity. This dichotomy of the drawbacks and plus points affords IL researchers a rather intriguing perspective as can be seen for different applications. Bacteria are essentially a good foundation to inspect IL toxicity as they have short generation times (Pham et al., 2010). Previous studies concluded that some pyridinium, imidazolium and quaternary ammonium ILs show toxicity towards a range of bacteria (Pernak et al., 2003, Pernak et al., 2004; Roslonkiewicz et al., 2005). It has been suggested that some quaternary ammonium compounds can even be considered for disinfection of medical equipment (Demberelnyamba et al., 2004). In this regard, Saadeh et al. (2009) synthesized tetrabutylammonium-based ILs and reported that they were effective against Gram-positive and Gram-negative bacteria. We had previously established that hydroxylammonium-based ILs exhibited toxicity to a wide spectrum of human pathogens and in some cases, showed inhibition effectiveness comparable to a standard

antibiotic, gentamicin (Hossain et al., 2011b). On this basis, it is postulated that hydroxyl-functionalized imidazolium ILs may also exhibit anti-microbial characteristics.

The aims of the present study are therefore to synthesize and characterize a series of new 1-(2-hydroxyethyl)-3-alkylimidazolium chloride ILs and to investigate their anti-microbial activities, particularly in the context of microbial viabilities as a function of IL concentrations. Four types of human pathogens, namely, *Escherichia coli*, *Aeromonas hydrophila, Listeria monocytogenes* and *Salmonella enterica* were selected to assess the potential toxicities of these ILs and their effectiveness as anti-microbial agents, which indirectly reflected their aquatic toxicities.

2. Materials and Methods

2.1. Synthesis of 1-(2-hydroxyethyl)-3-alkylimidazolium ILs

All the ILs were synthesized according to previous reported methods (Branco et al., 2002; Yeon et al., 2005; Hossain et al., 2011b). By way of example, ~0.14 mol of 1-methylimidazole (Sigma-Aldrich, USA, AR grade, 99%) and 0.2 mol of 2-chloroethanol (Merck KGaA, Darmstadt, Germany, synthesis grade) were added to a round-bottomed flask containing 200 mL of acetonitrile (Sigma-Aldrich, 98%). After fitting a reflux condenser, the flask was maintained at 343 K under nitrogen for 24 h with magnetic stirring. The reaction progress was monitored by a thin layer chromatography using aluminium sheets coated with silica gel, with methanol as the mobile phase. This product was kept at ~353 K under vacuum (1 Pa) overnight to remove volatiles and moisture. An analogous procedure was used to synthesize the other two ILs, using

91 0.11 mol imidazole (Merck, synthesis grade) and 0.6 mol 1-butylimidazole (Merck, synthesis
92 grade). The reactions employed and the structures of the ILs so obtained are represented in Fig.
93 1. All in all, three ILs were synthesized and characterized: 1-(2-hydroxylethyl)imidazolium
94 chloride, [2OHim][Cl], 1-(2-hydroxylethyl)-3-methylimidazolium chloride, [2OHimC][Cl] and
95 1-(2-hydroxylethyl)-3-butylimidazolium chloride, [2OHimC_4][Cl]. [2OHimC][Cl] was obtained
96 as a solid-like IL while the other two ILs were in liquid form.
97
98 *2.2. Characterization*
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100 The synthesized ILs were characterized using the Fourier-transform infrared (FTIR)
101 spectroscopy (Shimadzu 8400S), ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (Bruker
102 Avance, 400 MHz) and elemental analysis (Leco 932). For NMR, 5 mg of the sample was
103 dissolved in 0.7 mL of deuterated methanol (CD_3OD). The observed peaks (as seen in Table 1)
104 are abbreviated as s (singlet), d (doublet) t (triplet) and m (multiplet). Elemental analyses were
105 performed according to a standard procedure (ASTM – D5219, 2009). The solid samples (< 2
106 mg) were enclosed in a silver capsule whereas the liquid samples were analyzed in a silver
107 capsule containing a sorbit pad. Analyses were performed in triplicate and averaged.
108
109 *2.3. Anti-microbial activity*
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111 The ILs were assayed for anti-microbial activity against four registered microbial strains
112 obtained from the Institute of Medical Research (IMR), Kuala Lumpur, Malaysia. These were:
113 gram positive *Listeria monocytogenes* L 49 as well as gram negative *Escherichia coli*,

