Synthesis, characterization, antimicrobial activity and *In*-silico study of pyridinium based ionic liquids

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Antibacterial Working Procedure

Chemicals and Equipment

ILs are used as test chemical for antibacterial work and different concentrations were prepared using serial dilution technique taking one initial concentration as higher (1000 mM). Chemicals were dissolved in water or other suitable solvents, and they act as positive control. Nutrient agar was prepared according to the instruction leveled in the container and kept in freezer for longer use. Dilute alcohol or acetone were used to ensure septic environment during the culture preparation and bacterial inoculation. Laminar Air flow was confirmed all the time during the culture preparation and all the tests conducted. Incubator for bacterial growth was maintained at temperature 37 ± 1 °C.

Preparation of Bacterial Inoculums

The Gram Positive and Gram-Negative bacteria were pre-cultured in Nutrient Broth (NB) on overnight in incubator at 37° C. After incubation centrifuged at 10,000 rpm for 5 min, pellet was suspended in distilled water (DW). The Petri dishes were flooded, 50 mL of sterile distilled water, using sterile cotton buds, micro tips and forceps.

Preparation of IL solutions in different concentrations

The required amount of the sample was measured in digital balance with highly carefully so that no impurities were obtained. Then the required 1 ml distilled water was added and well shake for well soluble. Some of samples were not soluble in water. These are soluble in MeOH so that, methanol was required solvent for these samples.

Code no.	M. Wt.	Req. Wt.(g)	Code no.	M. Wt.	Req. Wt.(g)
IL-01	125.12	0.125	IL-12	275.14	0.275
IL-02	139.15	0.139	IL-13	185.14	0.185
IL-03	153.18	0.153	IL-14	199.18	0.199
IL-04	167.21	0.167	IL-15	213.32	0.213
IL-05	193.12	0.193	IL-16	227.34	0.227
IL-06	232.07	0.232	IL-17	253.13	0.253
IL-07	168.18	0.168	IL-!8	292.08	0.292
IL-08	182.20	0.182	IL-19	205.69	0.205
IL-09	196.21	0.196	IL-21	335.28	0.335
IL-10	210.23	0.210	IL-21	263.92	0.263
IL-11	236.19	0.236			

Table SA. List of ILs and the required weight for 1 M solution

The initial higher concentration 1000 m M was prepared for all ILs tested, which is then diluted to 500, 250, 100 and 50 m M using the solvent or water in them. The concentrations were then used for screening and MIC evaluation.

Antibacterial Screening via Well diffusion test

Media was spread in petri-plate at first and keep 40 minutes for solidification of media. Then bacterial inoculum was uniformly spread using sterile cotton swab or hockey stick on a sterile Petri dish Nutrient Agar media. Four serial wholes were made. Then four different compounds of 20 μ L with solution were taken in each whole in marking on petri-plate. 20 μ L of sample solution were added to each of the 3 wells (5 mm diameter holes cut in the agar gel, 40 mm apart from one another). Each plate contained a control as water or methanol with respect to solubility of solvent. The systems were incubated for 24 hours at 36 ±1°C, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in mm. Tests were performed in triplicate [242].

Determination of MIC

Determination of minimal inhibitory concentration (MIC) was conducted according to the standard procedure developed by the Clinical and Laboratory Standards Institute (CLSI), Pennsylvania, USA. The microbial strains were cultured on a Nutrient Agar media for 24 hours. The nutrient agar media was spread through the Petri-plates. The plates remained for 40 to 50 minute for solidification.

After solidification, the bacteria suspension was spread by hockey strike through all plates. Then six holes were made serially and put all the serial different concentration of ILs solutions was kept as 20 μ L per hole. Three replicates and seven different concentrations were studied for each IL. Microbial growth was visually determined after incubation for 24 hours at 37°C. The zone of Inhibition was recorded in mm.

