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Original Research

Ammonium chloride and urea-based deep eutectic solvent: toxicological and antioxidant profile

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Abstract

The present study examined the toxicity potential of ammonium chloride and urea (NCU) based deep eutectic solvent (DES). A homogeneous DES solution was obtained by heating at 60°C for 20 minutes. Fourier Transform Infrared Spectroscopy (FTIR) analysis was used to verify the components by identification of their functional groups. The toxicity profiling of ammonium-based DES was performed using *in vitro* cell lines (fibroblast growth factor) and microbes (fungi, gram positive and gram-negative bacteria) and *in vivo* model organisms (fish). DES was found to have the maximum zone of inhibition against tested bacteria (*Staphylococcus aureus*) and fungal strains (*Candida albicans*). The LC50 value of NCU DES had been reported as 105ppm against *Cyprinus carpio*. DES was found to have a higher percentage of cell viability at higher concentrations along with DPPH scavenging activity of 92%. In conclusion, ammonium chloride-urea based DES had been successfully formed and found less harmful at higher concentrations, thus can act as a promising solvent in different medical or pharmaceutical applications.

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Introduction: Due to growing concerns about environmental pollution by industrial activities, the scientific community has recently concentrated on creating new eco-friendly green solvents. Deep Eutectic Solvents (DESs), a new family of environment-friendly solvents, have caught researchers' interest as a potential replacement for traditional volatile chemicals and ionic liquids. A deep eutectic solvent combines two or more chemicals, producing a unique chemical composition with a lower melting point than each component alone[1]. They consist of an acceptor, which facilitates the formation of hydrogen bonds, and a hydrogen bond donor [2,3]. The lower melting point of DES than its parts is due to the charge delocalization caused by hydrogen bonding between the hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD). DESs are preferred green solvents because they are environmentally benign, inexpensive, recyclable, less toxic, non-flammable, highly viscous, and pure [4,5]. Deep eutectic solvents are used in bio catalysis, electrochemistry, the manufacture of nanomaterials, the manufacturing of polymers, separation and extraction operations, and biomedical procedures [6].

Deep eutectic solvents have a low toxicity range, but less is known about their characterization and uses, including their toxicity and biodegradability. As a result, their use in industrial and biological applications is limited. The toxicology of DES based on choline chloride (ChCl) was the subject of one of the studies. The cytotoxicity of the investigated DESs was noticeably greater than the cytotoxicity of their constituent parts, which indicated their unique toxicological activities. The fact that organic acids as HBD significantly contribute to the rising toxicity of these esoteric mixes served to confirm the notion that careful selection of NADESs components is required. However, compared to their DESs parents, NADESs appear to have less acute toxicity profiles overall [6]. Another study investigated the in vitro toxicological effects of three ChCl-based DESs using fish, human cell lines, wheat (phytotoxicity), and sewage microbes [7]. Before the industrial use of DESs, it is essential to evaluate the toxicity profile of these substances to comprehend their impacts on environmental safety and human health. [8].

There is still debate regarding the toxicity of these solvents despite the undeniable potential benefits of DESs. Therefore, it is essential to examine how these solvents affect organisms at various trophic levels to employ DESs more effectively in accordance with green chemistry. Model organisms were chosen based on prior research for toxicity testing against various chemicals [9–11]. In the current study, Gram negative (*Escherichia coli* and *Psedomonas aeroginosa*) and Gram positive (*Staphylococcus aureus* and *Listeria monocytogenes*) bacteria, fungi (*Candida albicans* and *Aspergillus niger*), and common carp fish (*Cyprinus carpio*) were all used to investigate the effectiveness of deep eutectic solvents. An *in vitro* cell viability test was performed on human fibroblast cell lines to ascertain their biocompatibility. Additionally, the antioxidant DPPH analysis had been carried out to determine the free radical scavenging abilities of DESs.

Materials and Methods: Preparation of DES: Hydrogen bond donor (Urea; U) and dry salt (Ammonium chloride; NC) were combined in DESs in a ratio of 1:4. The combination was heated in an oil bath at 80-100°C for 20 minutes while being continuously stirred to produce a homogeneous mixture that was claimed to be a eutectic solvent.

Fourier Transform Infrared Spectroscopy (FTIR) analysis: To verify their synthesis, the DESs were scanned using an FTIR spectrometer (Nicolet 6700, USA) with a detector in attenuated total reflection (ATR) and could measure wave numbers 4,000-400cm^{-1} over the course of 40 scans with resolution of 8cm^{-1}.

