UNIVERSITI PUTRA MALAYSIA

HISTOPATHOLOGICAL CHANGES IN LIVER, KIDNEYS AND TESTES OF MALE ICR MICE FED ANNONA MURICATA LEAVES ETHANOLIC EXTRACT (AMLE)

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ABSTRACT

Histopathological Changes in Liver, Kidneys and Testes of Male ICR Mice Fed Annona Muricata Leaves Ethanolic Extract (AMLE)

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Introduction: Medicinal plants have been exploited for their therapeutic values throughout human history and the rapid commercialisation of their products has been increasingly observed. *Annona muricata* is an example of a plant with various medicinal benefits that have been explored throughout past decades particularly for its anti-inflammatory, anticancer, and more recently, antifertility properties. Despite this, its toxicity profile is still not well-established. especially in the aspect of histology. In particular, there are limited histopathological profiles on the liver, kidney, and testes if animals were treated with AMLE after one spermatogenic cycle. The significant needs to evaluate the possible damage in selected organs lead to the design of this work. **Objective:** This study aimed to assess the toxicity effect of feeding *Annona muricata* leaves ethanol extract (AMLE) for 35 days on the liver, kidneys, and testes of male mice via histopathological examination and evaluation of the changes in relative organ weights (ROW). **Methodology:** Twenty male ICR mice were divided into four groups; group A acts as control with distilled water whilst treatment groups B, C and D were orally administered with AMLE at dose levels of 100 mg/kg (low dose), 200 mg/kg (medium dose) and 300 mg/kg (high dose) respectively for 35 days. Treatment was terminated on day 35 and mice were euthanised the next day. The liver, kidneys, and testes were harvested and weighed. The organs were subjected to histopathological processes which includes, tissue fixation, tissue processing, tissue embedding, and tissue sectioning to

obtain slides that were stained using haematoxylin-eosin (H&E) dye before microscopical examination. Histopathological scorings were given to any histological changes observed. **Results:** From the ROW evaluation, group D (5.74 ± 0.11) showed statistically significant difference in mean liver weights compared to group A (4.18 ± 0.21). From the histopathological analyses, only two of ten parameters in the treatment groups had significance difference when compared with group A. **Discussion:** The changes in the ROW and cellular injuries observed at the tissue level might not be pathologically influenced by AMLE consumption. This is supported by the insignificant differences in the histological findings between the treatment groups and control group A. **Conclusion:** Findings from this study suggest that feeding AMLE for 35 days at the mentioned dosages does not seem to induce toxicity in the liver, kidney, and testes in male mice. However, further toxicity assessment such as chronic studies of AMLE should be conducted to endorse prolonged consumption.

Keywords: Annona muricata leaves ethanol extract (AMLE), Histopathology, Liver, Kidney, Testes

ABSTRAK

Perubahan Histopatologi Hati, Ginjal dan Testis Mencit Jantan ICR yang Diberikan Ekstrak Etanol Daun *Annona Muricata* (AMLE)

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Pengenalan: Annona muricata adalah contoh tumbuhan dengan pelbagai khasiat perubatan yang telah dijelajahi sepanjang dekad yang lalu terutamanya kerana sifat anti-radang, antikanker, dan baru-baru ini, sifat antifertilitasnya. Walaupun begitu, profil ketoksikannya masih belum menyeluruhi; terutamanya dalam aspek histologi. Khususnya, terdapat profil histopatologi yang terhad pada hati, ginjal, dan testis jika haiwan diberi rawatan dengan AMLE sepanjang satu siklus spermatogenik. Keperluan penting untuk menilai kemungkinan kerosakan pada organ terpilih membawa kepada reka bentuk kajian ini. **Objektif:** Kajian ini bertujuan untuk menilai kesan ketoksikan memberi makan ekstrak etanol daun *Annona muricata* (AMLE) selama 35 hari pada hati, ginjal, dan testis tikus jantan melalui pemeriksaan histopatologi dan penilaian perubahan berat organ relatif (ROW). **Metodologi:** Dua puluh mencit jantan telah dibahagikan kepada empat kumpulan; kumpulan A (kumpulan kawalan) dengan air suling sementara kumpulan B, C dan D diberi AMLE pada tahap dos 100 mg / kg (dos rendah), 200 mg / kg (dos sederhana) dan 300 mg / kg (dos tinggi) selama 35 hari. Semua rawatan ditamatkan pada hari ke-35, dan mencit dimatikan keesokan harinya. Lalu, hati, ginjal, dan testis mencit ditimbang dan dijalani proses histopatologi yang melibatkan penetapan tisu, pemprosesan tisu, penyisipan tisu dan pemotongan tisu untuk mendapatkan slaid yang diwarnakan dengan perwarnaan haematoxylin-eosin sebelum pemeriksaan mikroskopik. Permarkahan histopatologi diberi untuk perubahan histologi yang diperhatikan bawah mikroskop. **Keputusan:** Dari penilaian ROW, kumpulan D (5.74 ± 0.11) menunjukkan perbezaan

yang signifikan secara statistik dalam min berat hati berbanding kumpulan A (4.18 ± 0.21). Dari analisis histopatologi, hanya dua dari sepuluh parameter dalam kumpulan rawatan yang mempunyai perbezaan yang signifikan jika dibandingkan dengan kumpulan A **Perbincangan**: Perubahan dalam ROW dan kecederaan sel yang diperhatikan pada tahap tisu mungkin tidak dipengaruhi secara patologi oleh penggunaan AMLE. Ini disokong oleh perbezaan yang tidak signifikan dalam penemuan histologi antara kumpulan rawatan dan kumpulan kawalan A. **Kesimpulan**: Penemuan dari kajian ini menunjukkan bahawa memberi makan AMLE selama 35 hari pada dos yang disebutkan sepertinya tidak menyebabkan keracunan pada hati, ginjal, dan testis pada tikus jantan. Walau bagaimanapun, penilaian ketoksikan lebih lanjut seperti kajian kronik AMLE harus dilakukan untuk menyokong penggunaan yang berpanjangan.

Kata kunci: Ekstrak etanol daun *Annona Muricata* (AMLE), Histopatologi, Hati, Ginjal, Testis

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µm	Micrometre or micron
AMLE	*Annona muricata* ethanolic leaf extract
AMMBE	*Annona muricata* methanolic bark extract
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BTB	Blood-testis barrier
DPX	Dibutyl Phthalate Xylene
EGFR	Epidermal Growth Factor Receptor
g	Gram
H&E	Haematoxylin and Eosin
IACUC	Institutional Animal Care and Use Committee
ICR	Institute of Cancer Research
ITIS	Integrated Taxonomic Information System
kg	Kilogram
LD_{50}	Median lethal dose
mL	Millilitre
RITA	Registry of Industrial Toxicology Animal-data

ROS	Reactive Oxygen Species
ROW	Relative Organ Weights
SEM	Standard Error of Mean
SPSS	Statistical Package for the Social Sciences
PMN	Polymorphonuclear cells
WHO	World Health Organization
β	Beta

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Medicinal plants have been researched for their promising therapeutic potentials to alleviate many human health challenges. Rapid commercialisation of herbal medicinal products as dietary supplements, non-prescriptive drugs, and home remedies has surge in recent years due to the increase public interest and marketability (Ekor, 2014). According to WHO (2002), market growth raises concerns regarding the safety and tolerability of herbal medicines imploring toxicological studies to be conducted. This corroborates with the key findings by WHO which discuss the increasing progress in the implementation of legislative and regulatory actions for traditional and complementary medicines (WHO, 2019).

Over the past three decades, soursop or *Annona muricata* has been gaining traction due to its bioactivity. In particular, numerous studies on the consumption of A. *muricata* leaves extracts reveal its diverse medicinal benefits.

A. *muricata* is a species under the Annonaceae family whose therapeutic potentials were described in the previous century (Baillon, 1869 as cited by Coria-Téllez et. al., (2018). Ethnobotanical studies disclosed the many uses of A. *muricata* which include treating fever, gastrointestinal and respiratory conditions, and malaria. Recent discoveries also highlight its potential in alleviating hypoglycaemia, hypertension, and as a treatment for cancer (Coria-Téllez et al., 2018b). These therapeutic properties are due to the plants' main bioactive constituent; acetogenins, alkaloids, and phenols (Rady et al., 2018).

Despite the considerable number of studies conducted on its pharmacological activities, the toxicity profile of A. *muricata* is still not well-established. The few toxicological studies conducted mainly underline the acute toxicity and neurotoxicity of A. *muricata*. Annonacin, a member of acetogenins, was identified to be neurotoxic by depleting the adenosine triphosphate (ATP) in brains (Champy et al., 2004; Escobar-Khondiker et al., 2007; Höllerhage et al., 2015; Lannuzel et al., 2003). As neurons require a high ATP level for metabolic demand, the potential for acetogenins to cause toxicity in other organs highly dependent on ATP availability such as the liver and kidneys, also require further analysis (El Bacha, 2010; Ramalho-Santos et al., 2009). Therefore, this study was conducted to evaluate the toxicity effects of feeding A. *muricata* leaves on the liver, kidneys, and testes of male mice via histopathological assessment and relative organ weight (ROW) calculations.

1.2 Problem Statement

A. *muricata* leaves which have been known for their many therapeutic values have an ambiguous toxicity profile yet to be explored. In particular, there is limited histological data on the effects of *Annona muricata* leaves ethanolic extract (AMLE) on the organs of metabolism that includes the liver and kidney (Chan et al., 2020). Furthermore, there is also no reported research on the possible histopathological changes in the testes of male mice after one spermatogenic cycle of 35 days given its potential anti-spermatogenic properties. One spermatogenic cycle was chosen to observe changes in spermatogenesis (from spermatogonia right up to the spermatozoa level) effects. Simultaneously, 35 days should suffice to observe possible histopathological changes in the liver and kidney as research reported before was conducted in a shorter period. Hence, characterisation of the possible tissue damages to the liver, kidneys, and testes should be carried out to discern the implication of consuming A. *muricata* leaves.

1.3 Objectives

1.3.1 General Objective

To assess the toxicity effect of feeding *Annona muricata* leaves ethanol extract (AMLE) on the liver, kidneys, and testes of male mice.

1.3.2 Specific Objectives

i. To evaluate the changes in relative organ weights in male mice after treatment of AMLE for one spermatogenic cycle.

ii. To identify the changes in tissue architecture on the liver, kidney, and testes of male mice after treatment of AMLE for one spermatogenic cycle via histopathological examination.

1.4 Research Questions

The research questions for this study include:

i. Does the treatment of AMLE cause changes in the relative organs weight in male mice?

ii. Does the treatment of AMLE cause histological changes in the liver, kidneys, and testes of male mice?

1.5 Hypothesis

It is hypothesized that there are no significant changes in the relative organ weights and histology of liver, kidney, and testes of AMLE-treated mice of dosage 100,200, and 300 mg/kg after 35 days of treatment.

1.6 Significance of the Study

The utilisation of herbal medicines as the choice of standard medical care showed an increase in its use in approximately 80% of the world population (Bodeker & Ong, 2005, as cited in Ekor, 2014). A. *muricata* leaves have diverse therapeutic values ranging from anti-inflammatory, anti-spermatogenic, anticancer and more which can be exploited for commercialisation (Chan et al., 2020; Oladipo et al., 2018). However, like any other drugs, there are potential harms that come with the therapeutic benefits. The findings of this study can contribute to the understanding of the possible toxicity effects of AMLE at the tissue level in mice.

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CHAPTER 2

LITERATURE REVIEW

2.1 *Annona muricata*

Annona muricata is an evergreen plant that is indigenous to the Central and South America and has since been cultivated throughout the tropics of Africa, Southeast Asia, and Pacific islands. This species which is known for its unique sweet and sour tasting fruits has many common names native to each country. In the English-speaking world, A. *muricata* is known as soursop whilst in Malaysia, it is known as 'durian belanda', 'durian maki', or durian benggala' to name a few. The plant is also recognised by names such as sasalapa (Samoa), sirsak (Indonesia), corossolier (French-speaking countries), graviola (Caribbean), and guayabano (Cambodia) (Badrie & Schauss, 2010). The taxonomic tree of this plant begins with its kingdom Plantae, followed by division Tracheophyta, class Magnoliopsida, order Magnoliales, family Annonaceae, genus *Annona* L, and species *Annona muricata* L. (ITIS, 2021) (Integrated Taxonomic Information System)

Figure 2.1 shows the tree of this plant which is about 5-10 metre tall and 15-83 cm in diameter and produces edible heart-shaped fruits. The dark green fruits

are made up of fusion of berries that creates creamy white flesh with 5-200 black seeds within (Badrie & Schauss, 2010; Morton, 1987).

Figure 2.1. Photographs of a soursop tree with the cross-section of its fruit. Adapted from Badrie & Schauss (2010) and (Adcox, 2017) (https://www.medicalnewstoday.com/articles/319720)

2.2 Ethnomedical Uses

The plant A. *muricata* is very versatile where its seed, fruit, leaf, root, or bark were reported to treat various ailments across the globe. Conventionally, the parts of the plant are prepared as a decoction and are applied in a variety of manner for medicinal gain. Table 2.1 enlists some of the traditional usage of this

species according to countries. In Malaysia, the decoctions of A. muricata leaves were used to topically remedy external parasites. Similarly, Boulogne et al. (2011) reported that the decoction of leaves is also topically applied to heal skin problems in Indonesia and the Pacific Islands and acts as local analgesic in Mauritius, Ecuador, and New Guinea. The leaves have also been observed for its use in treating cancer, malaria, diabetes, insomnia, hypertension, and headache (Coria-Téllez et al., 2018).

The fruits of A. *muricata* are also commonly digested to treat against internal parasites, rheumatism, diarrhoea, cancer, heart, and liver diseases. In addition, the seeds, leaves, and young fruits are utilised as an insect repellent, pesticides, and insecticides. Besides these few examples, there are still many ethnomedical uses that are yet to be documented (Abdul Wahab et al., 2018; Gavamukulya, Wamunyokoli, & El-Shemy, 2017).

Table 2.1. Ethnomedical uses of *Annona muricata* L.

Country or region	Medicinal uses	Parts of plant	Application	References
Cameroon	Malaria, antimicrobial, anthelmintic	Leaves	Oral	(Roger, Pierre-Marie, Igor, & Patrick, 2015)
Caribbean	Flu, indigestions, skin ailments, nerves, rash, palpitations	Leaves Bark	Not reported	(Boulogne et al., 2011)
Ecuador	Rheumatism	Leaves	Topical	(Tene et al., 2007)
Indonesia	Insecticide, dermatitis, malaria	Leaves	Not reported	(Badrie & Schauss, 2010)
Malaysia	Lice, hypertension, stomachache	Leaves Fruit	Oral	
Madagascar	Palpitations, malaria, liver disease	Leaves	Decoction	(Novy, 1997)
Nigeria	Prostate cancer, diabetes, rheumatism	Leaves Unripe fruit	Oral	(Pinto et al., 2005)
The Philippines	Lice, cancer, anthelmintic, hypertension, headache, diabetes	Leaves Fruits	Oral Decoction	(Badrie & Schauss, 2010; Langenberger et al., 2009)
Peru	Gastritis, diabetes, inflammation, cancer, flu, nerves	Leaves Fruits	Oral	(Badrie & Schauss, 2010; Bussmann et al., 2010)
Togo	Diabetes, malaria, hypertension	Leaves	Oral	(Ross, 2007)

2.3 Phytochemical constituents

As of 2017, there have been a total of 212 bioactive compounds isolated from various phytochemical studies. These studies usually employed the leaves of the plant as they are the parts of A. *muricata* commonly reported for ethnomedicine.

