Developing a Novel Wound Dressing Hydrogels Based on Deep Eutectic Solvent and Additives: Effectively Kill Bacteria.

By

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Declaration

I certify that this thesis does not have material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

Signed: Mansi Korat

Date: 6th May, 2024

List of abbreviations

Abbreviation	Definition
AFM	Atomic - Force Microscopy
ATCC	American Type Culture Collection
CA	Citric Acid
CFU	Colony Forming Unit
ChCl	Choline Chloride
DES	Deep Eutectic Solvent
FTIR	Fourier Transform Infrared Spectroscopy
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HPLC	High Performance Liquid Chromatography
ILs	Ionic Liquids
LA	Lactic Acid
LC-MS	Liquid Chromatography-Mass Spectrometry
MAE	Microwave-Assisted Extraction
MBC	Minimum Bactericidal Concentration
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant Staphylococcus Aureus
NADES	Natural Deep Eutectic Solvent
OD	Optimal Density
PC2	Physical Containment Two
PEF	Pulsed Electric Field Extraction

QTof	Quadrupole Time of Flight
SEM	Scanning Electron Microscopy
SFU	Supercritical Fluid Extraction
SWE	Subcritical Water Extraction
TEM	Transmission Electron Microscopy
UHPLC	Ultra-High Performance Liquid Chromatography
WHO	World Health Organization

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Abstract

Nowadays, wound management is a major concern for healthcare as it has considerable risk of developing infections and life-threating complications. Traditional wound dressing offers limited protection. Sometimes it fails to protect wound against bacterial infection. Antimicrobial resistance may lead to long-term debilitation in wound healing. Therefore, the demand for innovative antibacterial-resistant wound dressings has become increasingly evident. This study aims to design multifunctional wound dressing hydrogels based on NADES and bioactive additives. These hydrogels exhibit capacity to kill both Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-negative *Pseudomonas aeruginosa* (ATCC 15442) pathogens, which could promote wound healing.

Deep eutectic solvents (DESs) are synthesized through the collaboration of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD). Their notable feature lies in their lower melting points, attributed to the intricate interplay of hydrogen bonding, resulting in a homogeneous and transparent solution. In this study, three different DESs have been incorporated into hydrogels to evaluate their antibacterial activities. Two types of gelling agents including Pluronic F-127 and Xanthan are utilised to prepare DES hydrogels and compared their antibacterial properties. The antibacterial assay was conducted to assess the efficacy of various gels in terms of their antibacterial activity. Furthermore, bioactive compounds were extracted from the brown seaweed and analysed by using high-performance liquid chromatography (HPLC) coupled with quadrupole time-of-flight mass spectrometry (QTof). The brown seaweed extract was then incorporated into DES gels of which antibacterial activities were assessed.

In conclusion, the results show that DES solvent and gels show superior antibacterial activities for both Gram-positive and Gram-negative pathogens. Adding dopamine could enhance their antibacterial properties and bio-adhesive properties. This study highlights the potential of utilizing DES gels for the creation of antibacterial wound dressing materials, which could enhance wound healing processes.

1. Introduction

1.1 Wound healing

A wound is known as damage to skin caused by exterior injury or interior diseases, such as burn (Yang *et al.,* 2024). Wound healing is the process of replacing destroyed or damaged tissues by newly produced tissues. Human skin provides an effective barrier between the organism and the environment, prevents the spread of pathogens, and controls the unwanted loss of solutes and water (Jensen and Proksch, 2009).

There are two types of wounds: acute and chronic wounds. Acute wound recovers faster while chronic wounds take longer time (Raziyeva *et al.*, 2021). In the immediate aftermath of an injury, diverse intercellular and intracellular pathways are activated and orchestrated to restore tissue integrity and homeostasis. Normal wound healing passes through hemostasis phase (after injury), inflammation phase (just after tissue injury), proliferative phase, remodelling (formation of new tissues and blood vessels) (Bjarnsholt *et al.,* 2008).

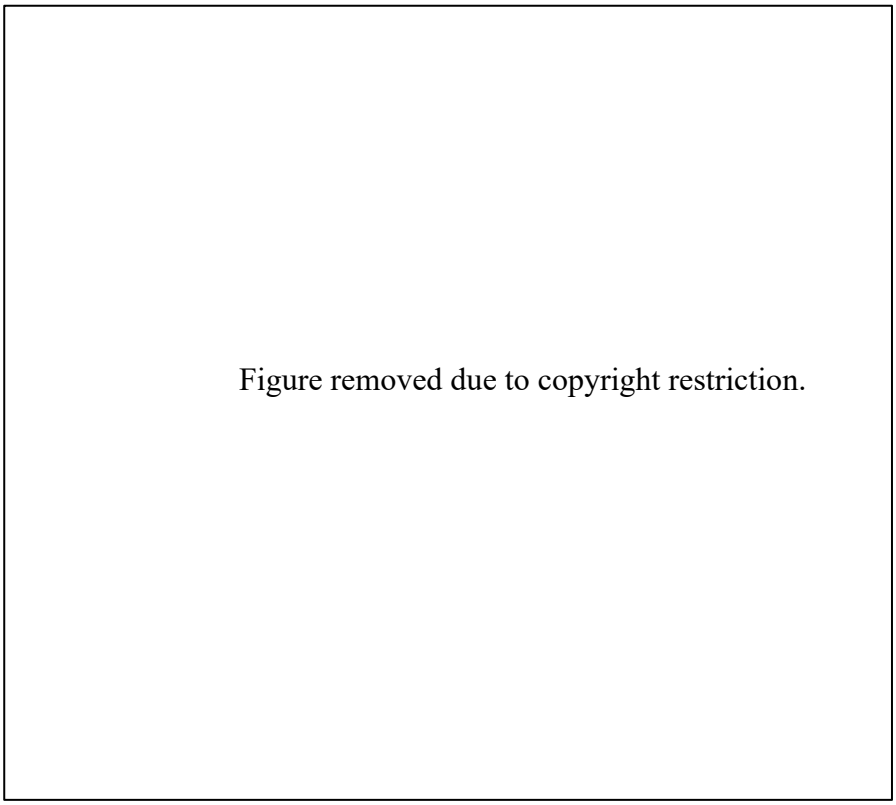

Figure 1. Image of four phases of wound healing process: 1. Haemostasis phase 2. Inflammatory phase 3. Proliferative phase and 4. Remodelling (Maynard and Collings, 2015).

- Phase 1: Hemostasis Phase

Hemostasis phase is the procedure where wound being closed by clotting. In the initial phase, blood vessels restrict to restrict the flow of blood. Afterwards, platelets are combined to seal the broken wall of blood vessel. At last, coagulation take place and reinforces the platelet plug with threads of fibrin that is known as molecular binding agent (Maynard and Collings, 2015). The hemostasis stage of wound healing occurs faster. The platelets adhere to the sub-endothelium surface within moment to break epithelial wall of blood vessel. Then, first fibrin strand starts to adhere. When fibrin mesh starts, the blood will transform from liquid to gel over coagulants and discharge prothrombin. The blood cells trapped by formation of clot.

Clotting plays significant role in wound healing, but it becomes problem if it breaks loose and travel through vessel it may cause stroke or heart attack (Wound Source, 2016).

- Phase 2: Inflammatory Phase

The second phase of wound healing is “Inflammation.” After an injury, blood vessels exposure transudate that cause swelling. Inflammation control bleeding and block the infection. The fluid helps to heal the wound and repair cells. In this phase, white blood cells called neutrophils enter the wound to remove bacteria, damaged cells, and organisms. When white blood cells leave, macrophages come to clearing debris. These cells help to growth factors and promote new tissue generation. This phase takes four to six days (Maynard and Collings, 2015).

- Phase 3: Proliferative Phase

In Proliferative phase, the wound is filled with advanced connective tissue that made up from collagen and extracellular matrix. The new blood vessels have been made so that granulation tissue can received enough oxygen and nutrients. The granulation tissue is red or pink in the healthy stage of wound healing. In addition to this, these granulation tissue does not bleed easily. If there are any dark coloured granulation tissues, then it is a sign of infection. The proliferative phase takes 4 to 24 days (Wound Source, 2016).

- Phase 4: Remodelling Phase

During the remodelling phase, the new tissues started gaining strength and flexibility. When collagen fibres reorganize, the tissue rebuilt and matures. The remodelling phase takes 21 days to 2 years it depends on wound (Maynard and Collings, 2015).

1.2 Wound dressing

As per the World health organization (WHO), 6.6 million people faced injuries and 300,000 deaths due to burns from fire, boiling water, and oil incidents (Walker and King, 2017). Therefore, it is important to take care of skin by using wound dressing. The main goal of wound dressing is to create an optimal environment that supports the natural healing process of the body while protecting the wound from further damage e.g. bacterial infections. Nowadays, distinct kinds of wound dressings and techniques are available in the market to manage wound.

1.2.1 Background of wound dressing

Wound dressing plays a vital role in healthcare as it can be applied to wound surface and promotes wound healing based on types of wounds. In 2500BC, people used clay tablet to treat wounds. Later in 1650BC, people washed the wound with milk or water then apply resin and honey (He *et al.,* 2021). Around 460-370BC, ancient Greece used wine and vinegar to clean wounds (Daunton *et al.,* 2012). Moreover, people used plant herbs, animal fat and honey to tissue engineered scaffolds (Boateng *et al.,* 2008). The modern wound dressing was developed in 20th century. Currently, there are various wound dressing available. For example, semipermeable dressing, plastic film dressing, hydrogels, calcium alginates, foam dressing, hydrocolloids, and paper adhesive tape (Yousefian *et al.,* 2023).

1.2.2 Challenges and limitation of traditional wound dressing

Traditional wound dressings like cotton wool, natural or synthetic bandages and gauze were developed to secure wound from infection and dehydration. It works as shield during initial phase of wound healing as it absorbs blood and exudates and clean the wound, but it is dry and cannot provide enough moist environment to wound (Fan *et al.,* 2014). Gauze dressing allows

moisture to evaporate which cause dehydration in wound bed (Radhakumary *et al.,* 2011). Traditional wound dressings are adhesive to skin, but it causes bleeding and tissue damage after it removed and give unnecessary pain to patient (Jones, 2006).

1.2.3 Hydrogel as an ideal wound dressing

It is ideal to assess mechanism of injury, risk of contamination, tissue damage, disability, and amount of tissue loss before using any wound dressing (Cox, 2023). The main function of wound dressing is to reduce the pain, apply compression of haemostasis, create, and keep moisture environment, to maintain mechanical stability, biocompatible, prevent secondary infection, stimulate the growth factors, protect wound from environment, and promote healing (Kokabi *et al.,* 2007). Modern dressing has potential to create moist environment around wound such as hydrocolloids, alginates, and hydrogels. It occurs in thin gel forms which improved wound healing faster (Radhakumary *et al.,* 2011).

Soft materials such as gels and colloids widely used in wound applications. The gels are semisolids that have soft to tough morphology. Gels have a bicomponent system in which liquid works as predominant component and gelator works as second component (Tomé and Mecerreyes, 2020). The three-dimensional polymer network can be seen in the morphology of hydrogel that is like natural extracellular matrix (Green and Elisseeff, 2016). This three-dimensional network helps hydrogel to absorb and retain huge amount of water which keeps moist environment around wound. Hydrogel has ability to rehydrate dead tissues, act as a barrier against microorganisms and allow oxygen to penetrate (de Lima *et al.,* 2018). It forms a physical barrier and hemostatic plugs at bleeding site that cessed the bleeding and carries antibacterial drugs to the wound (Wang *et al.,* 2020). Hence, hydrogel worked as a great wound dressing.

Hydrogels which are made up from natural or synthetic polymers had poor mechanical properties, lack antibacterial properties, and small equilibrium-swelling ratio (Fan *et al.,* 2014). Besides this, there are few things which needs to be considered while developing wound dressing like it should be able to absorb wound fluids and pus, adhesive, and act as a bacterial barrier. It is challenged to find an ideal hydrogel which is adhesive and act as a bacterial barrier.

1.3 DES-based hydrogel in wound healing

Ion gels are like hydrogels, but it has ionic liquids (ILs) as a primary component and a gelator or polymer as a secondary component which forms three-dimensional network. Deep eutectic solvents (DESs) are recognised as ionic liquids (ILs) because it has ionic nature, tunability, low flammability, low vapor pressure and wide liquid varieties (Smith *et al.*, 2014).

1.3.1 Deep eutectic solvents (DESs)

Deep eutectic solvents (DESs) are characterized as the new class of Ionic Liquids (ILs) that formulate with more than two compounds. It has ability to self-associate to form eutectic mixture at lower melting point. Mostly, they are liquids at room temperature (Botelho Junior *et al.,* 2022).

In 2003, first definition of DESs was given by Abbott and his co-workers (Abbott *et al.,* 2003). Afterwards, Choi *et al.* gave term called Natural deep eutectic solvents" (NADESs) (Choi *et al.,* 2011). It contains sugars, alcohols, polyols, choline derivatives and organic acids. DESs have mixture of two substances with melting point remarkably lower compared to starting material (Mecerreyes and Porcarelli, 2022). It can be derived from the quaternary ammonium salt with metal salt (HBA) and Hydrogen Bond Donor (HBD). The charge delocalization occurring between hydrogen bonding i.e., between halide ion (HBA) and HBD. Thus, it has

low melting point and lattice energy (Smith *et al.,* 2014). The most prominent HBA includes choline chloride (ChCl) and HBDs (Hydrogen Bond Donor) (Hydrogen Bond Donor) includes the natural compounds like sugars, alcohols, and organic acids (Paiva *et al.,* 2014). Moreover, mixture of HBD-HBA with intra- and inter- molecular interactions give resilience to hydrogen bond network which leads to a liquid formation (Araujo *et al.,* 2017). For that reason, the synthesis process is a quite easy as it does not require any reactions and purification steps. Figure 2**.,** shows chemical structures of DES components.

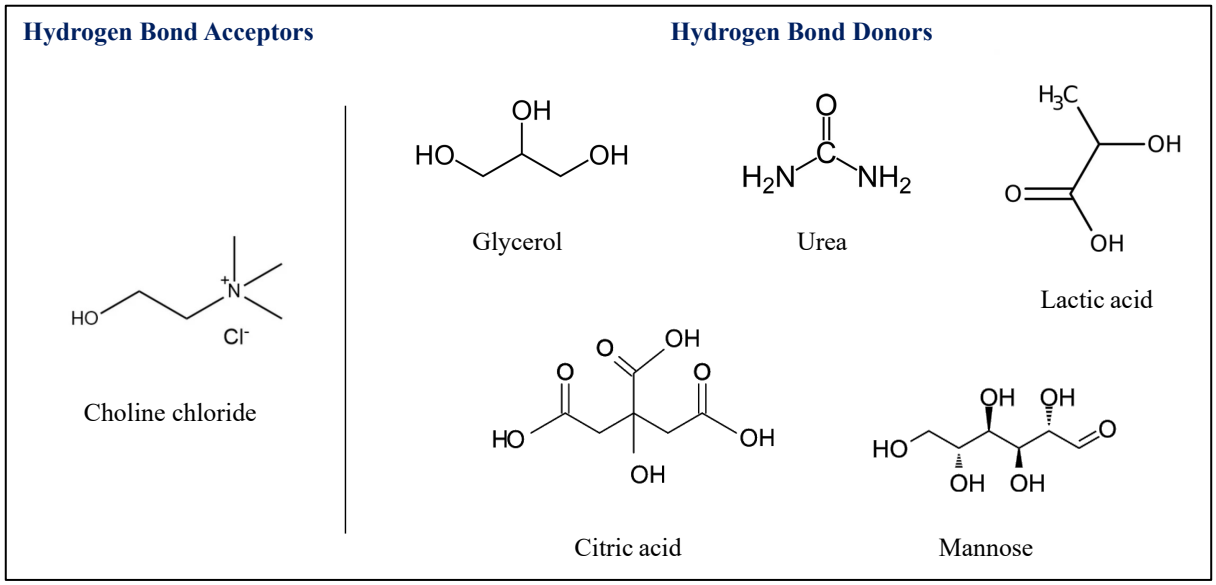

Figure 2. The chemical structure of Hydrogen Bond Acceptor (HBA) and Hydrogen Bond Donor (HBD) used in preparation of DESs.

Each prepared DESs have unique properties. Although, there are different HBA such as, metal salts (LiCl, $FeCl_3$, $ZnCl_2$) and other quaternary ammonium salt but still ChCl is the mostly used as an HBA. Additionally, glycerol, urea, lactic acid, citric acid, and mannose are widely used as an HBD,

1.3.2 Properties of DESs

A Deep Eutectic Solvent (DES) is an eco-friendly solvent which have unique properties like, low vapor pressure, non-flammability, chemical and thermal stability (Wang *et al.,* 2020). The preparation of DES is easy as just mixing of two components with medium heating (Smith *et al.,* 2014). DESs have several advantages like, easy to prepare and easy to available but it also has some disadvantages like, high viscosity, unfavourable toxicity, and solute solubility (Yadav and Pandey, 2014).

