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As Partial Fulfillment of the master's degree
In Food science – Option: *food security and quality assurance*

Theme:

Extraction of Chitosan and studying its antibacterial effect in vitro and in vivo combined with propolis

Held on Juin 25, in front of the jury composed of:

President	AZZI Nour Eddine	MAA	University of Tlemcen
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Dedications:

To all my loved ones

الملخص:

الهدف من هذا البحث هو استخلاص الشيتوزان من القشريات و اكتشاف خصائصه البيولوجية.حيث تم تقييم نشاطه المضاد للبكتيربا باستخدام طريقة انتشار الأقراص ضد سلالتين بكتيريتين هما المكورات العنقودية والعصيات القولونية حبث أظهرت النتائج تأثيرات مثبطة ملحوظة و عدم تكاثر الميكروبات. وفعالة حيث قدر تركيز المثبط االدنى(CMI) ب 2ملغ/مل. و تم اثباث ذلك في نتائج التركيز الأدنى المميت (CMB). كما تم تجربة الشيتوزان على الفئران لقييم سميته وفعاليته كمضاد حيوي عند استخدامه مع مستخلص العكبر. حيث حققت نتائج واعدة تأكد ان الجرعة المعطات 2000مغ/كغ غير سامة أما بالنسبة للعينات النسيجية فقد أظهرت أن اجتماع الشيتوزان و مصتخلص العكبر يعطي فعالية أفضل ضد الميكروبات.

الكلمات المفتاحية: الشيتوزان ، العكبر ، مضاد الميكروبات ، السمية ، الجسم الحي.

Abstract:

This study aims to extract chitosan from crustacean shells and investigate its biological properties. The antimicrobial activity of the chitosan extracts was evaluated using the disk diffusion method against two bacterial strains: *Staphylococcus aureus* and *Escherichia coli*. The results demonstrated significant inhibitory effects, preventing the proliferation of the pathogens. The microdilution method confirmed a minimum inhibitory concentration (MIC) of 0.1mg/L, which was further supported by the minimum bactericidal concentration (MBC). In addition, in vivo tests were conducted to assess the toxicity of chitosan and its efficacy as an antibiotic when combined with propolis. Promising results were obtained, confirming that the giving dose 2000 mg/kg is not toxic. As for the histological samples indicating potent antimicrobial activity for the combination of chitosan and propolis.

Key words: Chitosan, propolis, antimicrobial, toxicity, in vivo.

Resume

Cette étude vise à extraire le chitosane des carapaces de crustacés et à étudier ses propriétés biologiques. L'activité antimicrobienne des extraits de chitosane a été évaluée par la méthode de diffusion sur disque contre deux souches bactériennes: *Staphylococcus aureus* et *Escherichia coli*. Les résultats ont démontré des effets inhibiteurs significatifs, empêchant la prolifération des pathogènes. La méthode de microdilution a confirmé une concentration minimale inhibitrice (CMI) de 0,1mg/L, qui a été confirmée par la concentration bactéricide minimale (CBM). En outre, des tests in vivo ont été réalisés pour évaluer la toxicité du chitosane et son efficacité en tant qu'antibiotique lorsqu'il est associé à la propolis. Des résultats prometteurs ont été obtenus, confirmant que la dose de 2000 mg/kg n'est pas toxique. Quant aux échantillons histologiques, ils indiquent une puissante activité antimicrobienne pour la combinaison de chitosane et de propolis.

Mots clés: Chitosan, propolis, antimicrobien, toxicité, in vivo.

Abbreviation list:

- **HCl**: chlorohydric acid.
- **NaOH**: Sodium hydroxide.
- **DD**: Deacetylation degree.
- **MIC**: minimum inhibitory Concentration
- **MBC**: minimum bactericidal Concentration.
- **DPPH**: 1,1 diphenyl-2-pictylhydrazyle.
- **BHI**: Brain Heart Infusion.
- **DMSO**: dimethyl sulfoxide.
- **MH**: Mueller-Hinton.
- **Ch**: chitosan.
- **CDC:** Centers for Disease Control and Prevention
- **WHO:** World Health Organization
- **FDA:** Food and Drug Administration
- **DNA:** Deoxyribonucleic Acid
- **RNA** Ribonucleic Acid
- **SE_HPLC:** Size-Exclusion High-Performance Liquid Chromatography
- **OD:** Optical Density
- **CFU:** Colony-Forming Unit
- **CBC**: Complete Blood Count
- **WBC:** white blood cells
- **RBC:** Red blood cells
- **HGB:** Hemoglobin

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INTRODUCTION

Introduction:

The safety of food, the environment, and human health are the key issues in the food sector. Food deterioration and foodborne sickness are caused by oxidative reactions and microbe adhesion on abiotic surfaces. Over 7% of the global population suffers from a food-related ailment each year, according to the World Health Organization **(Mouhoub and al., 2022)**

Biopolymer nanofibrils possess remarkable mechanical properties, combining strength and toughness, and exhibit biological functions that interact with the surrounding environment. These properties arise from their hierarchical structures, which span from angstrom to hundreds of nanometer scales. To preserve these unique structural characteristics and harness the potential of these natural supramolecular assemblies, chitin nanofibrils, have garnered significant attention in recent years as the most abundant biopolymer nanofibrils on earth **(Ling and al., 2018)**. Chitin, a significant natural resource, is estimated to have an annual worldwide production of approximately 10^{10} to 10^{12} tons. It is derived from arthropods (insects and crustaceans), mollusks, and fungi. Primarily serving a structural role, chitin is predominantly sourced from crustacean shells due to their high content and widespread availability, making them the primary chitin source for commercial purposes. Chitin and its derivatives hold significant economic value due to their diverse applications in industries such as food, cosmetics, pharmaceuticals, textiles, wastewater treatment, and agriculture **(Gortari and Hours, 2013)**.

Cationic polymers show great potential as antibacterial agents due to their ability to effectively combat bacterial resistance, however, frequently result in limited biocompatibility compared to chitosan, a new biodegradable and biocompatible cationic antibacterial polymer can be seen in its ability to efficiently fight off a variety of pathogens at low inhibitory doses **(Si and al., 2021)**.

Natural substances like chitin and chitosan have the ability to control plant diseases in agriculture. These substances are poisonous to fungi, preventing their expansion and growth. Additionally, it has been discovered that they have activity against bacteria, viruses, and other pests. It has been demonstrated that when exposed to microbial diseases, host plants respond in a variety of defensive ways to fragments made of chitin and chitosan. Growing interest has been shown in using chitin, chitosan, and its derivatives in agricultural systems to reduce the detrimental effects of diseases on crop output and quality because of their capacity to strengthen host plant defenses **(El Hadrami and al., 2010)**.

Seafood, fish, and resources used in feed production constitute the main types of marine-derived food waste. Each year, the global trade of crustaceans results in the generation of 6 to 8 million tons of waste, specifically shells from shrimp, lobster, and/or crab, which possess significant value. To address this issue, a team of researchers with diverse technical expertise in chemical engineering design, chemistry, materials, predictive environmental sciences, and economics collaborated on a comprehensive project. Their objective was to develop a sustainable multiproduct pipeline for the biorefinery of these undesirable by-products. Through a standardized evaluation that encompassed purification performance, economic impact, and life cycle assessment, a viable business model was established, propelling the sector toward a sustainable ocean-based economy **(Vicente and al., 2022)**.

This master's thesis aims to investigate the potential applications of chitosan in the context of toxic infections caused by bacteria.

The first part of this thesis will focus on presenting the bibliographic data related to foodborne illness and production to the characterization of chitin and chitosan.

As for the second part, the experimental study will be exploring the methods applied during the progress of this thesis ranging from the extraction of chitosan to testing its toxicity and antimicrobial activity (in-vivo \ in-vitro).

The third part reports and discusses the results obtained based on the completed work.

Overall, this thesis will contribute to a better understanding of the properties and potential applications of chitin and chitosan as biopolymers for the treatment of toxic infections. The findings of this research could have significant implications for the development of alternative therapeutic approaches that are effective against antibiotic-resistant pathogens.

Chapter1:
food born Illness

I.1. Definition of foodborne illness:

Foodborne illness, also known as food poisoning, is a term used to describe diseases caused by the consumption of contaminated food or beverages. The symptoms of foodborne illness can range from mild, such as nausea and stomach cramps, to severe such as kidney failure and paralysis **(CDC, 2021)**.

Foodborne illness can be caused by a variety of pathogens, including bacteria, viruses, parasites, and toxins produced by these microorganisms **(Scallan and al., 2011)**. The most common bacterial causes of foodborne illness in the United States are *Salmonella, Campylobacter,* and *Escherichia coli* **(CDC, 2021).**

Food safety regulations, including proper food handling and preparation, have been implemented to reduce the incidence of foodborne illness. However, foodborne illness remains a significant public health concern, with an estimated 48 million cases occurring annually in the United States **(Scallan and al., 2011)**.

Food poisoning is a prevalent illness that is typically mild but occasionally deadly. It happens when a person consumes a meal or beverage that has been tainted with bacteria or a toxin. Food poisoning can very rarely result from toxins found in chemicals or herbicides **(Shlundt, J and Toyofuku.H., 2010)**.

I.2. Food poisoning:

Food poisoning is caused by the ingestion of contaminated foods and bacteria that proliferate in the food and/or in the digestive tract of the consumer. These germs may be pathogenic or normally recognized as non-pathogenic **(Bousseboua, 2005)**.

Food poisoning symptoms can include nausea, vomiting, diarrhea, abdominal pain, and fever. Food poisoning can occasionally be fatal, particularly in susceptible groups like young children, the elderly, and people with compromised immune systems **(WHO, 2015)**.

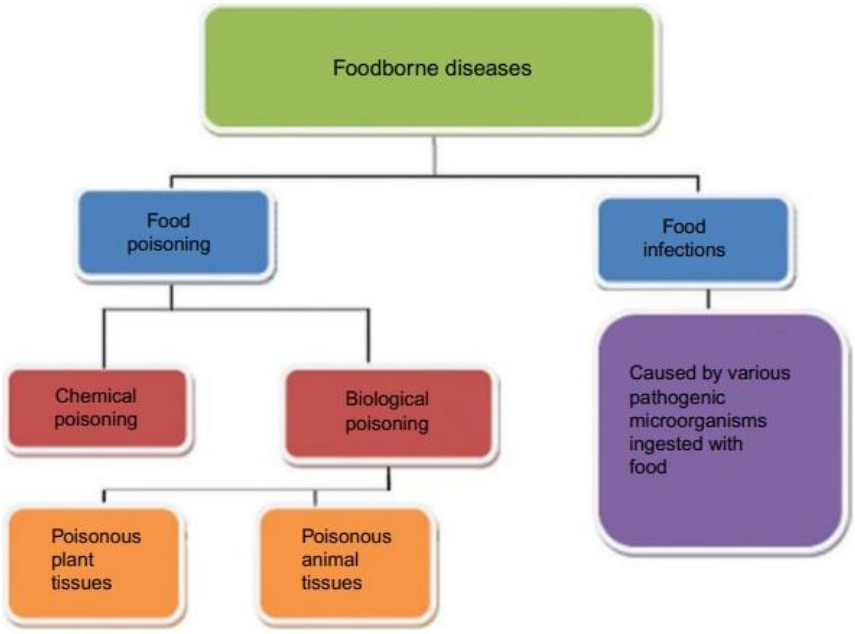

Figure 1: schematic causes of foodborne illness **(Bari & Yeasmin, 2018).**

I.3. Main food implicated in food contamination:

A wide variety of foods can become contaminated with bacteria, viruses, or other pathogens that can cause foodborne illness. Some of the most common sources of food contamination include:

- Raw or undercooked meat and poultry: These foods can be contaminated with bacteria such as *Salmonella*, *E. coli*, and *Campylobacter*.
- Raw or undercooked eggs: Eggs can be contaminated with Salmonella.
- Raw or undercooked seafood: Raw or undercooked seafood can be contaminated with bacteria and viruses such as Vibrio, norovirus, and hepatitis A.
- Raw sprouts: Raw sprouts can harbor bacteria such as Salmonella and E. coli.
- Unpasteurized milk and dairy products: These products can be contaminated with bacteria such as Listeria and Campylobacter.
- Fresh fruits and vegetables: These foods can be contaminated with bacteria such as Salmonella and E. coli, and viruses like norovirus.
- Processed foods: Processed foods such as deli meats, cheese, and canned goods can become contaminated during processing or packaging.

It is crucial to remember that if food is not handled, kept, or prepared correctly, it could get contaminated with pathogens. Following safe food handling procedures, such as frequently washing hands and surfaces, cooking food to the proper temperature, and quickly refrigerating perishable foods, is the best way to lower the risk of contracting a foodborne illness **(CDC, 2021).**

I.4. Origin of foodborne illness:

I.4.1. Physical and chemical:

Other chemical and physical agents from raw materials, machinery, the hands of operators, and the surrounding environment, in addition to the biological agents already stated, are the cause of food-borne disease **(FAO, 2007)**.

- **Physical:**

Slivers of glass, chunks of wood, rocks, metal shavings, wire, or tiny fragments of bone are just a few of the foreign objects that can cause harm when they are present in food **(FAO, 2007)**.

- **Chemical**:

According to **(Rhalem and Soulaymani 2009)** using different chemicals can be dangerous if they are not properly controlled or used improperly. We list a few of these chemical substances here:

- food additives: substances that are added to food to enhance its sensory, nutritional, and preservation properties.
- Residues of veterinary medicinal products: The problems with antibiotic-resistant microorganisms in humans have been caused by using antibiotics.
- Pesticides: can contaminate crops and the surrounding environment and can also persist in the soil and water for extended periods **(Guo and al., 2012)**.

Table1: Sources of environmental contamination in food **(Bari & Yeasmin, 2018).**

Origin	Food in Which a Contaminant Is Likely to Be Found	Possibility of Health Hazard From Amounts in Food, Assuming a Normal Varied Diet
Industrial:		
Mercury	Fish	Low
Lead	All foods, water	Low
Cadmium	Fish, shellfish, kidney	Very low
Polychlorinated biphenyls	Fish, poultry, milk, eggs	Very low
Agricultural:		
Pesticides	All foods	Low
Antibiotics	Milk	Very low
Hormones	Some poultry	Very low
Food processing:		
Cleaning agents Lubricants Packing materials Solvent residues Extraneous substances (rodent excreta, hair, insects, etc.)	Any processed food	Very low

I.4.2. Biological:

Microorganisms can be found in various parts of our environment including the air, food, and surfaces of objects. While some microorganisms are beneficial and do not pose a risk to consumers, others referred to as pathogens, can develop in food and lead to harmful consequences **(Borges,2014)**.

I.5. Viral foodborne illnesses:

foodborne infections caused by Norovirus, Hepatitis A, and Rotavirus, which can contaminate various foods such as shellfish, fruits, vegetables, and ready-to-eat foods and that's a significant global public health concern **(Muthusamy and Ranganathan, 2019)**. Viruses such as hepatitis A, Norwalk virus, Norovirus, and Calicivirus are the most significant culprits of foodborne illnesses. Unlike bacteria, viruses do not grow in food but can infect both humans and animals **(Ramanathan, 2010; Dervin, 2013)**.

