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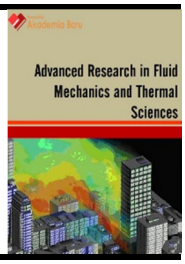

Synthesis and Characterization of New Cholinium-based Ionic Liquids for Antimicrobial Application

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ABSTRACT

A series of new cholinium-based ionic liquids (ILs) was synthesized by using simple neutralization reaction. The synthesized ILs were choline 3,5-dinitrosalicylate, choline gallate, choline s-(+)-2-(4-isobutylphenyl)propionate, choline behenate and choline peracetate. All the synthesized cholinium-based ILs were characterized by using FT-IR, ^{1}H and ^{13}C NMR, mass spectrometry, thermogravimetric and differential scanning calorimetry. Result obtained from all characterization techniques confirmed the formation of these cholinium-based ILs. These ILs were then tested for their anti-microbial properties. Anti-microbial test was carried out towards seven types of bacteria which consist of three Gram-positive bacteria, *Bacillus subtilis* (B29), *Staphylococcus aureus* (S276), *Staphylococcus epidermidis* (S273), two Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 15442) and *Escherichia coli* (E266) and two types of yeasts, *Candida albicans* (9002) and *Candida tropicalis* (A3). Streptomycin and Nystatin were used for all bacteria as standard. There is one cholinium-based IL shows excellent inhibition of microbes from growth. Choline peracetate was found to inhibit *Candida albicans* (9002) as good as the standard, Streptomycin which perform highest of inhibition zone diameter, 25 mm.

1. Introduction

Anti-microbials are widely used as an indicator to destroy or inhibit the growth of microorganisms. The acceptance of germ theory in 1800s make the understanding and development of antimicrobials increased tremendously [1]. This theory included anti-biosis, disinfection, preservation, sterilization and modern infection control.

Almost a century ago, ionic liquids (ILs) totally composed of ions, find many applications due to their interesting properties. The properties of ILs can be tuned based on sensible choice of anion and cation. Different ion substitution will give different properties to ILs [2]. Besides, ionic liquids

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also known as green solvents because of their characteristics low vapor pressure and non-flammability [3]. They have a low melting point which is below 100°C, high thermal stability and high conductivity.

Recently, ionic liquids were used in various applications of science and technology which depends on their properties. Interaction mechanism insights on the solvation of fullerene B80 with cholinium-based ILs was reported by Garcia and coworkers in 2015 using a novel density functional theory (DFT) method [4]. Other applications of ILs include separation processing, electrolytes for highly reversible lithium batteries and phase change materials for the storage of solar energy [5]. Cholinium-based ILs (i.e., cholinium sacchrinate, cholinium dihydrogen phosphate, cholinium lactate, cholinium citrate, and cholinium tartarate) had been successfully biodegraded the azo dyes [6]. In 2015, Clark and coworkers reported the extraction of DNA by magnetic ILs used for higher extraction efficiencies in larger DNA molecules [7].

Choline hydroxide is a basic constituent of lecithin that is found in many plants and animal organs. It is important as a precursor of acetylcholine, as a methyl donor in various metabolic processes, and in lipid metabolism. Choline hydroxide is a miscible compound in water. The application of choline hydroxide in aqueous solutions are useful in connection with electronic applications, such as positive photoresist developing agents, stripping photoresists, anisotropic etching agents, and washing agents for silicon wafers [8].

Dorland's Medical Dictionary for Health Consumers in 2007 defines antimicrobial is an agent for killing microorganisms or inhibiting their growth. The antimicrobial agent can be classified into three which are disinfectants, antibiotics and antiseptics. There are two types of test for antimicrobial activity which is Gram-negative and Gram-positive towards bacteria, fungi and algae. The examples of bacteria that widely used are *Escherichia coli* and *Bacillus subtilis* [9]. Gram-positive bacteria are bacteria that give a positive result in the Gram stain test and purple-colored can be seen through a microscope whereas Gram-negative bacteria cannot retain the violet stain after the decolorization step and red or pink-colored appeared.

Based on the previous research most of the researcher was used imidazolium based ILs to study the antimicrobial activity. However, this novel ILs needs to reduce its toxicity for further application [10-12]. Therefore, the main objective of this research was to synthesis new cholinium-based ionic liquids (ILs) with motivation to enhance the antimicrobial application by using different type of carboxylic acid. Cholinium based ILs was reported to be more biodegradable and less in toxicity which might be suitable to use for another application [13].

