UNIVERSITI PUTRA MALAYSIA

HISTOPATHOLOGICAL ASSESSMENT OF ACETAMINOPHEN-INDUCED LIVER INJURY AND REGENERATION RESPONSE FOLLOWING EDIBLE BIRD'S NEST ADMINISTRATION IN ICR AND BALB/C MICE MODEL

MUHAMMAD AZAM BIN FAZIL

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By

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Acetaminophen (APAP) overdose is known to induce liver injury in mice models and humans. Excessive accumulation of reactive metabolite, N-acetyl-p-benzoquinone-imine (NAPQI) increases oxidative stress in tissues, leading to centrilobular necrosis after exceeding the glutathione (GSH) threshold. Although liver recovery takes place after clearance of toxic insults, however, the degree of hepatic regeneration differs between mice strains. Therefore, the first aim of the study was a comparative assessment of APAP-induced liver injury (AILI) and subsequent hepatic regeneration of two mouse models (ICR and BALB/c). Meanwhile, edible bird's nest (EBN) is a well-known natural product with various health-enhancing properties including anti-oxidative and cell proliferative effects. However, the role of EBN from a toxicological perspective against APAP toxicity is lacking. Hence, the second objective aimed to assess the prophylactic and regenerative effect of EBN on AILI response in ICR mice. These studies hypothesized that both models respond differently, and self-regenerating hepatocytes could be prompted by prophylaxis EBN. For the first objective, 25 ICR and 20 BALB/c mice were grouped as controls and treatments of 5, 10, 24, and 48 hours post-APAP dosing (hpd) with 5 animals per each. Secondly, 80 ICR mice were assigned to groups of control, APAP (500 mg/kg) and seven days prophylactic of silymarin (200 mg/kg), and EBN (60, 120, and 250 mg/kg) followed by an APAP induction. Livers were harvested for histopathological assessment by haematoxylin and eosin (H&E) staining and proliferating cell nuclear antigen (PCNA) using immunohistochemistry. Results of the first experiment showed that APAP-treated BALB/c mice had an intense hepatocellular injury at 5 hpd than ICR mice that only exhibited damage at 10 hpd before both underwent almost complete regeneration after 24 hpd. The second study revealed significant differences in histological changes between APAP and prophylactic treatment groups at 10 hpd, with complete recovery of all groups observed at 24 hpd except for EBN 250 that sustained injuries. Hepatocytes proliferation was initiated at 5 hpd in silymarin, EBN 60 and EBN 120, while at 24 hpd, EBN 120 and 250 exhibited higher PCNA expressing hepatocytes. The hepatoprotective role was observed earlier in silymarin, EBN 60 and 120, while cellular proliferation was delayed in EBN 250. In

conclusion, all groups showed liver recovery after clearance of APAP insult at later time points but EBN 60 and 120 enhanced the hepatic proliferation as similar to silymarin. Therefore, this proves EBN 60 and 120 could act as prophylaxis liver supplements to accelerate the hepatic regeneration in AILI.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Master Sains

PENILAIAN HISTOPATOLOGI TERHADAP PENCETUSAN ACETAMINOPHEN KEPADA KECEDERAAN HATI DAN TINDAK BALAS PERTUMBUHAN SEMULA SELEPAS PEMAKANAN SARANG BURUNG BOLEH DIMAKAN DI DALAM MODEL MENCIT ICR DAN BALB/C