Antimicrobial activity assay: The bacterial strains employed in this investigation included, two gram-negative (*Escherichia coli* ATCC#2592, and *Pseudomonas aeruginosa* NCTC#10662) and two gram-positive strains (*Staphylococcus aureus* NCTC#6571, and *Listeria monocytogenes* ATCC#19115). Moreover, two fungal strains were also included for antifungal activity: Aspergillus *niger* ZGCL1 and *Candida albicans* ATCC#10231. The bacterial strains were grown in nutrient broth and incubated for 24 hours. The fungal strains were cultivated in potato dextrose agar and yeast extract broth and incubated for 96 hours at 37°C. The DES employed in this study was supposed to be evaluated for its antimicrobial capabilities using a disc diffusion test that measured the diameters of the inhibitory zone surrounding discs loaded with DES at different concentrations. Different serial dilutions of DES were made at concentrations of 10^{-1}-10^{-5}. Under sterile conditions, 30µl of the DESs solutions were put on the respective blank cellulose discs. The disks were incubated for 24 hours on a MacConkey agar, mannitol salt agar, Listeria selective agar and cetrimide agar plates with an overnight inoculum of *E. coli*, *S. aureus, L. monocytogenes* and *P. aeruginosa,* respectively. The discs loaded with DES solutions were cultured for 96 hours at 37°C on potato dextrose agar that had been seeded with *Aspergillus niger* and *Candida albicans* for the investigation of antifungal activity. Tetracycline discs were used as controls for antibacterial activity while for fungal strains nystatin discs were used as control group.

Fish toxicity profile of DESs: According to ISO 1982, reconstituted water was created with a conductivity of 7–7.5 mScm^{-1} and a total water hardness of 180–190 mg/L $CaCO_3$ for each container [12]. *Cyprinus carpio*, common carp fish, was chosen as the test subject because of its well-known status as a vertebrate model for acute toxicity assay in ecotoxicology. They were subjected to the test chemical for roughly 96 hours. For this, limit test with concentration of 100mg/L and a thorough test with concentrations between 10 and 100mg/L were carried out. The tests were carried out to ascertain the concentration at which 50% of the fish would die (LC50). Each container received an equal amount (5L) of reconstituting water (static system). The combinations were constructed, and six fish were added to the aquarium. The fish participated in 16-hour research in the daylight without food and then finally, the experiment was employed. A blank aquarium with no DES added was attributed as control group provided with same test conditions. The toxicity tests were performed in triplicates. Mortalities were noted and afterward removed from the container at 1, 24, 48, 72 and 96 hours. The LC50 value, the concentration that causes 50% of population to perish, was used to illustrate the toxicity of DES towards

Cyprinus carpio. The LC50 was calculated using US-Probit EP's analysis system based on the total number of death counts. The LC50 was found to be below 100mg/L in cases where the fatality occurred. However, LC50 was estimated to be higher than 100mg/L if there was no mortality. The composition and quality of water was tracked and recorded during the test [9]. The Microsoft Excel 365 ProPlus was employed to examine the data and calculate the LC50 value of the synthesized DES.

Cytotoxicity analysis of DES: Fibroblast cell lines (NIH/3T3) derived from mouse embryonic fibroblasts (ATCC#CRL-1658TM) were cultured in Dulbecco's modified Eagle's medium (DMEM) with addition of 100µg/ml of Penicillin/streptomycin (Sigma Aldrich, Life Sciences, USA) and 10% FBS (ThermoFisher Scientific, USA) and kept in T75 culture flasks in the humidified atmosphere. Cells at the exponential growth phase (90% confluency), with new media changes every two to three days, were employed for the cytotoxicity experiment. On the day of the seeding, the cells were counted using a haematocytometer and a microscope. NIH/3T3 cells were planted in 24 well plates at density of 5×10^4 per well in 100µl of culture medium after being diluted 1:10 with DMEM media. Following overnight incubation, DES samples diluted in DMEM at varying concentrations (0.1, 1, 10, 100, and 1000µg/100ml) were applied to NIH/3T3 cells. To examine the properties of cells produced with and without liquid samples, cells were cultivated on tissue culture plastic plates. Each well received 500µl of a 1mM Alamar Blue solution after 72 hours of exposure, and each well was then incubated for an additional 3 hours. The absorbance at 570nm was measured using a fluorescence plate reader (PR4100 Absorbance Microplate Reader BIO RAD, UK) [13].