2. Materials and Methods

A. Chemicals

Choline hydroxide (45 wt% aqueous solution) was purchased from ACROS Organics. 3,5-dinitrosalicylic acid, s-(+)-2-(4-isobutylphenyl) propionic acid and behenic acid were purchased from Sigma-Aldrich. Gallic acid was purchased from Merck Chemicals. Peracetic acid was purchased from R&M Chemicals. The solvents, diethyl ether (99% purity) and chloroform (99% purity) were purchased from Merck. All chemicals and materials are commercially available and of higher analytical grade unless otherwise specified. The chemicals were used without purification, unless otherwise stated.

B. Synthesis of Cholinium-based Ionic Liquids

Cholinium-based ILs were synthesized through neutralization of choline hydroxide with five

types of carboxylic acids. These ILs were synthesized according to the procedures reported by Taha *et al*., [14]. They reported the synthesis of choline hydroxide with Good's buffers. Scheme 1 shows the reaction mechanism of cholinium-based ILs. The product formed is choline carboxylate and water.

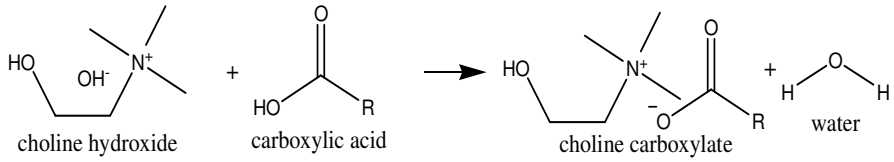


Fig. 1. Scheme 1 Reaction mechanism of choline-based ILs

Choline 3,5-dinitrosalicylate (1a). Choline 3,5-dinitrosalicylate was synthesized through neutralization of choline hydroxide with 3,5-dinitrosalicylic acid. 3,5-dinitrosalicylic acid (0.025 mol) was dissolved in 150 mL of distilled water with heating. It was added slowly into 0.025 mol of choline hydroxide. The mixture was stirred continuously for 12 h at room temperature. The solvent was removed using rotary evaporator, followed by vacuum drying at 70°C for 24 h. The sample was recrystallized in acetonitrile and diethyl ether. The yellow precipitate formed was dried. Table 1 shows the structure of five cholinium-based ILs.

Choline gallate (1b). Choline gallate was synthesized by using similar method as in **1a** but replacing 3,5-dinitrosalicylic acid with gallic acid.

Choline s-(+)-2-(4-isobutylphenyl)propionate (1c). Choline s-(+)-2-(4-isobutylphenyl)propionate was synthesized by using method as mentioned in **1a** but replacing 3,5-dinitrosalicylic acid with s-(+)-2-(4-isobutylphenyl)propionic acid. The sample was dissolved in 50 mL of chloroform and place in separator. The organic layer was then washed three times with 20 mL of distilled water. After separation, the organic layer was poured into round-bottomed flask and the solvent was evaporated under reduce pressure. The product was then dried under vacuum at 60°C for 24 h.

Choline behenate (1d). Choline behenate was synthesized by using the same method in **1a** but replacing 3,5-dinitrosalicylic acid with behenic acid. The solvent was removed by vacuum drying at 70°C for 3 days. For purification, the sample was washed with diethyl ether for three times. Then, the solvent was removed by vacuum drying at 65°C for 24 h.

Choline peracetate (1e). Choline peracetate was synthesized by using method in **1a** but replacing 3,5-dinitrosalicylic acid with peracetic acid. For purification, the sample was washed with diethyl ether for three times. Then, the solvent was removed by vacuum drying at 65°C for 24 h.

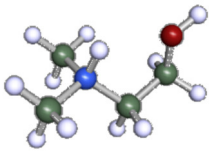
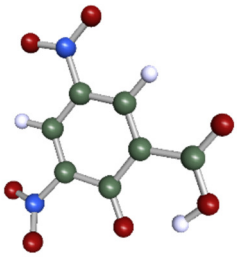
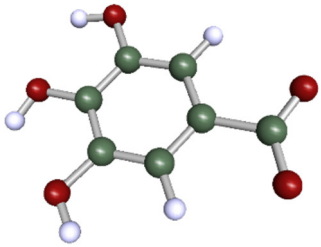
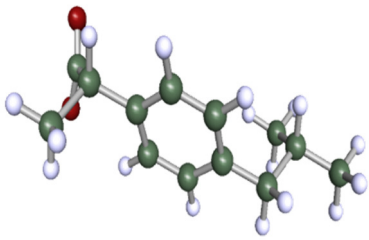
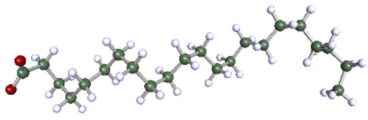
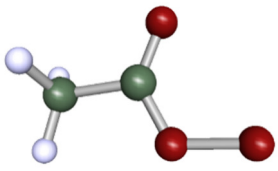
C. Characterization

