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Ecotoxicity of Pyridinium Based ILs Towards Guppy Fish and Four Bacterial Strains

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Abstract

Ionic liquids (ILs) are molten salts that possess low melting points and wide solvation properties. ILs attracted the attention of the academic and industrial professionals due remarkable properties such as low vapor pressure and thermal stability which make them more environmentally friendly as they can replace volatile organic solvents (VOCs) in many organic reactions. This has brought attention to broaden this research area to overcome the harmful emissions from VOCs in industry. Pyridinium ILs have a wide range of applications in various domains such as material sciences, organic and bioorganic syntheses, biotechnologies, nanotechnology and Enhanced Oil Recovery (EOR). However, available toxicity data in literature is yet scarce and hampers a large scale development. In this study, 18 synthesized ILs were tested against guppy fish and four bacterial strains. Two gram negative bacteria: *Salmonella enterica, Vibrio cholera* and two gram positive bacteria: *Listeria monocytogenes, Staphylococcus aureus* were chosen to represent each category of bacteria. Fish test was conducted using OECD guidelines and the "96-well plate" procedure was adopted for the bacterial test using (CLSI M100-S24). Results showed that long alkyl chain length ILs showed higher toxicity than short alkyl chain analogs on all the targeted strains. The highest toxic effect exhibited by pyridinium ILs towards guppy fish was moderately toxic and the rest varied between relatively harmless and practically nontoxic. The highest antibacterial effect indicated slightly toxic effect towards *Staphylococcus aureus* obtaining EC_{50} = 19.3 mg/L. In most cases, pyridinium ILs indicated relatively harmless and practically harmless effects towards bacteria after comparing the EC_{50} values obtained with Passino and Smith 1987 hazard ranking.

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1. Introduction

Ionic Liquids (ILs) are a new group of compounds which are composed of an organic cation with nitrogen-containing molecules as most representatives such as 1-methylimidazolium, N-alkyl pyridinium and of an organic/inorganic anion such as PF_6, BF_4, and CF_3COO^-. They possess many properties such as low vapor pressure, thermal stability and tunable characteristics based on their structure "altering the cation or anion" [1, 2].

Recently, ILs showed a good potential for green chemistry approach to replace volatile organic solvents "VOCs" which cause environmental pollution. ILs offer a good replacement for VOCs because of their negligible vapor pressure as well as a solvation medium for many organic and inorganic substances. Moreover, using ILs showed better productivity in many applications such as in catalysis or in organic reactions [3–9].

In Literature, few studies tested the toxicity of ILs using different techniques such as mutagenicity and enzymatic testing [10, 11] with different species tested such as Fish, bacteria, algae, mammalian organisms, fresh water snails and crustaceans [12–17]. Many factors were found to contribute to the overall toxicity effect of ILs towards organisms such as nature of the cationic moiety, anion species, biodegradability and lipophilicity of the compound [19, 20]. Previous studies observed that the alkyl chain length influenced greatly the results notably that short alkyl chain length ILs showed less toxicity effect than the longer alkyl chain length ILs [18–20].

Several studies on different types of ILs among pyridinium, imidazolium, ammonium and phosphonium have shown that the cationic species was the main responsible on the toxicity impact [15, 18, 23– 26] contrary to the anions which did not prove to exert any substantial influence on toxicity [27]. Pyridinium ILs were tested against many different species such as Zebrafish and *Daphnia magna* [28]. Stolte et al. [29] proved in his study that pyridinium ILs had the highest influence compared to imidazolium, 1-methylpiperidinium, and 4-methylmorpholinium on all the tested species such as marine bacterium *Vibrio fischeri*, the duckweed *Lemna minor*, and also the algae species *Scenedesmus vacuolatus*.

The interest of using pyridinium ILs is due to the wide range of applications in which pyridinium ILs have been used in different field such as nanotechnology, biotechnologies and in various organic reactions [30–32]. However, the toxicity data on pyridinium ILs is still deficient.

In this work, 18 pyridinium ILs were synthesized and investigated for evaluation of toxicity towards guppy fish and four strains of bacteria.