1.3.2.1 Viscosity

Viscosity of DESs is too high as compared to other molecular solvents because of hydrogen bond network between two components (Gabriele *et al.,* 2019). Still, DESs have been significantly used in various methods such as liquid-liquid extraction, gases absorption, and organic synthesis. Hydrogen bond network can be weakened by the addition of water that led to decrease in the viscosity of DESs.

1.3.3 Synthesis applications

DES can be synthesized by combining HBA (ChCl) and HBD (citric acid, glycerol, urea, lactic acid, and mannose). The stability of DES depends on structures of precursors and their molar ratio. In extraction process, DES needs to be stable at different temperature and pressure (Nam *et al.,* 2015).

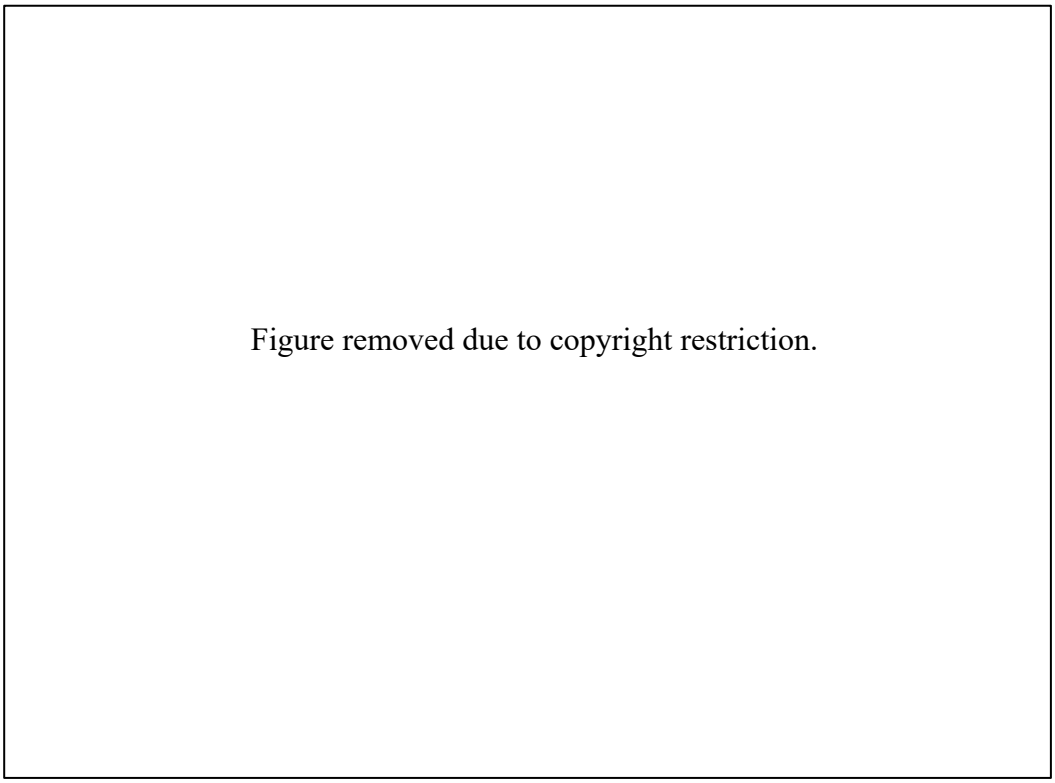

Figure 3. The image shows different methods for formation of NADES (Długosz, 2023).

Different methods are available for synthesis of DESs, as seen Figure 3., such as synthesis of DESs based on stirring and heating, vacuum evaporation, ultrasonic heating, grinding, and microwave-assisted and freeze-drying (Ferreira and Sarraguça, 2024). In the evaporation method, components has been mixed with water and evaporated at 50°C by using rotatory evaporator and resulting solution will place in desiccator with silica gel until it gives constant weight (Dai *et al.,* 2013). In microwave-assisted method, the precursors are placed in closed system where controlled power and temperature maintained. The material reacts with radiation and causes dipole rotation which leads to clash between molecules and between HBA and HBD (Zhu and Hang, 2013). Although, this technique gives higher yield with low energy consumption, but still huge amount of organic solvent needed (Ferreira and Sarraguça, 2024). Freeze drying method is based on freeze and drying aqueous solution and obtain clear viscous solution (Gomez *et al.,* 2018). This method is energy-sensitive but its time-consuming process.

Stirring and heating method involves mixing of two substances placed in vials with stirring bar and heated at 50-90°C until it becomes transparent solution (Dai *et al.,* 2013). In this study, stirring and heating method used for formation of DES as it has simplicity, provides even heat to whole the system, cheaper, and easy to prepare.

1.4 Brown algae used in hydrogel

Brown seaweeds are widely used in medical and food industries as it has rich source of bioactive compounds (Park *et al.,* 2018). Bioactive compounds mostly rely on their chemical, physical and biological properties which can affect wound microenvironment and promote wound healing (Li *et al.,* 2022). It has various biological effects such as anti-oxidative, anti-inflammatory, and antibacterial activities. Thus, the incorporation of bioactive compounds in wound dressing shows novel approach as it can prevent infection, act as an antibacterial agent, and revive tissue generation.

1.5 Bioactive compounds from brown seaweed

Marine algae contain abundant bioactive compounds such as phenolic compounds, fucoxanthin, lipids, vitamins, carotenoids, and polysaccharides (Wu *et al.,* 2023). Those compounds have antioxidant, antidiabetic, anticancer and antimicrobial properties. Marine algae categorised into three groups based on their pigmentation, chemical composition and nutritive: Brown (Phaeophyta), Red (Rhodophyta) and Green (Chlorophyta) (Hakim and Patel, 2020). Among them, brown algae are widely used because it contains bioactive compounds such as phlorotannin, fucoidans and fucoxanthin showing great antibacterial and antioxidant activity (Pádua *et al.,* 2015).

1.5.1 Phlorotannin

Phlorotannin is recognised as group of polyphenols based on phloroglucinol which exist in brown algae. They are available in soluble form or forming complexes within cell walls (Leyton *et al.,* 2016). They are well known for their anti-inflammatory, antibacterial, antidiabetic and antioxidant bioactivities (Montero *et al.,* 2016). Among marine brown algae, mainly *Ecklonia radiata*, *Ishige okamurae, Sargassum thunbergia, Hizikia fusiformis, Undaria pinnatifida* and *Laminaria japonica* contain phlorotannin (Li *et al.,* 2011). This study highlights the potential qualities and benefits of phlorotannin from brown seaweed *E. radiata* which is one of the most abundant species in South Australia.

1.5.2 Properties of phlorotannin

Phlorotannin is formed by polymerization of phloroglucinol (1,3,5- trihydroxybenzene) monomer units and biosynthesized by the polyketide pathway. Phlorotannin can be divided into four subclasses based on its linkages, such as fuhalols and phlorethols, fucols, fucophloroethols, and eckols (Li *et al.,* 2011).

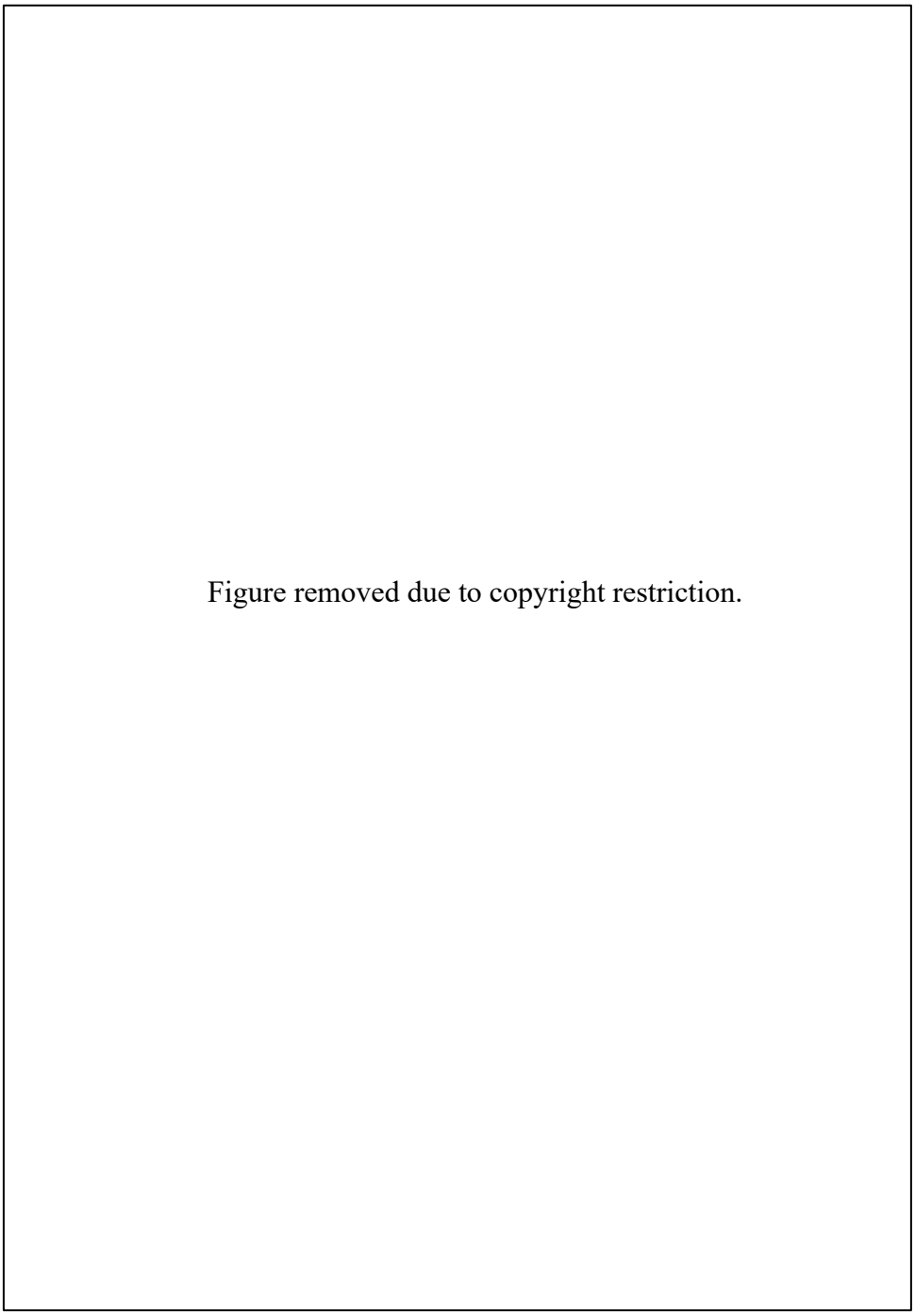

Figure 4. The chemical structures of phlorotannin from brown seaweed: (1) Phloroglucinol, (2) eckol, (3) Fucodiphloroethol G, (4) Phlrofucofuroeckol A, (5) 7-phloroeckol, (6) dieckol, and (7) 6,6'-bieckol (Li et al., 2011).

Phlorotannin is extracted and isolated from brown algae in different compounds. Phlorotannin can fight against bacterial biofilms as they can penetrate bacterial cell wall by making changes

in shape of cell membrane and causing cell death (Shannon and Abu-Ghannam, 2016). In addition to this, phlorotannin can eliminate bacterial reproduction (Pradhan *et al.,* 2022). Phlorotannin has a great antibacterial activity, but it unveils different sensitivity to Gram-positive bacteria and Gram-negative bacteria. It shows less sensitivity against Gram-negative compared to Gram-positive bacteria (Besednova *et al.,* 2020).

1.5.3 Extraction of bioactive compounds from brown seaweed

Extraction is the most critical part to derive bioactive compounds. Additionally, extraction methods, pre-treatment method, operating parameters also crucial to determine the quality and yield of extract (Daud *et al.,* 2022). Organic solvents used for extraction method such as, ethanol, acetone, methanol, and hexane (Bogolitsyn *et al.,* 2024). However, few organic solvents have drawbacks like utilization of large amount of solvent, degradation of oxygen-sensitive compounds and longer extraction time (Hermund *et al.,* 2023).

The main purpose of any extraction method is to recover high yields of targeted compounds from sample matrix. There are several methods to extract bioactive compounds. At first, hydrothermal extraction method is safer, and yield is higher, but it has lower content of active ingredients (Park *et al.,* 2023). Subcritical water extraction (SWE) is green extraction technology that works under critical temperature (374°C) and pressure of water (Zhang *et al.,* 2020). The main advantage of this method is that it has high extraction rate, it is heat sensitive and eco-friendly but still, it has limited industrial application due to high pressure equipment requirement (Mlyuka *et al.,* 2018).

Moreover, variety of extraction methods developed for extraction of bioactive compounds from algae such as supercritical fluid extraction (SFU) where bioactive compounds from brown

algae extracted by using different solubility of substance in supercritical state. It gives high yield and rich phenolic compounds (Sumampouw *et al.,* 2021). Ultra-sound extraction (UAE) method operates ultrasound energy and solvent to extract bioactive compounds (Kumar *et al.,* 2021). Solvent extraction method is used to separate compounds from a solution by extraction in another solvent. Microwave-assisted extraction (MAE) is fast method, and it reduces solvent consumption, but it is complex and laborious (Llompart *et al.,* 2019). Among them, solvent extraction method is used to extract bioactive compounds from *Ecklonia radiata* as its simple method, affordable and gives high yield of bioactive compounds.

1.6 Knowledge gap

There have been studies done on DESs based hydrogel, which gives a data on how DESs based hydrogel promotes wound faster. In one of the studies, they have made hydrogel from NADES and found that this hydrogel has self-healing ability and give long-lasting impact to wound. In addition to this, it provides greater wound protection (Chen *et al.*, 2018). But it does not have enough antibacterial properties. Another study shows that DESs based hydrogel shows 100% wound healing within 9 days. For that reason, this hydrogel represents as a first promising wound dressing which is based on DESs (Wang *et al.,* 2020). However, it has limited antibacterial activity. There is considerable need to develop DESs-based hydrogel with additives to enhance their antibacterial properties.

1.7 Aims and hypothesis

1.7.1 Hypothesis

The overreaching hypothesis of this project is to develop wound dressing hydrogel, which is based on DESs, and additives (bioactive compounds) will have excellent antimicrobial properties.

1.7.2 Aims

Following aims need to be achieved to test the hypothesis,

- **Aim 1:** To synthesize a range of NADESs and NADESs gels and evaluate their antibacterial properties.
- **Aim 2:** To extract and profile natural bioactive compounds from brown seaweed and assess its antibacterial activities.
- **Aim 3:** To fabricate NADES composite gel (NADESs + bioactive compounds) and evaluate their antibacterial properties.

1.8 Significance to Medical Biotechnology

Currently, wound management is a major concern for healthcare as it has substantial risk of infections and life-threating complications. Wound dressings which is available in the market, fails to be effective against bacterial infection such as, semipermeable dressing, plastic film dressing, hydrogels, calcium alginates, foam dressing, hydrocolloids, and paper adhesive tape (Yousefian *et al.,* 2023).

Wound healing includes healing promoters and use of antibacterial agents. However, there is limited evidence-based therapy available for all types of wounds (Monika *et al.,* 2022). Widespread use of antibiotic result in antibiotic-resistance bacteria which affect wound healing (Wright *et al.,* 1998). Antibiotic-resistance bacteria occurs due to overdose of antibiotic which makes bacteria to resistant to antibiotic (Baym *et al.,* 2016). There has been a considerable need to develop multifunctional wound dressing hydrogel which can kill Gram-positive and Gram-negative bacteria.

Furthermore, DESs and bioactive compounds widely recognised for their antibacterial activity and DESs are green solvent, environment friendly, easy to be prepared. Hence, in this study, DESs and bioactive compounds were combined and incorporated into a hydrogel to get excellent antibacterial activity. This hydrogel containing DES and bioactive compounds will be used in biomedical industry for prospective therapy.

2 Materials and methods

2.1 Research facilities and core service

In this study, zone of inhibition, MIC and MBC were done in College of Medicine and Public Health, at Flinders University. All the antibacterial assays were carried out in Physical Containment Two (PC2) facility in College of Medicine and Public Health, Flinders University. The Christ Rotational Vacuum Concentrator (RVC 2-25) and Freeze dryer (Virtis) were used in College of Medicine and Public Health, Flinders University. Additionally, LC-MS (Agilent) was used in College of Medicine and Public Health, Flinders University.