$$Viability = \frac{Absorbance_{sample} - Absorbance_{blank}}{Absorbance_{control} - Absorbance_{blank}} \times 100$$

DPPH Antioxidant Activity: The free radical scavenging activity of deep eutectic solvent was calculated using the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) test. The 0.004% w/v DPPH in 4ml methanol was added to an aliquot of 1ml of DES in methanol. The methanol and ascorbic acid were used as blank and standard, respectively [14]. The following formula was used to calculate the percentage of free radical scavenging activity.

$$DPPH\ Scavenging\ activity\ (\%) = \frac{A_{Blank} - A_{DES\ Sample}}{A_{Blank}} \times 100$$

Results and Discussion: Fourier transforms infrared spectroscopy (FTIR) analysis: FTIR spectroscopy is an analytical technique used to obtain infrared photoconductivity, absorption, and emission spectrums. It identifies a chemical compound's structure by identifying their functional groups and interactions between two molecules. Figure 1 showed the FTIR spectrum of ammonium chloride salt, urea, and respective DES. Urea, a diamine molecule, presented two sharp stretches of amine ($-NH_2$) group in the region of 3400-3200cm^{-1}. Moreover, a strong stretch of carbonyl (-CO) group at ~1670cm^{-1} had also been observed. Following the structural components of urea, a medium amine bending at 1587cm^{-1}, and medium cyanide group (-CN) stretches at 1150 and 1000cm^{-1} were observed [15] (Figure 1a). Ammonium chloride, an amine salt, presented broad amine (-NH) stretches at 3200-2800 cm^{-1}, and medium amine bends at 2000-1500 cm^{-1} which were accompanied with halo compounds at 700cm^{-1} (Figure 1b). The FTIR spectra of DESs presented bands with characteristic functional groups of the precursors employed in the study. The FTIR spectrum of deep eutectic solvent based on ammonium chloride and urea, showed a single broad peak in the region of 3600-3000 cm^{-1} which was suggestive of hydrogen bonding between two molecules [16]. However, a few shifts were observed in the final FTIR spectrum of DES in comparison to its components such as the medium bend of urea amine (-NH) group had been shifted from 1687cm^{-1} to 1591cm^{-1}, a medium cyanide stretch of urea had also been shifted from 1148cm^{-1} to 1156cm^{-1}. Both shifts in the spectrum were attributed as blue shifts because they had shifted the absorption spectra towards longer wavelengths. It had been stated that hydrogen bonding usually increased the polarity of molecule which in turn shifts the spectra [17].

Toxic effect of DESs on bacterial and fungal strains: A disk diffusion assay was carried out using the selected microbial strains to evaluate the antimicrobial potential of synthesized urea-based DES (Table 1). The deep eutectic solvent presented maximum antibacterial activity at the highest concentration (0.40±0.30cm***), along with dilution factors of 1:10 (0.40±0.30cm***) and 1:100 (0.43±0.28cm***) against gram-positive bacteria, *Staphylococcus aureus.* In the pure form, DES was found to be the least toxic against gram-negative bacteria, *Escherichia coli* (0.13±0.03cm***). Upon dilutions, the toxicity of deep eutectic solvent was found to be increased and it was maximum at 10^{-5} (0.28±0.04cm***), 10^{-1} (0.27±0.07cm***), and 10^{-3} (0.25±0.03cm***) dilutions (Figure 2). The fungal strain, *Aspergillus niger* presented no zone of inhibition when it was exposed to different concentrations of ammonium chloride: urea DES. Nevertheless, *Candida albicans* was found to be susceptible to DES at all concentrations except at 10^{-5} dilution. As shown in Table 1, the largest inhibition zone of *C. albicans* formed by NCU at 10^{-1} dilution was 0.66±0.12cm followed by 0.63±0.12cm at highest concentration. Overall, among all tested microbes NCU was more harmful to *C. alibicans* (Figure 3). Akayleh et al. had investigated the antimicrobial potential of capric acid, menthol, and Solutol™ along with their respective DES against two gram-positive and gram-negative bacterial strains. According to their results, DES possess antibacterial activity against *S. aureus, P. aeruginosa* and *C. albicans.* These results were comparable to our present study [18]. In 2018, a group of ten choline chloride-based DES had also been evaluated for their antimicrobial potential against different bacterial strains such as *E. coli, S. aureus, P. aeruginosa, S. typhimurium,* and *P. mirabilis. Candida albicans* was also used to check the antifungal activity of DES. In line with the results of present study, *P. mirabilis, S. typhimurium, S. aurus were found to be sensitive against DES* [19]. In another study, steroidal urea derivatives were synthesized, and their antibacterial activity was performed. The urea derivates were found toxic against both type of bacteria, gram negative and gram positive [20]. Urea and thiourea drivatives were also evaluated for their antibacterial activity and the compounds

showed antibacterial activity against all bacterial strains including *S. aureus* [21].

