SYNTHESIS OF IONIC LIQUIDS AND THEIR IN-VIVO AND IN-VITRO TOXICOLOGICAL ASSAY

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IN
ZOOLOGY

By

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RESEARCH COMPLETION CERTIFICATE

It is certified that the research work contained in this thesis entitled "Synthesis of Ionic Liquids and their in-vivo and in-vitro toxicological Assay Solvents and their toxicological effects on *Aspergillus niger*, and *Cyprinus carpio* fish", has been carried out and completed by " Zara Bashir" under my supervision during their Bachelor in Zoology program.

It is assured that the research work is original and has not been published anywhere else. All the changes suggested by the examiner were made and incorporated in this final copy.

Dr.Shamaila Inayat Nadeem **Dated: 16th June 2023**
Supervisor
Head of Department Zoology

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ABSTRACT

Deep eutectic solvents belong to the class of ionic liquids. They are called as "Designer solvents" because we can mold or change them according to our requirements. The synthesis of Ionic liquid (ILs) and them in-vivo and in-vitro toxicological activities were the main goals of this study. An ammonium-based salt tetrahexyl ammonium bromide (THAB) was taken as a hydrogen bond acceptor and decanoic acid (DA) was taken as a hydrogen bond donor to synthesize deep eutectic solvent. Both components were heated at 80^{o}C for 2 hours. A homogenous solution was obtained which was labelled as S13. The pure and diluted form of solvent was applied to bacteria, fungi and fish to test its toxicity. The solvent shows both positive and negative toxic effects on test organisms. In bacteria 10-3 didn't show any zone of inhibition on S.aureus and in *E.coli* on 10-2 it didn't show any zone of inhibition. DES was applied to pre cultured plates of *A.niger* an inhibition ring showed up after incubation and zero inhibition on cultured plates of *A.niger*. In full test in fishes, all dilutions except 10-5 resulted in mortalities.

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LIST OF ABREVIATION

DES	Deep eutectic solvent
NADES	Natural deep eutectic solvent
IL	Ionic liquid
HBD	Hydrogen bond donor
HBA	Hydrogen bond acceptor
NH_4Br	Ammonium bromide
CHCL	Choline chloride
St. error	Standard error
LC_{50}	Lethal concentration

CHAPTER I

INTRODUCTION

With their inherent toxicity and high volatility, typical organic solvents contribute to the evaporation of volatile compounds into the environment. Green technology deliberately seeks out alternative solvents to replace them. Throughout the preceding two decades, ionic liquids (ILs) have already drawn a lot of attention from the academic community, and the number of papers published in the scientific literature has significantly increased. However, the "greenness" of IL is frequently questioned, mostly because of its subpar biocompatibility, biodegradability, and sustainability. Deep Eutectic Solvents (DES), have emerged as a replacement for ILs (1). DESs are easily prepared using readily available and natural chemicals, making them seem like viable replacements for traditional organic solvents. They have great tenability as well (2). According to definitions given by researchers, DESs are a mixture of two or more substances, either liquid or solid, that, when combined, have a significant melting point depression and turn into liquid at room temperature (1).

1.1 DES solvents and their Physiochemical Properties:

Among the primary causes of the growing interest among researchers in deep eutectic solvents is their physicochemical makeup. Given the wide range of potential DESs' forming components, deep eutectic solvents appear to be chemically customizable, which implies they may be adapted for specific purposes in addition to having low volatility, low vapour pressure, chemical stability, thermal stability, and non-flammability (2). DESs have noticeably lower melting points than their pure constituent parts, which distinguishes them as a new type of mix. These substances show promise as low-cost "designer" solvents with a variety of customizable physicochemical features. The current research lacks a predictive understanding of the structure-property relationships in solvents. Their physicochemical characteristics and depressed melting points are attributed to complex hydrogen bonding (3).

Large, non-symmetric ions found in DESs have lower lattice energies and therefore low melting points. Quaternary ammonium salts are typically complexes with a hydrogen bond donor or metal salt in order to produce them. The drop in melting temperature of said mixturein contrast to the individual elements is caused by the delocalization of charge that results from hydrogen bonding, for example between a H-donor molecule and halide ion (1).

1.2 Relationship between Ionic Liquids and DES

Due to the fact that DESs have numerous similarities and differences with ILs they are now largely considered to be a new group of IL analogues. Even though DES and IL have frequently been used interchangeably in the literature, it is crucial to make it clear that these acronyms stand for two different types of solvents. DESs are complexes made by mixing Bronsted or Lewis bases to form a eutectic compound and acids that can comprise a number of cationic or/and anionic species, in comparison to ILs that are complexes produced from systems composed primarily of one type of distinct cation and anion. Here it is demonstrated that, despite the morphological similarities between DESs and other ILs, the chemical features of each of them point to very diverse potential application fields (1).

Non-flammability, excellent thermal and chemical stability, recyclability, low volatility, and strong solubilisation capacity for a number of chemicals are all characteristics that DES shares with ILs. Additionally, DESs are extremely tuneable as ILs, as their componentcompositions and molar ratios can be changed. Their primary advantages over ILs include cheaper production due to lower raw material prices, easier preparation and the elimination ofadditional stages of purification, and less negative environmental impact due to higher biodegradability and lower toxicity (4).

The fundamental shortcomings of conventional ILs, such as their non-biodegradability, high toxicity, complex manufacturing requirements, and high cost of the raw components, can be mitigated by the new generation of solvents known as DESs. Two safe, inexpensive, renewable, and biodegradable components that can combine to produce a eutectic mixture areall that are needed to create DESs (5).

1.3. Toxicological Assays for DESs:

DESs were employed as drug solubility vehicles for dose administration of rats and the manufacture of compostable polyesters with antimicrobial properties because it was documented in several articles that they are safe, environmentally friendly, and biodegradable solvents. The presumption that DESs are safe was based upon the material safety information sheets of each of their separate components, which show that the overall toxicological behaviour of DESs' separate components is non-toxic. Although tests on brine shrimp, as well as bacteria, revealed combinatorial effects of different DES components, more recent studies revealed certain harmful effects of DESs as well as the toxicity of specific DES components can differ from that of the resulting solution (6). Because it disregards the probability of a combinatorial effect following DES creation, this assumption is not necessarily true. Although DESs as solvents demonstrate high applicability in numerous reactions and processes, their biological and commercial applications are constrained by the absence of toxicity evidence (7). In order to confirm and study further such aspects, this research was designed to study the anti-fungal and anti-bacterial properties of DES along with its toxicity analysis on fish, anti- oxidant and cytotoxic aspects. All these properties were studied for a single DES i.e. Tetrahexyl ammonium bromide: Decanoic Acid (THABDA).

The anti-fungal activity was examined against one strains of fungi i.e., *Aspergillus niger*
A. niger is a fungus that looks dull or dark black and produces a number of crucial goods for the fermentation industry. It is a filamentous ascomycete that can grow quickly and tolerate a wide range of pH levels, making it one of the most significant global fungi involved in the post-harvest degradation of various substrates.

Two strains of bacteria were used to evaluate the anti-bacterial activity, one of which were Gram-positive (*Staphylococcus aureus*) and one of which were Gram-negative (*Escherichia coli*).

S. aureus bacteria are responsible for a wide range of clinical illnesses. The colonies exhibit typical beta haemolysis and are spherical, big (1-3 mm), convex, opaque, and yellow. *E. coli* plays a significant role in the typical gut micro biota of mammals, including humans. However, it is a notable foodborne pathogen that has an effect on humans globally and can cause serious sickness. Its colonies had a tint ranging from yellow to amber, and they caused very minor yellowish zones in the media. For the toxicity analysis on fish, the specie *Cyprinus carpio* was used. It is also known as the Common Carp. It is one of the most common and easily culture-able freshwater species and is an important test organism in toxicology.

RATIONALE

The main objective of chemical sciences targets the limiting eco-toxicity from those chemical compounds which are used in different industrial processes or as in product formulation and the elimination of waste, and efficiency in reactions. Safer alternatives and environmentally friendly solvents were given a lot of thought in previous years. The goal of this research is to evaluate the application of ILs in other fields of synthesis and extraction.

OBJECTIVES OF THE STUDY

Following were the aims of this research:

- The synthesis of Ionic Liquid (ILs).
- To test their anti-bacterial and anti-fungal activity.
- To test their effects on fish toxicity.

CHAPTER II

LITERATURE REVIEW

Ionic liquids are heat stable liquids that stay liquid at room temperature and have a low vapour pressure. At room temperature, these organic salts are in a liquid state that is reviewedto be effective "green" alternatives for traditional volatile solvents, as they are not flammable and do not evaporate (8). They are being utilized in several fields throughout the world and have a vast range of applications both in chemical and biochemical fields. ILs are widelyused as solvents for several reactions. During the past decade, remarkable work has been performed on the toxicological assays of ILs on the cells of various organisms including fish, bacteria, fungi, and humans.

Medical researchers have paid close attention to the cytotoxic, antibacterial, and poisonous characteristics of ionic liquids (ILs) in light of their potential use in drug manufacturing, medication delivery methods, and biomedical materials. The antibacterial response of a large number of microorganisms has been estimated using many antimicrobial activity actions for all kinds of ionic liquids. The precise mechanism underlying ILs' antibacterial action has not yet been thoroughly clarified. Alkyl chains, however, have typically been cited as the primary culprit, most likely because they disrupt the chemical structure of biological membranes (9).

Similar to this, a 2016 in another research the toxicity properties of choline chloride DESs were examined. This study assessed the toxicological effects of 10 DESs based on choline chloride (ChCl) on the C. carpio fish as well as four fungal strains: *L. tigrinus*, *P. chrysosporium*, *C. cylindracea*, *A. niger*. To create DESs, ChCl was mixed with substances from several chemical groups, including acids, sugars, alcohols, and others. $ChCl:ZnCl_2$ demonstrated the largest inhibitory zone diameter for the in vitro evaluated fungus growth among these DESs, led by that of the acid group. On *C. carpio* fish, another research was conducted to determine the severe toxicity and lethal concentration at 50% (LC_{50}) of related DESs. It was discovered that DES inhibitory range and LC_{50} differed from those of their individual components (10).

In 2016, the toxicological evaluation of ten choline-chloride based DESs against four of the fungi strains and the fish named *Cyprinus carpio* was carried out. To make DESs, ChCl was combined with materials from various chemical groups. This study concentrated on the individual DES components, their aqueous combination before DES production, and the DESs that were produced. To investigate the toxicity profile of these DESs, agar disc diffusion technique was used on fungal strains (*Candida cylindracea, Phanerochaete chrysosporium, Lentinus tigrinus* and *A. niger*). ChCl:ZnCl2 had the largest inhibitory zone diameter in vitro against fungi growth that was tested, followed by the acidic group. In addition, the acute toxicity was tested and LC_{50} was determined of the DESs on *C. carpio* fish. It was revealed that DESs inhibitory range and LC_{50} differed from those of its individual constituents. Additionally, it was discovered that DESs were less harmful than either its mixture or its constituent parts. ChCl:MADES had a much higher LC_{50}L than ChCl:MAMix. Moreover, a lower inhibition zone was shown by the DESs acidic group on fungi growth (7).