2.2 Materials

The following materials purchased from Sigma Aldrich: choline chloride (ChCl), lactic acid, citric acid, mannose, colistin, and Mueller Hinton broth for antibacterial assays. Vancomycin purchased from Cayman chemical company. Agar Bacteriological Grade carried out from the Chem Supply. Brown algae named *Ecklonia radiata* carried out from the Southern beach, South Australia. Pluronic F-127, Xanthan Gum and Dopamine were purchased from Sigma Aldrich for hydrogel preparation. Beside this, other reagents, or chemicals purchased from chemical supplier.

2.3 Synthesis of DES

DESs have been prepared by the following method: an hydrogen bond acceptor (HBA) choline chloride and hydrogen bond donor (HBD) lactic acid, mannose and citric acid (1:2, 1:1, and 1:2 respectively) were stirred on magnetic stir at 85°C for 1 to 2 hours at optimized molar ratio as mentioned in Table 1**.** until it forms clear solution (Wang *et al.,* 2020). The viscosity may influence the activity of DESs and to measure this different dilution of water (10%, 20%,40% and 50%) has been added to DESs.

Table 1. The table demonstrates the different components, molar ratio and water content which were used in formation of DESs.

Abbreviation	**Component A (Hydrogen Bond Acceptor)**	**Component B (Hydrogen Bond Donor)**	**Component C**	**Water content (%w/v)**	**Molar ratio**
DES 1	ChCl	Lactic acid	-	0	1:2
DES 2	ChCl	Lactic acid	Water	10	1:2:0.3
DES 3	ChCl	Lactic acid	Water	20	1:2:0.6
DES 4	ChCl	Lactic acid	Water	40	1:2:1.2
DES 5	ChCl	Lactic acid	Water	50	1:2:1.5
DES 6	ChCl	Mannose	-	0	1:1
DES 7	ChCl	Mannose	Water	10	1:1:0.2
DES 8	ChCl	Mannose	Water	20	1:1:0.4
DES 9	ChCl	Mannose	Water	40	1:1:0.8
DES 10	ChCl	Mannose	Water	50	1:1:1

DES 11	ChCl	Citric acid	-	0	1:2
DES 12	ChCl	Citric acid	Water	10	1:2:0.3
DES 13	ChCl	Citric acid	Water	20	1:2:0.6
DES 14	ChCl	Citric acid	Water	40	1:2:1.2
DES 15	ChCl	Citric acid	Water	50	1:2:1.5

2.4 Preparation of DES-based Hydrogel

2.4.1 Pluronic F-127 gel

DES-based hydrogel made by mixing 1.9g of Pluronic F-127 with 7.1 mL cold distilled water (5-10°C), and 1.0 mL propylene glycol. Later, the following formulation kept in refrigerator at 4°C overnight to ensure complete dissolution of Pluronic F-127 (Haidari *et al.,* 2020).

2.4.2 Xanthan gel

Xanthan gel prepared by 200 mg of Xanthan gum with 10% of water. The mixture continues stirred and heated at 80-85°C until Xanthan gum completely dissolved in water and formed gel (Kang *et al.*, 2019) .

2.5 Extraction of bioactive compounds from *Ecklonia species*

Brown algae *Ecklonia radiata* used to extract bioactive compounds. As seen in Figure 5**.,** 2500mg of brown algae mixed with 50 mL of 70% ethanol and solution stirred at 40°C for 1 hour and centrifuged at 3200 rpm for 20 min. Afterwards, same extraction procedure done with pellet and combined with the supernatant. The vacuum centrifugation has been used to evaporate the ethanol from the sample solution and the extract has been resuspended in water. The freeze-dry used to get bioactive compounds (Bogolitsyn *et al.*, 2024).

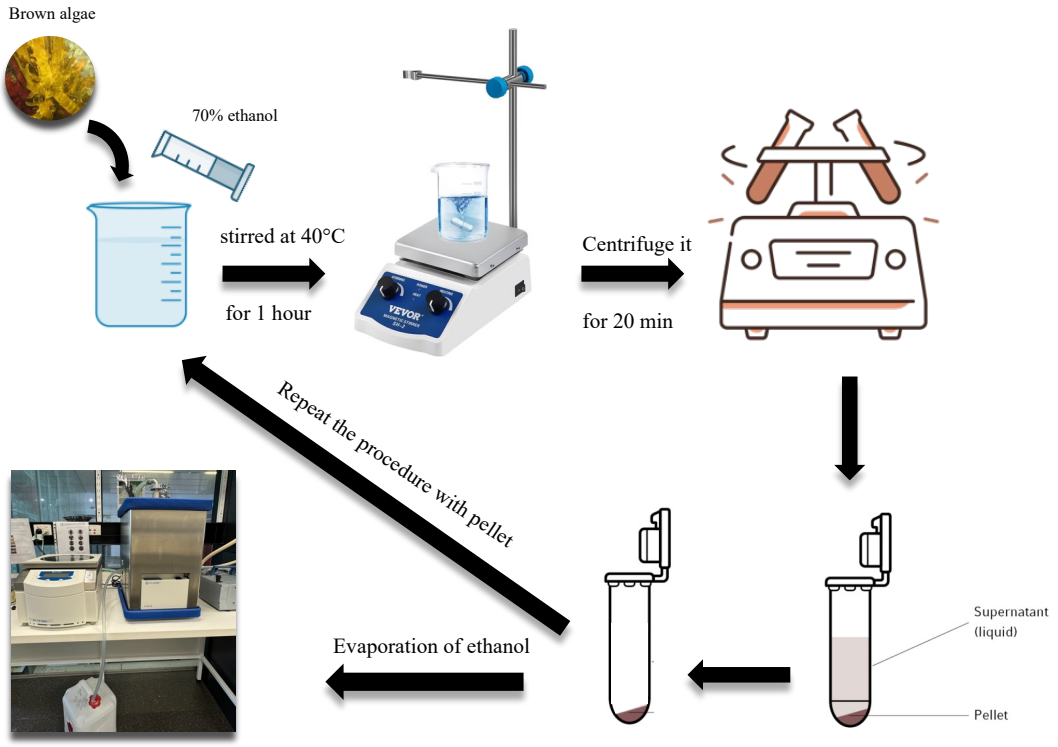

Figure 5. The image describes how bioactive compounds extracted from the brown algae by using solvent extraction method.

2.6 Sample preparation for Liquid Chromatography-Mass Spectrometry (LC-MS)

One mg of freeze-dried extract was dissolved in 50% methanol. After centrifugation at 3200 rpm for 10 min, 200 µL of supernatant is transferred to HPLC vials for LC-MS analysis. The phlorotannin compounds exist in the seaweed extracts were recognized by ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (Agilent 6540 Quadrupole Time-of-Flight (QTOF) mass spectrometer; Agilent Technologies, Santa Clara, CA, U.S.A.) (Clayton-Cuch *et al.*, 2024). In HPLC system, mobile phase involved 0.1% formic acid as solvent A and 90% acetonitrile in 0.1% formic acid as solvent B. It was suited with Agilent Zorbax Eclipse C18 column. The phenolic compounds separated by 2% from solvent B between 0 to 1 min, followed by 2%-30% of solvent B between 1 to 16 minutes. It increased

during 30% to 100% of solvent B during 16 to 18 minutes. At last, it risen to the 100% during 18-19 min and come back to 2% of solvent B between 19 to 21 minutes. Once separation completed, the column re-equilibrated 2% of solvent B for 5 minutes. The separation rate was 0.3 mL/min, and the column temperature was 40°C. The MS mode has been used for compound screening.

2.7 Antibacterial assays

2.7.1 Preparation of media

To prepare Mueller Hinton Broth (MHB), 21g of MHB powder dissolved in 1L Milli-Q water. Mixed it well and sterilized it in autoclave. To prepare Muller Hinton Agar, took 20 g of agar bacteriological grade and 21 g of Mueller Hinton Broth (MHB) and dissolved it in 1L Milli-Q water. Mixed it well and sterilized it in autoclave. Afterwards, pour the liquid in petri plates and waited until it solidifies.

2.7.2 Maintenance of culture and bacterial strain

S. aureus and *P. aeruginosa* were taken from the -80°C. One loopful suspension was streaked on Muller Hinton Agar plate and incubated it for 24 hours at 37°C in incubator. Next day, one colony has been suspended in 10 mL of sterilized MHB and incubate it for 18 hours at 37°C in shaking incubator. The bacterial culture which was incubated that has been diluted five-fold times to get an optical density 1×10^7 CFU/mL. The optical density (OD) has been measured at OD600nm.

2.7.3 Well diffusion assay

S. aureus and *P. aeruginosa* were inoculated in MHB overnight. Then, bacterial cultures were diluted to 1×10^8 CFU/mL. Afterwards, 100µL of bacterial strain pour onto MHA (Mueller Hinton Agar) plates and evenly spread by using sterile cotton swab. The 5 mm diameter of wells were punctured by using sterile cork borer. Then, 50µL of positive control, negative control and test sample has been added to each well. Incubate the plates for 24 hours at 37°C. The diameter of the zone of inhibition produced by sample measured in mm scale and recorded (Alsaud *et al.,* 2021). Well diffusion assay done by using triplicates (n=3).

2.7.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration (MIC_{50}) and Minimum Bactericidal Concentration (MBC) of test sample has been identified by serial dilution method and CFU count method (Holla *et al.,* 2012). *S. aureus* and *P. aeruginosa* were diluted to 1×10^7 CFU/mL at OD600nm. 95µL of MHB has been loaded in each well of 96-well plate and in first well 95µL of test sample has been added. Then, the sample has been two-fold serially diluted. At the end, 5µL of prepared bacterial culture has been added to each well and the final concentration of bacteria was 5×10^5 CFU/mL. Later, put it in shaking incubator for 24 hours at 37°C (Akbar *et al.,* 2023). Next day, the serially diluted sample were plated on MHA plates for MBC and incubated for CFU count method. Test has been done in triplicates (n=3).

2.8 Statical analysis

The data were expressed in mean ± standard deviation with three replicates. One-way ANOVA and Tukey test has been used to compare exposure groups by using GraphPad. Statical significance were * P < 0.05, ** P <0.01, *** P < 0.001, **** P < 0.0001.

3 Results and discussion

3.1 Preparation of different DESs

DESs have been prepared by mixing HBD (choline chloride) and HBA (mannose, lactic acid, or citric acid) with constant stirring at 85°C until it becomes clear and uniform solution (Figure 6). Several types of DESs have been prepared at different concentration and different molar ratios (Table 1**)**. The viscosity may influence the activity of the compounds and to measure this, DESs have been prepared in different dilutions of water to vary the viscosity.

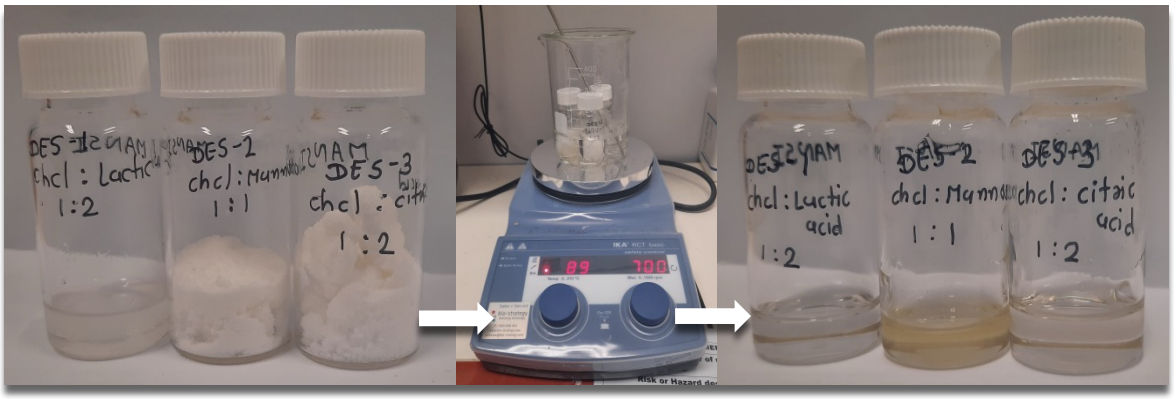

Figure 6. The image shows how DESs have been formed by constant stirring and heating at 85 °C for approximately 1 hour.

3.2 Antimicrobial study

3.2.1 Well Diffusion Assay

The antibacterial activity of prepared DESs have been seen by well diffusion assay. Choline choride:Lactic acid (ChCl:LA) showed as 35.33 ± 0.58 mm and 29.67 ± 1.53 mm higher antibacterial activity as compared to the other DESs against *S. aureus* and *P. aeruginosa*. ChCl:LA with 10% H_2O showed 32 ± 0 mm and 28.33 ± 1.15 mm of zones against *S. aureus* and *P. aeruginosa,* respectively. But there was notable change in ChCl:LA with 20% H_2O as it had better (33.67 ± 1.53 mm and 28.67 ± 0.58 mm) antibacterial activity compared to ChCl:LA with 10% H_2O against both bacteria. The reason for that is ChCl:LA without water or with low water content was too viscous to be able to freely diffuse into media. The zone of inhibition of ChCl:LA with 50% H_2O was smallest as seen in Figure 21**,** revealing that with increasing water content of DES, the antibacterial activities decrease for both *S. aureus* and *P. aeruginosa* bacteria.

Choline chloride:Mannose showed no antimicrobial activity against any of the tested microorganisms (Figure 22). This might be due to ChCl:Mannose solvent was too viscous to diffuse into agar media and mannose may not be organic acid in term of antibacterial activities. This result indicates that solvent had weak permeability towards the bacterial outer layer, which cause decrease in accumulation inside bacteria. Thus, ChCl:Mannose was not used in further experiments.

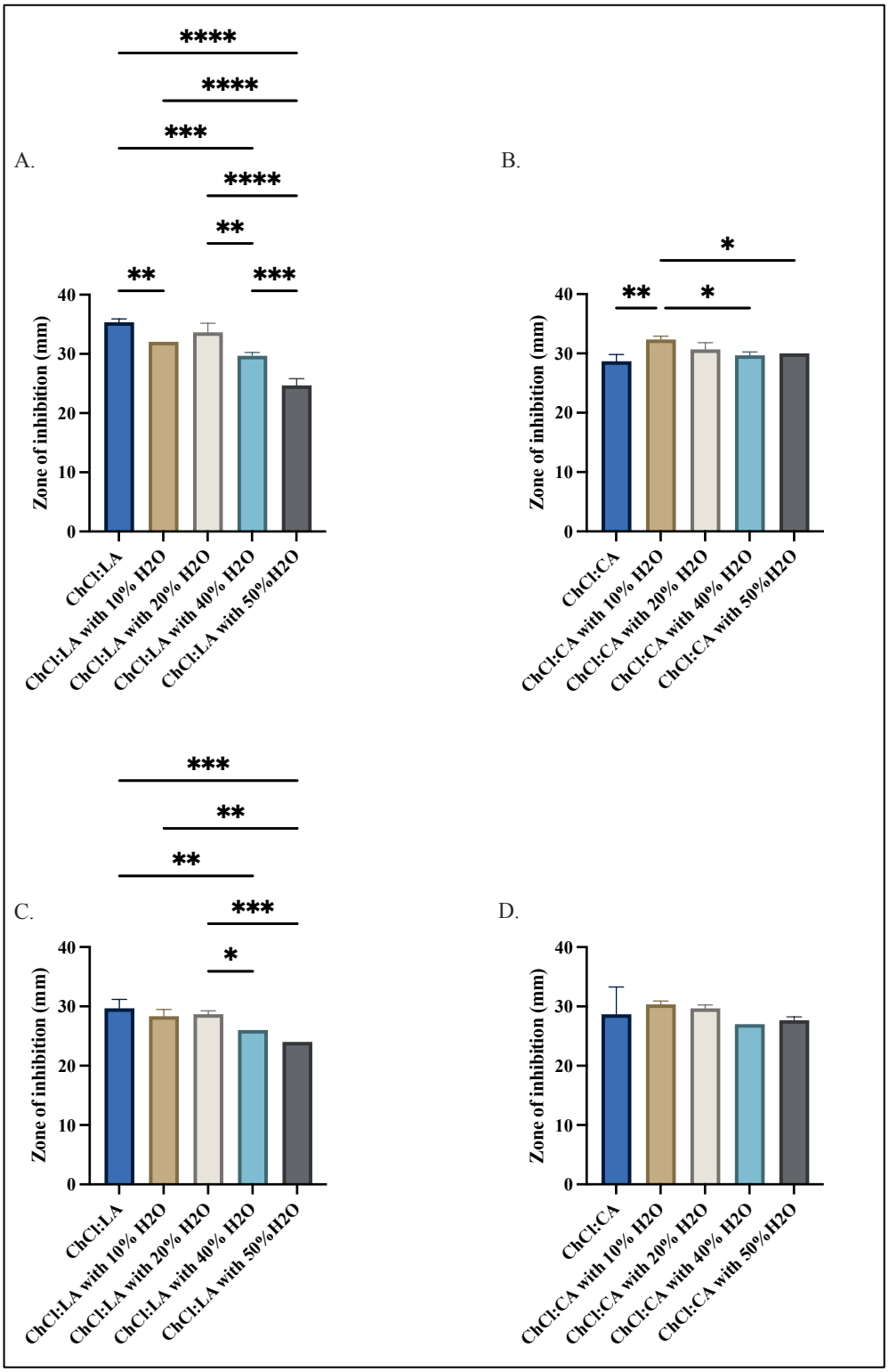

Figure 7. The zone of inhibition (mm) of different DESs A) ChCl:LA (with 10%,20%,40% and 50% H_2O concentrations) against S. aureus; B) ChCl:CA (with 10%,20%,40% and 50% H_2O concentrations) against S. aureus; C) ChCl:LA (with 10%,20%,40% and 50% H_2O concentrations) against P. aeruginosa; D) ChCl:CA (with 10%,20%,40% and 50% H_2O