Antifungal Screening Test

Aspergillus niger and *Rhizoplus azzahra* were used for evaluating the antifungal activity of all synthesized compounds. The antifungal activity was evaluated by Well diffusion method [244]. The media altered Potato dextrose broth (abbreviated "PDB") is formulated identically to PDA [245], omitting the agar. Common organisms that can be cultured on PDB are molds such as *Aspergillus niger and Rhizoplus azzahra* [246]. All synthesized compounds were dissolved in water or methanol basis on their solubility for making the concentration 1000 m M. The 100 μL

solution of ILs were taken in petri-plate. The Media of PDB was dispersed and solidified. A well of 5 mm were made in the middle of petri-plate using cork-borer and the fungal lead were place there [247]. The plates were then kept in incubator for 96 hours at 37° C. After 3 days the fungal growth in presence of ILs, were measured and analysed.

Name of Ionic	Molecular Structure 2D	Molecular Structure 3D
Liquids		
Pyridinium		
Formate IL-01	$\left[\begin{array}{c} & & \\ & & & \\ & & \\ & & \\ & & & \\ & & \\ & & \\ & & & \\ & & \\ & & \\ & & \\ & &$	$\checkmark \succ \checkmark$
Pyridinium		
Acetate IL-02	$\left[\begin{array}{c} \bullet\\ \bullet\\ H \end{array}\right] \left[\begin{array}{c} \bullet\\ \bullet\\ \bullet\\ H \end{array}\right]$	オキ
Pyridinium		
Propionate IL-03		
Pyridinium		
Butyrate IL-04		なな

Table S1A: Molecular Structure of Ionic Liquids (1-21)









Table S1B: Canonical SMILES ID of Ionic Liquids (1-21)

Name of Ionic Liquids	SMILES
Pyridinium Formate;	O=C[O-].C1=C[NH+]=CC=C1
IL-01	
Pyridinium Acetate;	CC([O-])=O.C1=C[NH+]=CC=C1
IL-02	
Pyridinium Propionate; IL-03	CCC([O-])=O.C1=C[NH+]=CC=C1
Pyridinium Butyrate;	CCCC([O-])=O.C1=C[NH+]=CC=C1
IL-04	
Pyridinium Trifluoroacetate;	FC(F)(F)C([O-])=O.C1=C[NH+]=CC=C1
IL-05	
Pyridinium 2-Bromopropionate; IL-06	CC(Br)C([O-])=O.C1=CC=CC=[NH+]1
4-Dimethyl Amino Pyridinium Formate; IL-07	CN(C)C1=CC=[NH+]C=C1.[O-]C=O
4-Dimethyl Amino Pyridinium Acetate;	CN(C)C1=CC=[NH+]C=C1.CC([O-])=O
IL-08	
4-Dimethyl Amino Pyridinium Propionate;	CN(C)C1=CC=[NH+]C=C1.CCC([O-])=O
IL-09	
4-Dimethyl Amino Pyridinium Butyrate;	CN(C)C1=CC=[NH+]C=C1.O=C([O-])CCC
1L-10 4 Dimethyl Amine Dyridiniym	CN(C)C1-CC-[N]H+]C-C1 EC(E)(C(E))
	$\bigcup_{i=1}^{i} \bigcup_{j=1}^{i} \bigcup_{i=1}^{i} \bigcup_{j=1}^{i} \bigcup_{j$
Influoroacetate;])=U)r
4-Dimethyl Amino Pyridinium 2-	CN(C)C1=CC=[NH+]C=C1.CC(Br)C([O-
])=O

Bromopropionate;	
IL-12	
2-Amino-3-Nitro Pyridinium Formate;	NC1=C([N+]([O-])=O)C=CC=[NH+]1.[O-
IL-13]C=0
2-Amino-3-Nitro Pyridinium Acetate;	NC1=C([N+]([O-
IL-14])=O)C=CC=[NH+]1.O=C(C)[O-]
2-Amino-3-Nitro Pyridinium Propionate;	NC1=C([N+]([O-
IL-15])=O)C=CC=[NH+]1.O=C(CC)[O-]
2-Amino-3-Nitro Pyridinium Butyrate IL-16	NC1=C([N+]([O-
])=O)C=CC=[NH+]1.CCCC([O-])=O
2-Amino-3-Nitro Pyridinium	NC1=C([N+]([O-
Trifluoroacetate IL-17])=O)C=CC=[NH+]1.O=C([O-])C(F)(F)F
2-Amino-3-Nitro Pyridinium 2-	O=[N+](C1=C(N)[NH+]=CC=C1)[O-
Bromopropionate IL-18].CC(Br)C([O-])=O
Benzyl Pyridinium Chloride IL-19	[N+]1(CC2=CC=CC=C2)=CC=CC=C1.[C1-]
Benzyl Pyridinium Trichloro Nickelate IL-20	Cl[Ni-
](Cl)Cl.[N+]1(CC2=CC=CC=C2)=CC=CC=C 1
Benzyl Pyridinium Acetate IL-21	CC([O-
])=O.[N+]1(CC2=CC=CC=C2)=CC=CC=C1

