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Ecotoxicity Study of Amino Acid Ionic Liquids towards *Danio rerio* Fish: Effect of Cations

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Abstract

Six Ionic Liquids (ILs) were prepared based on phosphonium, ammonium and choline cations, with two types of amino acids specifically taurine and phenylalanine as anions. Good yields were obtained in the synthesis of the ILs. The toxicity of the synthesized ILs was checked against the *Danio rerio* fish (OECD 203). All six tested Amino Acid Ionic Liquids (AAILs) shows remarkably low toxicity towards *Danio rerio* fish.

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Keywords: Ecotoxicity, Ionic Liquids, Amino Acid, Zebrafish, *Danio rerio*

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1. Introduction

Ionic liquids (ILs) have gained a limelight as an emerging green media for various types of applications in the last decade [1-6]. This class of material are ubiquitously studied in various arenas of modern organic and physical chemistry as well as in the biological field which includes; separation [7, 8], electrochemistry [9], organic synthesis [10, 11], biocatalysis [12-14] oil and gas [15], cellulose dissolution [16], biomass dissolution and conversion [17-19], pharmaceutical [20-22], drug delivery [23] and many more. An enthusiasm with vast progressive developments on the use of ILs in diverse platform entails a better understanding on the impact to the environment [24-26].

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Nomenclature

ILs	Ionic Liquids
VOC	Volatile organic compound
$[P_{4444}][Tau]$	Tetrabutyl phosphonium taurinate
$[N_{4444}][Tau]$	Tetrabutyl ammonium taurinate
[Ch][Tau]	Cholinium taurinate
$[P_{4444}][PheAla]$	Tetrabutyl phosphonium phenylalaninate
$[N_{4444}][PheAla]$	Tetrabutyl ammonium phenylalaninate
[Ch][PheAla]	Cholinium phenylalaninate

Ionic liquids are molten salt consist entirely of ions [27-30] that can be paired up by enormous number of cations and anions [31-33]. Since it can be made up from unlimited number of cations and anions, the desired physicochemical properties such as thermal stability, miscibility in water or organic solvent, electrochemical potential window, and electric conductivity can be fine-tuned depending on the field of interest [34, 35].

The *'green'* tag carried by this neoteric solvent predominantly lies on its extremely low or negligible vapor pressure [36, 37]. The solubility of these ILs highlights a concern on its release to water; potentially causing damage to aquatic organisms and ecosystems [38]. Other properties of ILs, include being resistant to photodegradation, bioaccumulation, water solubility and stability making them a threat to aquatic organisms [39]. This feature makes it possible to reduce the toxicity of the ILs generated, eventually reducing the undesirable effect of these chemical compounds on the environment [40]. For these reasons, proactive actions have been taken in evaluating the ecotoxicity of the ILs [41-43] and more new eco-friendly ILs that feature relatively low toxicity and biodegradability are actively designed.

With the inspiration for a better tomorrow, researchers around the world are striving towards making the use of ILs for a sustainable future. Several aspects have been highlighted as factors that contribute and influence the toxicity of ILs. These include but are not limited to; the nature of cations or anions, alkyl chain length as well as its functional group [35]. For instance, Couling and co-workers, 2005 [44] depicted that the trend of toxicity is closely related and driven by the type of cation used. The increased toxicity trend observed were as follows; ammonium < pyridinium < imidazolium < triazolium < tetrazolium. Anion in this study played a secondary role in toxicity with only minimal impact observed.

Herein, six ILs based on the choline, ammonium and phosphonium cations, with two types of amino acids anions had been synthesized via neutralization process. Choline with structural formula of N_{1112} was chosen based on its reported relatively low toxicity and biodegradability [45, 46, 47]. In comparison, two more non- aromatic cation namely N_{4444} and P_{4444} were selected to see the effects of different head group as well as its alkyl chain length. As for the selection of anion, Fukumoto et al, 2005 [48] proposed the use of naturally abundant feed stocks from amino acids family as source to design environmentally-friendly ionic liquids (ILs). In this study, two anions nominated as candidates specifically phenylalanine and taurine. Good yields were obtained in the synthesis of the ILs. The toxicity of the synthesized ILs was checked against the *Danio rerio* fish (OECD 203).