In 2016, researchers looked into the cytotoxic, genotoxic, and oxidative properties of 1- methyl 3-octyl Imidazolium chloride, another IL. In this study apoptosis and enzyme analysis was used to test the toxic profile of 1-methyl-3-octylimidazolium chloride ([C8mim]Cl) on fish (*Paramisgurnus dabryanus)*. It demonstrated that [C8mim]Cl was harmful to fish liver cells, causing cell damage, gene toxicity, and cytotoxicity. The three different comet characteristics, the four anti-oxidative enzyme activities, and the rates of apoptosis of the examined cells were all notably elevated with important differences. According to the study's findings, [C8mim]Cl may endanger aquatic creatures if it is unintentionally introduced into aquatic habitats (10).

In the year 2018, NADES were synthesized. These are the derivatives of DES. Because most NADES components are naturally occurring, they are known to be non-toxic; however, it is important to access their toxic profile along with biodegradability in research on their synthesis and application. The study aimed to examine the effect of newly synthesizedNADES on yeast (*C. albicans*), bacteria (*S. aureus, E. coli, P. aeruginosa, Salmonella typhi,* and *Proteus mirabilis*), and human cell lines (i.e., HeLa & MCF-7). Furthermore, the anti- oxidant activity of NADES was tested by using oxygen radical absorbance capacity (ORAC). Different responses between microorganisms and cell lines on toxicity were observed, and only the organic acid containing NADES was toxic to the test systems. The anti-oxidative activity was also observed in the NADES-containing compounds. The objective of thisinvestigation was to evaluate the anti-microbial, cytotoxicity, and anti-oxidant activities of synthetic NADES because they are frequently thought to be non-toxic. It was found that only the solvent containing organic acid showed toxicity to the testing, and toxicity responses varied between bacteria and cell types (11).

In order to obtain natural bioactive substances, DES has been investigated as an alternative to dangerous solvents. In the year 2020, choline chloride-based DES the was used to the evaluate extraction of phenolic from rosemary leaves. Initial screening outcomes for the DES examined revealed a total phenolic (TPC) up to 220% higher than the control. The main rosemary bio-compounds solubilities were estimated by COSMO-RS to be favourably linked with TPC. For further refinement, choline chloride:1,2-propanediol (CPH) was used. The TPC and antioxidant activity were 78 mg Gallic acid equivalent/g 80 mg Trolox equivalent/g and 80 mg respectively under the ideal circumstances. The improved extract's antibacterial activity showed a 39–51% suppression of all the tested microorganisms. In conclusion,rosemary was used to create an extract with potent antibacterial and antioxidant properties using CPH as a solvent (12).

The anti-microbial efficacy and toxicity of Imidazolium-based ILs were also examined in another study. It was established that dicationic ILs (DILs) based on Imidazolium are greatly desired and anticipated for application in live tissue in order to avoid fungal or bacterial infections. Studies revealed that DILs with a 10-carbon alkyl chain insertion were effective against the studied bacterial strains and yeasts. However, the majority of the DILs were hazardous and cytotoxic at 1 mM. DILs with alkyl chains shorter than ten carbons, incontrast, were efficacious against a few Candida species and bacteria, primarily *S. aureus*, and they manifested mild cytotoxicity (9).

NADES are a novel class of environmentally friendly solvents that can solubilize both natural and manmade compounds that have poor water solubility. NADES are combinations of two or three chemicals that play a role as both hydrogen bond donors and acceptors. Since mostof the components of NADES are derived from natural sources, it is believed that they are non-toxic and frequently display antibacterial action. In 2022, a study including menthol, capric acid, SolutolTM, and their related eutectic system was examined for potential antimicrobial effects on four distinct types of bacteria: *P. aeruginosa*, *B. subtilis*, *S. aureus* and *E.* coli and one fungus: *C. albicans*. As compared to each of their individual components, the NADES had a higher antibacterial effect, and the results also indicated that they had promised antimicrobial activity against *P. aeruginosa, S. aureus, and C. albicans,* no detectable antibacterial action of NADES was found against spore-forming *B. subtilis* (13).

Due to their exceptional qualities, DESs have become a hot issue in many scientific fields. They have been investigated in numerous applications. One of the most often utilized examples of these fluids is choline chloride DESs. However, it is crucial to ensure DESs' stability, reusability, and biocompatibility in order to use them in various applications. A

2022 investigation focused on the long-term stability of ChCl-based DESs created with urea, malonic acid, and glucose as hydrogen bond donors. Toxicological tests using bacterial strains (*P. aeruginosa*, *S. aureus*, and *E. coli*) were assessed to know the potential creation of hazardous by-products during prolonged heating. Since ChCl:urea DES was shown to be less hazardous to microorganisms and to have great long-term thermal stability, it can be regarded as a green solvent. At 100 °C, potential caramelization caused ChCl:glucose DES to begin to disintegrate, and degradation was accelerated at higher temperatures. Low antibacterial activities were noted, and the toxicity of this DES toward bacteria was not significantly affected by degradation. It was not advised to apply ChCl:malonic DES because it has been demonstrated to be unstable thermally due to malonic acid's esterification and breakdown. Additionally, in this investigation increased toxicity of this DES was noted compared to other DESs (7).

CHAPTER III

MATERIALS AND METHODS

3.1 PREPARATION OF DEEP EUTECTIC SOLVENT

3.1.1 Equipment Used

1. Laboratory Freezer
2. Analytical Balance
3. Beaker
4. Glass Rod
5. Magnetic Stirrer
6. Glass Vials
7. Oil Bath
8. Masking Tape

3.1.2 Chemicals Used

1. Vegetable Oil
2. Tetrahexyl Ammonium Bromide
3. Decanoic Acid

3.1.3 Methodology of DES Synthesis

3.1.4 Calculated Amount of Salt for 3ML Solution

DES	CHEMICALS USED	AMOUNT IN GRAMS	MOLAR RATIO
S13	$C_{24}H_{52}BrN$: $C_{10}H_{20}O_2$	4.35:3.34	1:2

An empty china dish was taken and weighed on an analytical balance and a reading was noted (R1). The salt was added to the same china dish and the weight was noted down (R2). Then R1 has subtracted from R2 the exact weight of the salt was obtained.

3.1.5 Methodology of Preparation of DES

In pre-sterilized flasks, 3.34 g of deconic acid (HBD) and 4.35 g of tetrahexyl ammonium bromide (HBA) were taken with a molar ratio of 2:1. To ensure that no moisture remains in the apparatus the whole apparatus was dried in the oven and the flask was heated at 80-100 °C in an oil bath on a hot plate for 2 hours. A glass rod was used to stir the mixture. A thermometer was suspended in the flask to note the temperature. A homogenous solution of white color was obtained. The temperature of the solution was kept at 0°C until it was frozen. After that, it was brought to room temperature. A homogenous solution was obtained which was labelled as S13.

STUDY OF TEST ORGANISMS

3.2.1 Model organisms

The model species used for this study were bacteria, fungi, and fish that are listed below:

1. **Bacteria**

E. coli is rod-shaped, facultative anaerobic bacteria that are frequently found in ground beef and water that has been tainted with faeces. The gram-positive, spherical bacteria, *Staphylococcus aureus* is commonly found in water and air. *S. aureus* is one of the most common pathogens responsible for antimicrobial resistance deaths.

2. **Fungus**

Aspergillus niger is a haploid filamentous fungus commonly found in food contaminants and causes the disease "black mould" on certain veggies and fruits.

3. **Fish**

One of the most frequently used species for the acute toxicity test is the common carp, *Cyprinus carpio*. It inhabits flooded places, shallow confined waters like lakes, oxbows, and water reservoirs, as well as the lower and middle streams of rivers.

3.2.2 Strain Collection

- The bacterial strain of *E. coli*, and *S. aureus* were collected from the pathology laboratory of the University of Veterinary and Animal Sciences.
- The fungal strain of *A. niger* was collected from the Government College University Lahore.
- The *C. carpio* fish was collected from the Manawa Fish Hatchery.

3.2 CULTURING OF BACTERIA

3.3.1 Equipment Used

1. Laminar Flow (Biosafety Cabinet)
2. Autoclave
3. Hot Air Oven
4. Incubator
5. Analytical Balance
6. Magnetic Stirrer Hot Plate
7. Test Tube Rack
8. Conical Flasks
9. Micropipette
10. Micro Tips
11. Test tube
12. Aluminium Foil
13. Cotton plug

Figure 3.3.1 (a) Laminar Flow **(b)** Autoclave **(c)** Incubator **(d)** Drying Oven

3.3.2 Chemical used

1. Ethanol
2. Distilled water
3. Nutrient Agar
4. Tryptone
5. Sodium chloride (NaCl)
6. Yeast extract
7. Agar powder

3.3.3 Strain used

- *Escherichia coli*
- *Staphylococcus aureus*

3.3.4 Preparation of media

A. Luria-Bertani Broth

Weighed amounts of 0.5 g sodium chloride (NaCl), 0.5 g yeast extract, and 1 g tryptone, and were weighed on an analytical balance and dissolved in 100 ml distilled water. The dissolution was done on a magnetic stirrer followed up by hot plating. Then the solution was covered aluminium foil and then autoclaved for 15 min at 121°C.

C. Luria-Bertani Agar

Calculated amounts of 2 g tryptone, 1 g yeast extract, 1 g NaCl and 3 g agar powder were dissolved in 200 ml distilled water. The dissolution was done by magnetic stirring followed up by hot plating. The mixture was covered by aluminium foil and was autoclaved for 15 minutes at 121°C.

D. Nutrient Agar

3.5 g of the calculated volume of nutrient agar was dissolved in 125 ml of distilled water by magnetic stirring. The mixture was covered by aluminium foil and was autoclaved for 15 minutes at 121°C.