*concentrations) against P. aeruginosa. (mean ± standard deviation, n=3) (Statistical analysis done by one-way ANOVA * P < 0.05, ** P <0.01, *** P < 0.001, **** P < 0.0001.)*

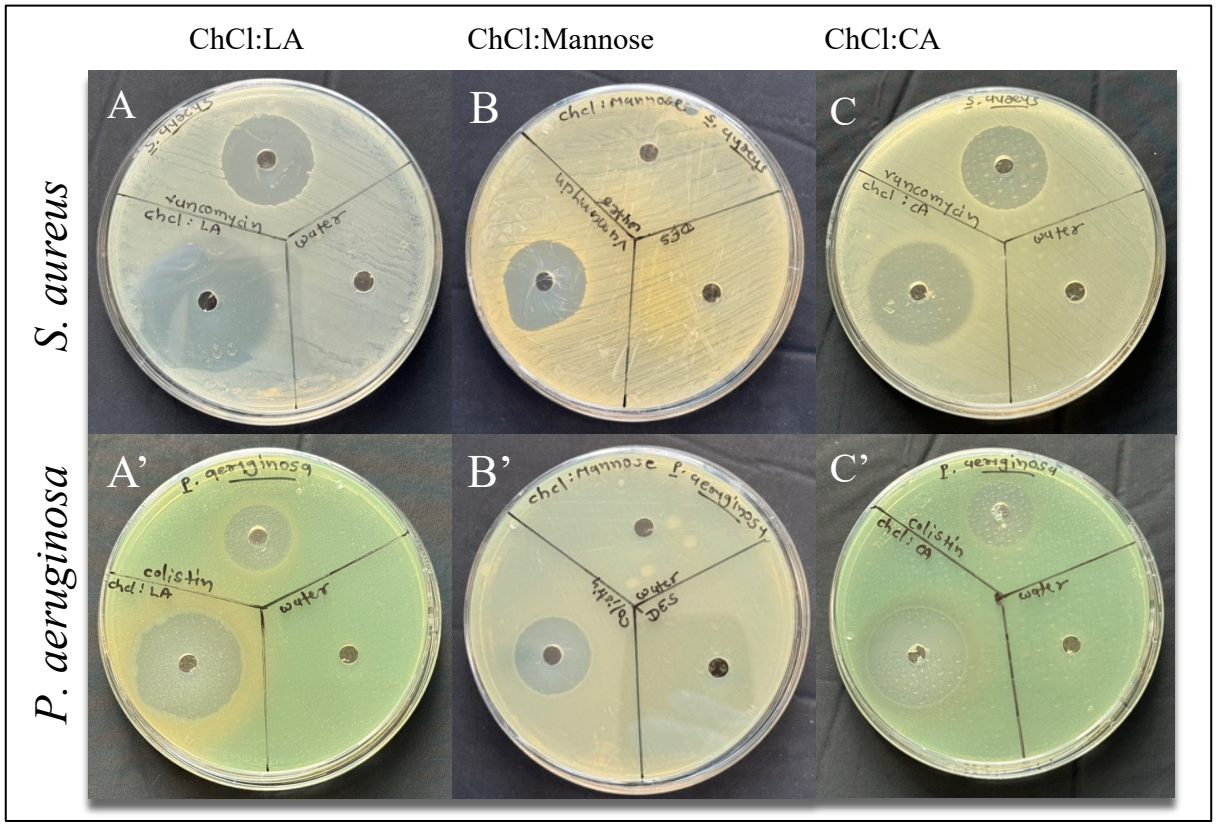

Figure 8. The zone of inhibition of DESs without water against S. aureus and P. aeruginosa bacteria (A, A') ChCl:LA (B, B') ChCl:Mannose (C, C') ChCl:CA (mean ± standard deviation, n=3).

Choline chloride: citric acid had higher zone of inhibition (28.67 ± 1.15 mm and 28.67 ± 4.62) as compared to ChCl:CA with 10% H_2O (32.33 ± 0.58 mm and 30.33 ± 0.58 mm) against *S. aureus* and *P. aeruginosa*. There was slight difference between ChCl:CA with 20% H_2O (30.67 ± 1.15 mm) and ChCl:CA with 40% H_2O (29.67 ± 0.58 mm) against *S. aureus* whereas against *P. aeruginosa,* ChCl:CA with 20% H_2O showed 29.67 ± 0.58 mm and ChCl:CA with 40% H_2O showed 27 ± 0 mm, respectively. ChCl:CA with 50% H_2O showed 30 ± 0 mm and 27.67 ± 0.58 mm against *S. aureus* and against *P. aeruginosa.* As seen in Figure 23, the zone of

inhibition of ChCl:CA was not clear and bigger as compared to ChCl:LA. So, the results clearly indicate that ChCl:LA had an excellent antibacterial activity as compared to ChCl:Mannose and ChCl:CA against both Gram-positive bacteria and Gram-negative bacteria.

3.2.2 MIC and MBC

MIC and MBC of DESs with both *S. aureus* and *P. aeruginosa* pathogens mentioned in Figure 9**.** As per the MIC results, Choline chloride:lactic acid (ChCl:LA) had excellent antimicrobial activities against *S. aureus* and *P. aeruginosa* with a MIC of 0.39% v/v. ChCl:LA with 10% H_2O, with 20% H_2O and with 40% H_2O showed similar antimicrobial activity against both bacteria (for reference the fold-change compared to 100% mentioned in Figure 24**.**), whereas ChCl:LA with 50% H_2O showed antimicrobial activity at a MIC of 0.39% v/v against both Gram-positive bacteria and Gram-negative bacteria. The results indicate that optimal antibacterial effect was constants even after 10%, 20%, 40% and 50% dilution with H_2O. The antibacterial activity remained same after changing the viscosity. So, water did not make any significant effect to DES.

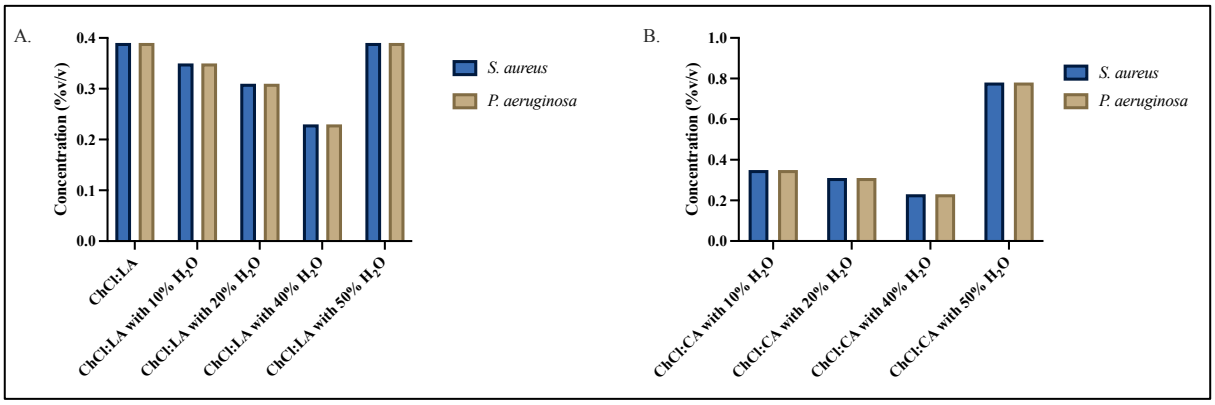

Figure 9. The MIC of DESs against S. aureus and P. aeruginosa. A) ChCl:LA at different concentration of H_2O and B) ChCl:CA at different concentration of H_2O (n=3) (standard error is 0).

Table 2. The table demonstrates the MIC and MBC of DESs against S. aureus and P. aeruginosa bacteria.

Sample	***S. aureus* (ATCC 25923)**		***P. aeruginosa* (ATCC 15442)**	
	MIC (% v/v)	MBC (% v/v)	MIC (% v/v)	MBC (% v/v)
ChCl:LA	0.39	0.78	0.39	0.78
ChCl:LA with 10% H_2O	0.35	0.70	0.35	0.70
ChCl:LA with 20% H_2O	0.31	0.63	0.31	0.63
ChCl:LA with 40% H_2O	0.23	0.47	0.23	0.47
ChCl:LA with 50% H_2O	0.39	0.78	0.39	0.78
ChCl:CA with 10% H_2O	0.35	0.70	0.35	0.70
ChCl:CA with 20% H_2O	0.31	0.63	0.31	0.63
ChCl:CA with 40% H_2O	0.23	0.47	0.23	0.47
ChCl:CA with 50% H_2O	0.78	1.56	0.78	1.56

Moving forward to choline chloride:citric acid (ChCl:CA), this solvent was too viscous to absorb through the pipette tips and small volume required for MIC, so it was hard to use this solvent in MIC. Therefore, different concentration of water used (10%, 20%, 40% and 50%) to determine MIC and MBC of ChCl:CA. ChCl:CA with 10% H_2O showed good antimicrobial activity (MIC = 0.35% v/v) against *S. aureus* and against *P. aeruginosa.* Meanwhile, ChCl:CA with 20% H_2O (MIC = 0.31% v/v) and ChCl:CA with 40% H_2O (MIC = 0.23% v/v) also showed similar antimicrobial activity against both bacteria (the fold-change compared to 100% mentioned in Figure 24**.** for reference), whilst ChCl:CA with 50% H_2O showed the lower antimicrobial activity with a MIC of 0.78% v/v against both the Gram-positive bacteria and Gram-negative bacteria. As shown in Figure 24, it has a smooth curve i.e., the antibacterial

activity initially increased with added water, up to optimal 40%, and then decreases with additional water. The increase was due to changing the initial viscosity, and then decrease was because ChCl:CA with 50% H_2O became too dilute to have significant effect. Among all DESs, ChCl:LA with 50% H_2O was selected for the further experiments as it was less viscous, easy to prepare and had excellent antibacterial activity against both bacteria.

3.3 Preparation of NADES-gel

The hydrogel was characterised for transitions of temperatures and mechanical properties. Pluronic F-127 hydrogel was completely dissolved in water and formed transparent clear solution after overnight freezing. When Pluronic F-127 hydrogel kept at 4°C, it was in liquid form but at room temperature it forms a hydrogel as seen in Figure 10**.**

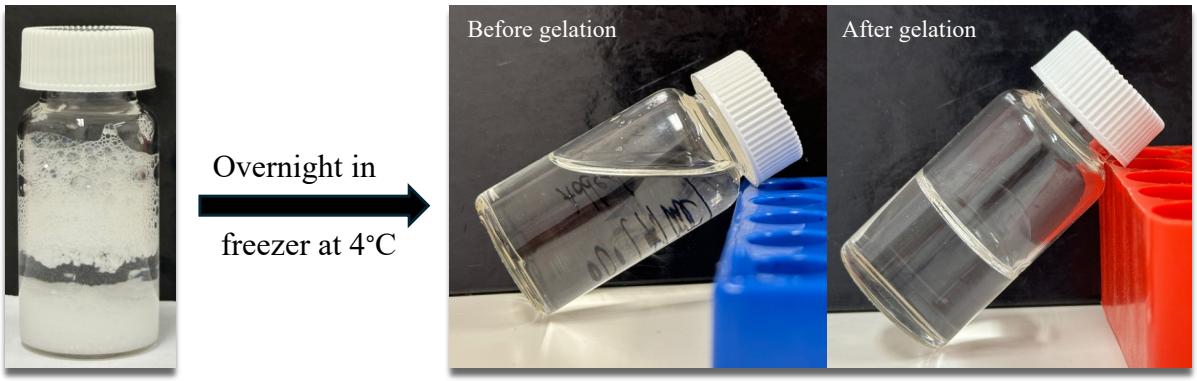

Figure 10. The image of formation of Pluronic F-127 gel by overnight freezing at 4 °C. The gel was in liquid form but at room temperature it solidified as gel.

The hydrogel formed by gelling agents with choline chloride-lactic acid (ChCl:LA). The different concentration of choline chloride-lactic acid (ChCl:LA) used in hydrogel to enhance antibacterial activity (10%, 50% and 90% of ChCl:LA used in hydrogel). The result shows variation in formation of hydrogel with ChCl:LA at different concentration. Pluronic F-127

with 10% ChCl:LA showed clear and uniform mixture and at room temperature it forms gel but by adding 50% ChCl:LA, it formed the gel at lower temperature. In contrast, the result shows that by adding 90% ChCl:LA in gel, Pluronic F-127 and ChCl:LA both were unable to completely dissolve in solution (Figure 25). It indicates that hydrogen bonding between ChCl:LA and Pluronic F-127 crosslinking so result in insoluble ChCl:LA in gel. Furthermore, to determine which component was responsible for antimicrobial activity, just 50% lactic acid and 50% choline chloride added to Pluronic F-127. and result mentioned in Figure 13**.**

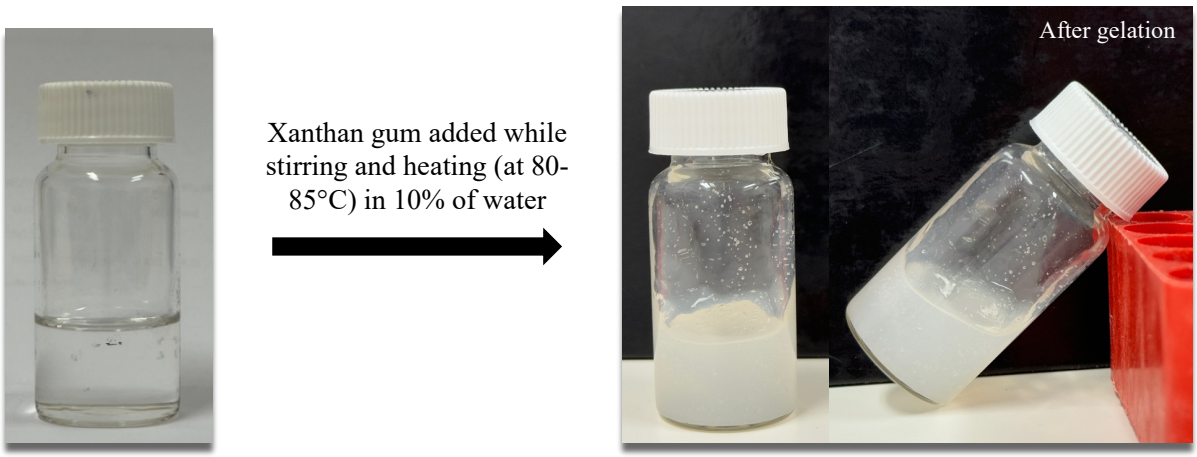

Figure 11. The image of formation of Xanthan gel by continuous stirring and heating at 80-85 ℃.

Xanthan based hydrogel was not transparent, but it was in gel formed after stirring and heating. To prepare Xanthan gum-based hydrogel, 10% of water added in total volume of gel and 200 mg Xanthan gum added while continuous string and heating (Figure 11). To add ChCl:LA in gel, 50% of water and 50% of ChCl:LA used and same procedure used in preparation of other gel (10% and 90%). As seen in Figure 26**,** adding different concentration of ChCl:LA made Xanthan hydrogel more viscous. Still, ChCl:LA and Xanthan completely dissolved in gel. Additionally, to check who has antibacterial activity from ChCl:LA, just 50% lactic acid and 50% choline chloride added in Xanthan gel and result mentioned in Figure 14**.**

3.4 Well diffusion assay for both gels

The antibacterial activity of Pluronic F-127 and Xanthan hydrogel seen by well diffusion assay. As seen in Figure 12 and Figure 14**.,** Pluronic F-127 and Xanthan itself showed no antibacterial activity but by adding DES (ChCl:LA) in hydrogel showed antimicrobial activity.

