Article

Antimicrobial Activity of Novel Deep Eutectic Solvents

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Abstract: Herein, we utilized several deep eutectic solvents (DES) that were based on hydrogen donors and hydrogen acceptors for their antibacterial application. These DES were tested for their bactericidal activities against Gram-positive (Streptococcus pyogenes, Bacillus cereus, Streptococcus pneumoniae, and methicillin-resistant Staphylococcus aureus) and Gram-negative (Escherichia coli K1, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Serratia marcescens) bacteria. Using lactate dehydrogenase assays, DES were evaluated for their cytopathic effects towards human cells. Results from antibacterial tests revealed that DES prepared from the combination of methyl-trioctylammonium chloride and glycerol (DES-4) and DES prepared from methyl-trioctylammonium chloride and fructose (DES-11) at a 2 µL dose showed broad-spectrum antibacterial behavior and had the highest bactericidal activity. Moreover, DES-4 showed 40% and 68% antibacterial activity against P. aeruginosa and E. coli K1, respectively. Similarly, DES-11 eliminated 65% and 61% E. coli K1 and P. aeruginosa, respectively. Among Gram-positive bacteria, DES-4 showed important antibacterial activity, inhibiting 75% of B. cereus and 51% of S. pneumoniae. Likewise, DES-11 depicted 70% B. cereus and 50% S. pneumoniae bactericidal effects. Finally, the DES showed limited cytotoxic properties against human cell lines with the exception of the DES prepared from Methyltrioctylammonium chloride and Citric acid (DES-10), which had 88% cytotoxic effects. These findings suggest that DES depict potent antibacterial efficacies and cause minimal damage to human cells. It can be concluded that the selected DES in this study could be utilized as valuable and novel antibacterial drugs against bacterial infections. In future work, the mechanisms for bactericides and the cytotoxicity effects of these DES will be investigated.

Keywords: antibiotic resistance; multi-drug resistance; deep eutectic solvents; antibacterial; cytotoxicity

1. Introduction

The morbidity and mortality associated with bacterial infections have remained significant despite advances in antimicrobial chemotherapy and supportive care [1]. In part, this is due to the ability of bacteria to develop resistance and it has become a major global health concern for humans, animals, and the environment. There are several factors that lead to antibacterial resistance including misuse of antibiotics in animals and humans, poor sanitary conditions, and leakage of non-metabolized antimicrobials and their derivatives into the environment [2–5]. Recent reports from Centers for Disease Control and Prevention (CDC) revealed that antibiotic resistance has resulted in over 23,000 mortalities annually in
the United States, 25,000 deaths in the European Union, 58,000 deaths of babies in India, 38,000 deaths in Thailand, and millions of cases of diseases and hospitalizations worldwide [6–9]. These findings suggest that there is an urgent need to identify novel antimicrobials and/or disinfection strategies to overcome bacterial infections [10]. Worryingly, research in drug development and discovery has fallen short in identifying novel antimicrobials [11].

DES are defined as the combination of two or more substances in specific mole ratio that display a considerable reduction in the melting point of the aggregates compared to those of the pure starting materials [12–16]. DES are primarily used as extraction solvents or as a vehicle for drug delivery. They are also used to extract biologically active molecules from a variety of biomass sources such as phenolic plant metabolites [17–19]. DES have been widely employed in the biomedical industry to dissolve and harvest biopolymers, as well as to preserve biomolecules such as cells, DNA, and G-quadruplexes [20,21]. Recent studies revealed that DES display remarkable antibacterial activity against pathogenic bacteria [22]. Hence, it has been suggested that DES have great potential to be used as antimicrobials for treatment and prevention purposes [23–28].

The objective of this study was to determine the antibacterial efficacy of DES against several Gram-negative and Gram-positive bacteria. In addition, the cytotoxic effects of these DES were assessed against human cells. This research provides insight into the potential potency of DES, which could be utilized as broad-spectrum antibacterials.