I.6. Parasitic foodborne illnesses:

Protozoa and helminths can contaminate food, usually through fecal contamination of water and food. In rare cases, carriers handling food can also lead to contamination. These parasites can cause more severe diarrhea in individuals with weakened immune systems. unspecified agents are among the leading causes of foodborne illness in the United States. Parasites such as "*Entamoeba histolytica*" (causing amoebiasis), *Giardia lamblia* (causing

giardiasis), *Cryptosporidium* (causing cryptosporidiosis), and *Cyclospora* (causing cyclosporiasis) are some of the various diseases caused by these parasites **(Scallan and al. 2011)**.

I.6.1 Bacterial foodborne illnesses:

Plenty of foods can become contaminated by bacteria **(Borges, 2014)**. *Salmonella, Listeria monocytogenes, and Ecoli* are just a few of the pathogenic bacteria that can cause serious foodborne infections and pose a danger to public health worldwide. All kinds of foods, including meat, poultry, eggs, dairy products, fruits, and veggies, can be contaminated by these bacteria. It can cause a variety of symptoms, some of which are mild to serious and even potentially fatal. Dehydration, nausea, vomiting, diarrhea, and abdominal pain are typical signs **(Bhunia and Potter, 2017; Tauxe, 2002)**.

I.7. Routes of transmission:

Considering food born illnesses are infections brought on by eating food tainted with dangerous bacteria, viruses, parasites, or toxins, foodborne illnesses are a major public health issue. People should be conscious of the diverse ways that foodborne illnesses can spread to lower their risk of getting sick **(CDC, 2021)**.

1. contaminated food: According to **Buchanan and Doyle (2015)**, food items such as raw or undercooked meat, poultry, eggs, seafood, and unpasteurized milk can be sources of contamination at any stage of food production, processing, storage, or handling. Another route of transmission is cross-contamination, which happens when harmful bacteria or other pathogens are transferred from one food to another through shared utensils, cutting boards, or other food contact surfaces **(Todd, 2013)**.

2. Contaminated water: another route that can lead to foodborne illnesses. Consuming water that has been contaminated with harmful pathogens can happen when the water used to wash produce or to prepare food is contaminated with bacteria or other pathogens **(WHO, 2015)**. Person-to-person transmission is another route, and some foodborne illnesses can be transmitted from person to person, usually through contact with fecal matter or vomit. This can happen when an infected person handles food without washing their hands properly, or if they contaminate food with their bodily fluids **(Todd, 2013)**.

3. Airborne transmission: Certain foodborne illnesses, such as norovirus, can potentially spread through airborne transmission. This may occur when an infected person vomits or has diarrhea, and the particles become airborne, leading to the spread of the illness **(Fernandez-Cruz and al., 2018)**.

I.8. Common symptoms of foodborne illness:

Depending on the type of pathogen involved and the severity of the infection, symptoms of foodborne illness can vary **(Fratamico, Bhunia, and Smith, 2005)**. Here are some of the most common symptoms of foodborne illness:

- Nausea and Vomiting: Nausea and vomiting are common symptoms of foodborne illness and are often caused by bacterial toxins **(FDA, 2018)**.

- Diarrhea: Diarrhea is another common symptom of foodborne illness and can be caused by a wide range of pathogens, including bacteria, viruses, and parasites **(CDC, 2021)**.

- Abdominal Pain and Cramping: Abdominal pain and cramping are common symptoms of many types of foodborne illnesses, including bacterial infections such as Salmonella and Campylobacter **(Mayo Clinic, 2021)**.

- Fever: Fever is a common symptom of many types of foodborne illnesses, including bacterial and viral infections **(CDC, 2021)**.

- Dehydration: Dehydration can occur from diarrhea and vomiting, which can lead to a loss of fluids and electrolytes **(WHO, 2018)**.

I.9. Impact of foodborne illness:

- Health effects: A variety of symptoms, from minor stomach distress to serious and life-threatening disorders including kidney failure, meningitis, or paralysis, can be brought on by foodborne illnesses. (Young children, expectant mothers, the elderly, and those with compromised immune systems are among those who are more susceptible to foodborne infections than others **(Buchanan and Doyle, 2015)**.

- Economic impact: Economic costs associated with foodborne illnesses can be high and include things like medical bills, missed wages, and the price of containing outbreaks and conducting investigations. Recalls of food products and a decline in customer confidence may cause business losses and supply chain disruption **(Gensheimer and Horowitz, 2015)**.

- Public health impact: Infectious infections can spread, and antibiotic-resistant bacteria can evolve because of foodborne illnesses, among other wider public health effects. Additionally, outbreaks can alter consumer behavior and raise demands for control and oversight by causing fear and distrust in the food chain **(CDC, 2020)**.

- Legal impact: In addition to other more extensive negative effects on public health, foodborne diseases can lead to the spread of infectious infections and the evolution of antibiotic-resistant bacteria. Furthermore, outbreaks can change consumer behavior and increase calls for oversight and control by engendering mistrust across the food chain **(Doyle and al., 2015)**.

I.10. Food safety regulations and standards:

The Algerian legal system has a substantial number of laws regarding protecting consumers from foodborne illnesses, ensuring the hygiene, safety, and safety of food, as well as measures and penalties for fraud prevention. Some of the most critical laws related to these areas are outlined below:

- Executive Decree No. 96-360 of September 8, 1996, on the conditions and labeling requirements for food products intended for human consumption, regulates the labeling and packaging of food products intended for human consumption. The decree also provides for the inspection and control of food labeling by competent authorities **(Official Journal of the People's Democratic Republic of Algeria, 1996)**.

- Executive Decree No. 04-210 of June 21, 2004, on the hygiene and safety conditions of foodstuffs, specifies the requirements for the design and maintenance of food premises, equipment, and utensils, as well as the rules for handling and processing foodstuffs. The decree also provides for the inspection and control of foodstuffs by competent authorities **(Official Journal of the People's Democratic Republic of Algeria, 2004)**.

- Law No. 06-01 of February 20, 2006, on consumer protection and the repression of fraud, establishes the National Agency for Consumer Protection (ANPC) to oversee and enforce its provisions **(Official Journal of the People's Democratic Republic of Algeria, 2006)**.

- Law No. 14-05 of February 24, 2014, on veterinary public health and food safety establishes the National Food Safety Agency (ANSAL) to monitor and ensure compliance with food safety standards **(Official Journal of the People's Democratic Republic of Algeria, 2014)**.

I.11. factors that promote the multiplication of involved germs:

1. Temperature: Maintaining an appropriate temperature is crucial in controlling bacterial multiplication. Bacteria tend to multiply rapidly within the range of 20°C to 45°C (68°F to 113°F) **(Jay and al., 2005)**. Hence, it is crucial to keep perishable food items at the correct temperature to impede bacterial growth.

2. Humidity: Moisture is a significant factor in bacterial growth as bacteria require it to multiply. Foods that are inadequately dried or have a high moisture content are more likely to support the growth of bacteria **(Jay and al., 2005)**.

Table2: Minimum water activity thresholds allowing the development of different types of microorganisms **(Castello.M and Zartarian.V., 2005).**

Minimum threshold required	Types of microorganisms that can develop
0.950 – 0.910	Normal bacteria
0.890	Hydrophilic molds
0.880 – 0.850	Normal yeasts
0.800	Normal or mesophilic molds
0.750	Halophilic bacteria
0.700 -0.650	Xerophilic molds
0.600	Osmophilic yeasts

3. The pH: is a crucial factor in bacterial growth as some bacteria thrive in acidic conditions, while others prefer basic conditions. The pH of food can impact its susceptibility to bacterial growth **(Jay and al., 2005)**.

4. nutrients: is another essential factor in bacterial growth. Bacteria require nutrients like protein, carbohydrates, and fats to grow and multiply **(Jay and al., 2005)**.

5. the presence of other microorganisms: The presence of other microorganisms can impact bacterial growth, as some microorganisms can inhibit the growth of other bacteria. The absence of these microorganisms can lead to the proliferation of unwanted bacteria **(Salminen and al., 2004)**.

I.11. prevention:

To ensure the safety of the food supply and consumer confidence, preventing foodborne illness is crucial. Effective strategies to prevent such illnesses include maintaining good personal hygiene, using safe food handling and storage practices, and implementing food safety management systems. According to the CDC and WHO in 2015, these approaches are essential **(CDC, 2021)**. and are also supported by other public health organizations and experts worldwide **(Hoorfar and al., 2018)**.

According to **(the Handbook of Foodborne Diseases 2018)**, the following measures can help prevent foodborne illness:

- Practice good personal hygiene, including washing hands thoroughly before handling food.
- Keep raw and cooked foods separate to prevent cross-contamination.
- Cook food thoroughly to kill any harmful bacteria or viruses.

- Store food at safe temperatures, both before and after cooking.
- Use safe water and raw materials to prepare food.
- Choose reputable food sources to minimize the risk of contaminated food.
- Keep kitchen surfaces and utensils clean and sanitized to prevent the spread of harmful bacteria.
- Wash fruits and vegetables thoroughly before consumption to remove any potential pathogens.
- Avoid consuming raw or undercooked meats and seafood, unpasteurized dairy products, and raw sprouts.

Chapter 2: Chitosan

I.1. Biopolymers:

II.1.1. Introduction:

In recent years, there has been a lot of attention on the term biopolymer, bio-based polymer, and biodegradable polymer in our society. However, it is crucial to understand the differences between them. A recent publication by the IUPAC has provided clarity on these terms. Biopolymers consist of biomacromolecules formed by living organisms, such as proteins, nucleic acids, and polysaccharides. On the other hand, a bio-based polymer is derived from biomass and can partially or completely replace fossil-based materials, offering environmental benefits **(Houili, 2019)**.

Another recent study published in Frontiers in Microbiology in 2021 by **(Nwodo and al.)** explored the use of microbial polysaccharides as a sustainable alternative to petroleum-based polymers in various applications, such as food packaging and biomedical devices. The study highlighted the unique properties of microbial polysaccharides, such as their biocompatibility and biodegradability, that make them attractive for use in these applications **(Nwodo and al., 2021)**.

II.1.2. Definition:

Biopolymers refer to a group of polymers synthesized by living organisms. They are complex molecules that encompass proteins, nucleic acids, and carbohydrates, all of which are essential for the proper functioning of living systems. Proteins consist of amino acid chains that adopt distinctive three-dimensional shapes, while nucleic acids like DNA and RNA serve as carriers of genetic information. Carbohydrates, on the other hand, are made up of long chains of simple sugars and perform functions like storing energy and providing structural support **(Alberts and al., 2002)**.

Biopolymers are indispensable constituents of every living organism and participate in a diverse array of biological procedures. These polymers serve as the structural scaffolding for cells, tissues, and organs, and function as enzymes, transporters, and signaling molecules **(Alberts and al. 2002).** Moreover, biopolymers have enormous potential in various applications, including biotechnology, medicine, and materials science **(Hu and al., 2016)**.

II.1.3. Classification:

Biopolymers can be classified into various categories based on their chemical composition, origin, and structure. Here are some examples of the classification of biopolymers with citations:

- Proteins: proteins are composed of extended sequences of amino acids. These vital molecules have various functions, including maintaining cellular structure, regulating enzymatic activity, and governing regulatory processes. Protein classification depends on their structure, function, and amino acid composition, which enables their organization into distinct groups **(Alberts and al., 2002)**.

- Nucleic acids: Nucleic acids are the genetic material of cells and are responsible for the storage and transmission of genetic information. They are composed of long chains of nucleotides and can be classified into two main types: DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) **(Alberts and al., 2002)**.

- Carbohydrates consist of extended sequences of simple sugars, for various purposes like energy storage and structural support. Carbohydrates can be categorized into multiple groups, based on their chemical structure, such as monosaccharides, disaccharides, and polysaccharides **(Klemm and al., 2011)**.

- Polysaccharides: Polysaccharides are complex carbohydrates composed of long chains of monosaccharides. They can be classified into several groups based on their chemical structure and origin, including cellulose, starch, chitin, and glycogen **(Klemm and al., 2011)**.

- Lipids: Lipids are a diverse group of molecules that are essential for cell structure and function. They can be classified into several groups based on their chemical structure, including fats, oils, waxes, and steroids **(Alberts and al., 2002)**.

These classifications provide a framework for understanding the diversity and complexity of biopolymers. They also highlight the separate roles that biopolymers play in living organisms and the potential applications of biopolymers in various fields.

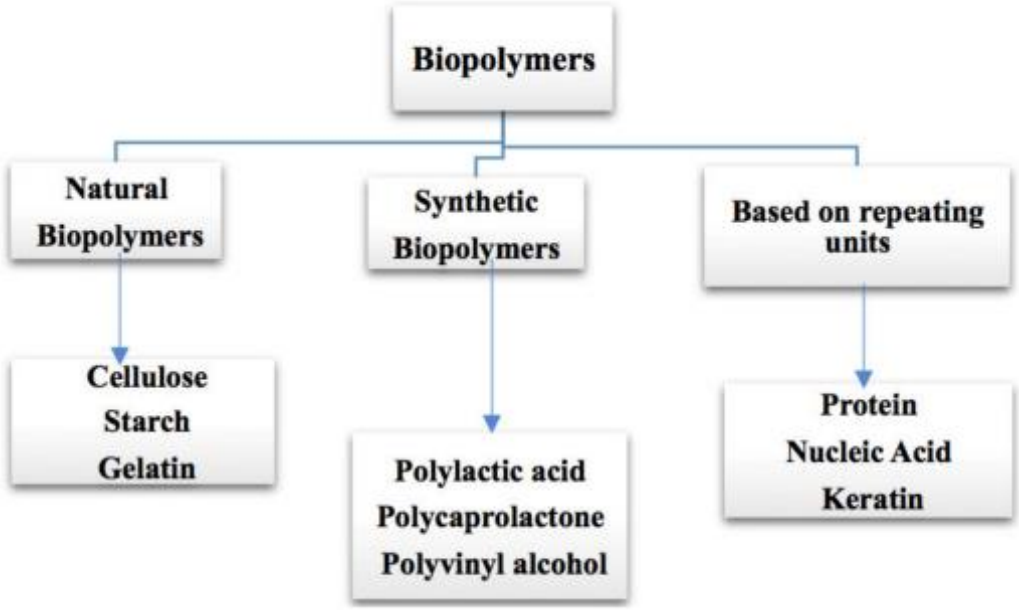

Figure 2: classification of biopolymers based on their origin **(Baranwal and al..,2022).**

II.2. Chitin:

Chitin is a renewable and abundant natural polymer that is easy to obtain. It is the second most prevalent natural polymer after cellulose. Chitins have an a-crystalline structure where the main chains are organized through strong intermolecular hydrogen bonds **(Tolaimate and al., 2000)**.

It is a linear homopolymer of high molecular weight consisting of N-acetylglucosamine (N-acetyl-2-amino-2-deoxy-D-glucopyranose) units linked by β-(1,4) bonds. The main derivative of chitin is chitosan, which is a copolymer of glucosamine and N-acetylglucosamine units linked by 1–4 glucosidic bonds. Chitin is the major component of crustacean shells and insects.,. **(Singh and al., 2017)**.

