IN VIVO TOXICOLOGICAL EVALUATION OF EDIBLE CHITOSANSTARCH FILM FOR FOOD PACKAGING

BY

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF TECHNOLOGY IN BIOCHEMISTRY

AUGUST, 2023.

DECLARATION

I hereby declare that this thesis titled: "***In vivo*** **Toxicological Evaluation of Edible Chitosan-starch Film for Food Packaging**" is a collection of my original research work and it has not been presented for any other qualification anywhere. Information from other sources (published or unpublished) has been duly acknowledged.

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CERTIFICATION

The thesis titled: "***In vivo*** **Toxicological Evaluation of Edible Chitosan-starch Film for Food Packaging**" by: **OKORIE, Maureen (MTech/SLS/2018/8558)** meets the regulations governing the award of the degree of MTech of the Federal University of Technology, Minna and it is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This research thesis is dedicated to God Almighty, for His unmerited Love, Mercy and All-sufficient Grace towards Me and for guiding me towards the successful completion of this research.

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ABSTRACT

The use of edible films made from chitosan as a food packaging material is on the increase but there is a dearth of information on the long-term toxic effect on experimental animals. This study aimed at evaluating the toxicity of edible chitosanstarch film in albino rats. Commercial rat diet was supplemented with 5–40 % portions of chitosan-starch films and proximate analysis was carried out using AOAC methods. Twenty-five rats were randomly distributed into five groups and fed the supplemented diets for 28 days. The daily feed intake and weekly body weight were taken. Hematological and biochemical parameters were determined using an auto-hematology analyzer and a semi-automatic biochemistry analyzer respectively. Histopathology of the liver, kidney and intestine was carried out. Chitosan was significantly ($p<0.05$) lower in all proximate components except for carbohydrates and fibre, and chitosanstarch film was significantly ($P>0.05$) higher in moisture, fat and ash compared to the supplemented diet and the control diet. Rats placed on 5 % edible chitosan-starch film had the highest feed intake and weight gain while those placed on 40 % had the least. The kidney and intestine body-weight ratios were significantly ($p<0.05$) lower in the rats on the supplemented diets compared to those placed on the control diet, but not different in the liver body-weight ratio. Haematological parameters varied significantly in experimental rats. Serum enzymes in rats fed the supplemented diets were significantly higher than control. The total protein in the diet groups was similar to those on the control diet but significantly ($p<0.05$) lower in albumin and ($P>0.05$) higher in bilirubin. The renal function parameters were significantly ($p<0.05$) higher in the experimental groups while lipid profile indices except HDL were lower when compared to the control rats. The liver showed normal intact hepatic cells, while the kidney and intestine showed degeneration of the glomeruli, capsular space, and connective tissue inflammation in rats fed diets above 5 % of edible chitosan-starch film. This study suggests that edible chitosan-starch films may be toxic to albino rats at levels higher than 5 % diet inclusion.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Food industries are responsible for providing consumers with food that is pleasant, fresh, and of high quality with health benefits (Tahir *et al*., 2019). However, the difficulty of satisfying consumer needs is crucial since no food can preserve its qualities and remain in ideal conditions owing to the natural degradation caused by chemical, physical, and biochemical changes (Kong and Singh, 2016). According to Roudaut and Debeaufort (2010), food spoilage is affected by a number of variables, including oxygen availability, temperature, relative humidity, water content, and pH. As a result of this, it is crucial to maintain the qualities of the food in addition to the characteristics of edible films and coatings. These qualities include the retention of nutritional value, and the preservation of physical and sensory qualities in accordance with established laws, like smell, taste, and texture, all of which work together to increase the shelf-life of the product (Remize, 2017).

Packaging is the most crucial stage after foods have undergone minimum or complete processing since it enables transportation from the manufacturers to the point of sale or distribution in good and acceptable condition. As a result, packaging helps to keep the majority of the food's physicochemical, functional, and organoleptic qualities (Marsh and Bugusu, 2007; Raheem, 2013). Packaging is perhaps the most pivotal factor for food preservation, due to its protective, preservative functions, providing the necessary product information, and enabling commercialization and distribution of the packaged product (Raheem, 2013). The nature of the food determines the characteristics of the packaging material. Several materials, including paper, cardboard, metal, glass, and plastic, have been used as packing materials (Deshwal *et al*., 2019). These conventional materials tend

to generate a lot of urban solid waste (USW). The recent data for 2018 from Mexico's Ministry of Environment and Natural Resources (SEMARNAT) shows that the country produces approximately 102,895 tonnes of USW of paper, cardboard, glass, and certain metals (aluminium) daily, of which only 10 % is recyclable. (SEMARNAT, 2019a; SEMARNAT, 2019b). As a substitute for

conventional, non-recyclable materials, packaging made of biodegradable materials has emerged in this way. Such biodegradable materials can preserve the product while being incredibly simple to produce, recycle, and degrade (Tavassoli-kafrani *et al*., 2016). To preserve quality and prolong the shelf life of minimally processed items, such as fruits and vegetables, the majority of biodegradable packaging uses ecologically friendly polymeric materials (Acevedo-Fani *et al*., 2017; Tahir *et al*., 2019). Additionally, as customer demands for healthy packaging alternatives and fresh, safe food ingredients increased, commercial edible films made for various food packaging systems have been discovered (Pavlath and Orts, 2009; Debeaufort and Voilley, 2009).

Edible films and coatings are referred to as any thin material used to wrap or coat food products and medicines to increase the shelf life of the product- which may be ingested simultaneously or removed before consumption. The edible film has two main considerations. The word ―edible‖ denotes, that films may be ingested together with the food and must meet and satisfy all properties of safe-food ingredients as described by the Food and Drug Administration (FDA) having Generally Recognized As Safe (GRAS) status (Erkmen and Bozoglu, 2016). Films indicate that the covering material should possess packaging qualities that limit the movement of gases and water vapour between the food and the outside environment while protecting the content from the outside environment. Typically, this material should not change the product's flavour, smell, or appearance. Due to these quality issues, film material should be as thin as appropriate

while yet possessing the necessary mechanical qualities to safeguard food. Food products are protected by edible films and coatings, which act as a barrier to gas and moisture migration. Recent advancements have made it possible to employ edible films with additional functional properties such as the encapsulation of aroma, volatiles, vitamins, flavouring agents, antimicrobials, and antioxidants (Han, 2014; Erkmen and Bozoglu, 2016). Additionally, they can raise the quality of food material by protecting them from deteriorations such as moisture loss, enzymatic browning reactions, microbial spoilage, and lipid oxidation as well as other physical, chemical, and microbiological deteriorations (Sun *et al*., 2022).

Film-forming material, plasticizers, and additives are the three main components of a typical edible film. Furthermore, to create a film-forming dispersion (FFD) for edible films, a suitable solvent is required. According to the natural solubility properties of film-forming materials, the most common solvents employed are water, diluted acids, alcohol, or aqueous alcohol. The main components that make edible films are proteins (such as whey proteins, soy proteins, collagen, gelatin, pea proteins etc.), polysaccharides (such as chitosan, starch, agar, pectin, cellulose etc.), lipids (such as waxes, acetoglycerides etc.), and combinations or mixtures of these (Han, 2014). Additionally, to enhance the protective qualities of edible films and coatings, functional additives such as plasticizers, antioxidants, vitamins, antimicrobial agents, essential oils, pigments, and chemical preservatives are utilized (Han, 2014). Most edible films are used to reduce food material moisture loss and respiration. After being food-grade, it has been discovered that moisture and gas barrier properties are the most crucial requirements for edible films and coatings for food materials (Erkmen and Bozoglu,
2016).

Chitosan is a linear amino polysaccharide having D-glucosamine and N-acetyl-Dglucosamine units that are derivative of chitin following deacetylation. Due to its exceptional qualities of antimicrobial activity, biocompatibility, biodegradability, chelating capability, etc., it has been used in various industries, including medicine, agriculture, food, textile, environment, and bioengineering (Mitelut *et al*., 2015; Hosseinnejad and Jafari, 2016; Verlee *et al*., 2017). Due to its solubility in acetic acid and hydrochloric acid, it has the capacity to form films. The characteristics of chitosanbased films, such as their antimicrobial activity, barrier property, antioxidant activity, mechanical property, optical property and thermal stability, have been modified by numerous researchers using techniques like casting, coating, layer-by-layer assembly, etc. In order to increase the combined advantages of the produced films, chitosan has been combined with other useful materials to create composite films. The produced films have been used to package a variety of products, including meat, fruit, and vegetables (Kuorwel *et al*., 2013; Romanazzi *et al*., 2017). It has demonstrated outstanding preservation properties and is quite promising as an alternative to conventional food packaging material. With the advancement of science and technology, there have been significant improvements in food packaging. Chitosan has received regulatory approval for use in food packaging from the FDA (UK) and for dietary uses from the governments of Japan, Italy, and Finland (Illum, 1998).

1.2 Statement of the Research Problem

Edible chitosan films are widely regarded as renewable, biodegradable substance and have been used to package different foods because they are found to be digestible (Jafarzadeh *et al*., 2020), with excellent preservative effects. Also, pharmaceutical industries apply edible films as adjuvants in controlled drug release systems (Brant *et al.*, 2014). Although these edible films are produced from natural resources (such as; chitosan,

and starch amongst others) and are quite promising, the risks of prolonged use and excess consumption of chitosan-based film are unknown (Douglas *et al*., 2015; Popescu *et al*., 2022). Also, there is a dearth of literature on the biochemical, haematological, and histoarchitectural organ changes in experimental animals fed edible chitosan-starch films.

1.3 Aim and Objectives of the Study

This research aimed at evaluating the toxicity of edible chitosan-starch film for food packaging.

Objectives

The objectives of this study were to determine the:

i. proximate composition of chitosan, edible chitosan-starch film and chitosanstarch film supplemented diets.

ii. haematological and biochemical parameters of albino rats placed on edible chitosan-starch film supplemented diets. iii. histopathology of the liver, kidney and small intestine of albino rats placed on edible chitosan-starch film supplemented diets.

1.4 Justification for the Study

Evaluation of haematological and biochemical parameters is an important and sensitive index, considered to be vital in toxicity studies (Arika *et al*., 2016). Available evidence has shown that the consumption of toxic substances can cause alterations in the haematological profile and biochemical indices (Arome and Chinedu, 2013; Zahmati and Saljooghi, 2016). Additionally, the use of experimental animals is most appropriate because of the similarities in the anatomical, physiological, and biochemical makeup compared to that of humans also the liver, kidney and intestine play key roles in metabolic

and digestive processes and assessment of the health status (Yang *et al*., 2014). Therefore, determining the haematological, biochemical, and histoarchitectural organ changes in albino rats placed on edible chitosan-starch films will provide information on the safety of edible chitosan-based films.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Food Packaging

Packaging becomes the most crucial phase once foods have undergone minimum or complete processing as it enables transportation of the packaged foods from the manufacturers to the point of sale or distribution in good and acceptable conditions. As a result, the packaging material helps to keep the majority of the food's physicochemical, functional, and organoleptic qualities (Marsh and Bugusu, 2007; Raheem, 2013). Furthermore, the packaging must not interact with the food/drug and should protect it from external harm of chemical, physical, and biological changes (Raheem, 2013). Chemical damage includes exposure to gases, moisture and light, physical changes encompass any harm caused by any shock or vibration, while biological damage is caused by the action of pathogens, insects, animals, or the senescence of the food itself (Marsh and Bugusu, 2007).

2.2 Types of Materials Used in Food Packaging

Several materials are used in food packaging, the main components used in packaging are glass, metal, plastic, paper, laminates, co-extrusion, and biodegradable polymers (Ojha *et al*., 2015).

2.2.1 Glass packaging

The first glass items for holding food are estimated to have emerged around 3000 BC, and glass has a long history in food packaging (Sarkar and Aparna, 2020). To create glass, a mixture of silica, sodium carbonate, calcium carbonate, and alumina (stabilizers) are heated to extremely high temperatures and then melted into a viscous liquid mass that is put into the molds. Broken glass is recovered and used as a raw element in the production of new glass. Glass vessels used in packaging food are typically surface-coated, which

prevents surface scratching or erosion and provides lubrication in the manufacturing process. Glass coatings increase durability and, in the end, decrease damage. Thicker glass can be used by producers due to the improved break resistance, which lowers weight and makes it easier to move and dispose of (Samir *et al*., 2022). Glass has many benefits for food packaging uses because it is inert to almost all foods. The soft drink and alcoholic beverage industries continue to be the major consumers of glass containers in the world. Glass is less and less used in pharmaceutical industries as rigid polymers continue to replace conventional glass packaging. But because glass packaging has a luxury image and is preferred by producers of alcoholic beverages, an ongoing increase in demand is anticipated (Samir *et al*., 2022).

2.2.2 Metal packaging

The most adaptable packaging material is metal. Excellent physical protection and barrier qualities, formability and decorative possibilities, recyclability, and customer approval are all combined in this product. Aluminium and steel are the two metals most frequently used in packaging. Metal aluminium, a light, silvery-white metal obtained from bauxite ore, where it coexists with oxygen as alumina, is frequently used to create cans, foil and laminated paper or plastic containers. Aluminum frequently has magnesium and manganese added to it to increase its tensile strength (Deshwal and Panjagari, 2020). Metal alloys can be used to make a variety of products, including food cans, can ends, plates, container tops, and closures. Additionally, it can be utilized to create substantial containers (like drums) for the mass selling and storing of ingredients or completed products (Yang *et al*., 2023). Beverage cans continue to be among the packaging groups with the fastest growth (beer, soft drinks and health drinks) (Fitrzyk and Niemiec, 2018).

2.2.3 Plastic packaging

Polymerization, also known as condensation polymerization or addition polymerization, is the process employed to create plastics. In polycondensation, low molecular weight leftovers like water and methanol are produced along with the development of the polymer chain through condensation interactions between molecules. Monomers with at least two functional groups, such as alcohol, amine, or carboxylic groups, are involved in polycondensation. Polyolefin, polyester, polyvinyl chloride, polyvinylidene chloride, polystyrene, polyamide, and ethylene vinyl alcohol is just a few of the polymers that are used to package food. Polyolefins and polyesters are the most widely used plastics for packaging, even though there are more than 30 different kinds of plastics (Groh *et al*., 2019). Polyesters are condensation polymers made from ester molecules produced by the interaction of carboxylic acid and alcohol. Examples of polyesters include polyethylene terephthalate (PET), polycarbonate, and polyethylene naphthalate (PEN). PET is polyester that is used in food packaging the most frequently, especially for drinks and mineral water. PET is increasingly used to manufacture plastic cups for carbonated beverages (Chitaka and Goga, 2023).

2.2.4 Laminates and co-extrusion packaging

Plastic materials can be formed as either a singular sheet or a composite of different plastics. Plastics can be combined in a variety of methods, including co-extrusion and laminating. The co-extrusion coating allows for the conventional requirements of "polyethylene coating" characteristics as a barrier and peelable sealing. It is applied to various materials. Lamination is the process of attaching two or more polymers to another substance, such as paper or aluminium, and this bonding is frequently accomplished using adhesives (Ojha *et al*., 2015). Both films are then moved between high-pressure rollers after the chemicals have been applied to the first film. Thermoplastics have also been

laminated without glue using laser technology. During the production of the film, two or more layers of liquid plastic are joined in coextrusion. The co-extrusion coating, used on various materials, allows for the addition of characteristics such as a barrier, peelable sealing, or others to the conventional requirements of "polyethylene coating." The ongoing use of packaging laminates in already established categories and their adoption into a broad variety of new goods, such as ready-to-eat foods, hot beverages, and personal care items, drove overall demand for these materials (Ojha *et al*., 2015).

2.2.5 Paper packaging

Trees are the primary supply of cellulose (or wood pulp), the material used to make paper. Paper can also be produced from resources like cotton, flax, esparto, straw, hemp, manilla, and jute in addition to wood pulp (Ojha *et al*., 2015). The method used to extract the fibres from the wood influences some of the pulp's characteristics. To create the finished paper product, the fibres are pulped, dyed, and treated with chemicals like slimicides and thickening agents. Corrugated boxes, milk cartons, folding cartons, purses and containers, cups, wrapping paper, tissue paper, and paper plates are all ubiquitous items made of paper or paperboard.

2.2.6 Paper laminates packaging

Coated or uncoated papers made from kraft and sulfite fibre make up paper laminates. To enhance different properties, they can be laminated with plastic, aluminium, or other substance having the same useful characteristics (Ojha *et al*., 2015). The paper can be coated with plastic to increase the water vapour transmission rate (WVTR) and make it heat-sealable. Laminating, however, significantly raises the price of paper. Packaging for dried goods like ready-to-serve soups, seasonings, and powdered herbs use laminated paper.

2.2.7 Biodegradable polymers packaging

These originate from replenishable stockpiles derived from microbial, animal, or marine food processing industry wastes. Biodegradable materials break down to create ecologically beneficial products like carbon dioxide, water, and high-quality compost in addition to reusable base materials (Moshood *et al*., 2022). For decades, cellulose and carbohydrates have been used to create biodegradable materials (Shaikh *et al*., 2021). The most prevalent cellulose-based biopolymer is cellophane. Amylose, hydroxylpropylated starch, and dextrin are examples of starch-based polymers that expand and distort when in contact with moisture. Polylactide, polyhydroxybuterate (PHB), polyhydroxyalkanoate (PHA), and a mixture of PHB and valeric acid (PHB/V) are additional starch-based polymers. Even when subjected to dampness, the polyactide produced by lactic acid bacteria fermentation of starch derivatives does not degrade (Ojo and Smidt, 2023). Microorganisms can also produce PHA, PHB, and PHB/V by fermenting carbohydrates. Additionally, chitosan, a biopolymer with many intriguing characteristics that have been used in numerous medicinal applications, can be used to form biodegradable films (Shaikh *et al*., 2021). Chitosan can be used as a biopolymer for creative, environmentally friendly packaging by reducing the brittleness of sheets and increasing membrane permeability. The production of composite materials, such as blends or alloys with other polymers is because of chitosan‘s numerous functional characteristics (Japanese Chitin and Chitosan Society, 1994).

2.3 Food Packaging and its Role in Food Preservation

Food packaging's primary objective is to maintain food safety while also meeting consumer demands, industry standards, and environmental requirements in a manner that is both economical and environmentally conservative (Goswami, 2019). Food packaging can delay product deterioration, preserve manufacturing benefits, increase shelf life, and

keep or improve food quality and safety. Packaging thus offers defense against three main categories of outside influences: chemical, biological, and physical (Alamri *et al*., 2021).

2.3.1 Chemical protection

Chemical protection reduces compositional alterations brought on by external factors like contact with gases (typically oxygen), moisture (gain or loss), or radiation (visible, infrared, or ultraviolet). Packaging serves as a barrier to lessen compositional changes that may occur as a consequence of environmental impacts, such as exposure to air, moisture, or light, in addition to protecting foods from external contaminants (Pascall *et al*., 2022). A chemical barrier can be produced using various packaging materials such as glass or metal because its closure mechanisms are often added to make filling and emptying easier. Glass and metals offer a nearly perfect barrier to chemicals and other environmental agents. Closure mechanisms might be made of materials that permit only very little leakage (Pascall *et al*., 2022).

2.3.2 Biological protection

The majority of edible film and coating features are important, but food biological protection is one of the most important since it directly affects a product's shelf-life. Bacterial or fungal microorganisms (as well as their derivatives) that can induce or accelerate putrefaction by the activity of their enzymes and byproducts formed during their metabolism in food must be blocked or eradicated (Holzapfel, 2015). Insects, mice, and other pests are kept out by biological protection, which creates a barrier between the packaged food and spoilage-causing microbes. Biological barriers also keep the circumstances necessary to prevent senescence (ripening and ageing) from occurring (Dodig *et al*., 2019). In addition, these barriers operate through a variety of processes, including blocking entry to the product, preventing odour transmission, and preserving the environment inside the package (Shaaban and Farouk, 2022).

2.3.3 Physical protection

Food is physically protected against mechanical harm by padding against stress and vibration experienced during transportation and delivery (Fernando *et al*., 2018). Physical barriers are frequently used as shipment receptacles and as packing for fragile goods like eggs and fresh fruits because they withstand impacts, abrasions, and crushing damage. They are typically made from paperboard and corrugated materials. The proper tangible packing also shields customers from a variety of risks (Marsh and Bugusu, 2007). For instance, child-resistant closures make it more difficult to obtain goods that could be hazardous. Additionally, the risk from shattered glass vessels has decreased as a result of the switch to plastic packaging for goods like shampoo and soda bottles.

2.3.4 Containment and food wastage reduction role

Containment and waste minimization are significant responsibilities that packaging plays in today's complex supply networks. According to estimates, 17 % of the food produced worldwide is wasted, with domestic waste accounting for 11 % of this total (UN Environment Programme, 2021). By extending the shelf life and increasing the product's utility, packaging can help decrease food waste (Pascall *et al*., 2022).

Resealable features, which enable many servings from a single container and prevent the product from being thrown away, help extend the usage of packaged food products (Ameripen, 2022). Packaging optimization measures can aid in the battle against food waste. People generally underestimate the value of proper packaging to minimize food loss and waste, and they frequently mistakenly believe that less packaging is more environmentally friendly than a container that preserves food longer (Wohner *et al*., 2019).

2.3.5 Marketing and information role

A product's packaging serves as its public visage and is frequently the only exposure customers have to the item before making a purchase (Fenko *et al*., 2010). Therefore, in a market that is competitive, packaging that is unique or creative can increase sales. The packaging may be made to improve the product's reputation and/or set it apart from the competitors (Bell *et al*., 2016). For instance, bigger labels could be used to fit recipes. Additionally, packaging informs the customer. The regulatory criteria for product identity, nutritional value, ingredient disclosure, net weight, and manufacturer information, for instance, are satisfied by container labelling. Also, the package communicates crucial details about the product, including cost, brand identity, and culinary directions (Fenko *et al*., 2010).

2.3.6 Traceability role

Traceability is described as "the ability to follow the movement of a food through specified stage(s) of production, processing, and distribution" by the Codex Alimentarius Commission (Codex Alimentarius Commission 2004). Improvement of supply management, facilitation of trace-back for food safety and quality reasons, and differentiation and marketing of products with subtle or undetectable quality characteristics are the three goals of traceability (Mahalik, 2014). Companies that produce food add distinctive identifiers to the container labels of their goods, enabling them to follow those foods as they are distributed. Codes can be scanned directly or automatically and come in a variety of forms (like printed barcodes or electronic radio frequency identity [RFID]).

2.3.7 Convenience role

Package invention is significantly influenced by convenience attributes like resealability, microwave ability, product visibility, and simplicity of access, handling, and disposal. As

a result, the packaging is essential in reducing the time and work needed to make and deliver food. The use of boil-in bags, microwaveable packaging, and oven-safe trays allows customers to prepare almost no food before cooking a complete dinner. New closure designs offer unique pouring features, resealability, and simplicity of access (Oliveira *et al*., 2022). For instance, a cookie producer recently unveiled a flexible bag with a portion that can be scored to allow entry to the cookies. Before a sale, a sheet with a peelable seal covers the hole and enables reclosure after it has been opened. Modern retail layouts that give customers the ease of one-stop buying and access to food from around the globe have been made possible by advancements in food packaging. These practical features boost a product's worth and competitive edge, but they may also affect how much and what kind of packaging waste needs to be disposed of (Mahalik, 2014).

2.3.8 Tamper indication role

Due to deliberate adulteration of food and medicinal goods, special packaging features have been developed to minimize or completely eradicate the risk of adulteration. Tamper-evident features cannot be readily changed, although any package can be compromised. Banding, unique membranes, breakaway closures, and special printing on container bottoms or composite cans, such as images or text that permanently change upon opening, are a few examples of tamper-evident features. Holograms are another form of special writing that is difficult to replicate. Tamper-evident packaging typically needs extra packaging materials, which makes disposal problems worse, but overall, the advantages exceed any disadvantages. A heat seal used on medical packaging that is chemically formulated to change colour when unsealed is an example of a tamperevident feature that does not require extra packaging materials (Marsh and Bugusu, 2007; Mahalik, 2014).

2.4 Food Packaging Laws and Regulations

There are rules that govern how packaging interacts with food products in terms of quality control (packaging-product relationship). Due to the wide range of packaging options, materials (like paper, glass, and plastic), presentations (like boats, boxes, and bags), and aggregates of presentations (like inks, adhesives, and seals), as well as the properties of the food products (like moisture, fat or alcohol content, pH, and freshness), these regulations are complex (Moran, 2012). The Food and Drug Administration (FDA) has stated that any potential packaging-product contamination may be related to the recycling of the packaging material, exposure to any hazardous materials from packaging solutions, or production during treatments (such as thermal or chemical). Similarly, reused packaging materials can retain such compounds confined for a very long period (FDA, 2019). Herein, the packaging must comply with five basic requirements in other to be made commercially available: it must not present any risks to human health, it must not alter the physicochemical composition of the food, it must not alter the organoleptic characteristics of the food, it must be made and handled in accordance with good manufacturing processes, and it must not give inaccurate information about the product it contains (Moran, 2012).

Also, laws created by the International Organization for Standardization (ISO) deal with laws pertaining to the creation, use, and distribution of packaging materials, such as ISO 18604:2013. (E). To be accepted, processed, and recycled as a new feedstock, various food packaging materials must adhere to the standards set out in such rules (ISO, 2013). While these standards aid in regulating the quality of food products by adapting packaging, there are also laws that govern the environmental impact of packaging waste production, which have limited the use of packaging that may be directly or indirectly responsible for polluting flora and fauna. While European Union legislation (regulation

EC 10/2011) only addresses things that may come into direct or indirect contact with food, such as production equipment, kitchen utensils used in the filling, and distribution containers and packing, since active and intelligent packaging (regulation EC 1935/2004) can only release substances that have been approved as food additives and must be accompanied by a certification of conformity, this rule specifies specific requirements for their usage (ISO, 2013). As a result of this, edible films and coatings have emerged as a potential and promising option to protect and even improve the quality of foods even while processing and during storage (Douglas *et al*., 2015).

2.5 Conventional Food Packaging Materials

Plastic is the most known packaging material, and many petrochemical-based materials are currently used due to their availability at a relatively low cost. However, environmental conservation regulations have strongly restricted their use as packaging materials since they are not fully recyclable or biodegradable (Siracusa *et al*., 2008). In the case of paper, it is used as packaging due to the fact that it comes from a biodegradable matter; nevertheless, being in contact with food loses its physical appearance and its protection. This is one of the main reasons to combine it with other materials, such as plastic and aluminium. Although, paper loses its biodegradable effect and the feature to be recycled (Khwaldia *et al*., 2014).

Glass is the oldest packaging material, it is commonly utilized since it has no odour and is chemically inert (Sarkar and Aparna, 2020). Modern glass-based packaging is thin and robust to sterilizing processes under high pressures and temperatures. Metals are the most adaptable material for all packaging types since they are completely recyclable and very durable. The two most prevalent metals are found to be aluminium and steel. Steel is solely utilized as a container, whereas aluminium may be moulded into the product. Metal

gaskets often offer protection against air, moisture, smells, and bacteria (Sarkar and Aparna, 2020).

Depending on these packaging materials, as well as their benefits and drawbacks as barrier types, packaging can take many forms. The sort of food or product that has to be preserved directly affects which ones are chosen. For instance, jams and sauces without any preservatives are typically packaged in glass containers since protection against biological agents is virtually always provided, allowing for their long-term preservation. For the most part, canned foods are packaged in aluminium cans to prevent odour exchange and microbiological contamination (Raheem, 2013). Milk, bread, and cookies are typically packaged in plasticized cardboard, which is sufficient protection for a short time, while other items with a limited shelf life, like bread, are typically packaged in paper.

2.6 Edible Films and Coatings as Packaging Materials

According to Ebuscado and Huber (2009), an edible film or coating is any material with a thickness of less than 0.3 mm that is produced from a mixture of biopolymers and other additives dispersed in an aqueous medium (Montalvo *et al*., 2012; Castro-Munoz and Gonzalez-Valdez, 2019; Morales-Jiménez *et al*., 2020). Some researchers refer to the term edible film and coating interchangeably, while others believe there is a difference because of how they are incorporated into the food product (Guimaraes *et al*., 2018). While the edible film is produced first and then attached to the product, the edible coating is formed directly on the food (Yai, 2008; Guimaraes *et al*., 2018). Notwithstanding, rigid matrices with identical properties are created in both circumstances (Guerrero *et al*., 2009; Tavassoli-Kafrani *et al*., 2016).