2. Materials and Methodology

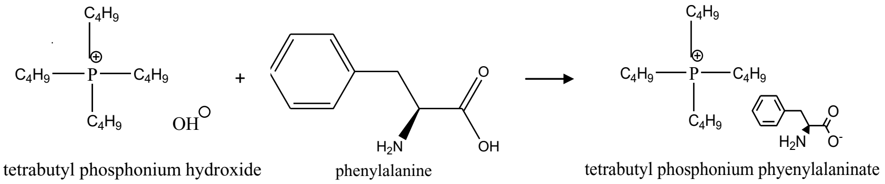
2.1. Synthesis and characterization of AAILs

2.1.1. Materials

Tetrabutylphosphonium hydroxide solution (40 wt %) was purchased from Aldrich, tetrabutylammonium hydroxide hydroxide (40 wt %) was purchased from Acros and cholinium hydroxide solution (45 wt %) was purchased from Sigma. L-Phenylalanine, L-Taurine and methanol for analysis were purchased from Merck. The fish species used for this study is the zebrafish (*Danio rerio*). The fish were supplied from an Aquatic Shop in Seri Iskandar, Perak. The fish were chosen based on the OECD 203 requirements, mainly molecular biology, neurobiology, genetics, cancer and drug discovery.

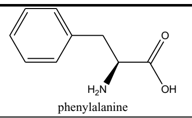
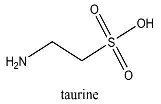
2.1.2. Synthesis of amino acid ionic liquid (AAIL)

Tetrabutylphosphonium phenylalaninate $[P_{4444}][Phe]$ was prepared according to Scheme 1. A mixture of tetrabutylphosphonium hydroxide solution (0.025 mol) and L-phenylalaninate (0.025 mol) was stirred at room temperature (25°C) for two days. After the reaction, methanol was added to wash the unreacted amino acid. The $[P_{4444}][Phe]$ was purified, weighed and characterized. Other five AAIL were synthesized by using the same procedure. Their structure and abbreviations are shown in Table 1.



Scheme 1: Schematic representation of the synthesis of $[P_{4444}][Phe]$ ILs

Table 1: AAIL names and abbreviations used

Cation	Anion	Ionic Liquids
Tetrabutylphosphonium $[P_{4444}]$		Tetrabutylphosphonium phenylalaninate
Tetrabutylammonium $[N_{4444}]$		Tetrabutylammonium phenylalaninate
Cholinium [Cho]		Cholinium phenylalaninate
Tetrabutylphosphonium $[P_{4444}]$		Tetrabutylphosphonium Taurinate
Tetrabutylammonium $[N_{4444}]$		Tetrabutylammonium Taurinate
Cholinium [Cho]		Cholinium Taurinate

2.1.3. Characterization method

^{1}HNMR and DEPT-Q NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer, using D_2O as the solvent. Thermogravimetric analysis (TGA) was performed on a STA 6000 (Perkin Elmer) under atmospheric conditions. Samples between 6 to 8 mg were heated from 303 to 773 K under a constant heating rate of 10K/min. Due to the hygroscopic properties of ILs, the water content measurement was measured using Karl Fisher coulometer (Mettler Toledo DL39).