The hydrogel without ChCl:LA (only Pluronic F-127 and Xanthan) has polymeric arrangements. It shows swelling capacity in water and holds >20% water without dissolving water. Moreover, Pluronic F-127 and Xanthan are gelling agent which helps to form a gel. In case of DES-based hydrogel, they are influenced by properties of DES. By adding excess amount (90%) of ChCl:LA, Pluronic F-127 and ChCl:LA were unable to dissolve in gel (Figure 25).

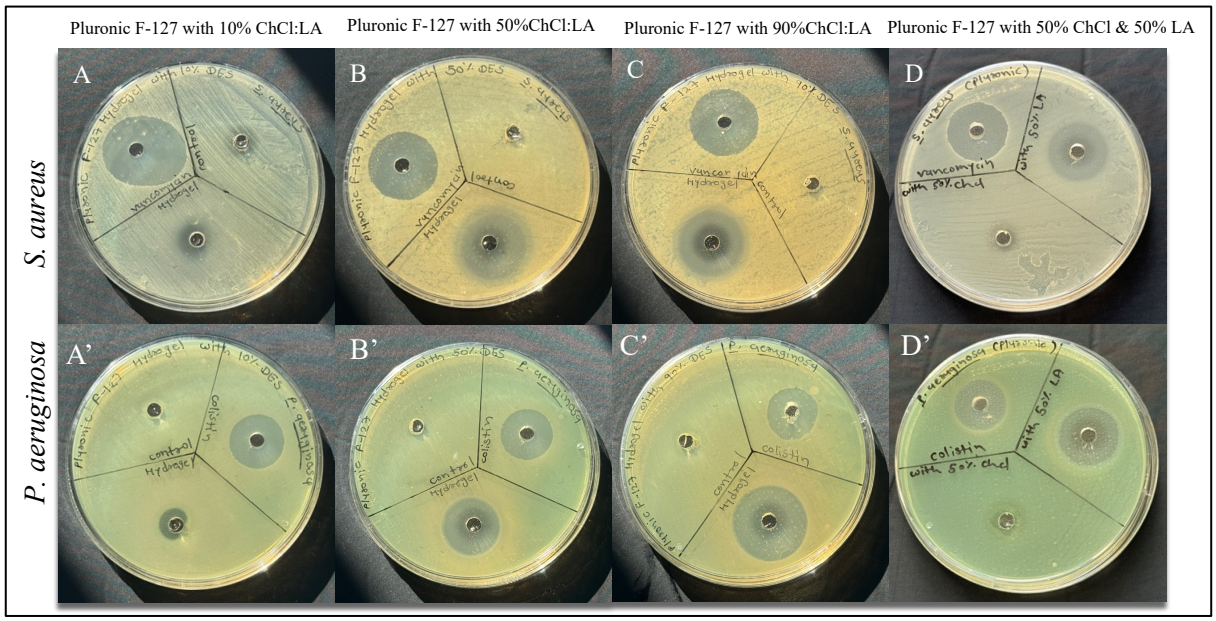

Figure 12. The zone of inhibition of Pluronic F-127 with ChCl:LA against S. aureus and P. aeruginosa bacteria (A,A') Pluronic F-127 with 10% ChCl:LA (B,B') Pluronic F-127 with 50% ChCl:LA (C,C') Pluronic F-127 with 90% ChCl:LA and; (D,D') Pluronic F-127 with

50% ChCl and with 50% LA (mean ± standard deviation, n=3) (as a control just Pluronic F-127 hydrogel used to compared it with ChCl:LA).

By adding 10% ChCl:LA in Pluronic F-127 showed 9.34 ± 0.58 mm and 9.67 ± 0.58 mm against *S. aureus* and *P. aeruginosa,* respectively. Pluronic F-127 with 50% ChCl:LA showed good antibacterial activity with *S. aureus* 23 ± 1 mm and with *P. aeruginosa* 19 ± 0.58 mm. Pluronic F-127 with 90% ChCl:LA showed excellent antibacterial activity as compared to others as it had 25 ± 0 mm of zone against both bacteria but ChCl:LA was not fully dissolved in Pluronic F-127 hydrogel. Therefore, this was not used in further experiment. The results indicate that Pluronic F-127 with 50% ChCl:LA had good antibacterial activity. To determine which component was responsible for this activity, just 50% lactic acid and 50% choline chloride added to Pluronic F-127. The result showed that choline chloride had no antimicrobial activity, but Lactic acid had excellent activity as seen in Figure 12**.** The 50% Lactic acid in Pluronic F-127 showed 20.33 ± 0.58 mm of zones against *S. aureus* and *P. aeruginosa.*

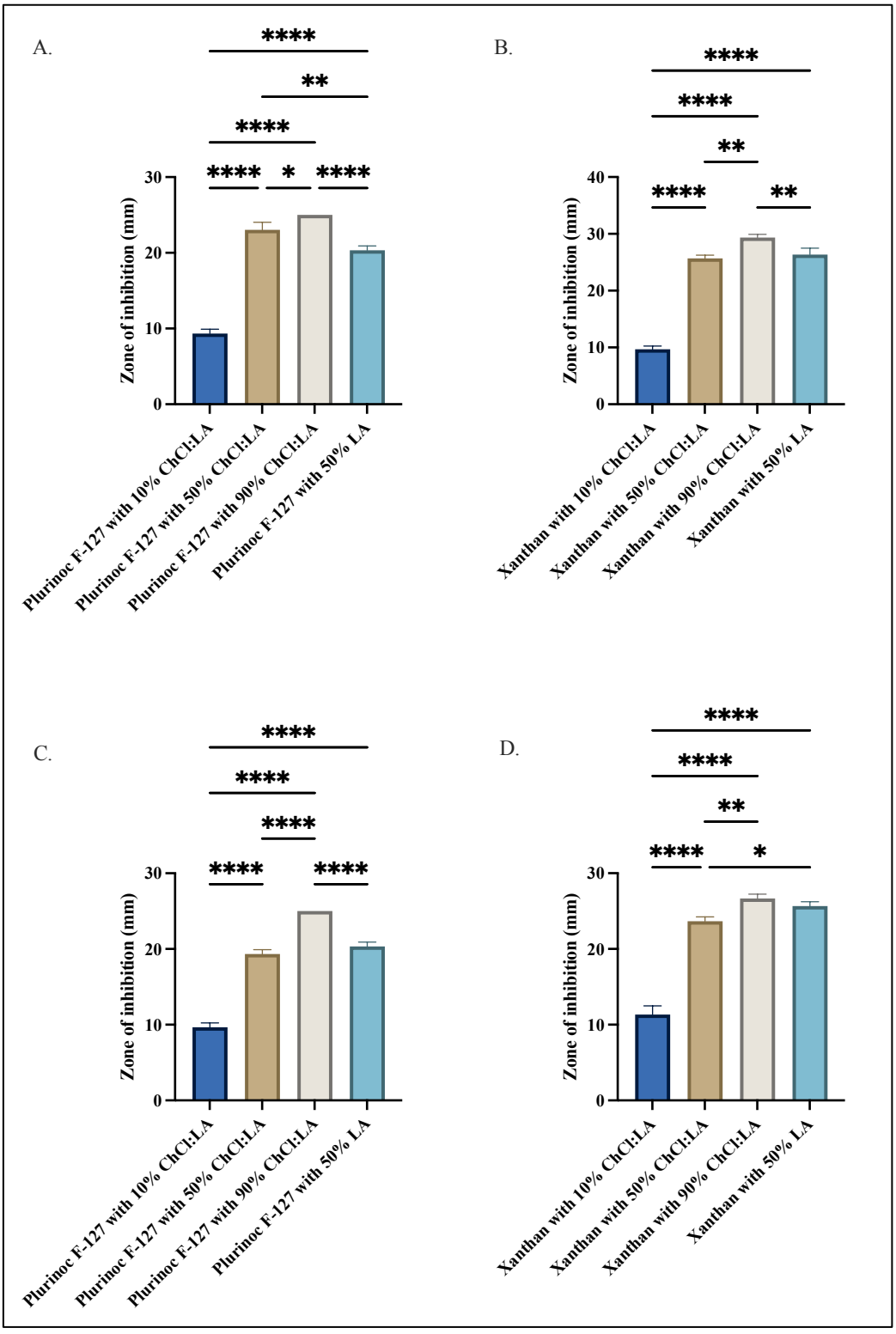

Figure 13. The zone of inhibition of Pluronic F-127 and Xanthan with ChCl:LA A) Pluronic F-127 with ChCl:LA (at 10%, 50% and 90% concentration) and 50% LA against S. aureus; B) Xanthan with ChCl:LA (at 10%, 50% and 90% concentration) and 50% LA against S. aureus; C) Pluronic F-127 with ChCl:LA (at 10%, 50% and 90% concentration) and 50% LA

*against P. aeruginosa; D) Xanthan with ChCl:LA (at 10%, 50% and 90% concentration) and 50% LA against P. aeruginosa. (mean ± standard deviation, n=3) (Statistical analysis done by one-way ANOVA * P < 0.05, ** P <0.01, *** P < 0.001, **** P < 0.0001.)*

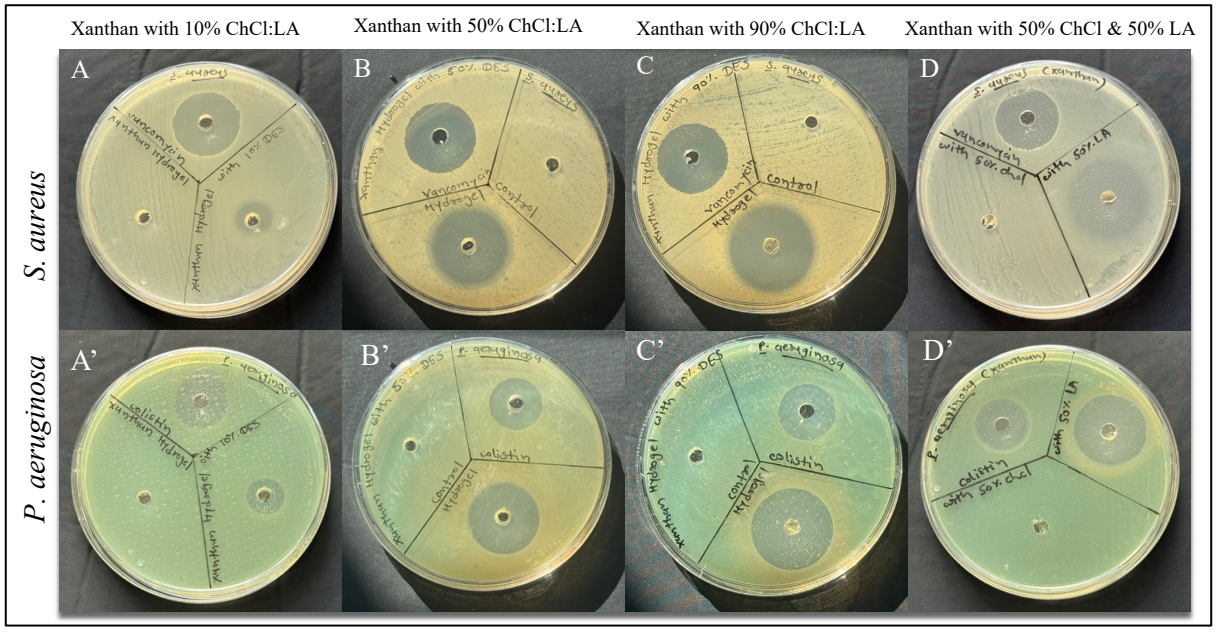

Figure 14. The image shows the zone of inhibition of Xanthan with ChCl:LA against S. aureus and P. aeruginosa bacteria (A,A') Xanthan with 10% ChCl:LA (B,B') Xanthan with 50% ChCl:LA (C,C') Xanthan with 90% ChCl:LA and (D,D') Xanthan with 50% ChCl and with 50% LA (mean ± standard deviation, n=3) (as a control just Xanthan hydrogel used to compared it with ChCl:LA).

Xanthan with 10% ChCl:LA showed 9.67 ± 0.58 mm and 11.33 ± 1.15 mm size of zone with *S. aureus* and *P. aeruginosa* whilst with 50% ChCl:LA, it had great antibacterial activity as it showed 25.67 ± 0.58 mm and 23.67 ± 0.58 mm zone against *S. aureus* and *P. aeruginosa*. Besides this, 90% ChCl:LA in Xanthan showed excellent antibacterial activity as compared to others as it had 29.33 ± 0.58 mm and 26.67 ± 0.58 mm zones against both Gram-positive and Gram-negative bacteria. To determine which component was responsible for this activity, just 50% lactic acid and 50% choline chloride added to the Xanthan hydrogel. The result showed

that choline chloride had no antimicrobial activity, but lactic acid had excellent activity as mentioned in Figure 14**.** The 50% lactic acid in Xanthan showed 26.33 ± 1.15 mm and 25.67 ± 0.58 mm of zones against *S. aureus* and *P. aeruginosa.* The results states that 90% ChCl:LA in Xanthan had excellent antibacterial activity but to compare Xanthan hydrogel with Pluronic F-127, 50% ChCl:LA was used in both gel for further experiments.

3.5 Profiling of bioactive compounds by using LC-QTof

The yield of methanol extract from brown algae was around 30.4 ± 2% (w/w). The composition of phlorotannin was analysed by using LC-QTof as shown in Figure 15 and Table 3**.** Fifteen phlorotannins were identified based on their accurate mass and database. The overall score of all identified compounds in database search is over 60, and the difference between theoretical mass and experimental mass is less than 5ppm, indicating good mass accuracy in the experiments and high confidence in compound assignments. All these 15 compounds belong to eckol-type phlorotannin, which has been reported to be rich in *Ecklonia* species (Okeke *et al.,* 2021). Figure 15**.** shows the top four abundant phlorotannins found in extract, which are eckol, eckmaxol I, dieckol II, and eckmaxol II. All these compounds have been reported to show excellent antimicrobial activities (Nagayama *et al.,* 2002).

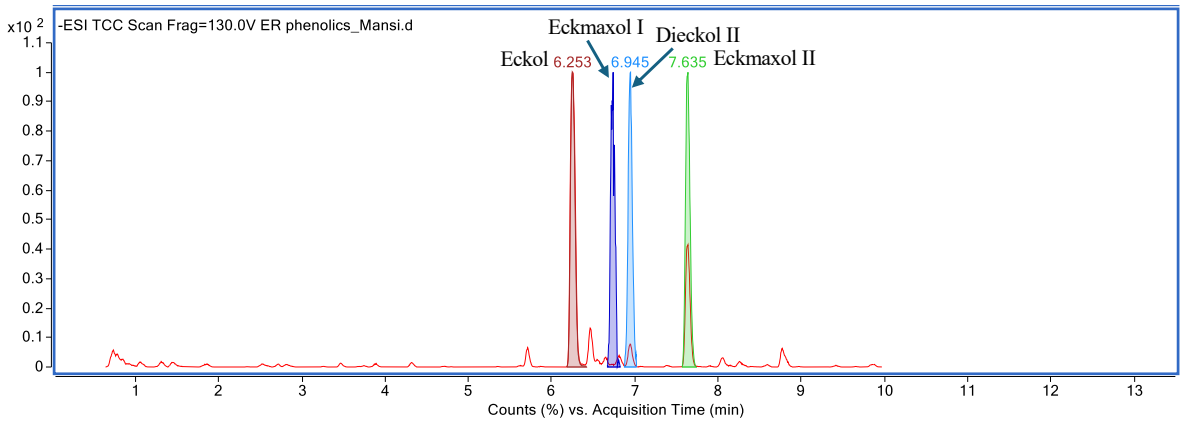

Figure 15. Extract ion chromatograph of top 4 abundant phlorotannin in methanol extract of E. radiata.

Table 3. Profile of phlorotannin in methanol extract from E. radiata seaweed.