Acute toxicity analysis on Fish and determination of LC_{50}: The acute toxicity of ammonium chloride-based DES was assessed against *Cyprinus carpio* fish by evaluating its lethal concentration 50 (LC_{50}) value. The fish were exposed to DES, which was found less harmful as their LC_{50} was 105mg/L which is greater than 100mg/L (OECD Guideline no. 203). There have been no previous investigations that have assessed the toxicity profile of ammonium based DESs on fish. Hence, limited data is available in comparison to our results. However, a few investigations were found where different DES had been mentioned. Such as, the fish toxicity profile of cholinium-based deep eutectic solvents had been reported in a study by Juneidi *et. al.* and it ranged from slightly toxic (10-100mg/L) to relatively harmless (>100mg/L) solvents. The DES solutions with Zinc chloride as a hydrogen bond donor were found most toxic followed by malonic acid containing DES with less toxicity (190mg/L), glycerol and ethylene glycol (>1000mg/L) with a relatively harmless nature [9]. Based on prior information, it can be hypothesized that there could be a strong hydrogen bonding present between DES components which was responsible for their low reactivity making them less harmful. Other studies also presented a significant difference in the toxicity profile of DESs component before and after the formation of solvents [7,22,23].

Cytotoxicity of DES in cell cultures*:** The cytotoxicity of synthesized ammonium chloride: urea was tested on NIH3T3 fibroblast cells in different concentrations ranging from 0.1 to 1000µg/100ml including a pure sample by Alamar Blue assay. The experiment was carried out for three days using liquid co-culturing, and the results were compared to the control (Figure 4). It had been observed that cell viability decreases gradually with decreasing concentration of DES. DES pure solution had a cell viability of 264% compared to the control (100%). At 100µg/100ml concentration, NCU had cell viability of 174%***. When the concentration decreased to 10µg/100ml, 1µg/100ml and 0.1µg/100ml, cell viability % had also been decreased for DES gradually.

In comparison to the control, DES showed a rise in cell viability % at its minimum concentration. The toxicity of ammonium chloride: urea-based DES on any organism has yet to be determined. However, Tetrabutylammonium chloride-based DESs were tested for toxicity on human cell lines, their finding was in line with the present study [24]. According to that study, two cell lines including keratinocytes HaCaT and tumor melanocytes MNT-1, had been evaluated for their compatibilities in cosmetic and pharmaceutical industries. In another study, five HBDs (hexanoic acid, butanoic acid and ethylene glycol, 1-propanol and urea) were tested for their role in common survivability along with three HBAs (cholinium chloride, tetramethylammonium chloride nd tetrabutylammonium chloride). Tetrabutylammonium chloride-based DES, on the other hand, was found to be harmful in both cell lines, while cholinium chloride and tetramethylammonium chloride-based DES was shown to be biocompatible. In contrast to the present study, a group of researchers looked at the cytotoxic activity of eleven NADES on fibroblast-like L929 cells and found that NADES containing acids (such as tartaric and citric acid) had the most apparent toxicity [10].

Evaluation of DES antioxidant activity: The pure and diluted DES samples were run through antioxidant analysis to measure their ability to scavenge free DPPH radicals and results were presented in figure 5. The antioxidant activity of the test sample and ascorbic acid (standard) was determined using a spectrophotometer. The results were presented as percentage inhibition of DPPH radical compared to ascorbic acid. All DES solutions showed concentration dependent antioxidant activity as the maximum activity was observed at highest concentration while minimum activity was observed with water inclusion of 1000µg/ml of DES. For DES dilutions, antioxidant activity was decreased in the following order $10^{-1}>10^{-3}>10^{-2}>10^{-4}>10^{-5}$. The antioxidant activities of the synthesized mixtures were assessed using the DPPH method, which revealed that NCU pure sample had the highest percentage inhibition of 92.33%, followed by 10^{-1} with 83.25%** inhibition against free radicals, as compared to standard (ascorbic acid) which had 91.66% inhibition. DPPH being a free stable radical accepts electron or hydrogen to attain a stable diamagnetic state. In the presence of a hydrogen donor thought to be an antioxidant, DPPH is scavenged and the reaction mixture color is changed from purple (DPPH) to yellow (diphenylpicryl hydrazine) with a decrease in absorbance [25]. Absorption is increased when a significant number of free radicals are available. NCU had more antioxidant properties against DPPH than the control which may be due to their different polarities and more reactivity for DPPH anion. A notable finding is that the antioxidant activity of the ammonium chloride DES system with a gradual increase in water content slightly outperformed the pure DES system. Given this information, it can be concluded that the physiochemical properties of pure DES may exert a big effect on the DPPH free radical scavenging effect.