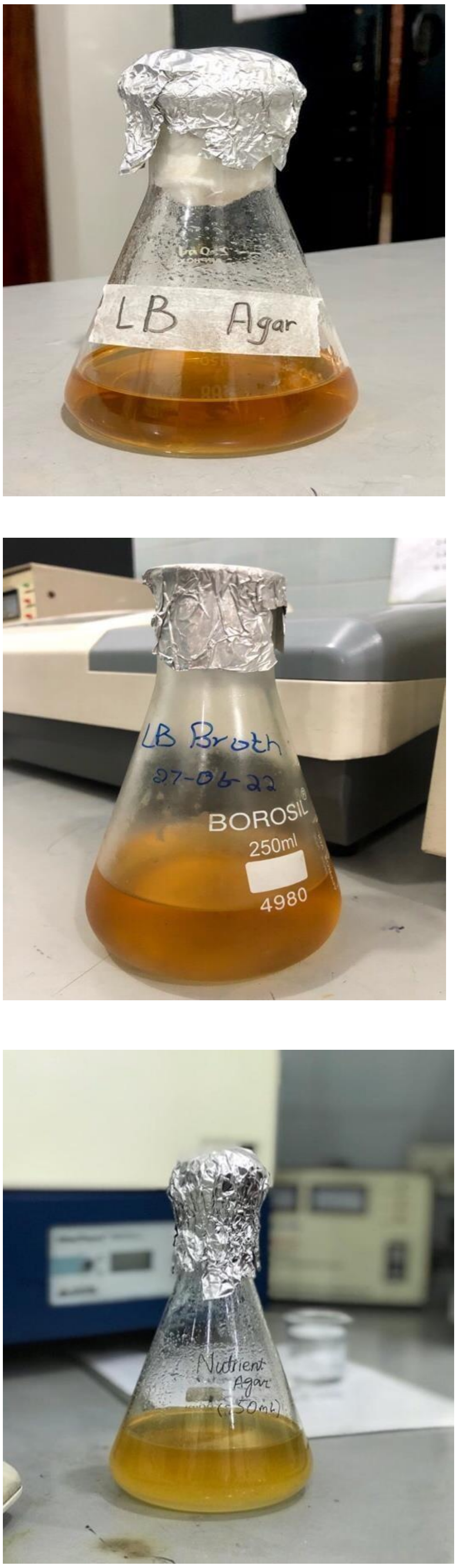


Figure 3.3.2 (a) L.B Agar **(b)** Nutrient Agar **(c)** L.B Broth

3.3.5 Preparation of Inoculum

Under aseptic conditions in the Laminar flow cabinet, autoclaved glass test tubes were set in the test tube rack. Four test tubes were labeled according to the type of bacterial strain and set in the rack. In each test tube 10 ml autoclaved L.B broth was pipetted out. With the help of a micro-pipet, 100 ul from each bacterial glycerol stock was inoculated in the broth. The rack was placed in an incubator at 37°C for 24 hours (14).

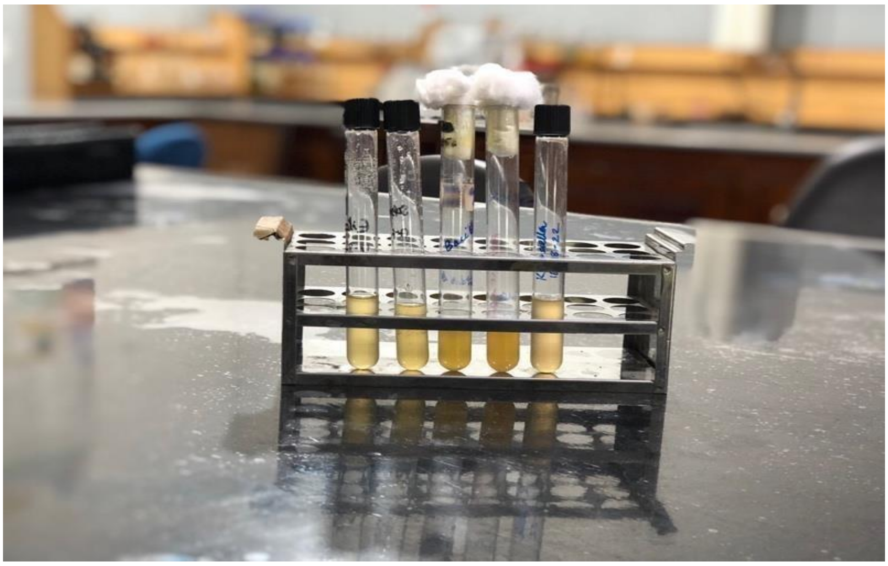


Figure 3.3.3 Inoculum in Glass Test-tubes

3.3.6 Cultivation of Bacteria

After the preparation of the media, it was poured into autoclaved petri plates in the Biosafety cabinet. Turbidity test confirmed bacterial growth in the cultured inoculum. Liquid inoculum of *E. coli* was applied to Luria-Bertani agar plates using two different sterilized swab sticks. *S. aureus* liquid inoculum were spread on Nutrient agar plates respectively by spreading method. The cultured plates were kept at inverted position in an incubator for 24 hours at 37°C (15).

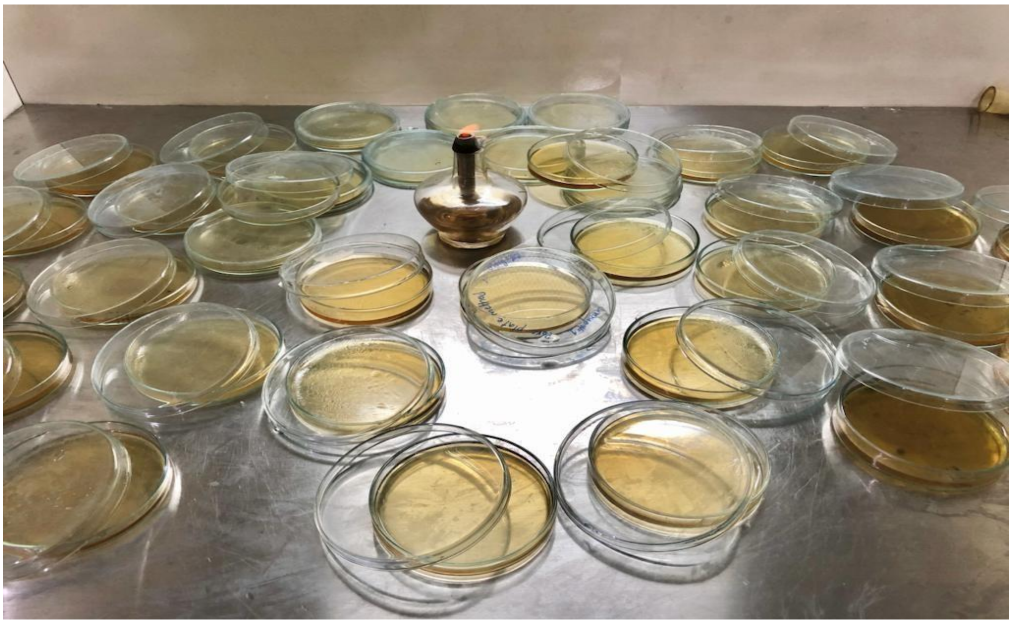


Figure 3.3.4 Setting of Luria Bertani Agar on petri plates

3.3 FUNGUS CULTURE

3.3.1 Equipment Required

1. Laminar Flow (Biosafety Cabinet)
2. Conical Flasks
3. Micropipette
4. Micro Tips
5. Autoclave
6. Hot Air Oven
7. Incubator
8. Analytical Balance
9. Magnetic Stirrer Hot Plate
10. Test Tube Rack
11. Test tubes
12. Cotton plug
13. Conical flasks
14. Inoculating loop
15. Petri plates
16. Spirit lamp

3.3.2 Chemical Used

1. Ethanol
2. Distilled water
3. Potato Dextrose Agar (PDA)
4. Nystatin
5. Potato Dextrose Broth

3.4.3 Strains Used

- *Aspergillus niger*

3.4.4 Preparation of Medium

A. Potato Dextrose Agar

9.75 g of the weighed amount of PDA was dissolved in 250 ml of distilled water in a flask. Agar was fully dissolved by magnetic stirring. The prepared solution was covered with a cotton plug followed by aluminium foil and autoclaved for 15 min at 121°C.

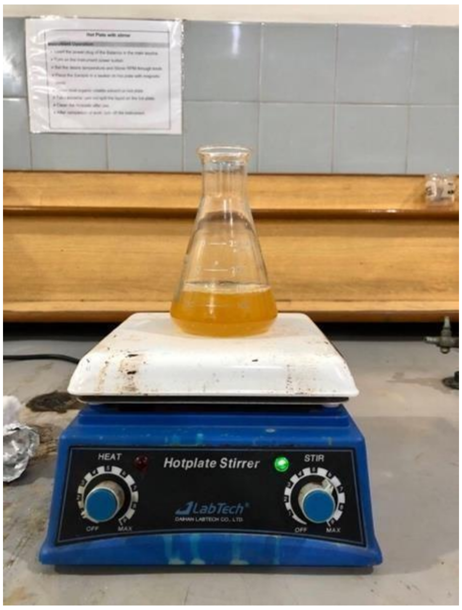


Figure 3.4.1 Magnetic stirring of Potato Dextrose Agar (PDA)

3.4.5 Preparation of Inoculum

After preparing aseptic conditions in the Laminar flow cabinet, autoclaved glass test tubes with caps were set in the test tube rack. Four test tubes were and labelled set in the rack, two for each fungal strain. 10 ml autoclaved Potato Dextrose broth was pipetted out in each test tube carefully. With the help of a sterilized inoculating loop, *A. niger* fungal spores were added in two test tubes. Then test tubes were closed and placed in an incubator at 37°C for 48hours (14).

3.4.6 Cultivation of Fungi

After the preparation of the media, it was poured into autoclaved Petri plates in the Laminar flow cabinet. The fungus growth in the incubated inoculum was confirmed. With the help of swab sticks, *A. niger* inoculum were spread on potato dextrose agar plates. Agar plates were put in an incubator for 48 hours at 37°C (15).

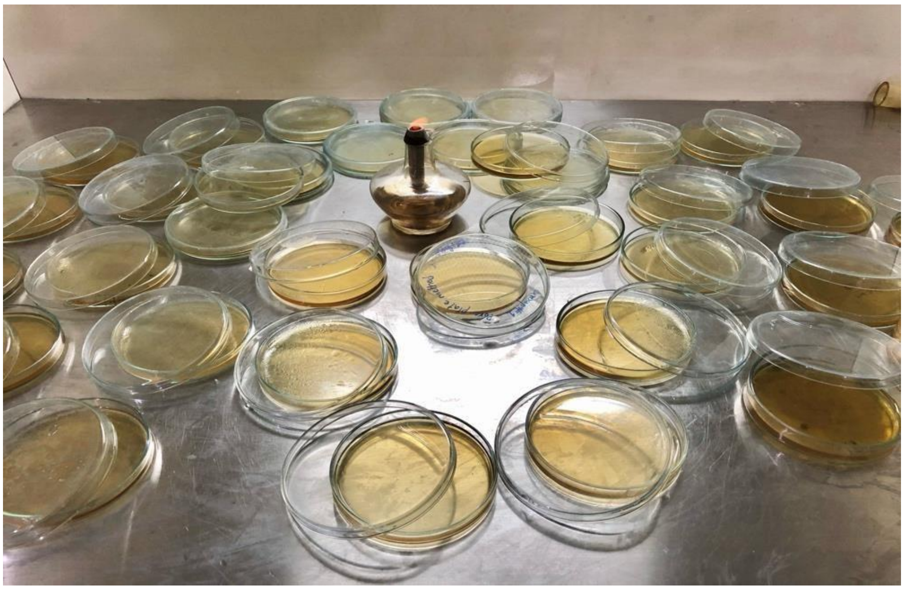


Figure 3.4.2 Setting of Potato Dextrose Agar on petri plates

3.4 TOXICOLOGICAL ASSAY OF DES ON BACTERIA & FUNGI

3.4.1 Equipment Used

1. Inoculated Agar plates
2. Forceps
3. Micropipette
4. Micro Tips
5. Spirit Lamp
6. Laminar Flow Cabinet
7. Incubator

8. Vortex
9. Autoclave
10. Whatman filter paper

3.4.2 Chemical Used

- Alcohol
- Ampicillin
- Kanamycin
- Gentamicin
- Nystatin
- Injected water

3.4.2 Acclimatization of Fish

Acclimatization was done to remove pathogens, germs, or waste from fish bodies. For this purpose, 2 plastic tubs of 50ml were taken. They were labelled as A and B. In tub A 30L water and 300g of NaCl were added whereas in tub B only 20L water was added. In each tub. 2 aerators were set to distribute oxygen evenly. In the tub B considered amount of CaCO; was added. As As soon as fish arrived, they were added into A tub with the help of a fishing net. After 5 minutes, fish were transferred to tub B. Tub B was covered with a net for 24 hours to acclimate the fish.