The instruments used for characterization are Shimadzu Europe-GCMS-QP5050A for mass spectrometry. The mass of the molecules was determined by measuring the mass-to-charge ratio (m/z) of its ion. Perkin Elmer-FTIR Spectrometer Spectrum RX1 was used to collect FTIR spectra via KBr disc procedure in frequency of 400-4000 cm^{-1}. The structures of each ionic liquid were checked by using Varian 500 MHz NMR spectrometer. The sample was dissolved in deuterated DMSO or chloroform solvent at room temperature. Tetramethylsaline (TMS) was used as the internal standard.

Thermal analysis was carried out using a Differential Scanning Calorimeter (DSC) Mettler Toledo DSC823 and Thermogravimetric Analysis (TGA). DSC was used to determine the phase transition temperature and enthalpy change of the sample. The heating and cooling cycle were carried out at the rate 10˚C/min with nitrogen flow 10ml/min. Thermal stability of the sample was measured over

a wide range of temperature which is 25 to 400˚C. Mettler-Toledo TGA/SDTA 851e TGA instrument measurements were conducted on alumina pans in nitrogen atmosphere at a heating rate of 10˚C/min.

Table 1
Structure of new choline-based ILs

Cation	Anion
 **cholinium**	 **1a**
	 **1b**
	 **1c**
	 **1d**
	 **1e**

D. Antimicrobial Activity

Antimicrobial or antifungal test was carried to determine the effectiveness of choline-based ILs in treating bacteria or fungal infections. The response (sensitivity/resistance) of microbes against antimicrobial compounds various to each other.

The test was carried out by placing 6mm diameter of paper disc containing IL sample onto a plate which microbes grew. The microbe culture was standardized to 0.5 Mc Farland standards which was approximately 10^8 cells. Streptomycin standard were used for each bacteria. Nystatin standard were used for yeast.

The plates were inverted and incubated at 30-37°C for 18-24-48 or until sufficient growth has occurred. After incubation, each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using sliding calipers or a ruler, which was held on the back of the inverted petri plate. The ILs samples were tested against *Bacillus subtilis* (B29), *Staphylococcus aureus* (S276), *Staphylococcus epidermidis* (S273), *Pseudomonas aeruginosa* (ATCC 15442) *Escherichia coli* (E266), *Candida albicans* (9002) and *Candida tropicalis* (A3).

3. Results and Discussion

A. Properties of Synthesized Ionic Liquids

Choline 3,5-dinitrosalicylate (1a). Yellow solid (Yield: 74.64%). FT-IR spectroscopy was used to characterize all the newly synthesized ILs. The absorption peak at around 3404.19 cm^{-1} was a characteristic of -OH stretching frequency of choline hydroxide [15]. The other peaks of choline hydroxide are at 1165.90 cm^{-1} which is a characteristic of C-N of aliphatic amines and 1068.77 cm^{-1} for C-O stretching of alcohols. The absorption peaks of 3,5-dinitrosalicylate are at 3090.08, 2878.10, 1683.83, 1600.26, 1534.18 and 1260.53 cm^{-1}, respectively. ^{1}H NMR (500MHz, DMSO) δ (ppm): 8.65 (d, 2H), 2.03 (s, 1H), 3.79 (t, 3H), 3.35 (t, 3H), 3.08 (s, 9H). ^{13}C NMR (500MHz, DMSO) δ (ppm): 169.47, 120.90, 167.41, 139.13, 126.12, 130.98, 130.56, 55.69, 67.49, 53.74−53.68. Mass spectrum for choline was at m/z 107 and m/z 58 and for 3,5-dinitrosalicylate was at m/z 211.

