UNIVERSITI MALAYSIA KELANTAN

Toxicity Study of Ubi Gadong Starch *(Dioscorea Hispida)* on Zebrafish *(Danio rerio)*

Tan Xin Pei

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DECLARATION

I declare that this thesis entitled "Toxicity Study of Ubi Gadong Starch *(Dioscorea Hispida)* on Zebrafish *(Danio rerio)*" is the results of my own research except as cited in the references.

Signature : 

Student's Name : TAN XIN PEI

Matrix No : J20A0636

Date : 29/2/2024

Verified by:

Supervisor Signature :

Supervisor's Name : Dr. ZUBAIDAH AIMI BINTI ABDUL HAMID

Stamp :

Date :

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Kajian Ketoksikan Kanji Ubi Gadong *(Dioscorea Hispida)* ke atas Ikan Zebra *(Danio rerio)*

ABSTRAK

Dioscorea hispida dennst *(D. hispida)* adalah sejenis keledek dalam keluarga Dioscoreacea yang terdapat dengan melimpah di negara-negara tropika dan sub-tropika. Ia biasanya terdapat di hutan tumbuh kedua dan tumbuh di kawasan teduh. Ubi mentah mengandungi dioskorin, sejenis protein yang boleh menyebabkan reaksi alergi pada manusia. Namun begitu, *D. hispida* mengandungi banyak kanji dan telah menjadi makanan asas di beberapa tempat serta digunakan secara tradisional dalam perubatan untuk pelbagai tujuan. Oleh itu, kajian ini bertujuan untuk menentukan toksisiti kanji yang terkandung dalam *D. hispida*. Penilaian toksisiti yang dilakukan termasuk Tahap Kesan Advers Tidak Diperhatikan (NAOEL) dan dos maut (LD50) kanji *Dioscorea Hispida*. Beberapa kepekatan yang berbeza, iaitu 0, 500, 1000, 1500, dan 2000mg/kg telah diberikan kepada ikan Zebra selama 24 jam. Kumpulan fungsi kanji *D. hispida* telah dikenal pasti menggunakan Spektroskopi Transformasi Fourier Infra-Red (FTIR) dan dicirikan menggunakan Mikroskop Elektron Pemindaian (SEM) dan Pencerobohan Sinar-X (XRD). Hasil kajian menunjukkan bahawa NAOEL adalah 500mg/kg, manakala nilai LD50% dianggarkan sebagai 1500mg/kg. Sertakan juga histopatologi dan hasil SEM dan XRD. Ginjal ikan Zebra didapati mengalami kesan histopatologi yang paling teruk.

Kata Kunci: Kanji Ubi Gadong, *Dioscorea Hispida* dennst, *Danio rerio*, Kajian Ketoksikan, LD50%, NOAEL

Toxicity Study of Ubi Gadong Starch *(Dioscorea Hispida)* on Zebrafish *(Danio rerio)*

ABSTRACT

Dioscorea hispida dennst (D.hispida) is a type of wild yam from the Dioscoreacea family that can be abundantly found in the tropical and subtropical country. The raw tubers contain dioscorine, an alkaloid that can cause allergic reactions for consumer. However, the *D. hispida* tuber contain abundant amount of starch, has become a staple food in some part of place and traditionally used in medicine for various purposes. Thus, this study aim to determine the toxicity of starch contained in *D. hispida* tuber. The functional group of *D. hispida* starch was identified by using Fourier Transform Infrared (FTIR) Spectroscopy and characterize using Scanning Electron Microscope (SEM), and X-ray Diffraction (XRD). Non- Observed Adverse Effect Level (NAOEL) and lethal dosage (LD50) of *D.Hispida* starch was conducted and Zebrafish (*Denio rerio*) was chosen as an animal model. Different concentrations of 0, 500, 1000, 1500, and 2000mg/kg were gavaged on Zebrafishes for 24 hours. The results showed that NOAEL is 500mg/kg, while LD50 value is 1500mg/kg. histopathology study showed that Kidney of zebrafish most severely affected in this study.

Keywords: Ubi Gadong Starch, *Dioscorea Hispida* dennst, *Danio rerio*, Toxicity Study, LD50%, NOAEL

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LIST OF ABBREVIATIONS

AGPase	ADP-glucose pyrophosphorylase	35
BE	Starch branching enzyme	35
C	Carbon	10
$C_{13}H_{19}O_2N$	Dioscorine	9
CN-	Cyanides	11
DBE	Starch debranching enzyme	35
DPPH	1,1-diphenyl-2-picrylhydrazyl	11
FET	Zebrafish Embryos	2
FTIR	Fourier Transforms Infrared Spectroscopy	6
HCN	Hydrogen cyanide	12
LC50	Lethal concentration	18
LD50	Lethal dose	2
NOAEL	Non-Observed Adverse Effect Level	2
OECD	Organization for Economic Cooperation and Development	2
SEM	Scanning Electron Microscopy	6
SS	Starch synthase	35
XRD	X-ray diffraction	6

LIST OF SYMBOLS

%	Percentage	2
°C	Celcius	26
cm	Centimetre	20
kg	kilogramme	27
mg	milligrammes	26
µl	microlitre	28

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Dioscorea Hispida plants that contain the alkaloid dioscorine compound in its tuber. The starch from *D. hispida* tubers is edible once the dioscorine compound was removed. Eventhough this plant contains a toxic compound, it has become an important food supply in some tropical regions. Besides, it's been used for medication purpose to treat constipation. *Dioscorea hispida* starch can be used in the production of adhesives, paper products, textiles, and biodegradable films. Starch can also be converted into modified starches with improved functional properties for specific industrial applications. Due to their absorbent and soothing properties, it can be used in cosmetics and personal care products. They can be incorporated into formulations such as creams, lotions, and dry shampoos to enhance texture, absorb excess moisture, and provide a soft, silky feel on the skin or hair. It can also serve as eco-friendly alternatives to conventional plastic packaging. They are biodegradable, renewable, and used to produce packaging materials with reduced environmental impact.

The zebrafish, also known as *Danio rerio*, is a tropical freshwater fish (Kent et al., 2020). Zebrafish increasingly being used in biological research as animal model organism due to its high physiological and external fertilization, genetic resemblance to mammals, transparent embryos and larvae, fast organogenesis, and simplicity of genetic manipulation (Khan & Alhewairini 2018).

Besides, its characteristics, especially in function system and organ make the zebrafish's genetic structure is startlingly like that of humans (Bozkurt, 2020). Toxicity research in zebrafish spans from determining the toxicity of bioactive chemicals or crude plant extracts to determining the best technique. The Organization for Economic Cooperation and Development (OECD) test guideline 236 testing methods for the use of zebrafish embryos (FET) were tested (Guideline, 1992). Thus, this research study the toxicity of starch in *Dioscorea hispida* by using zebrafish as an animal model.

Non-Observed Adverse Effect Level (NOAEL) is commonly used in toxicology and risk assessment to describe the highest dose or exposure level of a substance at which no adverse effects are observed in a test population under specified experimental conditions. To determine the NOAEL, toxicity tests are typically conducted on relevant animal species such as mice, rats, or other organisms. These tests involve administering various doses of the substance to the test subjects and closely monitoring them for any adverse effects (Pizzo & Benfenati, 2016). Adverse effects can encompass a range of physiological, biochemical, or histopathological changes.

Acute lethality is a commonly used indicator of the toxicity of a chemical product. The Lethal Dose 50 (LD50) is a measure typically used to determine the acute lethality hazard. It represents the median dose at which 50% of the test population is predicted to die (Morris-Schaffer & McCoy, 2020). To determine the LD50, a range of doses of the chemical product is administered to a test population, typically animals. The doses are often administered orally using an appropriate technique, such as an oral gavage or dosing via an infected tube. The test subjects are closely observed for indications of poisoning and mortality within a specified time, 24 hours. At this dose, 50% of test animal who received such a dose to die and 50% to survive (Kyhoiesh et al., 2021).

1.2 Problem Statement

Lack of information on the toxicity study of *Dioscorea Hispida* starch using zebrafish as animal model. Previously, the available literature on the toxicity of *Dioscorea Hispida* primarily focuses on its potential toxicity in terms of skin irritation and allergic reactions in humans. Studies evaluating the toxicity of *Dioscorea Hispida* on zebrafish or other aquatic organisms seem to be limited. Limiting research on *Dioscorea Hispida* starch will restrict its use in a variety of applications. However, limited study regarding the toxicity of *Dioscorea hispida* especially in the form of starch compound.

Some of the previous studies used for toxicity such as mice, rats, zebrafish embroyos and others that are not suitable for large scale study. While animal models, including mice and rats, have been valuable in scientific research and have provided insights into human physiology and disease mechanisms, there are inherent differences between species that can affect the applicability of results. These differences can include variances in genetics, physiology, metabolism, and immune responses. diseases are often complex and multifaceted, involving interactions between genetic, environmental, and lifestyle factors. Animal models, by their nature, often simplify these complexities and may not fully capture the intricacies of human diseases. Additionally, mice used in research studies typically lack the genetic diversity found in human populations, which further limits their representativeness for human conditions (LIDE Biotech).