The compounds identified are acetogenins, alkaloids, phenolic compounds, and others. Acetogenins are the most predominant compounds found in A. *muricata* with over 120 acetogenins isolated from the bark, leaves, pulp, seeds, and stems via ethanolic, methanolic, or other organic extraction. The compounds under this group have long aliphatic amino acid chain of 35-38 carbons as seen in Figure 2.2 depicting its characteristic bond with a γ-lactone α ring, determined number of oxygen groups, 1 or 2 tetrahydrofurans and a terminal substitution with β-unsaturated methyl. Some studies considered that acetogenins and alkaloids from A. *muricata* to have both therapeutic and cytotoxic potential (Abdul Wahab et al., 2018).

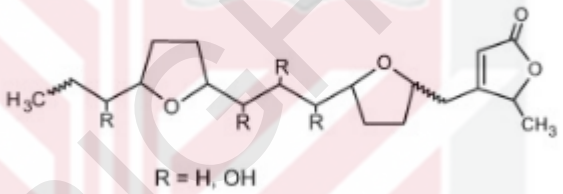


Figure 2.2. Chemical structures of a type of acetogenins found in *Annona muricata*. Adapted from (Coria-Téllez et al., 2018b).

There have been over 22 alkaloids isolated and identified in A. *muricata* with reticuline and coreximine being the most abundant. This naturally occurring compounds which are more commonly found in the leaves are derived from the transamination process and a previous in vitro study by Hasrat et al (1997) reported its affinity with the 5-HT1A receptors involved in the biosynthesis of dopamine. Thus, the alkaloids from this plant may induce stimulatory effects. A total of 37 phenolic compounds were also reported to be found in A. *muricata* with quercetin and gallic acid as the more prevalent. This group is considered as the main contributor to the plants' antioxidative property. Other reported

compounds also include megastifmanes, carotenoids, vitamins, volatile compounds, and essential oils. (Coria-Téllez et al., 2018b; Gavamukulya et al., 2017).

2.4 Pharmacological activities

There are numerous in vitro and in vivo pharmacological studies conducted to verify the biological activities of A. *muricata* over the past decades. These studies have demonstrated that the plant has anticancer, anti-inflammatory, antioxidant, anti-nociceptive, hypotensive, hypoglycaemic properties among others.

The ethanolic extract (100 mg/kg) and ethyl acetate (500 mg/kg) extract of A. *muricata* leaves have reported chemopreventive activities where they restore the colon total protein and inhibit the formation of lesions respectively in rats' colorectal carcinogenesis studies (Okolie, Agu, & Eze, 2013; Zorofchian Moghadamtousi et al., 2015). The extracts were reported to reduce the formation of malondialdehyde which is a radical commonly observed in colorectal cancer. Soursop also showed positive antitumorigenic activities where the aqueous and ethanolic extract (30-200 mg/kg) inhibit the growth of breast tumour (Dai et al., 2011), pancreatic tumour (Torres et al., 2012), and skin tumour (Hamizah et al., 2012). The mechanism behind this was suggested to be a combination of inhibitory signals to the metabolic, metastasis, cell cycle arrest, and necrosis induction pathways such as the downregulation of EGFR signalling

(Gavamukulya et al., 2017). Furthermore, the leaves' ethanolic and aqueous extracts also exhibited anti-inflammatory activities in rodents where they significantly reduce the induced oedema in dosage of 400 mg/kg and 1.5 mg/kg respectively. The activities are reported to be stemmed from arachidonic acid metabolites which are heavily involved in the inflammatory pathway similar to indomethacin (Poma, Requis, Gordillo, & Fuertes, 2011). Recently, A. *muricata* was also explored for its anti-spermatogenic activity by (Oladipo et al., 2018) where they concluded that the methanolic bark extract significantly lowers the sperm concentration and viability as well as affecting the morphology of sperm.

2.5 Toxicity

There are few data on the toxicity of *Annona muricata* where the studies mostly reported on the acute toxicity and the neurotoxicity of the plant (Chan et al., 2020). According to de Sousa et al (2010), the methanolic and ethanolic extracts of the leaves, flowers and pulp of the plant evaluated an oral LD_{50} of 1670 mg/kg whereas Florence et al. (2014) observed an oral LD_{50} of greater than 5000 mg/kg in aqueous extract. These values are considered to be non-toxic in accordance with the Organisation for Economic Co-operation and Development (OECD) guidelines as more than 71 cups daily needed to be consumed to reach lethality. A 1999 case study in Guadeloupe and London (New Caledonian and Caribbean patients) discovered a correlation between atypical Parkinsonism and A. *muricata*. This prompted murine studies on the isolated compounds of A. *muricata* and their capacity to induce neurotoxicity. Champy et al. (2004) reported that annonacin (a member of acetogenins) and reticuline (a member of

alkaloids) are neurotoxic and that annonacin is 100 times more toxic than 1-methyl-4-phenylpyridinium, a known neurotoxin causing parkinsonism in human and animals. However, the study also reported that the compounds of interest have low bioavailability and long-term exposure is needed for neurotoxicity to occur. The proposed mechanism behind the neurotoxicity observed are that annonacin cross the blood-brain barrier, enters the brain parenchyma, and seemed to inhibit the mitochondrial complex I enzyme which reduces ATP. The depletion of ATP contributes to neurodegeneration due to the plasma membrane disruption and mitochondrial damage caused by the increase of ROS (Escobar-Khondiker et al., 2007; Lannuzel et al., 2003; Nweke et al., 2019). Thus, it is hypothesized that the toxic compounds can also cross the blood-testis barrier leading to anti-fertility study by Oladipo et al. (2018) and the hypothesis of this study.

Toxicity in organs were reported in several studies of A. *muricata* leaves which are summarised in Table 2.2. Studies conducted by Utomo, Susilaningsih, & Armalina (2016), Bitar et al (2017), and Ekere, Ogunka-Nnoka, and Monago-Ighorodje (2019) observed normal histology of the mice hepatic and renal tissues after treatment with ethanolic and aqueous extracts. Interestingly in Ekere et al. (2019), the co-administration with zidovudine, an anticancer drug, showed an increase in the liver and kidney biochemical parameters. This indicates the imitation of hepatoprotective and nephroprotective properties of A. *muricata* owing to the many antioxidative and anti-inflammatory compounds (Akinnawo et al., 2005). These phenomenon can also be seen in Bitar et al.

(2017), where the co-administration of the aspirin improves the renal and hepatic tissue architectures. However, it was also reported that the liver glycogen was depleted after 3 days of treatment. This is indicative of the antidiabetic activity of A. *muricata* where the consumption can cause an increase in energy expenditure.

Arthur et al. (2011), EzejinduD. et al.(2015) and Sherif, Baba, and Abdullahi (2017) also reported similar findings but with anomalies at the higher concentrations of the extracts. At 250-1000 mg/kg, the tissues began to show mild histopathological changes (Sherif et al., 2017). EzejinduD. et al. (2015) reported mild hepatic histopathological changes in hepatic tissue at 0.4 and 0.6 mL concentrations. Arthur et al. (2011) reported that body and organ weights showed a decline at 1000 and 2500 mg/kg dose with significance changes in the albumin and creatinine readings indicating impaired renal function.

The above findings are contradicted by Nweke et al. (2019) who reported that there was significant ROW and histopathological changes occurring at a dose-dependent manner (100,200, and 300 mg/kg of AMLE). The explanation given by this study is similar to the neurotoxicity studies where brain and liver were dependent upon ATP availability

Table 2.2. Toxicity studies of *Annona muricata* leaves

Solvent	Dose	Test model	Results	References
Aqueous	500-2000 mg/kg/7 days	Mice	No changes in behaviour and normal histology of liver and kidney	(Utomo et al., 2016)
Aqueous	100-2500 mg/kg/7 days	Mice	No behavioural, haematologic, biochemical, gastrointestinal, changes in liver and kidney. Significant changes in the albumin and creatinine at 1000 mg/kg.	(Arthur et al., 2011)
Ethanol	0.2-0.6 mL/28 days	Rats	Mild histopathology changes of the liver at 0.4 mL and 0.6 mL	(EzejinduD. et al., 2015)
Methanol	10-1000 mg/kg/28 days	Rats	No significant changes in the ROW, biochemical and histological parameters in kidney and liver. Mild ballooning degeneration seen in hepatic tissues at 250-1000 mg/kg. Mild lymphocytic infiltrate in renal tissues at 500-1000 mg/kg.	(Sherif et al., 2017)
Ethanol	100-300 mg/kg/30 days	Mice	Increase in liver biochemical parameters. Dose-dependent changes in hepatic architecture.	(Nweke et al., 2019)
Ethanol	4.5 g/mL/42 days	Mice	Normal ROW and histology of liver and kidney Increase in liver and kidney biochemical parameters when co-administered with 100 mg/kg zidovudine	(Ekere et al., 2019)
Aqueous	100 mg/kg/3 days	Mice	Depleted liver glycogen. Mild renal and hepatic histopathology changes but shows normal histology when co-administered with aspirin Preliminary study with 600 mg/kg over 2 days observes lethality.	(Bitar et al., 2017)

2.6 Organs of Interest in Mice

The histology and physiology of liver, kidney, and testes in mice are similar to other mammals including humans. Thus, making them a suitable candidate for a histopathology study. Liver and kidney are chief metabolic organs with important physiological functions. Liver is primarily involved in the storing and releasing of nutrients as well as neutralising and eliminating toxic substances. The histological organisation in mice have similarities with other mammals with polygonal lobules as their functional unit consisting of cells and portal triad surrounding central venules. The liver is composed of different cells' populations. These include the hepatocytes, parenchymal cells that secretes bile and manufactures various proteins and lipids for export and innate immunity. The hepatocytes are non-stratified cuboidal epithelium with prominent nuclei. Additionally, the population also include resident cells such as Kupffer cells, stellate cells, and endothelial cells. The Kupffer cells and endothelial serves as defensive mechanism against bacteria, virus, and toxins with the latter also aiding in metabolic activities. Stellate cells store fats and heals injury, however prone injuries to the liver have the cells contributing to fibrosis instead (Baratta et al., 2009). Table 2.3 highlights the possible damages if the liver were to be affected by injurious agents.

Kidneys are bilateral organs that regulate the constituents and volume of the body fluids. The functional unit of the kidneys are the nephrons which composes of glomerulus (simple squamous epithelium) and tubules (simple cuboidal

epithelium). The former is also known as podocytes, specialised cells that enables the kidney to filter wastes and toxin for excretion. The latter functions to reabsorb the filtrate to maintain the optimal electrolyte balance. (Davidson, 2009). Table 2.3 highlights the possible damages if the liver were to be affected by injurious agents.

Testes were chosen in this study because a recent study by Oladipo et al (2018) on the spermatogenic activity of *Annona muricata* methanolic bark extract (AMMBE) revealed lesions in the testes of albino rats. This is also in conjunction with the previous study done by Raja Muhammad Iqbal which focused on antifertility effects of AMLE in mice which showed decreasing quality of sperm and libido after treatment over one spermatogenic cycle. As this is the continuation of said study, it can help determine whether the antifertility effects observed also resulted in toxicity. These studies in rodents can contribute to understanding the effects of consuming A. *muricata* towards male reproductive health.

Testes are composed of Sertoli, and germ cells packaged inside many seminiferous tubules separated by blood-testis barriers containing Leydig cells. These are required to fulfil the reproductive process of spermatogenesis which in mice takes 34.5 days or one spermatogenic cycle as seen in Figure 2.3 (Oakberg, 1956). Spermatogenesis is described when spermatogonia derived from primordial germ cells travel from the basal membrane to the lumen. These

cells will eventually develop into mature spermatozoon (spermiation) via strict multistage process of mitosis, meiosis, and differentiation. This can be seen in Figure 2.4 The Sertoli cells will act to support this process by secreting nourishing substances, repairing DNA, and forming BTB. The Leydig cells drives the process by creating the optimal environment via secretion of hormones (androgen, testosterone) (Xie et al., 2014). Any pathological changes caused by bacteria, virus, or toxins may result in impaired spermatogenesis and histological changes seen in Table 2.3. Accumulated injuries to the cells of liver, kidneys, and testes will result in numerous cell deaths thus hindering their many functions which are vital for survival.

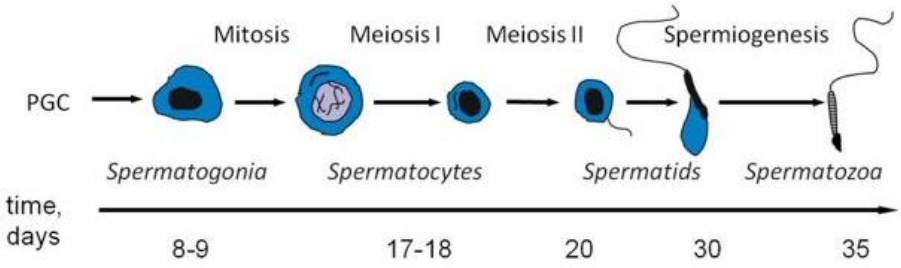


Figure 2.3. The time taken for one spermatogenic cycle in mice. Adapted from Margolin et al (2014).

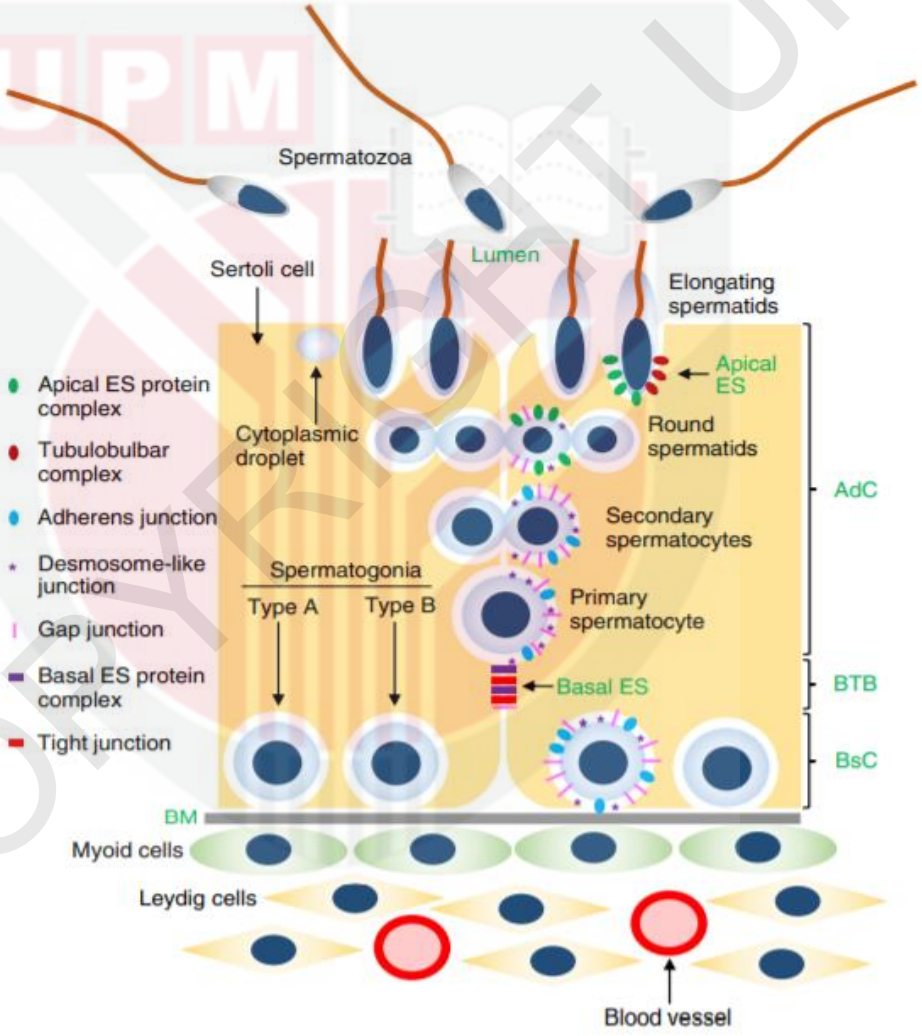


Figure 2.4. The process of spermatogenesis in mammals. Adapted from Nishimura & L'Hernault (2017).