114 *Aeromonas hydrophila* and *Salmonella enterica* S 1180. This test was carried out using the well-
115 diffusion method (Tagg and McGiven, 1971; Benkerroum et al., 2003). Muller-Hinton agar (20
116 mL) (Merck, Germany) was dissolved, melted and cooled to 55 °C and subsequently inoculated
117 with 1 mL of the bacterial suspension. The inoculated agar was transferred to a petri-plate and
118 allowed to cool. Upon solidification of the medium, 6 mm diameter holes were created on the
119 agar plate and 20 µL of the IL solution at different concentrations in deionized water were
120 poured into the wells. The plates were then incubated at 37 °C for 24 h or until visible growth
121 was established and the diameter of the inhibition-cleared zone around each well was
122 determined. The screening results were compared with a standard antibiotic, namely, gentamicin
123 (Atlantis Laboratory, Thailand).
124
125 *2.4. Determination of minimal inhibitory concentration (MIC)*
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127 Determination of minimal inhibitory concentration (MIC) was conducted according to the
128 standard procedure (CLSI-M07-A9, 2008) developed by the Clinical and Laboratory Standards
129 Institute (CLSI), Pennsylvania, USA. The microbial strains were cultured on a Muller-Hinton
130 broth (MHB) for 24 h. The ILs were added to the first two wells of two horizontal rows in the
131 96-well plate and twofold dilutions were made from the second set of wells while the last wells
132 were kept untreated. Three replicates and seven different concentrations were studied for each
133 IL. For turbid suspensions (optical density *ca* 0.1 to 0.3 at 530 nm), a 1:1000 dilution was used.
134 The final bacterial inocula ranged from 10^5 to 10^7 colony-forming units (CFU) per milliliter.
135 Gentamicin was used as a positive control while anhydrous dimethylsulfoxide (DMSO) (99.9%,
136 Sigma-Aldrich) was used as a negative control. Microbial growth was visually determined after

incubation for 24 h at 37°C. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC. The 96 well-plate was subsequently kept in an ELISA plate reader to establish the EC_{50} (effective concentration of IL required for 50 % toxicity within a specified exposure time) at wavelength 530 nm.

3. Results and Discussion

3.1. Characterization

The IR, ^{1}H-NMR and elemental analysis data are listed in Table 1. The IL product yields ranged from 88 to 95 %. For the ^{1}H-NMR spectrum of [2OHim][Cl], the peaks at δ 3.91 and 4.35 ppm indicate two two-proton triplets for $–CH_2OH$ and $–NCH_2$- of the side-alkyl chain of the imidazolium ring, respectively. The –CH protons at C-4 and C-5 provide two doublet-doublets at δ 7.53 (J = 0.16 Hz, 4-CH) and 7.67 (J = 0.28 Hz, 5-CH). The other two singlet peaks correspond to the –NH and –CH proton on C-2 at δ 8.80 and 9.08, respectively. the hydroxyl peak of the studied ILs disappeared, which could be explained by a dynamic proton exchange reaction between the labile proton of the –OH group for the ILs and the labile deuterium of the –OD group for the solvent CD_3OD. This can be confirmed by the appearance of a peak with weak intensity at 3.31 δ ppm, which may be attributed to the –OH group of CD_3OH. The other two ILs represent similar peaks that correspond to the necessary protons in the molecules which confirm the structures. Similar ILs have been studied and reported (Fraga-Dubreuil and Bazureau, 2003; Choi et al., 2007, Jalili et al., 2010) which can be further used to elucidate the structures of the present ILs.