The physiological differences between smaller animals and larger animals, including humans, can impact how a toxic substance affects different organs. For example, the metabolism and clearance of drugs or chemicals can vary between species due to differences in enzyme systems (Zhao et al., 2021). Additionally, the anatomy and physiology of certain organs may differ significantly between smaller animals and larger animals, which can affect the interpretation and relevance of toxicity data (Oehme, 2020). Different species can exhibit varying sensitivities to toxic substances. While mice and rats are commonly used in toxicity testing due to their availability, cost-effectiveness, and physiological similarities to humans, there can still be species-specific differences in susceptibility and response to toxicants. Therefore, findings from rodent studies may not always directly apply to larger animals or humans.

Zebrafish are important animal models due to their multiple benefits over other species. Zebrafish have a completely sequenced genome, facile genome modification, great fertility, a short generation period, fast embryonic development, and external fertilization. The transparent zebrafish embryo allows study to investigate many developmental phases beginning with embryogenesis. Furthermore, zebrafish develop whole organ systems, including the heart, gut, and blood arteries, demonstrating genetic tractability similarity to humans.

1.3 Objectives

The main objectives of the present study are:

1. To characterize the toxicity compound in of *D. hispida* starch.
2. To determine the level of toxicity in *Dioscorea Hispida* starch using Non-Observed Adverse Effect Level (NAOEL) and lethal dose (LD50) at different dosage concentration.
3. To investigate the effect of *Dioscorea Hispida* starch on zebrafish through histopathology analysis.

1.4 Scope of Study

In this research, the raw materials ubi gadong (*D. hispida*) was purchased at Tanah Merah in Kelantan. The ubi gadong was washed, peeling and slicing, Next, *D. hispida* was grinded into powder. After that, the toxicity study *D. hispida* starch on zebrafish were characterized using different ways, which are NOAEL, LD50, FTIR, SEM, XRD and histopathology studies. Besides that, the chemical and physical properties were tested.

1.5 Significances of Study

The significance of this research is to identify the toxicity effects on zebrafish. Information from this study be allow contribute to the development of scientic knowledge especially for *D. hispida* species. Besides the extraction and isolation of dioscorine content from *D. hispida* for use as an insecticide has practical implications. It could serve as an alternative to chemical insecticides. This could lead to a reduction in the dependency on chemical insecticides and would bring the benifical on human and environmental in the future.

CHAPTER 2

LITERITURE REVIEW

2.1 *Dioscorea Hispida (D. hispida)* Dennst

Dioscorea hispida dennst is a species of yam that belongs to the Dioscoreacea family. It is widely distributed in subtropical and tropical countries, typically found in second growth forests and shaded areas. Trichomes and thorns with a thick walled, broad range diameter, and stoutness covered the stem. *Dioscorea hispida* propagation is quite similar to that of other yams, which were traditionally reproduced vegetatively from big (setts), whole tubers (seed), and small (minisetts) tuber pieces. However, deep tubers of *D. hispida* have been shown to be more attractive than aerial tubers. As a result, aerial tubers can be utilized as replacement seedlings in *D. hispida* cultivation. Aerial tubers can be utilized as clean planting materials since they are typically free of soil-borne pests like nematodes (Wahid et al., 2014). The tuber is huge, bristly globose in form, and hairy, indicating that it belongs to the category of root crops. It can grow well in suitable environmental conditions with the light concentration humidity and temperature (Hazrati et al., 2021). *Dioscorea hispida* has long been used as a medicinal herb in Thailand. The dried tuber of this species has been utilized as a crude drugs in a Thai treatment known as Thoraneesanthakhat. It has long been used for treating constipation. Additionally, *D. hispida* has been traditionally used in medicine for various purposes, including treating inflammation, promoting wound healing, and providing relief from digestive issues. Morphologically, this plant grows by climbing the other plants. It has a

palmate compound leaf whose veins are reticulated. The *D. hispida* tuber has become an essential food in particular regions. The raw tubers contain dioscorine, a protein that can cause allergic reactions in humans. Figure 2.1 show *Dioscorea hispida* dennst tuber.

Figure 2.1 *Dioscorea Hispida* dennst tuber

(Source: Hazrati et al., 2020)

2.1.1 Chemical Composition of *D. hispida* Tuber

Dioscorine is an alkaloid compound that dissolves in deep water and has the molecular formula $C_{13}H_{19}O_2N$. Dioscorine has been reported to induce allergic reactions in human, including gastrointestinal symptoms, respiratory and skin rashes issues. *D. hispida* tuber contains numerous lignocellulosic, namely complex polymers of cellulose, hemicellulose and lignin (Hamid et al., 2019). Figure 2.1.1 show the compound structure of dioscorine in the *Dioscorea Hispida* tubers.

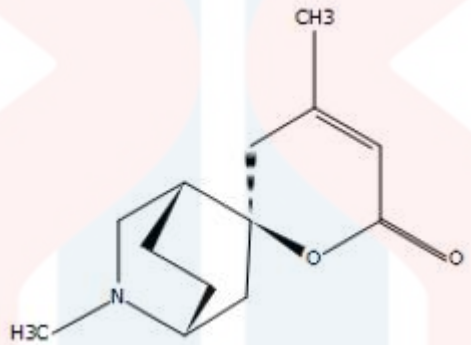

Figure 2.1.1 Dioscorea compound structure

(Source: Kamaruddin et al. 2020)

D. hispida tubers may contain phytochemical compounds with potential health benefits. These compounds, such as phenolic compounds and flavonoids, can act as antioxidants and have anti-inflammatory properties. Dioscorine features as a protein reserve in yam tubers. Diosgenin is a main precursor withinside the manufacturing of artificial steroids withinside the pharmaceutical industry. The organic sports of diosgenin, different steroidal saponins, and alkaloids had been examined in vitro. The anticancer bio-pastime of diosgenin is associated with the presence of hetero-sugar bonds and five.6-double bonds in its structure. The structural conformations at C-five

and C-25 carbon atoms additionally play a crucial position withinside the organic pastime of diosgenin (Raju and Rao, 2012).

Cellulose molecules are responsible for the structure of the plant. It is arranged as fibril bundles in common compounds, while hemicellulose and lignin fill the plant fibers. The concentration of lignocellulose can vary depending on the plant species. The variation is due to the different composition of genetic variation in plants. In general, the lignocellulosic components of a plant are 35-50% cellulose, 20-35% hemicellulose and 15-25% lignin. *Dioscorea hispida* tubers are rich in carbohydrates, particularly starch. Starch serves as the primary storage form of energy in yams and typically constitutes a significant portion of their composition. The tubers also contain dietary fiber, which contributes to their nutritional profile, can promote digestive health and help regulate blood sugar levels.

2.1.2 Toxicity of *D. Hispida* Tuber

Dioscorea Hispida species is a poisonous and commonly found wild among other Dioscorea species (Buenavista et al., 2021). *Dioscorea hispida* caused 252 poisonings and three deaths in Thailand (Sriapha et al., 2015), four poisonings and one death in the Philippines. Its toxicity is mainly due to the presence of the alkaloid dioscorine and cyanogenic glycosides, which make the toxicity dioscorea be there if taken directly or poorly processed. Dioscorine is a neurotoxic isoquinuclidine alkaloid that affects the neurological system and can result in acute kidney damage, toxic encephalopathy, and even death (Sasiwatpaisit et al, 2014). Additionally, being a cyanogenic plant, it can also cause neuropathy, metabolic disorders, amblyopia and paralysis. Importantly, toxic dioscorine consumption can result in nausea, vomiting, and nervous system dizziness (Sasiwatpaisit et al, 2014).

The *D. hispida* tuber contains a highly poisonous chemical that converts angiotensin to an enzyme inhibitor. In addition, the poisonous compounds of *D. hispida* tuber are ability to provide positive effects such as antimicrobial and therapeutic. Because of the presence of carboxylate ester, dioscorine belongs to the carbonyl family, a non-aromatic ring composed of five carbon atoms, one double bond ring and two oxygen atoms. Although dioscorine is toxic, it can also be used to treat hypertension by inhibiting the action of angiotensin - converting enzyme. Previous report discovered that dioscorine can control excessive blood pressure (Hsu et al., 2002) due to its capacity to scavenge hydroxyl free radicals and DPPH (1,1-diphenyl-2-picrylhydrazyl), and it can also increase immunological regulation.