Oleh

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Dos acetaminophen (APAP) yang berlebihan dapat mencetuskan kecederaan pada hati di dalam model mencit dan manusia. Lebihan metabolit reaktif, N-acetyl-p-benzoquinone-imine (NAPQI) menambahkan tekanan oksidatif pada tisu dan menyebabkan nekrosis sentrilobular apabila melepasi tahap ambang glutation (GSH). Walaupun pemulihan hati berlaku selepas toksik dibersihkan, namun, darjah percambahan semula hepatik berbeza mengikut jenis mencit. Oleh itu, sasaran pertama untuk kajian ini adalah menilai perbandingan terhadap pencetusan kecederaan hati oleh APAP (AILI) dan seterusnya penjanaan semula hepatik pada kedua-dua model mencit (ICR dan BALB/c). Sementara itu, sarang burung boleh dimakan (EBN) yang merupakan produk semula jadi dengan kepelbagaian ciri-ciri peningkatan kesihatan seperti kesan antioksida dan percambahan sel. Walau bagaimanapun, peranan EBN dalam perspektif toksikologi terhadap ketoksikan APAP masih lagi kurang. Maka, objektif kajian yang kedua adalah untuk menilai kesan profilaksis dan percambahan semula oleh EBN pada tindak balas AILI dalam model mencit ICR. Kajian ini menghipotesis tindak balas kedua-dua model mencit berbeza dan percambahan semula jadi hepatosit boleh ditingkatkan dengan profalaksis EBN. Untuk objektif pertama, 25 mencit ICR dan 20 mencit BALB/c dikelompokkan sebagai kawalan dan rawatan pada 5, 10, 24, dan 48 jam selepas dos APAP (hpd) dengan 5 ekor dalam setiap kumpulan. Keduanya, 80 ekor mencit ICR dibahagikan kepada kumpulan kawalan, APAP (500 mg/kg), dan profilaksis 7 hari oleh silymarin (200 mg/kg) dan EBN (60, 120 dan 250 mg/kg diikuti dengan rawatan APAP. Semua sampel hati dikumpulkan untuk penilaian histopatologi dengan pewarnaan haematoxylin and eosin (H&E) dan proliferasi antigen sel nuklear (PCNA) menggunakan immunohistokimia. Keputusan untuk eksperimen pertama menunjukan mencit BALB/c dirawat APAP mempunyai kecederaan hepatoselular yang tinggi pada 5 hpd berbanding mencit ICR yang menunjukan kecederaan yang teruk pada 10 hpd sebelum kedua-duanya melalui proses percambahan semula pada 24 hpd. Kajian kedua menunjukan perbezaan yang signifikan pada perubahan histologi antara APAP dan kumpulan rawatan profalaksis pada 10 hpd

bersama percambahan semula dengan sempurna dalam semua kumpulan pada 24 hpd kecuali untuk EBN 250 dengan mengekalkan kecederaan. Proliferasi hepatosit dicetuskan pada 5 hpd untuk kumpulan silymarin, EBN 60 dan EBN 120, manakala proliferasi untuk EBN 250 telah ditangguhkan. Konklusinya, semua kumpulan menunjukan pemulihan selepas pengurangan APAP pada titik masa yang akhir walaupun tanpa profilaksis EBN tetapi EBN 60 and 120 meningkatkan percambahan semula hepatik seperti silymarin. Ini membuktikan EBN 60 dan 120 dapat menjadi makanan tambahan secara profilaktik untuk mempercepatkan percambahan semula hepatik pada AILI.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

[^{3}H]-TdR	Tritiated thymidine
+ve	Positive
-ve	Negative
1BP	1-bromopropane
A.D	Anno domini
AASLD	American Association for the Study of Liver Diseases
ABTS	Azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]
AILI	APAP-induced liver injury
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APAP	Acetaminophen
AST	Aspartate aminotransferase
BrDU	Bromodeoxyuridine
BWT	Bodyweight
^oC	Degree Celsius
c-met	tyrosine-protein kinase Met
Caco-2	colonic adenocarcinoma cell line
CAT	Catalase
CCl_3-OO*	Trichlorometane
CCl_4	carbon tetrachloride
CNS	Central nervous system
Cyp1A2	Cytochrome P-4501A2
Cyp2E1	Cytochrome P-4502E1
CYP-450	Cytochrome P-450

DAB	3,3′-diaminobenzidine tetrahydrochloride
ddH_2O	Distilled water
DEN	Diethylnitrosamine
DILI	Drug-induced liver injury
DNA	Deoxyribonucleic acid
DPX	Dibutyl phthalate polystyrene xylene
EBN	Edible bird's nest
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
g	Gram
GalN	Galactosamine
galNAc	N-acetylgalactosamine
glcNAc	N-acetylglucosamine
GSH	Glutathione
Gsr	Glutathione reductase
GST	Glutathione S-transferase
Gsx	Glutathione oxidase
H_2O_2	Hydrogen peroxide
HAC	Human articular chondrocytes
H&E	Haematoxylin and eosin
HGF	Hepatic growth factor
hpd	Hours post APAP dosing
IACUC	Institutional of Animal Care and Use Committee
i.p	Intraperitoneal
IL-6	Interleukin-6
kg	Kilogram