Table S2: Results of Mannich Reaction with different ILs

Ionic Liquids	Quantity (gm)	Time (min)	Yield (%)
IL:05	0.20	10	91
IL:05	0.23	10	96.7
IL:11	0.23	30	85
IL:17	0.25	20	93

Reaction condition: Aldehyde:Amine:Cyclohexanone, (1:1:1) mole ratio; temperature at 25 °C; Two drops of water (0.06g) was added into ionic liquid to allow proper mixing.

Table S3: Data of zone of inhibition for antibacterial activity

Ionic	Bacillus	Staphyloc-	Sarcina	Salmonella	Eschericia	Pseudomonas
Liquids	cereus	occus aureus	lutea (+)	typhi	coli	aeruginosa
	(+)	(+)		(-)	(-)	(-)
IL:01	0	0	19±1	0	32±1	30±1
IL:02	0	0	24±1	0	0	22±1

IL:03	0	19±1	20±1	0	0	28±1
IL:04	0	0	0	0	27±1	0
IL:05	0	0	0	0	25±1	0
IL:06	18±1	16±1	22±1	15±1	27±1	21±1
IL:07	20±1	19±1	20±1	28±1	30±1	26±1
IL:08	16±1	22±1	21±1	24±1	25±1	21±1
IL:09	22±1	20±1	18±1	24±1	20±1	25±1
IL:10	30±1	35±1	31±1	36±1	33±1	31±1
IL:11	28±1	26±1	31±1	30±1	32±1	26±1
IL:12	24±1	22±1	20±1	28±1	28±1	25±1
IL:13	18±1	19±1	21±1	24±1	28±1	25±1
IL:14	23±1	17±1	16±1	24±1	25±1	28±1
IL:15	18±1	16±1	17±1	18±1	23±1	21±1
IL:16	19±1	15±1	14±1	16±1	15±1	21±1
IL:17	20±1	24±1	22±1	24±1	23±1	26±1
IL:18	22±1	21±1	24±1	18±1	25±1	23±1
IL:19	09±1	18±1	19±1	24±1	23±1	24±1
IL:20	18±1	19±1	17±1	23±1	18±1	20±1
IL:21	20±1	18±1	17±1	35±1	30±1	28±1

Table S4: Zone of growth observed from the antifungal test (Aspergillus niger)

Chemicals tested	Zone of growth	Percent of growth
Control	28 mm	100%
IL:01	20.5±1	78.57%
IL:02	19.0±1	67.80%
IL:03	25.0±1	89.28%
IL:04	14.5±1	51.78%
IL:05	12.5±1	46.67%
IL-06	25.5±1	91.07%
IL:07	22.0±1	78.85%
IL:08	18.0±1	64.28%
IL:09	17.0±1	60.71%
IL:10	23.0±1	82.14%
IL:11	18.5±1	64.28%
IL:12	27.0±1	96.62%
IL:13	25.0±1	89.28%
IL:14	5.0±1	17.85%
IL:15	12.7±1	45.53%
IL:16	14.8±1	52.75%

IL:17	22.7±1	81.25%
IL:18	26.5±1	92.85%
IL:19	15.5±1	53.35%
IL:20	18.5±1	66.07%
IL:21	26.2±1	93.75%

Table S5: Docking by Autodock vina (Swissdock)