160 The IR data confirm the functionality of the IL structures and are further corroborated by the
161 study done by Chang et al. (2007). The functionalities of the present ILs, such as hydroxyl
162 (OH), alkyl (-CH_3 and –CH_2-), tertiary and secondary amino groups (≡N and =NH), and
163 aromatic (CH) structures are confirmed by the IR spectroscopy (Fig. 2). The broad absorption
164 range between 3130 and 3350 cm^{-1} can be attributed to the presence of the hydroxyl group in the
165 structures. Spectral analysis of this broad absorption range is rather complicated due to the
166 presence of signals rising from the stretching modes of the water together with the hydroxyl
167 groups in the ILs. More likely with the observations of Chowdhury and Thynell (Chowdhury
168 and Thynell, 2006), the –C=C-, –C=N- and C-N stretching frequencies within the imidazolium
169 ring are observed at around 1560, 1450 and 1160 cm^{-1}, respectively. The C-H and C-N(1)-C
170 bending-in-plane (bip) modes appear around 1070 cm^{-1}, with the C-H bending-out-of–plane
171 (bop) modes occurring at 860 and 750 cm^{-1}. The intense peaks at 2950 and 2850 cm^{-1} can be
172 assigned to the -C-H in-plane stretching of the alkyl groups (Erdmenger et al., 2008). The low-
173 intensity band at 3070 cm^{-1} can be attributed to the =C-H stretching frequency (Chang et al.,
174 2007). The alkyl (-CH_3 and -CH_2-), NH, aromatic (CH) and hydroxyl (OH) structures detected
175 via ^{1}H-NMR are consistent with the IR data. The calculated carbon, hydrogen and nitrogen
176 percentages are in good agreement with the experimental values (Table 1).
177
178 *3.2. Anti-microbial activities of ILs*
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180 Agar diffusion technique (Tagg and McGiven, 1971; Benkerroum et al., 2003) was
181 employed for screening purpose. The anti-microbial activities of the synthesized ILs were
182 determined by measuring the inhibition zones on the agar plates. This zone is defined as the area

183 on the agar plate where microbial growth is inhibited by the test compound. Information from
184 the screening result was used to select the initial ILs concentration (250 mM) for the MIC test
185 and subsequently diluted to facilitate the investigation of the effect of the IL concentrations on
186 the microbial viabilities. The MICs for all ILs are estimated to be 125 mM with the exception of
187 [2OHim][Cl] and [2OHimC_4][Cl] against *Aeromonous hydrophilla* and *Escherichia coli*,
188 respectively, which record a MIC of 62.5 mM. The viabilities of the *Escherichia coli*,
189 *Aeromonas hydrophila, Listeria monocytogenes* and *Salmonella enterica* as a function of the IL
190 concentrations are shown in Fig. 3. In all cases, the viabilities of the growth decreased with
191 increasing concentrations as exhibited in the archetypal sigmoid curve except for with
192 *Salmonella enterica* which experienced gradual viability decline.

193 Table 2 shows the EC_{50} values calculated using the ELISA plate reader. These values ranged
194 from 23 to 73 mM of all ILs required to limit microbial viabilities to 50%. This interestingly
195 implied that the anti-microbial activities of all ILs were not as potent as compared to
196 gentamicin, which exhibits EC_{50} values *ca* 2 mM on all four microbial species. The three ILs
197 exhibited lower toxicities compared to gentamicin by factors ranging from approximately 12 to
198 36, which are rather significant. Indeed, such findings somewhat contrast our previous study
199 (Hossain et al., 2011b) in terms of IL anti-microbial potency whereby *2-hydroxy-N-(2-*
200 *hydroxyethyl)-N-methylethanaminium acetate* was found to exhibit toxicity levels (to *Vibrio*
201 *cholera* and *Listeria monocytogenes*) comparable with that of gentamicin, albeit it is
202 acknowledged that these two sets of ILs belong to different IL classifications.