2.8 Histopathology Examination

Histopathological examination is a tool to study the signs of a disease, or signs of toxicity. This is achieved by microscopically examining, identifying, interpreting, and grading of processed tissue sections that are fixed onto glass slides. To aid this process, the processed tissue sections are usually stained to distinguish the different cellular components. A routine stain used to discriminate between the nuclei and cytoplasm of cells is the Haematoxylin-Eosin staining (Gurcan et al., 2009). The grading of the tissue architectures can then be used to compare between normal (healthy) and abnormal tissues (diseased). The graded lesions can then be diagnosed. With the accompaniment of gross macroscopic observations, ROW evaluation, biochemical and haematological parameters, the conclusion of a study can be drawn.

According to Ruehl-Fehlert et al. (2003), the organs of interest subjected to histopathology examination of a toxicology study varies according to the objectives of the study. There are no guidelines that highlight certain organs are more susceptible to the toxic effects of a drug. However, organs such as the heart, brain, liver, kidneys, adrenal glands, and testes are recommended to be assessed for its toxicity (Sellers et al., 2007). Each organ is also assessed by its histological parameters or lesions that are chosen by previous related studies and recommendations. These are stated in Table 2.3.

Table 2.3. Diagnostic parameters of selected organs in a toxicity study.

Organs	Diagnostic Parameters	References
Liver	Interstitial oedema Sinusoid congestion Hepatocellular necrosis and apoptosis Hepatocyte vacuolation Polymorphonuclear cells infiltration Glycogen accumulation Ballooning degeneration Hepatocellular hypertrophy and hyperplasia Fatty changes Acidophil bodies	(Kubiak et al., 2010; Society of Toxicologic Pathology, 2021; National Toxicology Program, 2014)
Kidneys	Interstitial oedema (oedematous vessel cuffs, peritubular oedema, lymphatic dilation) Epithelial changes (paracellular spaces, brush border loss, tubular dilation and apoptosis) Tubular degeneration (tubular necrosis, epithelium slough, loss of tubular and cellular architecture, detached basal membrane) Capillary congestion Polymorphonuclear cells infiltration	
Testes	Tubular vacuolation Breakdown of blood-testis barrier Fibrosis Necrosis Tubular dilation Germ cell atrophy and degeneration	

CHAPTER 3

MATERIALS AND METHOD

3.1 Ethics Consideration

The examination and confirmation of the research, as well as the study protocol of animal ethics, were approved by the Institutional Animal Care and Use Committee (IACUC) and adhere strictly to conduct animal experiments in Universiti Putra Malaysia (UPM). The referral number is UPM/IACUC/AUP-R068/2014. The AMLE extract is a gift from Associate Professor Dr. Roslida Abdul Hamid.

3.2 Animals

Twenty sexually matured male mice of the ICR strain weighing 30-40 g were used in this experiment. For the duration of the study, the mice were acclimatised beforehand and accommodated with clean standard mice cages bedded with pine shavings. They were kept in a 12-hour light and dark cycle in a colony room and allocated with water and food *ad libitum*.

3.3 Parameters Studied

The parameters that are taken into consideration for this study includes the relative weights of the harvested organs (ROW) (see Appendix A for raw data of body weights, organ weights, and calculated ROW).

$$\text{Relative organ weight} = \left(\frac{Organ\ weight}{Body\ weight}\ x\ 100 \right)$$

In addition, each organ has a distinguished list of histological parameters that are examined under a microscope. For the liver, polymorphonuclear (PMN) infiltration, collagen deposition, ballooning hepatocytes, and the presence of acidophil bodies can indicate toxicity. Toxicity in kidneys can be marked by PMN infiltration, tubular necrosis, and interstitial oedema. Testes toxicity is marked by the absence of sperm cells, tubule vacuolation and breakdown of blood-testis barrier (BTB) in the seminiferous tubules.

3.4 Experimental Design

The twenty male mice were randomly and equally divided into four groups, each consisting of 5 mice. Group A which was given distilled water acts as the control group. The treatment groups B, C and D were administered with AMLE at dose levels of 100 mg/kg, 200 mg/kg, and 300 mg/kg respectively for 35 days or 1 spermatogenic cycle as seen in Figure 3.1 (Bitar et al., 2017; Ezejindu et

al., 2015; Nweke et al., 2019). These treatments were administered orally via oral gavage. These doses were also chosen with the consideration of the index LD_{50} of AMLE that was determined to be 1670 mg/kg (de Sousa et al., 2010). The treatments were terminated on day 35 and after 24 hours, the mice were euthanised via chloroform inhalation for 3 to 5 minutes. The dissection performed is followed by the excision, weighing, and fixation of the livers, kidneys, and testes in 10% formaldehyde solution for histological analysis

Treatment Protocol			
Control Group A	**Distilled water**	**Oral Gavage**	**Standard laboratory conditions (light, temperature, humidity, *ad libitum* food and water)**
Treatment Group B	**100 mg/kg/day AMLE**		
Treatment Group C	**200 mg/kg/day AMLE**		
Treatment Group D	**300 mg/kg/day AMLE**		
35 days period			

Figure 3.1. The treatment protocol of the twenty mice that were equally divided into four groups.

3.5 Histopathological Examination

The excised organs were histologically examined by the method described by (Slaoui and Fiette (2011). The tissues of interest underwent fixation, trimming, pre-embedding, embedding, sectioning, staining, and mounting before they can be examined under a microscope as summarised in Figure 3.2. Firstly, fixation

of the organs was attained by immersing them in 10% neutral-buffered formaldehyde to avoid autolysis. The fixed tissues now can be trimmed adequately to acquire the correct size and orientation per the standardised rules from RITA (Registry of Industrial Toxicology Animal-data). The trimming of each organ is explicitly described and is suitable for toxicological purposes (Ruehl-Fehlert et al., 2003). The trimmed tissues were placed in plastic cassettes in a fixative container. Pre-embedding of the trimmed tissues were then run overnight with the aid of Leica TP1020 Automatic Benchtop Tissue Processor as seen in Figure 3.3.

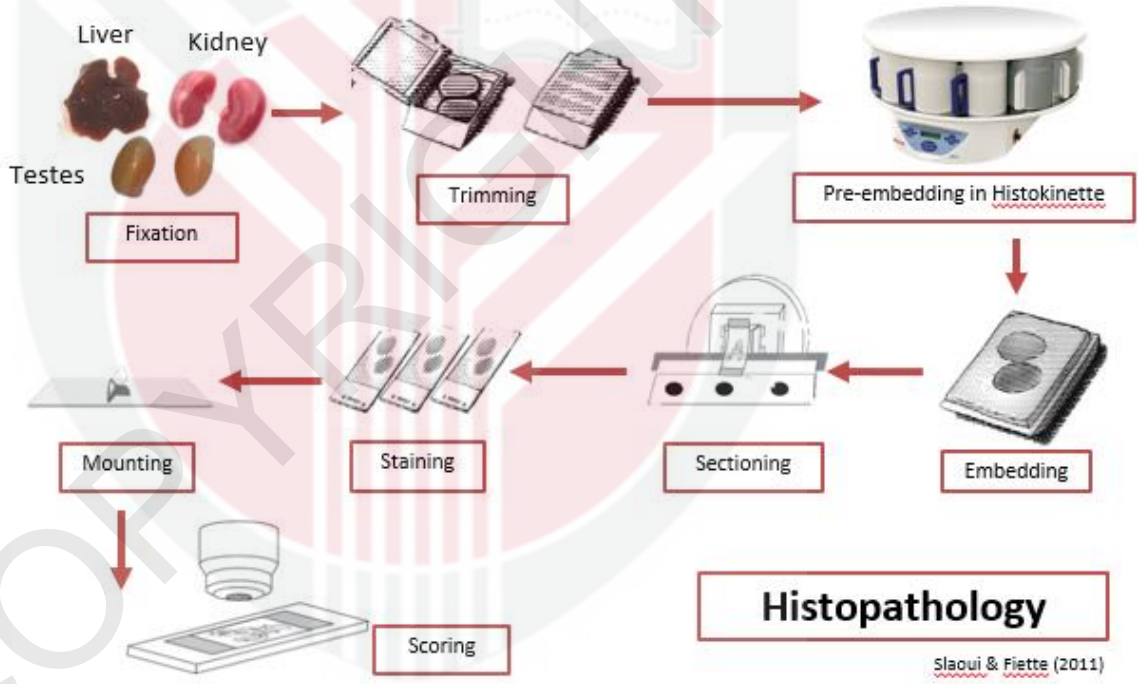


Figure 3.2. Flowchart of the histology procedure.

Figure 3.3. Leica TP1020 Automatic Benchtop Tissue Processor. Adapted from (https://www.leicabiosystems.com/histology-equipment/tissue-processors/products/leica-tp1020/)

This equipment serves to gradually substitute the water content of the tissues with paraffin wax since the latter provides enough rigidity for sectioning whilst maintaining the morphological structures. This automated process was achieved by dehydrating the tissues in ascending concentrations of alcohol (70%,95%, absolute alcohol) followed by clearing with xylene and infiltration of paraffin wax. Xylene is used as the ante medium between alcohol and paraffin wax as it is miscible in both. Subsequently, the tissues were embedded in paraffin wax inside a metal base mould in proper orientations to form tissue blocks (Leica EG1160, Germany) which were trimmed and sectioned using a rotary microtome. The ribbons of tissue sections were standardised to 4-5 μm with constant replacement of the microtome blade to ensure the thinness and quality. The tissue sections were then fished out onto microscope slides from a 45°C water bath to stretch the tissues for optical enhancement. Next, the dried slides with firmly adhered tissue sections were subjected to automated Haematoxylin and Eosin (H & E) staining using Tissue-Tek Prisma® Plus Automated Slide Stainer as seen in Figure 3.4. The protocol stipulates the rehydration of tissues in descending concentrations of alcohol and deparaffinization of the slides before staining since both dyes are water-soluble over the period of 4 hours. The

staining provides contrast in order to observe the tissue structures; whereby the basic haematoxylin dyes the acidic nuclei blue, and acidic eosin dyes the basic components of the cytoplasm orange-red in colour. The tissues were once again dehydrated for the next step.

Figure 3.4. Tissue-Tek Prisma® Plus Automated Slide Stainer. Adapted from (https://www.sakuraus.com/Products/Staining/Tissue-Tek-Prisma-Plus.html)

The stained tissue sections were further protected and optically enhanced prior to the microscopic examination via mounting of the slides with coverslips using neutral dibutyl phthalate xylene (DPX) medium. They were allowed to dry overnight within a fume hood. The tissue sections were then examined at 400x total magnification with a compound microscope equipped with digital imaging software for photomicrography as seen in Figure 3.5 and subsequent analysis which involves guided scoring of the histological parameters.

Figure 3.5. Optical microscopes Leica DM2500 & DM2500 LED. Adapted from (https://www.leica-microsystems.com/products/light-microscopes/p/leica-dm2500/)

3.6 Statistical Analysis

The relative organ weights (ROW) were subjected to the One-Way Analysis of Variance (ANOVA) test followed by post hoc comparisons using Tukey's test. These are performed using the Statistical Package for the Social Sciences 2.0 (IBM SPSS Inc. USA) with the data expressed as Mean ± SEM and significance level of $p < 0.05$ in consideration. The scoring data of the histological parameters were subjected to the non-parametric Kruskal-Wallis H test followed by Mann-Whitney U post hoc for comparison of mean rank scores at ($p < 0.05$) for all pairs.

CHAPTER 4

RESULTS

The ROW was subjected to the One-Way Analysis of Variance (ANOVA) test followed by Tukey's post hoc comparisons to calculate their means and standard error of means (SEM). The calculated values were then analysed to identify any significant differences at p-value less than 0.05 ($p < 0.05$) by comparing the means of treatment groups B, C, D against control group A. The statistical analysis identified only two statistically significant differences in the ROW of the treatment groups in comparison to the control group. The scoring data of the histological parameters were subjected to the non-parametric Kruskal-Wallis H test followed by Mann-Whitney U post hoc for comparison of mean rank scores at ($p < 0.05$) for all pairs. With the exception of these parameters [liver (ballooning hepatocytes), and kidney (interstitial oedema)], histopathological examination revealed no statistically significant differences between the treatment groups and control group.

4.1 Relative Organ Weights of Mice

Table 4.1. Effects of AMLE on male ICR mice relative organ weights after 35 days treatment.

ROW	Groups			
	A (Control)	B (100 mg/kg)	C (200 mg/kg)	D (300 mg/kg)
Liver	5.743 ± 0.109^{a}	6.672 ± 0.331	5.907 ± 0.249	4.180 ± 0.212^{a}
Kidney	0.983 ± 0.252^{b}	1.331 ± 0.836^{b}	1.088 ± 0.492	0.861 ± 0.258
Testes	0.625 ± 0.790	0.492 ± 0.351	0.618 ± 0.120	0.558 ± 0.328

Note: Data are presented as means ± SEM. [ab] Superscripts are prescribed on the mean value that are statistically significant at ($p < 0.05$) compared with control group.

The mean values of the weights of the liver and kidney of both control and experimental groups treated with AMLE is indicated in Table 4.1. There was a statistically significant difference ($p < 0.05$) between groups as determined by one-way ANOVA in liver ($F(3,16) = 19.142$) and kidney ($F(3,16) = 14.916$). Following post hoc comparisons, the ROW in liver revealed to be significantly decreased ($p < 0.05$) in treatment group D (4.180 ± 0.212) as compared to control group A (5.743 ± 0.109). However, the ROW in kidney revealed to be significantly increased ($p < 0.05$) in treatment group B (1.331 ± 0.836) as compared to control group A (0.983 ± 0.252). There were no other statistically significant differences. Further details on the statistical analysis can be found in Appendix B.

4.2 Histopathological Findings

4.2.1 Histopathology of Liver

Kruskal-Wallis test was conducted to examine the differences of liver histological parameters which include the presence of PMN infiltration, collagen deposition, acidophil bodies, and ballooning hepatocytes. No significant differences were found between treatment groups and control group in these parameters. However, the test revealed that there was a statistically significant difference ($p < 0.05$) in presence of ballooning hepatocytes between different A. *muricata* dosage, H (3) = 19 with a mean rank score of 3.00 for Group A, 10.50 for Group B and Group C, and 18.00 for Group D. Post-hoc Mann-Whitney U test reported that group B (mean rank = 8), group C (mean rank = 8), and group D (mean rank = 8) had higher scoring compared to control group A (mean rank = 3) as seen in Appendix G. The test indicated that these differences were statistically significant, $U < 0.01$, z = -3, p = 0.008 (see Appendix C for histopathological scoring data and Appendix D for SPSS output for liver histopathology).

The histopathology microphotographs in Figure 4.1A showed that control group A has normal histology whilst mice in group B and C showed a few ballooning hepatocytes (Figure 4.1B and 4.1C). In addition, mice treated with 300 mg/kg of AMLE showed many ballooning hepatocytes (Figure 4.1D). Although not indicated in the figure below, it was observed that there were also tubular vacuolation of hepatocytes in many of the photomicrographs of the three treatment groups. Furthermore, in photomicrographs of group D, binucleated and pyknotic nuclei were occasionally observed.