The consequences of dioscorine poisoning can cause several diseases and disorders, and without proper treatment, it can lead to death. Only free cyanides (CN-) are toxic, and if the hydrolysis process are not perform, the glycoside remains stable and foods made from food material

are safe. In addition, daily consumption of *Dioscorea hispida* products with dangerous cyanogenic concentrations as a staple food can cause chronic dioscorine toxicity as a long-term effect (Cooke and Maduagwu, 1978). According to the World Health Organization's 1988 standard, adequate processing may decrease all cyanogens in cassava products to below the permissible level of 10 mg HCN equivalent per kg body weight (Mlingi, Bainbridge, Poulter, & Rosling, 1995).

2.2 Animal Model for Toxicity Study

Animals were utilised as early models for studying disease pathogenesis, treatment, and progression. The afore mentioned use gave development to the field of investigative toxicology, in which animals are used as substitutes to predict probable negative health outcomes from chemical exposures in humans. This technique was opposed for some scientific, ethical, and philosophical reasons, yet using animal models to assess hazards and risks to humans is still the preferred method for protecting human health. Fish, birds, and mammals are used for scientific and medical study. Animal toxicity studies typically are conducted in rats, mice, rabbits, dogs, and fish. The rodent (mice and rat) are make up approximately 95% of all laboratory animals, with they are the most commonly used animal in toxicity study research. They have long been favored as preferred species for biomedical research due to their notable similarities in anatomy, genetics, and physiology to humans. These similarities make them valuable for studying various aspects of human biology and disease. Benefits of rodents include their small size, ease of maintenance, short life cycle, and abundant genetic resources.

Table 2.2: Toxicity Study of Ubi Gadong Starch *(Dioscorea Hispida)* on Zebrafish *(Danio rerio)*

Raw Materials	Animal Model	Methods	Assessment	Source
Carica papaya (CP) leaf	Sprague - Dawley (SD) rats	Gavage procedure	Acute Toxicity Study	Halim et al., 2011
Ginseng & Ashwagandha	Rats	Gavage procedure	Subacute Toxicity Study, LD50	Aphale et al., 1998
Caralluma dalzielii N. E. Brown	Mice & rats	Gavage procedure	Acute and Subacute Toxicity Test	Ugwah-Oguejiofor et al., 2019
Dioscorea antaly	Oryzias Latipes Embryos	Gavage procedure	LD50	Rakotobe et al., 2010
Dioscorea alata	Zebrafish (Danio rerio) Embryos	Immerse procedure	Fish Embryos Toxicity	Raherjang et al., 2021
Baby Aloe Powder (BAP)	Sprague-Dawley (SD) rats	Gavage procedure	NOAEL	Kwack et al., 2014

2.2.1 Zebrafish *(Danio rerio)* as Animal Model for Toxicity Study

In 1822, Francis Hamilton first documented the existance of the zebrafish *(Danio rerio)*, which is biologically a member of the family Cyprinidae of the order Cyprinidae (Spence et al., 2007). The zebrafish is a tiny tropical freshwater fish that originated in northern India's Ganges River (Tavares and Santos Lopes, 2013). It has an average lifespan of 3.5 years. However, some survive for up to 5.5 years. There are female and male zebrafish, the female zebrafish have a protruding belly (whitish in colour), a blueish-white cast, and silver stripes between blue stripes. Whereas male zebrafish are slenderer and have a golden sheen on their belly, deeper blue stripes, and a pinkish-yellow cast (Menke et al., 2011). They normally grow to be around 2-3 inches (5-7.5 cm) in length.

The zebrafish *(Danio rerio)* have extraordinary regeneration abilities. They have the ability to regenerate fins, heart tissue, and even sections of their spinal cord, making them an important model for researching tissue regeneration and healing (González-Rosa et al., 2017). It is an appropriate model for screening medications for possible utility in treating human disorders since its circulatory and digestive systems have similar morphology and physicology. The zebrafish genome has been completely sequenced. Zebrafish provide a major data set for a vertebrate animal, allowing researchers to link genetic, and biochemical to behavioral observations, functional, and high-performance structural. Zebrafish share a significant degree of genetic similarity with humans, with approximately 70% of human genes having orthologs in zebrafish. This genetic similarity allows for the investigation of conserved toxicological pathways and the extrapolation of results to human health.

Zebrafish also have organ systems analogous to those in humans, including the cardiovascular, nervous, digestive, and immune systems. This similarity enables the assessment of

organ-specific toxic effects and the evaluation of potential adverse outcomes. Additionally, zebrafish exhibit similar processes of drug absorption, distribution, metabolism, and excretion as humans. They also possess detoxification mechanisms similar to those found in mammals. These features make zebrafish a valuable model for studying pharmacokinetics and Toxicokinetics of compounds. The adult Zebrafish are utilized as experimental models. Zebrafish are amenable to large-scale study and it is suitable for toxicity study.

Scientists have been using zebrafish as a model species for almost 200 years. Every fish species has its own variety of advantages and disadvantages. Zebrafish has been popular in importance as a model in recent years due to its adaptability for a variety of research domains. The zebrafish *(Danio rerio)* has become increasingly useful as a toxicological model for fast in vivo studies and developmental toxicity tests. There are several advantages to using zebrafish *(Danio rerio)* as an animal model for toxicity studies (Ray et al., 2017). Zebrafish has significant genetic similarity to mammals, strong phenotypes, and high throughput genetic and chemical screening, making it a valuable tool for evaluating in vivo toxicity (Caballero and Candiracci, 2018). The zebrafish embryo toxicity model stands at the forefront of toxicology research, owing to its brief investigation period, rapid life cycle, remarkable fertility, transparent embryos, and genetic data similarity (Modarresa et al., 2020).

This are some advantages zebrafish as an animal model for toxicity study:

I. Rapid embryogenesis in 6 days

II. Rapid generation cycle (2 to 3 months)

III. Characterized developmental stages very well.

Figure 2.2.1 Zebrafish *(Danio rerio)*

(Source: McKie, 2013)

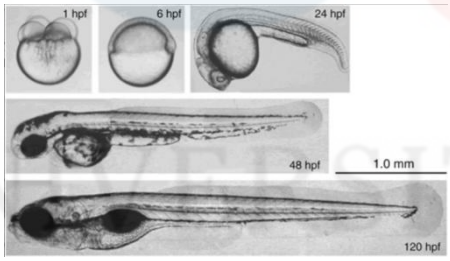

Figure 2.2.2 Zebrafish Rapid Development

(Source: Zhang et al., 2003)

2.3 Toxicity study (Lethal Dosage 50, NOAEL)

Previous report, scientists created standardized processes for evaluating potentially hazardous compounds to assure good scientific methodologies and the development of high-quality data that are crucial for assessing human hazards and risks. Toxicity testing in animals model used to determine potential detrimental effects from an agent's exposure and to generate dose-response relationships that allow for the evaluation of reactions at additional exposures (Council, 2006). The main purpose of the toxicity test was to identify a chemical's median lethal dosage (LD50), which is the amount that kills 50% of test animals.

The lethal dosage 50 (LD50) is a toxicological parameter used to estimate the possible impact of a poisonous material on several types of organisms. It gives an objective metric for comparing and ranking chemical toxicity. When comparing LD50 values, lower values are considered more toxic since they indicate that a lesser amount of the toxin is necessary to cause death. The LD50 is determined by the route of entrance into the organism being examined. Today, toxicity tests are now used to determine dose regimens for longer-term toxicity testing and to investigate the consequences of acute exposure more thoroughly (Council, 2006). The test chemical or preparation can be administered to the animal, by subcutaneously or by inhalation. The parameters that describe the findings of different tests so that they are comparable are lethal dosage (LD50) and lethal concentration 50 (LC50). The term LD50 refers to the dose that kills 50% of the test population. To compare acute toxicity, LD50 values are employed. It is measured in milligrammes per kilogramme (mg/kg) of test animal body weight (Mansouri et al., 2021).

According to Dorato and Engledhart (2005), no observed adverse effevt level (NOAEL) is defined as the highest experimental point which has no adverse effect observed. NOAEL is used to predict the adverse events in human or animal in non-clinical safety testing, identifying the toxic in human and also determining and predicting the dose response pattern by using animal toxicological studies. Nevertheless, the existing toxicological studies for the deriavation of NOAELs for dermal and inhalation routes are generally lacking for most pesticides. Therefore, the NOAELs are typically derived using oral toxicity studies and extrapolated for different routes of exposure when needed (Salem and Katz, 2006).

The NOAEL serves as an important reference point in risk assessment, as it helps establish the highest level of exposure to a substance that can be considered safe without causing adverse effects. It is crucial in determining safety thresholds and setting guidelines for human and environmental exposure to potentially harmful substances. In toxicology studies, several parameters are considered to identify the NOAEL. These parameters include the dose-response relationship, the precision of the measurements, the range of natural variation (controls), the biological plausibility of the observed effects, and statistical significance (Pandiri et al., 2017).