LPS	Lipopolysaccharide
µL	Millilitre
MDA	Malondialdehyde
mg	Milligram
mL	Millilitre
NAC	N-acetyl-cysteine
NaCl	Sodium chloride
NANA	N-acetylneuraminic acid
NAPQI	N-acetyl-p-benzo-quinine-imine
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
OECD	Organizational of Economic Corporation and Development
PCNA	Proliferating cell nuclear antigen
PH	Partial hepatectomy
p.s.i	Pound per square inch
ROS	Reduced reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Science
TAA	Thioacetamide
TAASO	Thioacetamide S-oxide
TNF-α	Tumour necrosis factor-alpha
TRS	Target retrieval solution
TSST	Toxic shock syndrome toxin
VEGF	Vascular epidermal growth factor

CHAPTER 1

INTRODUCTION

1.1 Background of study

Acetaminophen (APAP) has been used as an ideal toxicant to induce liver injury in a xenobiotic study (Ostapowicz et al., 2002). Liver injury is exhibited by hepatocellular necrosis as a result of APAP overdose due to excessive production of a reactive metabolite of APAP that is known as N-acetyl-p-benzo-quinone-imine (NAPQI). Accumulation of NAPQI occurs when exceeding the capacity of the antioxidant threshold due to depletion of glutathione (GSH), a scavenger within hepatocytes, that responsible to detoxify the reactive metabolites (Botta et al., 2006). Then, excessive NAPQI will form a covalent bond with intracellular protein for the formation of protein adducts in mitochondria leading to the occurrence of oxidative stress. As a consequence, the toxic oxygen species generate oxidation to critical cellular protein, deoxyribonucleic acid (DNA), and lipid that later on contribute to hepatocytes necrosis. However, hepatocytes are capable to proliferate by entering the mitotic cell cycle to replace the damaged and dead cells. Hence, the recovery of liver injury has proven to take place after the clearance of toxic insult due to the restitution of the hepatic GSH level.

Many studies have been conducted to obtain a relevant animal model that resembles APAP-induced liver injury (AILI) in humans. Laboratory rodents have been recognised as the most suitable study models in preclinical studies of drug hepatotoxicity (Jaeschke et al., 2014). However, laboratory rat was represented as a poor study model to demonstrate hepatotoxicity as it showed resistance towards APAP intoxication (McGill et al., 2012) due to the absence of mitochondrial dysfunction and reduced protein adducts in mitochondria (McGill et al., 2012). Meanwhile, laboratory mouse has been known to be a relevant study model of AILI as it resembles the responses of APAP toxicity in the human body (Jaeschke, 2015) with the exhibition of mitochondrial damage and DNA fragmentation induced by APAP intoxication in mice (Jollow et al., 1973). The different responses of mice gender on APAP toxicity are also studied where male mice were found to be more susceptible to hepatotoxicity than female counterpart due to induction of glutamate-cysteine ligase that correlate with faster recovery of GSH (Ramachandran & Jaeschke, 2017).

Nowadays, natural products have become favourable to some people. To overcome certain adverse effects of xenobiotics, people may shift to alternative medicinal products. One of the nutritious and natural products that have gained more attention recently is the edible bird's nest (EBN). This glutinous EBN is made from the saliva of swiftlets of the *Apopidae* family mainly from the white nest swiftlets (*Aerodramus fuciphagus*) and black nest swiftlets (*Aerodramus maximus*) (Babji et al., 2015; Sheldon, 2011). The blooming EBN industry has become an excellent opportunity to assure a lucrative return for the producers, as there is a high demand for EBN in the world's market. The value of EBN is worth the labour efforts of the farmer and harvester with the range of RM 4,000

to RM 5,000 per kilogram for raw EBN and RM 6,000 to RM 8,000 per kilogram for cleaned EBN (Mohamad Shukri et al., 2018). Artificial birdhouses built by farmers can attract swiftlets to build their nest as an alternative way for nesting instead of caves. South-East Asian countries are widely known region as EBN consumer and the highest producer of EBN globally in which Malaysia is one of the main EBN producers alongside Indonesia and Thailand.