Conclusion: The present study was done to synthesize ammonium chloride-based DES (ammonium chloride: Urea) and assess its toxicity profile both *in vivo* and *in vitro*. Because of its homogeneity, the DES prepared was outstanding. This study looked at the effects of synthesized DES on the bacteria (both gram-negative and gram-positive), fungi, fish, cell viability and antioxidant activity. Ammonium chloride and urea-based solvents yielded both favorable and negative outcomes. The deep eutectic solvent, NCU, was shown to have antimicrobial properties against *E. coli, S. aureus* and *C. albicans*. Compared to the control, NCU exhibits a greater cell proliferation percentage in their pure form. The antioxidant activity of DES was also determined using the DPPH assay and the % inhibition was found to be 92.33%. The hydrogen bonding in the formation of DES proved to be responsible for its distinctive behaviour in terms of toxicity level towards different organisms and cells.

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Table. 1. Antimicrobial activity of DES (Mean (cm)±SEM) with different dilution factors to *E. coli, S. aureus, P. aeruginosa, L. monocytogenes, A. niger, and C. albicans.*

	E. coli	*P. aeruginosa*	*S. aureus*	*L. monocytogenes*	*C. albicans*	*A. niger*
Pure	0.13±0.03***	0.10±0.06	0.40±0.30***	0.07±0.07***	0.63±0.12	0.00±0.00
10^{-1}	0.27±0.03***	0.07±0.07	0.40±0.30***	0.00±0.00***	0.66±0.12	0.00±0.00
10^{-2}	0.20±0.00***	0.07±0.07	0.43±0.28***	0.00±0.00***	0.10±0.10	0.00±0.00
10^{-3}	0.25±0.03***	0.07±0.07	0.03±0.03***	0.00±0.00***	0.13±0.13	0.00±0.00
10^{-4}	0.20±0.00***	0.03±0.03	0.17±0.12***	0.00±0.00***	0.56±0.47	0.00±0.00
10^{-5}	0.28±0.04***	0.13±0.07	0.13±0.04***	0.00±0.00***	0.00±0.00	0.00±0.00

SEM=Standard Error Mean, 10^{-1}-10^{-5} dilutions of pure DES increased with the factor of 10, starting from 1:10 followed by 1:100, 1:1000, 1:10000, and 1:100000. The p-value less than 0.05, 0.01 and 0.001 are denoted by *, ** and ***, respectively.