2. Materials and Methods

2.1. Materials and Instrumentation

The following materials were used in this study: methyl-trioctylammonium chloride (≥97%, Sigma-Aldrich, Darmstadt, Germany), 2-hydroxy benzoic acid (≥99.0%, Sigma-Aldrich, Darmstadt, Germany), naphthoic acid (≥96%, Sigma-Aldrich, Darmstadt, Germany), malonic acid (≥99%, Merck, Darmstadt, Germany), glycerol (≥99.5%, LabChem, Zelienople, PA, USA), dodecanoic acid (≥98%, Sigma-Aldrich, Darmstadt, Germany), 4-tert-butylbenzoic acid (≥99%, Sigma Aldrich, Darmstadt, Germany), glucose (≥98%, LabChem, Zelienople, PA, USA), ethylene glycol (100%, LabChem, Zelienople, PA, USA), urea (≥99%, LabChem, Zelienople, PA, USA), fructose (≥99%, Sigma-Aldrich, Darmstadt, Germany), yeast extract (Sigma-Aldrich, Darmstadt, Germany), protease peptone (Sigma-Aldrich, Darmstadt, Germany), bacteriological agar (Sigma-Aldrich, Darmstadt, Germany), and phosphate-buffered saline (Sigma-Aldrich, Darmstadt, Germany). A GENESYS spectrophotometer (Thermo Fisher, Waltham, MA, USA) was used to adjust bacterial cultures’ optical density. A New Brunswick shaker incubator (Eppendorf, The Netherlands) was used for culture incubation. The DES and their components were analyzed using a PerkinElmer transform infrared spectrophotometer (FTIR) (Thermo Scientific, Waltham, MA, USA). Muller Hinton broth (Himedia, India) was used for MIC_{50} analysis HeLa cells (CCL-2) were purchased from the American Type Culture Collection (ATCC). The Roswell Park Memorial Institute (RPMI) (Sigma-Aldrich, Darmstadt, Germany) was used to culture human cells. A cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN, USA) was used for the in vitro cytotoxicity analysis.

2.2. Preparation of Deep Eutectic Solvents

All the DES were prepared as described earlier [29–31]. Methyl-trioctylammonium chloride as a hydrogen-bond acceptor (HBA) was stirred at 60 °C for 1 h and heated with several hydrogen-bond donor (HBD) compounds until a uniform liquid mixture was achieved. The composition, structure, chemical names, and mole ratio for each DES are detailed in Table 1.
Table 1. Deep eutectic solvents, their structures, and molar ratios.

<table>
<thead>
<tr>
<th>DES</th>
<th>HBA</th>
<th>HBD</th>
<th>Composition of DES in Mole Fraction (HBAs:HBs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Chemical Name</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemical Formula</td>
<td>Chemical Formula</td>
<td></td>
</tr>
<tr>
<td>DES-1</td>
<td>2-Hydroxy benzoic acid</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>DES-2</td>
<td>1-Naphthoic acid</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>DES-3</td>
<td>Malonic acid</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>DES-4</td>
<td>Glycerol</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>DES-5</td>
<td>Dodecanoic acid</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>DES-7</td>
<td>Glucose</td>
<td>1:2</td>
<td></td>
</tr>
</tbody>
</table>

Methyltrioctylammonium chloride or Aliquat 336
<table>
<thead>
<tr>
<th>DES</th>
<th>HBA</th>
<th>HBD</th>
<th>Composition of DES in Mole Fraction (HBAs:HBDs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Chemical Name</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemical Formula</td>
<td>Chemical Formula</td>
<td></td>
</tr>
<tr>
<td>DES-8</td>
<td>Ethylene Glycol</td>
<td>Ethylene Glycol</td>
<td>1:1</td>
</tr>
<tr>
<td>DES-10</td>
<td>Citric acid</td>
<td>Citric acid</td>
<td>1:1</td>
</tr>
<tr>
<td>DES-11</td>
<td>Fructose</td>
<td>Fructose</td>
<td>1:1.25</td>
</tr>
</tbody>
</table>
2.3. Fourier Transform Infrared Spectroscopy

To determine the functional groups of separate components and synthesized DES, Fourier transform infrared spectroscopy (FTIR) was performed using the pressed potassium bromide (KBr) pellet method [32]. The variations in the functional groups before and after DES formation were analyzed by taking the average of 64 scans at 4 cm\(^{-1}\) resolution to obtain the spectra between 500 and 4000 cm\(^{-1}\) using a PerkinElmer Fourier transform infrared spectrophotometer (FTIR) (Thermo Scientific, Waltham, MA, USA).