EBN has been originally introduced as Chinese traditional medicine to be consumed for the benefits of health thousands of years ago. However, the health-promoting effects of EBN are based on anecdotal, historical, and observational reports since the 17th century (Vimala et al., 2012). Due to this discovery, EBN becomes one of the interest points in natural medicine products. It was first scientifically studied by Ng et al. (1986) who reported the potential effects of EBN in cell proliferation of the immune cells. Thereafter, many scientific studies were conducted to prove the benefits of EBN with the improvement of life quality by various health promising components such as high protein and carbohydrates, including glycoprotein, sialic acid, and other essential trace elements like zinc, iron, manganese, magnesium, calcium, and sodium (Marcone, 2005; Norhayati et al., 2010). A recent study also believed that it helps to reduce oxidative stress (Yida et al., 2014), increase cognitive function (Careena et al., 2018), enhance cell proliferation (Albishtue et al., 2018) and antiviral agent (Akmal et al., 2017).

1.2 Problem statement

The effect of APAP toxicity in humans and mice are almost undistinguishable. However, the extensively used laboratory mice in AILI have become a crucial reason to understand the different reactions of various mouse strains. Differences in mouse strain contribute to the variation in responses of APAP hepatotoxicity as each strain possess a distinct genetic line-up (Shahid & Subhan, 2014). Outbred stocks were known as genetically undefined mice with a heterogeneous genetic line that resembles humans (Chia et al., 2005). Inbred, however, possibly exhibit a high similarity of APAP toxicity response as inbred possess homogenous genetic line-up (Festing, 2010). Diverse mice strain is a challenge for researchers in choosing the suitable mouse model to ensure data validation for drug hepatotoxicity in humans. Even though the response of different strains of mice is still variable, toxic insult will be metabolised and cleared out throughout the body. Subsequently, recovery of the liver has taken place that could be observed by the regeneration of hepatocytes. Thus, the first part of this study is focusing on the assessment of histological changes of APAP–induced liver injury in different strains of ICR and BALB/c mice based on an AILI grading score system. The degree of hepatic regeneration is also evaluated after the clearance of APAP toxicity.

Positive effects of EBN on health have been noticed in toxicology and pharmacology studies especially as antioxidant and cell proliferative agent (Li et al., 2016). However, studies on the effects of EBN on hepatocytes regeneration after APAP overdose are still scarce and need to be further elucidated to provide a better understanding of the potential of EBN as a supplementary enhancer for liver health. The current study is necessary to prove the proliferation of hepatocytes to determine the degree of the hepatoprotective potential of EBN in AILI. We hypothesise that pre-treatment of mice with EBN extract

enables to enhance the rate of liver cell proliferation following injury. Therefore, in the second part of the study, we aim to explore the hepatoprotective effects of EBN extract in AILI mice.

1.3 Objectives of research

The main objective of this study is to assess the protective role and regenerative enhancement effects of edible bird's nest (EBN) on AILI in mice model
The specific objectives of this study are:

1. to assess and compare the histopathological responses of AILI in ICR and BALB/c mice
2. to assess the degree of protection and hepatic cell regeneration conferred by EBN prophylactic treatment against AILI in ICR mice through histological assessment and proliferation cell nuclear antigen (PCNA) immunostaining

1.4 Hypothesis

The null hypothesis of this study is the histological features of AILI in both outbred and inbred mice are the same with both strains reveal subsequent liver regeneration at later time points after clearance of toxicity whereby the regenerative process is not accelerated by the prophylactic effect of EBN extract.

As the alternative hypothesis, the histological features of AILI in both strains would be significantly different and the regeneration process will occur at a later time point whereby the regeneration process is enhanced by the prophylactic effect of EBN extract.