203 Another trend is apparent; an increase in the chain length of alkyl substituents correlates
204 with an increased microbial growth inhibition whereby the toxicity level of the studied ILs is:
205 [2OHimC_4][Cl] (highest) > [2OHimC][Cl] > [2OHim][Cl] (lowest). This observation was

consistent with previous ILs toxicity studies (Pernak et al., 2003, 2004; Docherty and Kulpa, 2005) in which an increase in the chain length of alkyl substituents on the cation ring increases IL toxicity for the imidazolium-based ILs. *Listeria monocytogenes* seems to be the least susceptible to the present ILs among all the studied microbial species since their EC_{50} values are the highest.

4. Conclusions

1-(2-hydroxylethyl)imidazolium chloride, 1-(2-hydroxylethyl)-3-methylimidazolium chloride, and 1-(2-hydroxylethyl)-3-butylimidazolium chloride ILs were synthesized and characterized, and their anti-microbial activities established. These ILs, though capable of inhibiting microbial growth, were not comparable with that of gentamicin in terms of anti-microbial potency. The EC_{50} values of the ILs also indicated a straightforward dependence on the chain length of alkyl substituents on the cation ring. The findings from this study can be used for better design of imidazolium-based ILs with consideration of their aquatic toxicities.

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Figure captions

Fig. 1. Synthesis reaction and structures of the present ILs.

Fig. 2. IR spectra of the present ILs.

Fig. 3.Viabilities of (a) *Escherichia coli*, (b) *Aeromonas hydrophila,* (c) *Listeria monocytogenes* and (d) *Salmonella enterica* as a function of ILs concentrations (■[2OHim][Cl]; ▲[2OHimC][Cl]; ◆[2OHimC_4][Cl]).

360 **Table 1.** FTIR and NMR spectroscopic and elemental data for the synthesized ILs.

Ionic liquid	MW (g/mol)	Yield (%)	FTIR (cm^{-1})	^{1}H-NMR (δ ppm)	Elemental analysis (%)	
					Experimental	Calculated
[2OHim][Cl]	148.5	86	626.8, 759.9, 829.3, 1066.6, 1163.0, 1446.5, 1581.5, 1629.7, 2846.7, 2958.6, 3143.8, 3357.8	3.91 (t, 2H, $\underline{C}H_2OH$), 4.35 (t, 2H, N^+-CH_2), 7.53 (dd, *J*=0.16 Hz, 4-CH), 7.67 (dd, *J*=0.28 Hz, 5-CH), 8.80 (s, 1H, 3-NH), 9.08 (s, 1H, 2-CH)	C 39.81 H 5.96 N 20.12	C 40.40 H 6.06 N 18.85
[2OHimC][Cl]	162.5	95	754.1, 867.9, 1072.3, 1164.9, 1448.4, 1568.0, 3072.4, 3145.7, 3232.5	3.90-3.93 (t, 2H, $\underline{C}H_2$-OH), 4.00 (s, 3H, CH_3-N), 4.36-4.39 (t, 2H, CH_2-N), 7.62 (s, 1H, 4-CH), 7.68 (s, 1H, -5CH), 9.01 (s, 1H, 2-CH)	C 44.53 H 7.03 N 17.11	C 44.30 H 6.77 N 17.23
[2OHimC_4][Cl]	204.5	88	752.2, 869.8, 1076.2, 1163.0, 1456.2, 1562.2, 2871.8, 3068.5, 3137.9, 3232.5	1.01 (t, 3H, CH_3), 1.39-1.42 (m, 2H, CH_2), 1.88-1.95 (m, 2H, CH_2), 3.90-3.92 (t, 2H, $\underline{C}H_2$-OH), 4.29 (t, 2H, CH_2-N), 4.36 (t, 2H, CH_2-N), 7.70 (s, 1H, 4-CH), 7.72 (s, 1H, 5-CH), 9.10 (s, 1H, 2-CH)	C 52.16 H 8.97 N 12.98	C 52.81 H 8.31 N 13.69

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363 **Table 2.** EC_{50} values calculated using ELISA plate reader and expressed in mM.