2. Materials and Methods

1.1. *Synthesis of pyridinium ILs*

1.1.1 *Quaternization*

17 pyridinium ILs were synthesized by Quaternization method using 1-bromoutane, 1-bromohexane, 1-bromooctane, 1-bromodecane, 1-bromododecane, 1-bromotetradecane along with Pyridine, 3-methylpyridine, 4-methylpyridine, 3,4-dimethylpyridine according to the established method [33]. All chemicals were bought from merck (Germany) and used as received. The reactants were added in a reflux flask in 1:1 mole ratio under nitrogen. The reaction time varied from 2-4 days according to the alkyl chain length. The reaction was heated at 60°C for the long alkyl chain ILs such as octyl-, decyl-, dodecyl-, tetradecyl- to enhance the completion of the reaction. The product was washed four to five times with ethyl acetate and then the product was further dried with rotary

evaporator at 55°C and 300 mbar. Then the product was dried on a vacuum line for 3 days at 70°C. Lastly, ILs structures and purities were examined by ^1H and ^{13}C NMR spectroscopy.

1.1.2 *Microwave-assisted synthesis*

"1-octyl-3,4-dimethylpyridinium bromide" was synthesized using 3,4-dimethylpyridine and 1-chlorooctane in a 1:1 mole ratio. The reaction time lasted 60 minutes using power 700 watt, temperature 170°C and 20 minutes ramping time. The product was washed four to five times with ethyl acetate and then dried using rotary evaporator at 55°C and 300 mbar. A further purification method was adopted in which the product was added to grinded charcoal in equal amounts and the mixture was washed with methanol for 3 hours. The product was filtered from charcoal and dried on rotary evaporator at 45°C and 350 mbar. Lastly, the product was further dried using vacuum line for three days at 60°C affording the final product [34].

1.2. *Fish acute toxicity test*

The test was performed according to the established method OECD guidelines [35]. Guppy fish (*Poecilia reticulata*) was purchased and brought to the lab within 15 minutes in plastic bags that has sufficient air and enough water for the fish to survive. Fish were transferred into tanks in which two air pumps were attached and food was given twice daily for acclimation of the fish for 12 days. Fish tanks were acclimated under normal illumination daily from 12-16 h. Temperature of the water was maintained at 23 ± 2°C as well as the dissolved oxygen = 5-7 ppm and the pH = 7. All parameters were checked daily and measured as well as the fish health and state. Fish displaying any abnormal behaviour or mortality was removed immediately.

After acclimation period, 10 healthy fishes were transferred to a 6.5 L tank containing 5 L of water. Fishes were considered healthy if they showed healthy swimming style and normal behavior while fishes exhibiting weak movements or sickness were removed immediately. All tanks were connected to air pumps and triplicate of each IL dose were prepared and dissolved in the water prior to fish transfer. No food was given throughout the test period and even before starting the test by 24 h. Fishes' behavior was thoroughly monitored for 96 h for any abnormalities or mortalities to be recorded. Fish was considered dead if no movement was observed or if no reaction was observed by touching the caudal peduncle. At least three concentrations were used for each IL. The median lethal concentration (LC_{50}) which is the concentration that kills 50% of the population in the test was calculated for each IL. All results were compared with the U.S. Fish and Wildlife Service (USFWS) which is the standard ranking for hazards as shown in Table 1 [36].

Table 1. Toxicity levels Published by U.S. Fish and wildlife service.

Toxicity level	LC_{50} (*mg/L*)
Super toxic	0.01-0.1
Highly toxic	0.1-1
Moderately toxic	1-10
Slightly toxic	10-100
Practically nontoxic	100-1000
Relatively harmless	>1000

1.3. *Antimicrobial testing*

All ILs were evaluated for toxicity using 4 bacterial strains: *Staphylococcus aureus* (Sa) S38, *Salmonella enterica* (Se) S1570, *Listeria monocytogenes* (Lm) L62, *Vibrio cholera* (Vc) V148. Bacterial strains were brought from institute of medical Research (IMR), Kuala Lumpur. Two bacterial strains are gram positive (Sa and Lm) and the other two represented the gram negative bacteria (Vc and Se). The 96-well plate technique was adopted to measure the antibacterial activity of each IL using CLSI M100-S24 guidelines [37]. Effective concentration (EC_{50}) which is the concentration that gives half maximal response from the population was obtained for each IL. All EC_{50} values were compared with the standard values of hazard ranking by Passino and smith 1987 as shown in Table 2 [38].

Table 2. Passino and Smith 1987 standard in ranking the hazards expressed in ug/mL.