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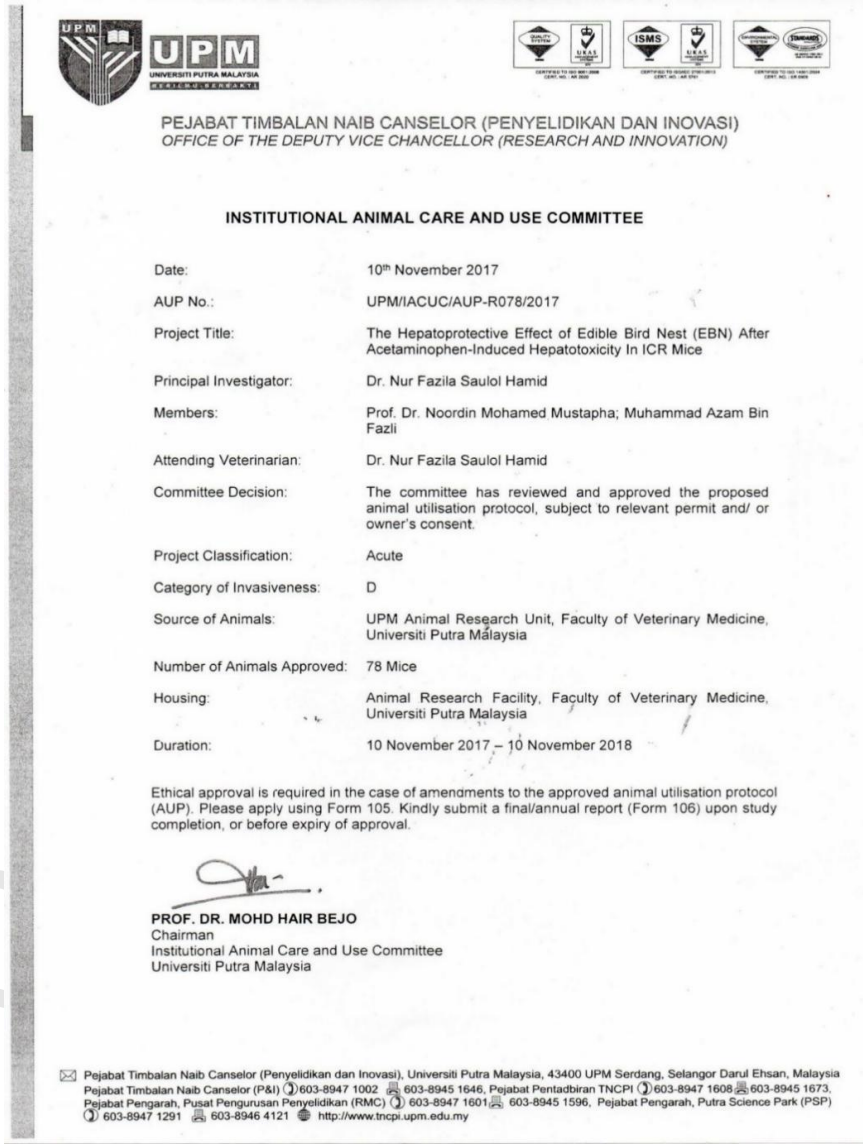
APPENDICES

Appendix A

Approval letter of ethical committee (IACUC), UPM



UPM
UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	10th November 2017
AUP No.:	UPM/IACUC/AUP-R078/2017
Project Title:	The Hepatoprotective Effect of Edible Bird Nest (EBN) After Acetaminophen-Induced Hepatotoxicity In ICR Mice
Principal Investigator:	Dr. Nur Fazila Saulol Hamid
Members:	Prof. Dr. Noordin Mohamed Mustapha; Muhammad Azam Bin Fazli
Attending Veterinarian:	Dr. Nur Fazila Saulol Hamid
Committee Decision:	The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.
Project Classification:	Acute
Category of Invasiveness:	D
Source of Animals:	UPM Animal Research Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia
Number of Animals Approved:	78 Mice
Housing:	Animal Research Facility, Faculty of Veterinary Medicine, Universiti Putra Malaysia
Duration:	10 November 2017 – 10 November 2018

Ethical approval is required in the case of amendments to the approved animal utilisation protocol (AUP). Please apply using Form 105. Kindly submit a final/annual report (Form 106) upon study completion, or before expiry of approval.

PROF. DR. MOHD HAIR BEJO
Chairman
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) 603-8947 1002 603-8945 1646, Pejabat Pentadbiran TNCPI 603-8947 1608 603-8945 1673,
Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-8947 1601, 603-8945 1596, Pejabat Pengarah, Putra Science Park (PSP)
603-8947 1291 603-8946 4121 http://www.tncpi.upm.edu.my

Figure 24. Institutional of Animal Care and Use Committee (IACUC) approval for the animal utilisation protocol in this study.