2.6.1 Characteristics of edible films

The primary properties of edible films include the following; protection against UV light (Douglas *et al*., 2015), transportation of solutes (such as salts, additives, and pigments), water vapour, organic vapour (such as aromas and solvents), and gases (such as oxygen, carbon dioxide, nitrogen, and ethylene) between food and the atmosphere (Falguera *et al*., 2011; Douglas *et al*., 2015), create a barrier against mechanical damage (such as dents or cuts) (Guimaraes *et al*., 2018), to increase the shelf-life of the packaged product (Falguera *et al*., 2011), bioactive components (such as antioxidants) (Sánchez *et al*., 2015; Salvia-Trujillo *et al*., 2017), have an antimicrobial effect against bacterial reproduction and fungal contamination (e.g. silver nanoparticles) (SalviaTrujillo *et al*., 2017; Kra'sniewska *et al*., 2020), healthy microorganisms (example, probiotics) that confer benefits to the consumer and biodegradable natural materials (Guimaraes *et al*., 2018).

2.7 Film-forming Materials

In order to create a continuous framework of films or coatings, many bio-based polymers have been researched. The most prevalent class of biopolymers employed in the creation of edible materials are hydrocolloids, which include both polysaccharides and proteins. Sources include plants, animals, and microbes. The most widely used polysaccharides in the fabrication of edible films and coatings are cellulose derivatives, starches, alginates, pectin, chitosan, pullulan, and carrageenan, while the most widely used proteins are soybean proteins, wheat gluten, corn zein, sunflower proteins, gelatin, whey, casein, and keratin (Falguera *et al*., 2011). Although the nature of these filmforming materials is hydrophilic in nature, they tend to retain moisture readily, as a result of this, various oils and fats are added to the hydrocolloid matrix to improve their water vapour barrier

qualities. Glycerol, wax, triglycerides, acetylated monoglycerides, free fatty acids, and vegetable oils are the most often used (Galus and Kadzińska, 2016).

2.8 Proteins-based Films

In comparison to polysaccharide films, protein films from plants (soy and zein proteins) and animals (milk and whey protein) have greater mechanical characteristics, an improved carbon dioxide barrier, and an improved oxygen barrier. Although, protein films often have poor water vapour properties due to their hydrophilic nature, compared to polysaccharide films which do not (Galus and Kadzińska, 2016). According to studies, the characteristics of protein films are influenced by the concentration, composition, size, and form of the plasticizers (Shroti and Saini, 2022). Proteins that are soluble in water generate coatings of variable solubility depending on the protein and the circumstances surrounding coating development and treatment, as opposed to proteins that are insoluble in water, such as maize zein and wheat gluten, which make insoluble coatings. Soy protein is not regarded as a hydrocolloid since the majority of its protein is not soluble in water but is soluble in diluted neutral salt solutions.

2.8.1 Gelatin films

Gelatin is made by thermally denaturing collagen that is extracted from fish skin, bones, and animal skin (Gómez-Estaca *et al*., 2011). Gelatins' physical characteristics are influenced by their amino acid makeup as well as their molecular weight distribution. At temperatures over 40 °C, gelatin is rapidly soluble in water, generating a viscous solution of randomly coiled linear polypeptide chains. When a gelatin solution is cooled to approximately 20 °C, collagen-like helices are formed however, these helices are not very long and just cover a portion of the material, forming a gel. According to Renard *et al.* (2006), mammalian gelatins often have better physical characteristics and thermostability

than the majority of fish gelatins, which has been attributed mostly to their greater amino acid content (Gómez-Estaca *et al*., 2011). Up until the 1960s, the use of mammalian gelatin in the creation of edible films or coatings was extensively researched, leading to several patents, mostly in the pharmaceutical industry. Gelatins' characteristics and capacity to produce films are closely correlated with their molecular weight, that is, the higher the average molecular weight the better the film quality (Acevedo *et al*., 2010). The molecular weight distribution is mostly influenced by the extraction method and degree of collagen cross-linking.

2.8.2 Whey proteins

Totally water-soluble coatings are produced from whey protein isolate, however, coatings containing insoluble protein are produced by heat-denatured solutions of whey protein isolate (Sothornvit, 2014). Whey protein isolate (WPI) has a protein concentration of 90 % while whey protein concentrate (WPC) manufactured industrially has a protein level 80 %. (Acevedo *et al*., 2010). Over the past decade, whey proteins have been the focus of much research. The moisture barrier of films made of whey proteins, which are clear, flexible, flavourless, and colourless, is weak (Ramos *et al*., 2012). Additionally, protein-based films have low oxygen permeability and a high aroma barrier. Since whey protein films made without any plasticizers added are exceedingly fragile, adding plasticizers gives the films flexibility while also increasing their water vapour permeability. Young's modulus and tensile strength of edible whey protein films decreased with increased plasticizer content, although elongation increased (Sothornvit, 2014). Hydrophobic materials, such as lipids, can be added to increase water vapour permeability (Bourtoom, 2009).

2.9 Lipid Films

A combination of natural wax, surfactants, and acetylated monoglycerides are used as protective coatings (Debeaufort and Voilley, 2009). Beeswax and paraffin wax are the most efficient lipid compounds. Due to their relatively low polarity, the main purpose of a lipid coating is to prevent the transfer of moisture. In contrast, the hydrophobic property of lipids results in the formation of thicker and more fragile films. They must thus be combined with substances that form films, such as proteins or derivatives of cellulose (Pavlath and Orts, 2009). In general, water vapour permeability decreases as hydrophobicity phase concentration increases. To give mechanical strength, lipid-based films are frequently supported on a polymer structural matrix, typically a polysaccharide.

2.9.1 Waxes and paraffin

The solid hydrocarbon mixture that results after ethylene catalytic polymerization is what makes up paraffin wax, which is made from the distillate fraction of crude oil. On raw fruit, vegetables, and cheese, paraffin wax is acceptable. Carnauba wax is an exudate from the leaves of palm trees (*Copoernica cerifera*). Honeybees create beeswax or white wax. Liquid paraffin and naphthenic hydrocarbon are the main components of mineral oil (Dhall, 2013). Wax is used to improve the surface look of various foods (e.g. the sheen on sweets) and as a barrier layer against gas and moisture (on the skin of fresh fruits). They are deemed edible when used in thin layers but must be removed if placed as a thick coating (like some cheese). The most effective edible materials for acting as a humidity barrier are waxes, particularly paraffin, carnauba, *candelilla*, and bee wax (Bourtoom, 2009).

2.9.2 Acetoglyceride films

1-Stearodiacetin is produced when glycerol monosterate is acetylated by acetic anhydride. This acetylated monoglyceride has the exceptional ability to transform from a molten state

into a flexible, wax-like solid (Pavlath and Orts, 2009). Majority of lipids can only be stretched to around 102 % of their original length in the solid form before they start to break. Although, to the exclusion of methyl or ethyl cellulose, acetylated glycerol monostearate may be stretched up to 800 % of its original length. This film has a substantially lower water vapour permeability than polysaccharide films. Chicken and beef cuts have been coated with acetylated monoglycerides to prevent moisture loss while storing (Douglas *et al*., 2015).

2.9.3 Shellac resins

The ant *Lacciferiacca* secretes shellac resins, which are made up of a complex blend of aliphatic alicyclic hydroxyl acid polymers. Alkaline solutions and alcohols both dissolve this resin. Shellac is only allowed as an indirect food additive in adhesives and food packaging, not as a GRAS material. Only a small variety of research on foods has been recorded, and it is mostly employed in pharmaceutical sector coatings (Taqi *et al*., 2013). After the volatiles from the crude resin was distilled, the remnants from the oleoresins of the pine tree were used to make resin. Many coatings for citrus and other fruits employ resin and its derivatives (Baldwin *et al*., 2011). These coatings were created largely to impart high-gloss at the time of the buyer's inspection, which is often after the coating has been applied. Fruit coated with substances create an extra barrier through which gases must travel. Coatings have varying impacts on gas exchange because of differences in their gas permeance and capacity to plug holes in the peel (Taqi *et al*., 2013). Compared to citrus fruits with wax coats, fruits coated with shellac and wood resin often have lower internal oxygen and greater internal CO_2 and ethanol concentrations (Maftoonazad *et al*., 2008). In turn, a high ethanol concentration is a sign of an off flavour (Bourtoom, 2009). Additionally, wood resin- and shellac-based treatments have a tendency to raise the occurrence of postharvest pitting (Bai and Plotto, 2011).

2.10 Polysaccharides-based Films

Polysaccharides-based films have been used to produce edible films and coatings, these materials have been used independently or as composites which is a combination of two or more materials to produce an edible coating.

2.10.1 Agar films

Agar is a hydrophilic colloid made of a combination of agarose and agaropectin that may be used to create reversible gels by simply cooling a hot aqueous solution. Agar gel melts when heated and returns to its original state when cooled. Due to its ability to form extremely hard gels at very low concentrations and the ease of the extraction process (Azeredo *et al*., 2010), agar has been used extensively as a gelling agent in the food industry. The typical gel temperatures of agar for 1.5 % solution are in the range 35–45 ºC (Kumar and Fotedar, 2009). Agar has been utilized sparingly as edible film due to a poor ageing process while being highly biodegradable and having a high gelling capacity. Likewise, agar's crystallinity is altered by photodegradation as well as changes in the environment's temperature and humidity, which results in the development of micro-fractures and polymer embrittlement (Azeredo *et al*., 2010). However, it was noted that agar-based film outperforms cassava starch film in terms of moisture barrier performance (Kamaruddin *et al.,* 2022).

2.10.2 Alginate films

Alginate can also be thought of as a source of dietary fibre because it is an indigestible biomaterial produced by brown seaweeds (Phaeophyceae, primarily Laminaria). Due to its special colloidal qualities, which include thickening, stabilizing, suspending, filmforming, gel-producing, and emulsion stabilizing, alginate has the ability to make biopolymer films or coating components (Synytsya *et al*., 2015). The ability of alginate solutions to gel in the presence of calcium is a desirable quality. Due to their hydrophilic

nature, edible films made from alginate create sturdy films but have poor water resistance (Maizura *et al*., 2007). When compared to synthetic films, the water permeability and mechanical characteristics might be thought of as intermediate (Synytsya *et al*., 2015). Edible films made of alginate are used to add additives and antibacterial agents. Applying probiotics and oil ingredients like oregano oil and garlic oil yields positive benefits. By combining starch and alginate to create edible film Jiménez *et al*. (2012) investigated how to enhance the mechanical characteristics of the film.

2.10.3 Carrageenan films

Carrageenan is a class of partly sulfated galactan-containing linear water-soluble polymers with significant potential as a material for making films (Dhanapal *et al*., 2012). These sulphated polysaccharides are extracted out of the red seaweed‘s cell walls (Rhodophyceae). Carrageenan production varies among seaweeds. The functionality of carrageenan is determined by the locations and numbers of the sulphate ester groups, which coupled with the anhydrogalactose bridge allow it to be divided into three main types: κ, ι and λ. Because they have the ability to form thermoreversible gels when cooled below the critical temperature, the carrageenans κ- and ι contain the 3, 6-anhyro units and are utilized as gelling agents. λ carrageenan is a thickening polymer that exclusively contains sulfated galactose units and no anhydrogalactose bridge (Dhanapal *et al*., 2012). Carrageenan is already used as edible coatings in many areas of the food industries, including sausage casings, dry solids foods, fatty foods, application to fresh and frozen meat, poultry, and fish to avoid surface dehydration. This method for the gelation of carrageenan occurs during moderate drying, resulting in the creation of a three-dimensional network of polysaccharide double helices and a solid film following solvent evaporation (Karbowiak *et al*., 2006). Furthermore, it was discovered that carrageenan films are less opaque than starch-based films.

2.11 Cellulose Derivatives

Cellulose derivatives are polysaccharides composed of linear chains of β (1-4) glucosidic units with methyl, hyroxypropyl or carboxyl substituents. Only four cellulose derivative forms are used for edible coatings or films: Hydroxypropyl cellulose (E463; HPC), Hydroxypropyl methylcellulose (E464; HPMC), Carboxymethylcellulose (E466; CMC) or Methylcellulose (E461; MC), Cellulose derivatives exhibit thermo-gelation therefore when suspensions are heated, they form a gel whereas they return to originally consistency when cooled (Wandrey *et al*., 2010). Films made from aqueous solutions of MC, HPMC, HPC, and CMC have a tendency to be flexible, translucent, flavourless, colourless, and tasteless, with mild barriers to oxygen. They also tend to be resistant to oils and fats. Some fruits and vegetables have been covered in edible coatings composed of CMC, MC, HPC, and HPMC to act as barriers against the transmission of oxygen, oil, or moisture (Karimi *et al*., 2012).

2.11.1 Chitin derivatives

In 1811, chitin was discovered by a French scientist Henri Braconnot, who gave it the name fungine. Odier discovered the same substance in plants and insects in 1823 and gave it the name "chitin." Chitin is the second most prevalent natural polysaccharide on the globe, behind cellulose. Chitin and cellulose share a similar chemical composition (Figure 2.1) and biological properties. Both polymers primarily function as structural compounds that support cell and body surfaces: cellulose thickens plant cell walls, while chitin increases the mechanical strength of fungal cell walls and arthropod exoskeletons (Kostag and El Seoud, 2021).

At least 1.1 X 10^{13} kg of chitin is thought to be present in the biosphere. However, due to its insoluble nature in the majority of solvents and relative difficulty in isolating it in pure form from natural sources under feasible economic circumstances, its application has

been restricted. Chitin is a nitrogenous polymer that is white, rigid, and inelastic and is present in both the interior and exoskeleton components of invertebrates. It is a Nacetylglucosamine linear cationic polymer with a β-1,4-linkage.

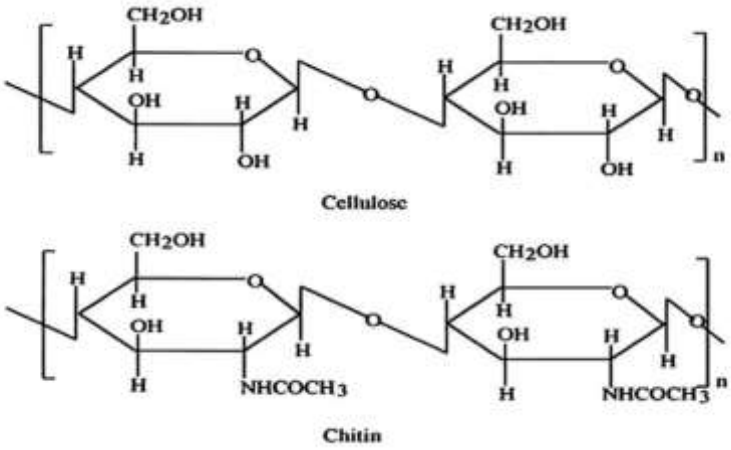


Figure 2.1: Chemical Structure of Chitin and Cellulose Source:

Ravi-Kumar, 2000.

Chitin exists in three polymorphic forms, α, β, and γ, however α-chitin is the most abundant in nature (Khoushab and Yamabhai, 2010). The arrangement of the chains is found to depend on the origin of the chitin. α-Chitin is present in fungal and yeast cell walls, insect cuticles, eggshells of nematodes and rotifers, the radulae of mollusks, and cuticles of arthropods. This form of chitin is also present in krill, lobster, crab tendons and shells, and shrimp shells, as well as in other marine organisms such as the harpoons of cone snails and the filaments ejected by Phacocystis seaweed. β-Chitin is found in the pen and cuticle of squid and the diatom Thalassiosira fluviatilis. In α-chitin, sheets are formed by intermolecular hydrogen bonding in parallel chains. Inter chain hydrogen bonding occurs between sheets in different directions. There is also intermolecular hydrogen bonding between CH_2OH groups, which is believed to be the cause for the lack

of swelling of α-chitin in water. β-Chitin has a monoclinic unit cell with polysaccharide chains attached in a parallel manner (Chawla *et al*., 2014). In β-chitin, hydrogen bonding occurs only within sheets, not between sheets as in α-chitin. This is thought to be responsible for the swelling of α-chitin, as water can be included between the sheets. γ-Chitin is said to be a combination of α and β structure rather than a third polymorph (Chawla *et al*., 2014). Chitin has <10 % degree of acetylation, 7 % nitrogen content, nitrogen/carbon ratio of 0.146, and molecular weight of 2.5 X 10^6 Da corresponding to a degree of polymerization of ca. 5,000–10,000, which differ in the arrangement of their molecular chains.

The membrane-integral enzyme chitin synthase, a member of the glycosyl transferase family, catalyses‘ the process that joins monomers of N-acetylglucosamine to form chitin. UDP-N-acetylglucosamine must be the substrate for the polymerization and divalent cations must be the cofactors. Three separate phases may be identified in the synthesis of chitin. The cytoplasmic site-facing catalytic domain of chitin synthase creates the polymer in the first phase. The second phase entails the discharge of the developing polymer into the extracellular space once it has crossed the membrane. The third phase results in the formation of crystalline microfibrils that fluctuate in width and length when single monomers spontaneously combine (Merzendorfer, 2006).

In the chitin crystal structure, the chains form hydrogen-bonded sheets linked by C=O and H-N- groups. In addition, each chain has intramolecular hydrogen bonds between the neighbouring sugar rings: the carbonyl group bonds to the hydroxyl group on C6. There is also a second hydrogen bond between the OH-group on C3 and the ring oxygen, similar to that in cellulose (Kostag and El Seoud, 2021). The substantial hydrogen bonding in Figure (2.1) increases the chitin chain's rigidity. Chitin is a structural component that is cross-linked to other substances in nature, with the chitin of diatoms being the sole known

exception. In particular, in crustaceans and insects, the chitin microfibrils interact with other sugars, proteins, glycoproteins, and proteoglycans to produce fungal septa and cell walls as well as arthropod cuticles and peritrophic matrices (Kostag and El Seoud, 2021). Chitin is linked to proteins in mammals, but it is linked to glucans, mannans, or other polysaccharides in the cell walls of fungi. It is found covalently attached to glucans in the walls of several fungi, either directly or through peptide bridges (Chawla *et al*., 2014). Chitin is constantly linked to certain proteins by both covalent and non-covalent bonding in insects and other invertebrates to generate the observed organized structures.

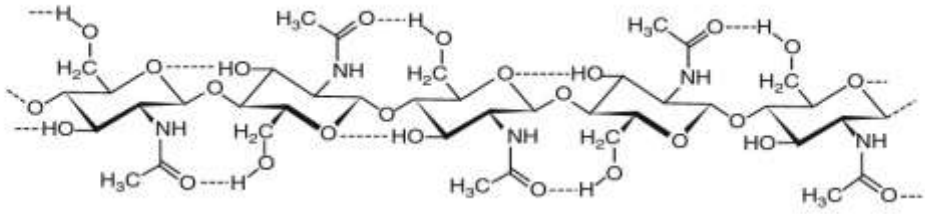


Figure 2.2: Chemical Structure of Chitin Shown with Its Intramolecular Hydrogen Bonds (Dotted Lines)

Source: Ravi-Kumar, 2000.

2.11.2 Chitosan derivatives

Chitosan is mostly generated from crustacean shells and it is the most prevalent naturally occurring polymer in nature after cellulose (Azuma *et al*., 2015).

Deacetylating chitin produces chitosan. It is made up of a D-glucosamine connected to N-acetyl D-glucosamine by β-1,4-glycosidic linkage (Figure 2.3). The method used to prepare chitosan molecules affects how these subunits are distributed. The degree of deacetylation in chitosan varies from 40 % to 98 %, and its molecular weight varies from 5 X 10^4 Da and 2 X 10^6 Da.

Chitosan is the subject of many studies because it is seen to have considerable futuristic promise and to offer enormous possibilities for structural alteration to confer desired qualities and functions. Chitosan has a wide range of applications in several sectors because of the reactive amino groups present at the C2 atom and the hydroxyl groups present at the C3 and C6 atoms. Chitosan‘s excellent biocompatibility, outstanding biodegradability, ecological safety, and a variety of biological activities, including low immunogenicity and antibacterial activity, have created many potentials for further research. Chitosan is extracted from insects, yeast, mushrooms, fungi's cell walls, and marine shellfish including crab, lobster, krill, cuttlefish, shrimp, and squid pens (Jo *et al*., 2011). Chitin, which is covalently bonded to proteins, certain metals, and carotenoids, forms the outer protective covering of shellfish. Proteins make up 40 % of crustacean shells, together with calcium carbonate, 30 % chitin, and pigments such as astaxanthin, canthaxanthin, lutein, and beta-carotene. These ratios change depending on the species and the season. Chitin and chitosan are mostly produced commercially from discarded shrimp, prawns, and crab. The number of shrimps and prawns being processed has greatly increased as a result of the rise in shellfish consumption and the growth of aquaculture, and this has resulted in increased waste that may be used to produce chitin and chitosan. Mycelium waste from fermentation operations is still a sizable and unexplored potential source of chitin and chitosan (Jo *et al*., 2011).

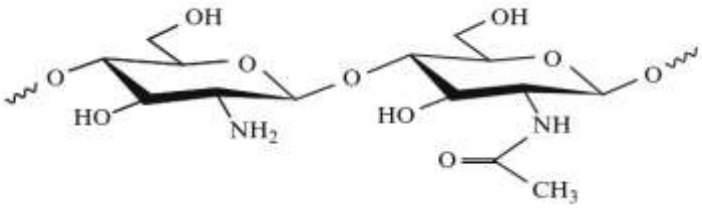


Figure 2.3: Chemical Structure of Chitosan

Source: Ravi-Kumar, 2000

2.12 Production of Chitosan

The majority of chitosan that is manufactured and sold worldwide is produced in the United States, Japan, Norway, Thailand, India, Australia, China, and Poland. The process of making chitosan includes a number of phases, including preparing the chitin from biological material and then deacetylating it to produce chitosan. Demineralization, deproteinization, decolouration, and deacetylation are the fundamental stages that make up the standard production of chitosan from crab shells. Demineralization and deproteinization stages of chitosan production can be altered in terms of order. The primary raw material for the commercial manufacturing of chitosan is the exoskeleton of crustaceans. The outline below shows a typical chitosan production process.

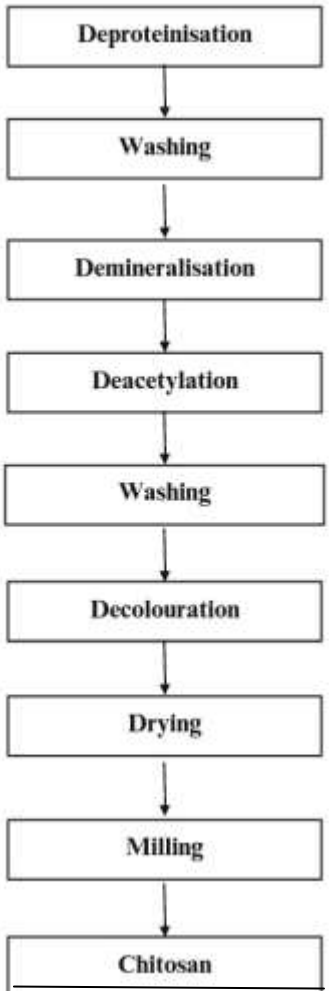


Figure 2.4: Outline for Chitosan Production Methods Source:

Chawla *et al*., 2015.

All types of crustaceans have different amounts of minerals in their exoskeletons. In order to dissolve calcium carbonates and calcium chloride, demineralization is often done at room temperature with agitation using acids such as hydrochloric acid, nitric acid, acetic acid, or formic acid (10 %). However, hydrochloric acid, which is utilized at a concentration of 0.2 M for 48 h at a temperature of 100 °C, is the recommended acid. Calcium chloride is produced in significant volumes when demineralization occurs for 1 hour at room temperature using diluted (8 %) hydrochloric acid. The typical solid-tosolvent ratio is 1:15 (w/v). An indication of how well the demineralization process occurred is determined by the amount of ash in the demineralized shell.

Chitin naturally exists in association with protein. Stable complexes are created when the protein forms covalent interactions with chitin via aspartyl, histidyl, or both residues (Chawla *et al*., 2015). Alkaline treatment is typically used to deproteinize chitin. The shells are subjected to sodium or potassium hydroxide treatments at temperatures of 65 °C with a minimum shell-to-alkali ratio of 1:4 for a period of 12 hours. When these circumstances exist, the protein separates from the solid portion of the shrimp waste. To improve the deproteinization efficiency, a solid-to-alkali solution ratio of 1/20 is utilized in conjunction with appropriate agitation. The procedure is typically carried out in a nitrogen atmosphere and with sodium borohydride ($NaBH_4$) present to avoid oxidation of the products. The protein hydrolysate is simply removed when the deproteinization stage is finished by separating the particles from the protein slurry by filtering. Depolymerization and deacetylation are the results of protracted alkaline treatment under severe circumstances.

Chitin is a coloured material that is produced from shell debris following demineralization and deproteinization. The chitin must be bleached or decoloured to produce cream-coloured chitin powder in order for it to be used commercially (Chawla and Kanatt, 2014). Complexes of chitin and the pigment found in crab shells are formed after decolouration. According to Fox (1973), the red kelp crab's exoskeletal chitin contained one 4-keto-β-carotene derivative and three 4 4′-diketo-β-carotene derivatives that were tightly linked. Among crab species, the degree of chitin and pigment varies. Solvents and/or oxidants are used to decolourize the leftovers. The chemical that is employed during the decolourization process should not have an impact on the physicochemical or functional characteristics of chitin and chitosan. Based on the dry shell, Chawla and Kanatt (2014) were able to create near-white crawfish chitin by extracting it with acetone, drying it for

two hours at room temperature, and then bleaching it for five minutes with a 0.315 % (v/v) sodium hypochloride solution (carrying 5.25 % accessible chlorine).

The process of deacetylation involves removing the acetyl group from chitin to produce chitosan. The temperature and time of deacetylation, alkali concentration, previous treatments used to isolate the chitin, atmosphere (air or nitrogen), the ratio of the chitin to the alkali solution, the density of the chitin, and the size of the chitin particles are some important factors that influence the extent of deacetylation. The optimal deacetylation process condition should result in chitosan that does not deteriorate and is soluble in diluted acetic acid in the shortest amount of time, taking into account all these as necessary requirements (Pellis *et al*., 2022). Since the polysaccharide cannot be hydrolyzed without the N-acetyl groups being removed, alkaline techniques must be used for N-deacetylation. Due to the resistance of groups imposed by the trans arrangement of the C2-C3 substituent in the sugar ring, severe alkaline hydrolysis treatments are necessary. It is typically accomplished by treating the polymer with concentrated sodium or potassium hydroxide solution (60 %) at a temperature of 140 °C for 30 minutes using a solid-to-solvent ratio of 1:10 (w/v) (Pellis *et al*., 2022). The recommended alkali is sodium hydroxide. After deacetylation, the chitosan is thoroughly washed to eliminate any alkali, and then it is dried to produce flakes which contains protein and ash. Environmental contamination, erratic molecular weights, and varying levels of acetylation are only a few drawbacks of chemically produced chitosan.

The chitin's quality might be negatively impacted by conventional, severe extraction methods. New techniques are being created to extract chitin from crustacean waste in place of conventional demineralization and deproteinization. There has been a lot of research done on the usage of enzymes in the deproteinization process. Using

Aspergillus Niger to deproteinize shrimp waste, washing, drying, and demineralizing it using acetic or lactic acid generated by fermentation from affordable biomass like cheese whey have all been documented (Rinaudo, 2006). Demineralization has been carried out using a variety of bacteria, including *Bacillus subtilis, Lactobacillus helveticus, Pseudomonas aeruginosa, Lactobacillus paracasei, Lecanicillium fungicola,* and *Penicillium chrysogenum* (Pellis *et al*., 2022). Organic salts like calcium lactate, which is easily removed from media by washing off, are precipitated by these microbes. The proteolytic activities of certain organisms are also used to carry out deproteinization. The calcium and potassium lactates may be used as food preservatives, whereas the calcium, magnesium, and potassium acetates produced as byproducts are proposed as potential de-icing agents. Commercial potential exists for enzymatic deacetylation employing fungal chitin deacetylase.

2.12.1 Characterization of prepared chitosan and its properties

One of a macromolecule's most fundamental properties is its molecular weight. Understanding polysaccharides' uses and their function in biological systems depends critically on having knowledge of their molecular weight. Methods including chromatography, light scattering (Pellis *et al*., 2022), and viscometry may be used to estimate the molecular weight of chitosan, which is highly dependent on the deacetylation conditions. The simplest and most often used method for determining the molecular weight of chitosan is viscometry. However, because the approach depends on the relationship between the intrinsic viscosity and molecular weight values, it has the drawback of not being absolutely accurate. The molecular weight of chitosan, which ranges from 10,000 to 1,000,000 Da, is sold commercially.