2.2. Ecotoxicity study of AAILs

2.2.1. Acute toxicity towards zebrafish, Danio rerio

2.2.1.1. Test Method

Acute toxicity of the six ILs towards zebrafish, *Danio rerio* was assessed by measuring their lethal effect after 96 hours exposure in a static test. Prior to the toxicity test, the fish were acclimated in the laboratory for at least 7 days. Fish were fed 3-7 times per week until 24 hours before the test. The fish food nutrients was fulfilled accordingly; crude protein 35%, crude fat 5%, crude fibre 5%, crude ash 10% and moisture 10%. The fish were observed carefully daily for signs of disease, stress, physical damage, and mortality. Dead and abnormal fish were immediately removed upon observation. Tests proceeded with a limit test performed at the concentration of 100 mg/L in order to demonstrate that the LC_{50} was greater than this concentration. If mortality occurred in the limit test, the full LC_{50} study was then carried out. All tests were performed according to the OECD Guideline no. 203 (OECD, 1992). The water temperature was kept at 23 ± 1°C, and fish were acclimatized for 12-16 hours under normal laboratory illumination. No food was provided during the test. On the day of experiment, 10 fishes were placed in 5 L-plastic aquaria containing test or control solution (rearing water) and aerated to restore the concentration of dissolved oxygen to at least 90% of its air saturation value. ILs was directly dissolved in rearing water without co-solvents or vehicles. The behavior of the fish was monitored closely and dead fish were removed immediately. The numbers of dead fish for each concentration were recorded after 24, 48, 72, and 96 hours. Fish were considered dead if no noticeable physical movement observed (e.g. gill movements) and produce no reaction towards touching of the caudal peduncle. The theoretical work (PROBIT analysis) (ASTM-E1847, 2008) was conducted based on the observations and the results after four days (96 hours).

3. Results and Discussions

3.1. Synthesis and characterization of AAILs

Six AAILs were successfully synthesized and characterized.

I. Tetrabutylphosphonium taurinate [P_{4444}][Tau]

The resultant ionic liquid is a transparent viscous liquid.

^{1}H NMR: δ 0.778 – 0.807 (*t*, 12H, -CH_3), δ 1.288 – 1.437 (*m*, 16H, -CH_2), δ 1.998 – 2.057 (*m*, 8H, -CH_2) δ 2.870 – 2.884 (*m*, 4H, -CH_2) and δ 3.211 (*s*, 1H, -OH). ^{13}C DEPT-Q NMR: δ 12.51 (CH_3), δ 17.34 (CH_2), δ 17.73 (CH_2), δ 22.61 – 23.30 (CH_2), δ 36.44 (CH_2) and δ 53.06 (CH_2); TGA- 91.57% (Decomposition temperature, T_G= 346.46°C); Water content = 0.86 wt. %, Refractive index, RI = 1.48

II. Tetrabutylammonium taurinate [N_{4444}][Tau]

The resultant ionic liquid is a transparent viscous liquid.

^{1}H NMR: δ 0.813 – 0.842 (*t*, 12H, -CH_3), δ 1.203 – 1.275 (*m*, 8H, -CH_2), δ 1.526 (*s*, 8H, -CH_2), δ 2.881 (*s*, 4H, -OH) and δ 3.057 – 3.090 (*m*, 8H, -CH_2). ^{13}C DEPT-Q NMR: δ 12.84 (CH_3), δ 19.10 (CH_2), δ 23.06 (CH_2), δ 36.49 (CH_2), δ 53.13 (CH_2) and δ 58.00 – 58.01 (CH_2); TGA- 96.37% (Decomposition temperature, T_G= 218.16°C); Water content = 0.52 wt. %, Refractive index, RI = 1.47

III. Cholinium taurinate [Cho][Tau]

The synthesized IL was yielded as transparent solid.

^{1}H NMR (500 MHz, Deuterium oxide-D_2O): δ 2.867 – 2.898 (*m*, 4H, CH_2), δ 3.064 (*s*, 9H, CH_3), δ 3.365 – 3.386 (*m*, 2H, CH_2) and δ 3.912 – 3.931 (*m*, 2H, CH_2);^{13}C DEPT-Q NMR: (125 MHz, Deuterium oxide-D_2O): δ 36.42 (CH_3), δ 53.14 (CH_2), δ 53.72 – 53.78 (CH), δ 55.52 (CH_2) and δ 67.38 – 67.42 (CH_2); TGA- 93.90% (Decomposition temperature, T_G= 188.05°C); Water content = 2.49 wt. %, Refractive index, RI = 1.48

IV. Tetrabutylphosphonium phenylalaninate [P_{4444}][PheAla]

The resultant ionic liquid is a yellowish viscous liquid.