No	Compound name	Formula	m/z	Experimental Mass	Theoretical Mass	Diff (DB, ppm)	Overall score
1	Fucodiphlorethol I	$C_{24}H_{18}O_{12}$	497.0714	498.0789	498.0798	-1.95	94.51
2	Fucophloroethol II	$C_{18}H_{14}O_{9}$	373.0556	374.0628	374.0638	-2.53	95.34
3	Fucodiphlorethol III	$C_{24}H_{18}O_{12}$	497.0715	498.0787	498.0798	-2.25	88.05
4	Eckol	$C_{18}H_{12}O_{9}$	371.0402	372.0474	372.0481	-1.92	98.18
5	Phloroeckol I	$C_{24}H_{16}O_{12}$	495.0555	496.0625	496.0642	-3.36	61.26
6	Phloroeckol II	$C_{24}H_{16}O_{12}$	495.0558	496.063	496.0642	-2.34	96.81
7	Eckmaxol I	$C_{36}H_{24}O_{18}$	743.0869	744.0942	744.0963	-2.79	93.2
8	Dieckol I	$C_{36}H_{22}O_{18}$	741.0714	742.0788	742.0806	-2.47	94.03
9	Dieckol II	$C_{36}H_{22}O_{18}$	741.0713	742.0786	742.0806	-2.77	94.37
10	Bifuhalol	$C_{12}H_{10}O_{7}$	265.0347	266.0419	266.0427	-2.86	83.17
11	Eckmaxol II	$C_{36}H_{24}O_{18}$	743.0878	744.0949	744.0963	-1.77	97.74
12	Dieckol III	$C_{36}H_{22}O_{18}$	741.0716	742.0788	742.0806	-2.45	95.94
13	Dioxinodehydroeckol	$C_{18}H_{10}O_{9}$	369.0241	370.0314	370.0325	-2.88	78.46
14	Dieckol IV	$C_{36}H_{22}O_{18}$	741.0723	742.0795	742.0806	-1.55	67.03
15	Phlorofucofuroeckol A	$C_{30}H_{18}O_{14}$	601.0608	602.068	602.0697	-2.77	89.86

3.6 Antibacterial assay for extract

3.6.1 Well diffusion assay

The antibacterial activity of extract has been seen by well diffusion assay. As shown in Figure 16**,** it did not show any zones against any of bacteria at 64 mg/ml concentration. This might be due to low concentration of extracted bioactive compounds. The bioactive compounds obtained from *E. radiata* shows 10.33 ± 1.15 mm of zone of inhibition at 128 mg/ml concentration against *S. aureus* whereas it did not show any antibacterial activity against *P. aeruginosa.* With 256 mg/ml, it showed good antimicrobial activity as zone size increased up to 12.33 ± 0.58 mm and 8.33 ± 1.15 mm against both bacteria (Figure 27).

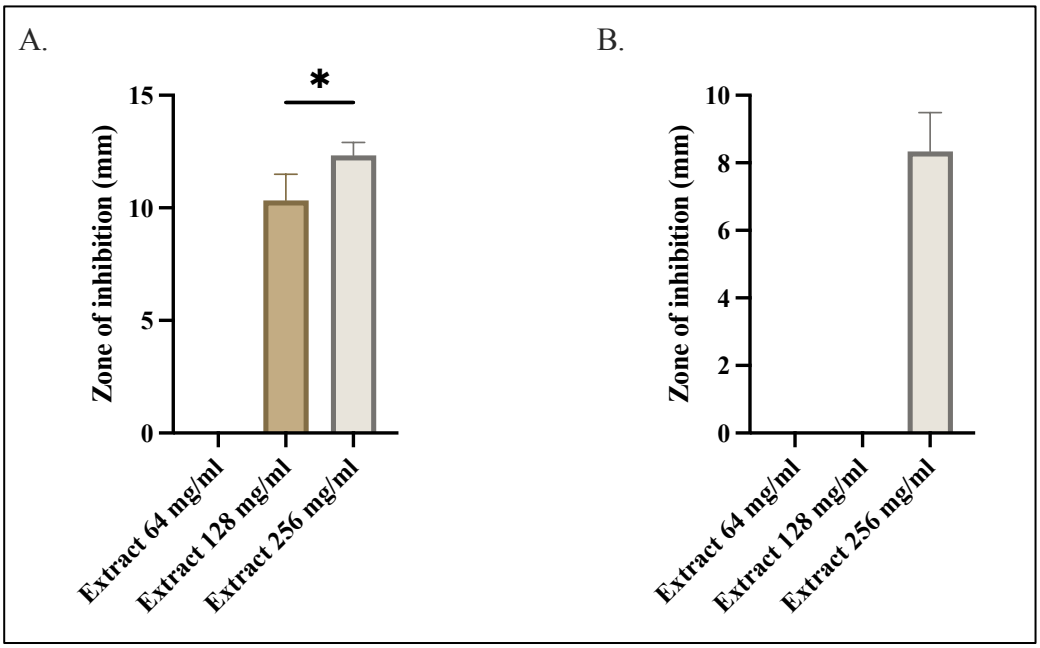

*Figure 16. The zone of inhibition of extract at different concentrations: 64 mg/ml, 128 mg/ml, and 256 mg/ml A) shows against S. aureus and B) shows against P. aeruginosa bacteria. (mean ± standard deviation, n=3) (Statistical analysis done by one-way ANOVA * P < 0.05.)*

3.6.2 MIC and MBC

According to MIC result, MIC of extract was 2 mg/ml against *S. aureus* whereas it showed 4 mg/ml against *P. aeruginosa* bacteria (Figure 17). It says that extracted bioactive compounds had a significant antibacterial activity against Gram-positive bacteria *S. aureus* as compared to Gram-negative bacteria *P. aeruginosa.* As mentioned earlier, it was less sensitive to Gram-negative bacteria.

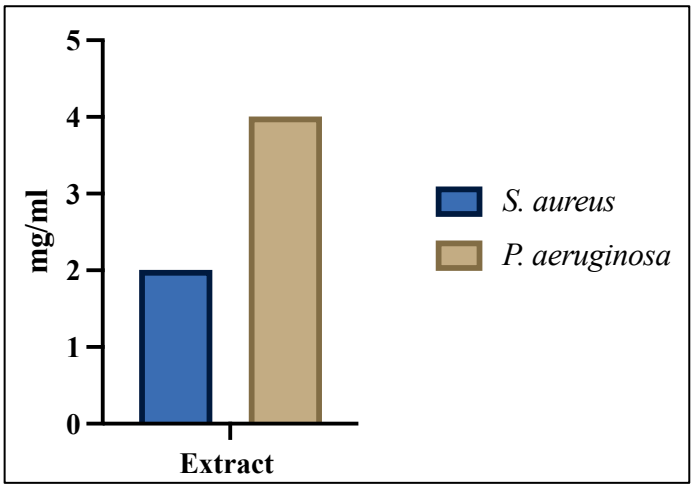

Figure 17. The MIC of extracted bioactive compounds against S. aureus and P. aeruginosa (n=3) (standard error is 0).

Table 4. The table illustrates the MIC of extracted bioactive compounds against S. aureus and P. aeruginosa.

Sample	***S. aureus* (ATCC 25923)**		***P. aeruginosa* (ATCC 15442)**	
	MIC	MBC	MIC	MBC
Extract	2 mg/ml	4 mg/ml	4 mg/ml	8 mg/ml

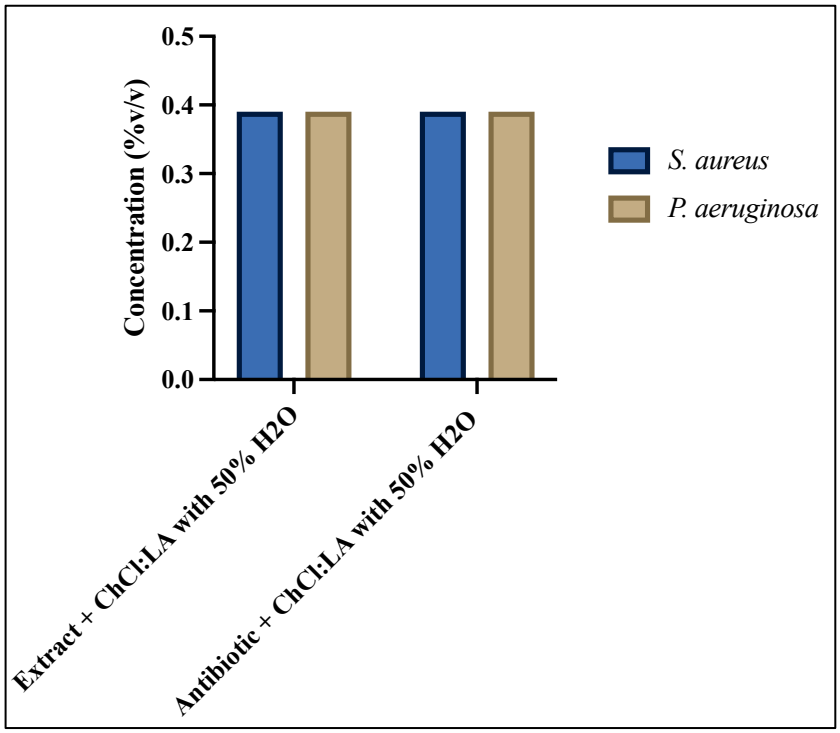

Figure 18. The MIC of A) extracted bioactive compounds + ChCl:LA with 50% H_2O; B) antibiotic + ChCl:LA with 50% H_2O against S. aureus and P. aeruginosa (n=3) (standard error is 0).

ChCl:LA with 50% H_2O has been added to check if by mixing extract and ChCl:LA with 50% H2O give excellent antibacterial activity or not. As per Figure 18**.,** extract and ChCl:LA with 50% H_2O showed 0.39 %v/v against *S. aureus* and *P. aeruginosa* (refer Figure 24 that shows fold-change compared to 100%). For comparative analysis, ChCl:LA combined with antibiotic to see the difference but there was no significant change in antibacterial activity.

Overall, the MIC of ChCl:LA with 50% H_2O remained constant even after combined with the extract and antibiotic. There were chances of handling error during experiment, or another reason might be that bioactive compounds were not stable to inhibit bacteria completely. But by combining ChCl:LA with 50% H_2O with bioactive compounds does not diminish its efficacy. In terms of antibacterial activity, ChCl:LA with 50% H_2O did not lose its original

antibacterial activity. Bioactive compounds still used for the further experiments as it can be good for the wound healing. Although, it does not have antibacterial activity as compared to ChCl:LA with 50% H_2O. But many studies showed that bioactive compounds had significant antibacterial activity.

3.7 Preparation of DES based hydrogel with bioactive compounds

The bioactive compounds loaded in 50% ChCl:LA based Pluronic F-127 and Xanthan hydrogel. Moreover, as an additives dopamine has been added to make it more adhesive which means hydrogel has more hydrogen bonds that increased the cross-linking of the hydrogel (Wang *et al.*, 2020). As seen in Figure 19**.,** dopamine made both the hydrogel adhesive.

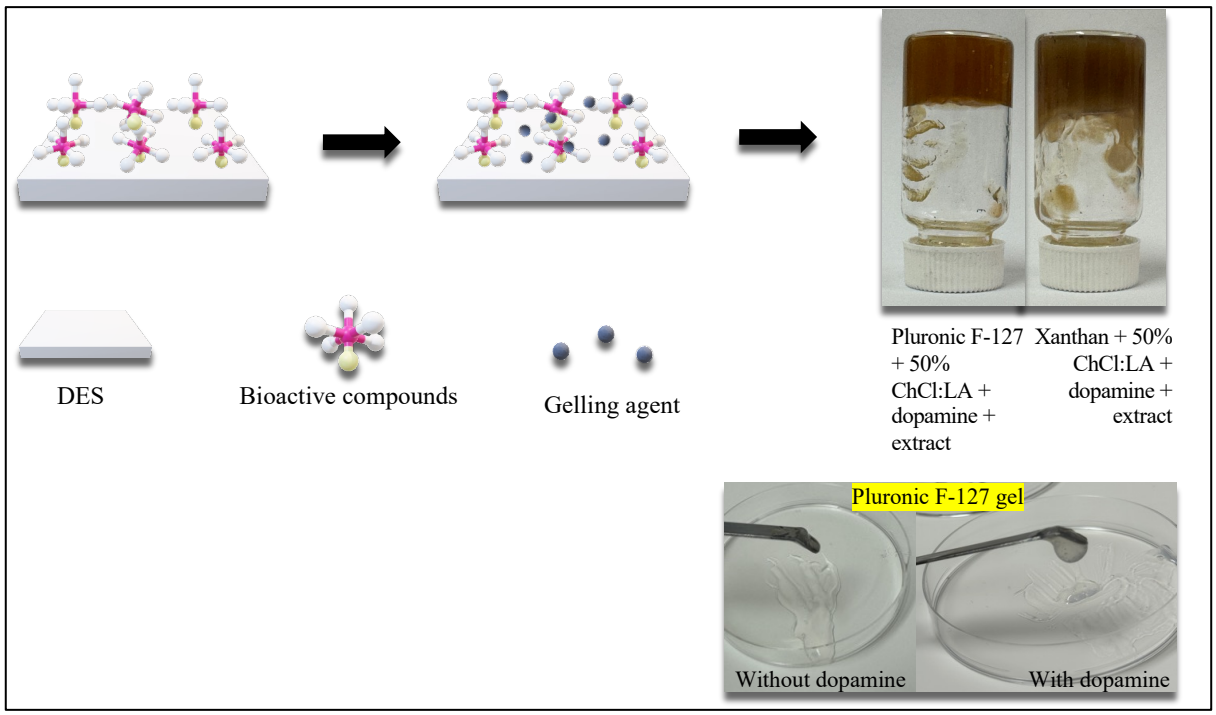

Figure 19. Schematic representation of how ChCl:LA with 50% H_2O, bioactive compounds, and dopamine (gelling agent) have been incorporated in hydrogel to develop multifunctional gel.

3.7.1 Antibacterial assay of loaded gel

After adding 50% ChCl:LA in Pluronic F-127 and Xanthan hydrogel, dopamine, and extract were added to enhance the adhesiveness and antibacterial activity of hydrogel. Pluronic F-127 and dopamine and Pluronic F-127 and extract used to investigate any of them were responsible for antibacterial activity or not. Pluronic F-127 with dopamine showed 23.33 ± 1.15 mm and 16.67 ± 0.58 mm against *S. aureus* and *P. aeruginosa*. As seen in Figure 28**.,** dopamine undergoes oxidation reaction and reactive oxygen generated that can kill both *S. aureus* and *P. aeruginosa* bacteria (Singh *et al*., 2021). Pluronic F-127 with extract showed no antibacterial activity against any bacteria (Figure 28). Pluronic F-127 with 50% ChCl:LA, dopamine and extract showed 22.67 ± 1.53 mm and 20.33 ± 1.53 mm against *S. aureus* and *P. aeruginosa*. As shown in Figure 20., Pluronic F-127 with 50% ChCl:LA showed good antibacterial activity with *S. aureus* 23 ± 1 mm and with *P. aeruginosa* 19 ± 0.58 mm whereas Pluronic F-127 with 50% ChCl:LA, dopamine and extract showed 22.67 ± 1.53 mm and 20.33 ± 1.53 mm against *S. aureus* and *P. aeruginosa*. The results state that by adding additives (50% ChCl:LA and bioactive compounds) in Pluronic F-127 gel did not make dramatic difference as it decrease against *S. aureus* and slightly increase against *P. aeruginosa.*

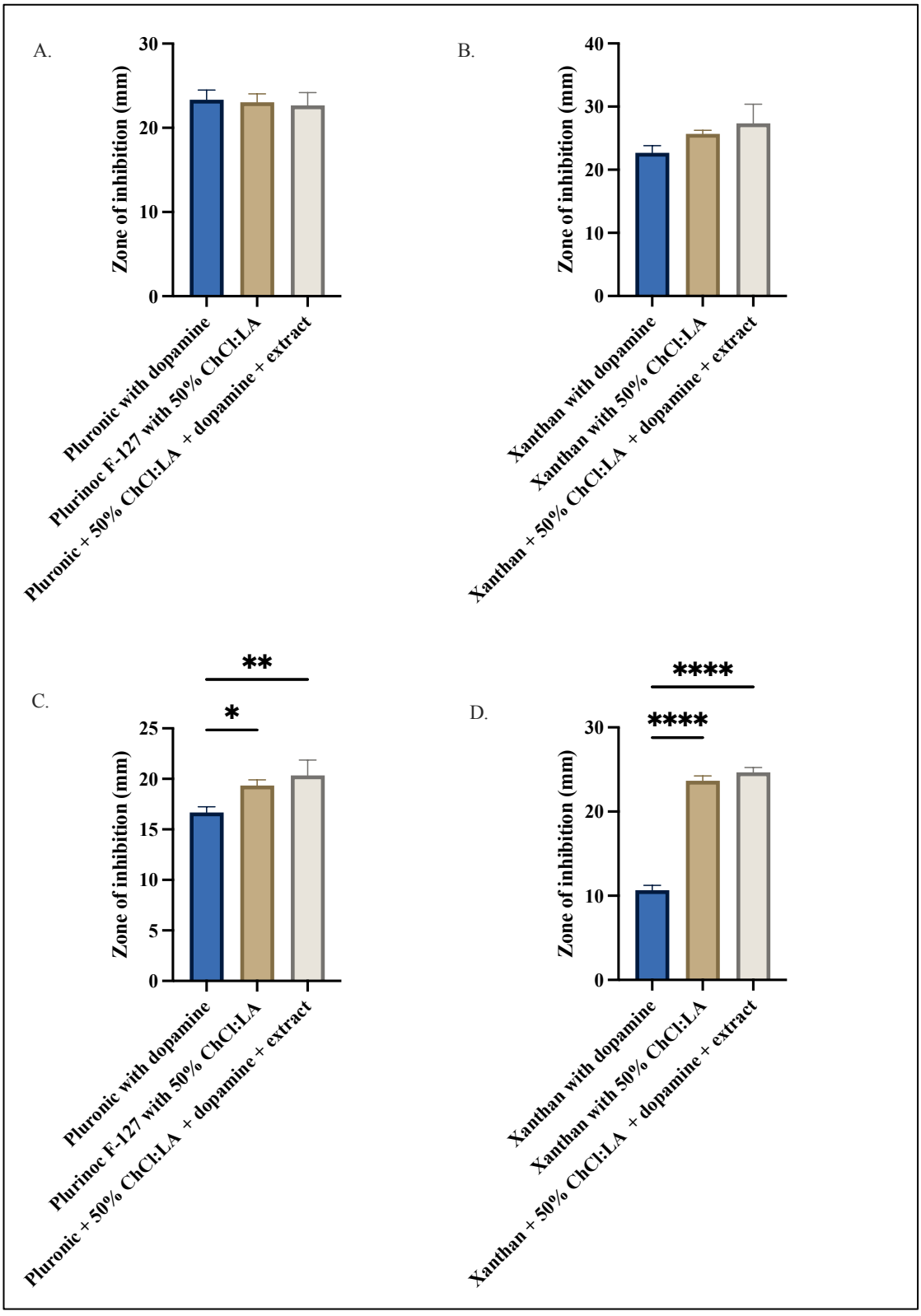

Figure 20. The zone of inhibition of A) Pluronic F-127 hydrogel with additives against S. aureus; B) Xanthan hydrogel with additives against S. aureus; C) Pluronic F-127 hydrogel with additives against P. aeruginosa and D) Xanthan hydrogel with additives against P.