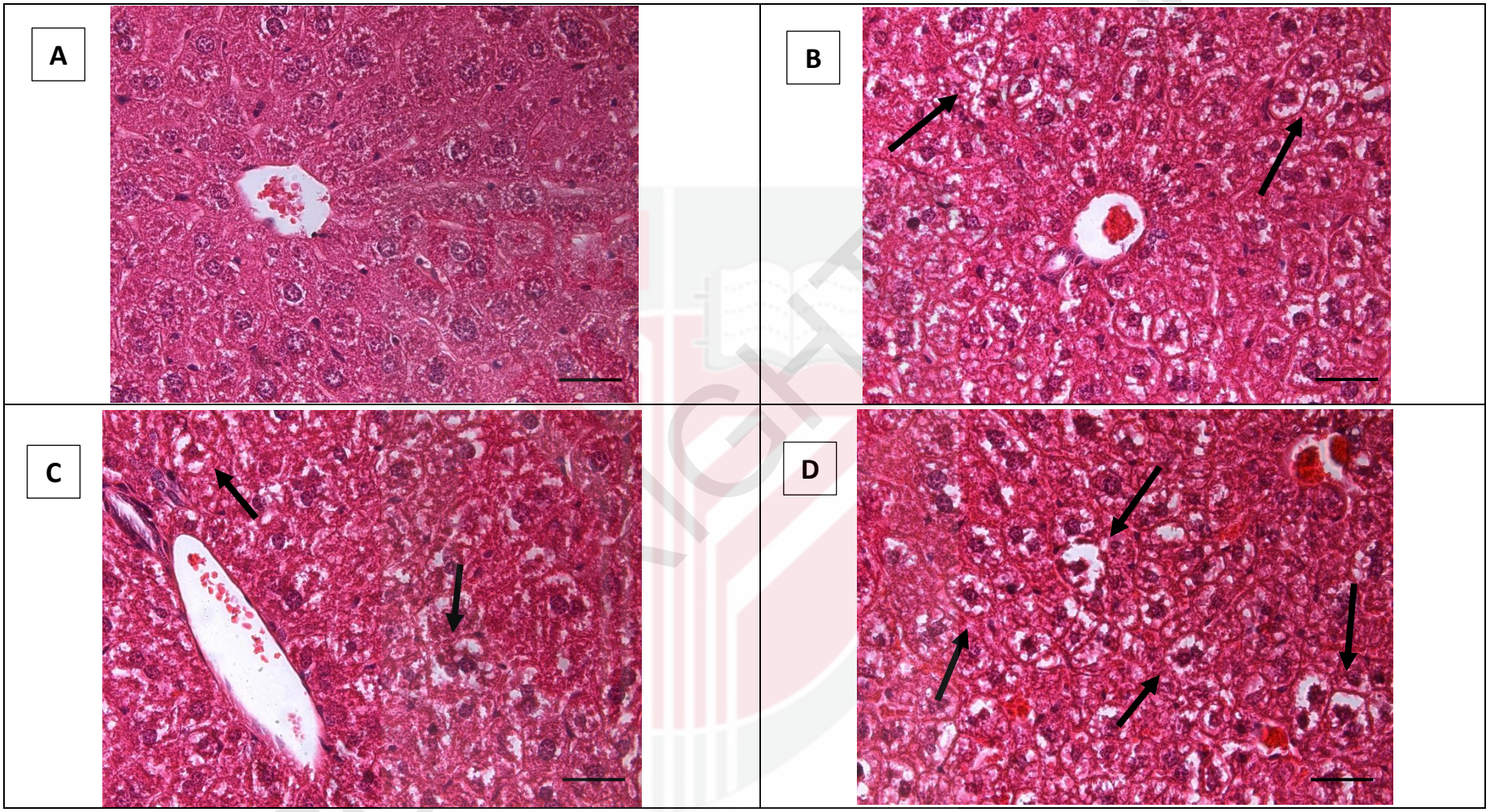


Figure 4.1. (A) Photomicrograph of liver section from control group A showing normal histology. (B) and (C) photomicrographs of liver sections from mice treated with 100 and 200 mg/kg of AMLE. (D) photomicrograph of liver section from mice treated with 300 mg/kg of AMLE. Arrows showing areas of ballooning degenerations (magnification: 40x, scale bar: 50 μm).

4.2.2 Histopathology of Kidney

Kruskal-Wallis test was conducted to examine the differences of histological parameters of the kidneys which include the presence of PMN infiltration, tubular necrosis, and interstitial oedema. No significant differences were found between treatment groups and control group in these parameters. However, the test revealed that there was a statistically significant difference ($p < 0.05$) in the presence of interstitial oedema between different A. *muricata* dosage, H (3) = 19 with a mean rank score of 3.00 for Group A, 10.50 for Group B and Group C, and 18.00 for Group D. Post-hoc Mann-Whitney U test reported that group B (mean rank = 8), group C (mean rank = 8), and group D (mean rank = 8) had higher scoring compared to control group A (mean rank = 3) as seen in Appendix G. The test indicated that these differences were statistically significant, $U < 0.01$, z = -3, $p = 0.008$ (see Appendix C for histopathological scoring data and Appendix E for SPSS output for kidney histopathology).

The histopathology microphotographs in Figure 4.2A showed that control group A has normal histology of kidney with few appearances of interstitial oedema whilst mice in group B and C showed higher appearances of interstitial oedema than group A (Figure 4.2B and 4.2C). Mice in group D also showed numerous interstitial oedema (Figure 4.2D). Although tubular necrosis was not observed, tubular vacuolation was visible in photomicrographs of group C and D.

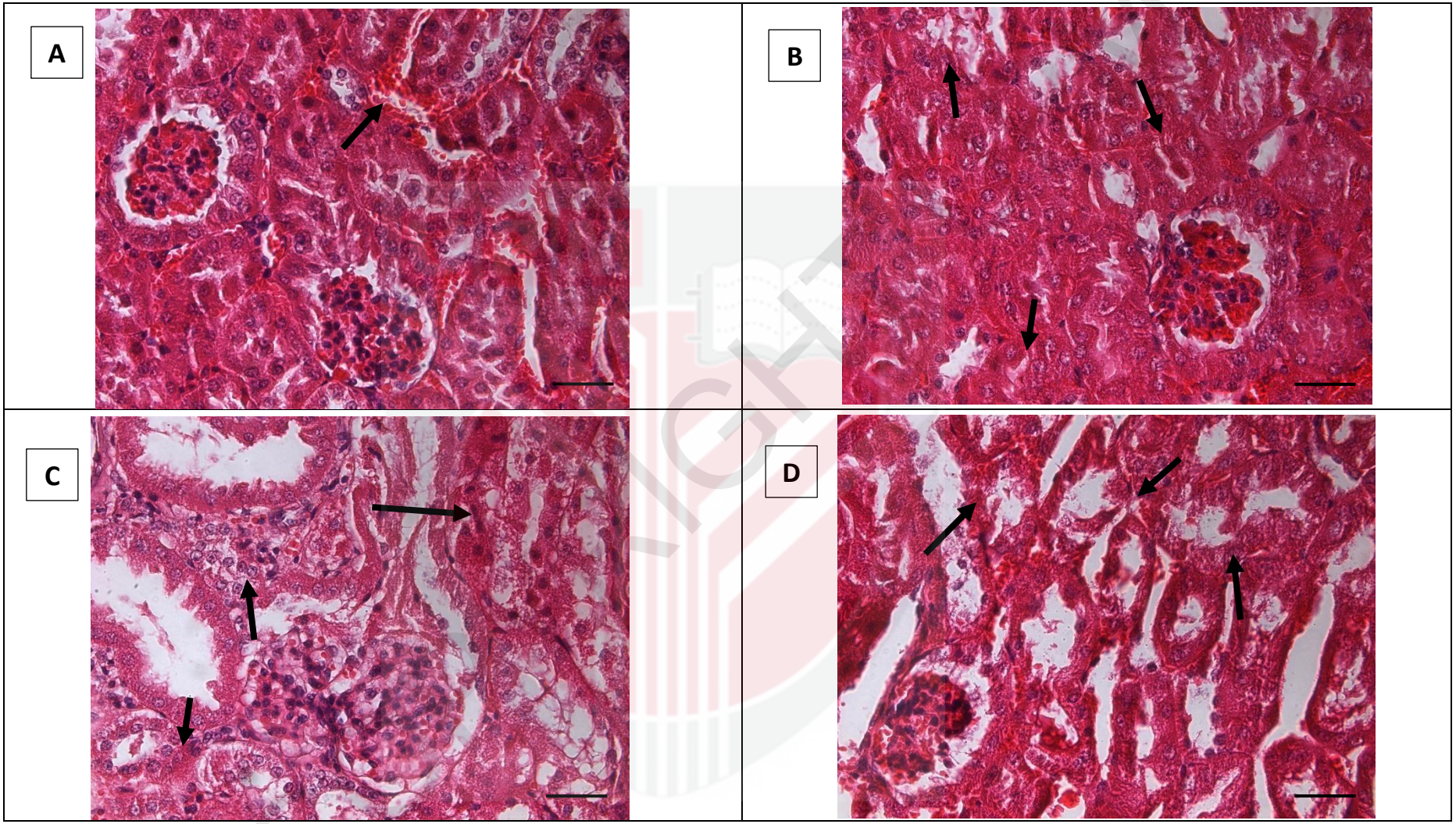


Figure 4.2. (A) Photomicrograph of kidney section from control group A showing normal histology with few interstitial oedemas. (B) and (C) photomicrographs of kidney sections from mice treated with 100 and 200 mg/kg of AMLE. (D) photomicrograph of kidney section from mice treated with 300 mg/kg of AMLE. Arrows showing areas of interstitial oedemas (magnification: 40x, scale bar: 50 μm).

4.2.3 Histopathology of Testes

Kruskal-Wallis test was conducted to examine the differences of the histological parameters of the testes which include the presence of sperm cells, tubular vacuolation, and BTB breakdown. No significant differences were found between treatment groups and control group for the presence of sperm cells. However, the test revealed that there was a statistically significant difference ($p < 0.05$) in the presence of tubular vacuolation between different A. *muricata* dosage, H (3) = 8.586, $p = 0.035$, with a mean rank score of 9.30 for Group A, 7.10 for Group B, 8.80 for Group C, and 16.80 for Group D. The test also showed that there was a statistically significant difference ($p < 0.05$) in BTB breakdown between different A. *muricata* dosage, H (3) = 9.929, p = 0.019, with a mean rank score of 9.50 for Group A, 5.90 for Group B, 9.60 for Group C, and 17.00 for Group D. However, in the post-hoc Mann-Whitney U test, it revealed that the significance difference detected earlier was not in association with control group A. The test reported significant differences ($p < 0.05$) in tubular vacuolation and BTB breakdown between group B and C when compared to group D (see Appendix C for histopathological scoring data and Appendix F for SPSS output for testes histopathology).

Histopathology microphotographs in Figure 4.3A, 4.3B, and 4.3C showed that at the dosages of 0, 100, and 200 mg/kg of AMLE, the histopathology examination revealed a high degree of tubular vacuolation and BTB breakdown. Group 300 mg/kg of AMLE showed an even higher degree of tubular vacuolation and BTB breakdown (Figure 4.3D).

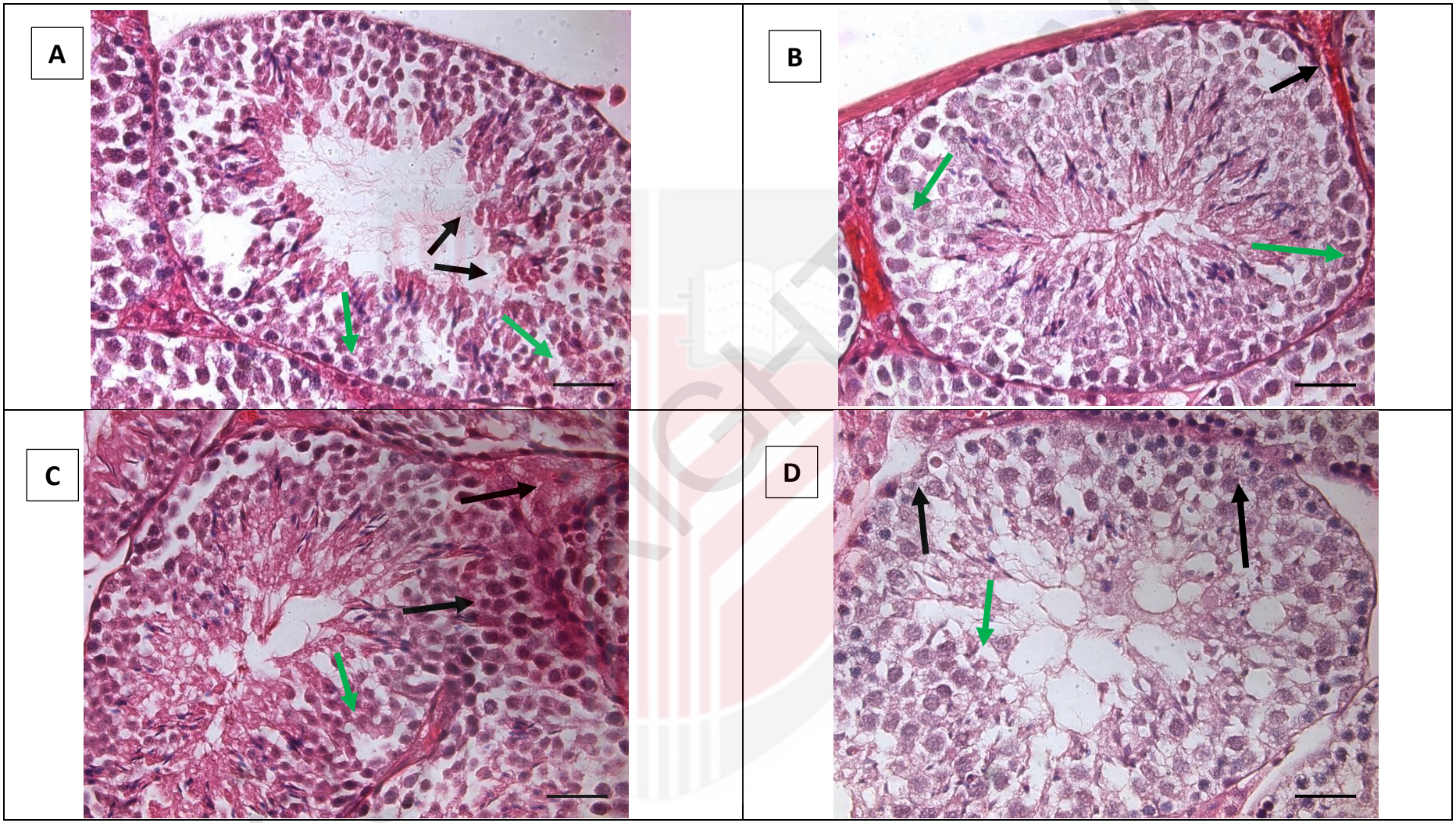


Figure 4.3. (A) (B) (C) (D) Photomicrograph of testes section mice treated with 0, 100, 200 and 300 mg/kg of AMLE respectively showing high degrees of BTB breakdown and tubular vacuolation in Sertoli cells. Arrows showing particular areas of BTB breakdown (black) and tubular vacuolation (green) (magnification: 40x, scale bar: 50 μm).

CHAPTER 5

DISCUSSION AND LIMITATIONS

Annona muricata is an ideal example of a medicinal plant that has various biological activities beneficial to humans such as antimicrobial, anticancer, anti-inflammatory and others as reviewed by Coria-Téllez et al (2018). Due to the pretext of being more affordable and available as well as less toxic compared to orthodox drugs, self-prescription of medicinal plants like A. *muricata* are worryingly common. The increasing interest in the plants' therapeutic properties prompted this study where the potential toxicity of AMLE in male mice after treatment for 35 days or one spermatogenic cycle was evaluated. The type of data that were considered to determine the hypotheses include relative organ weights and histopathological scoring. The findings of this study support the hypothesis stated in Chapter 1. This study is also a continuation of Raja Muhammad Iqbal's work in assessing the antifertility effects of AMLE in mice. His research focused on the sperm quality and libido of mice and observed a fall in sperm quality after consumption of AMLE. Therefore, this study also aims to investigate whether the fall in sperm quality correlates with testes toxicity.