Toxicity level	Rank
Less than 0.01	Super toxic
0.01 to 0.1	Extremely toxic
0.1 to 1	Highly toxic
1 to 10	Moderately toxic
10 to 100	Slightly toxic
100 to 1000	practically harmless
Greater than 1000	Relatively harmless

3. Results and Discussion

3.1. Influence of the alkyl chain length on toxicity

The influence of ILs on toxicity was studied towards guppy fish and bacteria with varying alkyl chain length from butyl- to tetradecyl- ILs. Moreover, different cationic moieties derived from pyridinium ILs such as pyridinium, 3-methylpyridinium, 4-methylpyridinium and 3,4-dimethylpyridinium were used to assess the influence on toxicity of added methyl group(s) on different positions in the pyridine ring.

Results found on the effects of the alkyl chain length on toxicity in terms of LC_{50} values are displayed hereafter Fig. 1(a-d). Whatever the presence or not of added methyl(s) group on the pyridinium group, it is obvious that higher values of LC_{50} are obtained for short alkyl chain ILs and that these value dramatically decrease as the alkyl chain length tends to increase. In each graph, each cation is illustrated showing the effect of the varying alkyl chain length on the LC_{50} value. For example, in Fig. 1(b) $3MPyC_{12}Br$ and $3MPyC_{10}Br$ obtained LC_{50} = 93.3 mg/L and 426.6 mg/L respectively, while $3MPyC_6Br$ obtained 1174.9 mg/L which indicates that C12 has the highest influence on toxicity while C6 exhibited the lowest influence. All pyridinium cations: 4-methylpyridinium, pyridinium, 3,4-dimethylpyridnium, and 3-methylpyridinium demonstrated the same behavior with all tested organisms emphasizing that the presence of an added methyl group directly bonded to the pyridinium moiety does not impact toxicity levels exerted by the side alkyl chain length. The highest toxic effect indicated towards guppy fish was moderately toxic which was obtained by tetradecyl- “C14” IL. The rest indicated slightly toxic, practically nontoxic and relatively harmless toxicity levels. All LC_{50} values are demonstrated in Table 3.

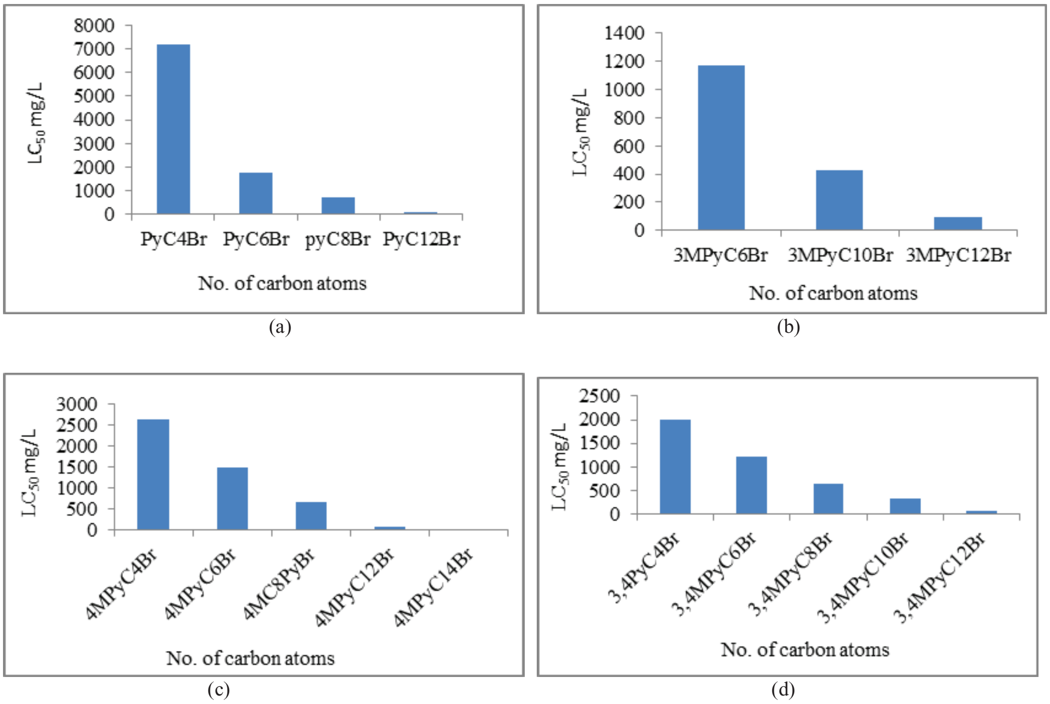
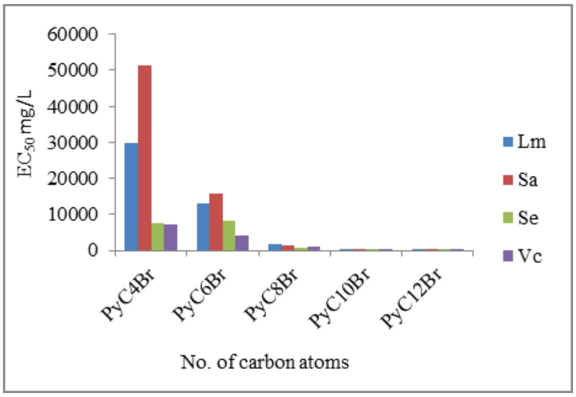