In proportion to its molecular weight and concentration, chitosan tends to becomes more viscous. Viscosity likewise increases with increasing deacetylation levels (Aranaz *et al*.,

2021). High and low deacetylated chitosan have distinct conformations in aqueous solution. Due to the charge repulsion in the molecule, when chitosan is heavily deacetylated, it adopts an expanded conformation and a more flexible chain. The low charge density in the polymer chain, however, causes the chitosan molecule to take on a rod-like or coiled form at low levels of deacetylation. Temperature and other variables, such as concentration, can also have an impact on how viscous a chitosan solution is. The viscosity increases as the temperature drops and the chitosan content rises. Because of depolymerization, chitosan's viscosity falls as demineralization time increases (Aranaz *et al*., 2021). Similarly, Chawla and Kanatt (2014) showed that physical (grinding, heating, autoclaving, ultrasonication) and chemical (ozone) treatments have a significant impact on chitosan viscosity. It is discovered that the viscosity of chitosan solution held at 4 °C is largely stable.

The degree of chitosan's deacetylation determines how soluble it is. High levels of deacetylation indicate increased solubility, whereas low levels indicate poor solubility (Pellis *et al*., 2022). Due to substantially weaker intermolecular hydrogen bonds brought on by the main chains' parallel orientation, it exhibits swelling properties. Chitosan solubility is influenced by the proportion of acetylated and non-acetylated Dglucosamine units and the quantity of protonated amino groups in the polymeric chain. Comparing it to other neutral or negatively charged polysaccharides, its cationic nature sets it apart. Chitosan has an amino group and a strong base with a pKa value of 6.3. The charged state and characteristics of chitosan are significantly changed by the pH of the solution (Yi *et al*., 2005). Also, because amines become protonated and positively charged at low pH, chitosan is a cationic polyelectrolyte that is water soluble. On the other hand, once the pH rises above 6, the polymer loses its charge and becomes insoluble due to the deprotonation of chitosan amines. Precipitation or gelation is more likely to happen at higher pH levels,

and the chitosan solution forms a poly-ion complex with an anionic hydrocolloid as a result, causing gel to develop (Yi *et al*., 2005). At a pH of 6.5, the soluble-insoluble transition takes place at the pKa value. At low pH levels, chitosan may readily produce quaternary nitrogen salts. Therefore, chitosan may be broken down by organic acids like acetic, formic, and lactic acids. Chitosan is most frequently dissolved in 1 % acetic acid, which has a pH of around 4.0 (Sikorski *et al*., 2021).

Additionally, chitosan is soluble in nitric acid and 1 % hydrochloric acid, but it is insoluble in sulfuric and phosphoric acids. The degree of deacetylation, ionic concentration, pH, the kind of acid used for protonation, the distribution of acetyl groups throughout the chain, the conditions of separation and drying of the
polysaccharide, and all of these factors affect the solubility of chitosan. Chitosan's usage in the food, cosmetics, agricultural, and health industries is constrained by its large molecular weight, which leads to low solubility at neutral pH and its high solution viscosity (Xia *et al*., 2011).

Many physiochemical and biological aspects of chitosans, including crystallinity, hydrophilicity, degradation, and cell responsiveness, are influenced by the degree of deacetylation (DD), which has frequently been identified as a key parameter. In other to achieve maximum deacetylation, chitosan is often treated with alkali and increasing time and temperature. The acetyl group of chitin combines with NaOH during the deacetylation process to create an amine group. This reversible reaction is skewed in the direction of the forward reaction by creating more chitosan when the concentration of NaOH is raised. Deacetylation will rise as a result. Deacetylation is the random removal of acetyl groups from polymers, leaving behind a final polymer with a distribution of acetyl glucosamine and glucosamine units. The percentage of D-glucosamine and Nacetyl D-glucosamine determines the biopolymer's deacetylation, which determines whether it

is chitin or chitosan. A variety of techniques have been reported for the determination of the degree of deacetylation of chitosan such as; spectroscopy (infrared, ultraviolet, or 1H, 13C, 15N nuclear magnetic resonance), conventional methods (various types of titration, conductometry, potentiometry, ninhydrin assay, adsorption of free amino groups of chitosan by picric acid), and destructive methods (elementary analysis or acid or enzymatic hydrolysis of chitin or chitosan) followed by colourimetric methods or high-performance liquid chromatography, pyrolysis gas chromatography, and thermal analysis using differential scanning calorimetry. Of these, 1H NMR has been proven to be the most straightforward, quick, and accurate than many of the other methods (Rinaudo, 2006).

The crystallinity of chitosan is one of the primary physical factors that determine its functional qualities (Lizardi-Mendoza *et al*., 2016). It has been discovered that crystallinity affects metal sorption. Lizardi-Mendoza *et al*. (2016) concluded that sorption was only feasible in the amorphous domains and not in the crystalline domains and discovered that the crystallinity of chitosan regulated the sorption rate and total absorption of uranyl. The accessibility of the amine groups in the polymer can also be influenced by its crystallinity. By using X-ray diffraction (XRD), which records the pattern created when X-rays diffraction through a crystal's closely spaced atomic lattice is studied to identify the structure of the lattice, the crystallinity of chitosan is ascertained.

The capacity of chitosan to sequester metal ions is superior to chitins. It chelates several transition metal ions and possesses reactive amino and hydroxyl groups. Chelation is influenced by both the location of the amino group and the number of amino acids present. The kind of cation is crucial to the interaction process (Kong *et al*., 2022). The development of complexes between metal ions and chitosan has been attributed to a number of processes, including adsorption, ion exchange, and chelation. Under

heterogeneous circumstances, chitosan functions as a poly(monodentate) ligand at pH less than 6, but at higher pH, it behaves as a poly(bidentate) ligand forming chelates.

The type of contact that predominates depends on the metal, its chemistry, and the pH. However, complexes can also form in solution when two amino groups from the same chain or from distinct chains are coordinated to the same metal ion.

2.12.2 Applications of chitosan

Numerous studies on the uses of chitosan are being conducted by academic and industry specialists. This is demonstrated in several pertinent academic publications and patents on the topic. Agriculture, food processing, biotechnology, chemistry, cosmetics, dentistry, medicine, textiles, veterinary medicine, and environmental sciences are just a few of the industries where chitosan and its derivatives are used. The gel-forming capability, high adsorption capacity, biodegradability, and antibacterial characteristics of chitosan—all of which are crucial for its commercial applications—are due to the polyelectrolyte's nature and the presence of reactive functional groups.

2.12.3 Antimicrobial activity

Chitosan displays broad-spectrum antimicrobial activity against bacteria, moulds and yeasts. It is effective against both Gram-positive and Gram-negative foodborne microorganisms, including *Aeromonas hydrophila, Bacillus cereus, B. licheniformis, B. subtilis, Clostridium perfringens, Brochothrix spp., Enterobacter sakazakii, Lactobacillus spp., Listeria monocytogenes, Pseudomonas spp., Salmonella typhimurium, S. enteritidis, Serratia liquefaciens, Staphylococcus aureus, and Escherichia coli* O157H7; the yeasts *Candida, Saccharomyces*, and *Rhodotorula;* and the molds *Aspergillus, Penicillium*, and *Rhizopus*. The chitosan and its derivatives are effective against plant pathogenic bacteria such as; *A. tumefaciens, C.*

fascians, E. amylovora, E. carotovora, P. solanacearum, and S. lutea and *fungi A. alternata, B. fabae, F. oxysporum, P. digitatum, P. debaryanum, and R. solani* (Venugopal, 2011). There are several elements that contribute to the fact that the precise mechanism of chitosan's antibacterial action is not entirely understood. To describe how chitosan works as an antibacterial, three models have been presented. The most convincing theory contends that the polycationic composition of chitosan is what causes it to have an antibacterial action. Chitosan's NH_2 groups in the C2 position protonate in an acidic environment to produce NH^{3+}, which binds to negatively charged carboxylate (-COO-) groups on the surface of bacterial and fungal cell surfaces, disrupting the barrier properties of the microorganisms' outer membranes and allowing cell components to leak out (Venugopal, 2011). Studies using electron microscopy that demonstrate the binding of chitosan to bacterial outer membranes provide credence to this idea (Raafat *et al*., 2008). The ratios of unprotonated and protonated amino groups depend on the pH of the microenvironment in which chitosan operates. 50 % of amino groups are protonated at pH pKa. The positively charged amino group provides 90 % at pH 5.5 and 99 % at pH 4.5. Below pH 6.0, where chitosan is most soluble and the protonated form predominates, the antibacterial efficacy of chitosan seems to be at its maximum.

According to Sebti *et al*. (2005), the second hypothesized mechanism is based on chitosan's capacity to bond with microbial DNA and block the production of mRNA and proteins. According to this theory, chitosan molecules can get past the bacterial cell wall, which is made up of many layers of cross-linked murein, and get to the plasma membrane. Confocal laser scanning microscopy has shown that *E. coli* exposed to chitosan under various circumstances contains chitosan oligomers, a chain with limited numbers of monomer units, supporting this notion.

Chitosan's capacity to chelate metals is the basis of the third mechanism. It is widely known that chitosan has good metal-binding abilities; the amine groups in the chitosan molecules are in charge of the absorption of metal cations through chelation; this results in decreased microbial growth and toxin generation (Goy *et al*., 2009). This method is probably more effective at high pH levels when positive ions are bonded to chitosan because the amine groups are unprotonated and the electron pair on the amine nitrogen is accessible for donation to metal ions. Fungal growth is hampered by chitosan's capacity to create a gas-impermeable covering. According to El-Ghaouth and colleagues (1992), it blocks a variety of developmental processes, including mycelial growth, sporulation, spore viability and germination, and the synthesis of fungal virulence factors. In varying degrees, it has been reported that chitosan derivatives like Ntrimethyl, sulfonated chitosan, and chitose oligomers have antibacterial effects on
Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, S. epidermidis, Klebsiella pneumonia, and *Proteus vulgaris*.

Chitosan's antibacterial action is influenced by the target organism, pH of the solution, degree of deacylation, and molecular weight. Chitosan's antibacterial action rises with increasing molecular weight. When comparing results from various research, it is challenging to identify a clear relationship between the molecular weight and the antibacterial activity of chitosan. This is mostly due to the fact that numerous researchers have referred to low MW (LMW) and high MW (HMW) chitosan by an ambiguous phrase without specifying the MW in question. According to several findings, MW has either favourable, negative, or neutral effects on chitosan's antibacterial activity (Badawy and Rabea, 2011).

The quantity of chitosan that has been deacetylated directly correlates with the antibacterial activity of chitosan. A higher level of deacetylation results in more amino

groups being present in chitosan. As a result, chitosan dissolves entirely in water and has a higher proportion of protonated amino groups in an acidic environment, which increases the likelihood that it would interact with negatively charged microbe cell walls. The pH has a significant impact on chitosan's antibacterial action. Along with the "hurdle effect" of causing acid stress on the target organisms, there is an increase in the amount of protonated amino groups on chitosan at lower pH levels (Badawy and Rabea, 2011). The antibacterial activity of chitosan is also influenced by the temperature of incubation. Compared to refrigeration temperatures, it has been demonstrated that a higher temperature (37 °C) increases its antibacterial action (Kong *et al*., 2010).

Chitosan's antimicrobial properties are suppressed by divalent cations in the following order: $Ba^{+2} > Ca^{+2} > Mg^{+2}$. According to a theory by Badawy and Rabea (2011), the cations form complexes with chitosan, reducing the number of amino groups accessible for chitosan to have a bactericidal effect. For a variety of industrial applications, chitosan is an appropriate antibacterial polymer due to a number of its properties. These qualities include being readily and abundantly available, having long-term storage stability at the application temperature, not decomposing or emitting toxic products, not being toxic or irritating to handlers, and being biocidal to a wide range of pathogenic microorganisms.

2.12.4 Antioxidant activity

The strong antioxidant activity of chitosan and its derivatives has been observed. Due to their capacity to bind metals, they are able to scavenge free radicals and regulate lipid oxidation. According to Venugopal (2011), the molecular weight, viscosity, and level of deacetylation of chitin and chitosan determine their antioxidant properties.

2.13 Chitosan as Edible Coating for Fruits and Vegetables

The application of edible films and coatings helps to increase the shelf life and quality of food items. For this reason, edible films made of cellulose and proteins are now being employed. They do an excellent job of lowering O_2 and CO_2 partial pressures, but they are less effective in transferring moisture between food and the environment. Chitosan can be used to create strong, durable, flexible, semi-permeable films that can be used as food wrappers to increase the shelf life of food. During postharvest storage, fruits and vegetables go through a number of physiological changes. Among these include tissue softening, a rise in sugar levels, the breakdown of chlorophyll, and the creation and breakdown of flavour compounds with volatile chemicals. As a certain amount of respiration activity is necessary to keep plant tissues from senescing and dying, controlling the respiration rate significantly increases the storage capacity and shelf life of fresh products.

The most significant quality factors influencing marketability in minimally processed agricultural goods are appearance, colour, texture, flavour, nutritional value, and microbiological quality. Therefore, effective management of these quality variations is necessary for these goods to be commercially viable. Chitosan film may enhance microbiological quality, preserve colour and flavour characteristics, reduce moisture loss and drip formation, and prevent moisture loss, all of which can increase the shelf life of a range of fruits and vegetables. Produce can be dipped in a diluted solution of chitosan and diluted acetic acid as opposed to being wrapped in a chitosan film. In order to increase the effectiveness of the therapy, the approach also permits the use of substances such as Vitamin E, rosemary, oleoresin, calcium, and potassium (Aider, 2010). According to Aider (2010), strawberries, bell peppers, cucumbers, peaches, pears, and kiwifruit, all show the treatment's effectiveness.

2.14 Haematological Studies

Haematology refers to the study of the numbers and morphology of the cellular elements of the blood – the red cells (erythrocytes), white cells (leucocytes), and the platelets (thrombocytes) and the use of these results in the diagnosis and monitoring of disease (Merck Manual, 2012). Haematological studies are useful in the diagnosis of many diseases as well as the investigation of the extent of damage to blood (Togun *et al*., 2007). Haematological studies are of ecological and physiological interest in helping to understand the relationship of blood characteristics to the environment (Isaac *et al*., 2013). Haematological parameters are good indicators of the physiological status of animals (Etim, 2014). Haematological parameters are those parameters that are related to the blood and blood-forming organs (Bamishaiye *et al*., 2009). Blood act as a pathological reflector of the status of exposed animals to a toxicant and other conditions (Olafedehan *et al*., 2010). As reported by Isaac *et al*. (2013) animals with good blood composition are likely to show good performance. Laboratory tests on the blood are vital tools that help detect any deviation from normal in the animal or human body (Ogunbajo *et al*., 2009).

The examination of blood gives the opportunity to investigate the presence of several metabolites and other constituents in the body of animals and it plays a vital role in the physiological, nutritional and pathological status of an organism (Doyle, 2006). According to Olafedehan *et al*. (2010) examining blood for its constituents can provide important information for the diagnosis and prognosis of diseases in animals. Blood constituents change in relation to the physiological conditions of health (Togun *et al*., 2007). These changes are of value in assessing the response of animals to various physiological situations (Etim, 2014). According to Afolabi *et al*. (2010), changes in haematological parameters are often used to determine various statuses of the body and to determine stresses due to environmental, nutritional and/or pathological factors

2.15 Haematological Components and Their Functions

Blood which is a vital special circulatory tissue is composed of cells suspended in a fluid intercellular substance (plasma) with the major function of maintaining homeostasis (Isaac *et al*., 2013). Haematological components, which consist of red blood cells, white blood cells or leucocytes, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration are valuable in monitoring feed toxicity, especially with feed constituents that affect the blood as well as the health status of experimental animals (Oyawoye and Ogunkunle, 2004).

2.15.1 Red blood cells

Red blood cells (erythrocytes) serve as a carrier of haemoglobin. It is this haemoglobin that reacts with oxygen carried in the blood to form oxyhaemoglobin during respiration (Chineke *et al*., 2006). According to Isaac *et al*. (2013), red blood cell is involved in the transport of oxygen and carbon dioxide in the body. Thus, a reduced red blood cell count implies a reduction in the level of oxygen that would be carried to the tissues as well as the level of carbon dioxide returned to the lungs (Ugwuene, 2011; Isaac *et al*, 2013; Soetan *et al*., 2013).

2.15.2 White blood cells

The major functions of the white blood cell and its differentials are to fight infections, defend the body by phagocytosis against invasion by foreign organisms and produce or at least transport and distribute antibodies in the immune response. Thus, animals with low white blood cells are exposed to a high risk of disease infection, while those with high counts are capable of generating antibodies in the process of phagocytosis and have a high degree of resistance to diseases (Soetan *et al*., 2013) and enhance adaptability to

local environmental and disease prevalent conditions (Kabir *et al*., 2011; Iwuji and Herbert, 2012; Okunlola *et al*., 2012; Isaac *et al*., 2013).

2.15.3 Platelets counts

Blood platelets are implicated in blood clotting. Low platelet concentration suggests that the process of clot-formation (blood clotting) will be prolonged resulting in excessive loss of blood in the case of injury. They are cytoplasmic molecules derived from the bone marrow megakaryocytes (Machlus *et al*., 2017) entering the bloodstream and missing the nucleus of cells. They are only present in mammals where they work in havetasis. Platelet plug formation (primary hemostasis) involves the activation of the coagulation cascade resulting in the deposition and linking of fibrins (secondary hemostasis). A low platelet concentration disorder called thrombocytopenia results from either a decline in development or elevated damage. An enormous concentration of platelets called thrombocytosis can be either congenital, reactive or as a result of unguided growth.

2.15.4 Packed cell volume

Packed Cell Volume (PCV) which is also known as haematocrit (Ht or Hct) or erythrocyte volume fraction (EVF), is the percentage (%) of red blood cells in the blood (Purves *et al*., 2003). According to Isaac *et al*. (2013), Packed Cell Volume is involved in the transport of oxygen and absorbed nutrients. Increased Packed Cell Volume shows better transportation and thus results in increased primary and secondary polycythemia. Haemoglobin is the iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates (Etim, 2014) with the exception of the fish family, channichthyldae (Sidell and O‘ Brien, 2006) as well as tissues of invertebrates. Haemoglobin has the physiological function of transporting oxygen to tissues of the animal for oxidation of ingested food so as to release energy for other body functions as well as transport carbon dioxide out of the body of animals (Ugwuene, 2011; Omiyale *et al*., 2012; Isaac *et al*.,

2013; Soetan *et al*., 2013). According to Peters *et al*. (2011), previous reports stated that Packed Cell Volume, haemoglobin and mean corpuscular haemoglobin are major indices for evaluating circulatory erythrocytes, and are significant in the diagnosis of anaemia and also serve as useful indices of the bone marrow capacity to produce red blood cells as in mammals (Chineke *et al*., 2006). Furthermore, Chineke *et al*. (2006) posited that high Packed Cell Volume (PCV) reading indicated either an increase in the number of Red Blood Cells (RBCs) or a reduction in circulating plasma volume. Mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration indicate blood level conditions. A low level is an indication of anaemia (Etim, 2014).

2.16 Biochemical Parameters

The Liver Function Test (LFT), Renal Function Test (RFT), and Lipid Profile (LP), which are crucial in the evaluation of health status, are biochemical markers. According to Fassett *et al*. (2010) and Yang *et al*. (2014), the liver and kidney are important for metabolic processes and determining health status.

2.17 Liver Function Parameters

The liver is frequently attacked by toxic substances due to its crucial role in the body. The evaluation of AST, ALT, ALP, protein, albumin, and bilirubin all provide credible indicators of the health status of the liver, despite their limited sensitivity and specificity (Fassett *et al*., 2011; Champion *et al*., 2012; Yang *et al*., 2014). For example, in a toxic environment, it has been observed that blood levels of AST and ALT increase significantly (Yang *et al*., 2014; Nosrati *et al*., 2017), possibly as a result of the degeneration of liver cells (Yang *et al*., 2014). Although ALT has been extensively utilized as a more specific marker to measure suspected liver cell damage, this is because ALT is abundantly present in the cytoplasm of liver cells (Champion *et al*., 2012; Yang *et al*., 2014).

2.17.1 Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST), a widely distributed enzyme, is found in the heart, liver, muscles, and kidneys, among other tissues and erythrocytes. Myocardial infarction, viral hepatitis, and muscular dystrophy are a few disorders that affect these tissues and are associated with elevated blood levels (Bezerra *et al*., 2014). Serum AST is raised after myocardial infarction and peaks two days after the start. There have been shown to be cytoplasmic and mitochondrial AST isoenzymes. The mitochondrial isoenzyme and the cytoplasmic isoenzyme have both been found in the sera of patients with coronary and hepatobiliary disorders, whereas only the cytoplasmic isoenzyme appears in normal blood (Yang *et al*., 2014).

2.17.2 Alanine aminotransferase (ALT)

The alanine aminotransferase (ALT) enzyme is found throughout the body, with concentrations being highest in the liver and lowest in the kidneys, heart, skeletal muscles, pancreas, and lungs. Hepatitis, cirrhosis, obstructive jaundice, liver cancer, and persistent alcohol misuse are all associated with elevated blood ALT levels. In individuals with a simple myocardial infarction, ALT is only marginally raised (Fassett *et al*., 2011). ALT is the more liver-specific enzyme, even though both AST and ALT are raised anytime disease processes damage the integrity of liver cells. Additionally, increases in ALT activity last longer than increases in AST activity.

2.17.3 Alkaline phosphatase (ALP)

A wide range of physiological and non-physiologic phosphoric acid esters are hydrolyzed by alkaline phosphatase (ALP) in an alkaline media (pH optimum 10). Alkaline phosphatase in normal sera comes from the liver and biliary tracts. Normal alkaline phosphatase levels vary with age, being higher in kids and teenagers than in adults. For

diagnosing cholestasis and obstructive jaundice, ALP is one of the preferred assays. In several disorders, including hepatitis, cirrhosis, cancer, and bone ailments, elevated levels are observed (Lowe *et al*., 2022).

2.17.4 Serum total protein

Acid-base balance and appropriate water transport between tissues and blood are both maintained by serum proteins, which are mostly synthesized in the liver. Both the amount of total protein and the ratio of various fractions may alter independently under certain pathological circumstances. Dehydration brought on by vomiting or diarrhoea may be accompanied by hyperproteinemia. In multiple myeloma, the levels of total protein also rise. According to Nosrati *et al.* (2017), pathological circumstances such as nephrotic syndrome, haemorrhage, sprue, and salt retention as well as chronic lowprotein diets can result in hypoproteinemia.

2.17.5 Albumin test

The main serum protein in healthy people is albumin. It binds and solubilizes several substances, including calcium and bilirubin, and it maintains the plasma colloidal osmotic pressure. Dehydration is frequently the cause of elevated serum albumin concentrations. The diagnostic importance of hyperalbuminemia is negligible. In several disorders, such as malabsorption, liver, kidney, severe burns, infections, cancer, and some genetic defects, hypoalbuminemia is particularly prevalent. Low plasma oncotic pressure in severe hypoalbuminemia (less than 2.5 g/dL) permits water to leak from blood capillaries into tissues, causing oedema (Moman *et al.*, 2022).

2.17.6 Total bilirubin

Humans produce 250 to 300 mg of bilirubin on average per day from a variety of sources, of which 85 % comes from haemoglobin released from senescent erythrocytes that are

destroyed in the reticuloendothelial system. The remaining 15 % is created through the degradation of other heme-containing proteins such as cytochromes and myoglobin as well as the destruction of erythrocytes in the bone marrow. Bilirubin is produced in peripheral organs and then transferred to the liver along with albumin. Bilirubin is coupled with glucuronic acid in the liver in order to be solubilized, transported through the bile duct, and eliminated through the digestive system. Unconjugated (indirect) bilirubin levels rise in the blood as a result of diseases or disorders that, due to hemolytic processes, create bilirubin more quickly than the liver can handle it. Both conjugated (direct) and unconjugated (indirect) bilirubin levels increase in the blood as a result of bile duct blockage or damage to the hepatocellular structure (Kalakonda *et al*., 2022).

2.18 Renal Function Parameters

Renal function parameters constitute; Urea, creatinine, and the electrolytes (Chloride, potassium, sodium and bicarbonate) Creatinine, a diagnostic of chronic renal dysfunction, is the most reliable renal marker and only rises after the major renal function is lost (Gounden *et al*., 2022). Urea, an indicator of acute renal dysfunction, is the first acute marker after renal injury.

2.18.1 Urea test

The primary byproduct of protein nitrogen metabolism is urea. It is produced in the liver during the urea cycle and eliminated through the kidneys. Urea levels in the blood are influenced by protein consumption, protein catabolism, and kidney function. Elevated urea levels can happen as a result of renal dysfunction, as well as in conditions including diabetes, infection, congestive heart failure, and several liver conditions. The most common screening test for renal function, along with serum creatinine, is the measurement of blood urea nitrogen (Gounden *et al*., 2022).

2.18.2 Creatinine test

The pancreas, liver, and kidney all produce creatinine. It travels via the blood to many organs, including the brain and muscles, where it is phosphorylated to become phosphocreatinine (Forbes *et al*., 2022). Daily conversion of some of the free creatinine in muscle to creatinine occurs, and the amount of creatinine generated is proportional to the mass of the muscle. The pace at which a person excretes creatinine is generally consistent in the absence of renal disease. As a result, measuring creatinine clearance is helpful in identifying renal disease and determining the degree of renal function impairment. Patients with renal dysfunction, particularly those with reduced glomerular filtration, have higher blood levels of both creatinine and urea. Increases in blood urea levels typically occur before increases in serum creatinine in the early stages of renal disease. However, dehydration, nutrition, and protein metabolism can all have an impact on blood urea levels. However, serum creatinine levels often remain stable and are unaffected by these variables. As a result, serum creatinine is a far more credible indicator of renal function than serum urea (Gounden *et al*., 2022).

2.18.3 Potassium test

The main cation in the intracellular fluid is potassium. Due to its effect on muscular action, it is also a crucial component of extracellular fluid. Its intracellular function is similar to its extracellular function in that it affects water retention, acid-base balance, and osmotic pressure. Hyperkalemia, or elevated potassium levels, are frequently linked to renal failure, dehydration shock, or adrenal insufficiency (Simon *et al*., 2023). Malnutrition, a negative nitrogen balance, gastrointestinal fluid losses, and adrenal cortex hyperactivity are all linked to decreased potassium levels (hypokalemia). Prior deproteinization of serum or plasma material was necessary for the colourimetric

techniques for potassium or sodium determination previously published. The direct spectrophotometric detection of potassium in blood or plasma is an improved approach.

2.18.4 Sodium test

The main cation in the extracellular fluid is sodium. It is crucial for maintaining the osmotic pressure and appropriate water distribution throughout the different fluid compartments. The major source of body salt is sodium chloride present in consumed meals. Only about one-third of the total body‘s sodium is contained in the skeleton since most of it is contained in the extracellular body fluid (Arneson and Brickell, 2007).

Hyponatremia (low serum sodium level) is present in a range of disorders such as; severe polyuria, metabolic acidosis, Addison‘s disease, diarrhoea, and renal tubular disease. The following conditions are associated with hypernatremia (an increased blood sodium level); hyperadrenalism, severe dehydration, diabetic coma following insulin therapy, and excessive treatment with sodium salts.

2.18.5 Chloride test

The extracellular anion that is most prevalent is chloride. It works in conjunction with Natrium Chloride to maintain osmotic pressure, the anion-cation balance, and therefore the water distribution in the extracellular fluid compartment. Reduced plasma Cl-concentrations (hypochloremia) can be brought on by metabolic acidosis, which is brought on by either an increase in the generation or a decrease in the secretion of organic acids, salt-losing nephritis, persistent gastric secretion, protracted vomiting, and other conditions. Increased plasma Cl-concentrations (hyperchloremia) can be caused by

dehydration, renal tubular acidosis, acute renal failure, hyperfunction of the adrenocortical glands, salicylate intoxication, metabolic acidosis brought on by protracted diarrhoea, and loss of sodium bicarbonate (Sharma *et al*., 2023). To assess the anion gap in serum and/or urine, chloride is frequently analyzed together with Natrium and Calcium. In the early assessment of hyperchloremic metabolic acidosis, the urinary anion gap is helpful. The thiocyanate approach is less affected by the presence of bromide than measurement with an ion-selective electrode because of the different reactivity equivalents of chloride and bromide.

2.18.6 Bicarbonate test

The diagnosis of the acid-base balance in the blood is made by bicarbonate measurement. Increased and decreasing levels signify diseases linked to respiratory and metabolic system abnormalities. A reliable NADH analogue technique and phosphoenolpyruvate carboxylase (PEPC) was utilized in an enzyme test. Based on sodium carbonate, this approach has proven primary standard-compliant (Burger and Schaller, 2022).

2.19 Lipid Profile

Lipid profile includes tests such as; total cholesterol, triglycerides (TG), high-density lipoproteins (HDL) and low-density lipoproteins (LDL) are evaluated to check for any risks of cardiovascular disease (Nosrati *et al*., 2017).

2.19.1 Total cholesterol

The determination of blood cholesterol levels is crucial for the diagnosis and categorization of hyperlipoproteinemia as well as serving as a marker of biliary, intestinal, and liver function. Diabetes, nephritic syndrome, and hypothyroidism are among the conditions that can lead to elevated cholesterol levels. Correlations between the

prevalence of coronary artery diseases and elevated blood cholesterol levels are strong. Normal cholesterol levels are impacted by stress, age, gender, hormonal balance, and pregnancy. Low levels are linked to serious liver disorders and hyperthyroidism (Nosrati *et al*., 2017).