aeruginosa (mean ± standard deviation, n=3). (Statistical analysis done by one-way ANOVA

** P < 0.05, ** P <0.01, *** P < 0.001, **** P < 0.0001)*

In case of Xanthan, Xanthan with dopamine had 22.67 ± 1.15 mm and 10.67 ± 0.58 mm with *S. aureus* and *P. aeruginosa* bacteria. Pluronic F-127 did not show any activity with extract but Xanthan showed 7.67 ± 0.58 mm zone with *S. aureus* whereas it showed no activity with *P. aeruginosa* (Figure 29). Xanthan with 50% ChCl:LA had great antibacterial activity as it showed 25.67 ± 0.58 mm and 23.67 ± 0.58 mm zone against *S. aureus* and *P. aeruginosa* whereas it showed 27.33 ± 3.05 mm and 24.67 ± 0.58 mm zones against *S. aureus* and *P. aeruginosa* in Xanthan with additives (50% ChCl:LA, dopamine and extract).

The results indicate that Pluronic F-127 with 50% ChCl:LA, dopamine and extract had good antibacterial activity but Xanthan with 50% ChCl:LA, dopamine and extract had excellent antibacterial activity. As per result, solvent (50% ChCl:LA) itself had 24.67 ± 1.15 mm and 24 ± 0 mm zones but when it combined with dopamine and extracted bioactive compounds it gave 27.33 ± 3.05 and 24.66 ± 0.58 mm zones in xanthan gel. Hence, it proves that combining ChCl:LA and bioactive compounds enhance the antibacterial activity of gel. Additionally, this hydrogel has better adhesiveness so it can easily stick to any surface.

4 Conclusion

The Pluronic F-127 and Xanthan hydrogel were prepared with different concentration of ChCl:LA then it was tested for antibacterial activity and result compared with without presence of ChCl:LA (with only Pluronic F-127 and Xanthan gel). Afterwards, dopamine and extract (bioactive compounds) loaded Pluronic F-127 and Xanthan hydrogel with 50% DES gel.

In conclusion, this study developed a novel Deep eutectic solvent (DES) based hydrogel. Among all the DESs which tested in this study, ChCl:LA had an excellent antibacterial activity. To enhance antibacterial performance, bioactive compounds extracted from brown seaweed and profiling has been done to identify the bioactive compounds. Afterwards, Pluronic F-127 and Xanthan hydrogel composite with 50% ChCl:LA, dopamine, and extract (bioactive compounds) and it shows higher antibacterial activity compared to the hydrogel without ChCl:LA and extract against Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-negative *Pseudomonas aeruginosa* (ATCC 15442) pathogens. This hydrogel can be used in wound dressing as an alternative to existing DES-based hydrogel to promotes wound healing.

Future direction

This hydrogel is not only limited to wound dressing but also could be used in health care applications. Further techniques for example, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Atomic - Force Microscopy (AFM), Fourier Transform Infrared Spectroscopy (FTIR) can be used to understand formation and characteristics of ChCl:LA. Structural characterization of hydrogel is one of significant part to know morphology and structure of gel. Therefore, SEM, TEM, AFM and Confocal Microscopy (CM) can be used to characterize the structure of hydrogel and obtain detailed visual images.

Add on to this, this study only checked antibacterial property of the hydrogel, but further study needed to check antibiofilm property of ChCl:LA. Furthermore, various additives can be added to enhance the antibacterial properties of this hydrogel such as, silver nanoparticles have good antibacterial properties. Additionally, this study only used Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-negative *Pseudomonas aeruginosa* (ATCC 15442)

pathogens, but further study needs to be done with antibiotic resistance bacteria. For example, with Methicillin-resistance *Staphylococcus aureus* (MRSA) as it difficult to treat.

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APPENDICES

Appendix 1. Antibacterial assay of DESs

Table 5. The zone of inhibition of different DESs at different concentration (10%,20%,40% and 50%) against S. aureus and P. aeruginosa.

	Test Bacteria	
Sample	***S. aureus*** **(ATCC 25923)**	***P. aeruginosa*** **(ATCC 15442)**
DES 1 ChCl:LA	35.33 mm	29.67 mm
DES 2 ChCl:LA with 10% H_2O	32 mm	28.33 mm
DES 3 ChCl:LA with 20% H_2O	33.67 mm	28.67 mm
DES 4 ChCl:LA with 40% H_2O	29.67 mm	26 mm
DES 5 ChCl:LA with 50% H_2O	24.67 mm	24 mm
DES 6 ChCl:Mannose	No zone	No zone
DES 7 ChCl:Mannose with 10% H_2O	No zone	No zone
DES 8 ChCl:Mannose with 20% H_2O	No zone	No zone
DES 9 ChCl:Mannose with 40% H_2O	No zone	No zone
DES 10 ChCl:Mannose with 50% H_2O	No zone	No zone
DES 11 ChCl:CA	28.67 mm	28.67 mm
DES 12 ChCl:CA with 10% H_2O	32.33 mm	30.33 mm
DES 13 ChCl:CA with 20% H_2O	30.67 mm	29.67 mm

DES 14 ChCl:CA with 40% H_2O	29.67 mm	27 mm
DES 15 ChCl:CA with 50% H_2O	30 mm	27.67 mm

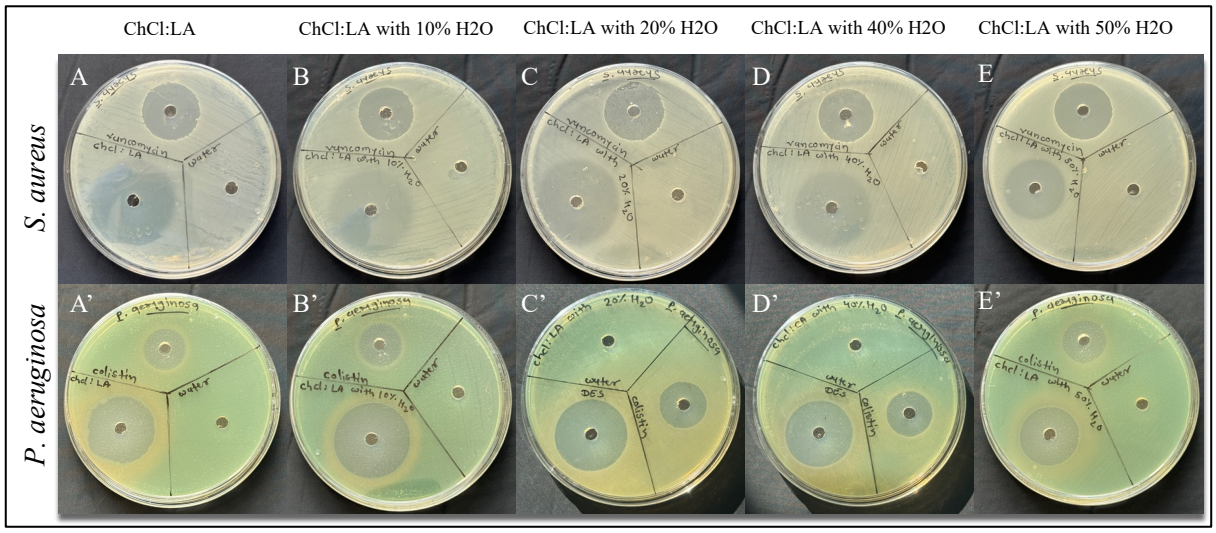

Figure 21. The zone of inhibition of ChCl:LA against S. aureus and P. aeruginosa bacteria (A,A') ChCl:LA (B,B') ChCl:LA with 10% H_2O (C,C') ChCl:LA with 20% H_2O (D,D') ChCl:LA with 40% H_2O and (E,E') ChCl:LA with 50% H_2O (mean ± standard deviation, n=3).

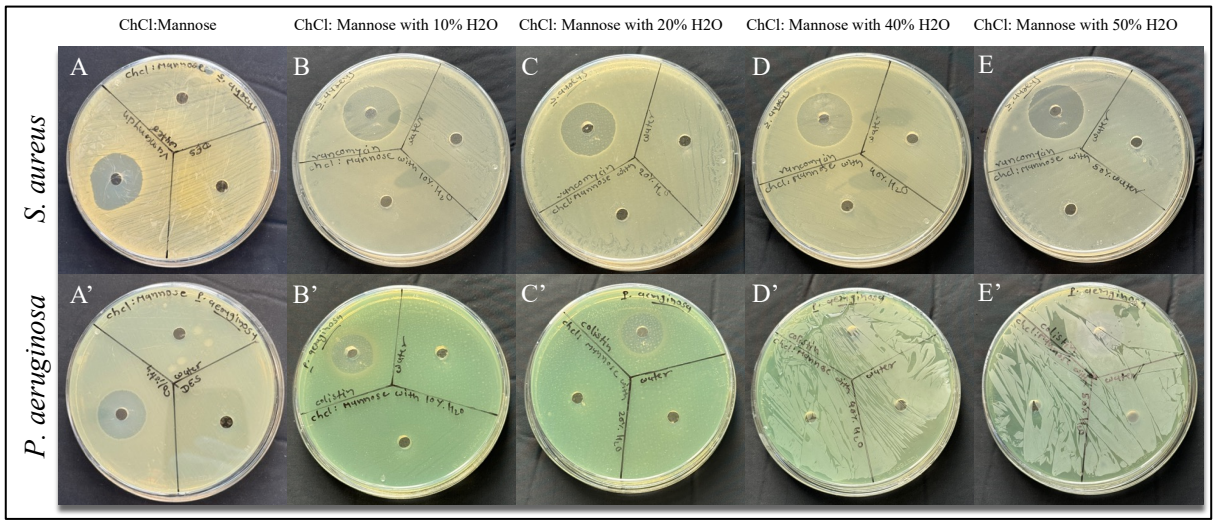

Figure 22. The zone of inhibition of ChCl:Mannose against S. aureus and P. aeruginosa bacteria (A,A') ChCl: Mannose (B,B') ChCl: Mannose with 10% H_2O (C,C') ChCl: Mannose with 20% H_2O (D,D') ChCl: Mannose with 40% H_2O and (E,E') ChCl: Mannose with 50% H_2O (mean ± standard deviation, n=3).

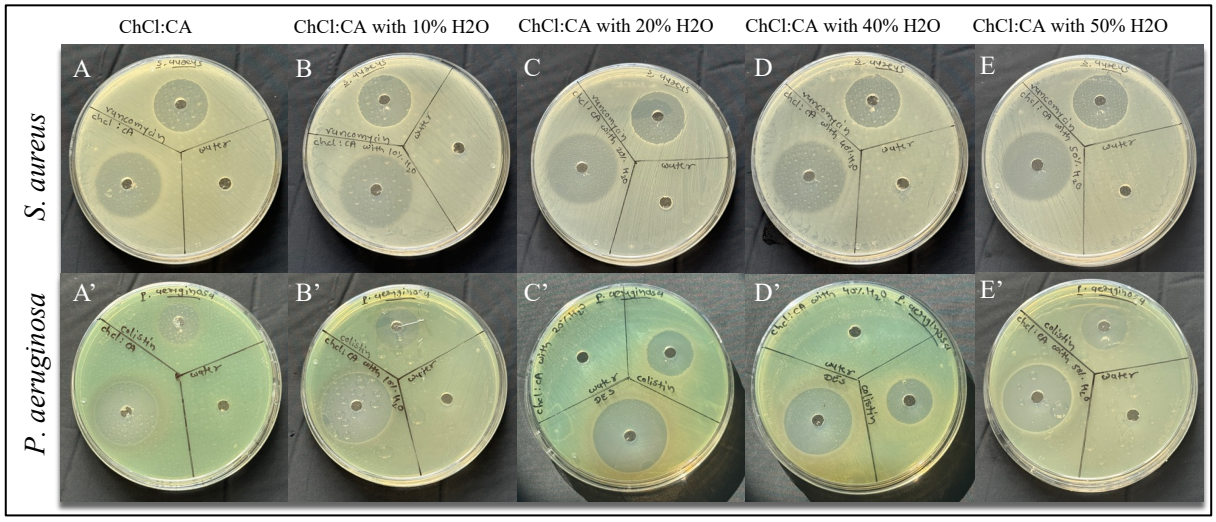

Figure 23. The zone of inhibition of ChCl:CA against S. aureus and P. aeruginosa bacteria (A,A') ChCl:CA (B,B') ChCl:CA with 10% H_2O (C,C') ChCl:CA with 20% H_2O (D,D') ChCl:CA with 40% H_2O and (E,E') ChCl:CA with 50% H_2O (mean ± standard deviation, n=3).

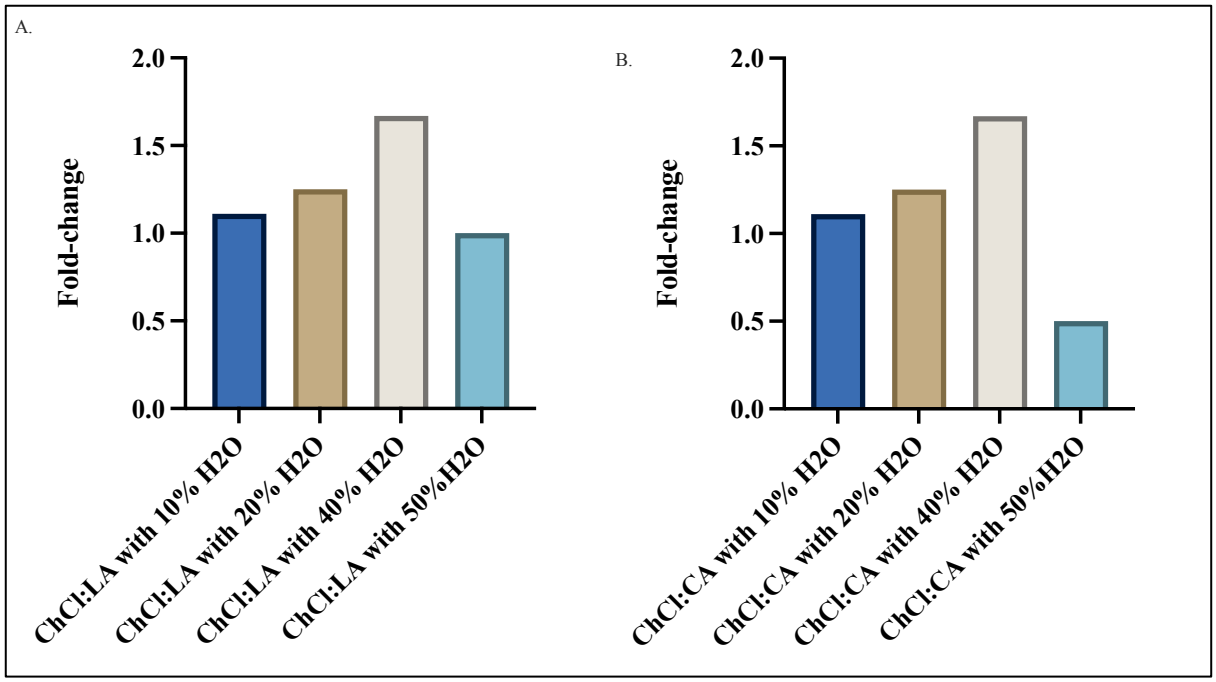

*Figure 24. The fold-change of DESs to compare the final concentration of well. A) shows ChCl:LA fold-change compared to 100% B) shows ChCl:CA fold-change compared to 100%. (*Note: DES without H_2O (100%) i.e., baseline viscosity. For example, ChCl:LA without H_2O (100%) concentration divided with ChCl: LA with 10% H_2O to get final MIC of that well).*

Table 6. Table illustrate the fold-change of MIC data compared to 100% of ChCl:LA.