Liver, kidney, and testes were used in assessing the ROW and histopathological alteration caused by AMLE. Liver and kidney are chief metabolic organs with important physiological functions where the former is involved with biotransformation

of drugs (detoxification and hydrophilization) and the latter serves to filter wastes including toxin for excretion. Therefore, any toxicity from exogenous sources can disrupt the histological architecture of these organs and cause histopathology (Garza, Park, & Kocz, 2020). Testes were chosen in this study because a recent study by Oladipo et al (2018) on the spermatogenic activity of AMMBE revealed lesions in the testes of albino rats. This can contribute to understanding the effects of consuming A. *muricata* towards male reproductive health.

A sensitive indicator for the overall health status of an animal is the changes in their body weight as mentioned by Arthur et al (2011) in their study. However, to distinguish the direct toxicity towards particular organs will need some adjustment. This is because the compounds in AMLE could indirectly affect the weight of organs through overall body weight changes thus making it difficult to discern specific toxicities in liver, kidney, and testes (Lazic, Semenova, & Williams, 2020). Relative organ weights were used to overcome this by using calculated ratios as shown in Appendix A. ROW are judged as a sensitive indicator of organ toxicity induced by chemicals even when there is no presence of morphological changes (Mandal, Loeffler, Salamat, & Fritsch, 2012). Miyauchi et al (2013) mentioned in their study that any increase in organ weight may signify normal metabolism or histopathology of organs. The latter which can include chemical-induced toxicity, abnormal mass, and contusions may be differentiated via histopathological examination.

From the results in Table 4.1, there were no significant differences in the ROW when comparing between the treatment groups and the control group. Nonetheless, there were two irregularities found through the statistical analysis. Firstly, there is a statistically significant decrease of the liver ROW in treatment group D (300 mg/kg) when compared to the control. This observation may not be attributed to the effect of AMLE as none of the previous toxicity studies of A. *muricata* inclusive of a ROW evaluation reported similar findings. The decrease in liver weight suggest underlying pathological conditions of the mice instead (Miyauchi et al., 2013). The cause of this condition is unknown but may be speculated due to the inadequate welfare of the mice or possible trauma during the course of the experimentation. Secondly, there was a statistically significant increase in the kidney weight in treatment group B (100 mg/kg) when compared to the control. This suggest presence of outlier(s) in the raw data since group C (200 mg/kg) and group D (300 mg/kg) are insignificance compared to control. This is further supported by the SPSS output seen in Appendix B where the kidney ROW data violated the homogeneity of variances.

To strengthen the correlation between the findings above and the hypotheses, histopathology examination were employed. Histopathology examination is the gold standard for tissue examination where the features often associated with tissue injury are assessed through the lens of a microscope. Based on the result of this study, only two of ten histological parameters were found to have statistically significant differences that were then refuted by the photomicrograph analyses which observed normal and preserved architecture of selected organs after treatment with AMLE.

In liver, out of the four histological parameters considered (presence of PMN infiltration, collagen deposition, acidophil bodies, and ballooning hepatocytes), only ballooning hepatocytes showed statistically significant difference ($p < 0.05$) in all treatment groups when compared to the control. The tendency for ballooning degeneration of hepatocytes significantly increases for each dose when compared to control group which can be visualised through Figure 4.1. Ballooning hepatocytes refers to the pale and swollen hepatocytes which manifest due to severe cell injury with subsequent depletion of ATP and rise in intracellular calcium. This eventually leads to the disruption of the hepatocytes' internal structure (Patel & Windon, 2021). Acetogenins, a class of compounds found in A. *muricata*, have been reported to inhibit mitochondrial complex I enzyme which reduces ATP. The depletion of ATP contributes to cell degeneration due to the plasma membrane disruption and mitochondrial damage caused by the increase of ROS (Escobar-Khondiker et al., 2007; Lannuzel et al., 2003; Nweke et al., 2019). This is consequential especially in organs such as liver, kidney, and brain whose performances are highly dependent upon ATP availability; studies on neurotoxicity caused by A. *muricata* by Champy et al (2004) and Escobar-Khondiker et al (2007) are in agreement with this. The ballooning degeneration observed in this study may be influenced from AMLE consumption however it is not certain as the liver ROW and other histological lesions indicating toxicity were not significantly influenced. This is supported by EzejinduD. et al (2015), Sherif et al (2017) and Utomo et al (2016) which showed similar results in their A. *muricata* toxicity studies with dosage ranging from 250-2000 mg/kg in mice and 0.2-0.6 mL in rats' model.

In kidneys, out of three histological parameters considered (presence of PMN infiltration, tubular necrosis, and interstitial oedema), only interstitial oedema had statistically significant difference ($p < 0.05$) in all treatment groups when compared to the control. The tendency for renal interstitial oedema significantly increases for each dose when compared to control group. Interstitial oedema refers to the accumulation of fluid in the interstitial spaces leading to increase in interstitial pressure which reduces perfusion thus activity of kidney (Scallan, Huxley, & Korthuis, 2010). The underlying pathogenesis behind the lesions are not understood and can be attributed to multitude of factors such as ischaemia, immunological reaction, infection, and many more (Dixon, Winearls, & Dunnill, 1981). These photomicrographs findings contradict the histopathology studies conducted by Ekere et al (2019), Sherif et al (2017) and Utomo et al (2016) which shows normal renal histology when treated with A. *muricata* at 250-2000 mg/kg dosage range. Thus, the interstitial oedema observed may not be influenced from AMLE consumption as corroborated by the insignificant changes of other histological parameters and the kidney ROW.

Interestingly, vacuolated hepatocytes and vacuolated renal tubules indicating hydropic changes were also observed in the tissues procured from this study. This is interpreted as areas of reversible cellular injury as hydropic changes usually indicate the onset of cellular degeneration in response to injurious agents (Urbana-Champaign, 2021) However, it is difficult to ascertain the origin of this vacuolated lesions since they are frequently observed as artifactual vacuolation caused by autolysis and poor fixation (National Toxicology Program, 2014). Therefore, they might not contribute to the

hypotheses; the insignificant changes in liver and kidney ROW further substantiate this. To surmise, it is difficult to determine the cause for the lesions observed in the hepatic and renal tissues (ballooning degeneration and interstitial oedema respectively). Nonetheless, they were not likely pathological in nature since the lesions does not amount to changes in the ROW. This is supported by Akinnawo et al (2005) and Ekere et al (2019) that suggested the normal architecture of the liver and kidneys are maintained due to the antioxidative and anti-inflammatory properties of the compounds in A. *muricata* extracts.

In testes, none of the three histological parameters considered (presence of sperm cells, tubular vacuolation, and BTB breakdown) had statistically significant difference ($p < 0.05$) in all treatment groups when compared to the control. The analysis of the photomicrographs showed that all groups including the control observed a high degree of hydropic changes and BTB breakdown which indicates reversible cell injury. Blood-testis barrier creates an immune-privileged environment for the developing sperm cells and is formed from the linings of Sertoli cells. When tubular vacuolation or hydropic change are persistent to the Sertoli cells, it compromises this barrier which can be due to an autoimmune response (Cheng & Mruk, 2012). The findings in this study suggests that the histopathology examination is considered insignificant since the control group was also compromised. Since there are no A. *muricata* toxicity studies yet reporting on the histology of testes, it is questionable to determine the effect of AMLE based on these particular findings. However, the insignificant ROW statistics of testes in this study does infer that there is no toxicity.

There were some limitations to this study where the tissues obtained for the histopathological examination may be damaged since there were delay in its fixation process and exposure to prolonged fixation (approximately a year) due to the sudden COVID restriction. When there are errors in this step, it cannot be remedied and will affect the finished products (Singhal et al., 2017). The COVID restriction also restricted the time spent in the laboratory and therefore limits the type of stains to experiment with. For example, collagen deposits are better observed with Sirius red staining. Moreover, the histopathological scoring was done via photomicrographs only which limit the overall observations.

To summarise, the histopathological and ROW results on the selected organs i.e., liver, kidney, and testes showed non-significant histopathology changes between the control and treatment groups. For one spermatogenic cycle of AMLE administration, the toxicity effects seen in mice were all reversible. Therefore, the consumption of AMLE is shown to be safe for antifertility purpose in mice. These findings supported the hypothesis of this study. This is further corroborated by Arthur et al (2011), Ekere et al (2019), and Utomo et al (2016) whose description of studies can be found in Chapter 2.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

In this present study, the changes in relative organ weights and many of the key histopathological parameters were found not to have significant differences when comparing between the treatment groups and control group. Thus, it can be concluded that the consumption of AMLE for 35 days or one spermatogenic cycle at 100 mg/kg, 200 mg/kg, and 300 mg/kg dosages observed non-significant histopathological changes in the liver, kidney, and testes in male mice. The reversible toxicity effects suggest that if the treatment is stopped, the mice will revert to their normal condition. Therefore, it is safe for mice to consume AMLE for 35 days.

The inclusion of gross observations of organs, biochemical and haematological assessments is encouraged to reinforce the findings of this study. Other future recommendations also include conducting further toxicity assessment such as sub chronic and chronic studies of AMLE to confirm the safety profile thus endorsing the prolonged consumption of *Annona muricata*.

REFERENCES

Abdul Wahab, S. M., Jantan, I., Haque, M., & Arshad, L. (2018). Exploring the leaves of Annona muricata L. as a source of potential anti-inflammatory and anticancer agents. *Frontiers in Pharmacology*, *9*, 661.

Adcox, M. (2017). What is graviola and how is it used? *Medicalnewstoday.Com*. Retrieved from https://www.medicalnewstoday.com/articles/319720

Akinnawo, O. O., Taiwo, V. O., Ketiku, A. O., & Ogunbiyi, J. O. (2005). Weight changes and organ pathology in rats given edible larvae of Cirina forda (Westwood). *African Journal of Biomedical Research*, *8*(1), 35–39.

Arthur, Woode, Terlabi, & Larbie. (2011). Evaluation of acute and subchronic toxicity of Annona muricata (Linn.) aqueous extract in animals. *European Journal of Experimental Biology*, *1*.

Badrie, N., & Schauss, A. G. (2010). Soursop (Annona muricata L.): Composition, Nutritional Value, Medicinal Uses, and Toxicology. *Bioactive Foods in Promoting Health*, 621–643. https://doi.org/10.1016/B978-0-12-374628-3.00039-6

Baillon, H. (1869). *Histoire des plantes: Tome premier... Dessins de Faguet* (Vol. 1). Librairie Hachette.

Baratta, J. L., Ngo, A., Lopez, B., Kasabwalla, N., Longmuir, K. J., & Robertson, R. T. (2009). Cellular organization of normal mouse liver: a histological, quantitative immunocytochemical, and fine structural analysis. *Histochemistry and cell biology*, *131*(6), 713–726. https://doi.org/10.1007/s00418-009-0577-1

Bitar, R., Fakhoury, R., Fahmi, R., & Borjac, J. (2017). Histopathological Effects of the Annona muricata Aqueous Leaves Extract on the Liver and Kidneys of Albino Mice. *Translational Medicine*, *07*(02). https://doi.org/10.4172/2161-1025.1000194

Bodeker, G., & Ong, C.-K. (2005). *WHO global atlas of traditional, complementary and alternative medicine* (Vol. 1). World Health Organization.

Boulogne, I., Germosén-Robineau, L., Ozier-Lafontaine, H., Fleury, M., & Loranger-Merciris, G. (2011). TRAMIL ethnopharmalogical survey in Les Saintes (Guadeloupe, French West Indies): a comparative study. *Journal of Ethnopharmacology*, *133*(3), 1039–1050.

Bussmann, R. W., Malca-García, G., Glenn, A., Sharon, D., Chait, G., Díaz, D., … Guardado, G. (2010). Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterial remedies. *Journal of Ethnopharmacology*, *132*(1),

101–108.

Champy, P., Höglinger, G. U., Féger, J., Gleye, C., Hocquemiller, R., Laurens, A., … Lombès, A. (2004). Annonacin, a lipophilic inhibitor of mitochondrial complex I, induces nigral and striatal neurodegeneration in rats: possible relevance for atypical parkinsonism in Guadeloupe. *Journal of Neurochemistry*, *88*(1), 63–69.

Chan, W.-J. J., McLachlan, A. J., Hanrahan, J. R., & Harnett, J. E. (2020). The safety and tolerability of Annona muricata leaf extract: a systematic review. *Journal of Pharmacy and Pharmacology*, *72*(1), 1–16. https://doi.org/https://doi.org/10.1111/jphp.13182

Cheng, C. Y., & Mruk, D. D. (2012). The blood-testis barrier and its implications for male contraception. *Pharmacological Reviews*, *64*(1), 16–64. https://doi.org/10.1124/pr.110.002790

Coria-Téllez, A. V., Montalvo-Gónzalez, E., Yahia, E. M., & Obledo-Vázquez, E. N. (2018a). Annona muricata: A comprehensive review on its traditional medicinal uses, phytochemicals, pharmacological activities, mechanisms of action and toxicity. *Arabian Journal of Chemistry*, *11*(5), 662–691. https://doi.org/10.1016/J.ARABJC.2016.01.004

Coria-Téllez, A. V, Montalvo-Gónzalez, E., Yahia, E. M., & Obledo-Vázquez, E. N. (2018b). Annona muricata: A comprehensive review on its traditional medicinal uses, phytochemicals, pharmacological activities, mechanisms of action and toxicity. *Arabian Journal of Chemistry*, *11*(5), 662–691. https://doi.org/https://doi.org/10.1016/j.arabjc.2016.01.004

Dai, Y., Hogan, S., Schmelz, E. M., Ju, Y. H., Canning, C., & Zhou, K. (2011). Selective growth inhibition of human breast cancer cells by graviola fruit extract in vitro and in vivo involving downregulation of EGFR expression. *Nutrition and Cancer*, *63*(5), 795–801.

de Sousa, O. V., Vieira, G. D.-V., de Jesus R. G. de Pinho, J., Yamamoto, C. H., & Alves, M. S. (2010). Antinociceptive and antiinflammatory activities of the ethanol extract of Annona muricata L. leaves in animal models. *International Journal of Molecular Sciences*, *11*(5), 2067–2078. https://doi.org/10.3390/ijms11052067

de Sousa, O. V., Vieira, G. D.-V., de Jesus R G de Pinho, J., Yamamoto, C. H., & Alves, M. S. (2010). Antinociceptive and anti-inflammatory activities of the ethanol extract of Annona muricata L. leaves in animal models. *International Journal of Molecular Sciences*, *11*(5), 2067–2078. https://doi.org/10.3390/ijms11052067

Davidson, A. J. (2009, January 15). *Mouse kidney development*. StemBook; The Stem Cell Research Community, StemBook doi/10.3824/stembook.1.34.1. https://www.stembook.org/node/532

Dixon, A. J., Winearls, C. G., & Dunnill, M. S. (1981). Interstitial nephritis. *Journal of Clinical Pathology*, *34*(6), 616–624.

Ekere, O. U., Ogunka-Nnoka, C. U., & Monago-Ighorodje, C. C. (2019). Hepatotoxicity and Nephrotoxicity Studies of Ethanol Extracts of Annona muricata Leaves and Fagara Zanthoxyloide Roots on Zidovudine-Induced Wistar Rats. *International Journal of Toxicology*, *4*(1), 45–53.