Fig. 1. Shows the effect of the alkyl chain length with different pyridinium cations (a) pyridinium cations; (b) 3-methylpyridine; (c) 4-methylpyridine; (d) 3,4dimethylpyridine.

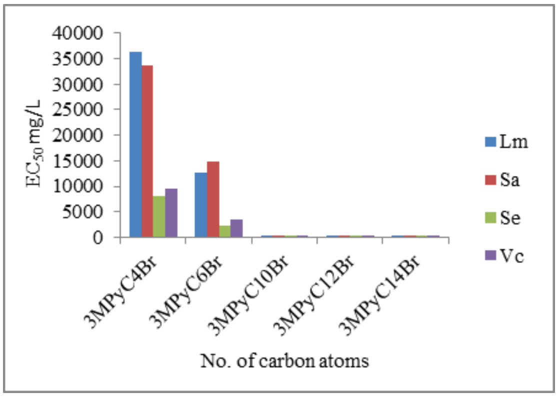
Table 3 Shows all the calculated LC_{50} of the tested Pyridinium ILs.

NO.	Name of ILs	Acronym	LC_{50} (*mg/L*)
1	1-butyl-3,4-dimethylpyridinium bromide	$3,4MPyC_4Br$	2000.0
2	1-hexyl-3,4-dimethylpyridinium bromide	$3,4MPyC_6Br$	1225.0
3	1-octyl-3,4-dimethylpyridinium bromide	$3,4MPyC_8Br$	650.0
4	1-decyl-3,4-dimethylpyridinium bromide	$3,4PyC_{10}Br$	323.6
5	1-dodecyl-3,4-dimethylpyridinium bromide	$3,4PyC_{12}Br$	63.1
6	1-butyl-4-methylpyridinium bromide	$4MPyC_4Br$	2630.3
7	1-hexyl-4-methylpyridinium bromide	$4MPyC_6Br$	1500.0
8	1-octyl-4-methylpyridinium bromide	$4MPyC_8Br$	676.1
9	1-dodecyl-4-methylpyridinium bromide	$4MPyC_{12}Br$	80.0
10	1-tetradecyl-4-methylpyridinium bromide	$4MPyC_{14}Br$	9.5
11	1-hexyl-3-methylpyridinium bromide	$3MPyC_6Br$	1174.9
12	1-decyl-3-methylpyridinium bromide	$3MPyC_{10}Br$	426.6
13	1-dodecyl-3-methylpyridinium bromide	$3MPyC_{12}Br$	93.3
14	1-butylpyridinium bromide	PyC_4Br	7211.1
15	1-hexylpyridinium bromide	PyC_6Br	1771.0
16	1-octylpyridinium bromide	PyC_8Br	699.8
17	1-dodecylpyridinium bromide	$PyC_{12}Br$	91.8
18	1-dodecylpyridinium chloride	$PyC_{12}Cl$	83.8

For Bacteria, long alkyl chain ILs "octyl-, decyl-, dodecyl-, tetradecyl-" showed higher antibacterial activity, i.e. low EC_{50} values, than short chain ILs "butyl- and hexyl-", i.e. high EC_{50} values, with all tested strains as shown in Fig. 2(a-d). All pyridinium cations showed the same trend with all bacterial strains. When found EC_{50} values are compared with Passino and Smith 1987 standards, pyridinium ILs exhibit slightly toxic effect from C12 and C14. The highest antibacterial activity manifested was detected from “1-dodecyl-3-methylpyridinium bromide” $3MPyC_{12}Br$ towards Sa obtaining 19.3 mg/L followed by the EC_{50} obtained from the same IL towards Se = 21.9 mg/L which was explained by the intrinsic resistance of the gram negative bacteria Se. The rest of ILs C4, C6, C8, C10 indicated practically harmless and relatively harmless effects.