Figure 3.5.1: Acclimatization of Common carp

3.4.3 Serial Dilution of DES

Serial dilution of S13 solvent was prepared in small Eppendorf tubes. A micro pipet ranging from 1ul to 1ml was used for this process. The number of dilutions prepared for solvent was five ranging from 10^{-1} to 10^{-5}. Five small, autoclaved Eppendorf was labelled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. In each Eppendorf, 900 ul (9 ml) of distilled water was added. 100 ul (1 ml) of solvent added to10^{-1}tube. It was shaken vigorously by using a vortex and then 100 ul (1 ml) from 10^{-1} was transferred to a 10^{-2} tube. This process is repeated until the dilution reaches10^{-5} degrees. To avoid error, the tip was changed after each dilution transfer.

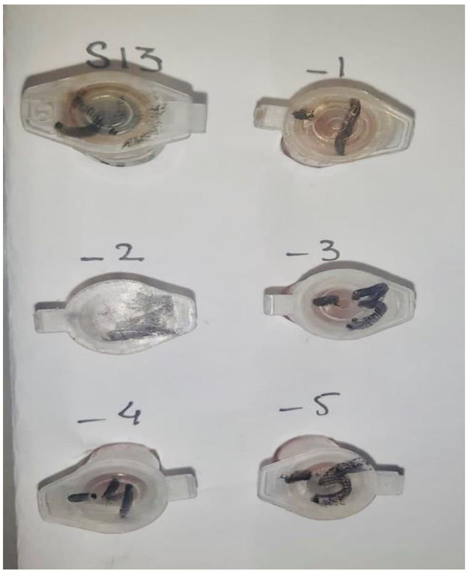


Figure 3.4.3 Serial dilution of S13

3.4.4 Assay of DES Toxicity on Bacteria

In Laminar flow cabinet, aseptic conditions were prepared for the application of toxicity assay on bacteria. Each cultured plate of *E. coli,* and *S. aureus*, and were divided into four equal parts by drawing lines on the opposite side of the plate with the help of permanent marker. 5ul of pure solvent and control group was poured onto the autoclaved disc with the help of a micro-pipet and soaked for up to five minutes. By using sterilized forceps, the solvent discs were placed on the three parts of each inoculated plate while the particular antibiotic disc was put on the fourth part (16).

Following that, serial dilutions ranging from 10^{-1} to 10^{-5} were applied to the pre-cultured plate designated as the experimental group. The filter paper was soaked in each dilution with the help of sterilized forceps on the cultured plate of *E. coli,* and *S. aureus.* The plates were correctly labelled with the type of dilution that was used on them. The plates were kept in an incubator at inverted position for 24 hours. After, this period it was possible to observe the zone of inhibition (17).

3.4.5 Assay of DES Toxicity on Fungi

In Laminar flow cabinet, aseptic conditions were prepared for the application of toxicity assay on fungi. The cultured plate of *A. niger* were divided into four equal parts by drawing lines on the opposite side of the plate with the help of permanent marker. 5ul of pure solvent and Nystatin was poured onto the autoclaved disc with the help of a micro-pipet and soaked for up to five minutes. By using sterilized forceps, the solvent disc was placed on the three parts of each inoculated plate while the Nystatin disc was put on the fourth part.

Following that, serial dilutions ranging from 10^{-1} to 10^{-5} were applied to the pre-cultured plate designated as the experimental group. The filter paper was soaked in each dilution with the help of sterilized forceps on the cultured plate of *A. niger*. The plates were correctly labelled with the type of dilution that was used on them. The plates were kept in an incubator at inverted position for 48 hours at 37 ^{o}C . After, this period inhibitory zone appeared (17).

3.5 TOXICOLOGICAL TEST OF DES ON FISH

3.5.1 Equipment used.

1. Glass aquariums
2. Aerators
3. Fishing net
4. Measuring cylinders
5. Thermometer
6. Conductivity and pH metres
7. Micro pipet
8. White tips
9. Plastic tubs
10. Analytical balance

Figure 3.5.1: Weighing fish on analytical balance.

3.5.2 Assay of DES Toxicity on Fish

The lethal effect of solvent on the fish measured after exposure in a 96-hour static system. The OECD Guideline 203 (18) was followed in the conduct of each test. The reconstituted water had a pH range of 7-7.5, a temperature of 23°C, a conductivity of 7-7.5 S/cm, and a total hardness of 180-190 mg $CaCO_3$ when it was manufactured in line with international standards.

This water prepared the 2-L glass aquariums. Pure form of solvent and it's all dilutions were tested. For this, a limit test with a concentration of 100 mg/L and a full test with lower levels were conducted. The primary limit test was used to determine the LC_{50} and whether it was high or low by combining each component at a concentration of 100 mg/L. A comprehensive LC_{50} test was then carried out for that chemical at lower dosages when mortality was found during the limit test (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) (7).

The dead fish were then taken out of the aquariums after the mortality rates were noted at 1, 24, 48, 72, and 96 hours. Each test material received three repetitions. Throughout the test, the tests water composition and quality were examined and noted. The overall number of fatalities made it possible to determine the deadly concentration of 50 using Correlation analysis. In this study, LC_{50} was found to be less than 100 mg/L in cases where fatality occurred. The LC_{50} estimated using Correlation analysis using the overall number of fatalities after 96 hours. However, the LC_{50} in this case was regarded as being >100 mg/L if there was no mortality (7).

3.6 DATA ANALYSIS

The anti-microbial data was analysed by using Microsoft Excel. To evaluate the toxicity of DES on fish, LC_{50} was calculated by using Probit analysis on MS Excel. Cytotoxicity and antioxidant activity was calculated by using Graph Pad Prism version 5.

CHAPTER IV

RESULTS

DES toxicity was analysed by using disc diffusion method on bacterial and fungal strains.

4.1 EVALUATION OF DES ON BACTERIAL STRAINS

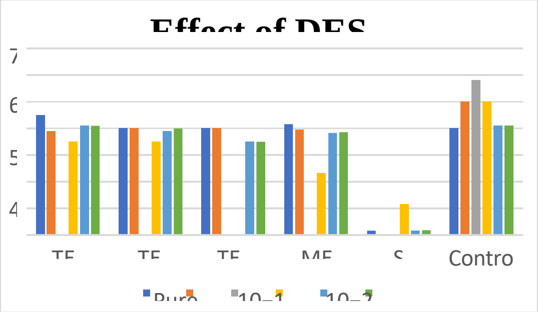
4.1.1 Escherichia coli

The pure form of solvent and it's all dilutions except 10^{-2} showed toxicity on *E. coli.*

Table 1: Anti-microbial activity of S13 on *E. coli.*

Zone of inhibition (mm)						
DES	**TEST 1**	**TES 2**	**TEST 3**	**MEAN**	**SE**	**Control Group (Amp)**
Pure	4.5	4	4	4.16	0.16	4
10^{-1}	3.9	4	4	3.96	0.03	5
10^{-2}	0	0	0	0	0	5.8
10^{-3}	3.5	3.5	0	2.33	1.16	5
10^{-4}	4.1	3.9	3.5	3.83	0.17	4.1
10^{-5}	4.1	4	3.5	3.86	0.18	4.1

These values can be visualized in following graph:



Graph 1: Analysis of anti-microbial activity of S13 on *E. coli*

4.1.2 Staphylococcus aureus

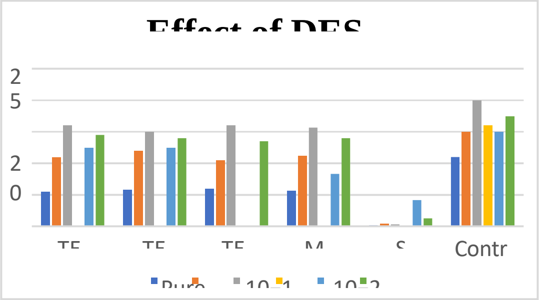
The pure form of solvent and it's all dilutions except 10^{-3} showed toxicity on *S. aureus*. The control group was Gentamycin.

Table 2: Anti-microbial activity of S13 on *S. aureus*.

Zone of inhibition (mm)						
DES	**TEST 1**	**TES 2**	**TEST 3**	**MEAN**	**SE**	**Control Group (Gen)**
Pure	5.5	5.8	6	5.67	0.14	11
10^{-1}	11	12	10.5	11.16	0.44	15
10^{-2}	16	15	16	15.66	0.33	20
10^{-3}	0	0	0	0	0	16
10^{-4}	12.5	12.5	0	8.33	4.16	15
10^{-5}	14.5	14	13.5	14	1.27	17.5

The values include radius of paper disc (i.e 3mm)

These values can be visualized in following graph:



Graph 2: Analysis of anti-microbial activity of S13 on *S. aureus*.

4.2 TOXICITY ASSAY ON FUNGAL STRAINS

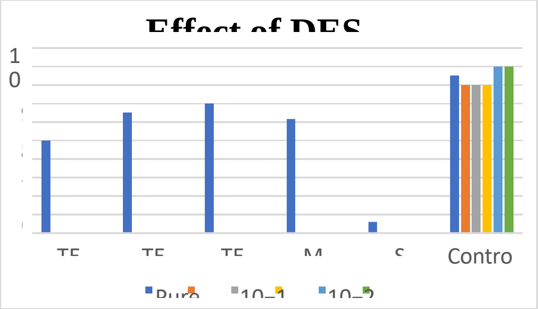
4.2.1 Aspergillus niger

The pure form of solvent created zone of inhibition on *A. niger*. However, its dilution didn't show toxicity on *A. niger*. The control group was Nystatin.

Table 3: Anti-microbial activity of S13 on *A. niger*.

Zone of inhibition (mm)						
DES	**TEST 1**	**TES 2**	**TEST 3**	**MEAN**	**SE**	**Control Group (Nys)**
Pure	5	6.5	7	6.16	0.60	8.5
10^{-1}	0	0	0	0	0	8
10^{-2}	0	0	0	0	0	8
10^{-3}	0	0	0	0	0	8
10^{-4}	0	0	0	0	0	9
10^{-5}	0	0	0	0	0	9

Thesis values can be visualized in following graph:



Graph 3: Analysis of anti-fungal activity of S13 on *A. niger*.

4.3 EVALUATION OF DES ON FISH

The given DES was very harmful to *C. carpio.* The LC_{50} calculated for S13 toxicity towards fish was less than 100mg/L. In limit test, within an hour of introduction of pure solvent to fish aquarium, all fish died. Therefore, full test was conducted showing that all dilutions except 10^{-5} were toxic to *C. carpio.*

Table 4: Showing LC_{50} (mg/L) values of S13.