2.4 Characterization of starch structure

2.4.1 Fourier Transforms Infrared (FTIR) Spectroscopy

The FTIR was performed in the range of 400 - 4000 cm^{-1} with a resolution of 4 cm^{-1}. The FTIR was used to analyse the spectra of *Dioscorea hispida* tuber. The FTIR graph was shown that the peak region of 3200-3500 cm^{-1} was related to the hydroxyl group and the bond stretch of O-H. In the graph, the peak 3270 cm^{-1} was the O-H stretching and peak 2927 cm^{-1} related to C-H bond stretching. The peak 928.78 cm^{-1} could be a β-D-grape pyranose or a β-D-galactose because these components were having a range from 905.0 – 876.0 cm^{-1}. All these peak, 1016.30, 1078.67 and 1150.32 cm^{-1} can be an alcohol C-OH group or C-O-C stretch vibration of β-1,4 glucosidase and β-1,4 mannoside. The peak range from 1440.00 – 1395.00 cm^{-1} was C-O-H carboxylic acid hence the peak 1408.30 cm^{-1} has the potential to be it since it lied in the range. The peak 1644.94 cm^{-1} was a C=O stretching vibration of a cellulose.

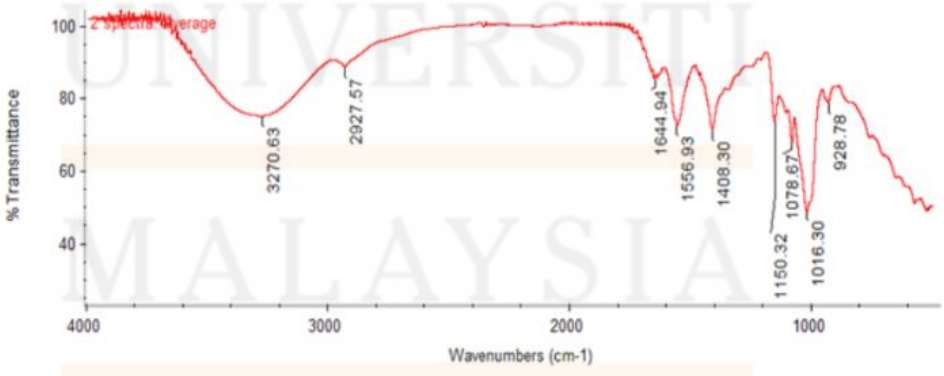

Figure 2.3.1: FTIR spectrum of *D. hispida* tuber

(Source: Hamid, et al., 2019)

2.4.2 Scanning Electron Microscope (SEM)

Scanning Electron Microscopy (SEM) is a specialized technique that focuses on examining the surface morphology of solid samples using a high-energy electron beam. This process yields a diverse array of signals from different depths within the solid sample. The surface composition and topography of the specimen are determined. The outcome of this analysis is presented in the form of images, which can be displayed on a computer screen. Figure 2.3.2 show the working principle of SEM.

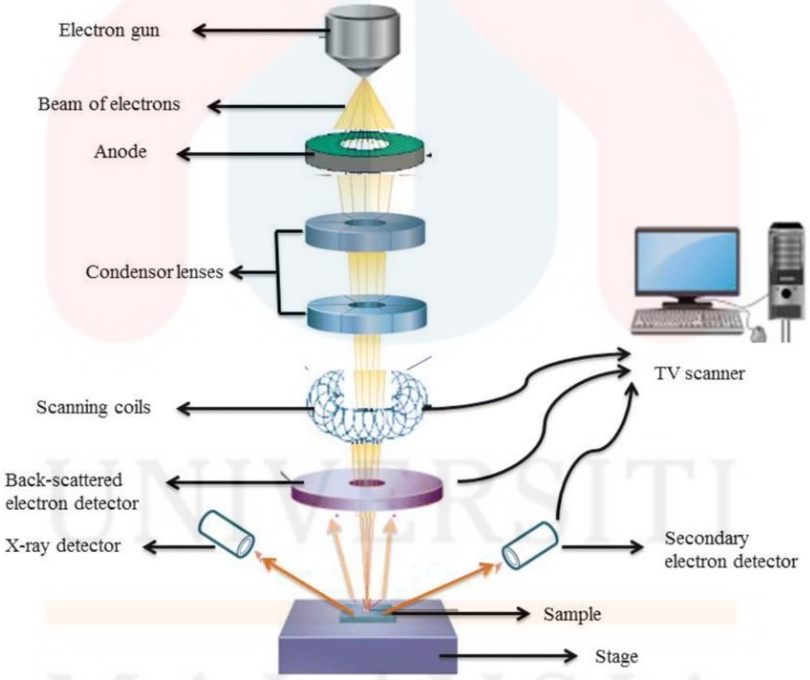

Figure 2.3.2: Working principle of SEM

(Source: Munir, et al., 2021)

2.4.3 X-ray Diffraction (XRD)

X-ray diffraction (XRD) is indeed a technique used to analyze the atomic and molecular structure of materials. By exposing a crystalline material to X-rays and measuring the resulting diffraction pattern, valuable information about the material's internal structure can be obtained (Giannini et al., 2016). The process of X-ray diffraction occurs when X-rays interact with the atoms in the material. These interactions cause the X-rays to scatter in different directions due to the periodic arrangement of atoms in the crystal lattice. The scattered X-rays interfere constructively and destructively, creating a distinct diffraction pattern. The diffraction pattern is typically recorded as a series of peaks, and the angles and intensities of these peaks contain information about the crystallographic structure, phase composition, crystal size, and orientation of the material. By comparing the diffraction pattern with known reference patterns or using mathematical analysis methods, researchers can determine the types of crystals present in the sample and deduce their arrangement within the lattice.

The diffraction effect occurs when electromagnetic radiation interacts with periodic structures that have geometric variations on the scale of the radiation's wavelength. In the case of X-rays, their wavelengths are on the order of 0.15 to 0.4 nanometers, which corresponds to interatomic distances in crystals and molecules. This wavelength range falls within the X-ray region of the electromagnetic spectrum, typically with photon energies between 3 and 8 kiloelectron volts (keV) (Zone & Zone). When crystalline and molecular structures are exposed to X-rays, phenomena such as constructive and destructive interference occur. This is because X-rays can interact with the electrons in the material, leading to scattering and diffraction patterns that depend on the arrangement of atoms in the crystal or molecule.

X-rays are generated by accelerating electrons with kinetic energies in the keV range. In laboratory X-ray tubes, electrons are emitted from a cathode filament and accelerated towards an anode plate made of a specific metal of high purity. When the accelerated electrons strike the anode, X-rays are produced. The emission spectrum of X-rays from the anode consists of a continuous component called Bremsstrahlung and discrete lines that correspond to the characteristic X-ray emissions of the target material's chemical elements (Alekseev et al., 2015). Commonly used materials for the anode in X-ray tubes include copper, chromium, molybdenum, or other metals.

2.4.4 Histopathology Studies

Histopathology studies play a crucial role in understanding the effects of substances on model animals and assessing organ failures and internal conditions. By analyzing tissue samples at a microscopic level, histopathology provides valuable insights into cellular changes, organ damage, and the overall impact of substances on the animal's body. Liver is a major site for the metabolism and detoxification of toxic compounds in zebrafish, as well as in many other organisms. Zebrafish hepatocytes, which are the primary functional cells of the liver, play a crucial role in metabolizing and eliminating various substances, including toxins. Zebrafish liver tissue can vary depending on the nature of the toxic compound, the duration and dosage of exposure, and individual variability.

Previous studies have shown that exposure to methylparabens, which are harmful compounds commonly used as preservatives in various products, can cause hepatotoxicity in zebrafish. Hepatotoxicity refers to liver damage or toxicity. In the case of methylparaben exposure, the histopathological assessment revealed the formation of vacuoles within hepatocytes. Vacuolization refers to the development of fluid-filled spaces within the cell. This indicates degeneration of cell processes and metabolic dysfunction (Hu et al., 2022).

Venom from snakes has been found to alter the liver tissue structure in zebrafish. The histopathological assessment revealed vacuolization in hepatocytes, similar to the effects observed with methylparaben exposure. However, doubts can arise because a reduction in glycogen levels can also lead to vacuolization. Nevertheless, zebrafish, known for their high activity levels and metabolism, tend to exhibit excessive cytoplasmic vacuolization in hepatocytes. This suggests that the vacuolization indicates degeneration of cell processes and metabolic dysfunction (Tozzi et al., 2022). In addition to liver effects, histopathology studies have identified changes in the zebrafish kidney following exposure to snake venom. The venom caused loss of cellular contour, tubular

hyaline degeneration, and necrosis in various parts of the zebrafish kidney. These findings indicate damage and dysfunction in the kidney tissues.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials, ubi gadong (*D. hispida*), zebrafish, tricaine mesylate (MS222) solution, 10% formalin buffer, Feeding Syringe (10mL), 2F Catheter (22-G), UV-Sterilized Sponge, Aerator, Anti-Chlorine.