Ionic Liquids	Aspergill us niger PDB ID (2BJH)	Bacillus cereus PDB ID (1QS1)	E. coli PDB ID (1JG0)	Pseudom onas aeruginos a PDB ID (4ESH)	Salmonel la typhi PDB ID (1A5A)	Sarcina lutea PDB ID (1GWF)	Staphyloco ccus aureus PDB ID (4CJN)
IL-01	-3.99	-4.28	-4.14	-4.24	-4.07	-4.36	-3.75
IL-02	-3.98	-4.28	-3.69	-4.41	-4.07	-4.33	-3.74
IL-03	-3.97	-4.26	-4.15	-4.55	-4.08	-4.32	-3.77
IL-04	-3.74	-3.81	-3.49	-3.95	-3.47	-3.68	-3.48
IL-05	-3.96	-4.28	-4.12	-4.56	-4.07	-4.36	-3.74
IL-06	-3.98	-4.28	-4.15	-4.54	-4.08	-4.31	-3.80
IL-07	-4.82	-5.12	-4.61	-4.99	-4.56	-3.66	-4.03
IL-08	-4.81	-5.11	-4.59	-4.95	-4.54	-5.13	-4.06
IL-09	-4.86	-5.08	-4.61	-4.99	-4.56	-5.15	-4.03
IL-10	-4.82	-5.08	-4.61	-4.99	-4.56	-5.12	-4.15
IL-11	-4.81	-5.11	-4.59	-4.98	-4.56	-5.12	-4.05
IL-12	-4.82	-5.12	-4.60	-4.97	-4.56	-5.06	-4.13
IL-13	-5.39	-6.14	-5.32	-5.91	-5.41	-5.59	-4.17
IL-14	-5.38	-6.11	-5.32	-5.67	-5.41	-5.42	-4.69
IL-15	-5.39	-6.14	-5.32	-5.92	-5.39	-5.50	-4.85
IL-16	-5.39	-6.14	-5.33	-5.91	-5.42	-5.46	-4.85
IL-17	-5.39	-6.14	-5.33	-5.91	-5.39	-5.39	-4.86
IL-18	-5.37	-6.11	-5.32	-5.92	-5.41	-5.40	-5.33
IL-19	-6.09	-7.14	-5.51	-6.81	-6.42	-7.29	-5.79
IL-20	-6.07	-7.19	-5.67	-6.69	-6.42	-7.29	-5.14
IL-21	-6.06	-7.01	-5.53	-6.72	-6.41	-7.32	-5.09

PDB ID	Х	Y	Z
2BJH	26	24	42
1QS1	30	22	40
1JG0	40	23	28
4ESH	25	27	40
1A5A	30	20	45
1GWF	40	27	25
4CJN	27	27	29

Table S5A: Parameters for molecular docking (Grid box Dimension)

Table S6: In silico Pharmacokinetic predictions of synthesized ILs (1-21)

Ionic Liquid s	H.I.A.	BBB	P-1 Gp. I.	R.O.T.	CYP450 2C9 Substrat e	CYP450 1A2 Inhibitor	Caco-2 permea bility	Sub- cellular localiza tion	P-2 Gp. S.
IL-01	0.9376	No	No	No	No	No	0.8478	Mitoch ondria	No
IL-02	0.9632	No	No	No	No	No	0.8312	Mitoch ondria	No
IL-03	0.9677	No	No	No	No	No	0.8010	Mitoch ondria	No
IL-04	0.9694	No	No	No	No	No	0.7698	Mitoch ondria	No
IL-05	0.9823	Yes	No	No	No	Yes	0.7355	Mitoch ondria	No
IL-06	0.9795	No	No	No	No	No	1.5542	Mitoch ondria	No
IL-07	0.7930	No	No	No	No	No	0.7245	Mitoch ondria	No
IL-08	0.7768	No	No	No	No	No	0.7185	Mitoch ondria	No
IL-09	0.7963	No	No	No	No	No	0.6715	Mitoch ondria	No
IL-10	0.8048	No	No	No	No	No	0.6315	Mitoch ondria	No
IL-11	0.9359	Yes	No	No	No	No	0.6232	Mitoch ondria	No
IL-12	0.8665	No	No	No	No	No	0.6627	Mitoch ondria	No
IL-13	0.6342	No	No	No	No	Yes	0.5452	Mitoch ondria	No
IL-14	0.7492	No	No	No	No	Yes	0.5124	Mitoch ondria	No