Solvent	Synthesized solvent	LC_{50} (mg/ L)	Hazard Ranking
S13	$C_{24}H_{52}BrN: C_{10}H_{20}O_2$	<100	-

The solvent was found to be practically harmful according to the Passino and Smith (1987) hazard ranking (19).

CHAPTER V

DISCUSSION

Toxicity test on bacteria

The DES gave both positive and negative results in terms of their toxic effect. A zone of inhibition was detected in each of the plates after the incubation period. A scale was used to measure the zone of inhibition. In this study, one gram-positive bacteria: *S. aureus* and one gram-negative bacteria: *E. coli* were tested against the solvent.

In this research study, S13 has shown positive toxic results on the cultured plates of *S. aureus*, *E. coli*. The zone of inhibition created by pure S13 on gram-negative bacteria: *E. coli* was noted to be 4.16 mm. On *E. coli*, S13 dilutions 10^{-1}, 10^{-3}, 10^{-4}, and 10^{-5} created 3.69 mm, 2.3 mm, 3.8 mm, and 3.5 mm inhibitory zones respectively. However, 10^{-2} didn't create a zone of inhibition on the cultured plate of E. coli. The control group was ampicillin in the case of *E. coli*.

S13 has shown positive toxic results on the growth of one gram positive bacteria: *S. aureus*. The pure form of S13 created 7.4 mm zone of inhibition on *S. aureus*. On S13 dilutions: 10^{-1}, 10^{-2}, 10^{-3} 10^{-4}, and 10^{-5} created 11.6 mm, 15 mm, 8 mm and 14 mm inhibitory zonesrespectively on the cultured plate of *S. aureus*. However, 10^{-3} didn't create any zone of inhibition on *S. aureus*. Investigation in 2013 the cytotoxicity and toxicity of phosphonium- based deep eutectic solvents (DESs) with three hydrogen bond donors, using brine two Gram positive and two Gram negative bacteria (20).

Toxicity test on fungi

The DES prepared was excellent due to their homogeneous properties. The liquid has a lower melting point as compared to the individual components used in preparation.

In the present study, S13 has shown both negative and positive toxic results on the fungal strains of *A. niger*. The inhibitory zone created by pure form of solvent on *A. niger* was measured to be 6.1 mm. However, S13 dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) did not create any inhibitory zone on the cultured plates of *A. niger*. Nystatin was used as the control group for fungal strain.

The results showed that when DES was applied to pre-culture plates of *A. niger* an inhibition ring showed up after incubation. A scale was used to measure the diameter of the inhibition zone. A clear ring could be seen around the filter paper, and fungi growth was stunted.

Toxicity test on fish

The tests findings indicated that, the DES was reported to be practically harmful, as death occurred during the limit test, and the full test was carried out. In (1987) 19 classes of compounds from the Great Lakes and Lake St. Clair, we have given a hazard ranking. Arene halides, phthalate esters, chlorinated camphene, polyromantic hydrocarbons, chlorinated fused polycyclic, nitrogen-containing compounds, alkyl halides, cyclic alkanes, silicon- containing compounds, and heterocyclic nitrogen compounds are the ten most dangerous classes.(19) To ascertain the sub-lethal effects of these substances, chronic bioassays should be performed (19). In accordance with OECD Guideline No. 203, the DES that was found to be practically harmless is not subjected to a full test. Whereas, in the case when mortality occurs during the limit test, a full test with at least five different concentrations (i.e. 10^{-1}, 10^{-2},10^{-3}, 10^{-4}, and 10^{-5}) has to be performed.

The DES synthesized was tetrahexyl ammonium bromide with decanoic acid. In full test, all dilutions except 10^{-5} resulted in mortalities. The solvent had an LC_{50}, less than 100 mg/L, and was considered practically toxic to *C. carpio.*

According to another study S. mossambicus was exposed to toxic and sub lethal concentrations of diammonium phosphate, resulting in 100% mortality and reduced feeding rate and growth rate (21).

Throughout the experiment, physical factors: water conductivity, hardness temperature, pH, were maintained. Throughout the experiment, all aquariums under observation noticed a slight change in ph. The pH values recorded during the test period were within the desired range of 4 to 8. One might argue that mortalities could also result in a change in ph. But in a full test, it can be assessed clearly that deaths occurred because of testing DES and not under influence of ph. Such change in pH is supported by study conducted by Chervova and Lapshin. (2005) on *Cyprinus carpio* fish to evaluate its sensitivity toward amino acids. The change in pH had no effect on mortality response of the tested organism. All aquariums maintained normal conductivity and temperature (22).

CONCLUSION

- Due to their homogeneous nature, the DESS that we prepared were excellent. Compared to the individual ingredients used to prepare them, these liquids had lower melting points.
- The effect of each was investigated by creating their serial dilutions, which revealed both positive and negative results for the solvents made from Ammonium Bromide
-
- Studying the DES's range of action was the goal of the serial dilution preparation. The range of dilutions was 10^{-1} to 10^{-5}.
- In *A. niger,* S13 all noted both favourable and unfavourable toxic results.
- In *Cyprinus carpio* fish, the solvents were discovered to be essentially harmful.

RECOMMENDATIONS

- It is recommended that a comparative analysis of ammonium based DESS can be done with Cholinium based DESs.
- It is recommended that the HBDs used can be of different type other than decanoic acids such as carbohydrates and amides.

LIMITATIONS

- Only one solvent could be synthesized in the given time. Due high cost of chemicals.