II.2.1. Chemical structure:

Chitin is an organic polysaccharide polymer produced through a cyclization process of modified glucose. Its molecular formula is (C8H13O5N) n. Unlike cellulose, which is made up of D-glucose, chitin is composed of a polymer of N-acetyl-D-glucosamine **(Hossin and al., 2021**.

ß-glucan is a polysaccharide made up of glucose molecules that are closely associated with chitin. Both chitin-glucan and chitin polymers are insoluble in many solvents but can swell in water. They are also biodegradable when certain enzymes are present **(Bornet and Teissedre, 2005)**.

16% of chitin units are deacetylated. Chitin exists in three different forms: α, β, and γ. α-chitin is the most common and accessible form. In α-chitin, the molecules are arranged antiparallel, which allows for strong hydrogen bonding. β-chitin has a parallel molecular arrangement, leading to weaker intermolecular forces. The only difference between α and β chitin is the way chain stacks are arranged. γ-chitin has characteristics of both α and β forms and is considered a variant of the α family due to its equivalent properties... **(Singh and al., 2017)**.

II.2.2. chitin sources:

For over a century, scientists have reported the presence of chitin in various organisms. Initially, zoologists named all yellow-brown hard structures chitin without chemical analysis, sometimes generating misleading data. Nowadays, it is estimated that a massive portion of the chitin produced in the biosphere is present in the oceans. It is found in aquatic species belonging to phyla such as Cnidaria, Entoprocta, Bryozoa, Porifera, and Mollusca. Moreover, chitin has also been detected in fungi, algae, and Onychophora (velvet worms). However, the most easily accessible sources of chitin are the exoskeletons of Arthropoda, including insects, arachnids, myriapods, and crustaceans **(Bastiaens and al., 2020)**.

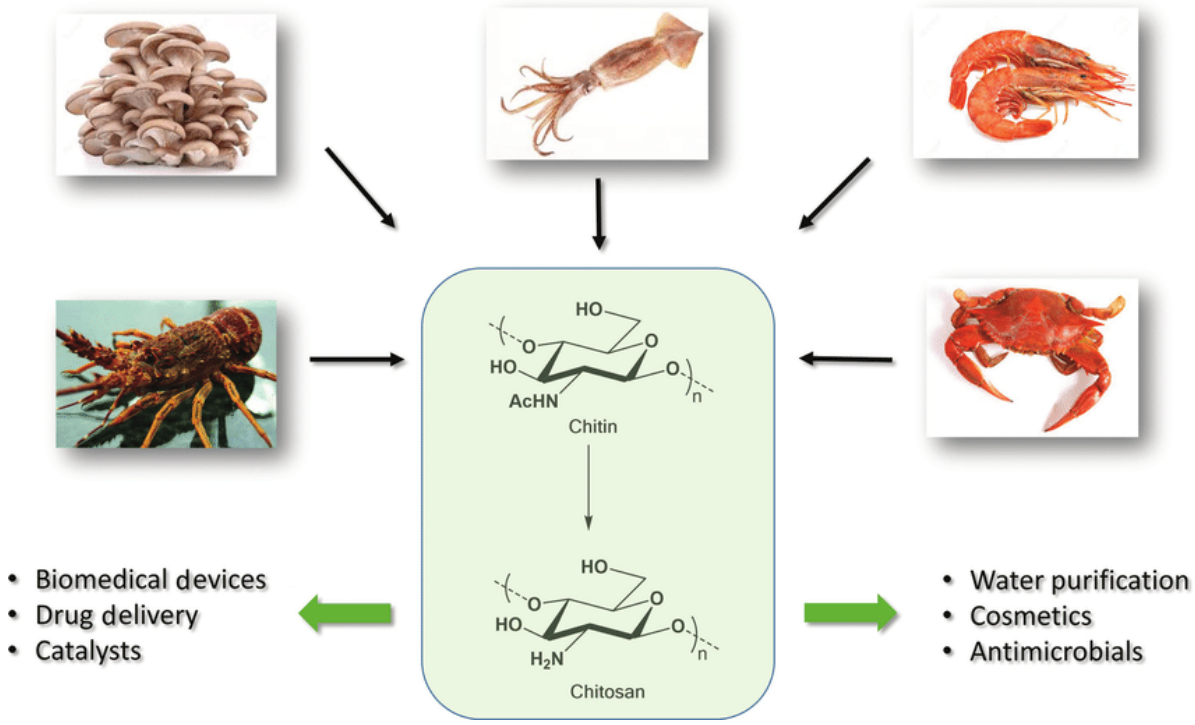

Figure 3: main sources of chitin and chitosan **(Jardine and al.,2017).**

II.3. Chitosan:

Chitosan is derived from chitin through a process called N-deacetylation and is a copolymer of glucosamine and N-acetylglucosamine that are linked by specific chemical bonds. While chitosan is a fiber as cellulose, it exhibits distinct properties such as the ability to form films and unique optical structures **(Youcefi and Riazi, 2012)**.

II.3.1. Chemical structure:

Chitosan is a type of linear polysaccharide that is made up of a copolymer of GlcN and GlcNAc units that are connected through (1→4) glycosidic bonds. Although it can be found naturally in Mucoraceae fungi, it is commonly produced on an industrial scale by subjecting chitin to thermochemical N-deacetylation processes **(Moussa and al.,2019)**.

The degree of deacetylation (DD) serves as the distinguishing factor between chitin and chitosan. Chitosan is typically defined as having a DD ranging from 60-70%, whereas chitin has a lower DD. Conversely, a DD above this range would also classify the compound as chitosan **(Aranaz and al,2009)**.

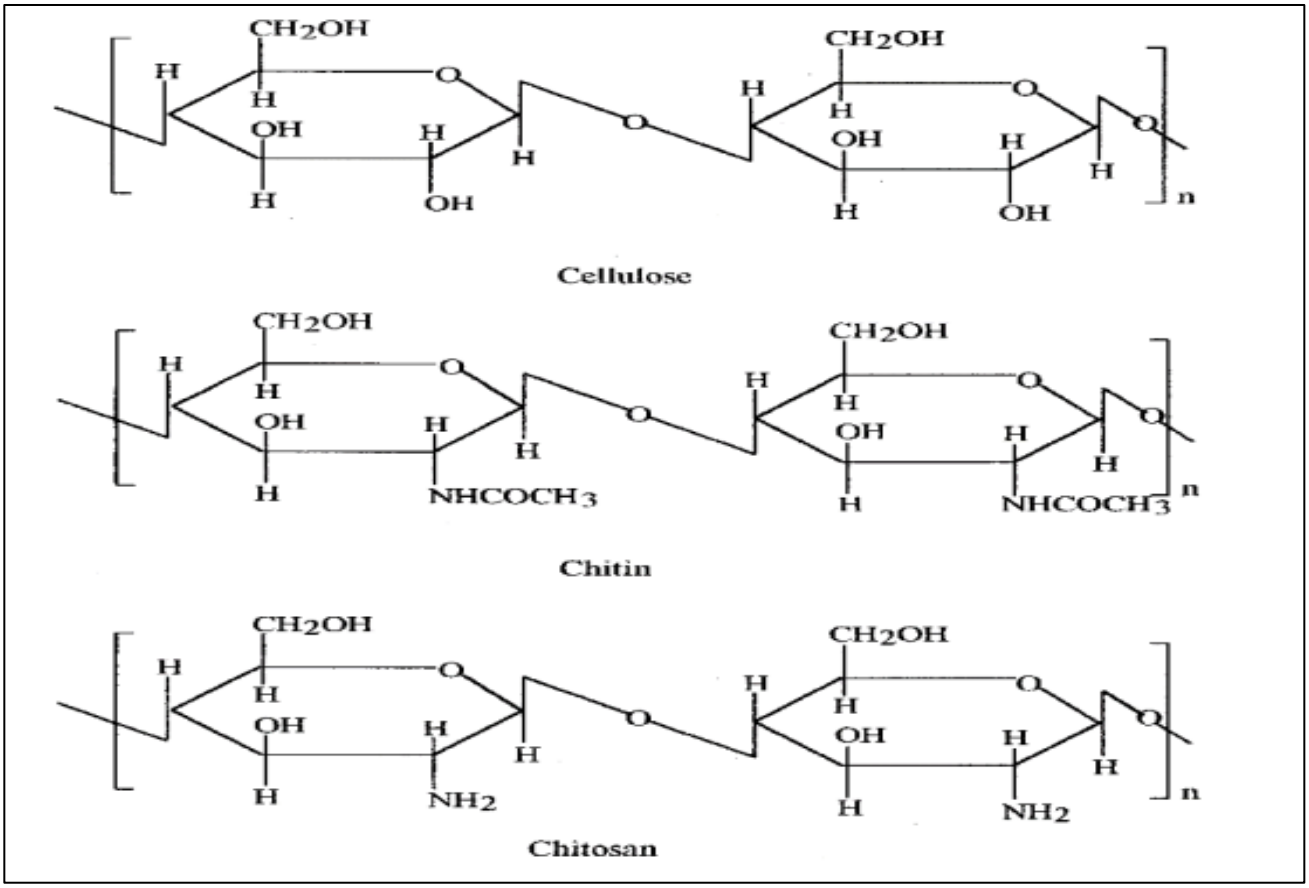

Figure 4: chemical structure of cellulose, chitin, and chitosan **(Ibram and al. 2020)**

II.3.2. Forms of chitosan:

- **Chitosan powder**: Chitosan is commonly sold as a powder, which can be used as a starting material for various applications, such as the production of films, coatings, and gels **(Hirano and al., 2018)**.

- **Chitosan hydrogels**: Hydrogels are three-dimensional networks of polymers that can absorb large amounts of water. Chitosan hydrogels have many potential applications, such as wound healing, drug delivery, and tissue engineering **(Shariatinia and al., 2021)**.

- **Chitosan beads**: Chitosan beads are spherical particles that can be used for controlled release applications, such as drug delivery or environmental remediation **(Guo and al., 2017)**.

- **Chitosan films**: Chitosan can be processed into films, which can be used for food packaging, wound dressings, and other applications where a thin, flexible material is required **(Liu and al., 2017)**.

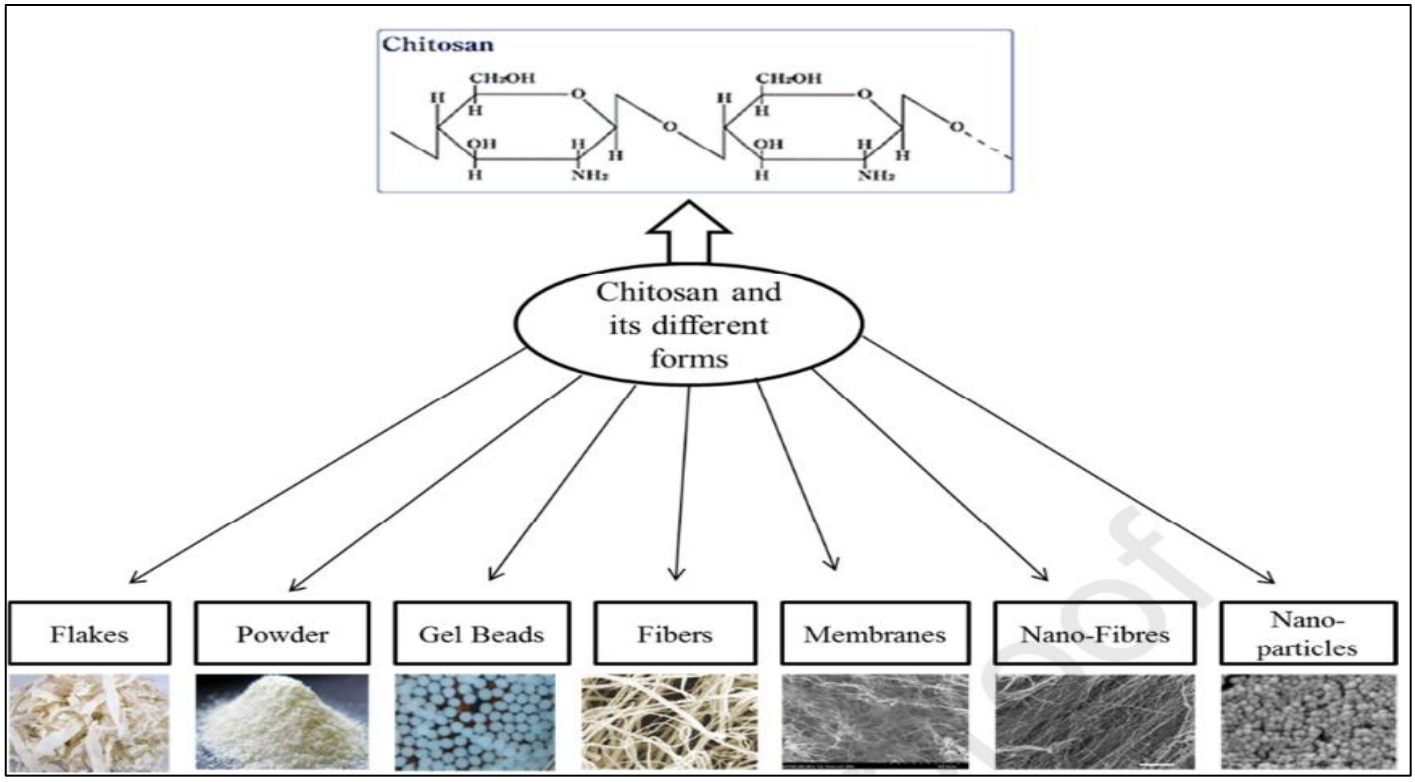

Figure 5: Brief description of different forms of chitosan **(Pal and al., 2019)**

- **Chitosan nanoparticles**: Chitosan nanoparticles are tiny particles that can be used for drug delivery, gene therapy, and imaging applications. They have unique properties such as a high surface area to volume ratio, which makes them effective carriers for drugs and other therapeutic agents **(Madi and al., 2019)**.

- **Chitosan fibers**: Chitosan fibers can be produced through electrospinning or other methods. They have potential applications in wound dressings, tissue engineering, and other biomedical applications **(Jalaja and al., 2017)**.

- **Chitosan scaffolds**: Chitosan scaffolds can be used to support the growth of cells and tissues in tissue engineering applications. They can be produced using various methods such as freeze-drying, salt-leaching, or electrospinning **(Shariatinia and al., 2021)**.