Appendix B

EBN Preparation Process

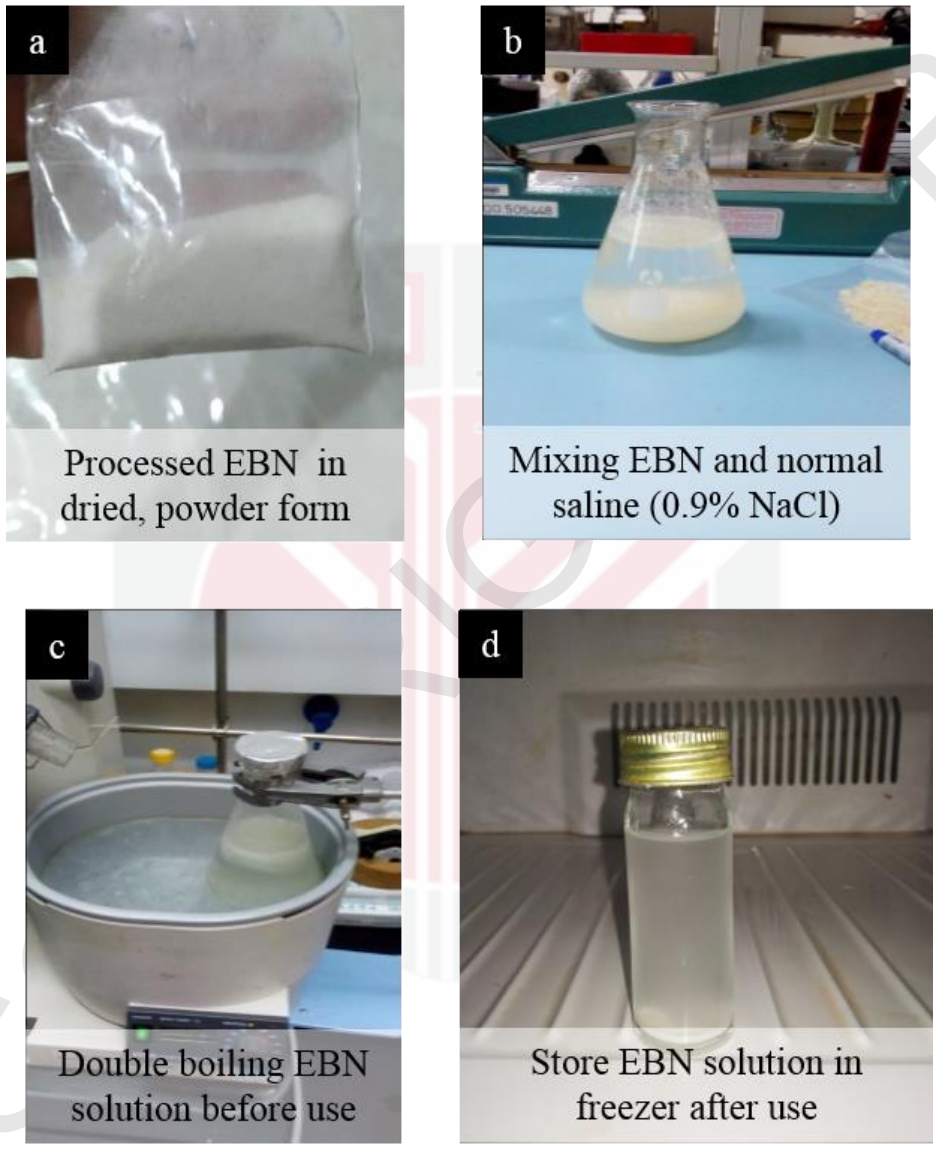


Figure 25. EBN preparation process for the prophylactic study of EBN consumption followed by AILI in ICR mice. (a) EBN was bought in powder form after being processed. (b) EBN powder was diluted with normal saline (0.9% NaCl) to obtain doses of 60, 120, and 250 mg/kg. (c) The mixed solution was double-boiled and let to cool at room temperature. (d) EBN solution was stored in a chiller of 4 ºC and will be heated to 40 ºC before it was instilled in the mouse using oral gavage.

Appendix C

Summary of histopathological description of the liver section

Table 2. Histopathological description of the liver section in each group after 5 hours of APAP induction. The histological features were summarised as a group according to the AILI mean scores calculated for each group.

Group	Score range (Mean ± SD)	Description
Control	0 (0)	Normal hepatocellular features with no histological abnormalities
APAP	0-0.5 (0.15±0.22)	Early hydropic degeneration with variably sized cytoplasmic vacuoles and showed minimal necrosis
Silymarin	0-0.25 (0.15±0.14)	Minimal to none centrilobular necrosis with variably size vacuoles in the centrilobular area
EBN 60	0.5-1 (0.75±0.25)	Minimal hepatocellular necrosis and early hydropic degeneration in the centrilobular area with few mitotic figures were seen
EBN 120	0-0.25 (0.05±0.11)	Scattered individual necrotic cells in the centrilobular area with variable size vacuolation and infiltration of inflammatory cells
EBN 250	1-3.5 (2.67±1.44)	Severe centrilobular necrosis and intense haemorrhage. Macrovascular steatosis was evident by large fat droplets

Table 3. Description of the histological finding of each group after 10 hours post APAP dosing (hpd). The histological descriptions were done based on the AILI mean scores in each group represented by a total of 5 animals per group.