2.4. Bacterial Cultures

Several Gram-positive (B. cereus, S. pneumoniae, methicillin-resistant S. aureus (MRSA), and S. pyogenes) and Gram-negative (K. pneumoniae, E. coli K1, P. aeruginosa, and S. marcescens) bacteria were used in this study (Table 2). MRSA and E. coli K1 were purchased from the Microbial Type Culture Collection (MTCC). All other bacteria in the study, such as B. cereus, S. pyogenes, S. pneumoniae, K. pneumoniae, P. aeruginosa and S. marcescens, were derived from clinical specimens, purchased from ATCC and MTCC (Table 2). These bacteria were grown aerobically in Luria–Bertani (LB) broth culture at 37 °C overnight [10,33].

Table 2. Types of bacteria used in this study together with their strain and source.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli K1</td>
<td>MTCC 710859</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 10145</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>MTCC 13880</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>ATCC 13883</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>ATCC 131621</td>
</tr>
<tr>
<td>Methicillin-resistant Staphylococcus aureus</td>
<td>MTCC 381123</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>ATCC 12344</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>ATCC 33400</td>
</tr>
</tbody>
</table>

2.5. Antibacterial Assays

DES were tested for their bactericidal properties against bacteria using antibacterial assays [34,35]. Briefly, the optical density of overnight-grown bacterial cultures was set to 0.22 at 595 nm using LB broth (approximately 1 × 10^8 CFU per mL). After this, 1 × 10^6 bacterial CFU (10 µL) were treated with 2 µL of DES for 2 h at 37 °C, and the final volume was adjusted to 200 µL with phosphate-buffered saline (PBS). All the DES were prepared by combining the methyl-trioctylammonium chloride and all the hydrogen-bond donors (Table 1), which were then used at a 2 µL dose in a final volume of 200 µL in the antibacterial assay. The treated cultures were then ten-fold serially diluted from 10^{-1} to 10^{-6}, and dilutions 10^{-3} to 10^{-6} were plated onto nutrient agar plates. The plates were then incubated at 37 °C for 24 h and the colony-forming units were estimated by counting the viable bacterial colonies. For controls, bacteria grown in phosphate-buffered saline (PBS) and bacteria in water were taken as the negative and solvent control, respectively, while bacteria incubated with gentamicin (100 µg/mL) were considered the positive control (PC). In some experiments, DES were tested at different doses to determine their MIC\(_{50}\) against E. coli K1 and B. cereus. The two-fold dilutions (0.5 µL, 1 µL, 2 µL, and 4 µL) of the tested DES were incubated with 1 × 10^4 bacteria/well in Muller Hinton broth using broth micro-dilution assays and the final volume was adjusted to 200 µL [22]. Briefly, all the DES were two-fold serially diluted in 100 µL of MHB broth in a 96-well plate and 100 µL of bacterial cultures having 1 × 10^4 bacterial cells and incubated for overnight at 37 °C. MHB alone was used as control. The next day, the optical density was measured using a spectrophotometer at 595 nm. The results from all treatments were compared with MHB alone and data were recorded.
2.6. In Vitro Host Cell Cytotoxicity

The cytotoxic effects of DES were evaluated by performing lactate dehydrogenase (LDH) assays [36,37]. Briefly, tests were accomplished in a 96-well plate having confluent monolayers of HeLa cells (P15). The cells were incubated with 2 µL of different DES at 37 °C with 5% CO2 and 95% humidity for 24 h. The next day, cell supernatant, having LDH and LDH kit reagents (cytotoxicity detection kit; Roche Diagnostics, Indianapolis, IN, USA), was combined equally to determine the cytotoxic effects by measuring the amount of LDH generated by HeLa cells. HeLa cells cultured in RPMI alone were treated as negative controls, and cells incubated with 0.1% triton X-100 (100 percent LDH release) were treated as positive controls. The following formula was used to calculate the percent cell cytotoxicity:

\[
\% \text{ cell cytotoxicity} = \frac{\text{sample value} - \text{negative control value}}{\text{positive control value} - \text{negative control value}} \times 100.
\]

2.7. Statistical Analysis

Student’s t-test was used to evaluate statistical significance in antibacterial investigations. To assure accuracy, all of the tests were conducted in triplicate. The results are expressed as the mean standard error of three biological replicates conducted in duplicates. \(p\)-values of less than 0.05 were deemed statistically significant. GraphPad Prism software was used to calculate the cytotoxic dose (MIC\textsubscript{50}).