2.19.2 Triglycerides test

The major lipids in human plasma are triglycerides; the other lipids include cholesterol, phospholipids, and non-esterified fatty acids (Feingold, 2021). Glycerol and fatty acids are esterified in the intestinal mucosa to create them. Patients with diabetes mellitus, liver blockage, nephrosis, and other disorders involving lipid metabolism are diagnosed and treated using measures of triglycerides. The diagnosis of hyperlipoproteinemia, as well as the prevention, detection, and follow-up of atherosclerosis, all depend on serum triglyceride levels.

2.19.3 High-density lipoproteins (HDL)

Measurements of high-density lipoprotein have been found to be helpful in determining the risk of coronary heart disease when combined with other lipid measurements (Nosrati *et al*., 2017). Increased levels of HDL reduce the risk of coronary heart disease because HDL is responsible for transporting cholesterol from peripheral cells back to the liver. Very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) are selectively precipitated from serum or plasma samples, and then the amount of cholesterol in the HDL-containing supernatant is determined (Juarez & Farzam, 2022).

2.19.4 Low-density lipoproteins (LDL)

The liver produces Low-Density Lipoproteins (LDL) by breaking down triglyceriderich Very Low-Density Lipoproteins (VLDLs) via a variety of lipolytic enzymes. There are certain LDL receptors that help liver parenchymal cells remove LDL from plasma. It has

been established that LDL is the primary source of the majority of the cholesterol found in atherosclerotic plaques. Because of this, the LDL-Cholesterol concentration is regarded as the most crucial clinical predictor for coronary atherosclerosis among all single measures. In therapy that targets lipid reduction to prevent atherosclerosis or slow its progression and avoid plaque rupture, accurate measurement of LDL-Cholesterol is crucial (Linton *et al*., 2019).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical

Chitosan was obtained from Beijing, Wisapple Biotech, Co., Ltd. All chemicals used was of General-Purpose Reagent (GPR) grade and a product of Sigma-Aldrich, USA.

3.1.2 Reagents

1. Aspartate aminotransferase (AST) reagent (R); Tris buffer (pH 7.7) 80 mmol/L, L-Aspartate 240 mmol/L, malate dehydrogenase (MDH) >450 U/L, lactate

dehydrogenase (LDH) >1200 U/L, Sodium Hydroxide 220 mmol/L, Sodium Azide 8 mmol/L, NADH >0.18 mmol/L and 2-α-ketoglutarate 18 mmol/L.

2. Alanine aminotransferase (ALT) reagent; Reagent 1 (R1) (Tris buffer (pH 7.40) 100 mmol/L, L-Alanine 1.4 mol/L, LDH ≥3500 U/L and sodium Azide 0.06 mmol/L) Reagent R (R2) (NADH ≥0.06 mmol/L, 2-α-ketoglutarate 4 mmol/L and Sodium Azide 8 mmol/L).
3. Alkaline phosphatase (ALP) reagent; Reagent 1 (R1) (2-Amino-2-Methyl-1Propanol (pH 10.3) 2.0 mol/L and $MgCl_2$ 2.0 mmol/L) Reagent 2 (R2) (ρNitrophenylphosphate 16 mmol/L).
4. Total protein reagent; R (sodium hydroxide 750 mmol/L, copper sulfate 12.0 mmol/L, sodium potassium tartarate 40.9 mmol/L and potassium iodide 19.8 mmol/L).
5. Albumin reagent; R (Succinate buffer 200 mmol/L, Bromocresol green 0.4 mmol/L and sodium azide 4.0 mmol/L).
6. Bilirubin reagent; R1 (Sulfanilic acid 31.0 mmol/l, HCL 0.20 N), R2 (Sodium nitrite 28.0 mmol/l), R3 (Caffeine 0.28 mol/l and Sodium Benzoate 0.55 mol/l), R4 (Tartarate 0.99 mol/l and Sodium hydroxide 2.0 N).
7. Urea reagent; R1 (Phosphate buffer (pH 8.0) 100 mmol/l, Sodium Salicylate 80 mmol/l, Sodium nitroprusside 6.0 mmo/l and EDTA 30.0 mmol/l), R2 (Urease >6000 U/I), R3 (Sodium hydroxide 400 mmol/l, sodium hypochlorite 20.0 mmol/l).
8. Creatinine reagent; R1 (Picric acid 25 mmol/L), R2 (Sodium hydroxide 0.4 mol/L).
9. Potassium reagent; R (Sodium Tetraphenylboron 2.1 mM, preservatives and thickening agents).

10. Sodium reagent; filtrate reagent (Uranyl Acetate 2.1 mM and Magnesium Acetate 20 mM), Acid reagent (dilute acetic acid), Sodium colour reagent (Potassium ferrocyanide, non-reactive stabilizers and fillers).
11. Chloride reagent; R (Hg II-thiocyanate 2 mmol/l, Fe III-nitrate 30 mmol/l and HNO_3 40 mmol/l).
12. Bicarbonate reagent; R1 (MgSO4 780 µmol/l, preservative, surfactant, inhibitor and sodium oxamate), R2 (phosphoenolpyruvate carboxylase (PEPC) ≥1.24 KU/l, MDH ≥33.2 KU/l, NADH ≥6.45 mmol/l, phosphoenolpyruvate (PEP) ≥21.1 mmol/l, MgSO4 370 µmol/l, buffer, stabilizer, preservative and surfactant).
13. Total cholesterol reagent; R (pipes buffer pH 7.0 50 mmol/L, phenol 30 mmol/L, sodium cholate 5.0 mmol/L, cholesterol esterase >250 U/L, cholesterol oxidase >500 U/L, peroxidase >2.0 KU/L, 4-amino-antipyrine 1.0 mmol/L and sodiumazide 8.0 mmol/L).
14. Triglycerides reagent; R (pipes buffer pH 7.0 50 mmol/L, 4-chlorophenol 6.0 mmol/L, magnesium aspartate >0.5 mmol/L, lipase >10 KU/L, peroxidase >2.0 KU/L, 4-aminoantipyrine 1.0 mmol/L, glycerol-3-phosphate oxidase >3.5 KU/L, glycerol kinase >750 U/L, ATP 1.0 mmol/L and sodium azide 8.0 mmol/L).
15. High Density Lipoprotein (HDL) reagents; R (Phosphotungstate 0.52 mmol/L and Magnesium chloride 30 mmol/L)
16. Low Density Lipoproteins (LDL) reagents; R1 (MES buffer (pH 6.5), Polyvinylsulfonic acid, polyrhyleneglycolmethylester, $MgCl_2$, Detergent, EDTA, 4aminoantipyrine, Cholesterol esterase, Cholesterol oxidase and Peroxidase, R2 (MES buffer (pH 6.5), EDTA, detergent TODB, N-Bis (4sulfobutyl)-3-methylaniline)

3.1.3 Scientific equipment

1. Auto Haematology Analyzer: Mindray BC-5300, Mainland, China.
2. Spectrophotometer: Semi-automatic Biochemistry analyzer-iChem-535shenzhen, Icubio Biomedical Technology Co., Ltd, China, RS232.
3. Centrifuge: LED PRP Centrifuge Machine, MedGroup SKU: MSLZL15.
4. Drying Oven: Laboratory Drying Oven at Rs 11000, Hot Air Oven in Chennai, ID: 20182319448.
5. Magnetic Stirrer: BEXCO Magnetic Stirrer with Hot Plate,
6. Soxhlet Extractor: Behr-Labor behrotest Soxhlet Extraction, 250 mL extraction, 500 mL round-bottom flask, 6 position, SKU: R256S, China.
7. Electronic Weighing Balance: Mini Pocket Digital Electronic Scale LCD Display 1000 g.

3.1.4 Plant sample

Cassava was obtained from the Ultra-Modern Market Minna, Niger State, Nigeria.

3.1.5 Experimental animals

Albino rats were obtained from Ahmadu Bello University animal house, Zaria, Kaduna State, Nigeria. The rats were aged 8-10 weeks and weighed 80-100 g. They were housed in plastic cages and maintained under standard laboratory conditions of 37 °C temperature and relative humidity between 40-45 °C. They were allowed to acclimatize to these conditions for a period of one week and fed rat chow and water *ad libitum*.

3.2 Methods

3.2.1 Starch extraction from cassava tuber

Fresh cassava tubers were peeled, washed, cut into cubes of approximately 1 cm in size, and then grinded for 5 minutes in a high-speed blender. The pulp was stirred for five minutes while being suspended in ten times as much water before being filtered through double-fold cheesecloth. The top liquid was decanted and discarded after the filter was let to stand for two hours to allow the starch to settle. Water was added to the sediment, and the mixture was stirred once more for five minutes. The filtrate underwent a second round of filtering as previously, and the starch was allowed to settle. The sediment (starch) was dried at 55 ^{o}C for one hour after decanting the top liquid (Kaur *et al*., 2016).

3.3 Preparation of Edible Chitosan-starch Films

Films were prepared by the casting process and dehydrating the suspension solution in Petri plates according to the method of Ossamulu *et al*. (2023). A suspension solution was

prepared by dissolving 1 g of chitosan in 100 ml of 2 % (v/v) of aqueous acetic acid solution (Singh *et al*., 2015). Cassava starch powder 1 g was dissolved in 75 ml of distilled water mixed with 75 ml of dissolved chitosan solution under controlled heating conditions (80 °C) with continuous stirring on a hot plate magnetic stirrer (Macro Scientific Works, India) until the gelatinization temperature was reached. To the mixture 0.6 g of glycerol was added and stirred for 30 minutes more for thorough mixing and removal of bubbles. The resulting solution was then filtered through a cheesecloth so as to remove undissolved materials and then dried at 40 °C for 48 h in an oven. The dried films obtained were peeled off manually.

3.4 Proximate Composition Analysis

The proximate composition of chitosan, edible chitosan-starch film and chitosan-starch film supplemented diets was carried out in triple replicates using the Association of Analytical Chemists (AOAC) standard procedures as reported by Opaleke *et al.* (2022) for moisture, total ash, crude fibre, and fat. Nitrogen was determined by the micro Kjeldahl method and the nitrogen content was converted to protein by multiplying by a factor of 6.25. Total carbohydrate content was estimated by _difference‘. All the proximate values were reported in percentage.

3.4.1 Determination of moisture

The oven-drying technique was used to determine the moisture content. In a clean, dried crucible (W1), 2 g of well-mixed samples were accurately weighed. The crucible was placed in an oven for 6 hours heated at 100 °C to achieve a consistent weight. The crucible was subsequently placed in a desiccator to cool for 30 minutes. It was weighed again (W2) after cooling.

3.4.2 Determination of ash

Empty crucible was heated to 550 ºC in a Muffle furnace for one hour, cooled in a desiccator, and then its weight was measured (W1). In a crucible (W2), 2 g of each sample (chitosan, edible chitosan-starch film, and the supplemented diets) were placed and heated until they were charred. Then, the crucibles were placed in a Muffle furnace to burn to ash for two hours at 550 ºC so as to become fully oxidized. The crucibles were cooled and weighed (W3).

3.4.3 Determination of crude protein

Kjeldahl's technique was used to determine the samples' protein content. A digestive flask containing 0.25 g of dried materials, 6 ml of concentrated H_2SO_4, and a trace of the Kjeldah-1 catalyst (10 g Na_2SO_4, 5 g $CuSO_4$, and 0.05 g selenium) was used. The contents of the flask were thoroughly mixed by swirling before being digested on the digestion block until the mixtures became clear (colourless or greenish). The digest was cooled before being transferred to a 100 ml volumetric flask, where it was given the correct volume by being mixed with distilled water. The Markham Distillation Apparatus was used for distilling the digest. Ten millilitres of the digest were put into the distillation tube, and ten millilitres of 40 % NaOH were added gradually in the same manner. The generated NH_3 was recovered as NH_4OH after distillation proceeded for at least 10 minutes. The distillate was subsequently titrated against typical 0.1 N HCL solutions until a pink colour appeared. The same procedures were followed with a blank as well.

3.4.4 Determination of crude fat

The Soxhlet equipment was used to determine crude fat using the ether extract technique. A fat-free thimble was used to hold the 2 g of the moisture-free sample before it was inserted into the extraction tube. Petroleum ether was placed into an apparatus-fitted

receiving flask that had been weighed, cleaned, and dried. The Soxhlet apparatus was assembled together, and after 6 hours of refluxing, the extract was placed into a clean glass dish and washed with ether that was then evaporated over a water bath. The dish was then dried in a desiccator after being placed at 105 °C to 110 °C for an hour.

3.4.5 Determination of crude fibre

A solution containing 1.25 g of H_2SO_4 per 100 ml was added to 2 g of the sample after it had been defatted with petroleum ether and heated under reflux for 30 minutes. The mixture was filtered through linen on a fluted filter paper, washed in boiling water until the washings were no longer acidic, transferred to a beaker, and boiled for 30 minutes with 200 ml of a solution containing 1.35 g of carbonate-free NaOH per 100 ml. The final residue was then filtered through a thin, closed pad of washed and ignited asbestos in a Gooch crucible, dried in an electric oven, cooled, and weighed before being incinerated.

3.4.6 Determination of carbohydrates content

The nitrogen-free method described by Association of Analytical Chemists (2012) was employed to determine the carbohydrate content. The carbohydrate is calculated as weight by difference between 100 and the summation of other proximate parameters as Nitrogen Free Extract (NFE).

3.5 Management of Experimental Animals

Albino rats were purchased from Ahmadu Bello University animal house, Zaria, Kaduna State, weighing 80-100 g. They were placed in plastic cages and fed rat chow and water *ad libitum* and kept in standard laboratory conditions for a week to acclimatize.

3.5.1 Experimental design (Sub-acute toxicity studies)

The rats were randomized into five groups (A-E) of five (5) animals each after the acclimatization period; the feeding regime lasted for a period of twenty-eight (28) days.

The chitosan-starch film supplemented diet table below shows the percentage (%) of edible chitosan-starch film supplemented in the rat diet for each group.

Table 3.1: Chitosan-Starch Film Supplemented Diets

Group	Number of Rats	% of Rat Chow	% of Edible Chitosan-Starch Film
A	5	100	0
B	5	95	5
C	5	90	10
D	5	80	20
E	5	60	40

3.6 Collection of Blood and Serum Preparation

Samples of blood were obtained using the procedure of Tauheed *et al*., (2021), the rats were sacrificed and whole samples of blood were obtained in clean EDTA sample bottles and plain sample bottles. The blood sample in the EDTA bottles was used for haematological analysis while biochemical analysis was performed using the blood in the non-EDTA containers. The blood samples were allowed to agglutinate for 30 minutes

before being centrifuged for 15 minutes at 3000 rpm to separate the plasma from the other cellular components for the biochemical analysis.

3.7 Determination of Haematological Parameters

Haematological studies of albino rats in the five (5) groups were determined with an automated haematological analyzer. The full blood count was obtained by the method described by Hoffbrand *et al*. (2006). Parameters such as; Hemoglobin (HB), Parked cell volume (PCV), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Total white blood cell count (TWBC), Platelets counts (PLC), Neutrophils (NEU), Lymphocytes (LYM), Embryoid body (EB), Red cell distribution width count (RDWC) and red blood cell count (RBC) were determined.

3.8 Determination of Liver Function Parameters

The levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), albumin, total protein (TP) and total bilirubin were determined using a commercial packaged kit (Spectrum kit). Specific procedures for the above determinations were carried out in compliance with the instructions of the test kit manufacturer.

3.8.1 Determination of aspartate aminotransferase (AST)

Assay Principle

The amino group is enzymatically transferred by AST present in the sample from Laspartate to the carbon atom of 2-α-ketoglutarate yielding oxaloacetate and L-glutamate.

Oxaloacetate in the presence of NADH and malate dehydrogenase (MDH) is reduced to L-malate. In this reaction, NADH is oxidized to NAD. The reaction is monitored by

measuring the rate of decrease in absorbance at 340 nm due to oxidation of NADH to NAD.

The addition of lactate dehydrogenase (LDH) to the reagent is necessary to achieve a rapid and complete reduction of endogenous pyruvate so that it does not interfere with the assay.

Procedure

Two test tubes were labelled macro and semi-micro respectively. 1.0 ml of R was added to the macro tube and 500 µl was added to the semi-micro tube, 100 µl of the specimen was added to the macro tube and 50 µl was added to the semi-micro tube. Both test tubes were mixed, the initial absorbance was read after 60 seconds and the timer was started simultaneously. The absorbance was read again after 60, 120 and 180 seconds.

The mean absorbance change per minute (ΔA/min) was determined at λ= 340nm (Yang *et al*., 2014).

3.8.2 Determination of alanine aminotransferase (ALT)

Assay Principle

The amino group is enzymatically transferred by ALT present in the sample from alanine to the carbon atom of 2-oxoglutarate yielding pyruvate and L-glutamate.

Pyruvate is reduced to lactate by LDH present in the reagent with the simultaneous oxidation of NADH to nicotinamide adenine dinucleotide (NAD). The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH.

Endogenous sample pyruvate is rapidly and completely reduced by LDH during the initial incubation period so that it does not interfere with the assay.

Procedure

In a test tube, 1 ml of working solution and 100 µl of the specimen were introduced. They were mixed and the initial absorbance was read after 30 seconds and the timer was started simultaneously. The absorbance was read again after 1, 2 and 3 minutes. The mean absorbance change per minute (ΔA/min) was determined at λ=340 nm (Fassett *et al*., 2010).

3.8.3 Determination of alkaline phosphatase (ALP)

Assay Principle

Alkaline phosphatase (ALP) hydrolyzes ρ-Nitrophenylphosphate (ρ-NPP) to ρNitrophenol and phosphate.

The increase of absorbance per minute at 405 nm is proportional to the enzyme activity.

Procedure

In a test tube, 1.0 ml of working solution and 100 ml of the specimen were introduced. They were mixed and initial absorbance was read after 1 minute and the timer was started simultaneously. The absorbance was read again after 1, 2 and 3 minutes. The mean absorbance change per minute (ΔA/min) was determined at λ=405nm (Lowe *et al*., 2022).

3.8.4 Determination of total Protein

Assay Principle

In an alkaline medium, copper reacts with the peptide bonds of proteins to form the characteristic pink-to-purple biuret complex. Sodium potassium tartrate prevents copper hydroxide precipitation, and potassium iodide prevents the auto-reduction of copper.

The colour intensity is directly proportional to the protein concentration. It is determined by measuring the increase in the absorbance at 546 nm.

Procedure

Three test tubes were labelled blank, standard and sample respectively. 1.0 ml of R was added to all the tubes. Then, 20 µl of the standard was added to the standard tube and 20 µl of the sample was added to the sample tube. The test tubes were mixed and incubated for 10 minutes at room temperature. The absorbance of the specimen ($A_{specimen}$) and standard ($A_{standard}$) against the reagent blank were measured after 30 minutes at λ= 546nm (Nosrati *et al.*, 2017).

3.8.5 Determination of albumin

Assay Principle

Measurement of albumin is based on its binding to the indicator dye bromocresol green (BCG) at pH 4.3 to form a blue-green coloured complex. The intensity of the blue-green colour is directly proportional to the concentration of albumin in the sample. It is determined by monitoring the increase in absorbance at 623 nm.

Procedure

Three test tubes were labelled blank, standard and specimen respectively. 2.5 ml of R was introduced into all tubes, then 10 µl of the standard was added to the standard test tube and 10 µl of the specimen was added into the specimen tube. They were mixed and incubated for 5 minutes at 25 °C. The absorbance of the specimen ($A_{specimen}$) and the standard ($A_{standard}$) against the reagent blank was measured after 60 minutes at λ=578nm (Moman *et al.*, 2022).

3.8.6 Determination of total bilirubin

Assay Principle

The total bilirubin concentration is determined in the presence of caffeine by the reaction with diazotized sulphanilic acid to produce an intensely coloured diazo dye (560-600 nm).

The intensity of colour of this dye formed is proportional to the concentration of total bilirubin. Direct bilirubin is determined in the absence of caffeine by the direct reaction with diazotized sulphanilic acid to form red-coloured azobilirubin, the color intensity of which measured at 546 nm is proportional to the concentration of the direct bilirubin in the sample.

Procedure

The test tubes were labelled sample blank and sample respectively. Two hundred (200) µl of R1 was added to all tubes, followed by 1 drop of R2 which was added to the sample tube, 1.0 ml of R3 was added to all tubes and 200 µl of the sample was added to both test tubes. The content was mixed and incubated for 10 minutes at 25 °C then; 1.0 ml of R4 was added to both tubes. They were mixed and incubated for 5 minutes at 25 °C. The absorbance of the sample (A_{sample}) against the sample blank at 578 nm was measured (Kalakonda *et al*., 2022).

3.9 Determination of Renal Function Parameters

Serum electrolytes (potassium, sodium, chloride and bicarbonate), serum urea and creatinine levels were determined using a test kit (Spectrum kit). Specific procedures for the above determinations were carried out in compliance with the instructions from the test kit manufacturer.

3.9.1 Determination of urea

Assay Principle

Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide.

The free ammonia in an alkaline pH and in the presence of indicator forms coloured complex proportional to the urea concentration in the specimen.

Procedure

Three test tubes were labelled blank, standard and specimen respectively. One (1.0) ml of R1 was added to all test tubes, followed by 50 µl of R2 was also added to all the test tubes, 10 µl of the standard was added to the standard tube and 10 µl of the sample was added to the specimen test tube. They were mixed and incubated for a period of 3 minutes at 37 °C. then, 200 µl of R3 was added into all the test tubes. They were mixed and incubated for a period of 5 minutes at 37 °C. The absorbance of the specimen ($A_{specimen}$) and standard ($A_{standard}$) against the reagent blank at 578 nm was measured (Gounden *et al*., 2022).

3.9.2 Determination of creatinine

Assay Principle

Creatinine reacts with picric acid under alkaline condition to form a yellow-red complex. The absorbance of the color produced, measured at a wavelength 492 nm, is directly proportional to creatinine concentration in the sample.

Creatinine Picric Acid Reagent contains a low concentration of picric acid, a chemical which, in its dry form, is flammable and potentially explosive.

Procedure

In a test tube, 1.0 ml of working solution and 100 µl of the standard were introduced.

They were mixed and after 30 seconds the absorbance A1 of the specimen was read.

Two minutes later, absorbance A2 of the standard at λ=492nm was read (Forbes *et al*., 2022).

3.9.3 Determination of potassium

Principle

The amount of potassium is determined by using sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension (Simon *et al*., 2023). The turbidity of which is proportional to potassium concentration in the range of 2-7 mEq/L.

Procedure

Test tubes were labelled standard, control, and sample and a blank was set; 1.0 mL of potassium reagent was pipette into all tubes. 0.01 mL (10 µl) of the sample was added to respective test tubes. It was mixed and left to sit at room temperature for 3 minutes. After 3 minutes the wavelength of the spectrophotometer was set to 500 nm. The absorbance of all tubes was read and recorded (Simon *et al*., 2023).

3.9.4 Determination of sodium

Principle

The present method is based on modifications of those first described by Maruna (1958) and Trinder (1951) in which sodium is precipitated as the triple salt, sodium magnesium uranyl acetate, with the excess uranium then being reacted with ferrocyanide, producing a chromophore whose absorbance varies inversely as the concentration of sodium in the test specimen.

Procedure

Filtrate preparation: Three test tubes were labelled blank, standard, control and sample respectively. One (1.0) ml of filtrate reagent was pipette into all test tubes. Fifty (50) µl of the sample was added to all test tubes and distilled water was added to the blank. All test tubes were shaken vigorously and mixed continuously for 3 minutes. The tubes were centrifuged at high speed (1,500 G) for 10 minutes.

Colour Development: Test tubes were labelled corresponding to the above filtrate tubes.

1.0 ml of acid reagent was pipette into all tubes. 50 µl of supernatant and 50 µl of colour reagent were added to respective tubes and mixed. Spectrophotometer was zeroed with distilled water at 550 nm. The absorbance of all tubes was read and recorded.

3.9.5 Determination of chloride

Assay Principle

Chloride ions and Hg II-thiocyanate form thiocyanate ions in an acidic medium. These ions react with HNO_3 and Fe III-ions and effect a red colour. The increasing extinction is directly proportional to the concentration of chloride ions (Sharma *et al*., 2023).

Procedure

Three test tubes were labelled blank, standard and sample respectively, 1 ml of R, 10 µl of the standard was added into the standard test tube and 10 µl of the sample was added into the sample tube. They were shaken to mix and left to stand for 5 minutes, then the absorbance, A standard and A sample against Reagent Blank was read at 492 nm (Sharma *et al*., 2023).

3.9.6 Determination of bicarbonate

Principle

A bicarbonate (HCO_3^-) test is part of an electrolyte panel, or metabolic panel used to identify or monitor an electrolyte imbalance or acid-base (pH) imbalance. This test measures the total amount of carbon dioxide (CO_2) in the blood, which occurs mostly in the form of HCO_3^-. Measuring HCO_3^- as part of an electrolyte or metabolic panel may also help diagnose acidosis or alkalosis.

This results in a conversion of CO_2 to bicarbonate (HCO_3^-) which then is included in the reaction. Therefore, the total CO_2 concentration is measured. The decrease of reduced

cofactor concentration is measured at 405 nm and is proportional to the concentration of total carbon dioxide in the sample.

Procedure

Three test tubes were labelled blank, standard and sample respectively, 10 µL of the standard was added to the standard test tube and 10 µL of the sample was added to the sample test tube. To all test tubes 1000 µL of reagent was added and mixed, the test tubes were incubated at 37 °C for 2 minutes and absorbance A1 was read, absorbance A2 was read after 10 minutes of incubation against the reagent blank at 405 nm.

3.10 Determination of Lipid Profile

Total cholesterol, triglycerides, high density lipoproteins (HDL) and low-density lipoproteins (LDL) levels were determined using a test kit (Spectrum kit). Specific procedures for the above determinations were carried out in compliance with the instructions of the test kit manufacturer.

3.10.1 Determination of total cholesterol

Assay Principle

Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase (CE) to cholesterol and free fatty acids. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase (CO) to cholest-4-en-3-one and hydrogen peroxide.

The hydrogen peroxide combines with phenol and 4-amino-antipyrine (4AAP) in the presence of peroxidase (POD) to form a chromophore (quinoneimine dye) which may be quantitated at 500-550 nm. For bichromatic analyzers the blank wavelength should be set to 600 or 650 nm.

Procedure

Three test tubes were labeled blank, standard and sample respectively. One (1.0) ml of R was added into all tubes, 10 µl of standard was added into the standard tube and 10 µl of sample was added into the sample tube. The test tubes were shaken to mix and was incubated for 10 minutes at 25 °C. The absorbance of specimen ($A_{specimen}$) and standard ($A_{standard}$) against reagent blank was read at 546 nm after 30 minutes (Nosrati *et al*., 2017).

3.10.2 Determination of triglycerides

Assay Principle

Triglycerides are hemolyzed by lipoprotein lipase (LPL) to glycerol. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2).

In the presence of peroxidase (POD), hydrogen peroxide effects the oxidative coupling of 4-chlorophenol and 4-aminoantipyrine (4AAP) to form a red color quinoneimine dye which is measured at 546 nm.

Procedure

Three test tubes were labeled blank, standard and sample respectively. One (1.0) ml of R was added all test tubes, 10 µl of the standard was added to the standard tube and 10µl of the sample was added to the sample tube. They were mixed and incubated for 10 minutes at 25 °C. The absorbance of specimen ($A_{specimen}$) and standard ($A_{standard}$) against reagent blank was measured after 30 minutes at 546 nm (Nosrati *et al*., 2017).

3.10.3 Determination of high-density lipoproteins (HDL)

Assay principle

Low density lipoproteins (LDL) and very low-density lipoproteins (VLDL) in sample precipitated with phosphotungstate and magnesium ions, after centrifugation, the cholesterol concentration in the HDL fraction, which remains in the supernatant, is determined.

Procedure

Precipitation

In a test tube 0.5 ml of the R and 0.2 ml of the specimen were added. They were mixed and incubated for 10 minutes at room temperature, then was centrifuged for 10 minutes at 4000 rpm. The supernatant was collected carefully.

Cholesterol- Liquizyme

Two test tubes labelled blank and specimen respectively, 50 ml of distilled water, 50 ml of the specimen supernatant and cholesterol reagent 1 ml each were pipetted into test tubes labelled blank and specimen. The test tubes were mixed and incubated for a period of 10 minutes at 25 °C. The absorbance of the specimen ($A_{specimen}$) against the reagent blank at 546 nm after 60 minutes was recorded (Nosrati *et al*., 2017).