II.3.3. Chitosan applications:

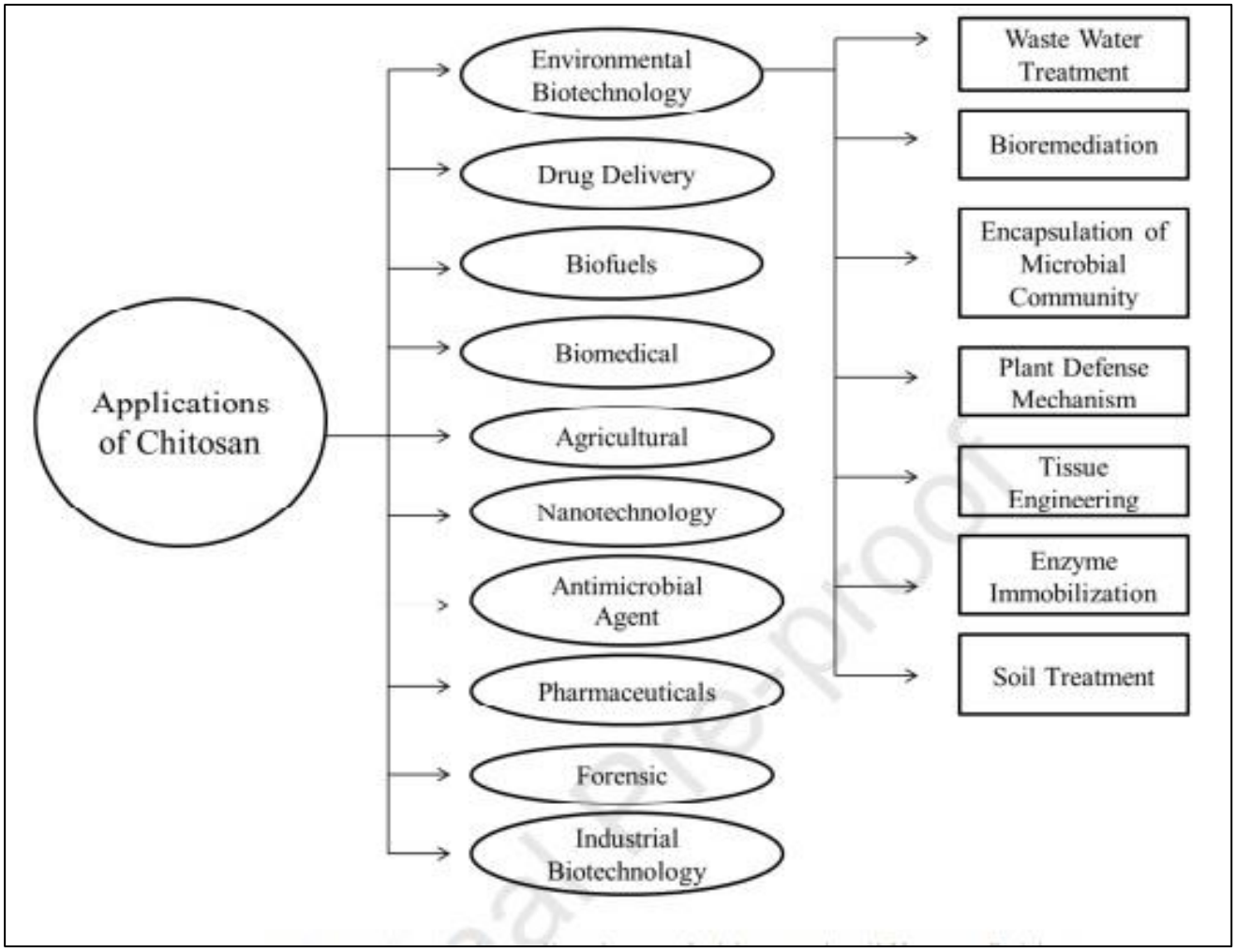

Figure 6: Diverse application of chitosan **(Pal and al. 2019)**

Chitosan solutions exhibit certain properties and have potential applications that depend on several parameters, including the degree of deacetylation, deacetylation pattern, molecular weight, and polydispersity index. It is important to consider these parameters when discussing the potential applications of this polymer. Furthermore, other factors, such as the degree of ionization, should also be considered when using chitosan solutions **(Giraldo and Rivas, 2021)**. Chitosan, as a biopolymer, has garnered considerable attention from researchers owing to its multiple potential applications in diverse fields, such as agriculture, the food industry, biomedicine, paper manufacturing, and the textile industry **(Youcefi and Riazi, 2012).**

II.3.3.1. Agriculture:

There has been a notable surge in research studies investigating the use of chitosan nanoparticles in agriculture, driven by the need for sustainable and environmentally friendly agrochemical products like fertilizers and pesticides. Like their role in medicine, chitosan nanoparticles are utilized as nano-carriers that improve the stability of active ingredients and enable controlled release **(Perez and Francois,2016)**. Chitosan is a promising polymer for the efficient delivery of agrochemicals and micronutrients in nanoparticle form. Due to their biocompatibility, biodegradability, non-toxicity, high permeability, cost-effectiveness, and excellent film-forming capacity, chitosan nanoparticles have been studied extensively as a platform for administering active ingredients in various applications. Moreover, chitosan exhibits broad-spectrum antimicrobial and insecticidal activities and can chelate different organic and inorganic compounds, making it highly suitable for improving the stability, solubility, and biocidal activity of fungicides or other chelated pesticides. Its non-toxic residues degrade at a rate that corresponds to its molecular weight and degree of deacetylation **(Kashyap and al., 2015)**.

As a result, agrochemical products can be applied at lower doses, requiring fewer treatments, reducing the risk of environmental contamination and toxic effects on non-targeted organisms **(Chen and Zeng, 2018).**

II.3.3.2. Food industry:

Chitosan has found its way into the food industry as a natural preservative and a substitute for fat in low-fat foods. Another application of chitosan is its use as a protective coating for fruits and vegetables to increase their storage life. Recently, researchers have demonstrated that chitosan can effectively restrain the growth of microorganisms in food products, which suggests that it can be a viable alternative to synthetic preservatives. One of the studies published in the Journal of Food Science and Technology in 2021 reported that chitosan coatings had a positive impact on the quality and storage life of tomatoes **(Khan and al., 2021)**. Chitosan has also been investigated as a fat substitute in yogurt. A recent study has demonstrated that incorporating chitosan in yogurt can enhance its texture and sensory properties. This finding suggests that chitosan has the potential to be used as an ingredient in the production of yogurt **(Yang and al.,2020)**.

II.3.3.3. in nutrition:

Chitosan has been found to enhance the bioavailability of extraintestinal tissues by reducing oxygen consumption and the absorption of dietary amino acids. It possesses a broad range of biological properties, which make it a promising candidate for various commercial applications. This review shows that the use of chitosan and its derivatives as food additives has positive effects on antimicrobial, antioxidant, immunoregulatory, and blood cholesterol-lowering properties. However, it's important to note that diverse types of chitosan have different biological properties and no single type can exhibit the complete range of its varied properties. Many studies have demonstrated the beneficial effects of chitosan, such as improved nutrient digestibility and growth performance **(Guan and al., 2019)**.

II.3.3.4. Food additives:

Chitosan-alginate nanoparticles' loading capacity has been identified as a valuable asset in the creation of innovative food additives. Researchers have utilized electrostatic deposition to prepare numerous multilayer emulsions that consist of gelatin particles and chitosan-alginate shells. Based on their findings, the emulsion's stability during storage and the droplets' stability in the gastric phase depends on the number of interfacial layers. These delivery systems that rely on alginate-chitosan have great potential in food products such as cheese and beverages due to their ability to mitigate fish oils' fishy taste and low water solubility **(Niculescu, 2022)**.

II.3.3.5. Pharmacy:

Pharmaceutical media including polymers, micelles, liposomes, and nanoparticles have received more attention recently. These systems have several benefits, including improved medication safety and efficacy. Depending on the kind of support, these systems may include hydrophobic and hydrophilic active substances. They can also provide therapeutic products with improved stability against chemical and enzymatic degradation, extended drug influence in the target tissue, increased bioavailability, and drug targeting by including certain ligands **(Yanat and Schroën, 2021)**.

II.3.3.6. Medicine:

Chitosan's capacity to improve blood coagulation and encourage the growth of granulation tissue is thought to be the basis for its wound-healing abilities. It also possesses antimicrobial qualities that might aid in infection prevention in both animal and human trials. Chitosan-based wound dressings have demonstrated the ability to accelerate healing and lower infection rates **(Sinha and al., 2020)**.

Additionally, chitosan can be employed to distribute medications. It is a potential substance for sustained-release drug administration since it can encapsulate medications and shield them from deterioration in the body **(Bajpai and al., 2021)**. A variety of medications, including growth hormones, antibiotics, and anticancer medicines, have been delivered using chitosan.

Chitosan is utilized in tissue engineering as a scaffold material for the regeneration of different tissues, such as bone, cartilage, and skin. It is a desirable material for tissue engineering applications because of its biocompatibility and capacity to support cell proliferation and differentiation **(Kaya and al., 2021)**.

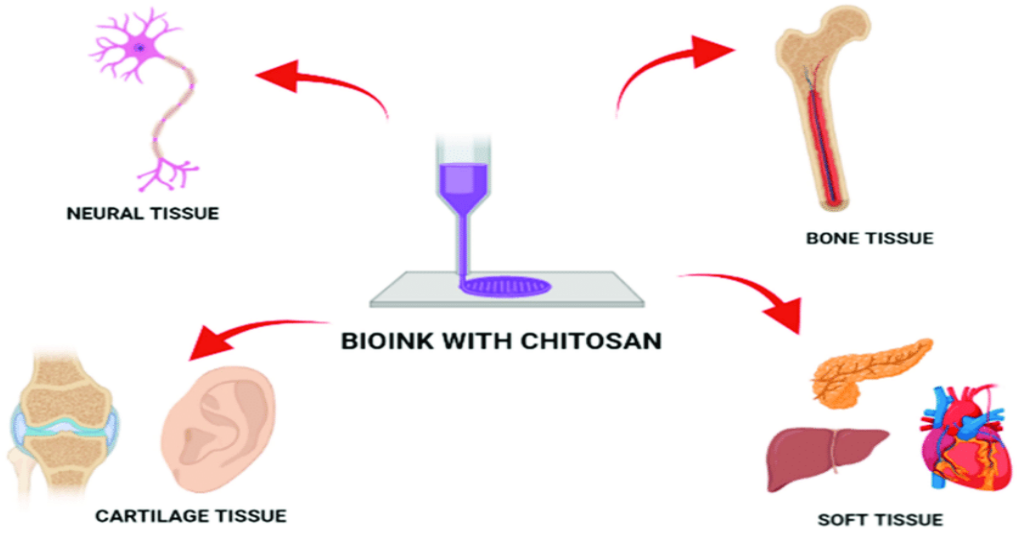

Figure 7: Application of chitosan bio-ink as a material for regenerative medicine **(Kołodziejska,2021)**

II.3.3.7. Cosmetics:

Chitosan can be dissolved in acidic aqueous solutions and can form various structures such as particles, films, scaffolds, and fibers in Micro, Nano, and Milli sizes. Its derivatives and chitin also possess numerous properties that make them valuable active ingredients in dental, skin, hair, and nail care, as well as in the cosmetic and cosmeceutical industries for delivering active ingredients. The physicochemical characteristics of the polymer are strongly associated with these beneficial properties **(Guan and al., 2019)**.

II.3.4. How does chitosan prevent foodborne illness:

- In food packaging: a study that appeared in the journal Food Packaging and Shelf Life showed that chitosan-based coatings may successfully inhibit the development of bacteria and mold on the surface of packed bread **(Basiak and al., 2021)**.
- As a coating: Chitosan coatings were successful in suppressing the development of *Salmonella* and *Listeria monocytogenes* on chicken breast fillets **(Shan and al., 2017)**. Also, in 2021 it was found that chitosan coatings could effectively inhibit the growth of bacteria and mold on the surface of fresh-cut carrots **(Ghasemzadeh and al., 2021)**.
- In food processing: To lower the danger of contamination, chitosan can also be utilized in the food processing industry. For instance, the journal Food Control showed that fresh-cut lettuce and spinach may be cleaned with chitosan to lower the number of pathogens like Salmonella and Listeria **(Yang and al., 2016)**.

II.3.5. Challenges and opportunities in the commercialization of chitosan-based products:

Chitosan's applicability is constrained in several fields, such as wastewater treatment, due to its poor solubility in neutral or alkaline environments. The utilization of chitosan derivatives, chemical modification, mixing with other polymers, and other strategies have all been explored to address this issue **(Kaur and Dhillon, 2018)**. Additionally, due to the unpredictability of the raw material and the employment of dangerous chemicals in the deproteinization and demineralization stages, the manufacture of chitosan from crustacean shells the main source of chitin can be expensive and difficult **(El Knidri and al., 2017)**.

The limited knowledge of its mechanisms of action and the absence of standardized procedures for its characterization and assessment present another difficulty in the commercialization of chitosan-based products **(Kumar and al., 2018)**.

II.3.6. Regulation and market size:

In 2022, the chitosan market was estimated to be worth USD 10.88 billion. From 2023 to 2030, it is expected to increase at a CAGR of 20.1%. The rise is linked to the rising demand for organically produced goods and their extensive use in medicinal, cosmetic, and waste-water treatment applications **(Grand View Research 2021).**

The FDA in the United States has granted chitosan the status of GRAS for use in biomedical applications as well as an additive in food. Italy and Finland have also approved it as an ingredient **(FDA, 2013)**.

Figure 8: market research report on the global chitosan market from 2017-2021 **(Picture: Business Wire).**

II.3.7. Chitin and chitosan extraction:

The extraction of chitin and chitosan is typically accomplished through either chemical or biological means. Chemical methods typically involve the use of powerful acids and bases to dissolve calcium carbonates and proteins **(El Knidri and al., 2018)**.

II.3.7.1. chemical Proceed:

High temperatures and concentrations of a potent alkaline solution are used in the chemical extraction of chitin and chitosan, which causes the breakage of polymer chains and intense deacetylation of chitosan. There are normally three basic phases in this procedure **(Santos and al., 2020)**.

the shells are powdered. Acid treatment is then applied to remove minerals from the powder. Weak acids like formic acid, or hydrochloric acid, can be used for this treatment. Hydrochloric acid (HCl) is commonly used for complete mineral removal. After demineralization, deproteinization is performed using sodium hydroxide (NaOH). The procedure is similar to the previous step, and the ratio of demineralized shells to NaOH depends on the species. Finally, the decolorization of chitin is achieved by treating it with acetone followed by sodium hypochlorite **(Abhinaya and al., 2021).**

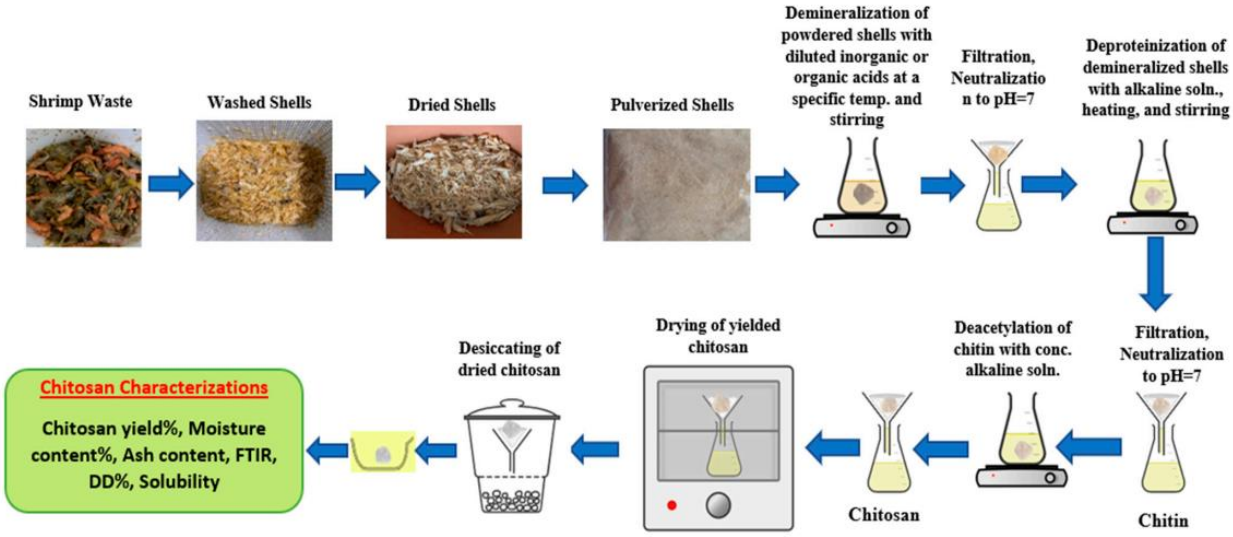

Figure 9: Chitin and chitosan chemical extraction **(Hosney and al., 2022).**

II.3.7.2. Biological Proceed:

The biological extraction approach promotes the synthesis of high-quality chitin using microorganisms to generate enzymes and organic acids at a low cost. This technology is more appealing than the chemical procedure since the production costs are lower **(Santos and al., 2020)**.