	[2OHim][Cl]	[2OHimC][Cl]	[2OHimC_4][Cl]	Gentamicin
Escherichia coli	58.44±4.07	43.40±2.54	23.67±2.22	2.00
Aeromonas hydrophila	65.37±1.82	35.54±1.14	31.21±2.51	1.97
Listeria monocytogenes	72.92±1.84	53.79±1.95	52.47±1.24	1.86
Salmonella enterica	56.16±3.56	50.84±1.72	45.15±4.27	1.92

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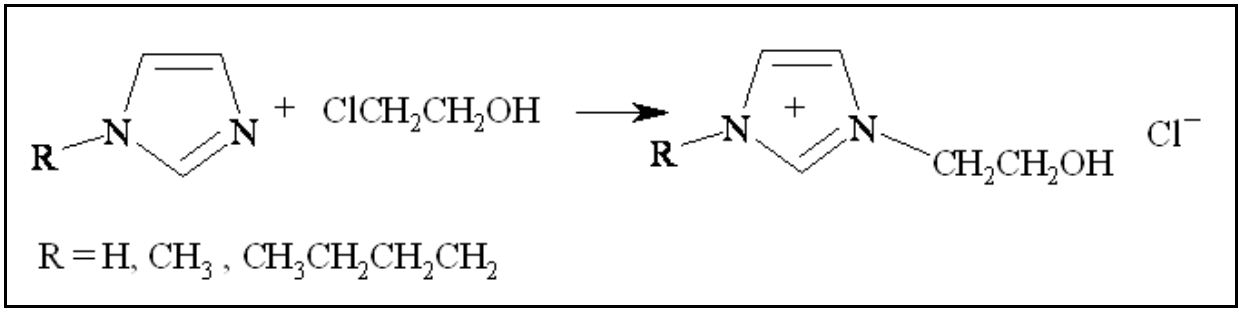
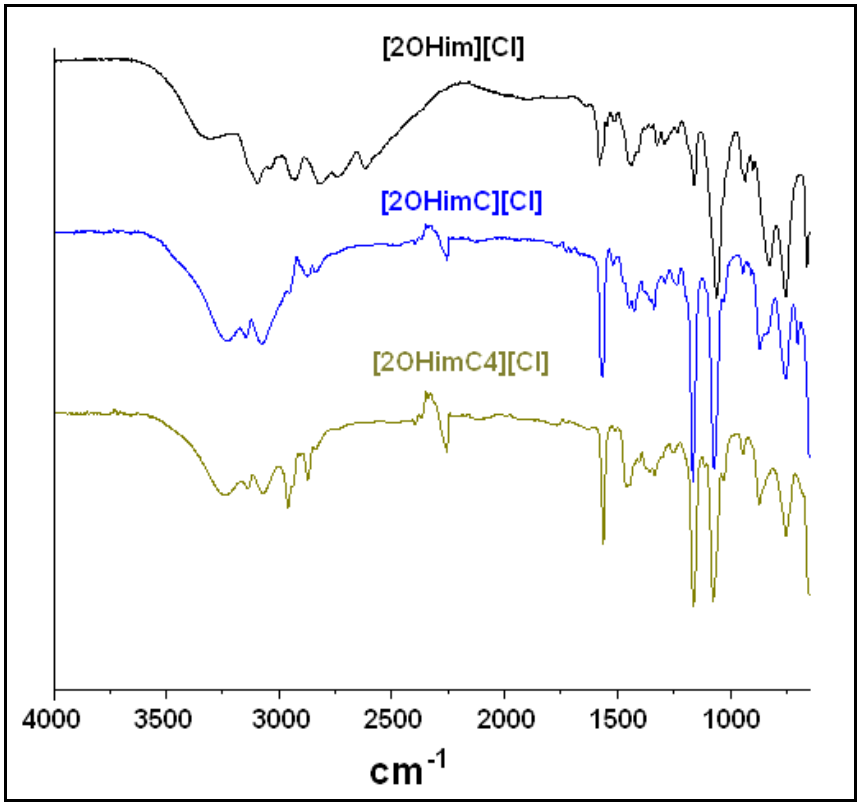


Fig. 1. Synthesis reaction and structures of the present ILs.