Sample	**Fold-change compared to 100%**
ChCl:LA with 10% H2O	1.11
ChCl:LA with 20% H2O	1.25
ChCl:LA with 40% H2O	1.67
ChCl:LA with 50%H2O	1.00

Table 7. Table illustrate the fold-change of MIC data compared to 100% of ChCl:CA.

Sample	**Fold-change compared to 100%**
ChCl:CA with 10% H2O	1.11
ChCl:CA with 20% H2O	1.25
ChCl:CA with 40% H2O	1.67
ChCl:CA with 50%H2O	0.5

Appendix 2. Well diffusion assay for DES based hydrogels.

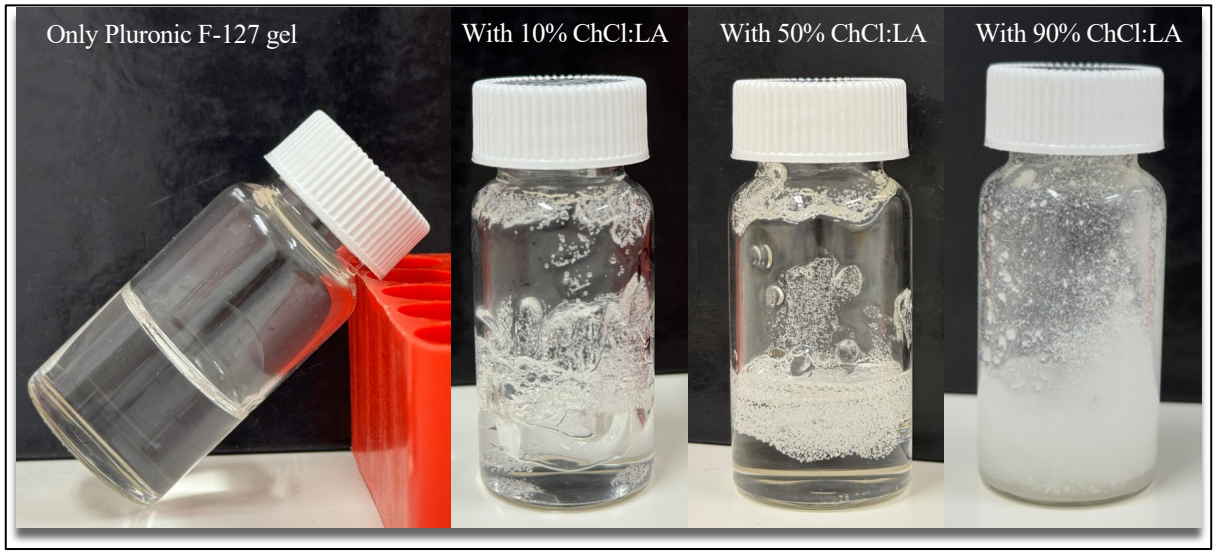

Figure 25. The image of Pluronic F-127 gel with only Pluronic F-127 gel, with 10% ChCl:LA, 50% ChCl:LA, and 90% ChCl:LA.

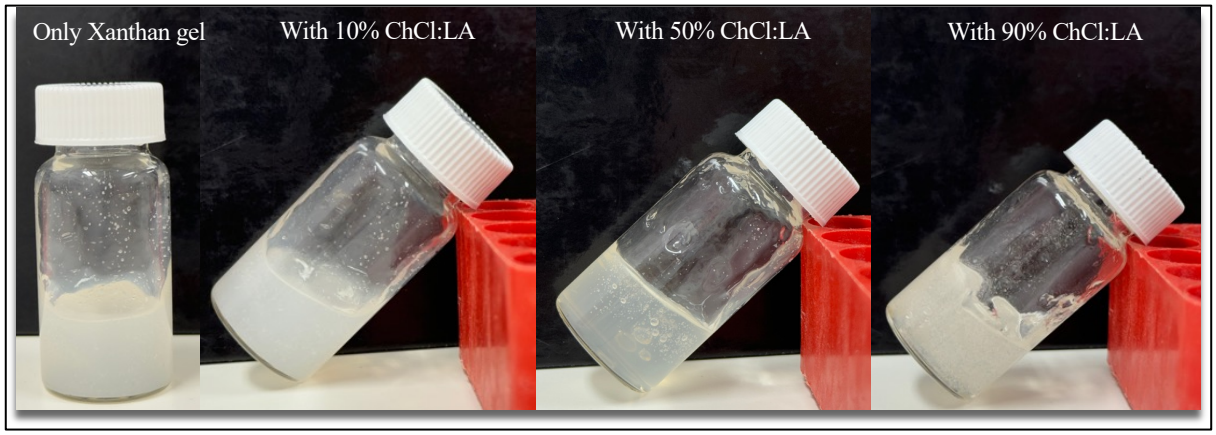

Figure 26. The image of Xanthan gel with only Xanthan gel, with 10% ChCl:LA, 50% ChCl:LA, and 90% ChCl:LA.

Table 8. The zone of inhibition of Pluronic F-127 with different concentration of ChCl:LA (10%, 50% and 90%) and with 50% LA against S. aureus and P. aeruginosa.

	Test Bacteria	
Sample	***S. aureus*** **(ATCC 25923)**	***P. aeruginosa*** **(ATCC 15442)**
Pluronic F-127 with 10% ChCl:LA	9.34 mm	9.67 mm
Pluronic F-127 with 50% ChCl:LA	23 mm	19.33 mm
Pluronic F-127 with 90% ChCl:LA	25 mm	25 mm
Pluronic F-127 with 50% LA	20.33 mm	20.33 mm

Table 9. The zone of inhibition of Xanthan with different concentration of ChCl:LA (10%, 50% and 90%) and with 50% LA against S. aureus and P. aeruginosa.

	Test Bacteria	
Sample	***S. aureus*** **(ATCC 25923)**	***P. aeruginosa*** **(ATCC 15442)**
Xanthan with 10% ChCl:LA	9.67 mm	11.33 mm
Xanthan with 50% ChCl:LA	25.67 mm	23.67 mm
Xanthan with 90% ChCl:LA	29.33 mm	26.67 mm
Xanthan with 50% LA	26.33 mm	25.67 mm

Appendix 3. Antibacterial assay of extract (Bioactive compounds)

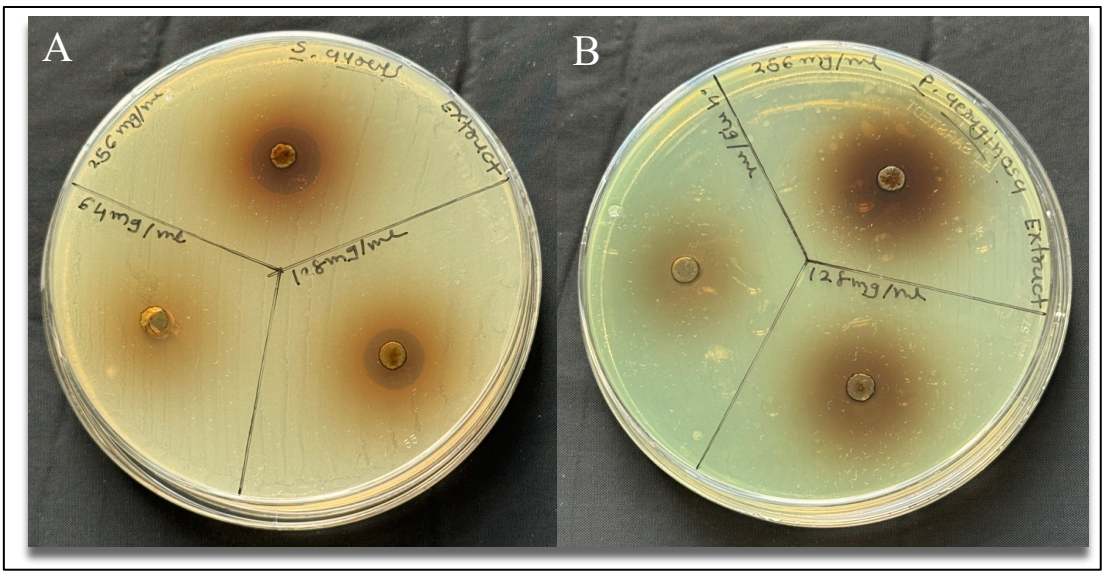

Figure 27. The zone of inhibition of extract at different concentrations: 64 mg/ml, 128 mg/ml, and 256 mg/ml ***(A)*** *shows against S. aureus and* ***(B)*** *shows against P. aeruginosa bacteria (mean ± standard deviation, n=3).*

Table 10. The table illustrates the zone of inhibition of different extract at different concentration (64 mg/ml, 128 mg/ml, and 256 mg/ml) against S. aureus and P. aeruginosa.

	Test Bacteria	
Extract at different concentration	***S. aureus*** **(ATCC 25923)**	***P. aeruginosa*** **(ATCC 15442)**
64 mg/ml	0 mm	0 mm
128 mg/ml	10.33 mm	0 mm
256 mg/ml	12.33 mm	8.33 mm

Table 11. The table demonstrates the MIC and MBC of DESs with additives against S. aureus and P. aeruginosa bacteria (n=3).

Sample	***S. aureus* (ATCC 25923)**		***P. aeruginosa* (ATCC 15442)**	
	MIC (%v/v)	MBC (%v/v)	MIC (%v/v)	MBC (%v/v)
Extract + ChCl:LA	0.39	0.78	0.39	0.78
Extract + ChCl:LA with 10% H2O	0.35	0.70	0.35	0.70
Extract + ChCl:LA with 50% H2O	0.39	0.78	0.39	0.78
ChCl:LA + antibiotic	0.39	0.78	0.39	0.78
ChCl:LA with 10% H2O + antibiotic	0.35	0.70	0.35	0.70
ChCl:LA with 20% H2O + antibiotic	0.31	0.63	0.31	0.63
ChCl:LA with 40% H2O + antibiotic	0.23	0.47	0.23	0.47
ChCl:LA with 50% H2O + antibiotic	0.39	0.78	0.39	0.78

(Vancomycin and Colistin used as an antibiotic for S. aureus and P. aeruginosa bacteria, respectively)

Appendix 4. Antibacterial assay of DES based hydrogel with additives

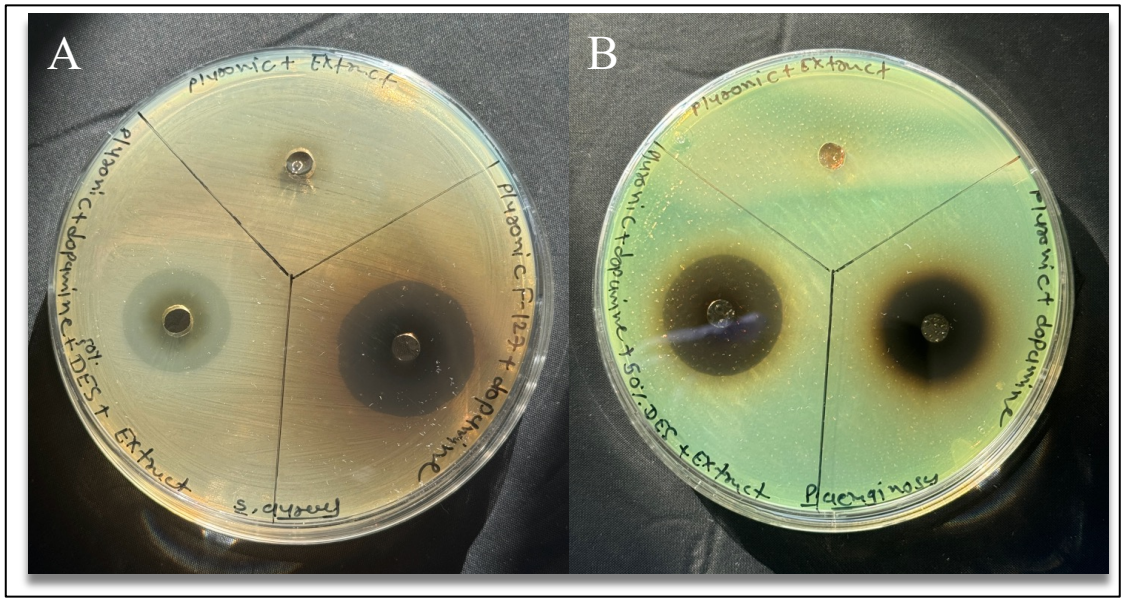

Figure 28. The zone of inhibition of Pluronic F-127 hydrogel with additives (with dopamine; with extract; and with 50% ChCl:LA + Dopamine + Extract): ***(A)*** *shows against S. aureus and* ***(B)*** *shows against P. aeruginosa bacteria (mean ± standard deviation, n=3).*

Table 12. The zone of inhibition of Pluronic F-127 with dopamine; with extract; and with 50% ChCl:LA + Dopamine + Extract against S. aureus and P. aeruginosa.

	Test Bacteria	
Sample	***S. aureus*** **(ATCC 25923)**	***P. aeruginosa*** **(ATCC 15442)**
Pluronic F-127 with Dopamine	23.33 mm	16.67 mm
Pluronic F-127 with Extract	0 mm	0 mm
Pluronic F-127 with 50% ChCl:LA + Dopamine + Extract	22.67 mm	20.33 mm

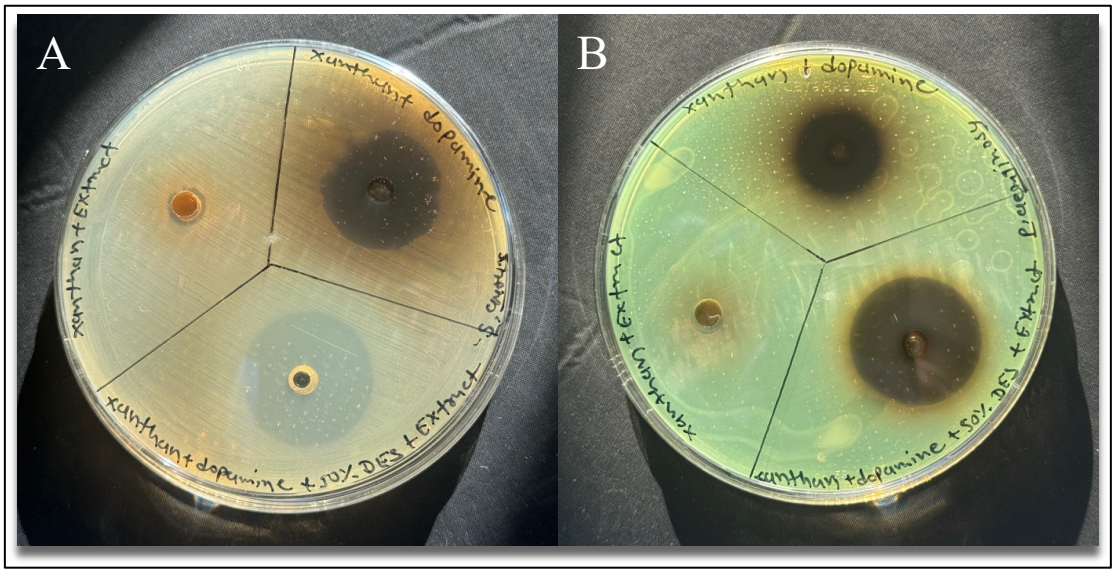

Figure 29. The zone of inhibition of Xanthan hydrogel with additives (with dopamine; with extract; and with 50% ChCl:LA + Dopamine + Extract): ***(A)*** *shows against S. aureus and* ***(B)*** *shows against P. aeruginosa bacteria (mean ± standard deviation, n=3).*

Table 13. The zone of inhibition of Xanthan with dopamine; with extract; and with 50% ChCl:LA + Dopamine + Extract against S. aureus and P. aeruginosa.

	Test Bacteria	
Sample	***S. aureus*** **(ATCC 25923)**	***P. aeruginosa*** **(ATCC 15442)**
Xanthan with dopamine	22.67 mm	10.67 mm
Xanthan with extract	7.67 mm	0 mm
Xanthan with 50% ChCl:LA + Dopamine + Extract	27.33 mm	24.67 mm