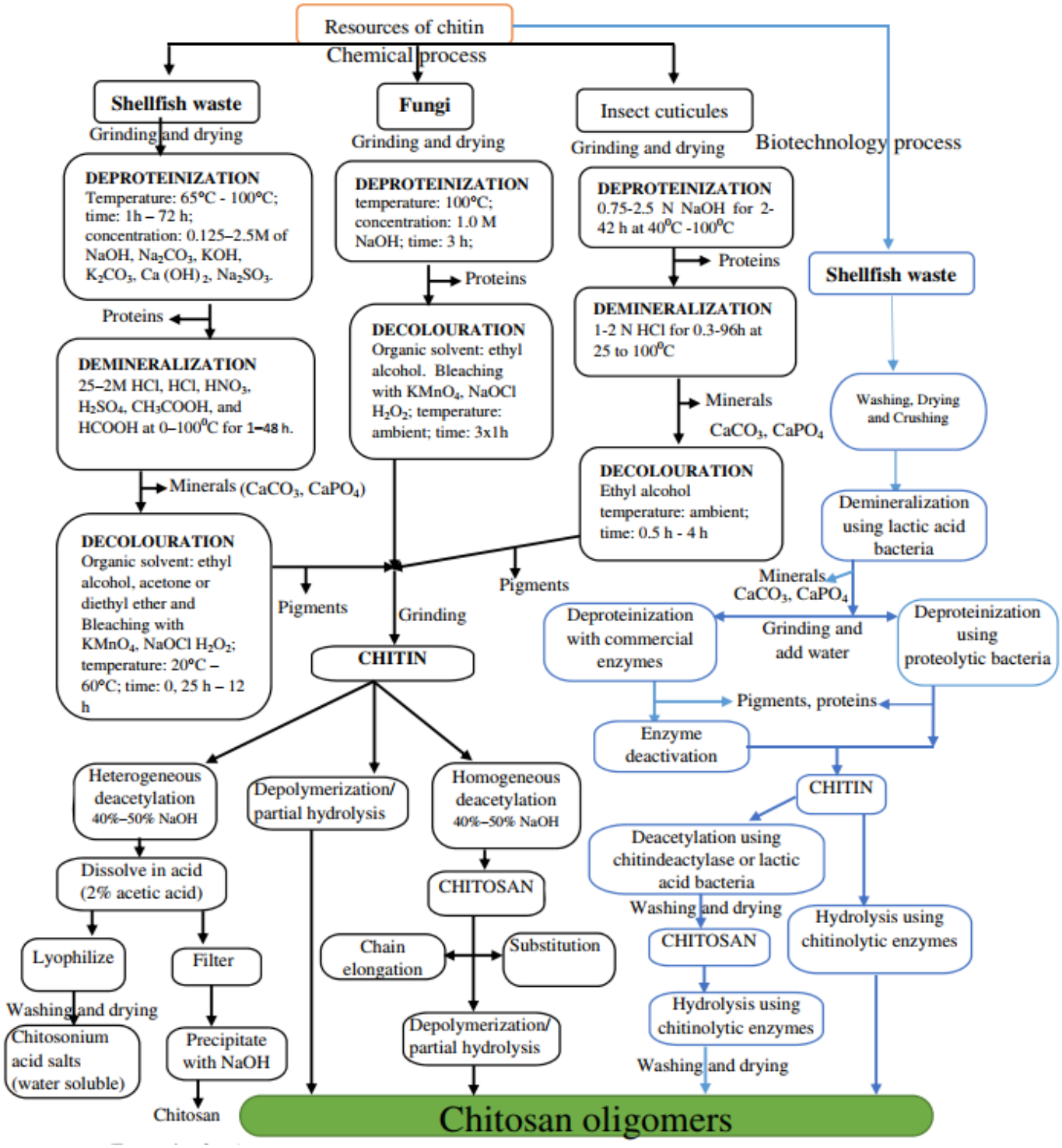

Figure 10: Flow chart of chemical and biological processes for chitin and chitosan **(Abhinaya and al., 2021).**

II.3.8. Chemical and physical properties:

II.3.8.1. Degree of deacetylation DD:

If the pH is less than 6-6.5, chitosan can dissolve and stay in solution in acidic conditions. However, most chitosan's become insoluble when the pH level rises beyond 6.5, which is problematic for biomedical applications that call for an aqueous solution **(Moran and al., 2018)**.

After studying the effect of three degrees of deacetylation (92%, 84%, and 73% DD) on water vapor permeability, it was found that the degree of deacetylation of chitosan does not significantly affect this property. However, the DD of chitosan has a more significant impact on the antimicrobial properties of chitosan films compared to their mechanical and barrier properties **(Wiles and al, 2000)**.

The chitosan polymer (DD) is a key factor to determine how biodegradable it is. Several enzymes, such as chitinases, chitosanases, and lysozymes, which specifically target acetylated groups, can break down chitosan. As a result, high DD (very deacetylated) chitosan polymers degrade more slowly than low DD (highly acetylated) polymers. The number of positive charges that a chitosan polymer can hold is linked to its DD **(Moran and al., 2018)**.

II.3.8.2. molecular weight:

The molecular mass of chitosan was determined using size exclusion high-performance liquid chromatography (SE-HPLC) **(Chang and al., 2015).** a decrease in molecular weight leads to a higher degree of color variation and film solubility **(Jiang and al., 2020)**.

Compared to low molecular chitosan weight, high molecular weight chitosan has better mechanical and barrier properties. The study indicates that the tensile strength of chitosan films increases as the molecular weight of the chitosan increases **(Chen and Hwa, 1996)**.

II.3.8.3 Viscosity:

Due to the handling challenges posed by excessively viscous solutions, polymer viscosity is a crucial technical characteristic. Even if it's not exact, a quick and easy approach can tell you how much of a solution has been hydrolyzed and how much of it has been emulsified. Chitosan's viscosity increases along with its molecular weight. As degradation may result in a decrease in viscosity during polymer storage, it can also be used to assess the durability of the polymer in solution **(Liu and al.,2020) ;(Aranaz and al., 2021)**.

Chitosan's degree of acetylation is one aspect that affects its viscosity; the more deacetylated it is, the more amine groups are free in it, increasing its soluble nature and viscosity. also depends on its concentration (greater concentration causes higher viscosity), temperature (higher temperature causes lower viscosity), and pH (lower pH causes higher viscosity) **(Zemmouri, 2008)**.

II.3.8.4. Solubility:

The solubility of chitosan is a critical parameter for its quality, and greater solubility leads to better chitosan. Several factors, such as temperature and deacetylation time, alkali concentration, the ratio of chitin to an alkaline solution, and particle size, significantly impact the solubility of chitosan **(Kumar, 2017)**

The solubility of chitosan was determined by measuring the solid content that is soluble in water (p/v) in the test solution. This solubility depends on a range of factors, including the molecular weight of the polymer, degree of acetylation, pH, temperature, and crystallinity of the polymer **(Aranaz and al., 2021).**

According to **(Chang and al., 2015)** It is measured by the following equation: ($S = 50 - m$)

S: Solubility in (mg/ml) the value of fifty is in (mg).
M: Dry weight of insoluble solid in(mg).
V: Volume of the sample equal to 5 ml.

In comparison to chitin, chitosan can be easily dissolved in some diluted acids, but as a result of its high crystallinity qualities, it can only be dissolved in polar acidic solutions. The solubility is influenced by the kind (organic or mineral) and quantity of the utilized acids **(Hazmi and al., 2021)**.

II.3.8.5. biocompatibility:

Chitin and chitosan cannot be found in mammals, but they can be broken down in vivo by various proteases, such as lysozyme, papain, and pepsin. As they degrade, they release non-toxic oligosaccharides of different lengths that can be incorporated into glycosaminoglycans, glycoproteins, metabolic pathways, or excreted. The rate of degradation appears to be influenced by the degree of crystallinity, which is controlled by the degree of deacetylation **(Aranaz and al., 2009)**.

II.3.8.6. Biodegradability

A detailed investigation demonstrated that the susceptibility to enzymatic degradation initially increased for DAs greater than 50%, followed by a decrease as the DA decreased, and there was no degradation observed for chitosan with a DA of 3 **(Shoenfeldt and al, 2002)**.

The effect of DA is evident in both in vitro (degradation by lysozymes) and in vivo (subcutaneous implantation) **(Dupasquier, 2011).**

II.3.9. Biological properties:

II.3.9.1. Antibacterial activity:

It is generally known that chitosan has antibacterial action against a range of bacteria, however, this activity is also restricted by chitosan's poor solubility, which is caused by its stiff crystalline structure. However, the existence of free amino groups allows producing more soluble molecules by generating chitosan through a variety of regulated chemical processes. Quaternization and hydrophilic substitution boost solubility by altering the structure. Studies using chitosan derivatives have shown that they have better antibacterial activity than chitosan itself at pH 7 against a variety of microorganisms, including *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa* **(Confederat and al., 2021)**.

Also, Increasing the degree of deacetylation in chitosan enhances its antimicrobial effectiveness **(Cuero, 1999)**.

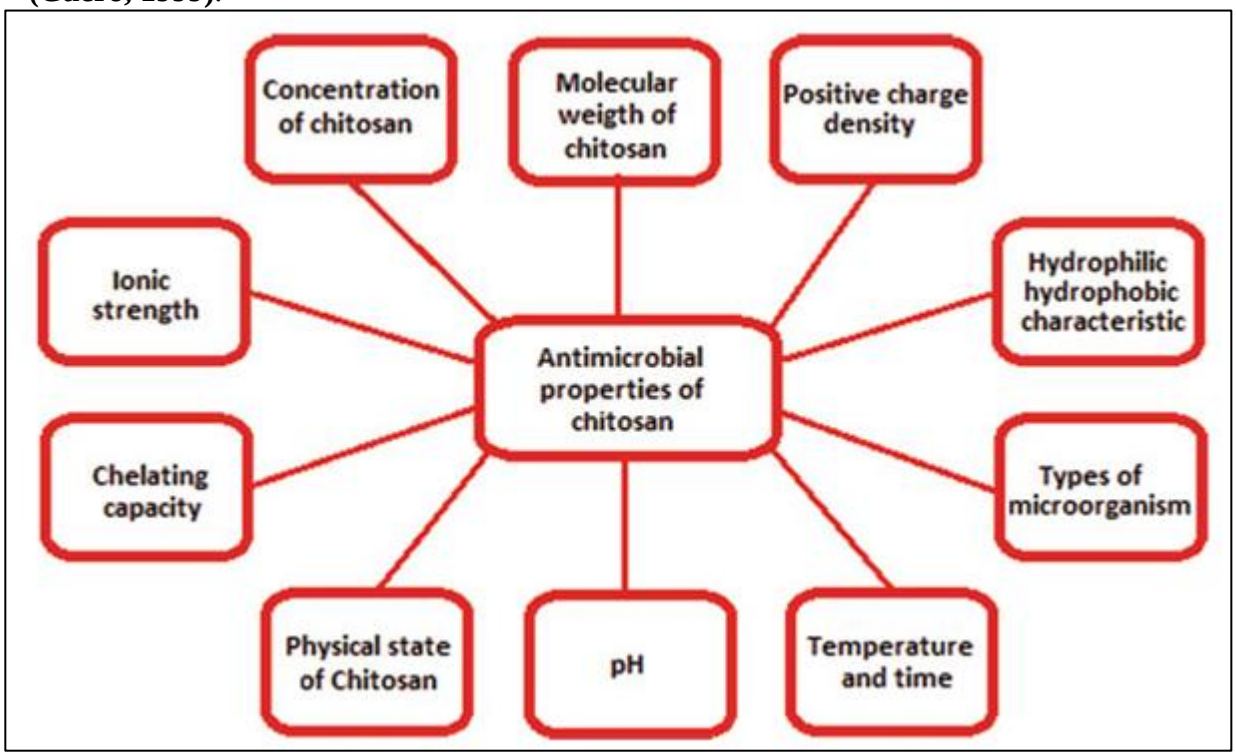

Figure 11: Factors affecting the antibacterial properties of chitosan **(H. YilmazAtay.2019).**

II.3.9.2. Antifungal:

Fungal infections are becoming more prevalent worldwide and pose a significant concern, especially among immunocompromised patients such as those with diabetes, HIV infection, various neoplasms, immunosuppressive therapy, and extended hospital stays in intensive care units. Whether occurring as a primary infection or superinfection after viral or bacterial infections, treating fungal infections can be challenging, and the outcomes are often unfavorable **(Confederat and al.,2021)**.

Candida, Aspergillus, Cryptococcus, and Pneumocystis are among the most commonly implicated fungal genera in human pathology, accounting for more than 90% of reported deaths due to fungal infections. The limited therapeutic options are available, and the high toxicity of existing drugs make treating these infections challenging. Furthermore, there is a significant interest in developing new antifungal agents that offer a more favorable safety profile **(Confederat and al.,2021)**.

Chitosan has been shown to exhibit important antifungal activity. Based on current data, fungi are more susceptible to chitosan's antimicrobial effects than bacteria. The antifungal activity of chitosan is influenced by a range of factors, including molecular weight, degree of deacetylation, chitosan concentration, and pH **(Confederat and al.,2021)**.

Chitosan's antifungal activity involves its ability to interact with cell membranes, altering their permeability. For instance, certain cations can inhibit fermentation with baker's yeast by preventing glucose entry through the yeast cell surface. Studies using UV absorption analysis have demonstrated that chitosan can cause a substantial loss of protein materials in Pythium arcandrum **(Rabea and al, 2003)**.