3.2 Methods

3.2.1 Experimental Animal

The experiment was carried out at an aquaculture lab which was located at Faculty of Agro-based Industry (FIAT). Wild types of Zebrafish were purchased from the local aquaculture facility, Sentosa Jaya Aquatic in Pasir Pekan, Wakaf Bahru, Kelantan Darul Naim. The optimum conditions of zebrafish *(Danio rerio)*: dissolved oxygen 97.9% (92.9% - 99.1%), pH 7.4 (7.0 - 7.6), temperature 25°C (24.7°C - 26.0°C), total ammonia 0.13mg L^{-1} (0.07mg L^{-1} - 0.18mg L^{-1}).

3.2.2 Preparation of *Dioscorea hispida starch*

Dioscorea hispida was obtained from Tanah Merah, Kelantan. *D. hispida* tuber was washed, peeled, and sliced diced into 1 to 2 cm size. Then, the slices were mixed with distilled water with ratio 1:3 in a beaker and blended into mesh. Filter out the starch slurry with filter cloth. Use aluminium foil to wrap the beaker and put it into the chiller for 3 hours to obtain the sediment starch. Then, dry it in the oven (50°C) and the dried starch ground into powder with used mortar and pestle. The yield starch content of D. hispida changed because of the extraction techniques. For this experiment, 100g of *D. hispida* was used for extracted starch. Finally, 20g of starch was produced after grounding. Adding 5 mL distilled water with different grams of starch will eventually produce different concentrations (Table 3.2.2).

Table 3.2.2 Different concentration with different *D. hispida* starch from 5mL distilled water

Distilled Water	Concentration (mg/kg)	Starch (g)
5 mL	0	0
	500	0.25
	1000	0.5
	1500	0.75
	2000	1

3.2.3 Lethal Dosage, LD50

45 zebrafish were put in fish tanks and fasted for 96 hours prior to experimentation. Fish were injected intraperitoneally with 5 µl of *Dioscorea hispida* starch with concentrations of 0, 500, 1000, 1500 and 2000 mg/kg. To determine the concentration that kills 50% of the fish, the mortality rate was calculated after 24 hour gavage procedure. The formula determined the mortality rate for different concentrations was shown in equation 1

$$Mortality\ Rate\ = \frac{Total\ number\ of\ dead\ Danio\ Rerio}{Total\ number\ of\ \ population} x\ 100\% \qquad \text{(Eg.1)}$$

3.2.4 Non- Observed Adverse Effect Level (NAOEL)

The zebrafish was treated with 5 different concentrations (0, 500, 1000, 1500 and 2000 mg/kg). Each concentration contained 5 zebrafish (5 x 5 = 25 fish). The survivals were counted, and the NOAEL of each concentration was calculated, based on the mortality rate of the fish (equation 2).

$$Mortality\ Rate\ = \frac{Total\ number\ of\ dead\ Danio\ Rerio}{Total\ number\ of\ \ population} x\ 100\% \qquad \text{(Eg.2)}$$

3.2.5 Fourier Transform Infrared Spectroscopy (FTIR)

The extract contained in the starch from *Dioscorea hispida* tuber was identified using FTIR infrared spectrometer (Bruker Vector 22, Lancashire, UK) in attenuated total reflectance (ATR) mode. The potassium bromide (KBr) disc technique was used to prepare the sample. Before use, samples were dried to a consistent weight in a 50°C oven for 24 hours. The specific peaks in the range 4000-400cm^{-1} were recorded. Each spectrum was captured with 16 scans at a resolution of 4cm^{-1} and at room temperature.

3.2.6 Scanning Electron Microscope (SEM)

The identification of *D. hispida* starch was conducted through Scanning Electron Micrograph (Hitachi S-3400N, Nara, Japan) analysis. The starch sample was coated with a thin layer of conducting material to enhance conductivity, allowing electrons to efficiently reflect off the specimen and produce the desired images. Imaging was performed at magnifications of ×1000, and ×2000. The determination of the average starch granule size was carried out at a magnification of x2000 using the Cell Sens application. This process provided detailed insights into the morphological characteristics of the starch granules.

3.2.7 X-ray Diffraction (XRD)

XRD analysis of prepared sample of *Dioscorea Hispida* dried tuber powder was done by Bruker D2-Phaser Machine with an angular range from 0° to 60° (2θ). Through application Diffrac Eva version 3.2, it interprets the XRD analysis degree of crystallinity and amorphous of the sample.

3.2.8 Histopathology Studies

Histopathology studies were carried out at the Histopathology laboratory at Pengkalan Chepa, Universiti Malaysia Kelantan. To prepare the fish samples, a solution with a high concentration of tricaine mesylate (MS222) (5µl) was prepared for the purpose to make the fish insensible. The sample zebrafish were placed in the disposable microcentrifuge tube after the fish had been submerged in the fluid. Next, immediately fill in the 10% formalin buffer. 4 fish samples with different concentrations were selected and sent to the Universiti Putra Malaysia Veterinary Lab.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR was performed to identified the functional group of *D. hispida* starch at the range of 400 - 4000 cm^{-1} with a resolution of 4 cm^{-1}. The FTIR spectra for raw *Dioscorea hispida* starch were presented in Figure 4.1, revealing characteristics of *D. hispida* starch.

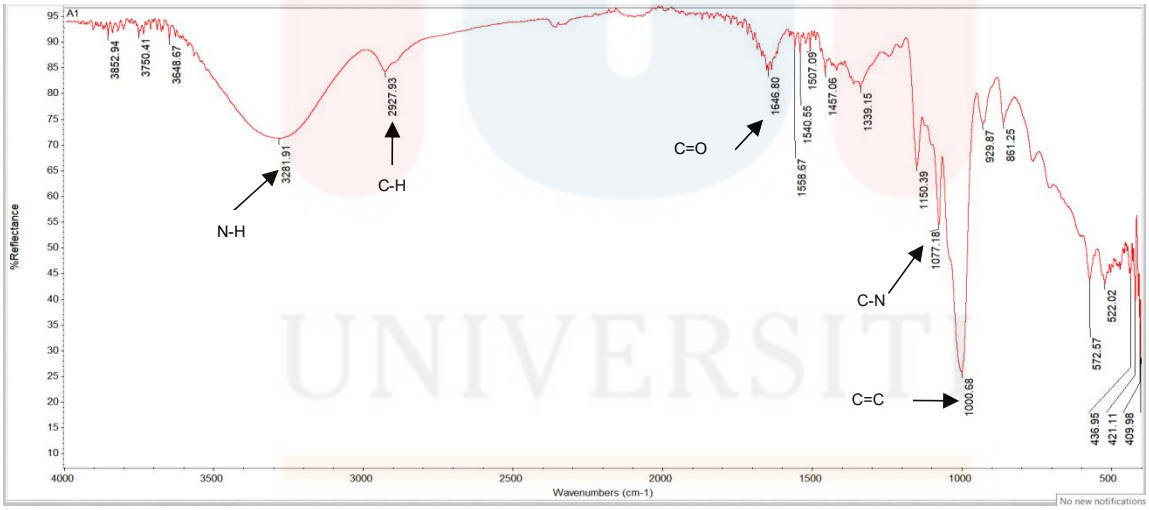

Figure 4.1: FTIR Spectrum of *D. hispida* starch

The analysis divided the curve into four main stages, each providing valuable insights into the molecular composition. In the first stage, occurring at wavenumbers below 800 cm^{-1}, functional groups associated with this lower range were examined. The second stage, spanning from 800 cm^{-1} to 1500 cm^{-1}, focused on mid-range wavenumbers, allowing for the analysis of different molecular vibrations or bonds. The spectra range between 1200 cm^{-1} - 950 cm^{-1} was related to the fingerprint

region of carbohydrates which specific for each polysaccharide. Main vibrations in this region include modes of bending and stretching of different functional groups, such C-O and C-C bonds, as well as modes of bending involving the hydrogen and carbon atoms in the molecules of carbohydrates. Specific substances, such as sugars like glucose and fructose, as well as more complex polysaccharides like cellulose, starch, and glycogen, might be found in this range. The third stage homed in on the C–H stretch area, ranging from 2800 cm^{-1} to 3000 cm^{-1}, where the stretching vibrations of carbon-hydrogen (C–H) bonds were explored. Finally, the last stage revealed intense peaks indicative of the presence of O–H groups. These hydroxyl groups were associated with the filler parts of the raw *Dioscorea hispida* starch, specifically found in cellulose, hemicellulose, and lignin. The systematic breakdown of the FTIR spectra in these stages facilitates a comprehensive interpretation of the molecular information embedded in the analyzed material.