3.10.4 Determination of low-density lipoproteins (LDL)

Assay Principle

The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol methyl ether (PEGME) coupled classic precipitation method with the improvements in using optimized quantities of PVS/PEGME and selected detergents. LDL, VLDL, and chylomicron (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER), whereas HDL reacts with the enzymes. Addition of Regent 2 (R2) containing a specific

detergent releases LDL from the PVS/PEGME complex. The released LDL reacts with the enzymes to produce H_2O_2 which is quantified by the Tinder reaction.

Procedure

Three test tubes were labelled bank, calibrator and sample. Three hundred (300) µL of R1 was added to all the test tubes, 4 µL calibrator was added to the calibrator test tube, 4 µL sample was added to the sample test tube, and they were mixed and incubated for 5 minutes at 37 °C. Then 100 µL of R2 was added to all tubes. They were mixed and immediately the absorbance (A1) of the samples and calibrator, against the blank was read, then the absorbance (A2) of the samples and calibrator after 5 minutes against the blank at 600 nm was read (Linton *et al*., 2019).

3.11 Histopathological Studies

Tissues collected from the animals were excised and dehydrated using grades of alcohol. Dehydration was followed by clearing the samples in two changes of Xylene. The tissue samples are then impregnated with two changes of molten paraffin wax, immersed, and blocked out. Finally, tissues were sectioned by microtomy and attached to the surface of a glass slide for further staining and microscopic examination. Paraffin sections of 3 µm thickness were stained with Hematoxylin and Eosin (HE) and mounted on a glass slide of a microscope (Fadia *et al*., 2022). The HE stained tissue sections were observed and photographed using an optical microscope (AX80, Olympus, Tokyo, Japan).

3.12 Statistical Analysis

The data obtained was presented as a mean ± standard error of three replicates and were subjected to analysis of variance (ANOVA) followed by post-hoc Duncan test for comparison of the means using SPSS version 23.0. The level of significance was accepted at $P<0.05$.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Proximate compositions of chitosan, edible chitosan-starch film and chitosanstarch film supplemented diets

The proximate compositions of chitosan, edible chitosan-starch film and chitosan-starch film supplemented diets are presented in Table 4.1. The moisture content of edible chitosan-starch film was significantly ($P<0.05$) higher than the moisture content in chitosan. The supplemented diets (B, C, D and E) were significantly ($P<0.05$) higher in moisture content than the control diet (A). The higher the film supplemented in the diets, the higher the moisture content, the same trend was observed for ash and lipid contents. However, a different pattern was observed for fibre, protein and carbohydrate contents, as edible chitosan-starch film was found to be low in these parameters when compared to chitosan. The higher the film supplemented in the diets the lower the fibre, protein and carbohydrate contents.

Table 4.1: Proximate Compositions of Chitosan, Edible Chitosan-Starch Film and Chitosan-Starch Film Supplemented Diets

Parameters (%)	*Chitosan-starch film supplemented diets					Edible Chitosan-Starch Film	Chitosan
	A	B	C	D	E		
Moisture	9.67±0.17^{a}	12.67±0.17^{b}	14.17±0.44^{c}	17.00±0.29^{d}	17.33±0.73^{de}	18.67±0.73^{e}	11.00±0.00^{a}
Ash	6.00±0.50^{b}	7.50±0.29^{c}	8.83±0.33^{d}	11.67±0.73^{e}	13.83±0.17^{f}	12.33±0.33^{e}	4.33±0.33^{a}
Fat	10.10±0.29^{b}	13.50±0.00^{c}	14.33±0.44^{cd}	15.33±0.44^{d}	14.17±0.17^{c}	25.33±0.44^{e}	8.50±0.29^{a}
Fibre	13.23±0.44^{d}	8.00±0.29^{b}	7.67±0.33^{b}	6.33±0.17^{a}	6.17±0.67a	7.50±0.00^{b}	10.17±0.33^{c}
Protein	13.73±0.12^{e}	12.69±0.06^{d}	12.45±0.10^{cd}	12.28±0.00^{c}	11.58±0.10^{b}	0.82±0.06^{a}	4.32±0.06^{f}
Carbohydrate	47.27±0.20^{d}	45.64±0.23^{d}	42.55±1.17^{c}	37.39±0.73^{b}	36.92±0.41^{b}	35.35±0.78^{a}	61.68±0.34^{e}

Values are Mean ± Standard Error of Mean of triplicate determinations.

Values with different superscripts between the samples are significantly different ($p<0.05$).

***A**: Control (100 % rat chow), **B**: 5 % film, **C**: 10 % film, **D**: 20 % film, **E**: 40 % film

4.1.2 Feed intake by albino rats placed on edible chitosan-starch film supplemented diets

Figure 4.1 shows the feed intake by albino rats placed on edible chitosan-starch film supplemented diets. The feed intake of the experimental rat groups and that of the control were not significantly different ($P>0.05$) in the first week, although there was a significant difference ($P<0.05$) in the feed intake at week 2, 3 and 4 for albino rats placed on the control and supplemented diets. Group B rats placed on 5 % edible chitosan-starch film had the highest feed intake in all the weeks while group E rats placed on 40 % edible film had the least feed intake.

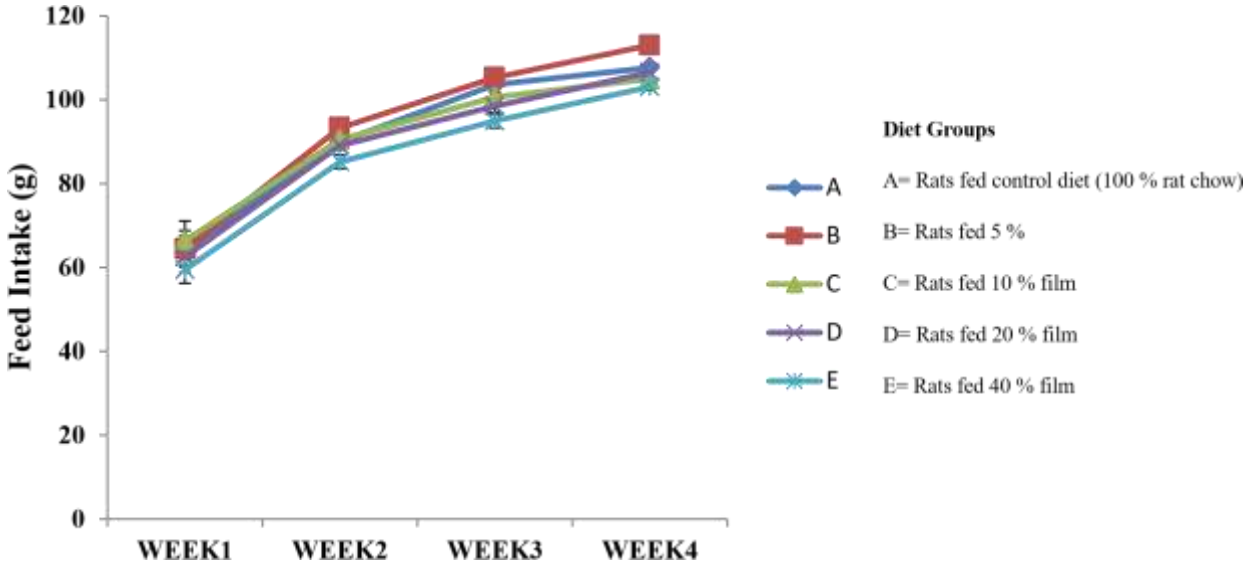


Figure 4.1: Feed Intake by Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

4.1.3 Body weight-gain of albino rats placed on edible chitosan-starch film supplemented diets

The body weight-gain of albino rats fed edible chitosan-starch film supplemented diets is presented in Figure 4.2. There was a significant change ($P<0.05$) in the weekly body weight-gain in the rats placed on the supplemented diets compared to those fed the

control diets. Group B rats fed 5 % edible chitosan-starch film had the highest body weight-gain in all the weeks.

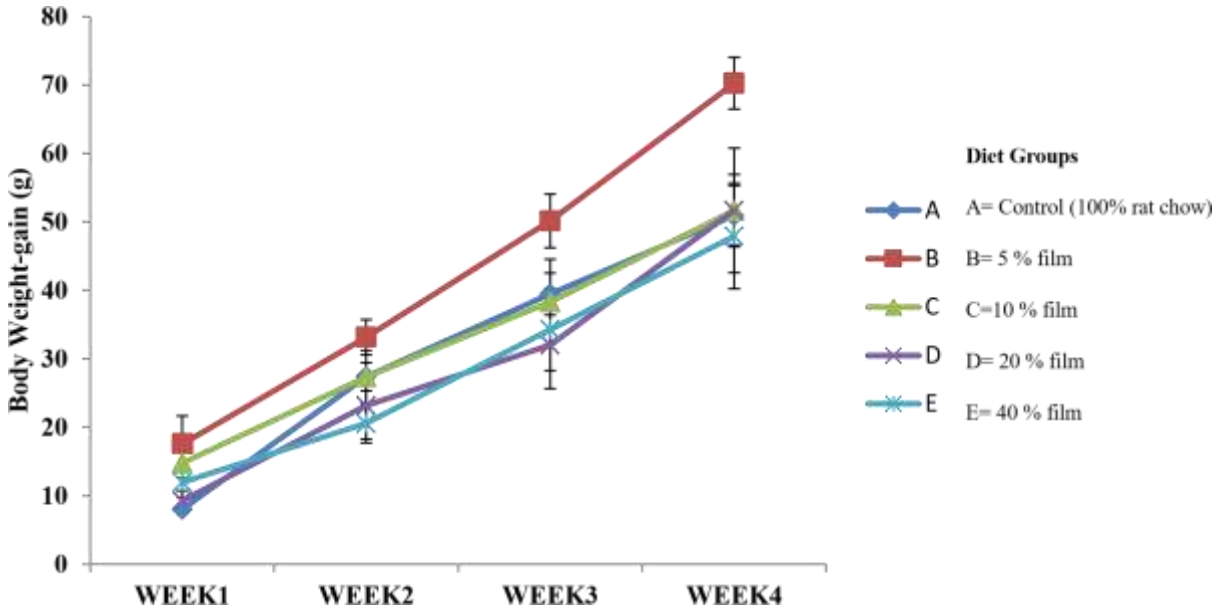
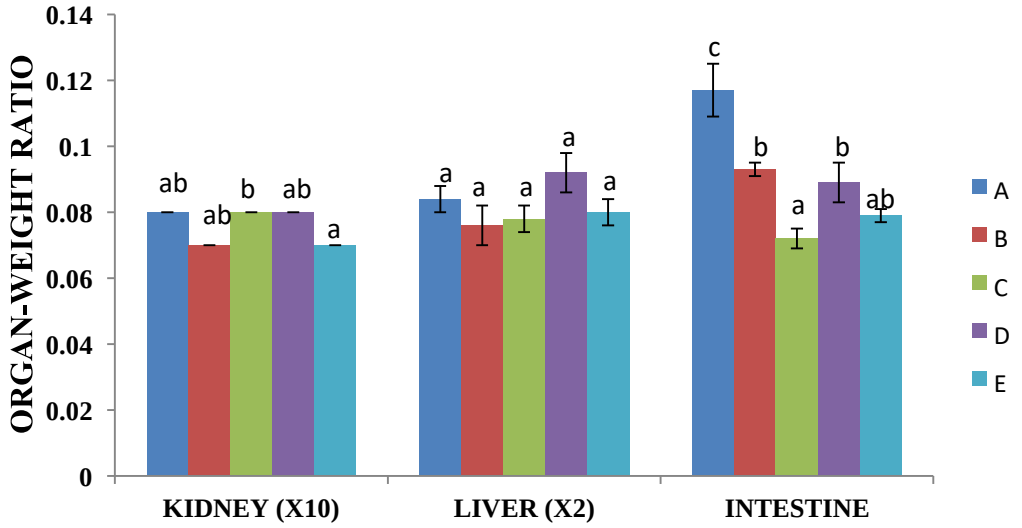


Figure 4.2: Body Weight-Gain of Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

4.1.4 Organ body-weight ratio of albino rats placed on edible chitosan-starch film supplemented diets

Figure 4.3 shows the kidney body-weight ratio, liver body-weight ratio and intestine body-weight ratio of albino rats placed on edible chitosan-starch film supplemented diets. The result show that there was significant difference ($P<0.05$) in the kidney bodyweight ratio and in the intestine body-weight ratio of rats on the experimented diets when compared to those placed on control diet. However, there was no significant difference ($P>0.05$) in the liver body-weight ratio of the albino rats.



Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

Figure 4.3: Organ-Weight Ratio of the Kidney, Liver and Intestine of Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets.

4.1.5 Haematological parameters of albino rats placed on edible chitosan-starch film supplemented diets

The hematological parameters of albino rats placed on edible chitosan-starch film supplemented diets are shown in Table 4.2. There were significant differences ($P<0.05$) in the HB, PCV, MCV, MCH, PLC, NEU, LYM, RDWC and RBC counts of albino rats placed on the supplemented diets when compared to those of the control, although there was no significant difference ($P>0.05$) in the EB, MCHC and TWBC counts.

Table 4.2: Hematological Parameters of Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

GROUPS	HB (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	TWBC (µL)	PLC (10^3/µL)	NEU (µL)	LYM (µL)	EB (µL)	RDWC (%)	RBC (10^6/µL)
A	5.62±0.64^{a}	17.20±1.85^{a}	54.00±1.56^{a}	19.80±0.86^{bc}	38.40±0.51^{a}	10.34±1.82^{a}	288.60±36.17^{a}	28.00±3.21^{bc}	63.80±3.56^{b}	8.60±1.78^{a}	30.62±0.31^{a}	2.92±0.37^{a}
B	7.34±0.19^{ab}	21.60±0.68^{ab}	57.00±1.41^{a}	18.40±0.68^{ab}	33.80±2.40^{a}	13.02±3.80^{a}	470.80±59.06^{b}	26.40±3.04^{ab}	60.20±4.81^{ab}	11.60±1.69^{a}	34.94±0.32^{cd}	3.82±0.12^{ab}
C	8.24±0.61^{b}	25.60±1.63^{b}	56.20±1.89^{a}	20.60±0.24^{c}	34.00±0.71^{a}	11.88±0.46^{a}	517.00±34.74^{b}	18.80±2.15^{a}	66.40±4.37^{b}	10.00±1.10^{a}	32.21±0.14^{b}	4.24±0.35^{bc}
D	8.14±0.95^{b}	25.78±2.72^{b}	56.00±0.95^{a}	17.40±0.51^{a}	35.60±0.71^{a}	13.04±1.51^{a}	556.80±70.30^{b}	36.20±3.93^{c}	47.40±3.17^{a}	10.60±1.21^{a}	35.98±0.66^{d}	4.96±0.51^{c}
E	6.44±0.98^{ab}	21.40±2.91^{ab}	55.00±1.92^{a}	18.80±0.37^{ab}	35.80±1.39^{a}	12.56±0.35^{a}	501.40±27.14^{b}	31.40±2.06^{bc}	58.80±5.29^{ab}	10.20±0.73^{a}	33.70±0.91^{bc}	4.16±0.22^{bc}

Values are Mean ± Standard Error of Mean of triplicate determinations.
Values with different superscripts between the samples are significantly different ($p<0.05$). **Keys:**

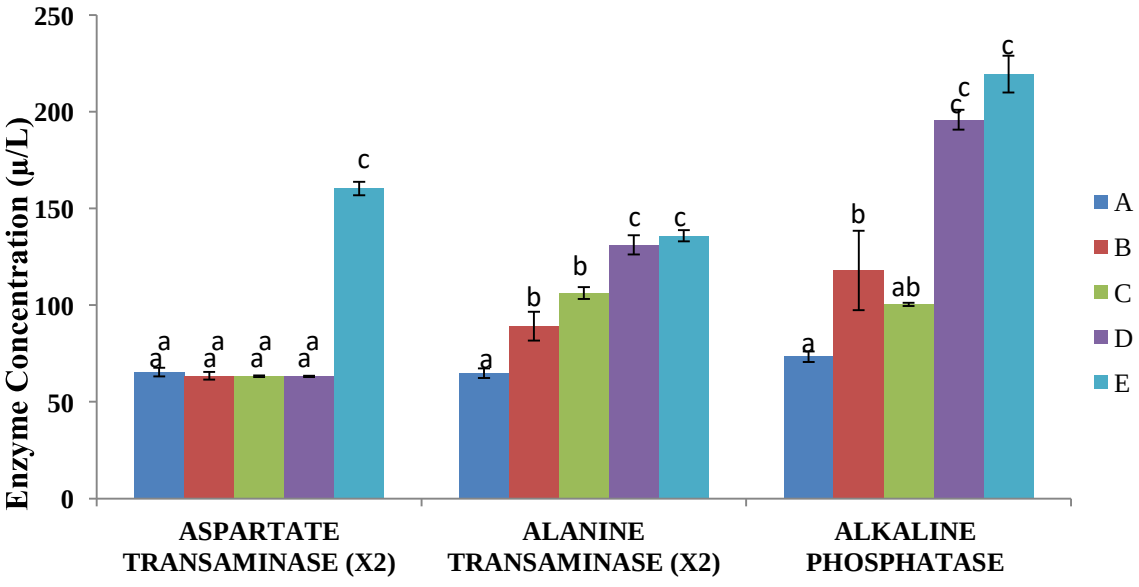
Haemoglobin (HB), Packed cell volume (PCV), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Total white blood cell count (TWBC), Platelets counts (PLC), Neutrophils (NEU), Lymphocytes (LYM), Embryoid body (EB), Red cell distribution width count (RDWC) and Red blood cell count (RBC). Diet Groups: **A**: Control diet (100 % rat chow), **B**: Rats placed on 5 % film, **C**: Rats placed on 10 % film, **D**: Rats placed on 20 % film, **E**: Rats placed on 40 % film.

4.1.6 Liver function parameters of albino rats placed on edible chitosan-starch film supplemented diets

Liver function parameters such as; serum enzymes (aspartate transaminase, alanine transaminase, and alkaline phosphatase), total protein, albumin and total bilirubin levels of albino rats placed on edible chitosan-starch film supplemented diets are presented in Figures 4.4-4.6.

4.1.7 Serum enzymes

Aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) levels in albino rats placed on edible chitosan-starch film are presented in Figure 4.4. The AST and ALP levels of rats fed the control diet (A) were significantly ($P<0.05$) lower than those fed the supplemented diets (B, C, D and E) whereas the ALT level of rats placed on the experimental diets was significantly ($P<0.05$) higher than those fed the control diet. An increase in the amount of film in the supplemented diet increased the ALT level in albino rats.

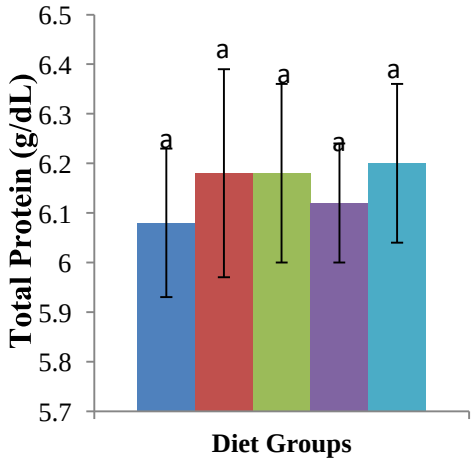


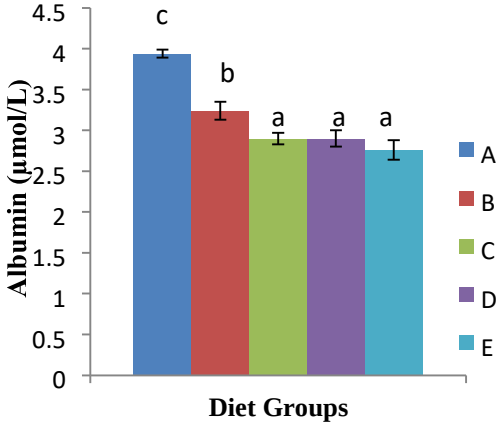
Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

Figure 4.4: Aspartate Transaminase, Alanine Transaminase and Alkaline Phosphatase Levels in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

4.1.8 Total serum protein and albumin levels

Total serum protein and albumin levels in albino rats placed on edible chitosan-starch film supplemented diets are presented in Figure 4.5a and 4.5b respectively. There was no significant difference (P>0.05) in the total protein level of rats placed on the supplemented diets and that of the control diet. However, the albumin level of rats placed on the supplemented diets was significantly (P<0.05) lower than those of the control.





Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

Figure 4.5a: Total Serum Protein Level in Albino Rats Placed on Edible ChitosanStarch Film Supplemented Diets

Figure 4.5b: Serum Albumin Level in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

4.1.9 Total bilirubin level

Figure 4.6 shows the total bilirubin level in albino rats placed on edible chitosan-starch film supplemented diets. The bilirubin level of rats placed on the supplemented diets was significantly higher ($P<0.05$) than the bilirubin level of rats placed on the control diet. An increase in the amount of film supplemented in the diets was observed to increase the total bilirubin level.

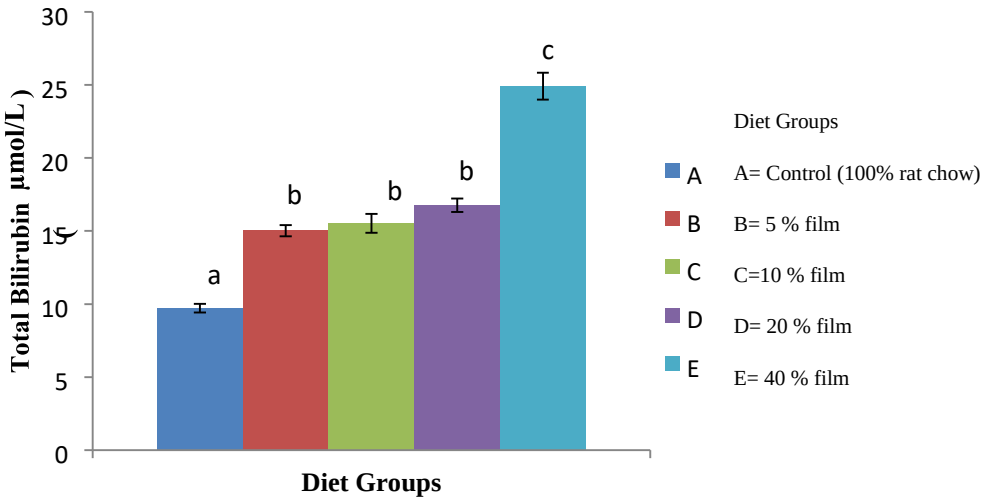


Figure 4.6: Total Bilirubin Level in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

4.1.10 Renal function parameters of albino rats placed on edible chitosan-starch film supplemented diets

Serum electrolytes (potassium, sodium, chloride and bicarbonate), serum urea and creatinine levels of albino rats placed on edible chitosan-starch film supplemented diets are shown in Figure 4.7-4.8 respectively.

4.1.11 Serum urea and creatinine levels

Urea and creatinine levels of albino rats placed on edible chitosan-starch film supplemented diets was significantly higher ($P<0.05$) than that of the control as shown in Figure 4.7a and 4.7b. It was observed that an increase in the film supplemented in the diets caused an increase the urea and creatinine levels.

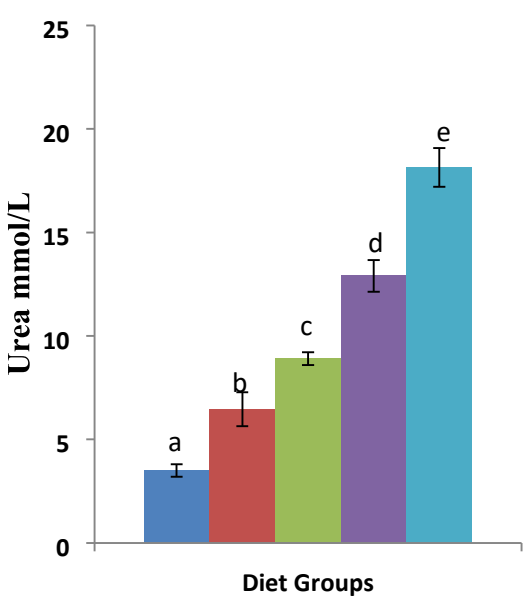


Figure 4.7a: Serum Urea Level in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

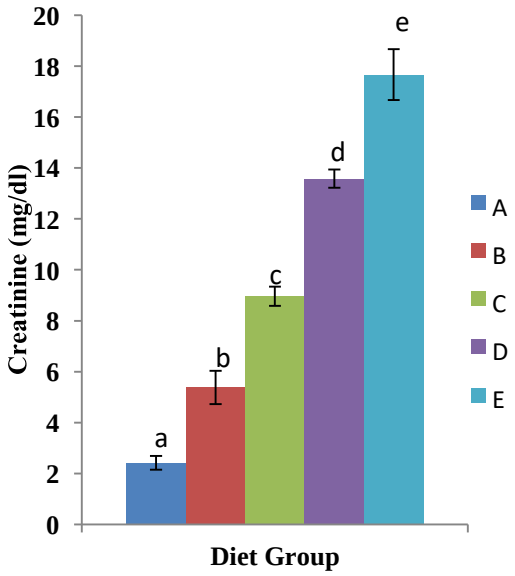


Figure 4.7b: Serum Creatinine Level in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

4.1.12 Serum electrolytes

Figure 4.8a and 4.8b show the serum electrolyte levels of albino rats placed on edible chitosan-starch film supplemented diets. The serum electrolyte levels in albino rats placed on edible chitosan-starch film supplemented diets were significantly different ($P<0.05$) from that of the control.

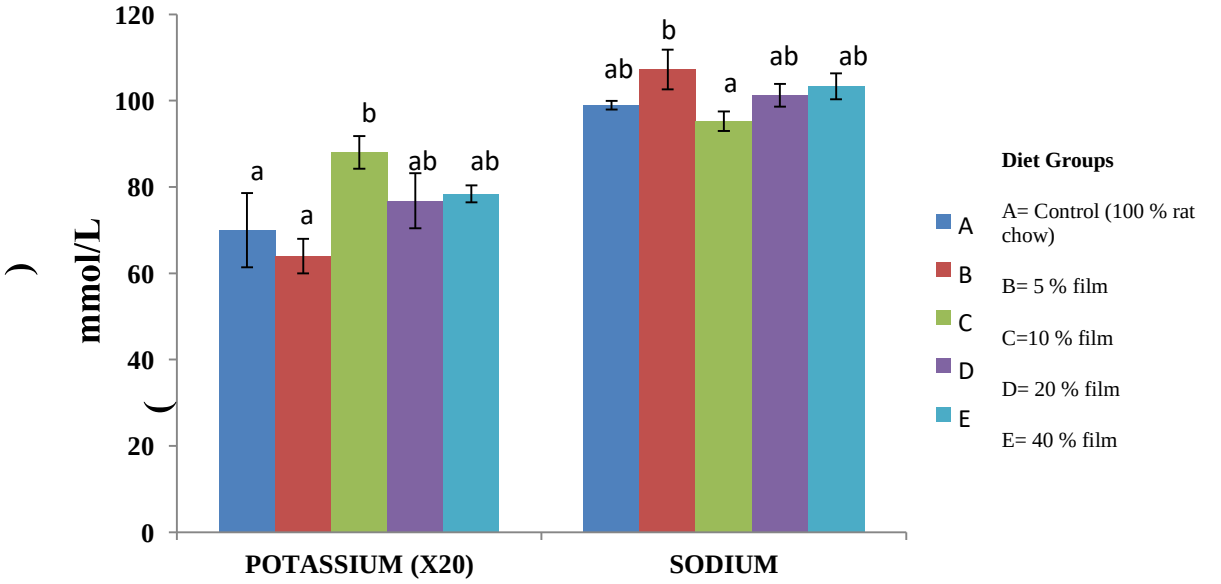


Figure 4.8a: Serum Potassium and Sodium Levels in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

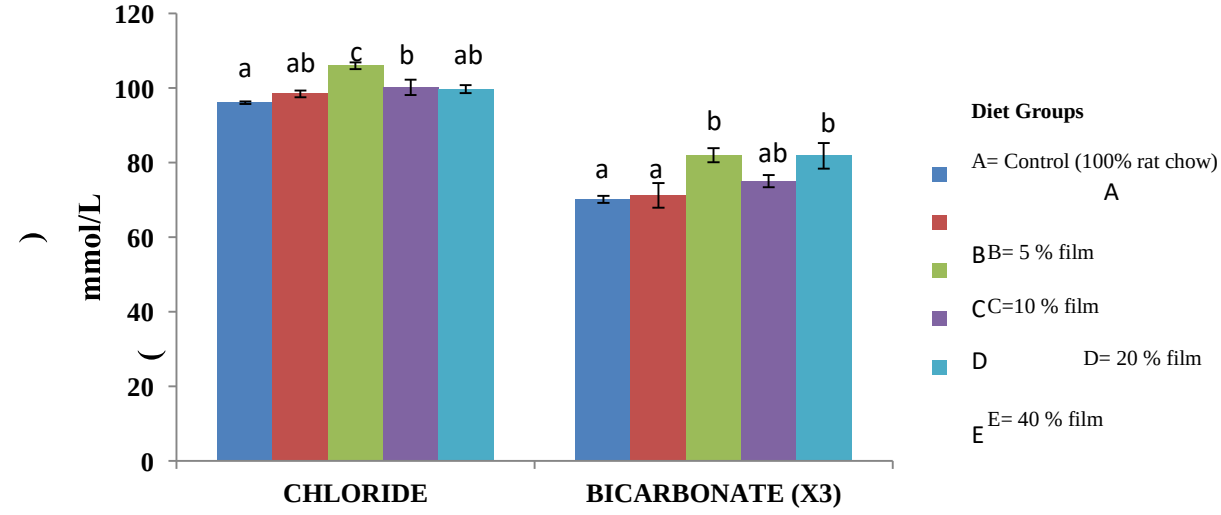


Figure 4.8b: Serum Chloride and Bicarbonate Levels in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

4.1.13 Lipid profile of albino rats placed on edible chitosan-starch film supplemented diets

Lipid profile of albino rats placed on edible chitosan-starch film is presented in Figures 4.9 and 4.10. Total cholesterol, triglycerides, high density lipoproteins (HDL) and lowdensity lipoproteins (LDL) levels were determined.