IL-15	0.7700	No	No	No	No	Yes	0.5204	Mitoch ondria	No
IL-16	0.7852	No	No	No	No	Yes	0.5511	Mitoch ondria	No
IL-17	0.8665	No	No	No	No	Yes	0.5188	Mitoch ondria	No
IL-18	0.8490	No	No	No	No	Yes	0.5053	Mitoch ondria	No
IL-19	0.8745	No	No	No	No	Yes	0.7878	Mitoch ondria	No
IL-20	0.9259	Yes	No	Yes	No	Yes	0.6354	Mitoch ondria	No
IL-21	0.8829	Yes	No	No	No	No	0.7652	Mitoch ondria	No

H.I.A.: Human Intestinal Absorption; BBB: Blood Brain Barrier; P-1 Gp. I.: P-1 Glycoprotein Inhibitor; R.O.T.: Renal Organic Transporter; P-2 Gp. S.: P-2 glycoprotein substrate;

Table	S7:	In	silico	prediction	of	the	Physico-chemical	properties	and	Biodegradation	of
Synthe	esized	d IL	s (1-21)							

Ionic Liquids	M.W.	TPSA	WLogP	Biodegradability
IL-01	125.13	54.27	-1.13	Ready
IL-02	139.15	54.27	-0.74	Ready
IL-03	153.18	54.27	-0.35	Ready
IL-04	167.21	54.27	0.04	Ready
IL-05	193.12	54.27	1.06	Not Ready
IL-06	232.07	54.27	0.02	Ready
IL-07	168.18	57.51	-1.07	Not Ready
IL-08	182.20	57.51	-0.68	Not Ready
IL-09	196.25	57.51	-0.29	Not Ready
IL-10	210.27	57.51	0.10	Not Ready
IL-11	236.19	57.51	1.13	Not Ready
IL-12	275.14	57.51	0.09	Not Ready
IL-13	185.14	126.11	-1.63	Not Ready
IL-14	199.16	126.11	-1.24	Not Ready
IL-15	213.19	126.11	-0.80	Not Ready
IL-16	227.34	126.11	-0.46	Not Ready
IL-17	253.13	126.11	0.56	Not Ready
IL-18	292.08	126.11	-0.48	Not Ready
IL-19	205.69	3.88	-0.97	Not Ready

IL-21 263.92 44.01 0.78 Ready	IL-20	335.28	3.88	4.09	Not Ready	
	IL-21	263.92	44.01	0.78	Ready	

[[M.W. = Molecular weight; TPSA = Topological Polar Surface Area]]

Ionic Liquids	Water Solubility LogS	Fish Toxicity pLC ₅₀	Rat acute toxicity	T. pyriformis toxicity (log ug/L)
		(mg/L)	LD_{50}	
			(mol/Kg)	
IL-01	-0.552	2.3049	1.6339	-0.8428
IL-02	-0.764	2.3835	1.7993	-0.7410
IL-03	-1.177	2.0228	1.6979	-0.3962
IL-04	-1.389	1.5838	2.0421	-0.0773
IL-05	-2.168	1.4113	2.2157	0.1679
IL-06	-2.174	1.6247	2.1065	0.0716
IL-07	-1.785	1.8704	2.5343	-0.1459
IL-08	-2.095	1.7549	2.5957	0.0855
IL-09	-2.491	1.5362	2.6356	0.2331
IL-10	-2.519	1.3573	2.6330	0.3422
IL-11	-3.397	1.2535	2.7181	0.1497
IL-12	-3.001	1.3722	2.5933	0.5309
IL-13	-2.747	1.6826	2.2317	0.3149
IL-14	-2.802	1.7235	2.2472	0.5775
IL-15	-3.116	1.5790	2.3669	0.6773
IL-16	-3.265	1.4194	2.4680	0.7488
IL-17	-3.591	1.3218	2.6353	0.8397
IL-18	-3.382	1.5017	2.3509	1.1014
IL-19	-2.929	0.7625	2.6823	0.8240
IL-20	-3.885	1.0173	2.6098	1.0402
IL-21	-2.345	1.3631	2.2848	0.3385

Table S8: In vivo predicted toxicity profiles of synthesized ILs (1-21)

[Based on the criterion of US EPA, the Acute oral Toxicity is classified in four categories based on LD50 Values.