Table 4.1 Summary of FTIR peakment of *D. hispida* starch

Wavelengths (cm^{-1})	**Compound (cm^{-1})**
1077.18	C-N stretch (aliphatic amines)
3281.91	N-H stretch (primary amines)
2927.93	C-H stretch
1000.68	C=C stretch
1646.80	C=O stretch (ester)

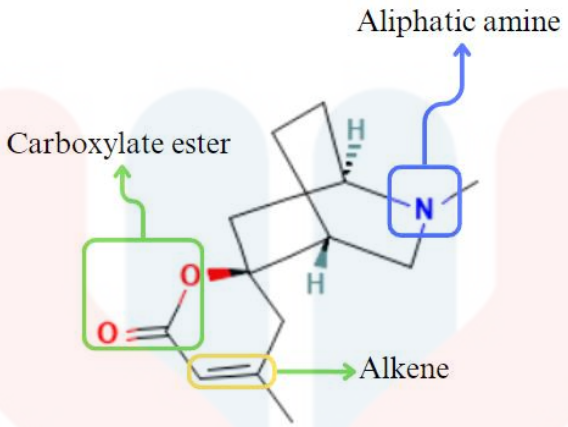

Figure 4.2 The dioscorine compound structure

4.2 Scanning Electron Microscope (SEM)

The starch exists on the *D. hispida* was examined by using SEM. The picture was taken at magnification × 1000 and × 2000. Figure 4.3 (a) and (b) show the Scanning Electron Micrograph (SEM) of *D. hispida* starch on magnification × 1000 and × 2000 respectively.

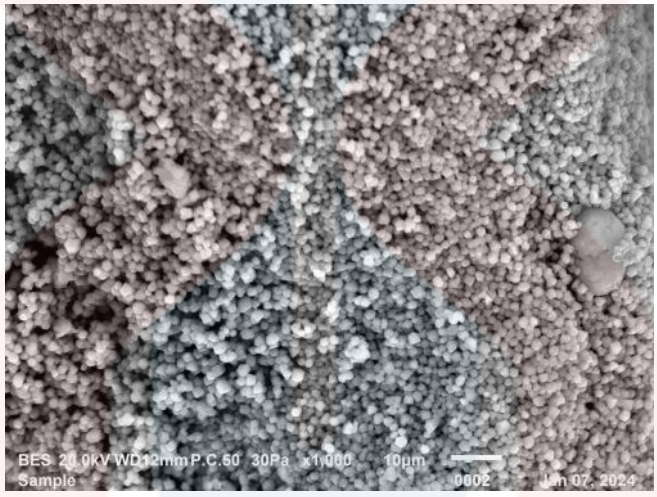

Figure 4.3 (a) Scanning Electron Micrograph (SEM) of *D. hispida* on magnification × 1000.

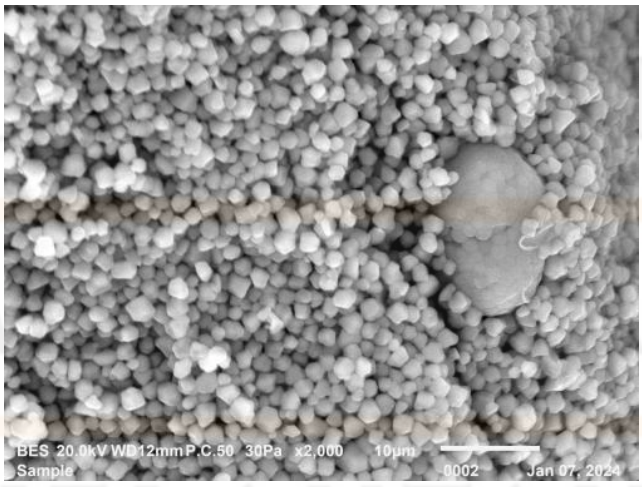

Figure 4.3 (b) Scanning Electron Micrograph (SEM) of *D. hispida* on magnification × 2000.

Scanning electron microscopy (SEM) analysis at 1000x magnification, shown in figure 4.3 (a), shows a closer look at the starch particles at a high magnification of 1000x, showing the size of the starch particles. Figure 4.3 (b) at 2000x magnification clearly shows the polyhedral shape of the starch particles. The starch granule was obtained from *D. hispida*, and the size range of the granules varied from 1.3 μm to 4.3 μm. The polyhedral shape observed in *Dioscorea hispida* starch is a unique feature and distinguishes it from other Dioscorea species (Hermiati et al., 2023). This difference is due to differences in biological sources and environmental growth conditions. In the existing literature, similar morphologies of starch granules have been reported in different Dioscorea species.

For example, Dioscorea species have three different shapes: triangular, ellipsoidal, and polyhedral (Shujun et al., 2006). Similarly, Dioscorea starch exhibited irregular shapes such as cake-shaped, oval, and crushed granules in previous studies. Toyosawa et al. (2016) investigated the biosynthesis of starch granules, which contain enzymes such as starch synthase (SS), starch branching enzyme (BE), ADP-glucose pyrophosphorylase (AGPase), and starch debranching enzyme (DPE), and elucidated the morphology of natural starch. This comprehensive study also considered plant physiological factors such as amylose content, light transmittance, swelling capacity, and water holding capacity, contributing to the overall understanding of the properties of Dioscorea starch granules.

The starch granules extracted from *Dioscorea alata*, also known as purple yam, have a round-oval form (Ochoa & Osorio-Tobón, 2023). The spherical structure was consistent with previous studies on the isolation of starch from different types of yams. Previous study by Santos et al. 2021 , the isolation of starch from purple yam *(Dioscorea trifida)* was investigated using steeping in alkaline pH (KS) and steeping in water (WS). The granule structure observed in that

study was described as round-oval and elongated. In another previous study, starch isolation from yam *(Dioscorea alata L.)* was performed using WS. The resulting starch granules were reported to have irregular elliptical and round-oval shapes (Oliveira et al., 2017).

4.3 X-ray Diffraction (XRD)

The crystallinity of *D. hispida* dried starch was examined by using XRD. Data was taken for the 2θ range 0° to 60°. Application Diffrac Eva version 3.2 use to interpret the XRD analysis degree of crystallinity and amorphous of the sample. Figure 4.1.5 show difractograms of *D. hispida*.

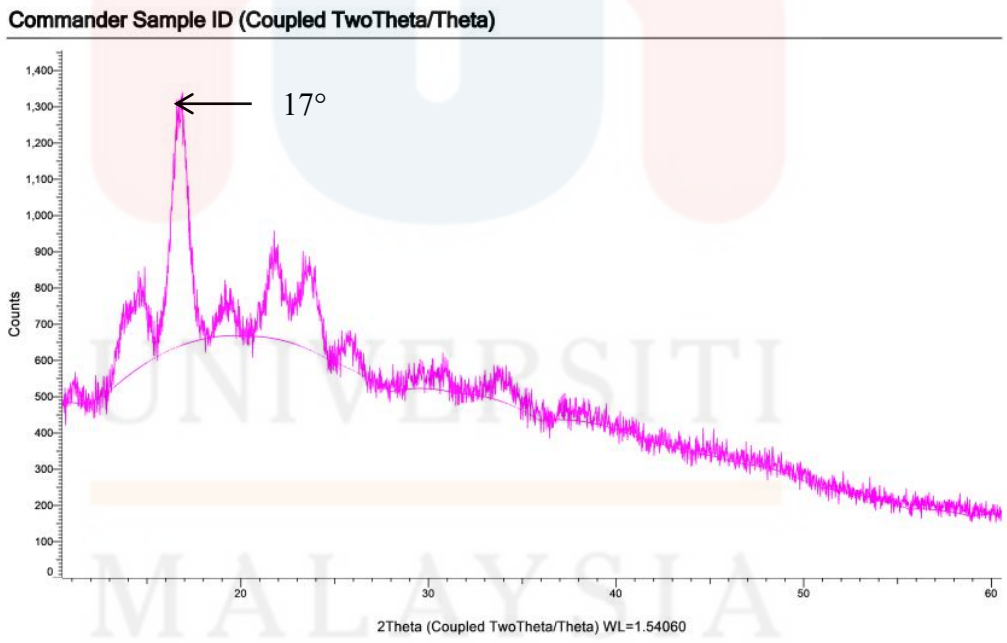

Figure 4.4 XRD difractograms of *D. hispida* starch

The phase analysis of *D. hispida* starch was conducted using the XRD (X-ray diffraction) technique. The XRD results revealed peaks at specific angles, indicating the presence of a semi-

crystalline structure known as the A-type starch semi-crystallinity. This pattern is typical for cereal starches, as noted in a study by Shamai et al. in 2003.

The diffractogram of *D. hispida* starch displayed six peaks at 2θ values of 15°, 17°, 20°, 22°, 24°, and 26°. Among these peaks, the one at 15° marked the starting point of the crystalline structure, while the peak at 17° was the highest and most prominent. Additionally, there was a flattening observed around 59°, indicating a characteristic feature of the diffraction pattern. Based on the figure 4.4 ,it can be inferred that *D. hispida* starch exhibits a semi-crystalline structure.