4.1.14 Total cholesterol and triglycerides levels

The total cholesterol and triglycerides levels in albino rats placed on edible chitosanstarch film supplemented diets are presented in Figure 4.9. The total cholesterol and triglycerides levels of rats placed on the supplemented diets were significantly lower ($P<0.05$) than those placed on the control.

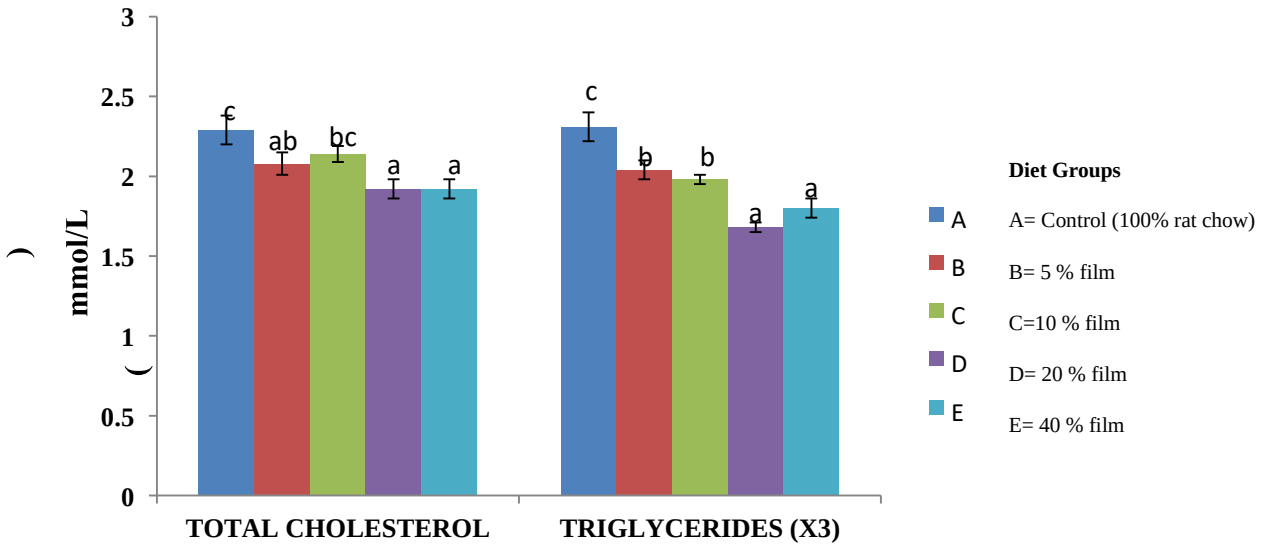


Figure 4.9: Total Cholesterol and Triglycerides Levels in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

4.1.15 High density lipoprotein (HDL) and low-density lipoprotein (LDL) levels

Figure 4.10 shows the high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels in the serum of albino rats placed on edible chitosan-starch film supplemented diets. There was no significant difference (P>0.05) in the HDL level of rats placed on the supplemented diets and that of the control. However, the LDL level of rats placed on the supplemented diets was significantly lower (P<0.05) than those placed on the control diet.

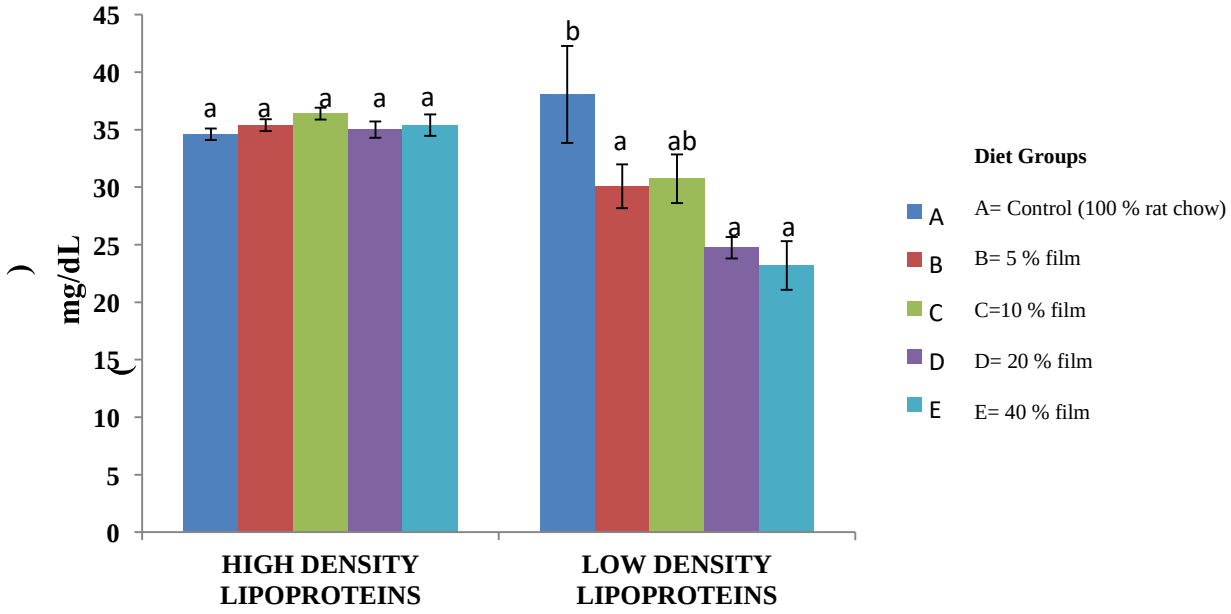


Figure 4.10: High density lipoprotein (HDL) and Low-density lipoprotein (LDL) Levels in Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

4.1.16 Histoarchitecture of the liver, kidney and intestine of albino rats placed on edible chitosan-starch film supplemented diets

Histoarchitecture of the liver, kidneys and intestines of albino rats placed on edible chitosan-starch film supplemented diets and those fed the control diet are presented in Plate I-XV.

4.1.17 Histoarchitecture of the liver

Plates I-V presents the histoarchitecture of the liver of rats placed on edible chitosanstarch film supplemented diets and those of the control diet. The results showed that no significant change was observed in the liver of rats placed on edible chitosan-starch film supplemented diets when compared to that placed on the control diet.

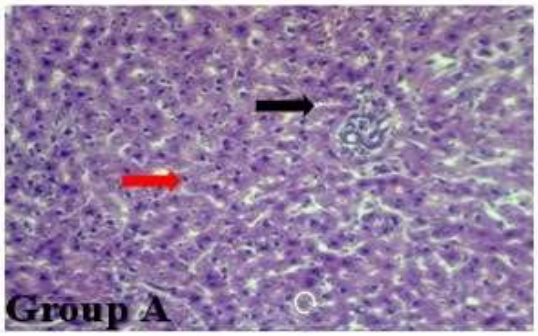


Plate 1: Photomicrograph of the Liver Tissue Showing Normal Histological Architecture

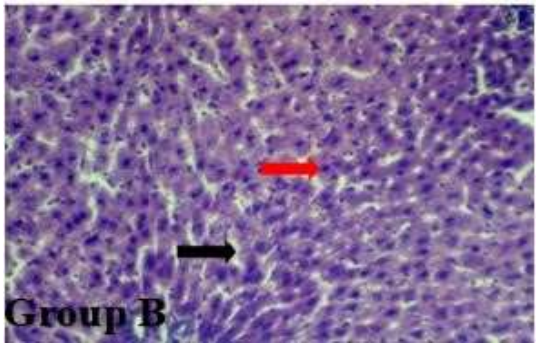


Plate II: Photomicrograph of the Liver Tissue Showing Unaltered Hepatic Cell

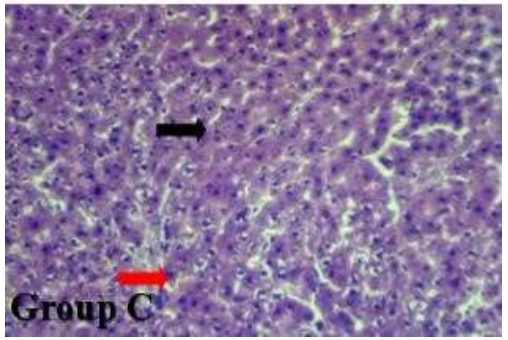


Plate III: Photomicrograph of the Liver with Intact Hepatic cells Tissue Showing Unaltered Hepatic Cells

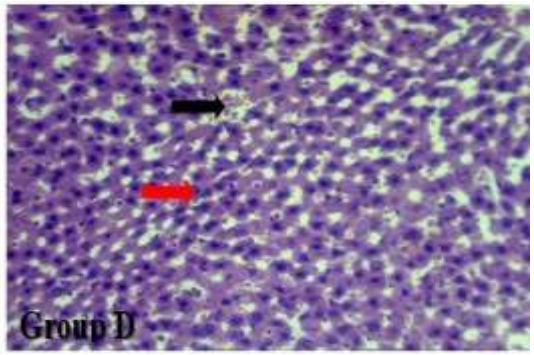


Plate IV: Photomicrograph of the Liver Tissue Showing Normal Histological Features

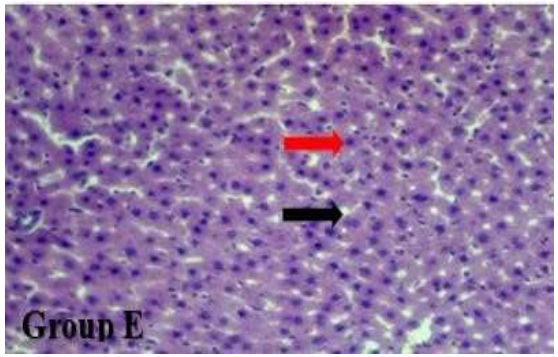


Plate V: Photomicrograph of the Liver Tissue Showing Unaltered Hepatic Cells

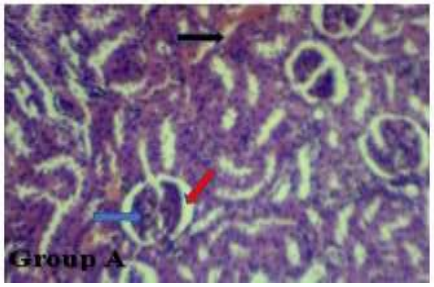
Plate I-V: Photomicrograph of the Liver Tissue (Mg x 40; Eosin/Haematoxylin)

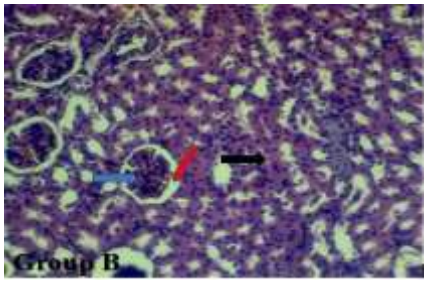
Black Arrow: Hepatocytes, **Red Arrow**: Hepatic sinusoids

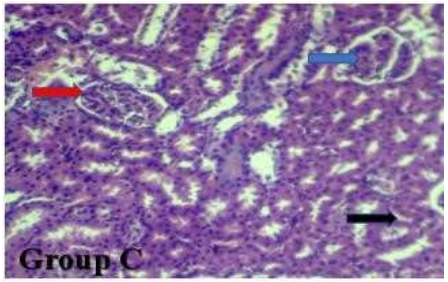
Diet Groups: **A**: Control diet (100 % rat chow), **B**: Rats placed on 5 % film, **C**: Rats placed on 10 % film, **D**: Rats placed on 20 % film, **E**: Rats placed on 40 % film [93]

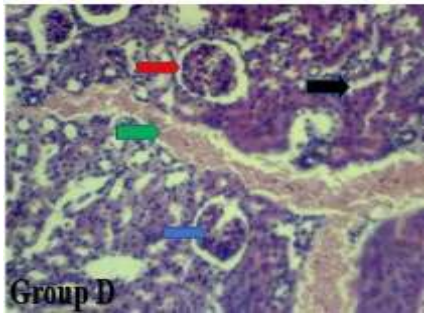
4.1.18 Histoarchitecture of the kidney

The histoarchitecture of the kidney of rats placed on edible chitosan-starch film supplemented diets and those of the control are showed in Plates VI-X. A significant change was observed in the kidney of albino rats placed on edible chitosan-starch film supplemented diets when compared to the kidney of that placed on the control diet.









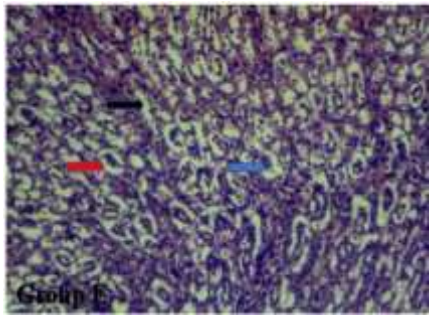


Plate IX: Photomicrograph of the Kidney Tissue Showing Normal Capsular and Glomeruli with Inflammation Sinusoids

Plate X: Photomicrograph of the Kidney Tissue Showing Degeneration of Capsular Space and Glomeruli

Plate VI: Photomicrograph of the kidney Tissue Showing Normal Histological Architecture with Intact Glomeruli and Capsular Space

Plate VII: Photomicrograph of the Kidney Tissue Showing Normal Glomeruli and Capsular Space

Plate VIII: Photomicrograph of the Kidney Tissue Showing Normal Capsular and Glomeruli Degeneration

Plate VI-X: Photomicrograph of the Kidney Tissue (Mg x 40; Eosin/Haematoxylin)

Blue Arrow: Glomerulus, **Red Arrow**: Capsular space, **Green Arrow**: Area of Inflammation, **Black Arrow**: Distal convoluted tubules.

Diet Groups: **A**: Control diet (100 % rat chow), **B**: Rats placed on 5 % film, **C**: Rats placed on 10 % film, **D**: Rats placed on 20 % film, **E**: Rats placed on 40 % film

4.1.19 Histoarchitecture of the intestine

The histoarchitecture of the intestines of albino rats placed on edible chitosan-starch film supplemented diets and those of the control are presented in Plates XI-XV. The result shows a significant change in the intestines of albino rats fed edible chitosanstarch film supplemented diets when compared to that of the control.

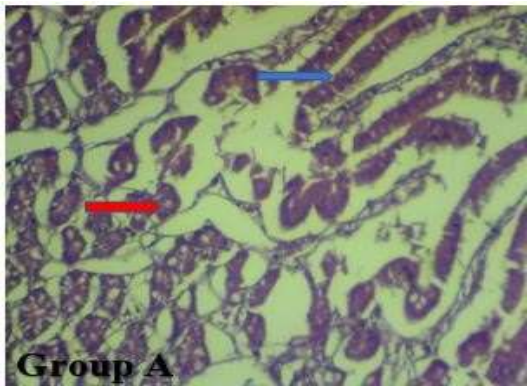


Plate XI: Photomicrograph of the Intestinal Tissue Showing Normal Histological Architecture with Intact Epithelial Cells

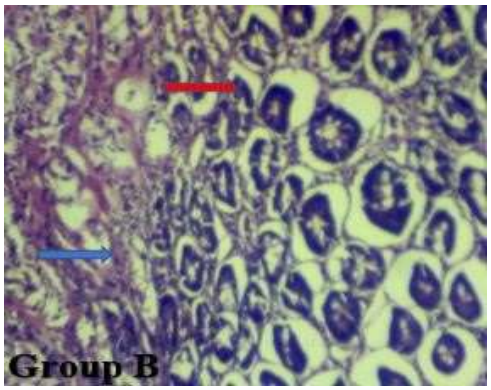


Plate XII: Photomicrograph of the Intestinal Tissue Showing Unaltered Histological Architecture

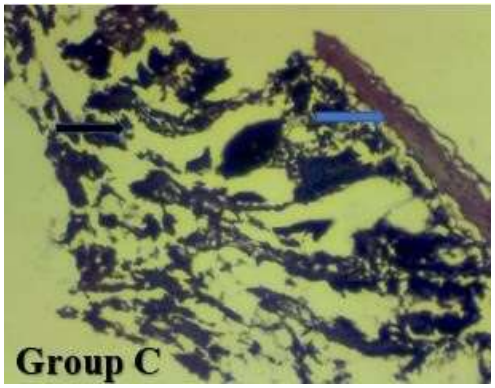


Plate XIII: Photomicrograph of the

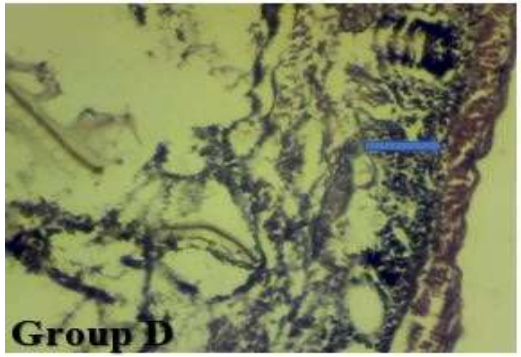


Plate XIV: Photomicrograph of the Intestinal Tissue Showing Connective Tissue Inflammation

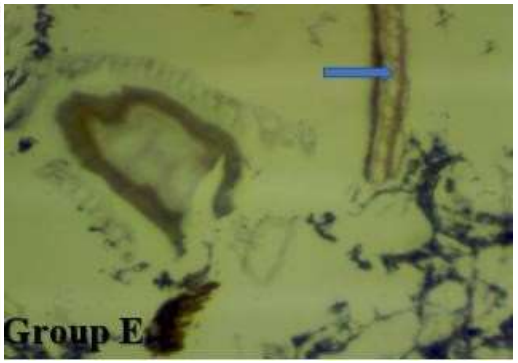


Plate XV: Photomicrograph of the Intestinal Tissue Showing Connective Tissue Inflammation

Intestinal Tissue Showing Slight Connective Tissue Degeneration

Plate XI-XV: Photomicrograph of the Instestine Tissue (Mg x 40; Eosin/Haematoxylin)

Blue arrow: Smooth muscle fibre, **Red arrow**: Lamina propria (Connective tissue)

Diet Groups : A: Control diet (100 % rat chow), B: Rats placed on 5 % film, C: Rats placed on 10 % film, D: Rats placed on 20 % film, E: Rats placed on 40 % film

4.2 Discussion

4.2.1 Proximate compositions

The high moisture content of edible chitosan–starch films compared to chitosan (Table 4.1) may be as a result of the positively charged chitosan chains which have wide hydration layers with high retention of water molecules which take part in the film structure (Bonilla *et al.,* 2014) The significantly higher moisture content of edible chitosan-starch film compared to chitosan is in agreement with the work of Apriliyain *et al*. (2020). The high moisture content may also be due to the glycerol component of the film which serves as the plasticizer, this agrees with the report of Singh *et al.,* (2015) who observed that the increase in the plasticizer concentration increased the moisture content of the film. This influence may be due to the high hygroscopic nature of the glycerol which assists in the formation of hydrogen bond with free –OH groups in water (Cerqueira *et al.,* 2012; Singh *et al.,* 2015). Another factor that may contribute to the high moisture content of the chitosan-starch film is the starch component of the film. This effect may be due to the presence of high amount of amylose content in starch (in film) which induces higher moisture sensitivity and thus affects the mechanical and barrier properties (Basiak *et al.,* 2017). Starch has also been reported to facilitate the retention of moisture content (due to its hydrophilic nature) in films (Thakur *et al.,* 2019).

The high moisture content of edible chitosan-starch film supplemented diets is expected because of the film that was supplemented in the diet. The moisture content of a film is an important index for packaging because the film acts as a barrier against water, air and gases from the external environment (Leceta, 2014). Films generally should be able to maintain moisture levels that exist around the packaged products. The moisture content present in a film gives a clue of its hydrophilic nature (Bourbon *et al.,* 2011). Apriliyain et al. (2020)

stated in their work that high moisture content in edible film will affect the resistance to air, water and gases from the atmosphere into the packaged product.

The ash content of a sample is the inorganic residue left after the organic matter has been burnt off (Ismail, 2017). The significantly high ash content observed in edible chitosanstarch film compared to chitosan is similar to the report of Martins da Costa *et al.* (2020). The resulting high ash content observed in edible chitosan-starch film may be as a result of the influence of the starch composite of the film due to the minerals in the fresh cassava tubers (Adeniji *et al*., 2007). The low ash content of chitosan is beneficial to produce films, because a starting material with resulting high ash content cannot give rise to a promising derived packaging material so a low ash content-starting material is better suited to produce long-lasting packaging material (Tajeddin, 2014). The higher ash content observed in the supplemented diets as compared to chitosan is as a result of the inclusion of chitosan-starch film in the diet.

Lipids are important in the diet as sources of energy, essential fatty acids and fat-soluble vitamins, which tend to be associated with fats. Lipids also contribute satiety, flavour, and palatability to the diet. The high lipid content observed in edible chitosan-starch film compared to that of chitosan may be as a result of the glycerol component of the film which serves as the plasticizer and this is in agreement with the work of Hazrol *et al*. (2020) who stated that a high glycerol concentration resulted in an increase in the lipid content, film thickness, moisture content and solubility of the film. The inclusion of edible chitosanstarch film in the diets would therefore explain the higher lipid content.

Chitosan contains non-proteinous nitrogen (Seed *et al*., 2003), the protein content observed in chitosan may be as a result of the high nitrogen content of chitosan (Aflukwa, *et al*., 2013) and therefore cannot add protein to the chitosan-starch film, the low protein content in edible chitosan-starch film resulted in the low protein contents observed in the supplemented diets.

The low protein content of edible chitosan-starch films is advantageous because, when the protein is low the microbial attack will be low too as bacterial spoilage occurs faster and more evidently in high-protein foods (Húngaro and Sant‘Ana, 2014).

One major importance of dietary fibre is its modulatory function of the intestinal tract. Foods that have high fibre content promote satiety, and are usually relatively low in calories compared to foods rich in other nutrients (Marlett *et al*., 2000). Fibre content in foods is made up of majorly cellulose, lignin and lignocellulose which are indigestible. The low fibre content observed in edible chitosan-starch film may be attributed to the fact that chitosan does not possess lignin or cellulose. Although crude fibre enhances digestibility, its presence in high level can trigger intestinal irritation, lower digestibility and depressed nutrient usage (Jimoh and Oladiji, 2005).

Carbohydrates are generally vital in the storage of energy reserves and the make-up of the structural framework of cells (Gerschenson *et al*., 2017). The high carbohydrate content in chitosan is expected because of the polysaccharide backbone of chitosan molecules (Li *et al*., 2020). However, the significant lower carbohydrate content of edible chitosan-starch film as compared to chitosan may be as a result of the glycerol component in the film which was used as the plasticizer. This could have also resulted in decreased carbohydrate content in the supplemented diets (Viegas *et al*., 2022; Jaderi *et al*., 2023).

4.2.2 Feed intake

Feed intake is a measure of an animal‘s efficiency of food utilization for maintenance and growth, and is defined as the difference between predicted feed intake and the leftovers (Green *et al*., 2013). The significant lower feed intake observed in Group C, D and E (rats fed 10 %, 20 % and 40 % edible chitosan-starch film respectively) may be attributed to the non-palatability of the feed mix. While, the highest feed intake as observed in Group B (rats fed 5 % edible chitosan-starch film) compared to the other groups could be as a result of the

low ratio of film supplemented in the feed. The higher the proportion of edible chitosan-starch in the diets the lower the feed intake, this result runs in agreement with the work of Alagbaoso *et al*. (2017).

4.2.3 Body weight-gain

Changes in body weight have been used by toxicologists to access the toxicity of a compound/mixture (Nirogi *et al*., 2014)**.** Significant higher body weight recorded in Group B (rats fed 5 % edible chitosan-starch film) is expected because Group B had the highest feed intake. It was reported by Iyeghe-Erakpotobor and Adeosun (2014), that feed intake is directly proportional to body weight-gain. However, a significant decrease was recorded in the body weight of Group C, D and E (rats fed 10 %, 20 % and 40 % edible chitosan-starch film respectively) which can be attributed to decreased feed intake. The body weight-gain followed the same pattern observed in the feed intake, the higher the proportion of edible chitosan-starch in the diets the lower the body weight-gain.

4.2.4 Organ body-weight ratio

Organ body-weight ratios between experimental and control rat groups have conventionally been employed in toxicological investigations to assess test substances that are potentially harmful, and it is a crucial quantitative endpoint in many toxicological research (Nirogi *et al*., 2014). The non-significant liver body-weight ratio in the experimental groups implies that the liver was not compromised with this diet, this may be due to the fact that chitosan, starch and glycerol can easily be tolerated by the liver (Tao *et al*., 2019). The significant decrease observed in the kidney body-weight ratio of rats placed on edible chitosan-starch film supplemented diets as compared to the control is in agreement with the findings of Queiroz *et al*. (2015), who stated that the intake of chitosan resulted in renal tissue accumulation of chitosan and promotes an increase in calcium excretion which cause kidney shrinkage. This may also explain the kidney shrinkage observed in rats placed on 540 % of

edible chitosan-starch film in this study. The shrinkage observed in the intestines of the rats placed on edible chitosan-starch film supplemented diets when compared to the control is not surprising since chitosan is made from chitin, and chitin is known to cause intestinal irritation (Nagatani *et al*., 2012). This effect increased across the rat groups, as group E rats placed on 40 % edible chitosan-starch film had highest severe shrinkage.

4.2.5 Haematological parameters

There are reports that have shown that the consumption of toxic substances/drugs can cause significant alterations in the hematological profile leading to various blood disorders (Arome and Chinedu, 2013; Arika *et al*., 2016; Zahmati and Saljooghi, 2016). The significant variations observed in the hematological parameters (HB, PCV, MCV, MCH, PLC, NEU, LYM, RDWC and RBC) did not follow a particular trend but varied differently within the experimental group when compared to the control. The nonsignificant variation recorded in the EB, MCHC and TWBC counts were similar to that reported by Ganesan *et al*. (2020). The non-significant difference of the TWBC, EB and MCHC indicates that the immune system is not compromised by the diet; as leucopoiesis that could otherwise have resulted in decreased immunity was not affected.

4.2.6 Biochemical parameters

The significant increase in the AST, ALT and ALP levels of albino rats placed on edible chitosan-starch film supplemented diets could suggest that the hepato-toxic effect of the film may have caused injury to the liver, destroyed the permeability of the membrane hence resulting in leakage of the enzymes into the blood. This may imply toxicity to the liver of albino rats fed edible chitosan-starch film supplemented diets. This result is in agreement with the work of Hanisa *et al*. (2011). The non-significant difference observed with the total protein levels shows that protein synthesis in albino rats is not affected by the film. Although, the significant difference in the albumin is because albumin is a more

sensitive indicator of protein deficiency than total protein (Gupta *et al*., 2011; Kassem, 2015). Lower than normal levels of albumin may indicate liver damage or disease which may be as a result of trauma, inflammatory conditions and malnutrition. A significantly elevated level of bilirubin is an important indicator of cholestatic liver damage in laboratory animals or could be a sign of biliary duct obstruction (Woodman, 1996). The total bilirubin in the experimental rats is in agreement with the report of Hanisa *et al.* (2011).

The significant increase in the urea and creatinine concentration could indicate impaired excretion by the kidneys according to Hanisa *et al*. (2011). This observed increase in urea concentration could be attributed to the nitrogen element present in chitosan structure (Zhang *et al*., 2019; Hammi *et al*., 2020). The higher the nitrogen, the more the urea and this may have resulted in increased creatinine concentration in the serum of the experimental rat groups. The significantly higher level of serum potassium, sodium, chloride and bicarbonate may be as a result of increased permeability to the kidney. The increased electrolytes concentrations recorded in this study is similar to the work of Yamada and Inaba (2021).