3. Results

3.1. DES Characterization

Figure 1a,b shows the FTIR spectra for synthesized DES (DES4 and DES11) and their components. The FTIR spectra show that the HBA (Aliquat 336) have peaks at various positions. The peak at 2927 cm\(^{-1}\) is dedicated to the –CH\(_3\) group. The peaks located at 1462 cm\(^{-1}\) and 1375 cm\(^{-1}\) are because of the quaternary ammonium group [38,39]. After DES formation with glycerol and fructose, the peaks become broader and move to 1468 cm\(^{-1}\) and 1378 cm\(^{-1}\) and their intensity decreases considerably (Figure 1a,b) [39]. Similarly, for other DES such as Figures S1–S4, the changes in their functional groups were observed after the formation of the DES from their corresponding HBA and HBD. This shifting of the ammonium group after mixing with glycerol and fructose with Aliquat 336 confirms the successful formation of the synthesized DES.

3.2. DES Presented Potent Bactericidal Effects against Multi-Drug-Resistant Pathogens

The results from bactericidal assays have shown that upon successful creation of the DES, all DES with the exception of DES-3 and DES-8 showed significant bacterial inhibition activity against \textit{P. aeruginosa} and \textit{E. coli K1} (\(p < 0.05\)) (Figure 2a,b). Of all the DES tested, DES-4 and DES-11 showed the highest bacterial inhibition properties against these two bacteria. DES-4 possessed 40% and 68% antibacterial properties against \textit{P. aeruginosa} and \textit{E. coli K1}, whereas DES-11 showed 65% and 61% bacterial inhibition effects against these bacteria (Figure 2a,b). Similarly, DES-1, DES-10, and DES-7 exhibited 48%, 45%, and 43% bactericidal effects against \textit{E. coli K1}, respectively (Figure 2a). DES-1 and DES-10 presented 39% and 38% bactericidal activities against \textit{P. aeruginosa} (Figure 2b). When tested against \textit{S. marcescens}, DES-11 and DES-7 showed 55% and 45% antibacterial activities (Figure 2c), while DES-1, DES-4, DES-7, and DES-11 revealed significant antibacterial properties against \textit{K. pneumoniae} (Figure 2d) upon their successful formation from their corresponding HBD and HBA.

Correspondingly, when tested against Gram-positive bacteria, all DES after their successful formation, excluding DES-3 and DES-8, demonstrated substantial bactericidal effects against \textit{B. cereus} (\(p < 0.05\)) (Figure 3a). DES-4 and DES-11 had the best bacterial inhibition properties, eradicating 75% and 70% of bacterial population. DES-1 and DES-2 had bactericidal activities of 53% and 46%, while DES-10 and DES-5 exhibited 51% and 43% antibacterial effects, respectively, against \textit{B. cereus} (Figure 3a). Against MRSA, only
DES-1, DES-2, DES-4, and DES-11 indicated significant antibacterial properties, while all other DES failed to show the activity (Figure 3b). All DES except DES-3, DES-7, and DES-8 revealed important bactericidal activity against *S. pyogenes* (Figure 3c). DES-4 eliminated 47% bacterial viability (Figure 3c). Consistently, DES-4 and DES-11 showed the highest bactericidal activity against *S. pneumoniae*, abolishing 51% and 50% bacterial growth (Figure 3d). Overall, DES revealed remarkable bactericidal efficacy against both the Gram-positive as well as Gram-negative superbugs. Table 3 summarizes the 50% minimum inhibitory concentration (MIC<sub>50</sub>) values of the DES using broth micro-dilution assays. All the DES were tested at their doses to assess MIC<sub>50</sub> as described in Materials and Methods. Out of all the DES, DES-4 (methyl-trioctylammonium chloride–glycerol) and DES-11 (Methyl-trioctylammonium chloride–fructose) presented MIC<sub>50</sub> values of 1.44 µL and 1.53 µL, respectively. Similarly, DES-1 (methyl-trioctylammonium chloride–2-hydroxy benzoic acid), DES-10 (methyl-trioctylammonium chloride–citric acid) and DES-7 (methyl-trioctylammonium chloride–glucose) showed MIC<sub>50</sub> values of 2.08 µL, 2.22 µL, and 2.32 µL, respectively, against *E. coli* K1 (Table 3). Against *B. cereus*, DES-4 and DES-11 exhibited MIC<sub>50</sub> values of 2.66 µL and 2.85 µL, whereas DES-1, DES-10 and DES-7 presented MIC<sub>50</sub> values of 3.77 µL, 3.92 µL, and >4 µL, respectively (Table 3).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** FTIR spectra for Aliquat 336, glycerol, fructose, and their synthesized DES (DES-4 and DES-11) before and after formation. (a) represents the FTIR spectra of individual components whereas (b) represents the FTIR spectra of two separate synthesized DESs.
trioctylammonium chloride–glucose) showed MIC 50 values of 2.08 µL, 2.22 µL, and 2.32 µL, respectively, against \textit{E. coli} K1 (Table 3). Against \textit{B. cereus}, DES-4 and DES-11 exhibited MIC 50 values of 2.66 µL and 2.85 µL, whereas DES-1, DES-10 and DES-7 presented MIC 50 values of 3.77 µL, 3.92 µL, and >4 µL, respectively (Table 3).