In a previous study by Ibrahim et al. in 2019, the XRD analysis of native corn starch showed five diffraction angles (2θ) with corresponding peaks at 15.14°, 17.4°, 18.6°, 20.11°, and 22.8°. The current study's findings align well with those reported by Paraginski et al. in 2014 for native corn starch. Paraginski et al. observed peaks at 15°, 17°, 18°, 20°, and 23°, which were effectively compatible with the results of the current study. The mentioned peaks were also largely consistent with the findings of Koo et al. in 2010, which identified peaks at 15.02°, 17.18°, and 23.73°.

4.4 Toxicity Studies

The table 4.4 showed that the total percentage of mortality rate in different concentration of *D. hispida* starch. The percentage were calculated to determine the median lethal dose and no observed adverse effect level (NOAEL). Mean mortality rate was 0%, 20%, 40%, 33.33%, 80% in the control treatment, 500, 1000, 1500, 2000 mg/kg of concentration starch and 45 zebrafish.

Table 4.4 Total mortality percentage of *Danio rerio* in different concentration of *Dioscorea hispida*

Concentration (mg/kg)	Total use zebrafish	Mortality rate (%)
0	5	0
500	5	20 (NOAEL)
1000	5	40
1500	15	33.33 (LD50)
2000	15	60

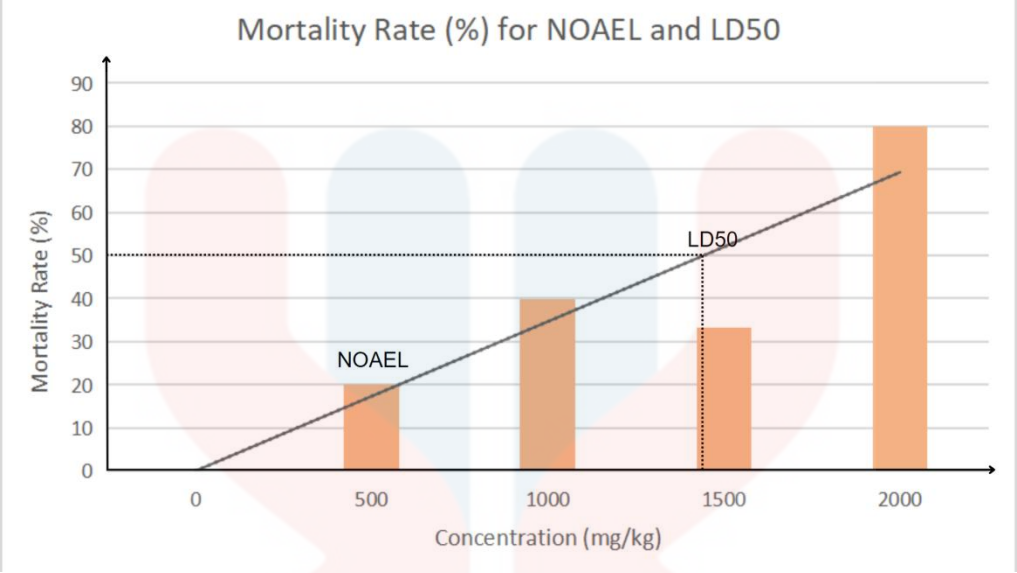

Figure 4.5 Mortality rate (%) for NOAEL and LD50

4.4.1 Non- Observed Adverse Effect Level (NAOEL)

A concentration-dependent mortality rate is evident from the result, and the determined LD50% zebrafish exposed to *Dioscorea hispida* starch. The *Dioscorea hispida* starch were gavaging for zebrafish at doses of 0, 500,1000, 1500, and 2000mg/kg for 24 hours. Therefore, the concentration-dependent toxicity of starch was highlighted by the discovery that the Non-Observed Adverse Effect Level (NOAEL) was 500mg/kg at which point only one fish out of five exhibited deaths. According to previous studies, *G. bimaculatus* has been used in determined NOAEL with doses of 0, 1000, 2000, 3000 mg/kg for four weeks. Finally, No Observed Adverse Effect Level (NOAEL) for *Gryllus bimaculatus* was 3000 mg/kg in rats (Yu et al., 2020).

Previous studies showed that in a 24-hour acute toxicity test, the No Observed Adverse Effect Level (NOAEL) and Lethal Concentration 50 (LC50) for D. rerio (zebrafish) to sodium hypochlorite (NaOCl) was determined. The test involved exposing the fish to various concentrations

of NaOCl for a specified duration. The exposure concentrations used in this study were 22.8, 28.5, 34.2, 39.0, 45.6, 51.3, and 57.0 mg/L. In the experiment, a total of 32 fish were exposed to concentrations corresponding to 40%, 30%, 20%, and 10% of the calculated LC50 concentration. Additionally, 32 fish were used as a control group and were not exposed to NaOCl. It was noted that from the 20% of the LC50 concentration onwards, swimming activity gradually decreased, indicating a dose-response relationship. However, the mean values of the 20% treated group were within the normal range of variation observed in the control group. Therefore, this concentration was considered as the No Observed Adverse Effect Level (NOAEL), implying that at this concentration, no adverse effects were observed in the swimming activity of the zebrafish (de Paiva Magalhaes et al, 2007).

4.4.2 Lethal Dosage, LD50%

This study aimed to determine the Lethal Dosage (LD50%) of *Dioscorea hispida* starch on zebrafish. The results indicate a concentration-dependent mortality rates, and the calculated LD50% value was determined to be 1500 mg/kg, 5 fish death out of 15 fish, signifying the dose at least less than 50% of the fish death to the toxic effects of starch. The observation in this study demonstrates a clear dose-dependent effect of *Dioscorea hispida* starch on zebrafish. If concentrations gradually increased, the incidence of mortality rate also increased.

Previous study by Nur-Nazifah et al., 20ll involved the experimental infection of 160 fingerlings and 80 adult tilapias with two bacterial pathogens, Streptococcus agalactiae and Staphylococcus aureus, to determine their LD50 values. Four different concentrations of Streptococcus agalactiae (10^9, 10^8, 10^7, and 10^6 CFU/mL) were used in the experiment. The fingerlings were divided into four groups, with each group consisting of 40 fingerlings. Similarly, the adult tilapias were divided into four groups, with each group consisting of 20 adults. For the fingerlings, groups 1, 2, 3, and 4 were exposed to the same concentrations of S. agalactiae, respectively, through immersion in a 2 L inoculum solution for 20 minutes. The adult groups were also exposed to the same concentrations of S. agalactiae, but through intraperitoneal injection at a rate of 1 mL of the inoculum per gram of body weight. The LD50 value for Group 1 fingerlings challenged with S. agalactiae was determined to be 2.9242×10^{20}, which was higher compared to Group 2 fingerlings challenged with S. aureus, with an LD50 value of 2.8665×10^{17}. These results indicated that S. aureus was more pathogenic compared to S. agalactiae, possibly due to the presence of S. aureus exotoxins. Group 3 fingerlings, which were challenged with both S. aureus and S. agalactiae, had an LD50 value of 4.9748×10^{11}. These findings suggest that the different

bacterial strains and their concentrations had varying pathogenicity in the fingerlings, with S. aureus being more lethal than S. agalactiae.

In the previous study, the LD50 (Lethal Dose 50) of cadmium was determined using a test that involved the intraperitoneal injection of hydrous CdCl2 in 30 male mice. The mice were randomly divided into 5 groups, with each group consisting of 5 mice. The mice in each group were injected intraperitoneally with increasing doses of cadmium (1mg/kg, 3mg/kg, 5mg/kg, 7mg/kg, and 9mg/kg) to determine the dose that would result in the death of 50% of the tested animals within a designated time frame of 24 and 48 hours. Multiple doses of cadmium were administered until the lethal dose was identified, which caused the death of half of the test mice population. A sixth group of mice served as the control group and received no treatment. The experiment was allowed to proceed for 24 and 48 hours, respectively. Based on the results, the LD50 values for cadmium at 24 and 48 hours were determined to be 5.98 mg/kg and 3.59 mg/kg, respectively. It is worth noting that no deaths were recorded among the control group, indicating that the deaths observed in the treated groups were attributable to the administration of cadmium (Hussen Ali, 2012).

4.5 Histophatology Studies

The zebrafish *(Danio rerio)* kidney is positioned within the abdomen along the vertebral column and encompasses a diverse array of tissues with multiple functions including hematopoiesis, immune response, endocrine regulation, and urinary excretion (Wolf et al., 2015). Figure 4.6 showed the morphological structure of zebrafish kidneys.