The significantly decreased total cholesterol and TG in the serum of experimental rats compared to the control may be as a result of the glycerol component of the film, because of the displacement of the lipids in the film with the glycerol used as plasticizer. The glycerol is therefore not free for the synthesis of cholesterol. This result agrees with the report of Tarique *et al*. (2021) who explained that the incorporation of glycerol as plasticizer decreased the density of edible films. The non-significant difference observed in the HDL level and significant lower LDL level in the serum of albino rats fed the supplemented diets when compared to those of the control, suggest that edible chitosanstarch film has a good effect in lowering LDL levels in albino rats. This further explains the fact that many

polysaccharides have the ability to reduce LDL (Castro-Muñoz and Gonzalez, 2019) thereby contributing to the reduction for the risk of cardiovascular diseases.

4.2.7 Histoarchitecture of organs

The liver of albino rats placed on edible chitosan-starch film supplemented diets did not show any abnormalities when compared to the control, this is because polysaccharides are easily broken down by the liver (Lovegrove *et al*., 2017). The pathology of the liver clearly showed that no toxicity sedimentation was found in the liver after administration of edible chitosan-starch film supplemented diets, this is in agreement with the work of Douglas *et al*. (2015). Toxicity mostly affects vital organs, especially the liver but this was not the case in this research as there was no trace of lesions found in the liver histoarchitecture. Also, this regular histological section of the liver shows well-arranged cells with normal hepatic sinusoids. However, the significant change of the glomeruli and capsular space degeneration with inflammation of the sinusoids in the kidney may be attributed to the kidney being over-worked because of the increased nitrogen in the chitosan resulting in the formation of urea and creatinine which needs to be excreted. This may have subjected the kidney to stress as a result of accumulation thereby leading to shrinkage (Queiroz *et al*.
2015). The proportion of edible chitosan-starch film supplemented in the rats‘ diets correlates with the severity of the damage as it was observed that group B rats placed on 5 % edible chitosan-starch film showed no significant change when compared to those placed on the control diet, while, the other rat groups placed on 10 %, 20 % and 40 % experienced capsular space and glomeruli degeneration. This, therefore, indicate that a higher proportion of edible chitosan-starch film supplemented in diets may cause kidney damage in albino rats.

The explanation of the observed connective tissue inflammation in the intestine of the experimental rats may be the same as for the low intestine body-weight ratio (section

4.1.4). Chitin the precursor of chitosan is a potent irritant of the intestinal mucosa (Nagatani *et al*., 2012). Increase of chitosan-starch film in the supplemented diets correlates to increased severity of intestinal inflammation (Plate I-XV).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The following conclusions could be made from this research:

Chitosan was significantly lower in all proximate components except carbohydrate and fibre, and chitosan-starch film was significantly higher in moisture, fat, and ash compared to the supplemented diets and the control diet.

Group B rats placed on 5 % edible chitosan-starch film supplemented diet had the highest feed intake and weight gain in all the weeks.

The kidney and intestine body-weight ratios were significantly lower in the rats on the supplemented diets.

The haematological parameters of albino rats placed on edible chitosan-starch films varied significantly within the experimental rat groups.

Significant alterations were observed in the biochemical indices (AST, ALT, ALP, albumin, total bilirubin, urea, creatinine, sodium, potassium, chloride, bicarbonate, total cholesterol, triglycerides, and LDL) of albino rats placed on edible chitosan-starch films.

Noticeable changes were also seen in the histoarchitecture of the kidney and intestine of albino rats placed on diets supplemented with more than 5 % edible chitosan-starch film.

Therefore, it can be concluded that edible chitosan-starch films may be considered toxic to albino rats at levels higher than 5 % diet inclusion.

5.2 Recommendation

It is recommended that further research be carried out on both sub-chronic and chronic toxicity studies for 1 % - 5 % edible chitosan-starch films supplemented diets in albino rats.

5.3 Contribution to Knowledge

This study has shown that at levels >5 % diet inclusion, edible chitosan-starch film could cause significant changes in the;

1. Biochemical indices (AST 80.14±1.71 µ/L, ALT 67.92±1.45 µ/L, ALP 219.36±9.52 µ/L, Albumin 2.76±0.12 µmol/L, Total bilirubin 24.92±0.92 µmol/L, Urea 18.14±0.94 mmol/L, Creatinine 17.66±0.42 mg/dL, Sodium 103.30±2.99 µmol/L, Potassium 3.92±0.10 µmol/L, bicarbonate 27.26±1.14 mmol/L, Chloride 99.70±1.09 mmol/L, Total cholesterol 1.92±0.06 mmol/L, Triglycerides 0.60±0.02 mmol/L and LDL 23.20±2.12 mg/dL),
2. Haematological parameters (HB 6.44±0.98 g/dL, PCV 21.40±2.91 %, MCV

55.00±1.92 fL, MCH 18.80±0.37 pg, PLC 501.40±27.14 10^3/µL, NEU 31.40±2.06 µL, LYM 58.80±5.29 µL, RDWC 33.70±0.91 % and RBC 4.16±0.22 10^6/µL) and

3. Histoarchitecture (kidney and intestine) of albino rats placed on diets supplemented with more than 5 % edible chitosan-starch film.

REFERENCES

Acevedo, C., Pedreschi, F., Enrione, J., Osorio, F. & Aguilera, J. M. (2010). *Food Hydrocolloid Edible Films and Coatings*. Nova Science Publishers, 77-93.

Acevedo-Fani, A., Soliva-Fortuny, R. & Martin, B. (2017). Nanoemulsions as edible coatings. *Current Opinion in Food Science*, 15, 43-49.

Adeniji, T. A., Sanni, L., Barimalaa, I. S. & Hart, A. D. (2007). Mineral composition of five improved varieties of cassava. *Nigerian Food Journal*, 25(2), 25.

Aflukwa, J. N., Okereke, C. & Odo, M. O. (2013). Evaluation of proximate and mineral contents of termite (*Trinervitermes germinatus*) from Abakaliki and Ndieze izzi, Ebonyi state, Nigeria. *American Journal of Food and Nutrition*, 3(3), 98-104.

Afolabi, K. D., Akinsoyinu, A. O., Olajide, R. & Akinleye, S. B. (2010). Haematological parameters of the Nigerian local grower chickens fed varying dietary levels of palm kernel cake. Proceedings of 35th Annual Conference of Nigerian Society for Animal Production, p.247.

Aider, M. (2010). Chitosan application for active bio-based films production and potential in the food industry [review]. *LWT Food Science and Technology*, 43, 837–842.

Alagbaoso, S. O, Nwosu, J. N., Njoku, N. E., Okoye, E. C., Eluchie, C. N. & Agunwa I. M. (2017). ―Haematology and Growth Study of Albino Rats Fed Varying Inclusions of Cooked *Canavalia plagiosperma* Piper Seed Meal Based-Diets.‖ Journal of Food and Nutrition Research, 5(9), 649-658.

Alamri, M., Qasem, A. A., Mohamed, A. A., Hussain, S., Ibraheem, M. A., Shamlan, G., Alqah, H. A. & Qasha, A. S. (2021). Food packaging's materials: A food safety perspective. *Saudi Journal of Biological Sciences*, 28(8), 4490-4499.

Ameripen. (2022). Ameripen Report: Quantifying the Value of Packaging as a Strategy to Prevent Food Waste in America. Available online: https://www.ameripen.org/page/foodwastereport (Accessed on 22 May 2023).

Apriliyan, M., Purwadi, P., Manab, A., Apriliyanti, M. & Ikhwan, A. (2020). Characteristics of Moisture Content, Swelling, Opacity and Transparency with Addition Chitosan as Edible Films/Coating Base on Casein. *Advance Journal of Food Science and Technology*, 18, 9-14.

Aranaz, I., Alcántara, A. R., Civera, M. C., Arias, C., Elorza, B., Heras Caballero, A. & Acosta, N. (2021). Chitosan: An Overview of Its Properties and Applications. *Polymers*, 13(19), 3256.

Arika, W., Nyamai, D., Musila, M., Ngugi, M. & Njagi, E. (2016). Hematological markers of *in vivo* toxicity. *Journal of Hematology and Thromboembolic Diseases*, 4(2), 4-10.

Arneson, W. & Brickell, J. (2007). Clinical chemistry— A laboratory perspective. F.A. Davis Company, Philadelphia, 55-63.

Arome, D. & Chinedu, E. (2013). A new method for determining acute toxicity in animal models. *International Journal of Toxicology*, 20(3), 224-226.

Association of Official Analytical Chemists (AOAC). (2012). Official Methods of Analysis. 17th edition. Washington, DC, USA, 115-119.

Azeredo, H., de Britto, D. & Assis, O. (2010). *Chitosan edible films and coatings: a review. Chitosan: manufacture, properties, and usage, ed*. by Davis SP. New York: Nova Science Publishers, 179-194.

Azuma, K., Izumi, R., Osaki, T., Ifuku, S., Morimoto, M., Saimoto, H. & Okamoto, Y. (2015). Chitin, Chitosan, and Its Derivatives for Wound Healing: Old and New Materials. *Journal of Functional Biomaterials*, *6*(1), 104-142.

Badawy, M. E. & Rabea, E. I. (2011). A biopolymer chitosan and its derivatives as promising antimicrobial agents against plant pathogens and their applications in crop protection. *International Journal of Carbohydrate Chemistry*, 29, 58.

Bai, J. & Plotto, A. (2011). Coatings for fresh fruits and vegetables. *Edible Coatings and Films to Improve Food Quality*, 185-242.

Baldwin, E. A., Hagenmaier, R. & Bai, J. (Eds.). (2011). *Edible coatings and films to improve food quality*. New York: CRC Press, 156-173.

Bamishaiye, E. I., Muhammad, N. O. & Bamishaiye, O. M. (2009). Haematological parameters of albino rats fed on tiger nuts (Cyperus esculentus) tuber oil mealbased diet. *The International Journal of Nutrition and Wellness*, 10(1), 95-109.

Basiak, E., Lenart, A. & Debeaufort, F. (2017). Effect of starch type on the physicochemical properties of edible films. *International Journal of Biological Macromolecules*, 98, 348-356.

Bell, A. F., Walton, K. L. & Tapsell, L. C. (2016). Easy to open? Exploring the ‗openability' of hospital food and beverage packaging by older adults. *Appetite*, 98, 125–132.

Bezerra, C., Sette, L. H. & Lopes, A. (2014). Liver enzymes serum levels in patients with chronic kidney disease on hemodialysis: A comprehensive review. *Clinics*, 69(4), 271-278.

Bonilla, J., Fortunati, E., Atarés, L., Chiralt, A. & Kenny, J. (2014). Physical, structural and antimicrobial properties of poly vinyl alcohol–chitosan biodegradable films. *Food Hydrocolloids*, 35, 463-470. Sobral, P. J., Menegalli, F. C., Hubinger, M. D. & Roques, M. A. (2001). Mechanical, water vapor barrier and thermal properties of gelatin based edible films. *Food Hydrocolloids*, 15(6), 423–432.

Bourbon, A. I., Pinheiro, A. C, Cerqueira, M. A., Rocha, C. M., Avides, M. C., Quintas, A. C. & Vicente, A. A. (2011). Physicochemical characterization of chitosanbased edible films incorporating bioactive compounds of different molecular weight. *Journal of Food Science,* 106, 111-118.

Bourtoom, T. (2009). Edible protein films: properties enhancement. *International Food Research Journal*, *16*(1), 1-9.

Brant, N. M., Gasparotto, F. M. & De Oliveira A. V. (2014). ―Cardiovascular protective effects of *Casearia sylvestris* Swartz in Swiss and C57BL/6 LDLr-null mice undergoing high fat diet.‖ *Journal of Ethnopharmacology*, 154(2), 419–427.

Burger, M. K. & Schaller, D. J. (2022). Metabolic Acidosis. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482146/ [Accessed 6 May 2023]

Castro-Muñoz, R. & Gonzalez, V. (2019). New trends in biopolymer-based membranes for preservation. *Molecules*, 24, 3584.

Cerqueira, M. S., Bartolomeu, T. J. & Vicente, A. (2012). Effect of glycerol and corn oil on physicochemical properties of polysaccharide films – A comparative study. *Food Hydrocolloids*, 27, 10-16.

Champion, V., Carbajal, R. & Lozar, J. (2012). Risk factors for developing transient neonatal cholestasis. *Journal of Pediatric Gastroenterology and Nutrition*, 55, 592–598.

Chawla, S. & Kanatt, S. (2014). Chitosan. *Polysaccharides*, 6(15), 1-24.

Chawla, S. P., Kanatt, S. R. & Sharma, A. K. (2015). Chitosan. In: Ramawat, K., Mérillon, JM. (eds) Polysaccharides. Springer, Cham, 89-97.

Chineke, C. A., Ologun, A. G. & Ikeobi, C. O. (2006). Haematological parameters in rabbit breeds and crosses in humid tropics. *Pakistan Journal of Biological Sciences*, 9(11), 2102-2106.

Chitaka, T. & Goga, T. (2023). The evolution of life cycle assessment in the food and beverage industry: A review. Cambridge Prisms: *Plastics*, 1-16.

Codex Alimentarius Commission. (2004). Report of the 20th session of the Codex committee on general principles. Joint FAO/WHO food standards programme. Paris, France, 44 p.

Debeaufort, F. & Voilley, A. (2009). Lipid-based edible films and coatings. In *Edible films and coatings for food applications*. New York: Springer, 135-168.

Deshwal, G. K. & Panjagari, N. R. (2020). Review on metal packaging: Materials, forms, food applications, safety and recyclability. *Journal of Food Science and Technology*, 57(7), 2377-2392.

Deshwal, G. K., Panjagari, N. R. & Alam, T. (2019). An overview of paper and paperbased food packaging materials: Health safety and environmental concerns. *Journal of Food Science and Technology*, 56(10), 4391-4403.

Dhall, R. K. (2013). Advances in edible coatings for fresh fruits and vegetables: A review. *Critical Reviews in Food Science and Nutrition*, *53*(5), 435-450.

Dhanapal, A., Rajamani, L. & Banu, M. S. (2012). Edible films from Polysaccharides. *Food Science and Quality Management*, 3, 9-17.

Dodig, S., Čepelak, I. & Pavić, I. (2019). Hallmarks of senescence and aging. *Biochemia Medica*, 29(3), 76-88.

Douglas, R. J., Lorena, N. B., Thiago, B. P., Leonardo, F. M., Francielli, G., Karla, M. R., Douglas, C. D., Emerson, L. B., Paulo, R. D. & Arquimedes, G. J. (2015). Ninety-Day Oral Toxicity Assessment of an Alternative Biopolymer for Controlled Release Drug Delivery Systems Obtained from Cassava Starch Acetate, *Evidence-Based Complementary and Alternative Medicine*, 7, 60-71.

Doyle, D. (2006). William Hewson (1739-74). The father of haematology. *British Journal of Haematology*, 133(4), 375-381

El Ghaouth, A., Aru, l. J., Grenier, J. & Asselin, A. (1992). Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. *Phytopathology*, 82, 398–402.

Embuscado, M. E. & Huber, K. C. (2009). Edible films and coatings for Food Applications; Springer Science and Business Media: New York, NY, USA, 11-18.

Erkmen, O. & Bozoglu, T. F. (2016). Food Microbiology: Principles into Practice. John Wiley and Sons Ltd, Chichester, UK, 458-460.

Etim, N. (2014). Haematological Parameters and Factors Affecting Their Values. *Agricultural Science*, 2, 37-47.

Fadia, B. S., Mokhtari-Soulimane, N., Meriem, B., Wacila, N., Zouleykha, B., Karima, R., Soulimane, T., Tofail, A. M., Townley, H. & Thorat, N. D. (2022). Histological Injury to Rat Brain, Liver, and Kidneys by Gold Nanoparticles is Dose-Dependent. *American Chemical Society Omega*, 7(24), 20656-20665.

Falguera, V., Quintero, J. P., Jiménez, A., Munoz, J. A. & Ibarz, A. (2011). Edible films and coatings: Structures, active functions and trends in their use. *Trends in Food Science and Technology*, 22(6), 292-303.

Fassett, R. G., Gobe, G. C. & Peake, J. M. (2010) Omega-3 polyunsaturated fatty acids in the treatment of kidney disease. *American Journal of Kidney Disease*, 56, 728–742.

Feingold, K. R. (2021) Introduction to Lipids and Lipoproteins. In: Feingold KR, Anawalt B, Blackman MR, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK305896/ [Accessed 7 May 2023]

Fenko, A., Schifferstein, H. N. & Hekkert, P. (2010). Shifts in Sensory Dominance between Various Stages of User-Product Interactions. *Applied Ergonomics*, 41, 34–40.

Fernando, I., Fei, J., Stanley, R. & Enshaei, H. (2018). Measurement and evaluation of the effect of vibration on fruits in transit-Review. *Packaging Technology and Science*, 31, 50-61.

Fitrzyk, A. & Niemiec, K. (2018). Quality Control in Aerosol Production. *Multidisciplinary Aspects of Production Engineering*, 1, 85-91.

Food and Drug Administration (FDA) US. (2019). Guidance for Industry: Use of Recycled Plastics in Food Packaging (Chemistry Considerations) Available online: https://www.fda.gov/regulatory-information/search-fda-guidancedocuments/guidance-industry-use-recycled-plastics-food-packaging-chemistryconsiderations [Accessed 15 July 2022]

Forbes, S. C., Cordingley, D. M., Cornish, S. M., Gualano, B., Roschel, H., Ostojic, S. M., Rawson, E. S., Roy, B. D., Prokopidis, K., Giannos, P. & Candow, D. G. (2022). Effects of Creatine Supplementation on Brain Function and Health. *Nutrients*, 14(5), 921-932.

Fox, D. L. (1973). Chitin-bound keto-carotenoids in a crustacean carapace. *Comparative Biochemistry and Physiology*, 44, 953–962.

Galus, S. & Kadzińska, J. (2016). Whey protein edible films modified with almond and walnut oils. *Food Hydrocolloids*, 52, 78-86.

Ganesan, A. R., Subramani, K., Balasubramanian, B., Liu, W. C., Arasu, M. V., AlDhabi, N. A. & Duraipandiyan, V. (2020). Evaluation of in vivo sub-chronic and heavy

metal toxicity of under-exploited seaweeds for food application. *Journal of King Saud University - Science*, 32(1), 1088-1095.

Gerschenson, L. N., Rojas, A. M. & Fissore, E. N. (2017). Carbohydrates. *Nutraceutical and Functional Food Components*, 39-51.

Gómez-Estaca, J., Gómez-Guillén, M. C., Fernández-Martín, F. & Montero, P. (2011). Effects of gelatin origin, bovine-hide and tuna-skin, on the properties of compound gelatin–chitosan films. *Food Hydrocolloids*, 25(6), 1461-1469.

Goswami, B. R. (2019). The role of food packaging. In book: Global Challenges and Innovation in Science and Management (pp. ICGCISM/AP-18). Publisher: KAAV Publications, 49-57.

Gounden, V., Bhatt, H. & Jialal, I. (2022). Renal Function Tests. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK507821/ [Accessed 18 July 2023]

Goy, R. C., De Britto, D. & Assis, O. B. (2009). A review of the antimicrobial activity of chitosan. *Polimeros* 19, 241–247.

Green, T. C., Jago, J. G., Macdonald, K. A. & Waghorn, G. C. (2013). Relationships between residual feed intake, average daily gain, and feeding behavior in growing dairy heifers. *Journal of Dairy Science*, 96(5), 3098-3107.

Groh, K. J., Backhaus, T., Carney-Almroth, B., Geueke, B., Inostroza, P. A., Lennquist, A., Leslie, H. A., Maffini, M., Slunge, D., Trasande, L., Warhurst, A. M. & Muncke, J. (2019). Overview of known plastic packaging-associated chemicals and their hazards. *Science of The Total Environment*, 651, 3253-3268.

Guerrero, L. I., Rosmini, M. R. & Armenta, R. E. (2009). *Tecnología de Productos de Origen Acuático.* LIMUSA; Mexico City, Mexico, 99-107.

Guimaraes, A., Abrunhosa, L., Pastrana, L. M. & Cerqueira, M. A. (2018). Edible films and coatings as carriers of living microorganisms: A new strategy towards bio preservation and healthier foods. *Comprehensive Review for Food Science and Food Safety*, 17(3), 594-614.

Gupta, S., Ahern, K., Nakhl, F. & Forte, F. (2011). Clinical usefulness of haptoglobin levels to evaluate hemolysis in recently transfused patients. *Advances in Hematology*, 43, 422-427.

Hammi, N., Chen, S., Dumeignil, F., Royer, S. & El Kadib, A. (2020). Chitosan as a sustainable precursor for nitrogen-containing carbon nanomaterials: synthesis and uses. *Materials Today Sustainability*, 10, 2589-2347.

Han, J. H. (2014). Innovation in food packaging. In: Han JH, Edible Films and Coatings: A Review. Academic Press, London, 213-255.

Hanisa, H., Noor, Z. A., Ahmad, T. S., Wan, W. M. & Somchit, M. N. (2011). Subacute Effects of Edible Film from Modified Sago Starch in Rats. *American Journal of Food Technology*, 6(8), 695-700.

Hazrol, M. D., Sapuan, S., Mohamed, Y., Mohd, Z., Zainudin, E. S., AbdulWahab, N. I., Ilyas, R. A., Harussani, M. M., Tarique, J., Asmawi, N. & Syafiq, R. M. (2020). Effect of Sorbitol and Glycerol Plasticizer and Concentration on Physical Properties of Corn Starch (*Zea Mays*) Biodegradable Films. *Natural Fibre Reinforced Polymer Composites*, 18-21.

Hoffbrand, A., Moss, P. & Pettit, J. (2006*).* Essential Haematology. Malden, Mass, USA, 35-42.

Holzapfel, W. (2015). Advances in Fermented Foods and Beverages. Improving Quality. *Technologies and Health Benefits*, 63-70.

Hosseinnejad, M. & Jafari, S. M. (2016). Evaluation of different factors affecting antimicrobial properties of chitosan. *International Journal of Biological Macromolecules*, 85, 467–475.

Húngaro H. M. & Sant'Ana, A. S. (2014). In Encyclopedia of Agriculture and Food Systems, 88-96.

Illum, L. (1998). Chitosan and Its Use as a Pharmaceutical Excipient. *Pharmaceutical Research*, 15, 1326-1331.

International Organization for Standardization (ISO). (2013). ISO 18604. Packaging and the Environment—Material Recycling. ISO; Geneva, Switzerland, pp. 1–11.

Isaac, L. J., Abah, G., Akpan, B. & Ekaette, I. U. (2013). Haematological properties of different breeds and sexes of rabbits. Proceedings of the 18th Annual Conference of Animal Science Association of Nigeria, 24-27.

Ismail, B. (2017). Ash Content Determination. *International Journal of Biological Macromolecules*, 7, 65–79.

Iwuji, T. C. & Herbert, U. (2012). Haematological and serum biochemical characteristics of rabbit bucks fed diets containing garcimiola kola seed meal.

Proceedings of 37th Annual Conference of Nigerian Society for Animal Production, 87-89.

Iyeghe-Erakpotobor, G. & Adeosun, T. (2014). Relationship between feed intake, weight gain, nutrient intake and digestibility of rabbits fed graded levels of sugarcane peel diets. *Journal of Animal Production Research*, 26, 20-32.

Jaderi, Z., Yazdi, F. T., Mortazavi, S. A. & Koocheki, A. (2023). Effects of glycerol and sorbitol on a novel biodegradable edible film based on Malva sylvestris flower gum. *Food Science & Nutrition*, 11(2), 991-1000.

Jafarzadeh, S., Jafari, S. M., Salehabadi, A., Nafchi, A. M., Uthaya Kumar, U. S. & Khalil, H. A. (2020). Biodegradable green packaging with antimicrobial functions based on the bioactive compounds from tropical plants and their byproducts. *Trends in Food Science & Technology*, 100, 262-277.

Japanese Chitin and Chitosan Society. (1994). Chitin and Chitosan Handbook. Tokyo, Gihodo, 460–483.

Jiménez, A., Fabra, M. J., Talens, P. & Chiralt, A. (2012). Edible and biodegradable starch films: a review. *Food and Bioprocess Technology, 5*(6), 2058-2076.

Jimoh, F. O. & Oladiji A. T. (2005). Preliminary studies on *Piliostigma thonningii* seeds: Preliminary analysis, mineral composition and phytochemical screening. *African Journal of Biotechnology*, 4, 1439-1442.

Jo, G. H., Park, R. D. & Jung, W. J. (2011). Enzymatic production of chitin from crustacean shell waste. In: Kim SK (ed) Chitin, Chitosan, Oligosaccharides and Their Derivatives. CRC Press/Taylor & Francis, Boca Raton, pp 37–45.

Juarez, F. M. & Farzam, K. (2022). Biochemistry, Very Low-Density Lipoprotein. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan. Available from: https://www.ncbi.nlm.nih.gov/books/NBK587400/ [Accessed 19 July 2023]

Kabir, M., Akpa, G. N., Nwagu, B. I., Adeyinka, I. A. & Bello, U. I. (2011). Sexual dimorphism, breed and age characteristics of rabbits in Zaria, Nigeria. Proceedings of the 16th Annual Conference of Animal Science Association of Nigeria, 133-137.

Kalakonda, A., Jenkins, B. A. & John, S. (2022). Physiology, Bilirubin. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK470290/ [Accessed 19 July 2023]

Kamaruddin, Z. H., Jumaidin, R., Ilyas, R. A., Selamat, M., Alamjuri, R., Yusof, M. D. & Fahmi, A. (2022). Biocomposite of Cassava Starch-Cymbopogan Citratus Fibre: Mechanical, Thermal and Biodegradation Properties. *Polymers*, 14, 1-19.

Karbowiak, T., Debeaufort, F., Champion, D. & Voilley, A. (2006). Wetting properties at the surface of iota-carrageenan-based edible films. *Journal of Colloid and Interface Science*, *294*(2), 400-410.

Karimi, Y., Maftoonazad, N., Ramaswamy, H. S., Prasher, S. O. & Marcotte, M. (2012). Application of hyperspectral technique for color classification avocados subjected to different treatments. *Food and Bioprocess Technology, 5*(1), 252264.

Kassem, J. M. (2015). Future Challenges of Whey Proteins. *International Journal of Dairy Science*, 10, 139-159.

Kaur, K., Ahluwalia, P. & Singh, H. (2016). Cassava: Extraction of starch and utilization of flour in bakery products. Project: Tuber Crops. *International Journal of Food and Fermentation Technol*ogy, 6(2), 351-355.

Khoushab, F. & Yamabhai, M. (2010). Chitin research revisited. *Marine Drugs*, 8, 1988–2012.

Khwaldia, K., Basta, A. H., Aloui, H. & El-Saied, H. (2014). Chitosan–caseinate bilayer coatings for paper packaging materials. *Carbohydrate Polymers*, 99, 508-516.

Kong, D., Foley, S. R. & Wilson, L. D. (2022). An Overview of Modified Chitosan Adsorbents for the Removal of Precious Metals Species from Aqueous Media. *Molecules*, 27(3), 978.

Kong, F. & Singh, R. P. (2016). Chemical deterioration and physical instability of foods and beverages. In Woodhead Publishing Series in Food Science, Technology and Nutrition; Subramaniam, 43-76.

Kong, M., Guang, X., Xing, C. K. & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *International Journal of Food Microbiology*, 144, 51–63.

Kostag, M. & El Seoud, O. A. (2021). Sustainable biomaterials based on cellulose, chitin and chitosan composites - A review. *Carbohydrate Polymer Technologies and Applications*, 2, 79-89.

Kra´sniewska, K., Galus, S. & Gniewosz, M. (2020). Biopolymers-based materials containing silver nanoparticles as active packaging for food applications- A review. *International Journal of Molecular Science*, 21(3), 698.

Kumar, V. & Fotedar, R. (2009). Agar extraction process for *Gracilariacliftonii*. *Carbohydrate Polymers*, *78*(4), 813-819.

Kuorwel, K. K., Cran, M. J., Sonneveld, K., Miltz, J. & Bigger, S. W. (2013). Migration of antimicrobial agents from starch-based films into a food simulant. *LWT - Food Science and Technology*, 50(2), 432-438.

Leceta, I. (2014). Structure–moisture sorption relation in chitosan thin films. *Materials Letters*, 128, 125-127.

Li, B., Elango, J. & Wu, W. (2020). Recent Advancement of Molecular Structure and Biomaterial Function of Chitosan from Marine Organisms for Pharmaceutical and Nutraceutical Application. *Applied Sciences*, 10(14), 4719-4730.

Linton, M. R., Yancey, P. G. & Davies, S. S. (2019). The Role of Lipids and Lipoproteins in Atherosclerosis. In: Feingold KR, Anawalt B, Blackman MR, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK343489/ [Accessed 19 July 2023]

Lizardi-Mendoza, J., Argüelles-Monal, W. & Goycoolea, F. (2016). Chemical Characteristics and Functional Properties of Chitosan. 10, 56-63.