II.3.9.3. Antioxidant:

The use of certain antioxidants has been limited in some countries due to potential health risks. As such, there has been an increased focus on studying the antioxidant activity of chitosan and its derivatives. Research has shown that chitosan and its derivatives exhibit antioxidant properties by removing oxygen radicals, including hydroxyl, superoxide, alkyl, and highly stable DPPH radicals, as observed in vitro testing **(Younes and Rinaudo, 2015)**.

Studies have shown that the antioxidant properties of chitosan are influenced by its degree of deacetylation and polymer concentration. The primary amino groups present in chitosan play a crucial role in scavenging free radicals by forming NH3+ groups. Of the four forms of amino groups, primary amino groups, amino groups, secondary amino groups, and Qatarized amino groups, the latter exhibits remarkable antioxidant activity against hydroxyl radicals. Specifically, deacetylated chitosan with low molecular weight exhibits significant antioxidant properties, making it a promising natural antioxidant **(Confederat and al., 2021)**.

II.3.9.4. Anti-inflammatory:

Research has shown that chitosan has anti-inflammatory effects. In vitro and in vivo studies have shown that chitosan can reduce inflammation by lowering the production of inflammatory cytokines and chemokines **(Chellappan and al., 2020)**. In vitro, research has also revealed that chitosan oligosaccharides, which are produced from chitosan, have anti-inflammatory characteristics **(Kim and al., 2019)**. Additionally, in vivo, research has shown that chitosan can reduce inflammation in a mouse model of sepsis by blocking the TLR4/NF-B signaling pathway as well as in a rat model of arthritis by enhancing the immunomodulatory effect of adipose-derived stem cells **(Cui and al., 2020)**.

It's interesting to note that chitosan nanoparticles have been demonstrated to have anti-inflammatory effects in plants **(Mohammed and al., 2017)**.

II.3.9.5. Crystallinity:

The degree of crystallinity is a key factor in affecting the accessibility to internal sites, water swelling, and diffusion of chitosan, among other features. Usually, crystallinity is assessed by X-ray diffraction techniques. Chitosan crystallizes in the orthorhombic system and is a semi-crystalline material. Chitosan I, which has a low degree of deacetylation and is in the salt form, is less ordered than chitosan II, which has a high degree of deacetylation and is in the amine form **(Aljawish, 2013)**.

Material and methods

Our work involves establishing a process for extracting chitinous products from shrimp shell waste, with the aim to produce a biopolymer called chitosan, and evaluate its contribution to mitigating foodborne illnesses, the study aims to assess the antimicrobial properties of chitosan against common foodborne pathogens. Additionally, it seeks to investigate the effectiveness of chitosan as a natural preservative in extending the shelf life of food products. and sensory evaluations to determine the efficacy and acceptability of chitosan-treated food samples.

III .1 Materials:

III .1.1 Chitosan origin:

The shrimp waste, originating from multiple fisheries in Tlemcen province, which we have collected, consists of shrimp shells that served as the raw material for chitin and chitosan extraction.

III .1.2 Propolis origin:

Samples of raw propolis were gathered from beekeepers in Tlemcen (bnisnous region) The samples were collected during autum, and were ere stored at a temperature of -4°C.

III .1.3 Pathogenic strains.:

the pathogenic strains used in this study are summarized in the table below

Table 3: strains description **(original).**

Stains	***Escherichia coli* ATCC 25922(-)**	***Staphylococcus aureus* ATCC 6538(+)**
Reference	Research laboratory LAMAABE,Tlemcen	Research laboratory. LAMAABE,Tlemcen
Culture mediums	BHI (réf. 804251, Laboratory Conda S.A, Madrid, Spain)	BHI (réf. 804251, LaboratoriConda S.A, Madrid, Spain)
Growth temperature.	37 °C	37 °C

III .1.4 culture medium:

The pathogenic bacteria, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 6538, were reactivated and cultured using 9 ml of Brain Heart Infusion broth (BHI) (reference 804251, Conda S.A. Laboratory, Madrid, Spain) obtained from pre-cultures. The incubation was carried out at a temperature of 37 °C for 24 hours.

The Mueller-Hinton medium (reference TM 325, TITAN BIOTECH LTD, Rajasthan, India) with a pH of 7.3 ± 0.2 and soft agar was used to evaluate the sensitivity of the selected test bacteria to chitosan extracts derived from shrimp shell. These culture media were sterilized in an autoclave (SANO clav) set at 121 °C for 15 minutes. The strains were cultured at least three times before the experiment.

III .1.5. Rats*:*

Rats have become a species of choice due to metabolic similarities with the human species, their small size, docile nature, short lifespan (2-3 years), and short gestation period. Our study focused on females to avoid variability between the two sexes, from the Wistar Albino (*Ratus norvegicus*) strain, aged 2 to 3 months and weighing between 200 and 300g provided by the laboratories of Mostaganem.

III .2. Methods:

III .2.1. The pre-treatment of shells:

Firstly, the shrimp shells were meticulously washed with tap water to meticulously remove any remnants of flesh and residual tissues. We repeated the operation 2 more times, then left it to dry under the sun's warmth for a duration of 12 hours. **(Padida and al., 2020; Al-dubakel & Alshatty, 2018)**.

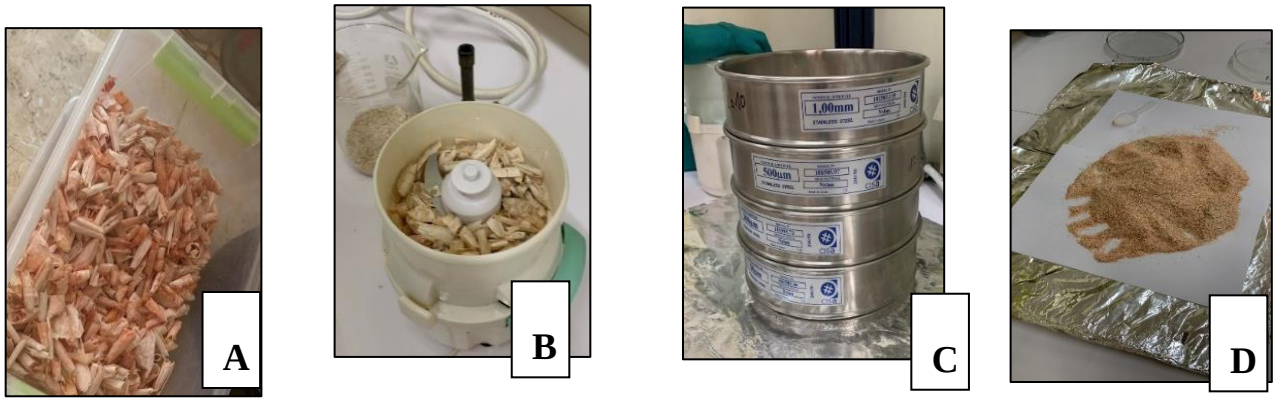

Figure 12: pretreatment steps of shrimp's shells **(original).**

A) dried shells B) Grinder

C) sieve D) carapace flour or powder

The dried shells were crushed and sieved through various diameters ranging from 500 µm to 150 µm,

III .2.2 Chitosan chemical extraction:

The extraction of chitosan from crustacean shells involves a complex process in 3 steps which are demineralization, deproteinization, and deacetylation. These processes require high temperatures and the use of strong acids and bases, along with breaking down the complex structure of the shell, extracting chitin, and subsequently converting it into chitosan. **(Varun and al., 2017)**

✓**Step1:** Acid demineralization

100g of dry powder from crushed shells (300-150 µm) will be dispersed in a 2N HCl solution. The solid-to-solvent ratio is 1:15 (w/v), meaning the weight of the dry powder from the shells divided by the volume of the diluted HCl solution is 1:15.

The solid/solvent mixture is then kept at 50°C under constant agitation for 30 minutes. The resulting product is subsequently recovered, filtered, and washed several times with distilled water until a neutral pH of 7 is obtained. The recovered product is then dried in an oven for 24 hours at 50°C.

- ✓ **Step 2:** deproteinization **(NaOH)**

The demineralized product is agitated in a 50% NaOH alkaline solution for 1 hour at a high temperature of 90°C. The ratio of the product to NaOH is 1:10 (w/v), meaning the weight of the dry powder from the shells divided by the volume of NaOH.

Afterward, the treated product is filtered to separate it from the alkaline solution. It is then washed with distilled water and finally dried in an oven at 50°C for at least 24 hours or until a stable weight is achieved.

- ✓ **Step 3:** deacetylation

Chitosan is obtained through the deacetylation of chitin. This process is typically carried out in a 50% NaOH solution at a temperature of 121°C for 30 minutes using an autoclave. The ratio used is 1:15 (w/v), meaning the weight of chitin to the volume of the NaOH solution.

After being acquired, the chitosan is filtered to remove it from the mixture. The sodium hydroxide (NaOH) is then rinsed away with distilled water until the pH of the wash water is neutral. Then washing and filtration are conducted to eliminate any residual impurities or acid residues (Kaur and Dhillon, 2018).

we can go for more steps such as drying, milling, or other techniques to reach the desired particle size or form

Based on (**Youcefi and Riazi, 2015)** protocol, we produced chitin and then transform it into chitosan as in the Pictures

Figure 13: Chitosan extraction steps **(original).**

III .2.3 Solubilization:

Based on **(Hekiem and al., 2021)** A solution of chitosan powder was prepared by dissolving 1 g of chitosan powder in 100 ml of acetic acid solution, with concentrations of acetic acid solvent (1%, (v/v)). Afterward, the solution was subjected to agitation for 4 to 8 hours on a hot plate set at 70°C.

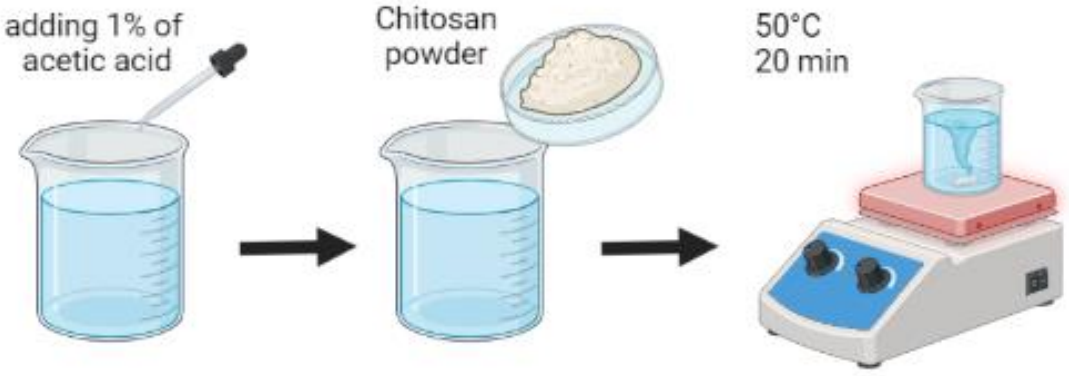

Figure 14: Chitosan solubilization protocol **(original).**

III .2.4 Propolis extraction:

To initiate the extraction process, 1 gram of finely ground propolis in the dark was dissolved in 100 milliliters of 70% ethanol. Subsequently, an ultrasonic probe was inserted into the solution, and the extraction took place for a duration of 15 minutes at a frequency of 20 kHz using the (R. Espinar, S.L.).

Following the extraction, the solution underwent centrifugation at a speed of 4000 revolutions per minute for 10 minutes. The supernatant, containing the desired components, was carefully separated from the sediment.

To refine the supernatant and eliminate any residual impurities, it was subjected to filtration using Whatman 4 filter paper. This filtration step effectively removed solid particles and ensured a purified supernatant solution, The propolis sample was re-extracted two more times using the same conditions, to extract the maximum possible number of bioactive compounds from the raw propolis **(Oroian and al., 2020).**

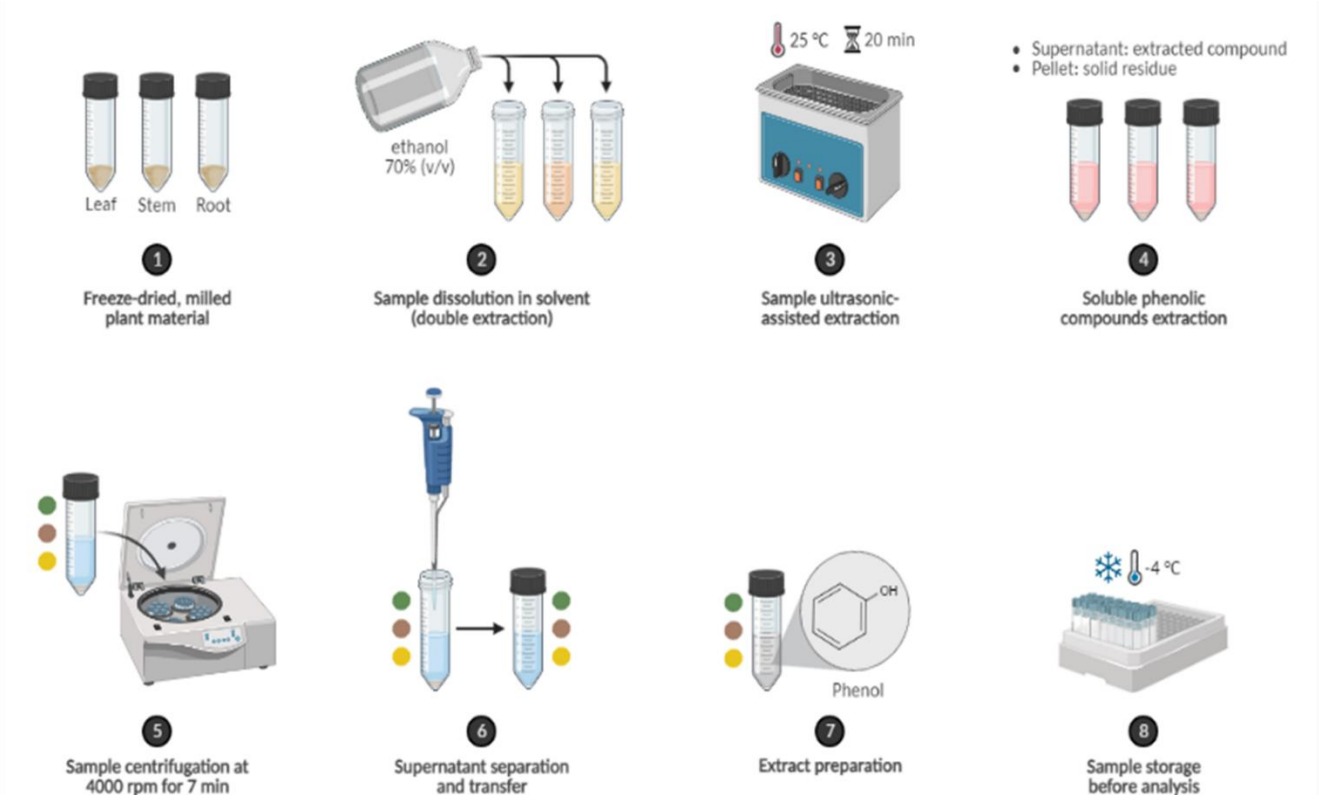

Figure 15: propolis ultra sound extraction method **(original).**

III .2.5 strains Revival and Preservation:

Colonies from the tubes containing the preserved strains on inclined agar were transferred into a sterile test tube containing 5 ml of nutrient broth. The test tube was incubated at 37°C, which is the optimal temperature for the growth of these bacteria. After 24 hours of incubation, the growth of the strains was assessed by observing the appearance of turbidity in the medium, indicating bacterial growth Strain

During the purification step, isolation of strains is achieved by streaking them onto agar plates prepared in Petri dishes, using specific media. Incubation of the plates is then performed at 37°C for 24 hours. The purity of the strains is determined by observing colonies that exhibit uniform size and appearance after the incubation period. To further confirm the purity, Gram staining is conducted, and a microscopic examination is carried out

To preserve purified strains, short-term and long-term preservation methods are commonly used. For short-term preservation, colonies are inoculated on inclined agar and stored at 4°C for a few weeks. For long-term preservation, cryoprotectants such as glycerol or dimethyl sulfoxide (DMSO) are added to the culture media, and the strains are stored at -20°C.