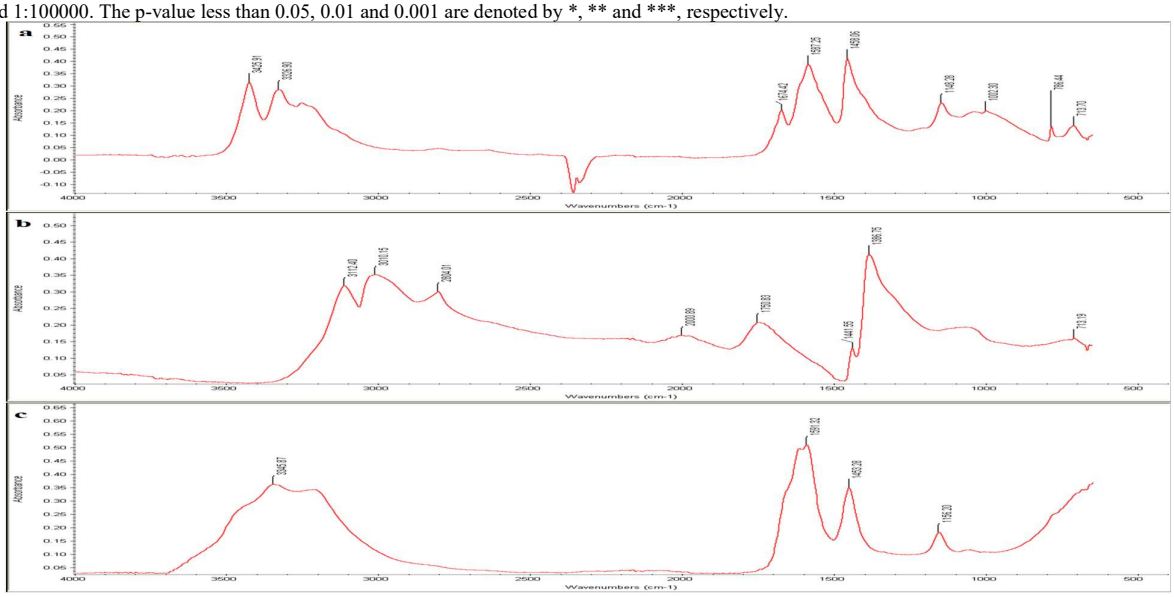

Fig. 1. FTIR analysis of DESs and their precursors. a) FTIR Spectrum of urea. b) FTIR Spectrum of ammonium chloride. c) FTIR Spectrum of Ammonium chloride: Urea, NCU deep eutectic solvent.

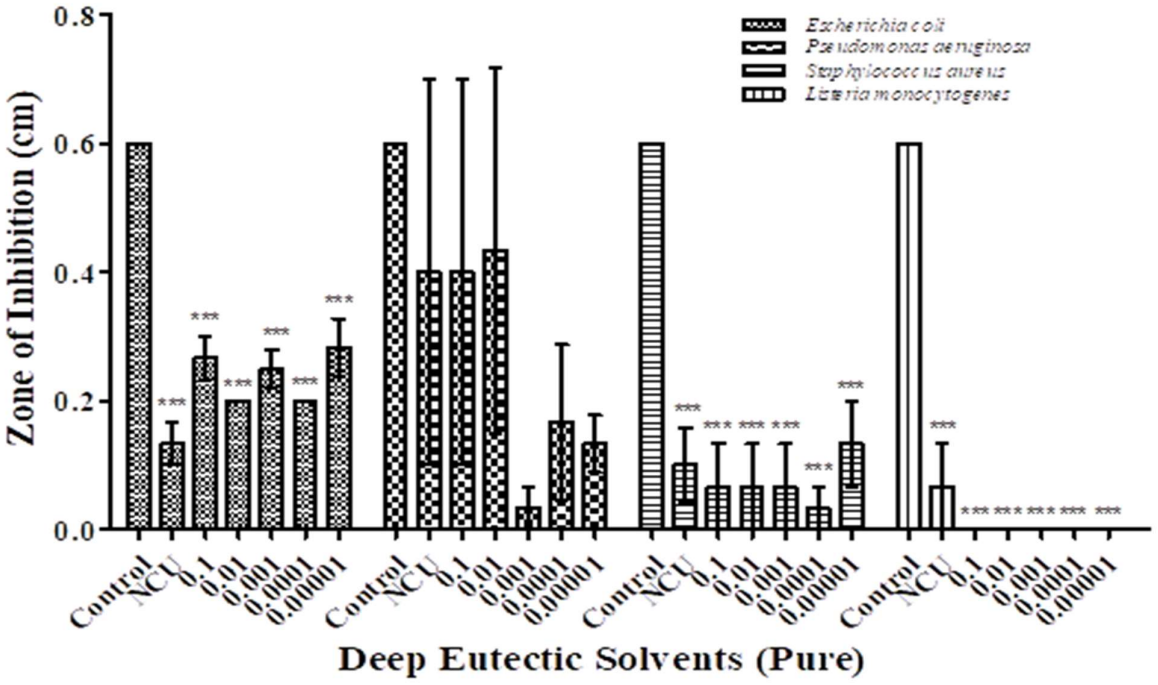

Fig. 2. Antibacterial activities of DESs against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Listeria monocytogenes.* Data is shown as mean ± standard error mean (SEM) where p-value< 0.05 is considered to be significant (as established by using ANOVA). The p-value less than 0.05, 0.01 and 0.001 are denoted by *, ** and ***, respectively.