Lovegrove, A., Edwards, C. H., Noni, I. D., Patel, H., El, S. N., Grassby, T., Zielke, C., Ulmius, M., Nilsson, L., Butterworth, P. J., Ellis, P. R. & Shewry, P. R. (2017). Role of polysaccharides in food, digestion, and health. *Critical Reviews in Food Science and Nutrition*, 57(2), 237-253.

Lowe, D., Sanvictores, T. & Zubair, M. (2022). Alkaline Phosphatase. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459201/ [Accessed 19 July 2023]

Machlus, K. R., Wu, S. K., Vijey, P., Soussou, T. S., Liu, Z. J., Shacham, E., Unger, T. J., Kashyap, T., Klebanov, B., Sola-Visner, M., Crochiere, M., Italiano, J. E. & Landesman, Y. (2017). Selinexor-induced thrombocytopenia results from inhibition of thrombopoietin signaling in early megakaryopoiesis. *Blood,* 130, 1132–1143.

Maftoonazad, N., Ramaswamy, H. S. & Marcotte, M. (2008). Shelf-life extension of peaches through sodium alginate and methyl cellulose edible coatings. *International Journal of Food Science & Technology*, *43*(6), 951-957.

Mahalik, N. P. (2014). Advances in packaging methods, processes and systems. *Challenges*, 5, 374–389.

Maizura, M., Fazilah, A., Norziah, M. H. & Karim, A. A. (2007). Antibacterial activity and mechanical properties of partially hydrolyzed sago starch–alginate edible film containing lemongrass oil. *Journal of Food Science*, *72*(6), 324-330.

Marlett, J. A., Kajs, T. M. & Fischer, M. H. (2000). An Unfermented Gel Component of Psyllium Seed Husk Promotes Laxation as a Lubricant in Humans. *American Journal of Clinical Nutrition,* 72, 784–789.

Marsh, K. & Bugusu, B. (2007). Food Packaging—Roles, Materials, and Environmental Issues. *Journal of Food Science*, 72(3), 39-55.

Martins da Costa, J. C., Lima Miki, K. S., da Silva Ramos, A. & Teixeira-Costa, B. E. (2020). Development of biodegradable films based on purple yam starch/chitosan for food application. *Heliyon*, 6(4), 37-41.

Maruna, R. F. (1958). Beitrag zur Serum-Natrium-Bestimmung: Eine kritische studie über kolorimetrische bestimmungen und angabe einer einfachen fotometrischen method. *Clinica Chimica Acta*, 2, 581-588.

Merck Manual. (2012). Haematologic reference ranges. Mareck Veterinary Manual. Retrieved from http://www.merckmanuals.com/ [Accessed 19 July 2023]

Merzendorfer, H. (2006). Insect chitin synthases: A review. *Journal of Comparative Physiology,* 176, 1–15.

Mitelut, A. C., Tanase, E. E., Popa, V. L. & Popa, M. E. (2015). Sustainable alternative for food packaging: Chitosan biopolymer- A Review. *AgroLife Scientific Journal*, 4(2), 52-61.

Moman, R. N., Gupta, N. & Varacallo, M. (2022). Physiology, Albumin. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459198/ [Accessed 22 July 2023]

Montalvo C., López M. A. & Palou, E. (2012) Películas comestibles de proteína: Características, propiedades y aplicaciones. *Temas Sel Ing Aliment*, 6, 32–46.

Morales-Jiménez, M., Gouveia, L., Yanez, F. J., Castro-Munoz, J. & Barragen-Huerta, B. E. (2020). Microalgae-based biopolymer as a potential bioactive film. *Coatings*, 10(2), 120.

Moran, F. (2012). Food packaging laws and regulation with particular emphasis on meat, poultry and fish. In Advances in Meat, Poultry and Seafood Packaging; Kerry, J.P., Ed.; Woodhead Publishing Limited: New Delhi, India, pp. 631–659.

Moshood, T. D., Nawanir, G., Mahmud, F., Mohamad, F., Ahmad, M. H. & AbdulGhani, A. (2022). Sustainability of biodegradable plastics: New problem or solution to solve the global plastic pollution? *Current Research in Green and Sustainable Chemistry*, 5, 273-287.

Nagatani, K., Wang, S., Llado, V., Lau, C. W., Li, Z., Mizoguchi, A., Nagler, C. R., Shibata, Y., Reinecker, C., Mora, J. R. & Mizoguchi, E. (2012). Chitinmicroparticles for the control of intestinal inflammation. *Inflammatory bowel diseases*, 18(9), 1698.

Nirogi, R., Goyal, V. K., Jana, S., Pandey, S. K. & Gothi, A. (2014). What suits best for organ weight analysis: Review of relationship between organ weight and body / brain weight for rodent toxicity studies. *International Journal of Pharmaceutical Sciences and Research*, 5(4), 1525-1532.

Nosrati, A., Alizadeh, A. & Hashemi-soteh, M. B. (2017). Hepatotoxicity and ALT/AST enzymes activities change in therapeutic and toxic doses consumption of acetaminophen in rats. *International Biological and Biomedical Journal*, 3 (3), 119-124.

Ogunbajo, S. O., Alemede, I. C., Adama, J. Y. & Abdullahi, J. (2009). Haematological parameters of Savannah brown does fed varying dietary levels of flamboyant tree seed meal. Proceedings of 34th Annual Conference of Nigerian Society for Animal production, p. 88-91.

Ojha, A., Akriti, S., Manvesh, S. & Ojha, S. (2015). Food packaging – materials and sustainability-A review. *Agricultural Reviews*, 36. 241.

Ojo, E. & Smidt, O. (2023). Lactic Acid: *A Comprehensive Review of Production to Purification*, 11, 1-37.

Okunlola, D. O., Olorunisomo, A. O., Aderinola, A. O., Agboola, A. S. & Omole, O. G. (2012). Haematology and serum quality of red Sokoto goats fed Baobab (Adansonia digitata) fruit meal as supplement to guinea grass (Panicum maximum). Proceedings of the 17th Annual Conference of Animal Science Association of Nigeria, p.427-433.

Olafedehan, C. O., Obun, A. M., Yusuf, M. K., Adewumi, O. O., Oladefedehan, A. O., Awofolaji, A. O. & Adeniji, A. A. (2010). Effects of residual cyanide in processed cassava peal meals on haematological and biochemical indices of growing rabbit. Proceedings of 35th Annual Conference of Nigerian Society for Animal Production, p.212.

Oliveira, V., Prell, M., Smallwood, D. & Frazão, E. (2022). Infant Formula Prices and Availability—Final Report to Congress. Available online: https://www.ers.usda.gov/webdocs/publications/43025/35759_efan02001.pdf?v=0 [Accessed on 22 May 2023].

Omiyale, C. A., Yisa, A. G. & Ali-Dunkrah, L. A. (2012). Haematological characteristics of Yankasa sheep fed fonio (Digitaria iburua) straw based diets. Proceedings of 37th Annual Conference of Nigerian Society for Animal Production, p. 87-89.

Opaleke, D. O., Salami, L. I., Ijabadeniyi, A. O. & Abimbola, A. (2022). Determination of Proximate Composition, Phytochemical Contents, Amino Acid Profile, and Functional Characteristics of *Landolphia togolana* Root Bark as a Potential Functional Food. Chiang Mai University. *Journal of Natural Sciences*, 21(1), 123-130.

Ossamulu, I. F., Akanya, H. O., Egwin, E. C. & Kabiru, A. Y. (2023). Improvement of shelf-life and nutrient quality of tomatoes and eggplant fruits using chitosanstarch composite coat. *Acta Scientiarum Polonorum, Technologia Alimentaria*, 22(1), 43-55.

Oyawoye, B. M. & Ogunkunle, H. N. (2004). *Biochemical and haematological reference values in normal experimental animals.* New York: Masson, p. 212218.

Pascall, M. A., DeAngelo, K., Richards, J. & Arensberg, M. B. (2022). Role and Importance of Functional Food Packaging in Specialized Products for Vulnerable Populations: Implications for Innovation and Policy Development for Sustainability. *Foods*, 11, 30-43.

Pavlath, A. E. & Orts, W. (2009). Edible films and coatings: why, what, and how? In *Edible films and coatings for food applications.* New York: Springer, 1-23.

Pellis, A., Guebitz, G. M. & Nyanhongo, G. S. (2022). Chitosan: Sources, Processing and Modification Techniques. *Gels*, 8(7), 76-89.

Peters, S. O., Gunn, H. H., Imumorin, I. G., Agaviezor, B. O. & Ikeobi, C. O. (2011). Haematological studies on frizzled and naked neck genotypes of Nigerian native chickens. *Tropical Animal Health Production*, 43(3), 631-638.

Popescu, A., Palade, L. M., Nicolae, C., Popa, E. E., Miteluț, A. C., Drăghici, M. C., Matei, F. & Popa, M. E. (2022). Chitosan-Based Edible Coatings Containing Essential Oils to Preserve the Shelf Life and Postharvest Quality Parameters of Organic Strawberries and Apples during Cold Storage. *Foods*, 11(21), 77-90.

Purves, W. K., Sadava, D., Orians, G. H. & Heller, H. C. (2003). Life: The science of Biology (7th ed., p.954). Sinauer Associates and W. H. Freeman, 43-51.

Queiroz, M. F., Teodosio, K. R., Sabry, D. A., Sassaki, G. L. & Oliveira, H. A. (2015). Does the Use of Chitosan Contribute to Oxalate Kidney Stone Formation? *Marine Drugs*, 13(1), 141-158.

Raafat, D., Bargen, K., Haas, A. & Sahl, H. G. (2008). Insights into the mode of action of chitosan as an antibacterial compound. Applied and Environmental Microbiology, 74, 3764–3773.

Raheem, D. (2013). Application of plastics and paper as food packaging materials- An overview. *Emirates Journal of Food and Agriculture*, 25(3), 177-188.

Ramos, Ó. L., Fernandes, J. C., Silva, S. I., Pintado, M. E. & Malcata, F. X. (2012). Edible films and coatings from whey proteins: a review on formulation, and on mechanical and bioactive properties. *Critical Reviews in Food Science and Nutrition*, 52(6), 533-552.

Ravi-Kumar, M. N. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, 46(1), 1-27.

Remize, F. (2017). Spore-forming bacteria. In The Microbiological Quality of Food: Foodborne Spoilers, pp. 99-120.

Renard, D., van de Velde, F. & Visschers, R. W. (2006). The gap between food gel structure, texture and perception. *Food Hydrocolloids*, *20*(4), 423-431.

Rinaudo, M. (2006). Chitin and chitosan: properties and applications. *Progress in Polymer Science*, 31, 603–632.

Romanazzi, G., Feliziani, E., Baños, S. B. & Sivakumar, D. (2017). Shelf-life extension of fresh fruit and vegetables by chitosan treatment, *Critical Reviews in Food Science and Nutrition*, 57(3), 579-601.

Roudaut, G. & Debeaufort, F. (2010). Moisture loss, gain and migration in foods and its impact on food quality. In Chemical Deterioration and Physical Instability of Food and Beverages, pp. 143-185.

Salvia-Trujillo, L., Soliva-Fortuny, R., Rojas-Grau, M. A., Mc Clements, D. J. & Martin-Belloso, O. (2017). Edible nanoemulsions as carriers of active ingredients: A review: *Annual Review of Food Science and Technology*, 8, 439466.

Samir, A., Ashour, F. H., Hakim, A. A. & Bassyouni, M. (2022). Recent advances in biodegradable polymers for sustainable applications. The *Nature Partner Journals Series Materials Degradation*, 6(1), 1-28.

Sánchez, A. D., Contreras-Esquivel, J. C., Nevárez-Moorillón, G. V. & Aguilar, C. N. (2015). Caracterización de películas comestibles a base de extractos pécticos y aceite esencial de limón Mexicano. *Journal of Food Science,* 13, 17–25.

Sarkar, S. & Aparna, K. (2020). Food Packaging and Storage. In book: Research Trends in Home Science and Extension 3, 27-51.

Sebti, I., Martial, A., Carnet-Pantiez, A., Grelier, S. & Coma, V. (2005). Chitosan polymer as bioactive coating and film against *Aspergillus Niger* contamination. *Journal of Food Science*, 70, 100–104.

Secretariat of the Environment and Natural Resources (SEMARNAT). (2019a). Interinstitutional Group announces Action Plan to comprehensively address the arrival of sargassum in the Mexican Caribbean. Retrieved from https://www.gob.mx/semarnat/prensa/grupo-interinstitucional-anuncia-plan-deaccion-para-atender-de-manera-integralarribo-de-sargazo-en-caribemexicano?idiom=es (Accessed 5th July 2021).

Secretariat of the Environment and Natural Resources (SEMARNAT). (2019b) Technical and Management Guidelines for Contingency Care caused by Sargassum in the

Mexican Caribbean and the Gulf of Mexico. Retrieved from https://www.gob.mx/semarnat/documentos/lineamientos-tecnicos-y-de-gestionpara-la-atencion-de-la-contingenciaocasionada-por-sargazo-en-el-caribemexicano-y-el-golfo-de-mexico (Accessed 5th July 2021).

Seed, N. K., Sekine, J., Hishinuma, M. & Hamana, K. (2003). Chitin and chitosan as possible novel nitrogen sources for ruminants. *Canadian Journal of Animal Science*, 83, 161-163.

Shaaban, H. & Farouk, A. (2022). Preservation and Packaging Food Recent Methods and Techniques: A Review. *Biomedical Journal of Scientific and Technical Research*, 46, 37680 - 37695.

Shaikh, S., Yaqoob, M. & Aggarwal, P. (2021). An overview of biodegradable packaging in the food industry. *Current Research in Food Science*, 4, 503-520.

Sharma, S., Hashmi, M. F. & Aggarwal, S. (2023). Hyperchloremic Acidosis. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482340/ [Accessed 6 May 2023]

Shroti, G. K. & Saini, C. S. (2022). Development of edible films from protein of brewer's spent grain: Effect of pH and protein concentration on physical, mechanical and barrier properties of films. *Applied Food Research*, 2(1), 10-13.

Sidell, B. D. & O‘ Brien, K. M. (2006). When bad things happen to good fish: the loss of haemoglobin and myoglobin expression in Antarctic icefishes. *The Journal of Experimental Biology*, 209, 1791-1802.

Sikorski, D., Gzyra-Jagieła, K. & Draczyński, Z. (2021). The Kinetics of Chitosan Degradation in Organic Acid Solutions. *Marine Drugs*, 19(5), 34-39.

Simon, L. V., Hashmi, M. F. & Farrell, M. W. (2023). Hyperkalemia. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK470284/ [Accessed 22 July 2023]

Singh, D., Shashi, A., Alok, M. & Kaushal, K. (2015). Advancement of chitosan-based nanoparticles for targeted drug delivery of antiulcer drugs. *World Journal of Pharmaceutical Sciences*, 4(1), 505–518.

Singh, R., Bhattacharya, B., Rhee, H. W. & Singh, P. K. (2015). Solid gellan gum polymer electrolyte for energy application. *International Journal of Hydrogen Energy*, 40(30), 9365–9372.

Siracusa, V., Rocculi, P., Romani, S. & Rosa, M. D. (2008). Biodegradable polymers for food packaging: A review. *Trends in Food Science and Technology*, 19(12), 634-643.

Soetan, K. O., Akinrinde, A. S. & Ajibade, T. O. (2013). Preliminary studies on the haematological parameters of cockerels fed raw and processed guinea corn (Sorghum bicolour). Proceedings of 38th Annual Conference of Nigerian Society for Animal Production, p. 49-52.

Sothornvit, R. (2014). Edible films and coatings for packaging applications. *Polymers for Packaging Applications, 173*, 24-30.

Sun, X., Wang, J., Dong, M., Zhang, H., Li, L. & Wang, L. (2022). Food spoilage, bioactive food fresh-keeping films and functional edible coatings: Research status, existing problems and development trend. *Trends in Food Science & Technology*, 119, 122-132.

Synytsya, A., Čopíková, J., Kim, W. J. & Park, Y. I. (2015). Cell Wall Polysaccharides of Marine Algae. In *Springer Handbook of Marine Biotechnology*. Springer Berlin Heidelberg, 543-590.

Tahir, H. E., Xiaobo, Z., Mahunu, G. K., Arslan, M., Abdalhai, M. & Zhihua, L. (2019). Recent developments in gum edible coating applications for fruits and vegetable preservation: A Review. *Carbohydrate Polymers*, 224, 115-141.

Tajeddin, B. (2014). Cellulose-Based Polymers for Packaging Applications. In book: Lignocellulosic Polymer Composites, 477-498.

Tao, W., Sun, W., Liu, L., Wang, G., Xiao, Z., Pei, X. & Wang, M. (2019). Chitosan Oligosaccharide Attenuates Nonalcoholic Fatty Liver Disease Induced by High Fat Diet through Reducing Lipid Accumulation, Inflammation and Oxidative Stress in C57BL/6 Mice. *Marine Drugs*, *17*(11), 66-79.

Taqi, A., Askar, K. A., Mutihac, L. & Stamatin, I. (2013). Effect of Laurusnobilis L. oil, Nigella sativa L. oil and oleic acid on the antimicrobial and physical properties of subsistence agriculture: the case of cassava/pectin based edible films. *Food and Agricultural Immunology*, *24*(2), 241-254.

Tarique, J., Sapuan, S. M. & Khalina, A. (2021). Effect of glycerol plasticizer loading on the physical, mechanical, thermal, and barrier properties of arrowroot (*Maranta arundinacea*) starch biopolymers. *Scientific Reports*, *11*, 66-71.

Tauheed, A. M., Mamman, M., Ahmed, A., Sani, N., Suleiman, M. M., Sulaiman, M. H. & Balogun, E. O. (2021). Acute, sub-acute, sub-chronic and chronic toxicity studies of four important Nigerian ethnomedicinal plants in rats. *Clinical Phytoscience*, 7(1), 1-12.

Tavassoli-Kafrani, E., Shekarchizadeh, H. & Masoudpour, B. M. (2016). Development of edible films and coatings from alginates and carrageenans. *Carbohydrate Polymer*, 137, 360-374.

Thakur, R., Pristijono, P., Scarlett, C. J., Bowyer, M., Singh, S. P. & Vuong, Q. V. (2019). Starch-based films: Major factors affecting their properties. *International Journal of Biological Macromolecules*, 9, 67-75.

Togun, V. A., Oseni, B. A., Ogundipe, J. A., Arewa, T. R., Hammed, A. A., Ajonijebu, D. C. & Mustapha, F. (2007). *Effects of chronic lead administration on the haematological parameters of rabbits – a preliminary study*. Proceedings of the 41st Conferences of the Agricultural Society of Nigeria, p. 341.

Trinder, P. (1951). A rapid method for the determination of sodium in serum. *Analyst*, 76, 596-599.

Ugwuene, M. C. (2011). Effect of Dietary Palm Kernel Meal for Maize on the Haematological and Serum Chemistry of Broiler Turkey. *Nigerian Journal of Animal Science*, 13, 93-103.

UN Environment Programme. (2021). UNEP Food Waste Index Report. Available online: https://www.unep.org/resources/ report/unep-food-waste-index-report2021 (Accessed on 22 May 2023).

Venugopal, V. (2011). Marine polysaccharides: food applications. CRC Press/Taylor & Francis Group, Boca Raton, 99-108.

Verlee, A., Mincke, S. & Stevens, C. V. (2017). Recent developments in antibacterial and antifungal chitosan and its derivatives. *Carbohydrate Polymers*, 164, 268283.

Viegas, I., Tavares, L. C., Elisabeth, P., Silva, E., Rito, J., Marandel, L., Palma, M., Rodrigo, R., Magnoni, L. & Panserat, S. (2022). On the Utilization of Dietary Glycerol in Carnivorous Fish - Part I: Insights Into Hepatic Carbohydrate Metabolism of Juvenile Rainbow Trout (Oncorhynchus mykiss) and European Seabass (Dicentrarchus labrax). 10, 79-86.

Wandrey, C., Bartkowiak, A. & Harding, S. E. (2010). Materials for encapsulation. In *Encapsulation technologies for active food ingredients and food processing.* New York: Springer, 31-100.

Wohner, B., Pauer, E., Heinrich, V. & Tacker, M. (2019). Packaging-related food losses and waste: An overview of drivers and issues. *Sustainability*, 11, 264

Woodman, D. D. (1996). Assessment of Hepatotoxicity. In: Animal Clinical Chemistry, A Primer for Toxicologist, Evans, G.O. (Ed.). Taylor and Francis, London, pp: 917-956.

Xia, W., Liu, P., Zhang, J. & Chen, J. (2011) Biological activities of chitosan and chitooligosaccharides. *Food Hydrocolloid*, 25, 170–179.

Yai, H. (2008). Edible films and coatings: Characteristics and properties. *International Food Research Journal*,15, 237–248.

Yamada, S. & Inaba, M. (2021). Potassium Metabolism and Management in Patients with CKD. *Nutrients*, 13(6), 61-75.

Yang, L., Li, X., Yang, L., Zhu, X., Wang, M., Song, Z., Liu, H. H., Sun, W., Dong, R. & Yue, J. (2023). Effect of Mg Contents on the Microstructure, Mechanical Properties and Cytocompatibility of Degradable Zn-0.5Mn-xMg Alloy. *Journal of Functional Biomaterial*, 14, 195.

Yang, X., Schnackenberg, L. K., Shi, Q. & Salminen, W. F. (2014). Hepatic toxicity biomarkers. *Biomakers in Toxicology*, pp. 241-259.

Yi, H., Wu, L. Q., Bentley, W. E., Ghodssi, R., Rubloff, G. W., Culver, J. N. & Gregory, F. P. (2005). Biofabrication with chitosan. *Biomacromolecules*, 6, 2881–2894.

Zahmati, M. & Saljooghi, A. S. (2016). The evaluation of deferasirox on haematological parameters after lead administration. *Asian Pacific Journal of Medical Toxicology*, 5, pp. 124-129.

Zhang, J., Tan, W., Wei, L., Chen, Y., Mi, Y., Sun, X., Li, Q., Dong, F. & Guo, Z. (2019). Synthesis of urea-functionalized chitosan derivatives for potential antifungal and antioxidant applications. *Carbohydrate Polymer*, 1(215), 108118.

APPENDICES Appendix A: Feed Intake by Albino Rats Placed on Edible Chitosan-Starch Film

Supplemented Diets

WEEKS (g)	A	B	C	D	E
WEEK 1	64.13±3.46^{a}	64.42±4.35^{a}	66.44±4.63^{a}	62.52±3.66^{a}	59.47±3.23^{a}
WEEK 2	89.95±1.58^{b}	93.28±1.46^{b}	90.53±0.99^{b}	89.08±0.99^{ab}	85.19±1.63^{a}
WEEK 3	103.63±0.93^{cd}	105.29±1.81^{d}	100.63±1.25^{bc}	98.51±1.61^{ab}	94.95±1.69^{a}
WEEK 4	107.67±0.96^{b}	113.00±1.19^{c}	104.95±1.97^{ab}	106.51±1.76^{ab}	103.10±0.87^{a}

Values are Mean ± Standard Error of Mean of triplicate determinations.

Values with different superscripts between the samples are significantly different ($p<0.05$).

Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

Appendix B: Body weight-gain of albino rats placed on Edible Chitosan-starch Film Supplemented Diets

WEEKS (g)	A	B	C	D	E
WEEK 1	8.02 ± 0.49^{a}	17.54 ± 4.12^{b}	14.76 ± 2.17^{ab}	9.27 ± 1.44^{a}	11.95 ± 2.24^{ab}
WEEK 2	27.38 ± 2.08^{ab}	33.13 ± 2.59^{b}	27.33 ± 3.91^{ab}	23.15 ± 4.88^{ab}	20.56 ± 2.83^{a}
WEEK 3	39.45 ± 3.06^{ab}	50.15 ± 3.92^{b}	38.31 ± 6.21^{ab}	32.03 ± 6.41^{a}	34.27 ± 6.01^{ab}
WEEK 4	50.94 ± 4.34^{ab}	70.25 ± 3.77^{b}	51.64 ± 5.27^{ab}	51.68 ± 9.10^{ab}	47.92 ± 7.72^{a}

Values are Mean ± Standard Error of Mean of triplicate determinations.
Values with different superscripts between the samples are significantly different ($p<0.05$).

Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

Appendix C: Organ Body-Weight Ratio of Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

ORGANS	A	B	C	D	E
KIDNEY	0.008 ± 0.000^{ab}	0.007 ± 0.000^{ab}	0.008 ± 0.000^{b}	0.008 ± 0.000^{ab}	0.007 ± 0.000^{a}
LIVER	0.042 ± 0.002^{a}	0.038 ± 0.003^{a}	0.039 ± 0.002^{a}	0.046 ± 0.003^{a}	0.040 ± 0.002^{a}
INTESTINE	0.117 ± 0.008^{c}	0.093 ± 0.002^{b}	0.072 ± 0.003^{a}	0.089 ± 0.006^{b}	0.079 ± 0.002^{ab}

Values are Mean ± Standard Error of Mean of triplicate determinations.
Values with different superscripts between the samples are significantly different ($p<0.05$).

Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

Appendix D: Liver Function Parameters of Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

PARAMETERS	A	B	C	D	E
AST (µ/L)	32.68±1.09[a]	31.74±0.97[a]	31.62±0.46[a]	31.58±0.37[a]	80.14±1.71[c]
ALT (µ/L)	32.42±1.24[a]	44.54±3.74[b]	53.12±2.99[b]	65.56±5.02[c]	67.92±1.45[c]
ALP (µ/L)	73.32±2.74[a]	117.90±20.44[b]	195.86±5.11[c]	100.34±0.86[ab]	219.36±9.52[c]
TP (g/dL)	6.08±0.15[a]	6.18±0.21[a]	6.18±0.18[a]	6.12±0.12[a]	6.20±0.16[a]
Albumin (µmol/L)	3.94±0.05[c]	3.24±0.11[b]	2.90±0.07[a]	2.90±0.10[a]	2.76±0.12[a]
TBil (µmol/L)	9.72±0.29[a]	15.02±0.39[b]	15.52±0.64[b]	16.76±0.47[b]	24.92±0.92[c]

Values are Mean ± Standard Error of Mean of triplicate determinations.
Values with different superscripts between the samples are significantly different ($p<0.05$).

Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

Appendix E: Renal Function Parameters of Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

PARAMETERS	A	B	C	D	E
UREA (mmol/L)	3.50±O.30^{a}	6.46±0.82^{b}	8.90±0.31^{c}	12.90±0.77^{d}	18.14±0.94^{e}
CREATININE (mg/dL)	2.42±0.27^{a}	5.38±0.65^{b}	8.96±0.38^{c}	13.58±0.36^{d}	17.66±0.42^{e}
POTASSIUM (µmol/L)	3.50±0.43^{a}	3.20±0.20^{a}	4.40±0.19^{b}	3.84±0.32^{ab}	3.92±0.10^{ab}
SODIUM (µmol/L)	98.94±1.03^{ab}	107.24±4.60^{b}	95.22±2.25^{a}	101.24±2.60^{ab}	103.30±2.99^{ab}
CHLORIDE (mmol/L)	96.04±0.31^{a}	98.42±0.91^{ab}	105.96±0.89^{c}	100.14±2.09^{b}	99.70±1.09^{ab}
BICARBONATE (mmol/L)	23.38±0.32^{a}	23.74±1.10^{a}	27.32±0.63^{b}	25.00±0.54^{ab}	27.26±1.14^{b}

Values are Mean ± Standard Error of Mean of triplicate determinations.
Values with different superscripts between the samples are significantly different ($p<0.05$).

Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.

Appendix F: Lipid Profile of Albino Rats Placed on Edible Chitosan-Starch Film Supplemented Diets

PARAMETERS	A	B	C	D	E
TCHOL (mmol/L)	2.29±0.09^{c}	2.08±0.07^{ab}	2.14±0.05^{bc}	1.92±0.06^{a}	1.92±0.06^{a}
TG (mmol/L)	0.77±0.03^{c}	0.68±0.02^{b}	0.66±0.01^{b}	0.56±0.01^{a}	0.60±0.02^{a}
HDL (mg/dL)	34.60±0.51^{a}	35.40±0.51^{a}	36.40±0.51^{a}	35.00±0.71^{a}	35.40±0.93^{a}
LDL (mg/dL)	38.06±4.21^{b}	30.08±1.90^{a}	30.74±2.12^{ab}	24.74±0.93^{a}	23.20±2.12^{a}

Values are Mean ± Standard Error of Mean of triplicate determinations.
Values with different superscripts between the samples are significantly different ($p<0.05$).

Diet Groups: **A**= Control (100 % rat chow), **B**= 5 % film, **C**=10 % film, **D**= 20 % film, **E**= 40 % film.