Choline gallate (1b). Brown solid (Yield: 69.59%). The absorption peak at around 3150.75 cm^{-1} was a characteristic of -OH stretching frequency of choline hydroxide although the peak slightly lower frequency than the theoretical value (3363 cm^{-1}) however the peak was strong and broad [15]. The other peaks of choline hydroxide are at 1192.28 cm^{-1} as a characteristic of C-N of aliphatic amines and 1086.65 cm^{-1} for C-O stretching of alcohols. C-H bending frequency of choline hydroxide was observed at 951.72 cm^{-1} which tally with the report by Kalmode *et al*., 2015. The absorption peaks of gallate are at 1686.34 cm^{-1} and 1605.36 cm^{-1}. ^{1}H NMR (500MHz, $CDCl_3$) δ (ppm): 6.88 (d, 2H), 5.00 (s, 1H), 2.47 (s, 1H), 3.77 (t, 3H), 3.46 (t, 3H), 3.32 (s, 9H). ^{13}C NMR (500MHz, $CDCl_3$) δ (ppm): 170.61, 124.63, 109.80, 144.51, 136.95, 55.67, 67.44, 53.85. Mass spectrum for choline was at m/z 106 and for gallate was at m/z 170 and m/z 125.

Choline s-(+)-2-(4-isobutylphenyl) propionate (1c). White solid (Yield: 35.24%). According to Kalmode *et al*., frequency of 3363 cm^{-1} was a characteristic of -OH stretching of choline hydroxide should be found in this sample, however the absorption peak for this -OH was missing. This might due to some other reactions. The other peaks of choline hydroxide are at 1227.59 cm^{-1} as a characteristic of C-N of aliphatic amines and 1279.15 cm^{-1} for C-O stretching of alcohols. The absorption peaks of s-(+)-2-(4-isobutylphenyl) propionate are at 2949.33, 2636.40, 1697.69 and 1459.88 cm^{-1}. ^{1}H NMR (500MHz, DMSO) δ (ppm): 3.81 (m,4H), 1.45 (d, 2H), 7.07 (s,1H), 2.51 (d, 2H), 2.22 (m), 1.01 (d, 2H), 2.00 (s, 1H), 3.97 (t, 3H), 3.43 (t, 3H), 3.30 (s, 9H). ^{13}C NMR (500MHz, DMSO)

δ (ppm): 176.01, 39.46, 22.67, 139.03, 127.62, 129.46, 140.05, 39.62, 30.15, 19.05. Mass spectrum for choline was at m/z 107 and for s-(+)-2-(4-isobutylphenyl) propionate was at m/z 206 and 161.

Choline behenate (1d). White solid (Yield: 67.10%). In this sample the absorption peak for this -OH is at 3364.90 cm^{-1} which is almost equal to the frequency at 3363 cm^{-1} [10]. The other peaks of choline hydroxide are at 1253.88 cm^{-1} was characteristic of C-N of aliphatic amines and 1084.13 cm^{-1} for C-O stretching of alcohols. ^{1}H NMR (500MHz, $CDCl_3$) δ (ppm): 0.86 (t, 3H), 1.24 (m, 4H), 1.56 (m, 4H), 2.22 (t, 2H), 1.94 (s, 1H), 3.62 (t, 3H), 3.30 (t, 3H), 3.26 (s, 9H). ^{13}C NMR (500MHz, $CDCl_3$) δ (ppm): 14.19, 22.77, 29.44−32.32, 25.91, 36.32, 179.23, 56.20, 68.40, 54.72. Mass spectrum for choline was at m/z 87 and for behenate was at m/z 340 and m/z 323.

Choline peracetate (1e). Colorless liquid (Yield: 71.43 %) The absorption peak at around 3248.00 cm^{-1} was a characteristic of -OH stretching frequency. The absorption peak at 1075.68 cm^{-1} was characteristic assign for C-O stretching and 950.92 cm^{-1} for C-H bending. ^{1}H NMR (500MHz, $CDCl_3$) δ (ppm): 1.82 (s, 1H), 1.99 (s, 1H), 3.96 (t, 3H), 3.48 (t, 3H), 3.18 (s, 9H). ^{13}C NMR (500MHz, $CDCl_3$) δ (ppm): 181.23, 23.41, 55.70, 67.49, 53.88−53.94. Mass spectrum for choline was at m/z 58 and for peracetate was at m/z 41.

B. Thermal Analysis

Melting temperature (T_m) of cholinium-based compounds were measured using DSC and the results were tabulated in Table 2. The selected combinations of anions and cations will contribute different characteristic properties of ILs [11]. In this research, all five compounds showed a T_m but only two compounds fulfill the characteristic of ILs with slightly low T_m which are below 100°C. Two compounds that showed melting temperatures below 100°C were **1c** and **1d**.