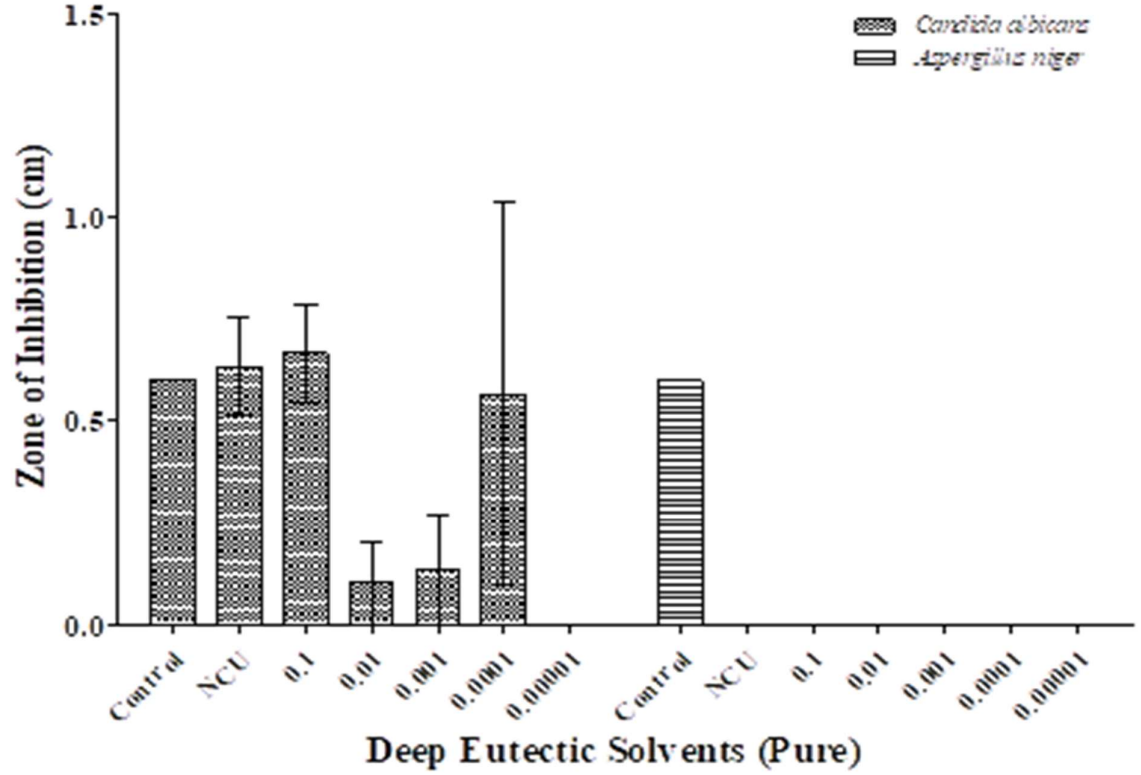

Fig. 3. Antifungal activities of DESs against *Aspergillus niger* and *Candida albicans.* Data is shown as mean ± standard error mean (SEM) where p-value< 0.05 is considered to be significant (as established by using ANOVA). The p-value less than 0.05, 0.01 and 0.001 are denoted by *, ** and ***, respectively.