Ekor, M. (2014). The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*, *4*, 177. https://doi.org/10.3389/fphar.2013.00177

El Bacha, T. (2010). Nutrient Metabolism, Human | Learn Science at Scitable. *Nature.Com*. Retrieved from https://www.nature.com/scitable/topicpage/dynamic-adaptation-of-nutrient-utilization-in-humans-14232807/

Escobar-Khondiker, M., Höllerhage, M., Muriel, M.-P., Champy, P., Bach, A., Depienne, C., … Yagi, T. (2007). Annonacin, a natural mitochondrial complex I inhibitor, causes tau pathology in cultured neurons. *Journal of Neuroscience*, *27*(29), 7827–7837.

EzejinduD., N., UdemezueO, O., ChukwujekwuI, E., UchefunaR, C., MadukaS, O., AkingboyeA, J., & EzejinduC, N. (2015). The Histological Effect of Ethanolic Leaf Extracts of Annona Muricata (Soursop) On Liver of Adult Wistar Rats. *International Journal of Health Sciences and Research*, *5*, 125–129.

Florence, N. T., Benoit, M. Z., Jonas, K., Alexandra, T., Désiré, D. D. P., Pierre, K., & Théophile, D. (2014). Antidiabetic and antioxidant effects of Annona muricata (Annonaceae), aqueous extract on streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology*, *151*(2), 784–790.

Garza, A. Z., Park, S. B., & Kocz, R. (2020). Drug Elimination. *PubMed*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK547662/

Gavamukulya, Y., Wamunyokoli, F., & El-Shemy, H. A. (2017). Annona muricata: Is the natural therapy to most disease conditions including cancer growing in our backyard? A systematic review of its research history and future prospects. *Asian Pacific Journal of Tropical Medicine*, *10*(9), 835–848. https://doi.org/https://doi.org/10.1016/j.apjtm.2017.08.009

Gurcan, M. N., Boucheron, L. E., Can, A., Madabhushi, A., Rajpoot, N. M., & Yener, B. (2009). Histopathological image analysis: a review. *IEEE Reviews in Biomedical Engineering*, *2*, 147–171. https://doi.org/10.1109/RBME.2009.2034865

Hamizah, S., Roslida, A. H., Fezah, O., Tan, K. L., Tor, Y. S., & Tan, C. I. (2012). Chemopreventive potential of Annona muricata L leaves on chemically-induced skin papillomagenesis in mice. *Asian Pacific Journal of Cancer Prevention*, *13*(6), 2533–

2539.

Hasrat, J. A., Pieters, L., De Backer, J.-P., Vauquelin, G., & Vlietinck, A. J. (1997). Screening of medicinal plants from Suriname for 5-HT1A ligands: Bioactive isoquinoline alkaloids from the fruit of Annona muricata. *Phytomedicine*, *4*(2), 133–140.

Höllerhage, M., Rösler, T. W., Berjas, M., Luo, R., Tran, K., Richards, K. M., … Godoy, H. T. (2015). Neurotoxicity of dietary supplements from Annonaceae species. *International Journal of Toxicology*, *34*(6), 543–550.

ITIS, I. T. I. S. (2021). ITIS Standard Report Page: Annona muricata. *Integrated Taxonomic Information System*. Retrieved from https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=18098#null

Kubiak, B. D., Albert, S. P., Gatto, L. A., Snyder, K. P., Maier, K. G., Vieau, C. J., … Nieman, G. F. (2010). Peritoneal Negative Pressure Therapy Prevents Multiple Organ Injury in a Chronic Porcine Sepsis and Ischemia/Reperfusion Model. *Shock*, *34*(5), 525–534. https://doi.org/10.1097/shk.0b013e3181e14cd2

Langenberger, G., Prigge, V., Martin, K., Belonias, B., & Sauerborn, J. (2009). Ethnobotanical knowledge of Philippine lowland farmers and its application in agroforestry. *Agroforestry Systems*, *76*(1), 173–194.

Lannuzel, A., Michel, P. P., Höglinger, G. U., Champy, P., Jousset, A., Medja, F., … Laurens, A. (2003). The mitochondrial complex I inhibitor annonacin is toxic to mesencephalic dopaminergic neurons by impairment of energy metabolism. *Neuroscience*, *121*(2), 287–296.

Lazic, S. E., Semenova, E., & Williams, D. P. (2020). Determining organ weight toxicity with Bayesian causal models: Improving on the analysis of relative organ weights. *Scientific Reports*, *10*(1), 6625. https://doi.org/10.1038/s41598-020-63465-y

Mandal, R., Loeffler, A. G., Salamat, S., & Fritsch, M. K. (2012). Organ weight changes associated with body mass index determined from a medical autopsy population. *The American Journal of Forensic Medicine and Pathology*, *33*(4), 382–389.

Margolin, G., Khil, P. P., Kim, J., Bellani, M. A., & Camerini-Otero, R. (2014). Integrated transcriptome analysis of mouse spermatogenesis. *BMC Genomics, 15*(1), 39. doi:10.1186/1471-2164-15-39

Miyauchi, S., Oshima, S., Asaka, M., Kawano, H., Torii, S., & Higuchi, M. (2013). Organ size increases with weight gain in power-trained athletes. *International Journal of Sport Nutrition and Exercise Metabolism*, *23*(6), 617–623.

Morton, J. F. (1987). *Fruits of warm climates.* JF Morton.

National Toxicology Program. (2014, October 23). Nonneoplastic Lesion Atlas - National Toxicology Program (M. F. Cesta, D. E. Malarkey, R. Herbert, A. Brix, & R. C. Sills, Eds.). Ntp.niehs.nih.gov. https://ntp.niehs.nih.gov/nnl/index.htm

National Toxicology Program (2014). Kidney, Renal Tubule - Vacuolation, Cytoplasmic - Nonneoplastic Lesion Atlas (M. F. Cesta, D. E. Malarkey, R. Herbert, A. Brix, & R. C. Sills, Eds.). *Ntp.Niehs.Nih.Gov*. Retrieved from https://ntp.niehs.nih.gov/nnl/urinary/kidney/rtvac/index.htm

Nishimura, H., & L'Hernault, S. W. (2017). Spermatogenesis. *Current Biology*, *27*(18). https://doi.org/10.1016/j.cub.2017.07.067

Novy, J. W. (1997). Medicinal plants of the eastern region of Madagascar. *Journal of Ethnopharmacology*, *55*(2), 119–126.

Nweke, E. O., Ndukwe, G. U., & Opara, J. K. (2019). Ethanolic extract of Annona muricata leaf and its effect on the liver. *Journal of Drug Delivery and Therapeutics*, *9*(3-s), 463–466. https://doi.org/10.22270/jddt.v9i3-s.2880

Okolie, N. P., Agu, K., & Eze, G. I. (2013). Protective effect of ethanolic leaf extract of Annona muricata Linn on some early events in cycasinduced colorectal carcinogenesis in rats. *Journal of Pharmaceutical and Scientific Innovation*, *2*(4), 14–21.

Oladipo, T., Adebiyi, A., Adetomiwa, A., Oladipo, O., Adeleye, O., & Olukunle, J. (2018). Evaluation of spermatogenic activity of Annona muricata methanolic bark extract in Albino rats. *Egyptian Journal of Veterinary Sciences*, *49*, 83–90. https://doi.org/10.21608/ejvs.2018.2836.1029

Patel, K. S., & Windon, A. L. (2021). Forms of hepatic injury. *PathologyOutlines*. Retrieved from https://www.pathologyoutlines.com/topic/liverpatternshepaticinjury.html

Pathology, S. of T. (2021). The Society of Toxicologic Pathology (STP). *Www.Toxpath.Org*. Retrieved from https://www.toxpath.org/ssndc.asp

Pinto, A., De, Q., Cordeiro, M., De Andrade, S. R. M., Ferreira, F., Filgueiras, H., … Kinpara, D. (2005). Annona muricata. *Annona Species. Taxonomy and Botany International Centre for Underutilised Crops. University of Southampton, UK*, 3–16.

Poma, E. M., Requis, E. R., Gordillo, G. C., & Fuertes, C. M. (2011). *Phytochemical study and anti-inflammatory activity of Annona muricata L.(Soursop) from Cuzco*.

Rady, I., Bloch, M. B., Chamcheu, R.-C. N., Banang Mbeumi, S., Anwar, M. R., Mohamed, H., … El Sayed, K. A. (2018). Anticancer properties of graviola (Annona muricata): a comprehensive mechanistic review. *Oxidative Medicine and Cellular Longevity*, *2018*.

Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P. C., Sousa, A. P., & Amaral, A. (2009). Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Human Reproduction Update*, *15*(5), 553–572.

Roger, T., Pierre-Marie, M., Igor, V. K., & Patrick, V. D. (2015). Phytochemical screening and antibacterial activity of medicinal plants used to treat typhoid fever in Bamboutos division, West Cameroon. *Journal of Applied Pharmaceutical Science*, *5*(06), 34–49.

Ross, I. A. (2007). *Medicinal plants of the world, volume 3: Chemical constituents, traditional and modern medicinal uses* (Vol. 3). Springer Science & Business Media.

Ruehl-Fehlert, C., Kittel, B., Morawietz, G., Deslex, P., Keenan, C., Mahrt, C. R., … Deschl, U. (2003). Revised guides for organ sampling and trimming in rats and mice – Part 1. *Experimental and Toxicologic Pathology*, *55*(2–3), 91–106. https://doi.org/10.1078/0940-2993-00311

Scallan, J., Huxley, V. H., & Korthuis, R. J. (2010). *Capillary Fluid Exchange: Regulation, Functions, and Pathology*. https://doi.org/10.4199/C00006ED1V01Y201002ISP003

Sellers, R. S., Mortan, D., Michael, B., Roome, N., Johnson, J. K., Yano, B. L., … Schafer, K. (2007). Society of Toxicologic Pathology Position Paper: Organ Weight Recommendations for Toxicology Studies. *Toxicologic Pathology*, *35*(5), 751–755. https://doi.org/10.1080/01926230701595300

Sherif, H. B., Baba, G., & Abdullahi, S. M. (2017). Acute and sub-chronic toxicity profile of Annona muricata (Sour sop) on wister albino rats. *Bayero Journal of Pure and Applied Sciences*, *10*(2), 57–63.

Singhal, P., Sreedhar, G., Singh, N. N., Sharma, D., Bannerji, S., & Gowhar, O. (2017). Evaluation of histomorphometric changes in tissue architecture due to fixation delay. *Journal of Oral and Maxillofacial Pathology : JOMFP*, *21*(1), 70–75. https://doi.org/10.4103/0973-029X.203771

Slaoui, M., & Fiette, L. (2011). Histopathology Procedures: From Tissue Sampling to Histopathological Evaluation. *Drug Safety Evaluation: Methods in Molecular Biology*, *691*, 69–82. https://doi.org/10.1007/978-1-60761-849-2_4

Tene, V., Malagon, O., Finzi, P. V., Vidari, G., Armijos, C., & Zaragoza, T. (2007). An ethnobotanical survey of medicinal plants used in Loja and Zamora-Chinchipe, Ecuador. *Journal of Ethnopharmacology*, *111*(1), 63–81.

Torres, M. P., Rachagani, S., Purohit, V., Pandey, P., Joshi, S., Moore, E. D., … Batra, S. K. (2012). Graviola: a novel promising natural-derived drug that inhibits tumorigenicity and metastasis of pancreatic cancer cells in vitro and in vivo through altering cell metabolism. *Cancer Letters*, *323*(1), 29–40.

Urbana-Champaign, U. of I. C. of M. at. (2021). Atlas of Pathology. *Www.Med.Illinois.Edu*. Retrieved from https://www.med.illinois.edu/m2/pathology/pathatlasf/Atlas01.html

Utomo, A. W., Susilaningsih, N., & Armalina, D. (2016). Acute toxicity test of soursop leaves (Annona muricata) on liver and kidney of switzerland mice. *Sains Medika: Jurnal Kedokteran Dan Kesehatan*, *6*(2), 48–51.

World Health Organisation. (2019). *WHO global report on traditional and complementary medicine 2019*. Retrieved from Geneva: World Health Organisation website: https://www.who.int/traditional-complementary-integrative-medicine/WhoGlobalReportOnTraditionalAndComplementaryMedicine2019.pdf?ua=1

World Health Organisation (2002). *WHO traditional medicine strategy 2002-2005 / World Health Organization*. Retrieved from http://www.who.int/medicines/library/trm/trm_strat_eng.pdf

Xie, B. G., Li, J., & Zhu, W. J. (2014). Pathological changes of testicular tissue in normal adult mice: A retrospective analysis. *Experimental and therapeutic medicine*, *7*(3), 654–656. https://doi.org/10.3892/etm.2014.1481

Zorofchian Moghadamtousi, S., Rouhollahi, E., Karimian, H., Fadaeinasab, M., Firoozinia, M., Ameen Abdulla, M., & Abdul Kadir, H. (2015). The chemopotential effect of Annona muricata leaves against azoxymethane-induced colonic aberrant crypt foci in rats and the apoptotic effect of acetogenin annomuricin E in HT-29 cells: a bioassay-guided approach. *PloS One*, *10*(4), e0122288.

APPENDICES

APPENDIX A

Raw Data of Organ Weights, Body Weights and Calculated ROW

	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA
1						MALE MICE ORGAN HARVEST WEIGHT																					
2	Control	Liver	Kidney		Testicles			t200	Liver	Kidney		testicles														
3		weight	R Weight	L Weigth	R Weight	L Weigth			2.22	0.4	0.45	0.4	0.37														
4		2.14	0.37	0.39	0.19	0.17			2.34	0.46	0.47	0.18	0.25			1											
5		2.13	0.38	0.38	0.2	0.19			2.36	0.42	0.41	0.19	0.15			2											
6		2.15	0.34	0.39	0.34	0.31			2.65	0.39	0.36	0.22	0.22			3											
7		2.16	0.37	0.37	0.25	0.21			1.93	0.44	0.37	0.2	0.2			4											
8		2.28	0.4	0.36	0.2	0.21										5										
9																										
10								t300	Liver	Kidney		testicles														
11	t100	Liver	Kidney		testicles				1.69	0.32	0.34	0.35	0.34		C(L)	T100(L)	T200(L)	T300(L)	C(RK)	T100(RK)	T200(RK)	T300(RK)	C(LK)	T100(LK)	T200(LK)	T300(LK)
12		1.8	0.36	0.38	0.2	0.21			1.61	0.3	0.28	0.14	0.18		5.988	5.659	6.086	4.232	1.035	1.132	1.096	0.801	1.091	1.195	1.234	0.851
13		2	0.51	0.51	0.15	0.16			1.34	0.35	0.39	0.18	0.18		5.821	6.268	6.255	4.858	1.039	1.598	1.230	0.905	1.039	1.598	1.256	0.845
14		2.28	0.4	0.39	0.14	0.16			1.45	0.31	0.34	0.2	0.15		5.886	7.294	5.958	3.527	0.931	1.280	1.060	0.921	1.068	1.248	1.035	1.027
15		2.1	0.38	0.35	0.15	0.15			1.57	0.3	0.29	0.17	0.16		5.365	6.684	6.298	4.103	0.919	1.209	0.927	0.877	0.919	1.114	0.856	0.962
16		2.23	0.43	0.41	0.13	0.15									5.656	7.456	4.940	4.179	0.992	1.438	1.126	0.799	0.893	1.371	0.947	0.772
17																	C(RT)	T100(RT)	T200(RT)	T300(RT)	C(LT)	T100(LT)	T200(LT)	T300(LT)		
18																	0.532	0.629	1.096	0.877	0.476	0.660	1.014	0.851		
19																	0.547	0.470	0.481	0.422	0.519	0.501	0.668	0.543		
20																	0.931	0.448	0.480	0.474	0.849	0.512	0.379	0.474		
21																	0.621	0.477	0.523	0.566	0.522	0.477	0.523	0.424		
22																	0.496	0.435	0.512	0.452	0.521	0.502	0.512	0.426		