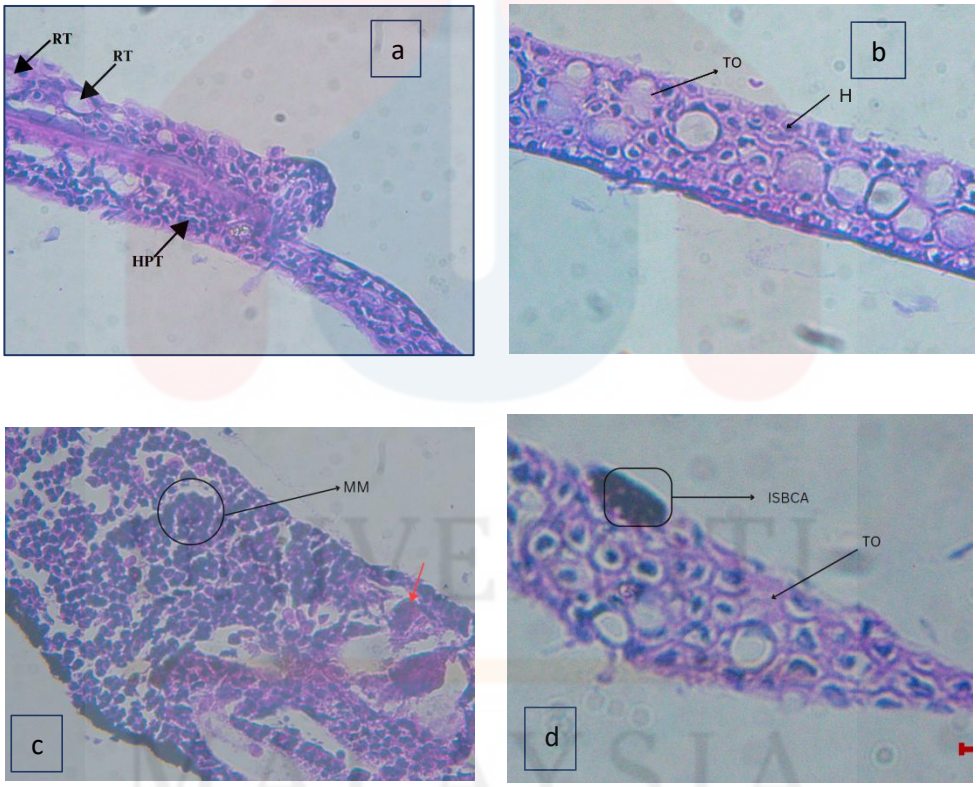

Figure 4.6 Morphological structure of zebrafish kidneys. (a) Kidney structure after injection 0mg/kg. (b) 500mg/kg. (c) 1500mg/kg. (d) 2000mg/kg.

Its primary responsibility is to eliminate excess water that enters through the mouth, while also serving as a site for waste filtration and maintenance of osmotic balance. The zebrafish kidney exhibits notable similarities to mammalian kidneys and is particularly susceptible to the effects of toxic compounds. The nephrons, the functional units of the kidney, consist of glomeruli, distal and proximal tubules, and are enveloped by the Bowman's capsule and epithelial cells. Additionally, the interstitial regions of the kidney contain hematopoietic cells, internal cells, and chromaffin cells (Dasmahapatra & Tchounwou, 2022).

In Figure 4.6 (b), (c), and (d), the kidneys of zebrafish exhibited various histological abnormalities, including lightweight, loss of cellular contour, and severe tubular hyaline degeneration, hypertrophy of tubular cells, tubular disorganization, glomerular and tubular degeneration, and increase in the space within the Bowman's capsule (ISBCA). The presence of melanoma macrophages (MM) (Figure 4.6c) was observed alongside other blood cells in the hematopoietic tissue (HPT) of the apical part of the kidney. Melanoma macrophages are phagocytic cells that contain pigments, including melanin, which gives them a brown-black appearance. When starch was injected into zebrafish, its immune system recogonizes it as a foreign and potentially hazardous substance. This causes an immunological response, resulting in the activation and migration of melano-macrophages towards the injection site. Melano-macrophages absorb the starch particles by phagocytosis, aiming to eliminate them from the fish's system (Figure 4.7). This process involves the creation of pseudopodia, which were cell membrane extensions that encircle and absorb starch particles. Melano-macrophages undergo melanization, which was the process by which they create and release melanin pigment (Wang et al., 2022).

These macrophages are likely associated with the presence or development of melanoma, a type of skin cancer characterized by the uncontrolled growth of melanocytes, the cells responsible

for producing melanin (Azar & Khoshnood). Melanin production serves several purposes, including the encapsulation of foreign particles to prevent their spread and the modulation of immune responses. The observation of melanoma macrophages within the kidney suggests potential metastasis or infiltration of melanoma cells into the renal tissue.

These findings indicate structural damage, cellular changes, and disruptions in kidney function. In contrast, the kidneys of fish in the control concentration did not show any toxicological alterations.

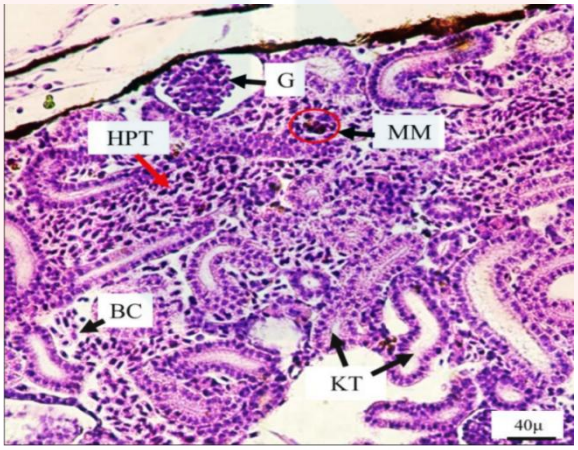

Figure 4.7 Histology of the apical part of the kidney in Zebrafish *(Danio rerio)*.

(Source: Wang et al., 2022)

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The toxicity study of *Dioscorea hispida* starch on Zebrafish *(Danio rerio)* provides insights into the potential toxic effects of this starch on the fish species. This study was to assess the impact of *D. hispida* starch exposure on mortality rates and organ function, with a specific focus on the kidneys. In this study, adult zebrafish were exposed to different concentrations of *D. hispida* starch for a designated period of 24 hours. The concentrations used (0, 500, 1000, 1500, and 2000 mg/kg) were determined based on preliminary experiments and relevant toxicity testing guidelines. The study aimed to determine the Non-Observed Adverse Effect Level (NOAEL) and the lethal dose (LD50%) of *D. hispida* starch on *Danio rerio*. To further analyze the properties of *D. hispida* starch, Fourier Transform Infrared (FTIR) Spectroscopy analysis was performed to identify the functional groups present in dioscorine found in *D. hispida*. This study also investigated the morphology and size of *D. hispida* starch using Scanning Electron Microscope (SEM). X-ray diffraction (XRD) analysis was conducted to differentiate the crystalline components present in the starch. Lastly, the study assessed the kidney effects of *D. hispida* starch exposure by examining the histology in zebrafish.

The results of this study indicate that the concentration-dependent toxicity of *D. hispida* starch was highlight by the discovery that the NOAEL was 500mg/kg on zebrafish. In addition, LD50 value was determined to be 1500 mg/kg. For the FTIR analysis showing at peak of C=O the bond between 1646.80 - 1660 cm^{-1} which was the main part of dioscrine as known as Carboxylate ester group. The starch of *D. hispida* was sucessfully characterize by SEM and XRD. SEM analysis show that the *D. hispida* starch has granule shape which was polygonal shape. XRD analysis show that confirms the crystalline nature of *D. hispida* starch. Lastly, the zebrafish *(Danio rerio)* kidney was positioned affected by *D. hispida* starch. In conclusion, the objective which was to determine the Non- Observed Adverse Effect Level (NAOEL) and lethal dose (LD50) of *Dioscorea hispida* starch using adult zebrafish *(Danio rerio)* at different dosage concentration and characterize the effect of *D. hispida* starch on zebrafish through histopathology analysis had been successfully archieved for this study.

5.2 Recommendations

First, to extract dioscorine from *D. hispida* starch, the researcher can explore different extraction methods and solvents. Additionally, it would be beneficial to investigate the effects of different drying methods and pretreatment of *D. hispida* tubers on dioscorine content and potential toxicity. Second, given that zebrafish are highly sensitive to water quality parameters, maintaining optimal water conditions is crucial during toxicity studies. The researcher should control and monitor parameters such as temperature, pH, ammonia levels, and dissolved oxygen to ensure consistent and suitable water quality for the fish during exposure to *D. hispida* starch. This approach minimizes potential confounding factors and ensures that any observed effects are primarily due to the starch exposure. Last, the researcher should carefully consider the tank size and environmental enrichment for housing the zebrafish during the study. Overcrowding can lead to stress, aggression, and compromised water quality, which may confound the interpretation of toxicity effects. Therefore, it is important to select an appropriate tank size that provides sufficient space for swimming and avoids overcrowding. Incorporating features such as plants, rocks, PVC pipes, or commercial tank decorations can offer hiding places and environmental enrichment, thereby promoting a more natural environment for the zebrafish and minimizing stress during exposure to *D. hispida* starch.

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