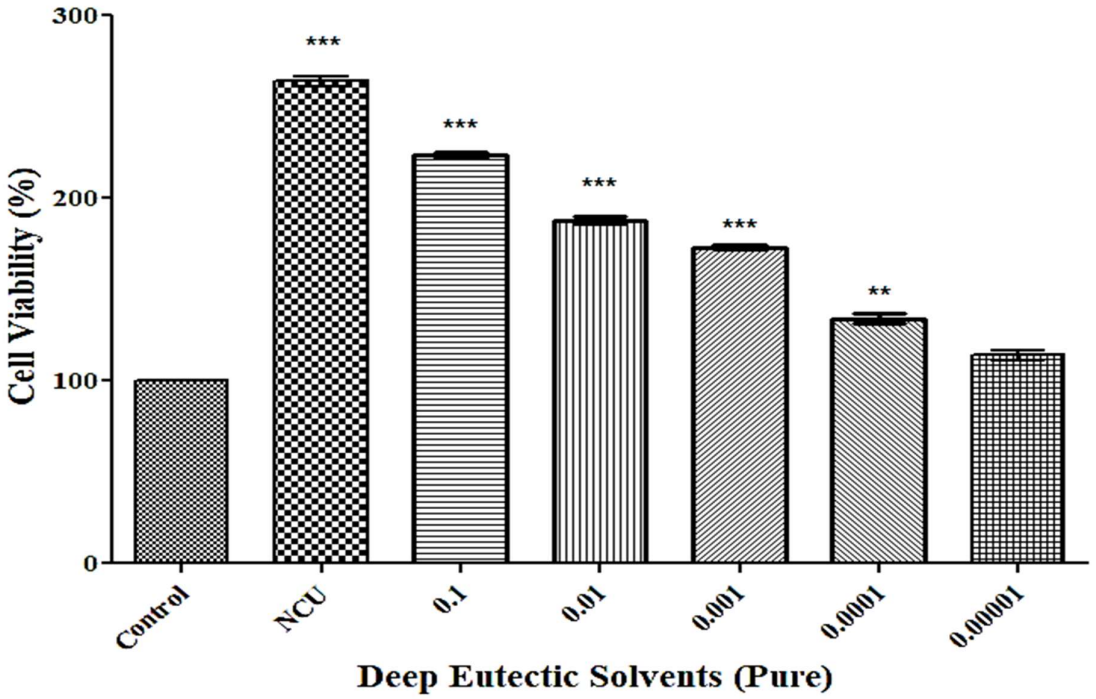

Fig. 4. Cell viability percentage of DES for NTH313 cells after 72h of exposure. Results are presented as triplicate experiments. Data is shown as mean ± standard error mean (SEM) where p-value < 0.05 is significant (as established by using ANOVA). The p-value less than 0.05, 0.01 and 0.001 are denoted by *, ** and ***, respectively.

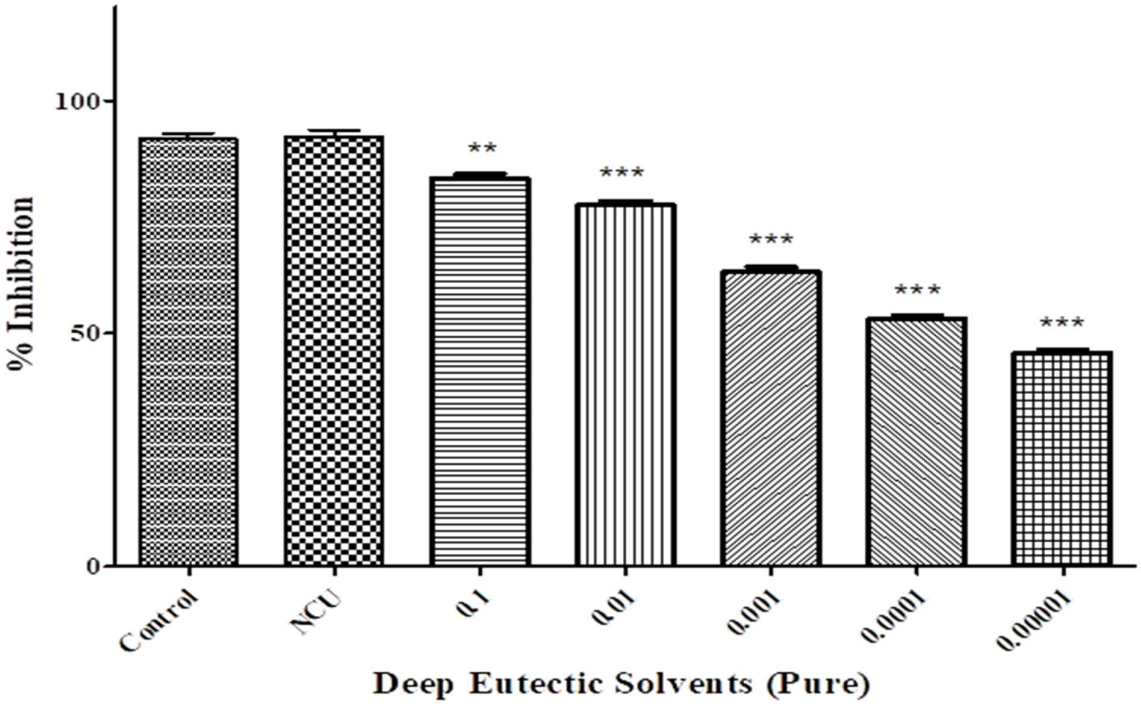

Fig. 5. Antioxidant activity of synthesized DES. Antioxidant % inhibition in different proportions from pure sample to 10^{-1}-10^{-5} dilutions. Data is shown as mean ± standard error mean (SEM) where Data is shown as mean ± standard error mean (SEM) where p-value < 0.05 is significant (as established by using ANOVA). The p-value less than 0.05, 0.01 and 0.001 are denoted by *, ** and ***, respectively.