In this study, young cultures (18-24 hours) in nutrient broth were used. The cultures were inoculated into inclined tubes and incubated for 24 hours at 37°C. After incubation, 3ml of BHIB (Brain Heart Infusion Broth) containing 15% glycerol was added to each tube. The mixture was vortexed, and 2 ml of the mixture was transferred to an Eppendorf tube. This process was repeated three times, resulting in three Eppendorf tubes preserved in the freezer at -4°C to minimize the risk of loss.

Note: Glycerol serves as a cryoprotectant, preventing cell rupture during storage.

III .2.6 Evaluation of the sensibility of pathogenic strains to chitosan extracts:

The determination was conducted utilizing the direct contact technique on agar medium, as described in the study by **(Bousmaha-Marroki and al.,2007)**.

- Paper discs, Wattman N°1, with a diameter of 6 mm were prepared and placed in a tightly closed glass jar that was sterilized in an autoclave.
- A 1% initial stock solution of chitosan was prepared and subsequently added to Muller-Hinton medium (MH, Conda Pronadisa, Spain) to achieve the desired concentration range.
- The resultant agar solutions were promptly mixed and poured into sterile plates.

- After that, disks were merged with strains of *Staphylococcus aureus* (ATCC 6538) and then inoculate in the disks in the plate at a temperature of 30°C for a duration of 24 hours.

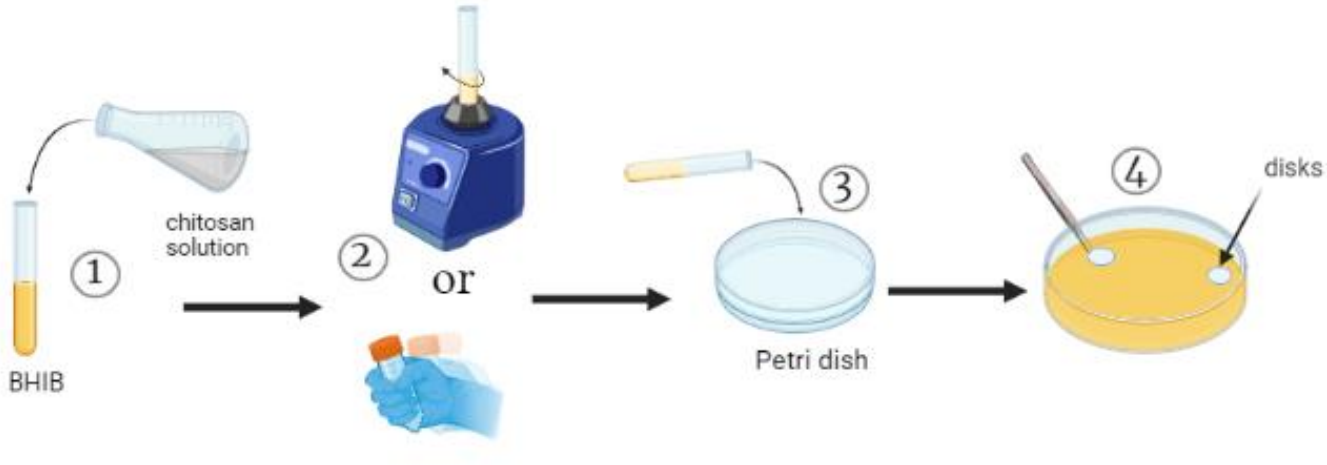

Figure 16: anti-microbial activity protocol **(original).**

III .2.6.1 MIC (Minimum Inhibitory Concentrations):

The MIC (Minimum Inhibitory Concentrations) is also determined against the target bacteria. The inoculum of each tested bacterium is obtained from an 8-hour incubation pre-culture, and the microbial load is adjusted to 108 CFU/ml based on a standard turbidity of 0.5 McFarland. The antibacterial effect of chitosan study at concentrations of 20%, 50%, 80%, and 100% chitosan *on Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 6538 was evaluated by determining the Minimum Inhibitory Concentration (MIC**) (Lima in 2020)**.

The inoculum is adjusted to 1-2x108 cells/mL (OD of 0.08 to 0.13) by measuring the optical density at a wavelength of 625 nm. The final cell concentration of 1-2x107 cells/mL is achieved by diluting the initial inoculum at a 1:10 ratio in physiological water. For the preparation of the microplate. The culture medium used is liquid Muller Hinton.

Chitosan was diluted in DMSO to achieve concentrations (1%,2%) 96-well plates (Iwaki brand, Asahi Techno Glass, Japan) were prepared as follows: 100 µl of BHI broth + 100 µl of the previously prepared serial dilutions + 100 µl of the inoculum. The last wells in the plate represented the negative and positive controls, and two replicates were included for repeatability testing. After 24 hours of incubation at 37 °C, the MIC value was defined as the lowest concentration of our extracts capable of inhibiting all bacterial growth, resulting in a complete absence of turbidity.

The degree of turbidity was evaluated by spectrophotometry at an absorbance of 630 nm using a microplate reader (ref. Microplate Reader, RT-2100C).

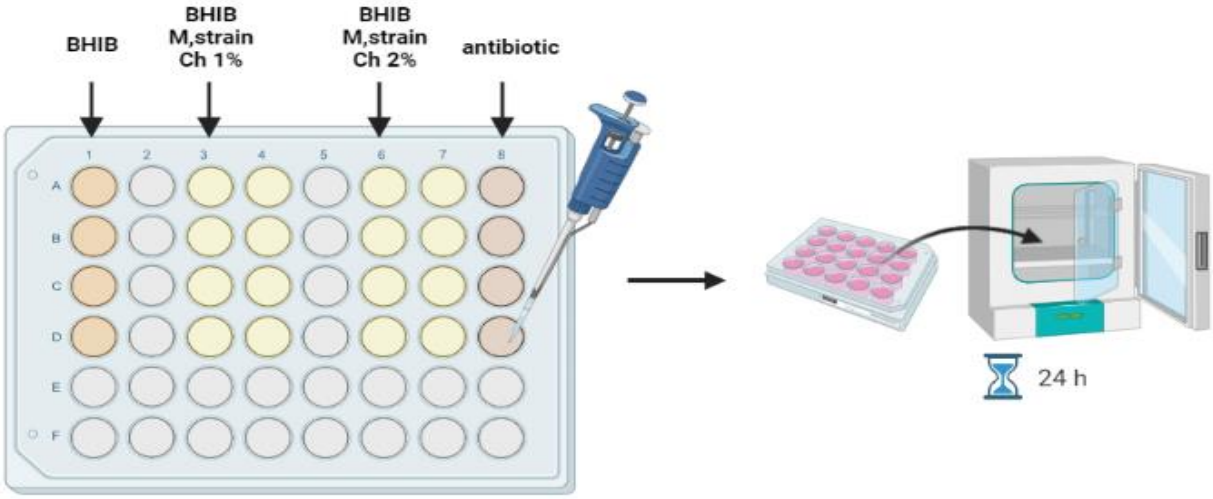

Figure 17: Chitosan MIC protocol **(original).**

III .2.6.2 MBC (minimum bactericidal concentration):

The MBC, or Minimum Bactericidal Concentration, is defined as the minimum concentration of the antibiotic required to eradicate 99.9% of the final cell concentration. Following the determination of the MIC (Minimum Inhibitory Concentration), the two wells containing antibiotic concentrations exceeding the MIC are selected for the determination of the MBC.

To perform this determination, a 20µL sample from each well, exhibiting no visible growth, will be aseptically transferred into Petri dishes containing Muller Hinton Agar (MHA) medium. The Petri dishes will be subsequently incubated at 35°C in a controlled environment for a duration of 20 hours. This technique enables us to assess the viability and culturability of the cells. The MBC is indicated by a colony count of less than three in the respective dish.

III .2.7 Rat treatment:

III .2.7.1 rat acclimation conditions:

Healthy rats were adopted from a local rat breeding located in mostaganem. The breeding of the animals took place within the animal facility of the Department of Biology, Faculty of Natural and Life Sciences, Earth and Universe Sciences, Abou Bekr Belkaïd University (Tlemcen,buhanak).

These animals are kept under favorable conditions: Temperature between 18 to 22°C, ambient humidity (40-70%), sufficient ventilation, 12H/12H light-dark cycle.

The cage was changed every day throughout the experimental period to ensure proper hygiene for the rats by replacing the sawdust bedding. The animals were fed a standard diet in the form of pellets, and the nestle waters used to hydrate them in the washed water bottles were constantly refreshed.

- Before the experience started, we sterilized the cages, water bottles, water, and rat diet.
- Note: The animals fasted before sacrifice.

Ethical clearance:

This research was equipped with ethical clearance obtained from the Health Research Ethics Commission, Faculty of Nature and Life Sciences, and Earth and Universe Sciences.

III .2.7.8. Creation of biofilms based on chitosan:

First, we take out dissolved chitosan in acetic acid, add plasticizer(glycerol) as an addition for more flexibility and to improve film properties, and stir for a viscous and homogeneous solution

Then we Pour the chitosan solution into a clean, flat surface or use a casting frame to obtain a uniform film thickness. Then we Spread the solution evenly using a blade or a suitable tool. The thickness of the film can be controlled by adjusting the amount of solution and the casting method.

We Dry the cast film under controlled conditions. This can be achieved by air-drying the film at room temperature or using an oven at a low temperature. The drying time will depend on the film thickness and environmental conditions. Once the film is completely dry, carefully peel it off the casting surface and handle it with care to avoid damage we Stored the chitosan film in a dry and airtight container to prevent moisture absorption **(Priyadarshi and Rhim, 2020)**.

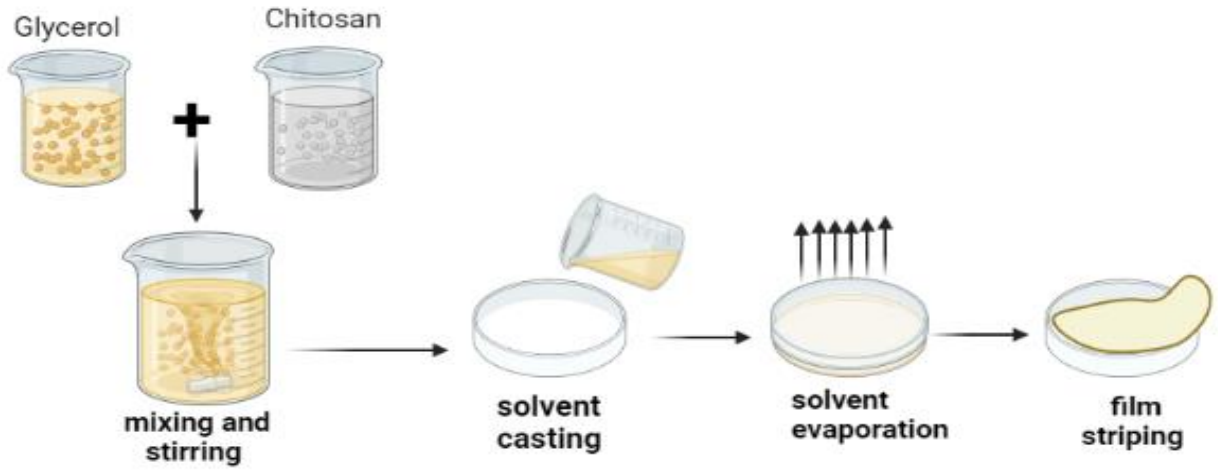

Figure 21: Bio films protocol **(original)**.

Results and discussions

IV.1 Extraction of chitosan Result:

As is shown in Figure (x), after the extraction we got chitin with a reddish color close to orange after deacetylation of chitin to produce chitosan to remove the reddish color to bright color during the removal of an acetyl group and we got "off white "color for chitosan same as **(Tamzi and al., 2020)**. As for the chitosan yield, we got 48% which is a little bit more than the average result.

Based on **(Hosney, and al., 2022)** the deacetylation alkali concentration is the most crucial parameter that affects chitosan yield, followed by the concentrations of acid and alkali used in demineralization and deproteinization.

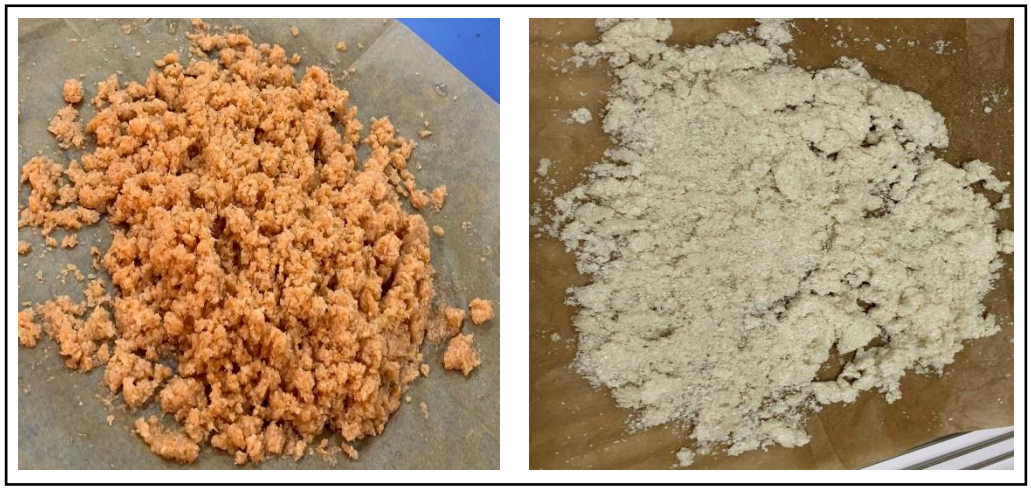

Figure 22: Chitin and chitosan during extraction **(original)**.

IV.2 Propolis extraction result:

Ultrasound-assisted extraction is considered an environmentally friendly and economically feasible alternative to traditional methods for extracting natural products. The advantages of UAE, such as reduced extraction and processing time and decreased consumption of energy and solvents, are primarily attributed to the mechanical effects of acoustic cavitation **(Bankova, Trusheva, and Popova, 2021**).