Table 2
Melting temperature (T_m) of choline-based ILs

Cholinium-based ILs	Melting temperature (T_m/°C)
1a*	111.72 (±2.0)
1b*	123.49 (±2.1)
1c	50.77 (±1.0)
1d	80.69 (±0.8)
1e*	105.14 (±1.7)

* Not considered as ILs due to high melting point (>100°C)

In comparison, **1b** showed a highest T_m which was 123.49°C, followed by **1a** and **1e** with T_m 111.72°C and 105.14°C, respectively. Thus, these three compounds will not be considered as ILs due to their melting temperature were above 100°C and considered as new choline-based salts. Other than that, **1c** (50.77°C) has a lowest T_m compared to all compounds and its T_m was lower than **1d** (80.69°C). In addition, all of cholinium-based compounds decompose at high temperature at a range 200°C to 300°C by thermogravimetric analysis (TGA).

C. Antimicrobial Activity

Seven strains of bacteria, three Gram-positive, *Bacillus subtilis* (B29), *Staphylococcus aureus* (S276), *Staphylococcus epidermidis* (S273), two Gram-negative, *Pseudomonas aeruginosa* (ATCC 15422) and *Escherichia coli* (E266) and two types of yeasts, Candida albicans (9002) and *Candida tropicalis* (A3) were used to assess the antimicrobial activity of the ILs that have been synthesized.

The minimum inhibitory concentration (MIC) of this cholinium-based ILs cannot be determined

due to low solubility of ILs in nutrient broth. Table 3 shows the inhibition zone diameter of ILs (mm) towards the bacteria. All the bacteria used were tested with standard, Streptomycin and Nystatin which will show positive inhibition zone diameter (mm) whereas water and DMSO will show zero inhibition zone diameters (mm).

In this study, only cholinium peracetate give some results whereas other cholinium-based compounds do not give any results. Among seven strains of bacteria, only three bacteria show positive results which are *Bacillus subtilis*, *Staphylococcus epidermidis* and *Candida albicans*. The highest result is *Candida albicans* with 25 mm diameter of inhibition of bacteria. Choline peracetate also show good inhibition similar to the standard. Choline 3,5-dinitrosalicylate, choline behenate, choline s-(+)-2-(4-isobutylphenyl) propionate and choline gallate give zero inhibition zone diameter towards all the bacteria.

Table 3
Inhibition zone diameter of choline-based compounds towards bacteria (mm)

Cholinium-based ILs	Inhibition zone diameter of choline-based ILs (mm) towards bacteria						
	B29	*S276*	*S273*	*ATCC 15422*	*E266*	*9002*	*A3*
1a	-	-	-	-	-	-	-
1b	-	-	-	-	-	-	-
1c	-	-	-	-	-	-	-
1d	-	-	-	-	-	-	-
1e	9	-	10	-	-	25	-
Std (+ve) (1 mg/ml)	19	20	22	20	23	25	19
H_2O (-ve) (1 mg/ml)	-	-	-	-	-	-	-
DMSO (-ve) (1 mg/ml)	-	-	-	-	-	-	-

4. Conclusion

The main target of this research is to synthesize a series of new cholinium-based ionic liquids (ILs) by neutralization reaction. Five selected carboxylic acids, 3,5-dinitrosalicylic acid, gallic acid, s-(+)-2-(4-isobutylphenyl)propionic acid, behenic acid and peracetic acid were added to choline hydroxide to form cholinium-based ILs. The synthesized cholinium-based compounds were cholinium 3,5-dinitrosalicylate, cholinium gallate, cholinium s-(+)-2-(4-isobutylphenyl) propionate, cholinium behenate and cholinium peracetate.

All the synthesized cholinium-based compounds were characterized by using infrared spectroscopy, ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry, thermogravimetric and differential scanning chromatography. Result obtained from all characterization techniques confirmed the formation of these choline-based compounds. Two cholinium-based ILs were successfully formed which were cholinium s-(+)-2-(4-isobutylphenyl) propionate and cholinium behenate due to low melting temperature below 100°C whereas the other three compounds cannot be classified as ILs but as new choline-based salts.

These cholinium-based compounds were then tested for their antimicrobial properties. Antimicrobial test was carried out towards seven types of bacteria which consist of three Gram-positive bacteria, *Bacillus subtilis* (B29), *Staphylococcus aureus* (S276), *Staphylococcus epidermidis* (S273), two Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 15442) and *Escherichia coli*

(E266) and two types of yeasts, *Candida albicans* (9002) and *Candida tropicalis* (A3). One of cholinium-based compounds shows excellent inhibition of microbes from growth. Streptomycin and Nystatin were used for all bacteria as standard. Cholinium peracetate was found to inhibit *Candida albicans* (9002) as good as the standard, Streptomycin by produce 25 mm inhibition zone diameter same with standard.

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