^{1}H NMR δ 0.780 – 0.794 (*t*, 12H, -CH_3), δ 1.285 – 1.413 (*m*, 16H, -CH_2), δ 1.964 – 2.023 (*m*, 8H, -CH_2), δ 2.7012 – 2.8812 (*m*, 2H, -CH_2), δ 3.379 – 3.382 (*t*, 1H, -CH) and δ 7.128 – 7.233 (*m*, 5H, -CH);^{13}C DEPT-Q NMR: δ 12.46 (CH_3), δ 17.31 – 17.70 (CH_2), δ 22.59 – 23.28 (CH_2), δ 40.23 (CH_2), δ 48.75 (CH), δ 126.64 (CH), δ 128.54 (CH), δ 129.34 (CH), δ 137.84 (CH_2) and δ 181.55 (C=O); TGA- 95.0% (Decomposition temperature, T_G= 296.54°C);
Water content = 0.70 wt. %, Refractive index, RI = 1.51

V. Tetrabutylammonium phenylalaninate [N_{4444}][PheAla]

The resultant ionic liquid is a yellowish solid.

^{1}H NMR δ 0.781 – 0.811 (*t*, 12H, -CH_3), δ 1.156 – 1.215 (*m*, 8H, -CH_2), δ 1.419 – 1.482 (*m*, 8H, -CH_2), δ 2.685 – 2.847 (*m*, 2H, -CH_2), δ 2.957 – 2.991 (*m*, 8H, -CH_2), δ 3.328 – 3.353 (*t*, 1H, -CH) and δ 7.126 – 7.242 (*m*, 5H, -CH). ^{13}C DEPT-Q NMR: δ 12.82 (CH_3), δ 19.08 (CH_2), δ 23.02 (CH_2), δ 40.59 (CH_2), δ 57.31 – 57.96 (CH), δ 126.61 – 129.41 (CH), δ 138.11 (CH_2) and δ 181.87 (C=O); TGA- 95.20% (Decomposition temperature, T_G= 171.29°C); Water content = 0.93 wt. %, Refractive index, RI = 1.51

VI. Cholinium phenylalaninate [Cho][PheAla]

The synthesized IL was yielded as orange-brownish viscous liquid.

^{1}H NMR δ 2.699 – 2.825 (*m*, 2H, CH_3), δ 2.998 (*s*, 9H, CH_3), δ 3.295 – 3.350 (*m*, 3H, CH_2, CH), δ 3.846 – 3.871 (*m*, 2H, CH_2) and δ 7.119 – 7.237 (*m*, 5H, CH). ^{13}C DEPT-Q NMR: δ 40.65 (CH_3), δ 53.68 – 53.14 (CH), δ 55.41 (CH_2), δ 57.34 (CH_2), δ 67.31 – 67.35 (CH_2), δ 126.57 – 129.36 (CH), δ 138.16 (C=O); TGA- 95.20% (Decomposition temperature, T_G= 190.81°C); Water content = 1.66 wt. %, Refractive index, RI = 1.53

3.2. Ecotoxicity of AAILs

3.2.1. Acute toxicity of AAILs towards zebrafish, Danio rerio

The concern on the environmental risk of ILs has emerged for several decades with regards to the water solubility and miscibility. ILs sips into the environment via accidental spills and industrial effluents [49]. The issue with the water solubility of ILs can be related to its ionic character with modifications of cations and anions [50, 51]. Recently, numerous ecotoxicity studies of amino acid based ILs towards organisms of various levels of organization have been discussed [52-59]. Although there have been studies on the ecotoxicity of ILs towards *Danio rerio*, [60-63], to the best of our knowledge, there have been no investigations on the ecotoxicity of amino acids based ILs against zebrafish, *Danio rerio*. In this study, the acute toxicity towards zebrafish of six ILs with different cations (phosphonium, ammonium and cholinium) and anions (phenylalaninate and taurinate) were assessed. All the ILs tested had 96 h LC_{50} values greater than 100 mg/L. Evidently, results depicted values of "practically non-toxic" with LC_{50} greater than 100 mg/L- according to the Acute Toxicity Rating Scale by Fish and Wildlife Service (FWS) (Table 3). Results of the limit test are shown in Table 2.