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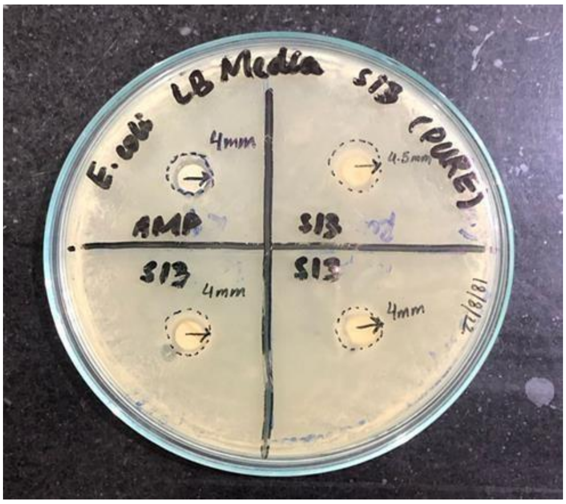
ANNEUXURES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S13
S13
S13
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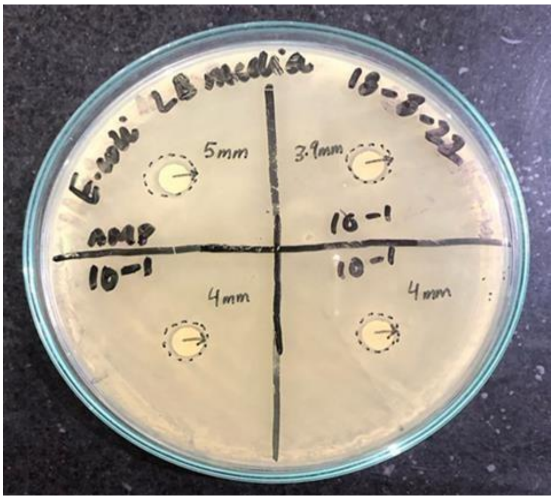
4mm
17/8/22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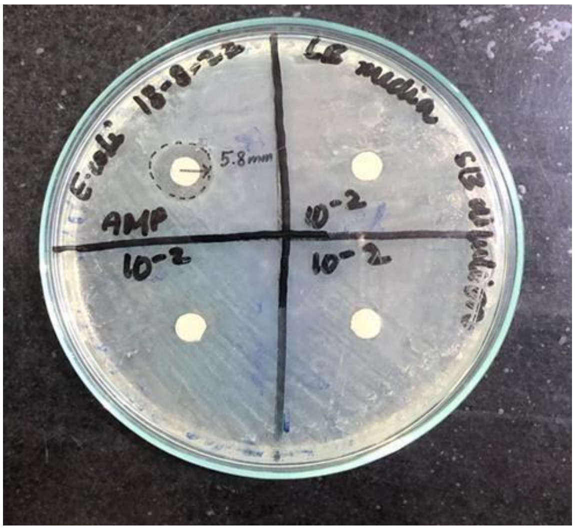
4mm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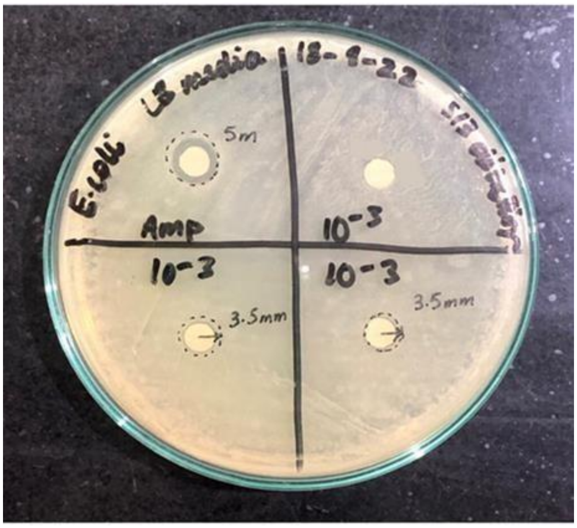
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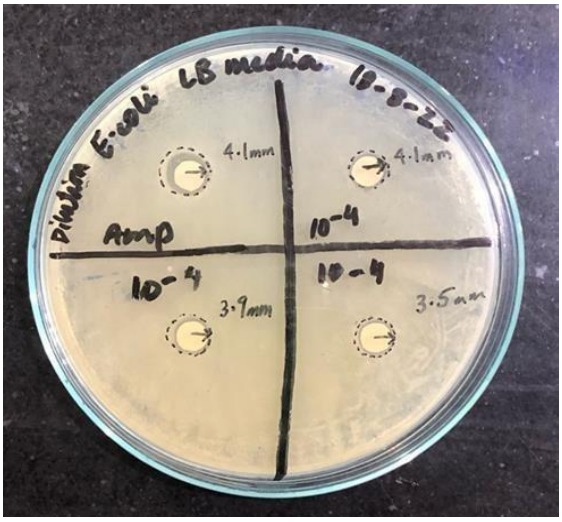
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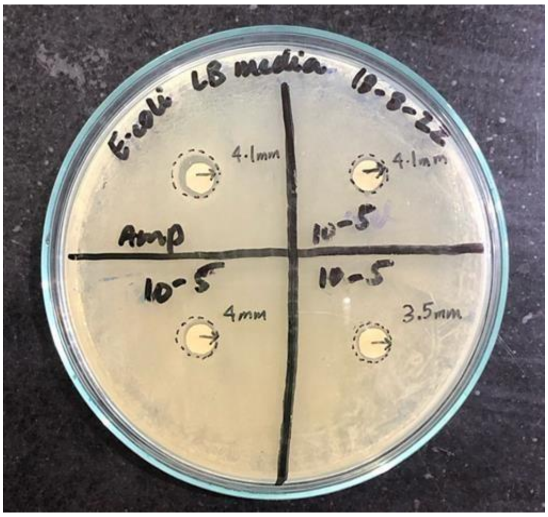
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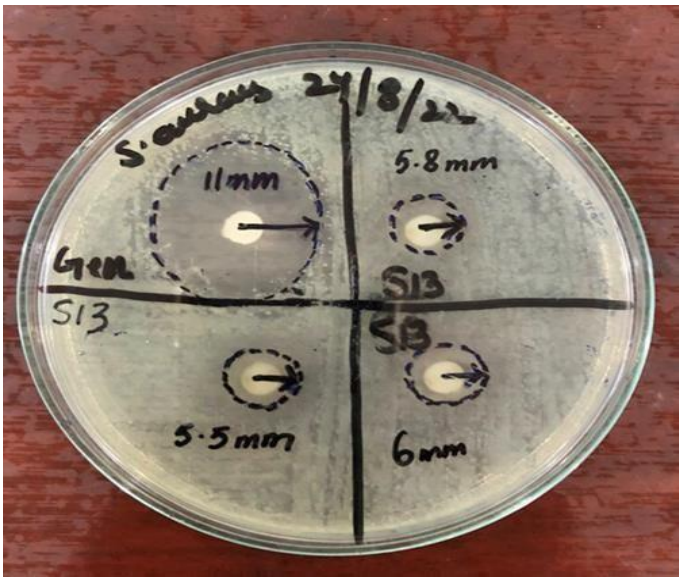
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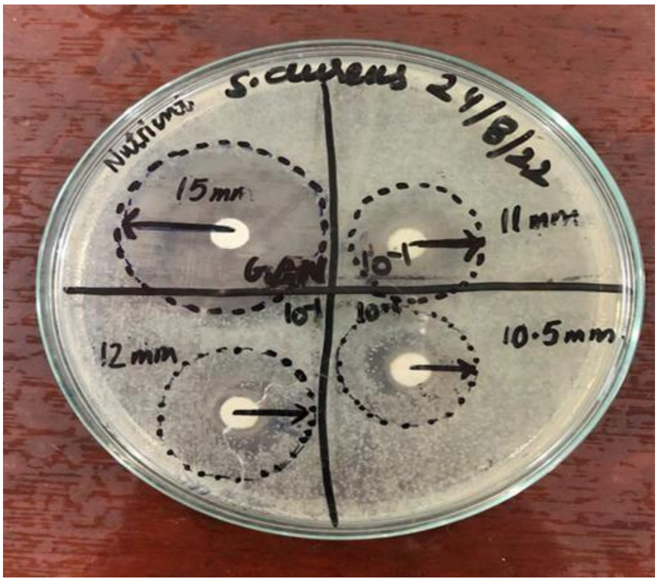
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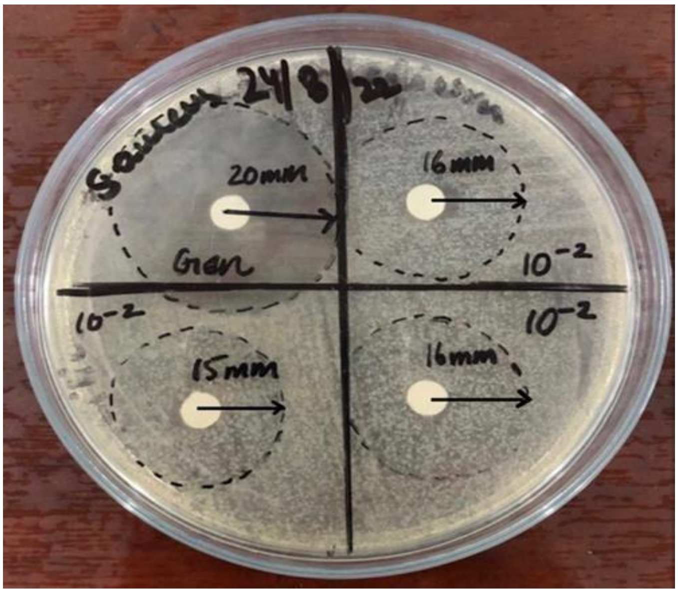
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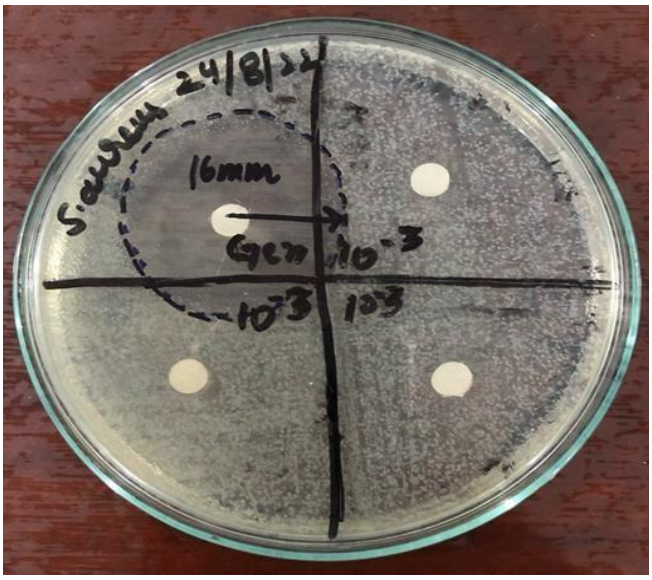
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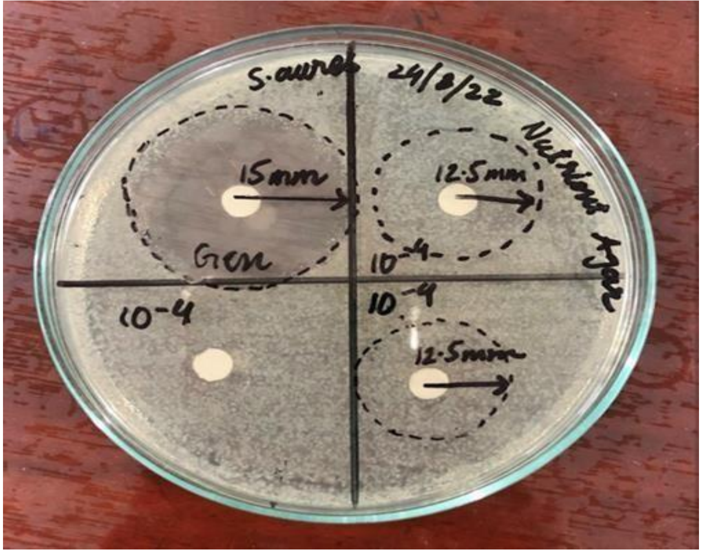
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10-4
10-4
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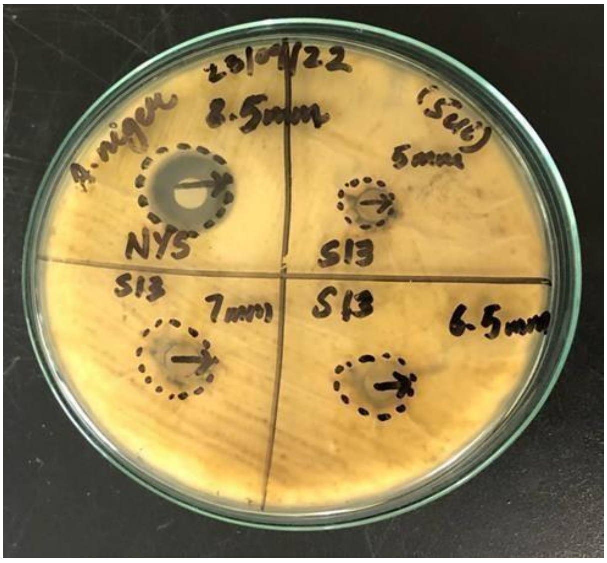
Gan
10-5
10-5
10-5
14mm
14.5mm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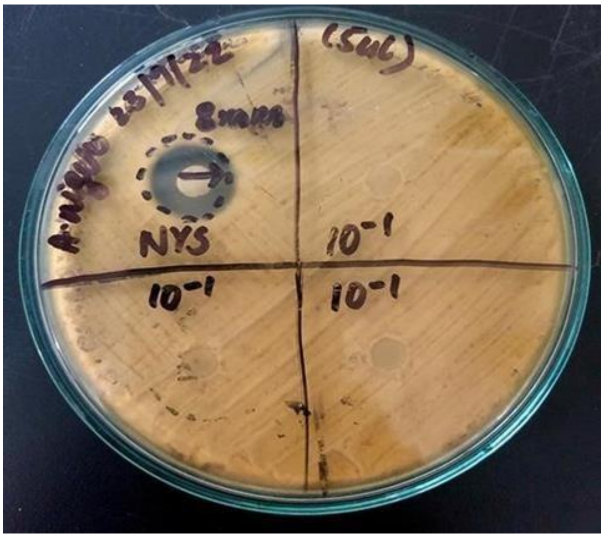
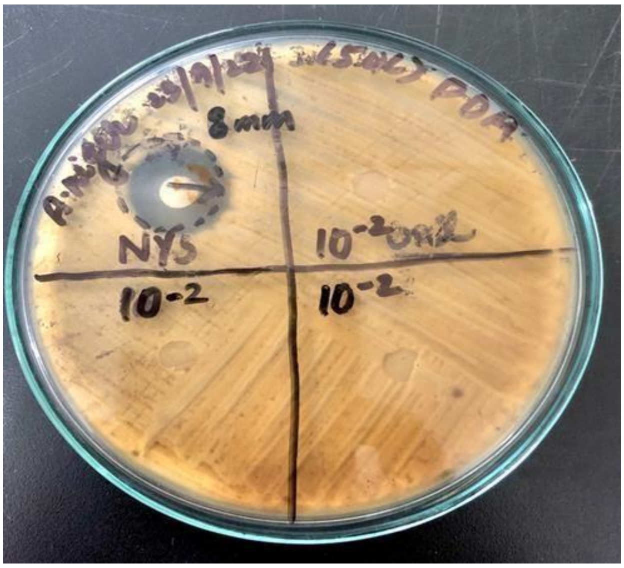
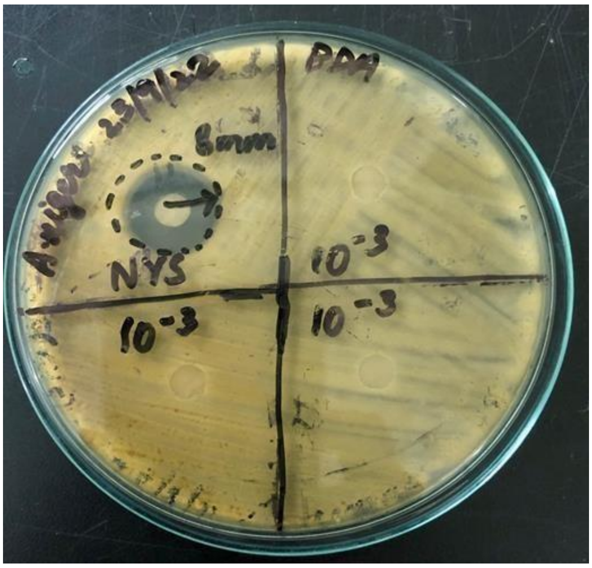
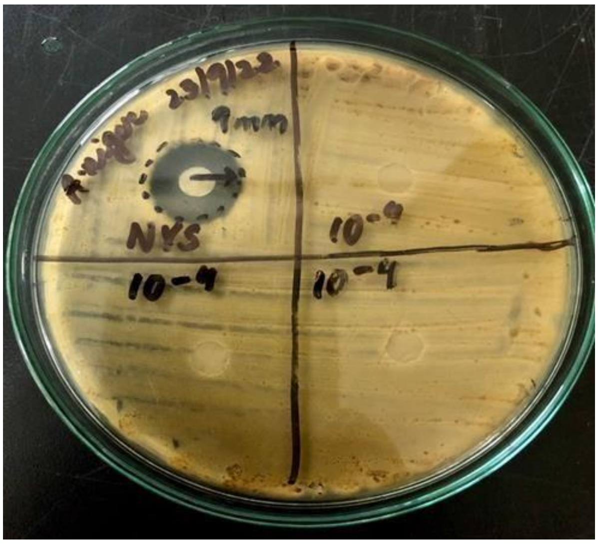
S13
S13
7mm
S13
6.5mm