401 

402 **Fig. 2.** IR spectra of the present ILs.

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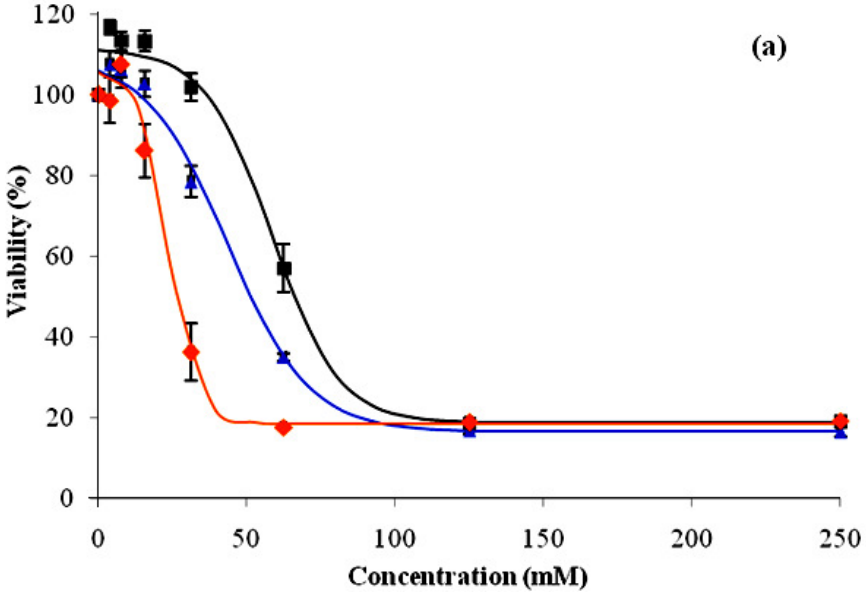

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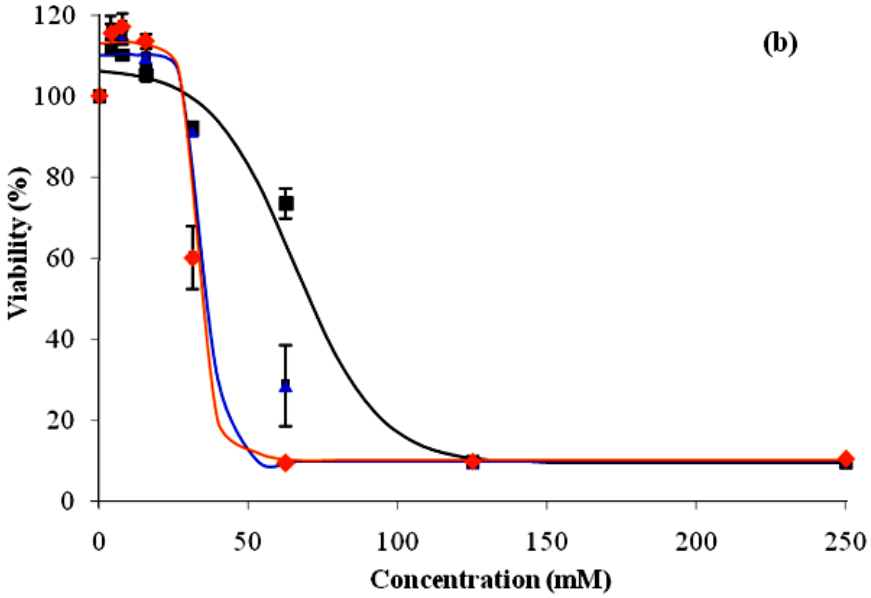