Furthermore, it has been shown that the composition of the extracted compounds varies significantly depending on factors such as the specific plant used, the extraction materials employed, the climate conditions, and the timing of collection **(Pobiega and al., 2019**). Notably, the EEP of Benisnous is observed to possess exceptional clarity, displaying a distinct mustard yellow hue.

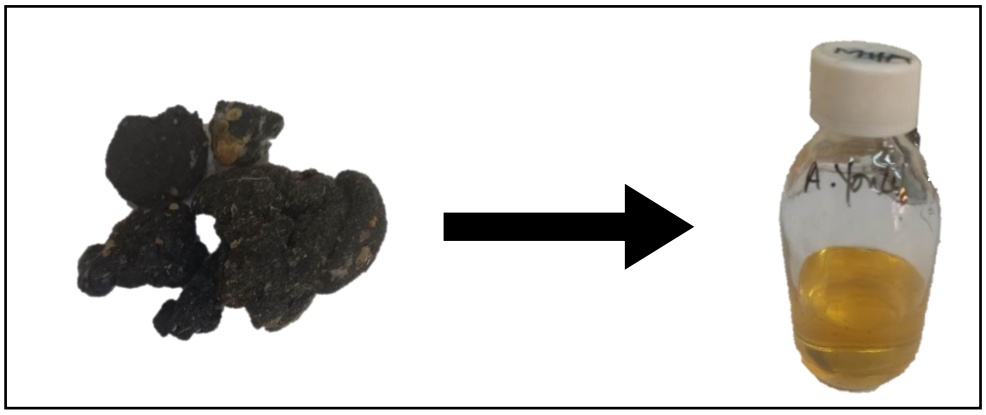

Figure 23: propolis before and after extraction (original)

IV.3 the sensibility of pathogenic strains to chitosan extracts:

After 24h of incubation, all the Petri wells didn't show any of microbial proliferation (total inhibition) same as **Shin and al., (2019).** This confirms the chitosan antibacterial activity against microbial strains.

Based on **(Yilmaz Atay, 2020).** The most common antibacterial action of chitosan is by binding to negatively charged bacterial cell walls, which disrupts the cell and changes membrane permeability, followed by attachment to DNA, which inhibits DNA replication and ultimately causes cell death. Another potential explanation is that chitosan functions as a chelating agent that specifically binds to components that produce toxins and prevents microbial development.

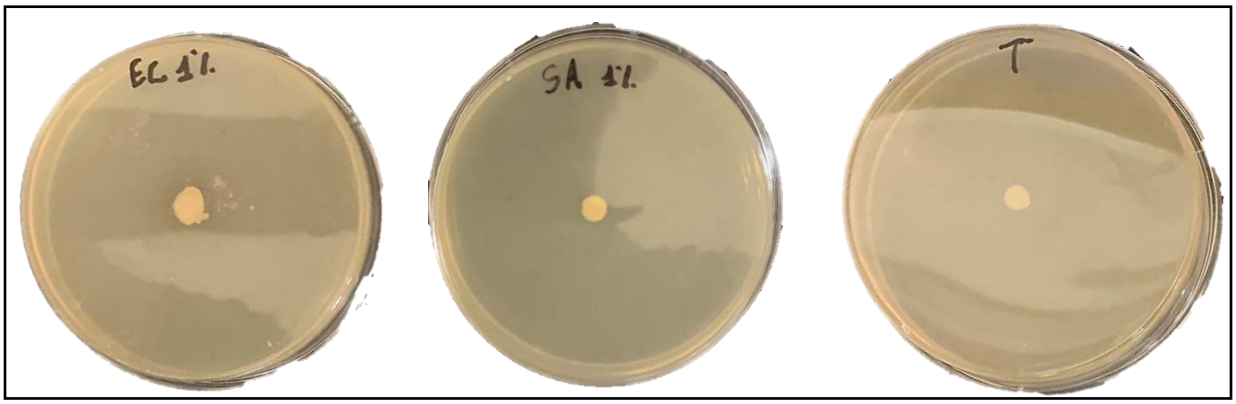

Figure 24: Petri well' after 24 of incubation (original).
1) *E. Coli* + chitosan 1% s. 2) *S.aureus*+ chitosan 1% 3)standard.

IV.4 MIC and MBC:

The result showed that the minimum inhibitory concentration for (1%) of chitosan is 0.2% for *Escherichia coli* ATCC 25922 and 0.4% for *Staphylococcus aureus* ATCC 6538.

perhaps the chitosan nanoparticle with a positive charge can interact with the cell surface or essential nutrients so, as to inhibit the growth of bacteria or can interfere with anionic channels. the antibacterial activity of chitosan under an acidic environment may result from its polycationic structure. Positively charged chitosan can bind to bacterial cell surface which is negatively charged and disrupt the normal functions of the membrane, e.g., by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells **(Abdeltwab and al., 2019)**.

The obtained result was the same as **Youcefi and Riazi (2015)**. who have elucidated the antibacterial efficacy of chitosan against *Staphylococcus aureus* ATCC 6538.

Based on **(Soleimani and al., 2015)** The results demonstrated the remarkable antibacterial activity of these nanoparticles against the tested strains. Among them, the standard strain of *Staphylococcus aureus* exhibited the highest sensitivity to the nanoparticles. Chitosan, known for its broad antimicrobial spectrum, displayed varying inhibitory efficiency against Gram-positives the most.

For the MBC there was no proliferation of microbial strains over the plates either for *Escherichia coli* ATCC 25922 or Staphylococcus *aureus* ATCC 6538. This confirms our MIC results from the previous test that are accurate and the inhibitory effects of chitosan on the growth of microorganisms.

In conclusion, chitosan and nano-chitosan showed anti-growth and anti-adherence effects against cariogenic bacteria in vitro. The results indicated the high potential of chitosan and nano-chitosan as anti-cariogenic agents, suggesting their potential application as dental biomaterials for the prevention of dental caries **(Aliasghari and al., 2016).**

IV.5. In vivo tests results:

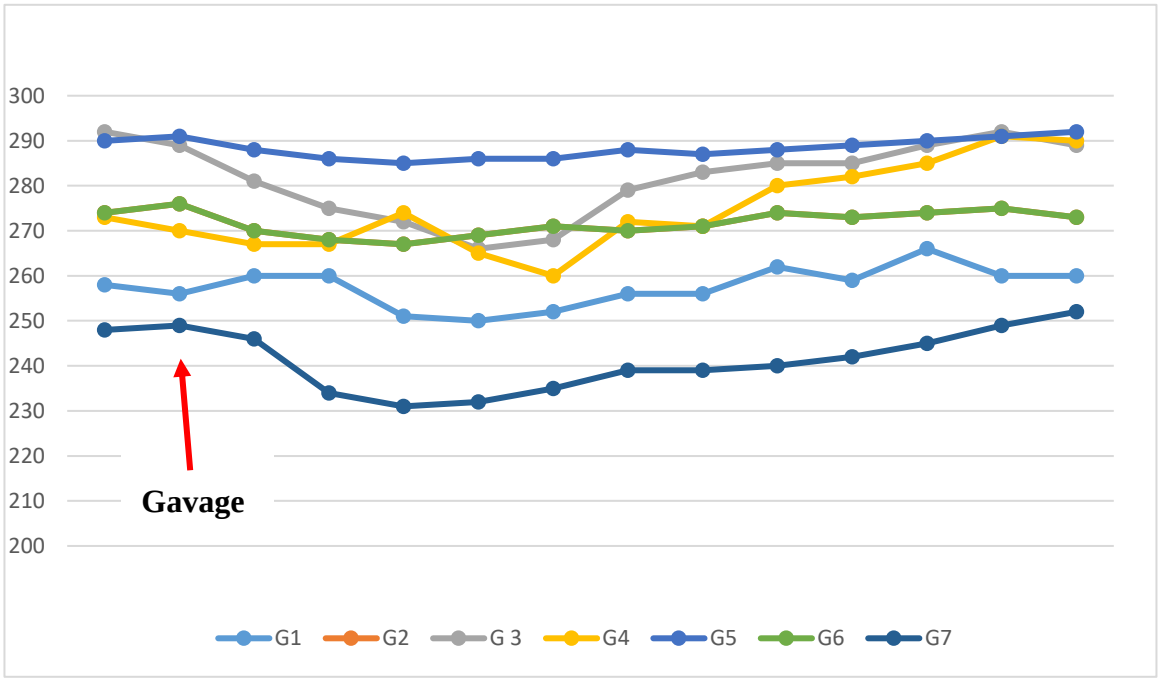

Figure25: weight changes during test period (original).

To study the toxicity of chitosan, histological sections of the (liver, kidney and intestine) of rats treated with the substance are shown, compared with those of untreated rats (control group). Alterations such as lesions, inflammation, cellular necrosis or other histological abnormalities may indicate potential toxicity. The following table shows the main tissue changes:

IV.6. Result of Bio-films:

ccording to bibliographic studies, it has been found that plasticizing chitosan is essential for enhancing its elasticity and preventing film breakage during application.

The study by **(Fernandez and Ingber, 2014)** provides further details on this aspect. Additionally, the choice of plasticizer and its concentration play significant roles in the successful application of chitosan as a coating or edible film.

the chitosan film tended to be brittle and prone to breakage, limiting its practical use. However, by introducing a suitable plasticizer, the film's elasticity was enhanced, allowing for easier handling and application.

Different plasticizers have varying effects on the film's properties, such as its flexibility, transparency, and barrier properties

Appearance:

The chitosan solutions, whether prepared with or without a plasticizer, exhibit transparency and are free from air bubbles. The resulting chitosan films are transparent, dense, and possess a smooth surface without any visible pores.

They are easy to demold from the petri dish after drying, similar to the chitosan films obtained by **(Chabbi and al., 2018).**

Figure 33: Bio-film (original)

Conclusion:

In the field of biological research, significant efforts have been made in recent years to explore new natural compounds, study their efficacy factors, and understand their mechanisms of action. In this context, our focus has turned to chitosan, a naturally occurring compound derived from the exoskeletons of crustaceans.

The main objective of this study is to sheds light on the valuable potential of chitosan, derived from underutilized marine biomass co-products, in combating foodborne toxin infections. The obtained results confirm the remarkable antibacterial properties of chitosan.

Chitosan, derived from chitin found in the exoskeletons of crustaceans, has garnered considerable attention due to its unique characteristics and biological activities. These include its antioxidant, antimicrobial, and anti-inflammatory activities, both in vitro and in vivo, against specific bacterial strains and demonstrated its potential.

For The antibacterial activity of chitosan was confirmed through direct contact testing with BHIB agar medium and estimation of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The results demonstrated that chitosan had an inhibitory effect on the two tested bacterial strains, namely *S. aureus* ATCC 6538 and *E. coli* ATCC 25922. No bacterial proliferation was observed around the chitosan-impregnated discs. These values confirm the effectiveness of chitosan in inhibiting bacterial growth. Furthermore, a Minimum Bactericidal Concentration (MBC) was determined, confirming that chitosan was also capable of killing bacteria.

Toxicity testing results for chitosan at doses of (2000 mg/kg) showed no signs of significant toxicity.

Following the administration of chitosan and propolis, the results of the in vivo antibacterial test there was a significant decrease in bacterial load, the histology cuts showed that the combined effect had the best result against *S. aureus.* also suggest that chitosan and propolis have beneficial effects on the health of treated rats in terms of improving blood parameters and restoring tissues affected by bacterial infection.

for the bioplastics made by chitosan, showed a great result for stability and flexibility and illustrated the great role of glycerol, this will help to reduce the reliance on non-renewable fossil fuels and mitigates the accumulation of plastic waste in landfills and ecosystems.

Furthermore, the study highlights the promising applications of chitosan in bioplastic production, with visually appealing and structurally consistent bioplastic materials.

These findings emphasize the potential of chitosan as a multifunctional compound with antibacterial, bioplastic, and therapeutic properties. The utilization of underexploited marine biomass co-products for chitosan extraction not only presents an innovative approach for waste valorization but also offers a sustainable solution for combating foodborne infections and promoting environmental well-being.

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الملخص:

الهدف من هذا البحث هو استخلاص الشيتوزان من القشريات و اكتشاف خصائصه البيولوجية.حيث تم تقييم نشاطه المضاد للبكتيريا باستخدام طريقة انتشار الأقراص ضد سلالتين بكتيريتين هما المكورات العنقودية والعصيات القولونية حبث أظهرت النتائج تأثيرات مثبطة ملحوظة و عدم تكاثر الميكروبات. وفعالة حيث قدر تركيز المثبط االدنى(CMI) ب 2ملغ/مل. و تم اثبات ذلك في نتائج التركيز الأدنى المميت (CMB). كما تم تجربة الشيتوزان على الفئران لقييم سميته وفعاليته كمضاد حيوي عند استخدامه مع مستخلص العكبر. حيث حققت نتائج واعدة تأكد ان الجرعة المعطات 2000مغ/كغ غير سامة أما بالنسبة للعينات النسيجية فقد أظهرت أن اجتماع الشيتوزان و مصتخلص العكبر يعطي فعالية أفضل ضد الميكروبات.

الكلمات المفتاحية: الشيتوزان ، العكبر ، مضاد الميكروبات ، السمية ، الجسم الحي.

Abstract:

This study aims to extract chitosan from crustacean shells and investigate its biological properties. The antimicrobial activity of the chitosan extracts was evaluated using the disk diffusion method against two bacterial strains: *Staphylococcus aureus* and *Escherichia coli*. The results demonstrated significant inhibitory effects, preventing the proliferation of the pathogens. The microdilution method confirmed a minimum inhibitory concentration (MIC) of 0.1mg/L, which was further supported by the minimum bactericidal concentration (MBC). In addition, in vivo tests were conducted to assess the toxicity of chitosan and its efficacy as an antibiotic when combined with propolis. Promising results were obtained, confirming that the giving dose 2000 mg/kg is not toxic. As for the histological samples indicating potent antimicrobial activity for the combination of chitosan and propolis.

Key words: Chitosan, propolis, antimicrobial, toxicity, in vivo.

Resume

Cette étude vise à extraire le chitosane des carapaces de crustacés et à étudier ses propriétés biologiques. L'activité antimicrobienne des extraits de chitosane a été évaluée par la méthode de diffusion sur disque contre deux souches bactériennes: *Staphylococcus aureus* et *Escherichia coli*. Les résultats ont démontré des effets inhibiteurs significatifs, empêchant la prolifération des pathogènes. La méthode de microdilution a confirmé une concentration minimale inhibitrice (CMI) de 0,1mg/L, qui a été confirmée par la concentration bactéricide minimale (CBM). En outre, des tests in vivo ont été réalisés pour évaluer la toxicité du chitosane et son efficacité en tant qu'antibiotique lorsqu'il est associé à la propolis. Des résultats prometteurs ont été obtenus, confirmant que la dose de 2000 mg/kg n'est pas toxique. Quant aux échantillons histologiques, ils indiquent une puissante activité antimicrobienne pour la combinaison de chitosane et de propolis.

Mots clés: Chitosan, propolis, antimicrobien, toxicité, in vivo