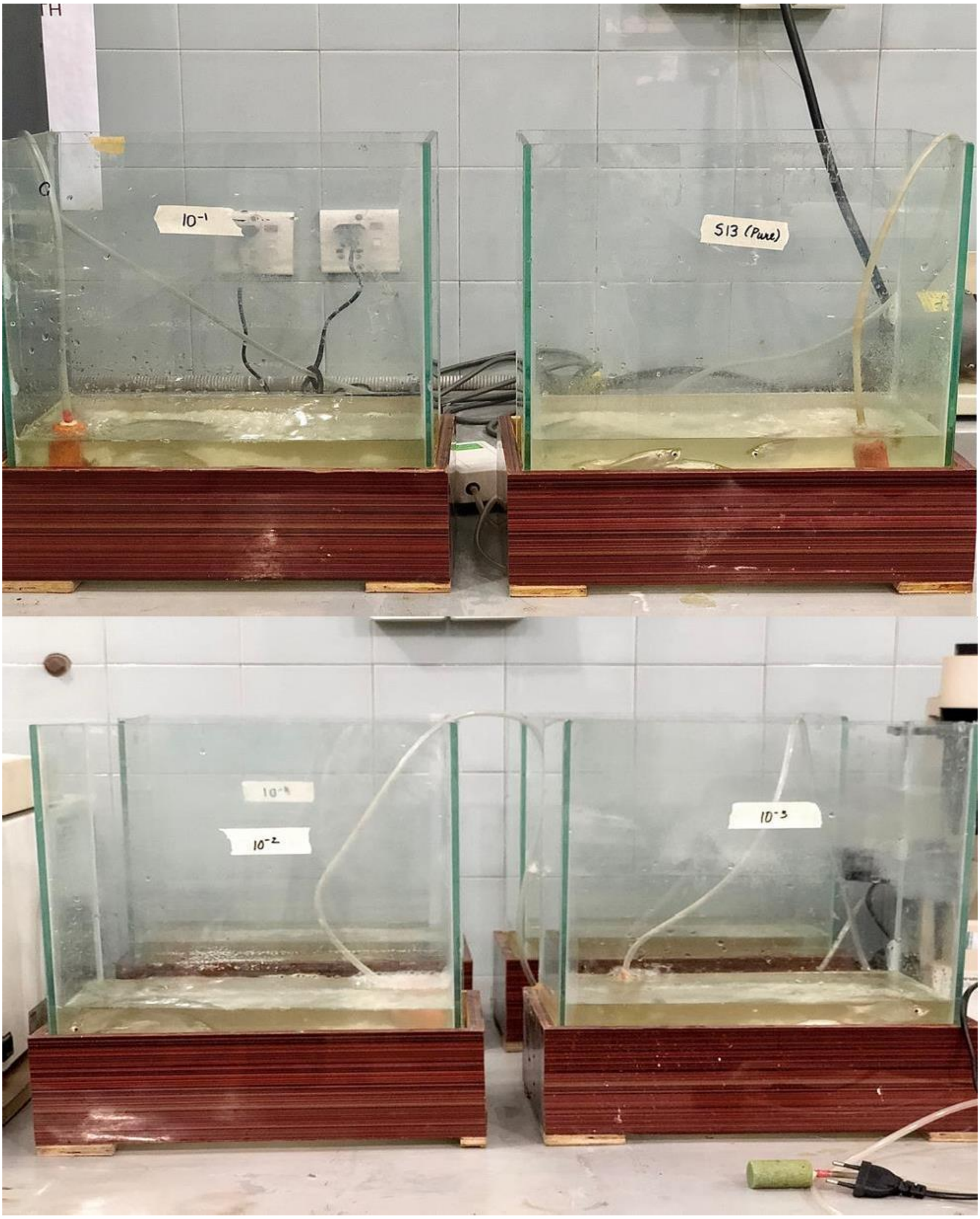

A. niger 23/9/22
(Sul)
8mm
NYS
10^{-1}
10^{-1}
10^{-1}

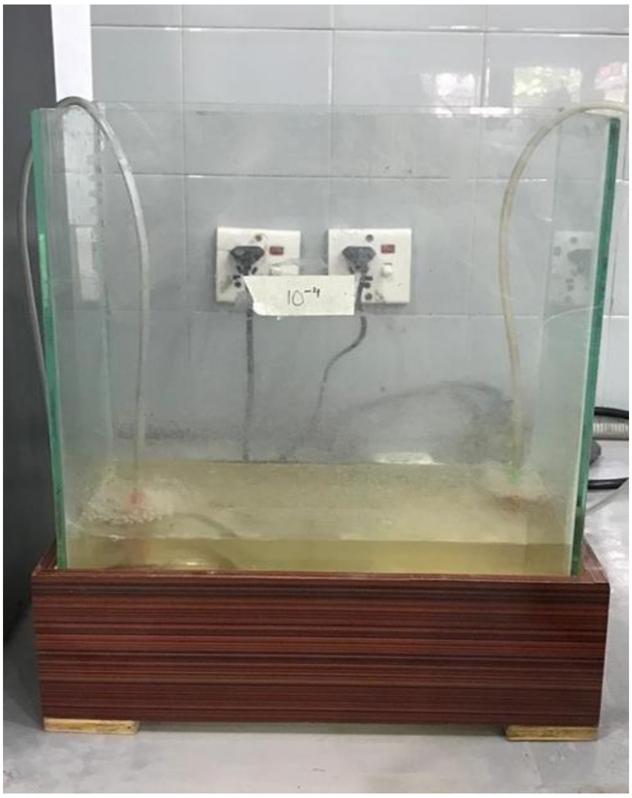

A. niger 23/9/22 (Sul) PDA
8mm
NYS
10^{-2} only
10^{-2}
10^{-2}


A. niger 23/9/22
PDA
8mm
NYS
10^{-3}
10^{-3}
10^{-3}


A. niger 23/9/22
9mm
NYS
10^{-4}
10^{-4}
10^{-4}

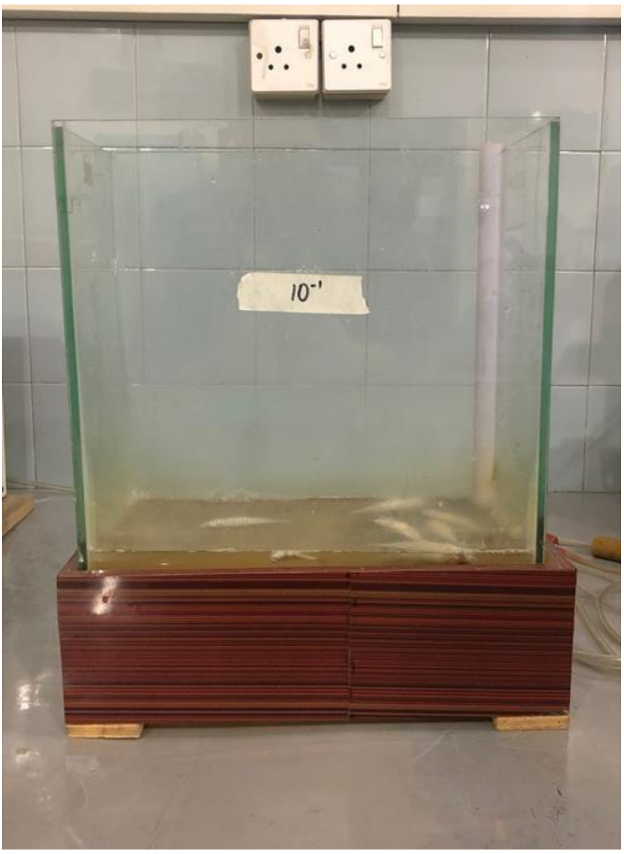

A. niger 23/9/22
8mm
NYS
10^{-5}
10^{-5}
10^{-5}

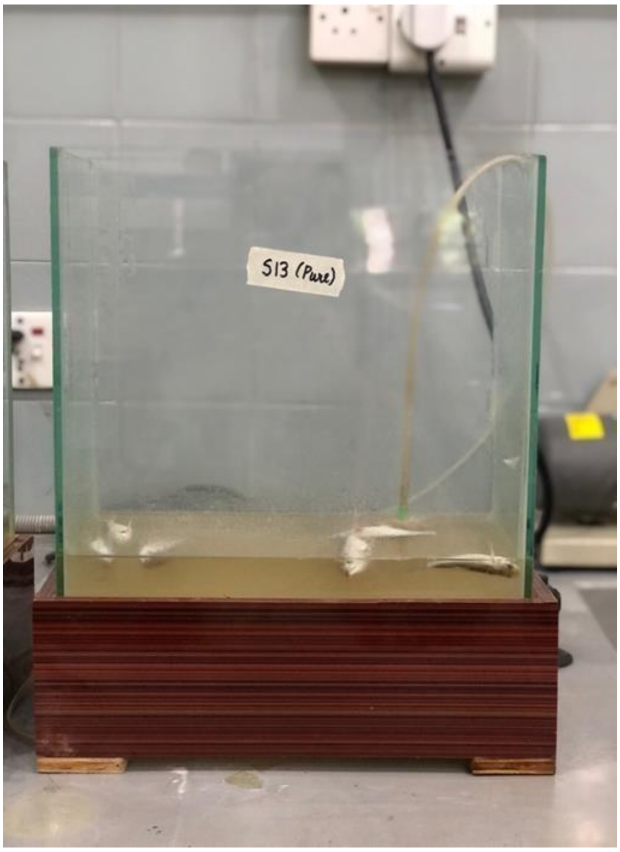