Figure 2. Bactericidal effects of DES against Gram-negative bacteria. (a) represents antibacterial effects towards \textit{E. coli} K1, (b) against \textit{P. aeruginosa}, (c) against \textit{S. marcescens}, and (d) against \textit{K. pneumoniae}. The results are shown as the means ± standard errors from three separate tests conducted in duplicate. (*) denotes $p \leq 0.05$.

3.3. DES Showed Limited Cytotoxicity against Human Cells

Results from LDH assays revealed that all the DES except DES-10 showed partial cytotoxic properties towards human cells. DES-11 and DES-4 showed the lowest cell cytotoxicity, showing 15% and 20% cytotoxicity against human cells ($p \leq 0.05$) (Figure 4). Similarly, DES-7 and DES-8 exhibited 25% and 27% cytotoxic effects, while DES-5, DES-1, DES-2, and DES-3 revealed 29%, 33%, 37%, and 40% cytotoxic properties, respectively, against human cells. DES-10 was potently cytotoxic and presented 88% cytotoxicity (Figure 4).
Figure 3. Antibacterial properties of DES against Gram-positive bacteria. (a) represents bactericidal effects against \( B. \) cereus, (b) represents bactericidal activity against MRSA, (c) against \( S. \) pyogenes, and (d) against \( S. \) pneumoniae. The data are articulated as the means ± standard errors from three independent trials carried out in duplicate. (*) signifies \( p \leq 0.05 \).

Table 3. 50% minimum inhibitory concentration (MIC\(_{50}\)) of deep eutectic solvents against bacteria (µL).