Group	Range score (Mean ± S.D)	Description
APAP	3.5-4 (3.75±0.29)	Extensive centrilobular necrosis and haemorrhage that spread out to almost ¾ of liver sections
Silymarin	0 (0)	Completely intact liver cells with no histological abnormalities
EBN 60	0-0.5 (0.25±0.25)	An extensive area of normal hepatocytes compared to individual necrotic cells with few presences of mitotic figures
EBN 120	0.25-1.5 (0.6±0.52)	Normal hepatocytes cover a broad area of liver sections and some scattered individual necrosis particularly the centrilobular area
EBN 250	0-2.5 (1.33±1.26)	Moderate to severe necrosis and haemorrhage at the centrilobular area

Table 4. The histological findings of APAP-induced ICR mice were described in each treatment group based on the AILI mean scores by assessing the hepatocytes changes for individual animals in the groups at 24 hpd.

Group	Range score (Mean ± S.D)	Description
APAP	0.5 (0.5±0)	Minimal cell damage and hydropic degeneration at the centrilobular area of the liver in most of the individual animals in the group, also some degree of mitotic figures
Silymarin	0-0.1 (0.07±0.06)	Normal hepatocytes present with no histological changes and a very minimal degree of hydropic
EBN 60	0-0.25 (0.06±0.13)	The liver section covered mostly with the normal architecture of liver cells and a very minimal degree of hydropic degeneration
EBN 120	0 (0)	Normal hepatocellular features with no histological abnormalities are observed
EBN 250	0-3.5 (1.17±1.75)	Continuous variable hepatocytes necrosis at the centrilobular area with extensive haemorrhage and mild macrovascular steatosis

BIODATA OF STUDENT

Muhammad Azam Bin Fazil is a post-graduate student in the Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang campus under the Department of Veterinary Pathology and Microbiology. His Master's study focuses on the research project of the histopathological assessment on acetaminophen-induced liver injury with subsequent regeneration and its response to the edible bird's nest extract in a mouse model. During the post-graduate candidature, an article research paper was published at Veterinary World Journal (Vet World) and another had been submitted which still in the process to be published. This student had participated in a poster presentation at the 10th Malaysian Association of Veterinary Pathology (MAVP) Scientific Conference at Hotel Casuarina @ Meru, Ipoh, Perak in 2018.

Before the post-graduate study, Muhammad Azam had been graduated from Universiti Malaysia Sarawak (UNIMAS) in Kota Samarahan, Sarawak. He enrolled in the Animal Science and Resources Management course from 2013 to 2016. During the final year project, he worked on a project entitled the effect of preservation and fixation process in bone and cartilage staining of juvenile tilapia specimens. After completing the study period, Muhammad Azam attended an internship at World Wide Fund for Nature (WWF) in the Turtle Conservation project at Kerteh, Terengganu for 3 months period and due in September 2016.

LIST OF PUBLICATIONS

Publications

Muhammad-Azam, F., Nur-Fazila, S. H., Ain-Fatin, R., Noordin, M.M., & Yimer, N. (2019). Histopathological changes of acetaminophen-induced liver injury and subsequent liver regeneration in BALB/C and ICR mice. *Veterinary World, 12*(11), 1682–1688.

Ain-Fatin R., Nur-Fazila S.H., Nur-Mahiza M.I., Yasmin A.R., Muhammad-Azam F. & Nur-Kuain H. (2020). Incidental findings of *Heterakis spumosa* and *Chirodiscoides caviae* with pinworms in Sprague Dawley rats. *Sains Malaysiana, 49*(5), 1097-1106.

Muhammad-Azam F., Nur-Fazila S.H., Ain-Fatin R., Noordin M.M., Yasmin A.R. & Yimer N. Prophylactic effect of edible bird's nest on acetaminophen-induced liver injury response in mice model. *Accepted in Pakistan Journal of Pharmaceutical Sciences.*

Proceeding

Poster presentation on "The effects of acetaminophen-induced liver injury in ICR mice" at 10th Malaysian Association of Veterinary Pathology (MAVP) Scientific Conference 2018.

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