Category I; \geq 50mg/kg, Category II; 50mg/kg < 500mg/kg, Category III; 500mg/kg < 5000mg/kg, Category IV; > 5000mg/kg.]



Table S9: Bioavailability Radar of ionic liquids (1-21)









Figure S1: UV-VIS spectrum of synthesized ionic liquid of IL: 05 (255 nm)



Figure S2: FT-IR spectrum of synthesized ionic liquid of IL: 05



Figure S3: ¹H-NMR spectrum of synthesized ionic liquid of IL: 05



Figure S4: ¹H-NMR spectrum of synthesized ionic liquid of IL: 05



Figure S5: UV-VIS spectrum of synthesized ionic liquid of IL: 06 (252 nm)



Figure S6: FT-IR spectrum of synthesized ionic liquid of IL: 06



Figure S7: ¹H-NMR spectrum of synthesized ionic liquid of IL: 06



Figure S8: ¹H-NMR spectrum of synthesized ionic liquid of IL: 06



Figure S9: ¹H-NMR spectrum of synthesized ionic liquid of IL: 06



Figure S10: ¹H-NMR spectrum of synthesized ionic liquid of IL: 06



Figure S11: UV-VIS spectrum of synthesized ionic liquid of IL: 11 (286 nm)



Figure S12: FT-IR spectrum of synthesized ionic liquid of IL: 11



Figure S13: ¹H-NMR spectrum of synthesized ionic liquid of IL: 11



Figure S14: ¹H-NMR spectrum of synthesized ionic liquid of IL: 11



Figure S15: UV-VIS spectrum of synthesized ionic liquid of IL: 12 (285 nm)



Figure S16: FT-IR spectrum of synthesized ionic liquid of IL: 12



Figure S17: ¹H-NMR spectrum of synthesized ionic liquid of IL: 12



Figure S18: ¹H-NMR spectrum of synthesized ionic liquid of IL: 12



Figure S19: UV-VIS spectrum of synthesized ionic liquid of IL: 17 (389 nm)



Figure S20: FT-IR spectrum of synthesized ionic liquid of IL: 17



Figure S21: ¹H-NMR spectrum of synthesized ionic liquid of IL: 17



Figure S22: ¹H-NMR spectrum of synthesized ionic liquid of IL: 17



Figure S23: UV-VIS spectrum of synthesized ionic liquid of IL: 18 (385 nm)



Figure S24: FT-IR spectrum of synthesized ionic liquid of IL: 18



Figure S25: ¹H-NMR spectrum of synthesized ionic liquid of IL: 18



Figure S26: ¹H-NMR spectrum of synthesized ionic liquid of IL: 18



Figure S27: ¹H-NMR spectrum of synthesized ionic liquid of IL: 18





Figure S28: UV-VIS spectrum of synthesized of IL-19 (258 nm)



Figure S29: FT-IR spectrum of synthesized ionic liquid of IL-19



Figure S30: ¹H-NMR spectrum of synthesized ionic liquid IL-19



Figure S31: ¹H-NMR spectrum of synthesized ionic liquid IL-19



Figure S32: UV-VIS spectrum of synthesized of Mannich Base Compound-01 (261 nm)



Figure S33: FT-IR spectrum of synthesized Mannich Base Compound MB:01



Figure S34: ¹H-NMR spectrum of synthesized Mannich compound of MB-01



Figure S35: ¹H-NMR spectrum of synthesized Mannich compound of MB-01



Figure S36: ¹H-NMR spectrum of synthesized Mannich compound of MB-01



Figure S37: ¹³C-NMR spectrum of synthesized Mannich compound of MB-01



Figure S38: ¹³C-NMR spectrum of synthesized Mannich compound of MB-01



Figure S39: ¹³C-NMR spectrum of synthesized Mannich compound of 13

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