<table>
<thead>
<tr>
<th>Deep Eutectic Solvent (DES)</th>
<th>( E. ) coli K1</th>
<th>( B. ) cereus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC(_{50})</td>
<td>MIC(_{50})</td>
</tr>
<tr>
<td>DES-1</td>
<td>2.08</td>
<td>3.77</td>
</tr>
<tr>
<td>DES-2</td>
<td>3.56</td>
<td>&gt;4</td>
</tr>
<tr>
<td>DES-3</td>
<td>&gt;4</td>
<td>&gt;4</td>
</tr>
<tr>
<td>DES-4</td>
<td>1.44</td>
<td>2.66</td>
</tr>
<tr>
<td>DES-5</td>
<td>2.85</td>
<td>&gt;4</td>
</tr>
<tr>
<td>DES-7</td>
<td>2.32</td>
<td>&gt;4</td>
</tr>
<tr>
<td>DES-8</td>
<td>&gt;4</td>
<td>&gt;4</td>
</tr>
<tr>
<td>DES-10</td>
<td>2.22</td>
<td>3.92</td>
</tr>
<tr>
<td>DES-11</td>
<td>1.53</td>
<td>2.85</td>
</tr>
</tbody>
</table>
Antibiotics have been extensively utilized to treat and prevent serious infections since the 1940s [40]. Over the years, chemical modification of natural compounds seems to be the primary method for discovering novel antibacterials for infections such as bacteria-associated pneumonia, dysentery, gonorrhea, and other bacterial ailments. Unfortunately, increased antibiotic resistance has rendered many of these drugs ineffective due to overprescription and inappropriate off-purpose use in animal husbandry. As a result, new approaches to the development of antibacterial agents are being developed [41,42]. In this study, different deep eutectic solvents were formed by reacting hydrogen-bond donors and acceptors. Antibacterial tests were used to evaluate the bactericidal properties of DES molecules. Additionally, the cytotoxicity profiles of synthesized DES against human cell lines were also determined using LDH assays. DES revealed broad-spectrum antibacterial properties against several bacteria. Our findings are consistent with previous research. For instance, fatty acid-based DES presented important antibacterial activity against S. epidermis, methicillin-resistant S. aureus, and methicillin-resistant Staphylococcus aureus [22]. In another study, natural DES were investigated for antimicrobial properties against several bacteria and yeast. The DES presented essential antibacterial activity against test bacteria and C. albicans [25]. Aroso et al. discovered that choline chloride- or menthol-based therapeutic DES have broad-spectrum antibacterial action [43].

Among all the DES tested, DES-4 showed potent broad-spectrum antibacterial activity against bacteria. DES-4 is formed by conjugating methyl-trioctylammonium chloride (Aliquat 336) with glycerol at a 1:1 molar ratio. Similarly, DES-11 formed by Aliquat 336 and fructose exerted consistent bactericidal activity against both the Gram-positive bacteria and Gram-negative isolates. Choline chloride-based DES showed noticeable antibacterial properties against Gram-positive and Gram-negative bacteria [28]. Grozdanova et al., in 2020, reported that medicinal plants’ extracts in combination with glycerol-based choline chloride with citric acid-1,2-propanediol (1:4) and 30% H$_2$O presented potent bactericidal effects and limited cytotoxic properties towards human cells [44]. In a recent study, choline chloride-based natural DES extracted from phenolic compounds revealed improved antibacterial activity against Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,

**Figure 4.** Deep eutectic solvents revealed limited cytotoxicity against HeLa cells. Human cells grown until confluence were treated with DES with 5% CO$_2$ and 95% humidity overnight at 37 °C. Cells alone in serum-free RPMI and with 0.1% Triton X-100 were considered as negative and positive controls, respectively. Data were investigated with Graph PadPrism software (8.0.2). The data are presented as mean ± SE of several independent trials conducted in duplicates. (*) denotes $p \leq 0.05$. 

### 4. Discussion

Antibiotics have been extensively utilized to treat and prevent serious infections since the 1940s [40]. Over the years, chemical modification of natural compounds seems to be the primary method for discovering novel antibacterials for infections such as bacteria-associated pneumonia, dysentery, gonorrhea, and other bacterial ailments. Unfortunately, increased antibiotic resistance has rendered many of these drugs ineffective due to overprescription and inappropriate off-purpose use in animal husbandry. As a result, new approaches to the development of antibacterial agents are being developed [41,42]. In this study, different deep eutectic solvents were formed by reacting hydrogen-bond donors and acceptors. Antibacterial tests were used to evaluate the bactericidal properties of DES molecules. Additionally, the cytotoxicity profiles of synthesized DES against human cell lines were also determined using LDH assays. DES revealed broad-spectrum antibacterial properties against several bacteria. Our findings are consistent with previous research. For instance, fatty acid-based DES presented important antibacterial activity against S. epidermis, methicillin-resistant S. aureus, and methicillin-resistant Staphylococcus aureus [22]. In another study, natural DES were investigated for antimicrobial properties against several bacteria and yeast. The DES presented essential antibacterial activity against test bacteria and C. albicans [25]. Aroso et al. discovered that choline chloride- or menthol-based therapeutic DES have broad-spectrum antibacterial action [43].