(a)



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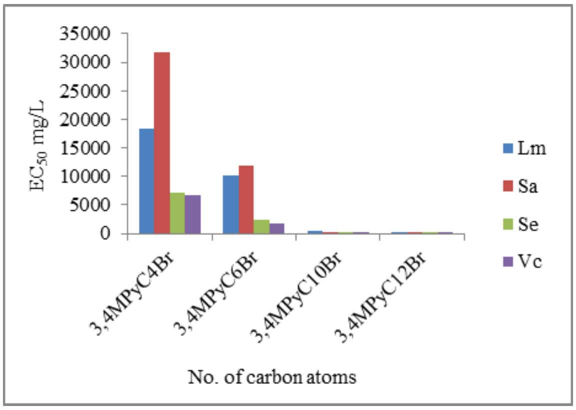

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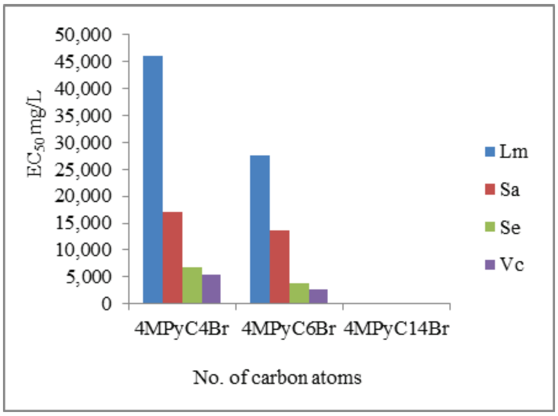

(d)

Fig. 2. Illustrates the effect of the alkyl chain length on the antibacterial activity. (a) pyridinium cations; (b) 3-methylpyridine; (c) 3,4-dimethylpyridine; (d) 4-methylpyridine.

3.2. The effect of anion moiety on toxicity

In Fig. 3, $PyC_{12}Cl$ indicated higher toxicity level than $PyC_{12}Br$ by obtaining less LC_{50} value which is 83.8 mg/L while $PyC_{12}Br$ obtained LC_{50} = 91.8 mg/L. The same anion effect was manifested with bacteria as well in which the EC_{50} values for three bacterial strains: Sa, Se, and Vc indicated higher toxicity influence of the $PyC_{12}Cl$

compared to $PyC_{12}Br$ as shown in Table 4. *Listeria monocytogenes* showed lower resistance to the toxic effect of $PyC_{12}Cl$ which was explained with the intrinsic resistance that gram negative bacteria may develop.

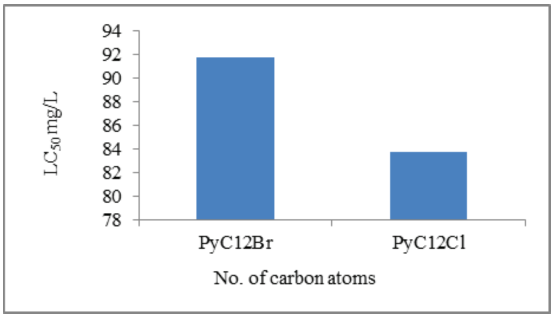

Fig. 3. Shows the anion effect on toxicity.

Table 4 Shows the EC_{50} values for PyC_4Br and PyC_4Cl in mg/L.

IL	Lm	Sa	Se	Vc
1-butylpyridinium chloride	47757.0	28388.6	7293.7	7362.4
1-butylpyridinium bromide	29684.9	51512.9	7684.1	7362.9

4. Conclusion

Pyridinium ILs are interesting compounds due to their various applications and by evaluating the toxicological impact on guppy fish and bacteria, results indicated that short alkyl chain length ILs exhibited relatively harmless and practically non-toxic effects. C4 and C6 ILs proved to be relatively harmless with $LC_{50} > 1000$ according to "Acute toxicity rating scale by Fish and Wildlife Service" (FWS). While a practically nontoxic effect was indicated from C8 and C10 ILs with LC_{50} that ranges from 100 to 1000, and only the C12 ILs manifested a slightly toxic effect towards guppy fish. For Bacteria, pyridinium ILs exhibited a slightly toxic antibacterial activity and this was the highest toxicity indication towards bacteria, while most of the results manifested practically harmless and relatively harmless indications. Eventually, more toxicity data is required to understand the mechanism of toxicity towards the tested organism and to enhance the utility of ILs in industry.

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