APPENDIX B

SPSS Output for ROW Changes

```
GET
  FILE='C:\Users\WIN10\OneDrive\Desktop\efwhypee\analysis\raw\bodyweight
dataset.sav'.
DATASET NAME DataSet1 WINDOW=FRONT.
ONEWAY Liver Kidney Testes BY Treatment
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /PLOT MEANS
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

```
[DataSet1] C:\Users\WIN10\OneDrive\Desktop\efwhypee\analysis\raw\bodyweight
dataset.sav
```

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean
						Lower Bound
Liver ROW	Control	5	5.74316	.243235	.108778	5.44115
	T100	5	6.67185	.740151	.331005	5.75283
	T200	5	5.90720	.557558	.249347	5.21490
	T300	5	4.17994	.472941	.211506	3.59271
	Total	20	5.62554	1.050177	.234827	5.13404
Kidney ROW	Control	5	.98317	.056402	.025224	.91314
	T100	5	1.33132	.187036	.083645	1.09909
	T200	5	1.08789	.109925	.049160	.95140
	T300	5	.86073	.057690	.025800	.78910
	Total	20	1.06578	.206940	.046273	.96893
Testicles ROW	Control	5	.62522	.176750	.079045	.40575
	T100	5	.49174	.078468	.035092	.39431
	T200	5	.61841	.267923	.119819	.28574
	T300	5	.55824	.185823	.083103	.32751
	Total	20	.57340	.182510	.040810	.48798

Descriptives

	95% Confidence Interval for Mean	Minimum	Maximum
	Upper Bound		

	Control	6.04518	5.365	5.988
	T100	7.59086	5.659	7.456
Liver ROW	T200	6.59950	4.940	6.298
	T300	4.76717	3.527	4.858
	Total	6.11704	3.527	7.456
	Control	1.05321	.919	1.039
	T100	1.56356	1.132	1.598
Kidney ROW	T200	1.22438	.927	1.230
	T300	.93236	.799	.921
	Total	1.16263	.799	1.598
	Control	.84468	.496	.931
	T100	.58917	.435	.629
Testicles ROW	T200	.95108	.480	1.096
	T300	.78897	.422	.877
	Total	.65882	.422	1.096

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Liver ROW	1.299	3	16	.309
Kidney ROW	3.570	3	16	.038
Testicles ROW	1.148	3	16	.360

At □ > 0.05, the p-values for the relative weight of kidney is smaller than alpha. It rejects the hypothesis, thus the homogeneity of variances are violated for this. Robust violation of homogeneity of variances, there is a risk of inaccurate output.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
	Between Groups	16.388	3	5.463	19.142	.000
Liver ROW	Within Groups	4.566	16	.285		
	Total	20.955	19			
	Between Groups	.599	3	.200	14.916	.000
Kidney ROW	Within Groups	.214	16	.013		
	Total	.814	19			
	Between Groups	.058	3	.019	.539	.663
Testicles ROW	Within Groups	.575	16	.036		
	Total	.633	19			

At alpha > 0.05, the means between groups are statistically significant for liver and kidney.

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.
Liver ROW	Control	T100	-.928683	.337865	.062
		T200	-.164039	.337865	.961
		T300	1.563225*	.337865	.001
	T100	Control	.928683	.337865	.062
		T200	.764645	.337865	.149
		T300	2.491908*	.337865	.000
	T200	Control	.164039	.337865	.961
		T100	-.764645	.337865	.149
		T300	1.727263*	.337865	.001
	T300	Control	-1.563225*	.337865	.001
		T100	-2.491908*	.337865	.000
		T200	-1.727263*	.337865	.001
Kidney ROW	Control	T100	-.348151*	.073195	.001
		T200	-.104714	.073195	.500
		T300	.122443	.073195	.369
	T100	Control	.348151*	.073195	.001
		T200	.243437*	.073195	.020
		T300	.470594*	.073195	.000
	T200	Control	.104714	.073195	.500
		T100	-.243437*	.073195	.020
		T300	.227157*	.073195	.031
	T300	Control	-.122443	.073195	.369
		T100	-.470594*	.073195	.000
		T200	-.227157*	.073195	.031
Testicles ROW	Control	T100	.133475	.119879	.687
		T200	.006807	.119879	1.000
		T300	.066972	.119879	.943
	T100	Control	-.133475	.119879	.687
		T200	-.126667	.119879	.720
		T300	-.066502	.119879	.944
	T200	Control	-.006807	.119879	1.000
		T100	.126667	.119879	.720
		T300	.060165	.119879	.957

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Treatment	(J) Treatment	95% Confidence Interval	
			Lower Bound	Upper Bound
Liver ROW	Control	T100	-1.89532	.03796
		T200	-1.13068	.80260
		T300	.59659*	2.52986
	T100	Control	-.03796	1.89532
		T200	-.20199	1.73128
		T300	1.52527*	3.45855
	T200	Control	-.80260	1.13068
		T100	-1.73128	.20199
		T300	.76062*	2.69390
	T300	Control	-2.52986*	-.59659
		T100	-3.45855*	-1.52527
		T200	-2.69390*	-.76062
Kidney ROW	Control	T100	-.55756*	-.13874
		T200	-.31413	.10470
		T300	-.08697	.33186
	T100	Control	.13874*	.55756
		T200	.03402*	.45285
		T300	.26118*	.68001
	T200	Control	-.10470	.31413
		T100	-.45285*	-.03402
		T300	.01774*	.43657
	T300	Control	-.33186	.08697
		T100	-.68001*	-.26118
		T200	-.43657*	-.01774
Testicles ROW	Control	T100	-.20950	.47645
		T200	-.33617	.34978
		T300	-.27600	.40995
	T100	Control	-.47645	.20950
		T200	-.46964	.21631
		T300	-.40948	.27648
	T200	Control	-.34978	.33617
		T100	-.21631	.46964
		T300	-.28281	.40314

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference	Std. Error	Sig.

			(I-J)		
Testicles ROW		Control	-.066972	.119879	.943
	T300	T100	.066502	.119879	.944
		T200	-.060165*	.119879	.957

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Treatment	(J) Treatment	95% Confidence Interval	
			Lower Bound	Upper Bound
Testicles ROW		Control	-.40995	.27600
	T300	T100	-.27648	.40948
		T200	-.40314*	.28281

*. The mean difference is significant at the 0.05 level.

At alpha > 0.05, there is a statistically significant difference in the means of ROW of liver between control and T300 treatment groups. The difference also exist in the ROW of kidney between the control and T100 treatment groups. There is no significant difference between the means of ROW in testicles across all groups.

Homogeneous Subsets

Liver ROW

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
T300	5	4.17994	
Control	5		5.74316
T200	5		5.90720
T100	5		6.67185
Sig.		1.000	.062

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Kidney ROW

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
T300	5	.86073		
Control	5	.98317	.98317	

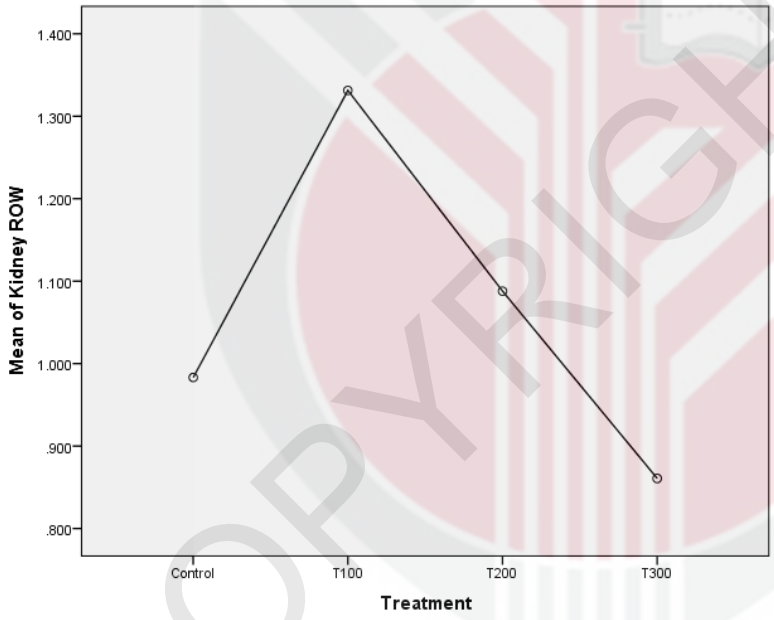
T200	5		1.08789	
T100	5			1.33132
Sig.		.369	.500	1.000

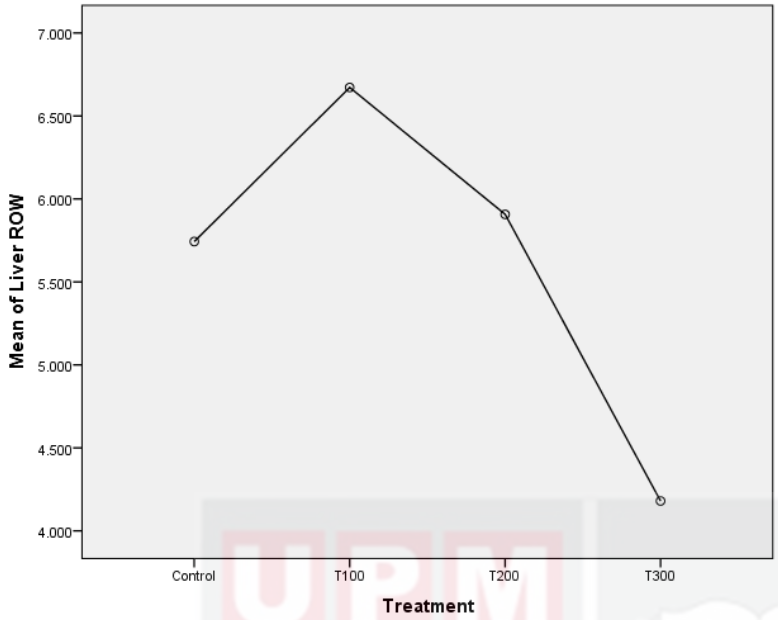
Testicles ROW

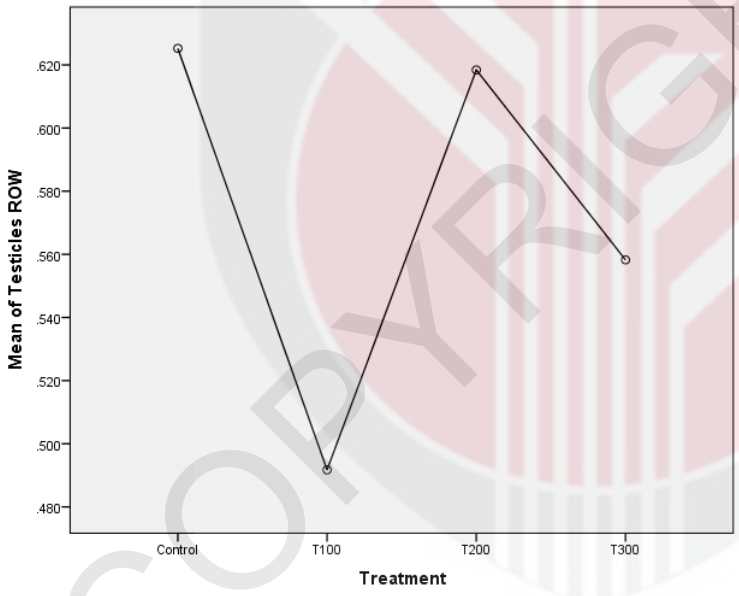
Tukey HSD

Treatment	N	Subset for alpha = 0.05
		1
T100	5	.49174
T300	5	.55824
T200	5	.61841
Control	5	.62522
Sig.		.687

Means Plots







APPENDIX C

Histopathological Scoring Data

Results											
	Liver				Kidney			Testes			
Groups	PMN infiltration	Collagen deposition	Ballooning hepatocytes	Acidophil bodies	PMN infiltration	Tubular necrosis	Interstitial oedema	Presence of sperm cells	Tubule vacuolation	BTB breakdown	Notes
Cx											
C1	None	None	None	None	None	None	Few	+	+++	+++	No tubular necrosis seen. Nuclei are normal shaped
C2	None	None	None	None	None	None	Few	+	none	none	
C3	None	None	None	None	None	None	Few	+	+++	+++	
C4	None	None	None	None	None	None	Few	+++	+	+	Interstial edema observed. Tubules are engorged, lumen of tubule becomes small

C5	None	None	None	None	None	None	Few	++	+++	+++	
Tx 100											
T1001	None	None	Few	None	None	None	Many	+	none	none	
T1002	None	None	Few	None	None	None	Many	+	+	+	Bowman's capsule loses its structure, becomes irregular shaped
T1003	None	None	Few	None	None	None	Many	++	++	++	
T1004	None	None	Few	None	None	None	Many	+	+++	++	
T1005	None	None	Few	None	None	None	Many	+	++	++	
Tx 200											
T2001	None	None	Few	None	None	None	Many	+	+++	++	
T2002	None	None	Few	None	None	None	Many	+	+	+	
T2003	None	None	Few	None	None	None	Many	++	+	++	
T2004	None	None	Few	None	None	None	Many	+	++	+++	
T2005	None	None	Few	None	None	None	Many	+	+++	+++	
Tx 300											
T3001	None	None	Many	None	None	None	Numerous	+	+++	+++	
T3002	None	None	Many	None	None	None	Numerous	+	+++	+++	
T3003	None	None	Many	None	None	None	Numerous	+	++++	++++	
T3004	None	None	Many	None	None	None	Numerous	+	++++	++++	

T3005	None	None	Many	None	None	None	Numerous	+	+++++	+++++	

Those are the findings from my observation:

1. Kidney
- T200K1, T300K2, T300K3- hydropic changes of the tubules
Interpretation: Consistent with reversible cellular injury

2. Liver
T100L3, T100L4, T200L2 until T200Lextra1, T300 (all images)- Hydropic changes of hepatocytes
T200L4, T300L3, T300L4- Occasional binucleated hepatocytes and occasional pyknotic nuclei
Interpretation: Reversible cellular injury with areas of irreversible injury/ cell death

3. Testes
T300 (all images) and some images of T200- hydropic changes
Interpretation: Consistent with reversible cellular injury

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Pathology Department

Faculty of Medicine and Health Sciences

Universiti Putra Malaysia

43400 Serdang

APPENDIX D

SPSS Output for Liver Histopathology

```
GET DATA /TYPE=XLSX
  /FILE='C:\Users\WIN10\OneDrive\Desktop\efwhypee\analysis\liver.xlsx'
  /SHEET=name 'Sheet1'
  /CELLRANGE=full
  /READNAMES=on
  /ASSUMEDSTRWIDTH=32767.
EXECUTE.
DATASET NAME DataSet1 WINDOW=FRONT.
NPAR TESTS
  /K-W=PMNinfiltration Collagendeposition Ballooninghepatocytes
Acidophilbodies BY Treatment(0 3)
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS.
```

NPar Tests

a. Based on availability of workspace memory.

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
PMN infiltration	20	.00	.000	0	0
Collagen deposition	20	.00	.000	0	0
Ballooning hepatocytes	20	1.00	.725	0	2
Acidophil bodies	20	.00	.000	0	0
Treatment	20	1.50	1.147	0	3

Kruskal-Wallis Test

Ranks

	Treatment	N	Mean Rank
PMN infiltration	Control	5	10.50
	T100	5	10.50
	T200	5	10.50
	T300	5	10.50
	Total	20	
Collagen deposition	Control	5	10.50
	T100	5	10.50
	T200	5	10.50
	T300	5	10.50
	Total	20	
Ballooning hepatocytes	Control	5	3.00
	T100	5	10.50
	T200	5	10.50

	T300	5	18.00
	Total	20	
Acidophil bodies	Control	5	10.50
	T100	5	10.50
	T200	5	10.50
	T300	5	10.50
	Total	20	

Test Statistics[a,b]

	PMN infiltration	Collagen deposition	Ballooning hepatocytes	Acidophil bodies
Chi-Square	.000	.000	19.000	.000
df	3	3	3	3
Asymp. Sig.	1.000	1.000	.000	1.000

a. Kruskal Wallis Test

b. Grouping Variable: Treatment

A Kruskal-Wallis test showed that there was a statistically significant difference in balloning hepatocytes between different A.muricata dosage, X(squared)(3) = 19, p < 0.05, with a mean rank score of 3.00 for Group A, 10.50 for Group B and Group C, and 18.00 for Group D.