Table 2 Limit test (100mg/L) of six ionic liquids towards *Danio rerio*

Ionic liquids	**LC_{50} 96 h zebrafish**
[P_{4444}][Phenylalaninate]	> 100 mg/L
[P_{4444}][Taurinate]	>100 mg/L
[N_{4444}][Phenylalaninate]	>100 mg/L
[N_{4444}][Taurinate]	>100 mg/L
[Cho][Phenylalaninate]	>100 mg/L
[Cho][Taurinate]	>100 mg/L

Table 3 Acute Toxicity Rating Scale by Fish and Wildlife Service (FWS)

Relative Toxicity	**Aquatic 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

A previous study by Pretty and team [60] on the toxicity of ILs against zebrafish demonstrated that ILs may cause a different effect on fish depending to their chemical structure. For instance, imidazolium, pyridinium and pyrrolidinium showed a LC_{50} more than 100 mg/L and could be considered non-toxic towards zebrafish. However, ammonium salts showed LC_{50} lower than that reported for organic solvents and tertiary amines. According to Pham et al. (2010) [38], in general, those data presented by Pretti and team [60] deduced that fish were less sensitive to ILs toxicity compared to other species in lower trophic levels. Later, Pretti and co-workers (2009) [61] conducted another study on the acute toxicity towards zebrafish and concluded that all the tested ILs which comprised of various cation head groups, side alkyl chain and counter anions were practically harmless with EC_{50} more than 100 mg/L.

Structure-toxicity relationship of ILs has been widely studied and discussed [41, 43, 64, 65] however these deal with a general assumption on toxicity and the behaviour of these compounds towards microorganisms. Real time values indicate the toxicity of ILs are vital in determining the impact of ILs towards fish. In our study, the cholinium cation and the butyl side chain on both of the ammonium and phosphonium cation led to a non-toxic nature towards the zebrafish. This is in agreement with Stolte and team (2007) [41], who carried out studies on the influence of different head groups, functionalised side chains and anions of the ILs towards aquatics (*Vibrio fischeri*, *Lemna* *minor* and *Scenedesmus vacuolatus*). They reported [41] that the reduced toxicity towards the test organisms can be achieved by the use of short functionalised side chains instead of the non-polar alkyl chains. They also proposed that the influence of the head group is of minor relevance to alter lipophilicity concomitantly the toxicity in most of the cases. Stolte and team [41] also mentioned that the toxicity was dependent on the lipophilicity of ILs cations in which, the side chain was the main factor in the changes of lipophilicity. The introduction of polar functionalised groups into a short side chain consistently diminished the observed toxicities in all three test systems in comparison to the butyl side chain. These data by our team was in lieu with [52] and [65] in which the increase in the alkyl chain length increased the toxicity of ILs towards the organisms tested.

With the abundant information on the effect of cation towards the toxicity of organisms [41, 58, 59, 64], the influence of anion has been less described. Matzke et al. (2007) [64] deduced that a toxic nature were mainly caused by the cationic species rather than the anionic ones. Stolte et al. (2007) [41] reported the insignificant effect of halide anions with different cations towards the toxicities. However, an increased in toxicity was observed when the cation was paired with the hydrophobic NTf_2 anion. Ghanem et al. (2015) [58] claimed that the amino acids based ILs, consisted of 1-(2-hydroxyethyl-3-methylimidazolium) cation with glycinate, serinate, alaninate and prolinate as counter anions were eco-friendly in the antimicrobial test against the green algae, *Scenedesmus quadricauda* and bioluminescent marine bacteria, *Vibrio fischeri*. These were due to the presence of a short hydroxyl functional group and the environmentally friendly nature of amino acid anions.

4. Conclusion

The six amino acid-based ILs were synthesized with 3 different cations, namely, phosphonium, ammonium and choline. All the ILs showed values of being practically non-toxic with LC_{50} values exceeding 100 mg/L.

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