10⁻¹
S13 (Pure)
10⁻⁴
10⁻²
10⁻³

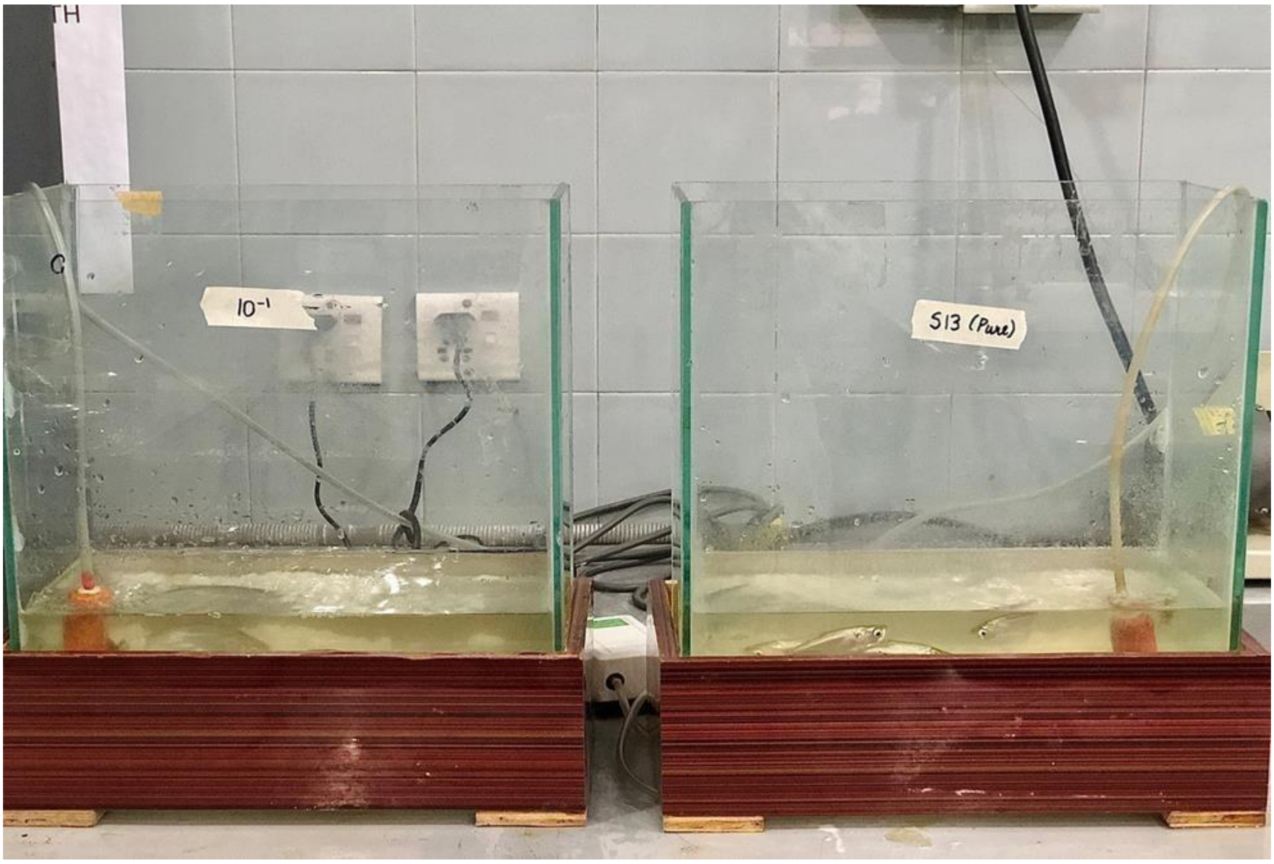

10^{-4}

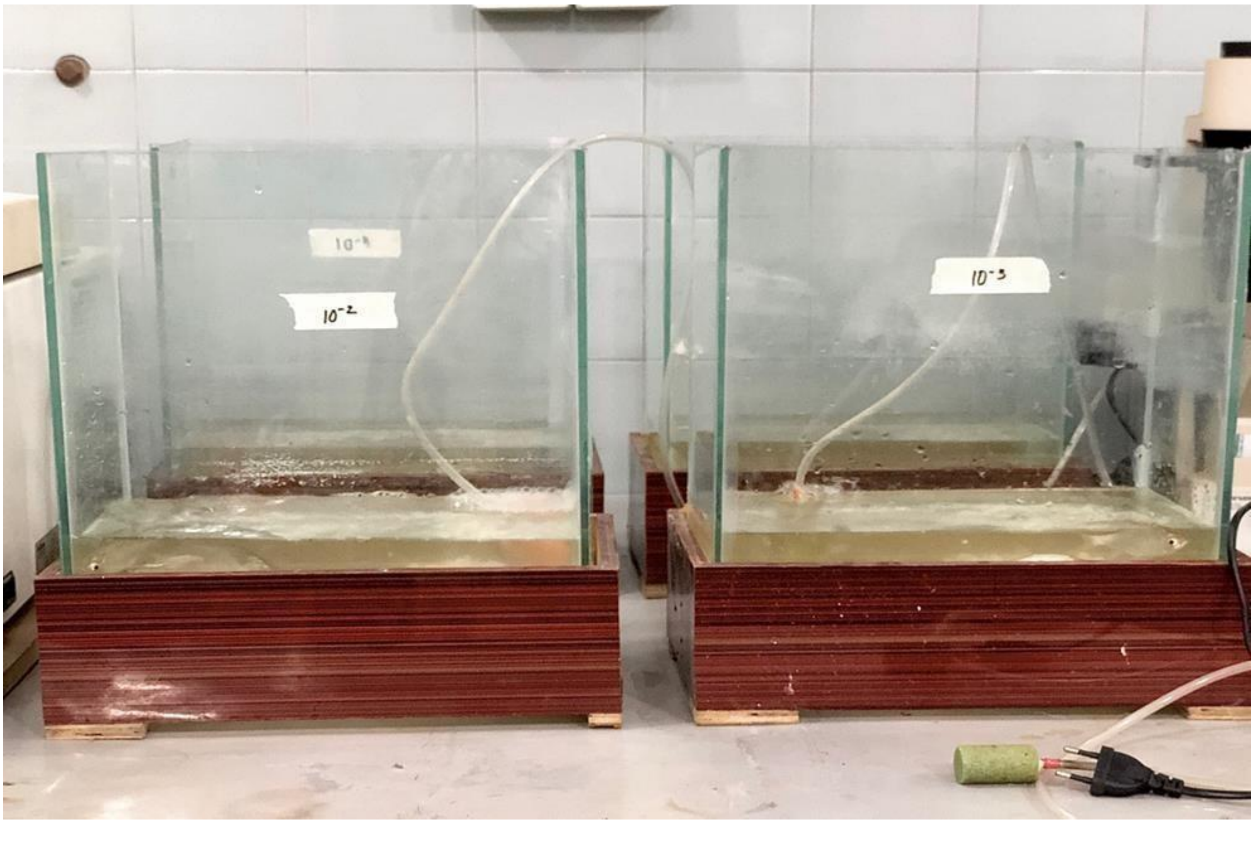

10^{-5}

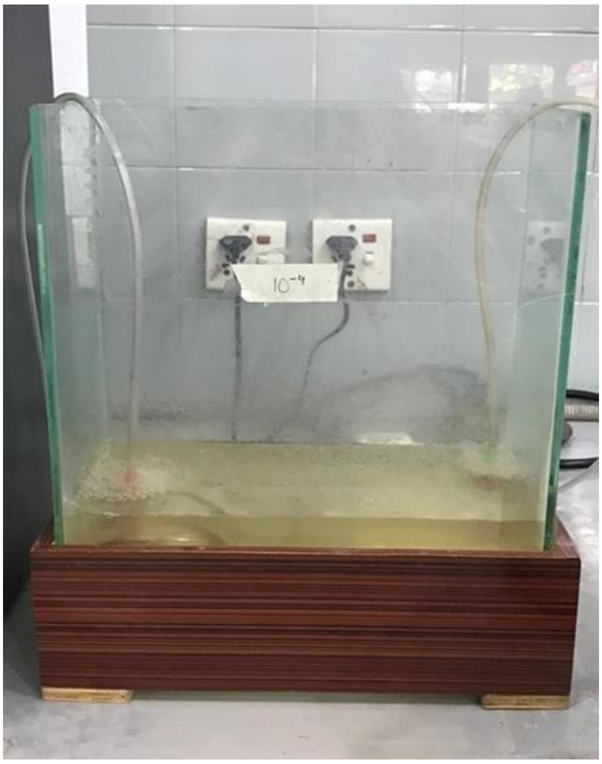
`


10^{-1}


S13 (Pure)


10^{-1}
S13 (Pure)


10^{-4}
10^{-2}
10^{-3}


10⁻⁴


10⁻⁵

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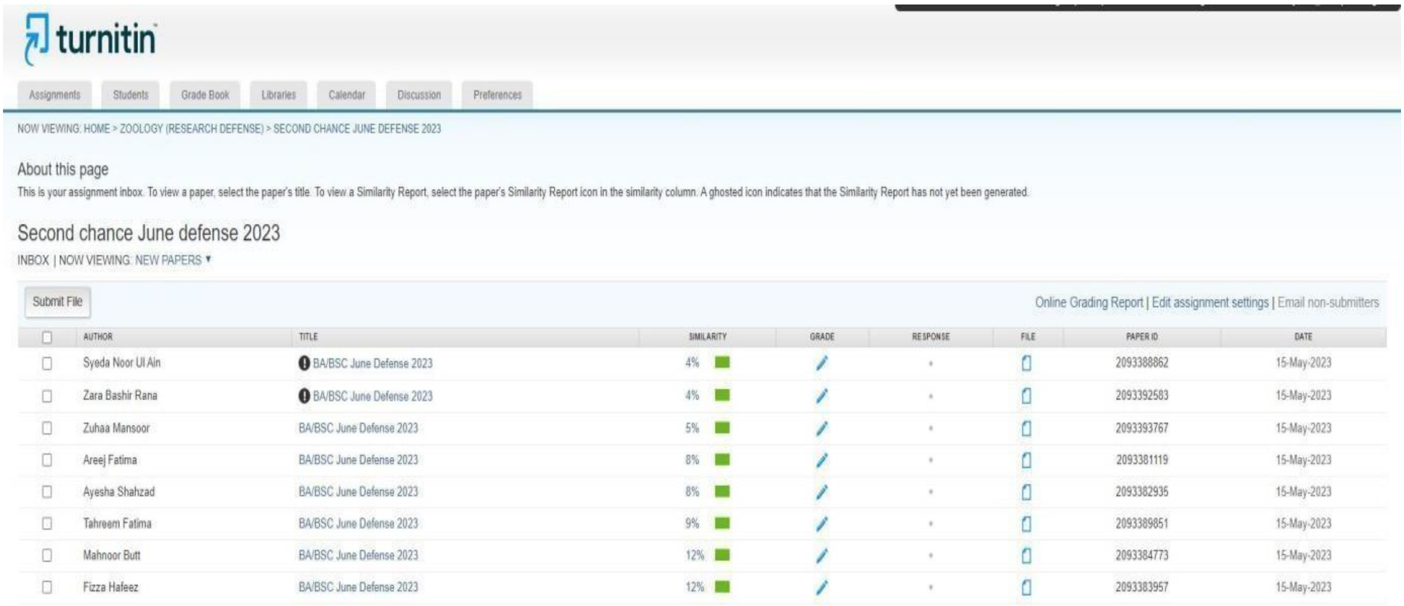

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