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and *Salmonella enterica* [45]. Phosphonium-based deep eutectic solvents produced worthy antibacterial effects against Gram-negative bacteria and Gram-positive pathogens [24]. The DES used in this study are hydrophobic and the hydrophobicity of the DES increases as the alkyl chain length grows. As a result, the hydrophobic phase of these DES may interact better with bacteria and exert antibacterial activity [46].

In the present study, DES were tested at different doses to determine the dose that can inhibit/kill 50% (MIC$_{50}$) of bacterial populations. Zhao et al. reported the broad-spectrum minimal inhibitory and bactericidal concentration of choline chloride-based DES [28]. The MIC$_{50}$ values in this study are quite promising, showing the minimum inhibitory strength at microliter doses, whereas the choline chloride-based DES revealed MIC and MBC at millimolar concentration. This indicates the significance of our synthesized DES. Similarly, in another study, Silva et al. reported the MIC and MBC of fatty acid-based DES against *Staphylococcus* species, which were relatively high against the test bacteria [22]. Further work, using the log-fold method, can also be carried out to understand the potency of these DES.

The biocompatibility of numerous natural DES has been evaluated against several bacterial species (*Salmonella enteritidis*, *S. aureus*, *Vibrio fischeri*, and *E. coli*), fungi, and cell lines [28,47]. Choline, being a component of vitamin B, plays a significant function in cellular metabolism; the majority of the natural DES investigated were based on choline [28,48]. In the present study, the biocompatibility of the DES was evaluated against HeLa cells and DES showed limited cytotoxicity towards human cells. Nonetheless, future studies to examine any anti-apoptotic effects, or cytotoxicity effects evaluated against other human cell lines, should be carried out in addition to in vivo studies. Interestingly, DES-4 and DES-11, with potent antibacterial activity, presented the least cytotoxic effects against human cells, according to our data. Rodriguez-Juan et al. reported the non-toxic effects of several choline chloride-based DES against several human cell lines [49]. However, recent research has indicated that different DES have the potential to be hazardous and cytotoxic [12,50]. These investigations do, in fact, reveal the toxicity of DES. For example, natural DES with organic acids, such as malonic acid, have been shown to have higher-toxicity profiles than sugar-based natural DES [51]. Moreover, the chain length of the DES determines their cytotoxicity profiles. The larger the chain length, the more toxic the DES [32]. The toxicity of ionic liquids (ILs) is known to be influenced by both their cationic and anionic natures, according to published research [53,54]. The size of the functional groups and the length of the alkyl chain have been revealed to be key determinants of the ILs’ toxicity [55,56]. The toxicity of ILs increases with the lengthening of the alkyl chain on cations. As the length of the alkyl chain grows from C7 to C12, it has been found that the toxicity of guanidinium-based ILs toward *Vibrio fischeri* increases [57]. Furthermore, Aliquat 336 is known to possess cytotoxicity to humans and aquatic organisms. However, incorporating certain functional groups onto the alkyl chain may reduce toxicity and augment their biodegradability [58,59].

In conclusion, methyl-trioctylammonium chloride-based DES were produced by coupling with different hydrogen-bond donor molecules. The DES showed remarkable bacterial inhibition properties against a panel of Gram-positive and Gram-negative bacteria. The DES revealed MIC$_{50}$ at micromolar dose against bacteria. Intriguingly, the DES exhibited limited cytotoxic effects against human cells. Our data supports the notion that methyl-trioctylammonium chloride-based DES are anticipated chemotherapeutic agents against bacteria. Future studies are needed to understand the specific mechanism of action and in vivo effects of these DES against pathogenic bacteria in addition to in vivo research and the construction of infection models for bacterial infections.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/scipharm91010009/s1, Figure S1: DES 2; Figure S2: DES 3; Figure S3: DES 7; Figure S4: DES 8.
Author Contributions: R.S. and N.A.K. conceived the idea. The materials and resources were provided by T.I., M.K., A.S.K., A.M.A. and H.A. N.A. performed all experimentation under the supervision of R.S., N.A.K. and R.S. and N.A. wrote the first draft of the manuscript. R.S., A.M.A., H.A., M.K., T.I. and N.A.K. corrected the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank the American University of Sharjah and the University of Sharjah for their contributions to this research.

Conflicts of Interest: There is no conflict of interest, and the article was submitted with the permission of all of the authors.

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