```
NPAR TESTS
  /M-W= Ballooninghepatocytes PMNinfiltration Collagendeposition
Acidophilbodies BY Treatment(0 1)
  /STATISTICS=DESCRIPTIVES
  /MISSING ANALYSIS.
```

NPar Tests

a. Based on availability of workspace memory.

```
[DataSet1]
```

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Ballooning hepatocytes	20	1.00	.725	0	2
PMN infiltration	20	.00	.000	0	0
Collagen deposition	20	.00	.000	0	0
Acidophil bodies	20	.00	.000	0	0
Treatment	20	1.50	1.147	0	3

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
Ballooning hepatocytes	Control	5	3.00	15.00
	T100	5	8.00	40.00
	Total	10		
PMN infiltration	Control	5	5.50	27.50
	T100	5	5.50	27.50
	Total	10		
Collagen deposition	Control	5	5.50	27.50
	T100	5	5.50	27.50
	Total	10		
Acidophil bodies	Control	5	5.50	27.50
	T100	5	5.50	27.50
	Total	10		

Test Statistics[a]

	Ballooning hepatocytes	PMN infiltration	Collagen deposition	Acidophil bodies
Mann-Whitney U	.000	12.500	12.500	12.500
Wilcoxon W	15.000	27.500	27.500	27.500
Z	-3.000	.000	.000	.000
Asymp. Sig. (2-tailed)	.003	1.000	1.000	1.000
Exact Sig. [2*(1-tailed Sig.)]	.008[b]	1.000[b]	1.000[b]	1.000[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There is a significant difference (U=0, p=0.008) between ballooning hepatocytes
for group B compared to group A.

```
NPAR TESTS
  /M-W= Ballooninghepatocytes PMNinfiltration Collagendeposition
Acidophilbodies BY Treatment(0 2)
  /STATISTICS=DESCRIPTIVES
```

```
  /MISSING ANALYSIS.
```

NPar Tests

a. Based on availability of workspace memory.

```
[DataSet1]
```

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
Ballooning hepatocytes	Control	5	3.00	15.00
	T200	5	8.00	40.00
	Total	10		
PMN infiltration	Control	5	5.50	27.50
	T200	5	5.50	27.50
	Total	10		
Collagen deposition	Control	5	5.50	27.50
	T200	5	5.50	27.50
	Total	10		
Acidophil bodies	Control	5	5.50	27.50
	T200	5	5.50	27.50
	Total	10		

Test Statistics[a]

	Ballooning hepatocytes	PMN infiltration	Collagen deposition	Acidophil bodies
Mann-Whitney U	.000	12.500	12.500	12.500
Wilcoxon W	15.000	27.500	27.500	27.500
Z	-3.000	.000	.000	.000
Asymp. Sig. (2-tailed)	.003	1.000	1.000	1.000
Exact Sig. [2*(1-tailed Sig.)]	.008[b]	1.000[b]	1.000[b]	1.000[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There is a significant difference (U=0, p=0.008) between ballooning hepatocytes
 for group C compared to group A.

```
NPAR TESTS
  /M-W= Ballooninghepatocytes PMNinfiltration Collagendeposition
Acidophilbodies BY Treatment(0 3)
```

```
  /STATISTICS=DESCRIPTIVES
  /MISSING ANALYSIS.
```

NPar Tests

a. Based on availability of workspace memory.

```
[DataSet1]
```

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
Ballooning hepatocytes	Control	5	3.00	15.00
	T300	5	8.00	40.00
	Total	10		
PMN infiltration	Control	5	5.50	27.50
	T300	5	5.50	27.50
	Total	10		
Collagen deposition	Control	5	5.50	27.50
	T300	5	5.50	27.50
	Total	10		
Acidophil bodies	Control	5	5.50	27.50
	T300	5	5.50	27.50
	Total	10		

Test Statistics[a]

	Ballooning hepatocytes	PMN infiltration	Collagen deposition	Acidophil bodies
Mann-Whitney U	.000	12.500	12.500	12.500
Wilcoxon W	15.000	27.500	27.500	27.500
Z	-3.000	.000	.000	.000
Asymp. Sig. (2-tailed)	.003	1.000	1.000	1.000
Exact Sig. [2*(1-tailed Sig.)]	.008[b]	1.000[b]	1.000[b]	1.000[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There is a significant difference (U=0, p=0.008) between ballooning hepatocytes
 for group D compared to group A.

APPENDIX E

SPSS Output for Kidney Histopathology

```
NEW FILE.
DATASET NAME DataSet2 WINDOW=FRONT.
DATASET ACTIVATE DataSet2.
DATASET CLOSE DataSet1.
GET DATA /TYPE=XLSX
  /FILE='C:\Users\WIN10\OneDrive\Desktop\efwhypee\analysis\kidney.xlsx'
  /SHEET=name 'Sheet1'
  /CELLRANGE=full
  /READNAMES=on
  /ASSUMEDSTRWIDTH=32767.
EXECUTE.
DATASET NAME DataSet3 WINDOW=FRONT.
DATASET ACTIVATE DataSet3.
DATASET CLOSE DataSet2.
NPAR TESTS
  /K-W=PMNinfiltration Tubularnecrosis Interstitialoedema BY Treatment(0 3)
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS.
```

NPar Tests

```
[DataSet3]
```

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
PMN infiltration	20	.00	.000	0	0
Tubular necrosis	20	.00	.000	0	0
Interstitial oedema	20	2.00	.725	1	3
Treatment	20	1.50	1.147	0	3

Kruskal-Wallis Test

Ranks

	Treatment	N	Mean Rank
PMN infiltration	0	5	10.50
	1	5	10.50
	2	5	10.50
	3	5	10.50
	Total	20	
Tubular necrosis	0	5	10.50
	1	5	10.50
	2	5	10.50
	3	5	10.50
	Total	20	
Interstitial oedema	0	5	3.00

	1	5	10.50
	2	5	10.50
	3	5	18.00
	Total	20	

Test Statistics[a,b]

	PMN infiltration	Tubular necrosis	Interstitial oedema
Chi-Square	.000	.000	19.000
df	3	3	3
Asymp. Sig.	1.000	1.000	.000

a. Kruskal Wallis Test

b. Grouping Variable: Treatment

A Kruskal-Wallis test showed that there was a statistically significant difference in interstitial oedema between different A.muricata dosage, X(squared)(3) = 19, p < 0.05, with a mean rank score of 3.00 for Group A, 10.50 for Group B and Group C, and 18.00 for Group D.

```
NPAR TESTS
  /M-W= PMNinfiltration Tubularnecrosis Interstitialoedema BY Treatment(0
1)
  /STATISTICS=DESCRIPTIVES
  /MISSING ANALYSIS.
```

NPar Tests

[DataSet3]

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
PMN infiltration	20	.00	.000	0	0
Tubular necrosis	20	.00	.000	0	0
Interstitial oedema	20	2.00	.725	1	3
Treatment	20	1.50	1.147	0	3

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
PMN infiltration	0	5	5.50	27.50
	1	5	5.50	27.50
	Total	10		
Tubular necrosis	0	5	5.50	27.50
	1	5	5.50	27.50
	Total	10		

	0	5	3.00	15.00
Interstitial oedema	1	5	8.00	40.00
	Total	10		

Test Statistics[a]

	PMN infiltration	Tubular necrosis	Interstitial oedema
Mann-Whitney U	12.500	12.500	.000
Wilcoxon W	27.500	27.500	15.000
Z	.000	.000	-3.000
Asymp. Sig. (2-tailed)	1.000	1.000	.003
Exact Sig. [2*(1-tailed Sig.)]	1.000[b]	1.000[b]	.008[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There is a significant difference (U=0, p=0.008) between interstitial oedema for group B compared to group A.

```
NPAR TESTS
  /M-W= PMNinfiltration Tubularnecrosis Interstitialoedema BY Treatment(0
2)
```

NPar Tests

[DataSet3]

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
	0	5	5.50	27.50
PMN infiltration	2	5	5.50	27.50
	Total	10		
	0	5	5.50	27.50
Tubular necrosis	2	5	5.50	27.50
	Total	10		
	0	5	3.00	15.00
Interstitial oedema	2	5	8.00	40.00
	Total	10		

Test Statistics[a]

	PMN infiltration	Tubular necrosis	Interstitial oedema
Mann-Whitney U	12.500	12.500	.000
Wilcoxon W	27.500	27.500	15.000

Z	.000	.000	-3.000
Asymp. Sig. (2-tailed)	1.000	1.000	.003
Exact Sig. [2*(1-tailed Sig.)]	1.000[b]	1.000[b]	.008[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There is a significant difference (U=0, p=0.008) between interstitial oedema for group C compared to group A.

```
NPAR TESTS
  /M-W= PMNinfiltration Tubularnecrosis Interstitialoedema BY Treatment(0
3)
  /STATISTICS=DESCRIPTIVES
  /MISSING ANALYSIS.
```

NPar Tests

```
[DataSet3]
```

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
PMN infiltration	0	5	5.50	27.50
	3	5	5.50	27.50
	Total	10		
Tubular necrosis	0	5	5.50	27.50
	3	5	5.50	27.50
	Total	10		
Interstitial oedema	0	5	3.00	15.00
	3	5	8.00	40.00
	Total	10		

Test Statistics[a]

	PMN infiltration	Tubular necrosis	Interstitial oedema
Mann-Whitney U	12.500	12.500	.000
Wilcoxon W	27.500	27.500	15.000
Z	.000	.000	-3.000
Asymp. Sig. (2-tailed)	1.000	1.000	.003
Exact Sig. [2*(1-tailed Sig.)]	1.000[b]	1.000[b]	.008[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There is a significant difference (U=0, p=0.008) between interstitial oedema for group D compared to group A.

APPENDIX F

SPSS Output for Testes Histopathology

```
NPAR TESTS
  /K-W=Presence Tubule BTB BY Treatment(0 3)
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS.
```

NPar Tests

```
[DataSet2]
```

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Presence	20	1.25	.550	1	3
Tubule	20	2.35	1.348	0	5
BTB	20	2.35	1.309	0	5
Treatment	20	1.50	1.147	0	3

Kruskal-Wallis Test

Ranks

	Treatment	N	Mean Rank
Presence	Control	5	12.70
	T100	5	10.40
	T200	5	10.40
	T300	5	8.50
	Total	20	
Tubule	Control	5	9.30
	T100	5	7.10
	T200	5	8.80
	T300	5	16.80
	Total	20	
BTB	Control	5	9.50
	T100	5	5.90
	T200	5	9.60
	T300	5	17.00
	Total	20	

Test Statistics[a,b]

	Presence	Tubule	BTB
Chi-Square	2.606	8.586	9.929
df	3	3	3

Asymp. Sig.	.456	.035	.019

a. Kruskal Wallis Test

b. Grouping Variable: Treatment

A Kruskal-Wallis test showed that there was a statistically significant difference in tubule vacuolation between different A.muricata dosage, X(squared)(3) = 8.586, p = 0.035, with a mean rank score of 9.30 for Group A, 7.10 for Group B, 8.80 for Group C, and 16.80 for Group D.

A Kruskal-Wallis test showed that there was a statistically significant difference in BTB breakdown between different A.muricata dosage, X(squared)(3) = 9.929, p = 0.019, with a mean rank score of 9.50 for Group A, 5.90 for Group B, 9.60 for Group C, and 17.00 for Group D.

There is a significant difference in the means of Tubule vacuolation and BTB breakdown as p-value is smaller than alpha.

```
NPAR TESTS
  /M-W= Presence Tubule BTB BY Treatment(0 1)
  /MISSING ANALYSIS.
```

NPar Tests

```
[DataSet2]
```

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
Presence	Control	5	6.10	30.50
	T100	5	4.90	24.50
	Total	10		
Tubule	Control	5	6.10	30.50
	T100	5	4.90	24.50
	Total	10		
BTB	Control	5	6.40	32.00
	T100	5	4.60	23.00
	Total	10		

Test Statistics[a]

	Presence	Tubule	BTB

Mann-Whitney U	9.500	9.500	8.000
Wilcoxon W	24.500	24.500	23.000
Z	-.775	-.653	-.970
Asymp. Sig. (2-tailed)	.439	.514	.332
Exact Sig. [2*(1-tailed Sig.)]	.548[b]	.548[b]	.421[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There is no significant difference between control and T100 in all 3 parameters.

```
NPAR TESTS
  /M-W= Presence Tubule BTB BY Treatment(0 2)
  /MISSING ANALYSIS.
```

NPar Tests

```
[DataSet2]
```

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
Presence	Control	5	6.10	30.50
	T200	5	4.90	24.50
	Total	10		
Tubule	Control	5	5.60	28.00
	T200	5	5.40	27.00
	Total	10		
BTB	Control	5	5.50	27.50
	T200	5	5.50	27.50
	Total	10		

Test Statistics[a]

	Presence	Tubule	BTB
Mann-Whitney U	9.500	12.000	12.500
Wilcoxon W	24.500	27.000	27.500
Z	-.775	-.113	.000
Asymp. Sig. (2-tailed)	.439	.910	1.000
Exact Sig. [2*(1-tailed Sig.)]	.548[b]	1.000[b]	1.000[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There is no significant difference between control and T200 in all 3 parameters.

```
NPAR TESTS
  /M-W= Presence Tubule BTB BY Treatment(0 3)
  /MISSING ANALYSIS.
```

NPar Tests

Mann-Whitney Test

Ranks

	Treatment	N	Mean Rank	Sum of Ranks
Presence	Control	5	6.50	32.50
	T300	5	4.50	22.50
	Total	10		
Tubule	Control	5	3.60	18.00
	T300	5	7.40	37.00
	Total	10		
BTB	Control	5	3.60	18.00
	T300	5	7.40	37.00
	Total	10		

Test Statistics[a]

	Presence	Tubule	BTB
Mann-Whitney U	7.500	3.000	3.000
Wilcoxon W	22.500	18.000	18.000
Z	-1.491	-2.124	-2.124
Asymp. Sig. (2-tailed)	.136	.034	.034
Exact Sig. [2*(1-tailed Sig.)]	.310[b]	.056[b]	.056[b]

a. Grouping Variable: Treatment

b. Not corrected for ties.

There are no significant differences between control and T300 (p-value > alpha)
(U=3, p= 0.056)and (U=7.5, p=0.310)

APPENDIX G

Effects of AMLE on male ICR mice histopathological parameters after 35 days of treatment.

Parameters	Groups							
	A (Control)		B (100 mg/kg)		C (200 mg/kg)		D (300 mg/kg)	
	Kruskal-Wallis	Mann-Whitney U	Kruskal-Wallis	Mann-Whitney U	Kruskal-Wallis	Mann-Whitney U	Kruskal-Wallis	Mann-Whitney U
Ballooning hepatocytes (liver)	3^{a}	3^{b}	10.5^{a}	8^{b}	10.5^{a}	8^{b}	18^{a}	8^{b}
Interstitial oedema (kidney)	3^{c}	3^{d}	10.5^{c}	8^{d}	10.5^{c}	8^{d}	18^{c}	8^{d}

Note: Data are presented as mean rank. ^{abcd} Superscripts are prescribed on the mean value that are statistically significant at ($p < 0.05$) compared with control group.