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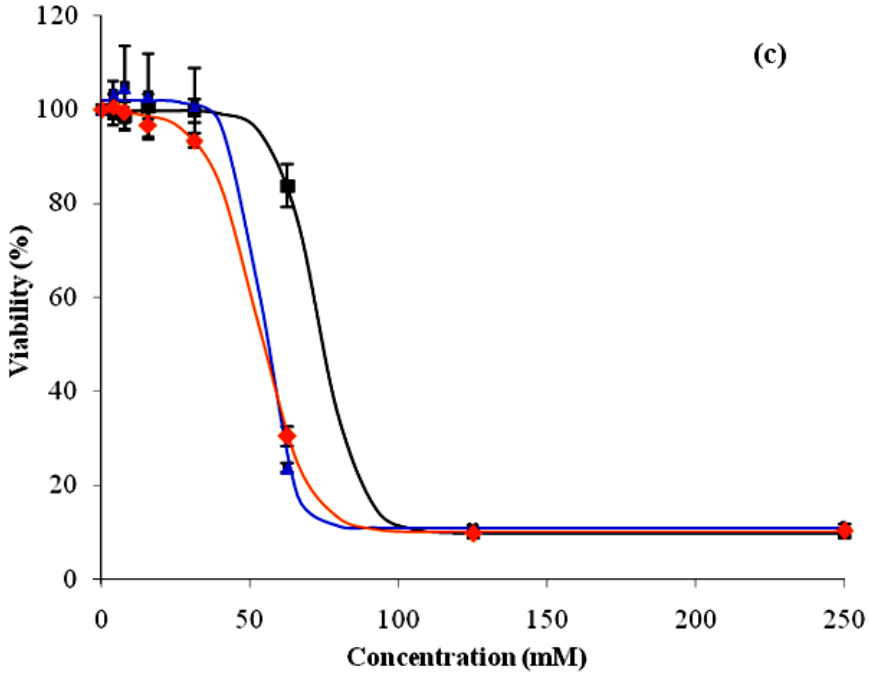

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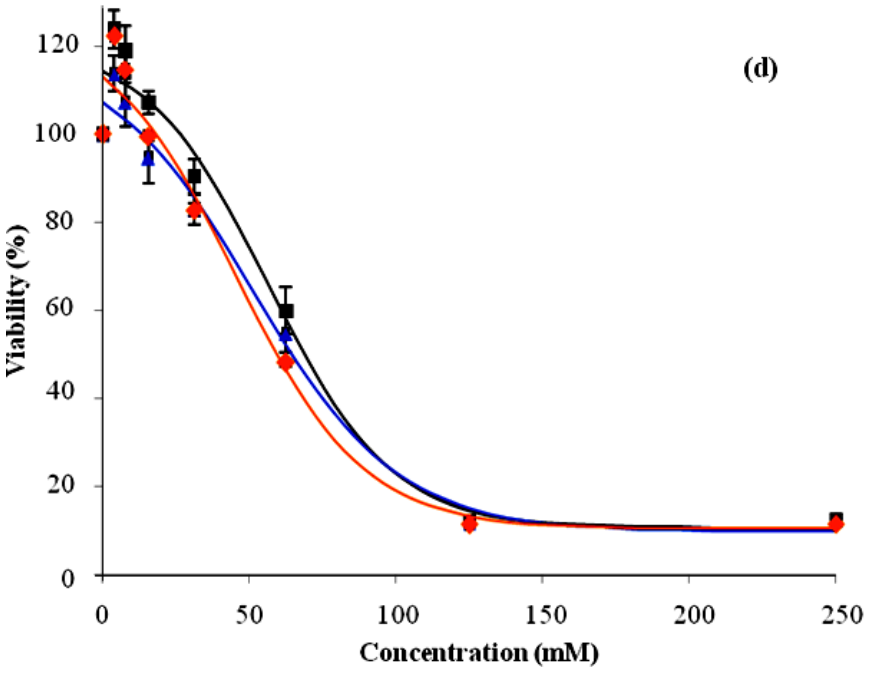

420

421 **Fig. 3**.Viabilities of (a) *Escherichia coli*, (b) *Aeromonas hydrophila,* (c) *Listeria monocytogenes*

422 and (d) *Salmonella enterica* as a function of ILs concentrations (■[2OHim][Cl]; ▲

423 [2OHimC][Cl]; ◆ [2OHimC_4][Cl]).