TOXICITY STUDIES OF 1'-S-1'-ACETOXYCHAVICOL ACETATE IN RAT MODEL

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TOXICITY STUDIES OF 1'-S-1 '-ACETOXYCHAVICOL ACETATE IN RAT MODEL

ABSTRACT

Natural compounds are the main source of therapeutic molecules for various diseases in this era, and most of the current medicines are derived from plants, microorganisms or animals. Nevertheless, the safety profile of these natural compounds has to be confirmed prior to their medicinal use. 1'-S-1'-acetoxychavicol acetate (ACA), a type of phenylpropanoid extracted from *Alpinia conchigera* Griff., is one of the natural compounds being investigated for its potential therapeutic purposes. ACA was previously reported to reduce tumour volume in athymic mice at the effective dose of 1.56 mg/kg body weight intraperitoneally and induce apoptotic death of tumour cells through the activation of caspase 3, 9, and 8 in the mitochondrial and Fas-dependent pathways and inactivation of the NF-κB pathway. However, a detailed toxicological profile for ACA has not yet been investigated. Therefore, a toxicity analysis involving intravenous ACA treatments in Sprague-Dawley male/female rats for an acute study of 2, 4 and 6.66 mg/kg doses of body weight for 14 days was conducted. In addition, a sub-acute study with weekly injections of 0.66, 1.33 and 2.22 mg/kg was also performed for 28 days. In these toxicity studies, ACA treatment did not affect behaviour, food/water intake or body weight, and showed insignificant changes in haematology and biochemical parameters or rat mortality. Thus, the LD_{50} value for ACA is higher than 6.66 mg/kg regardless of the sex of rats. Though there were mild inflammation of the kidneys and lobular hepatitis in sub-acute studies, these were not associated with significant functional adverse effects when compared with control rats. The no-observed-adverse-effect level (NOAEL) for intravenous ACA treatment in 28-day sub-acute studies was 2.22 mg/kg body weight for both male and female rats. The

findings show that intravenous ACA treatment at doses tested between 0.66 and 6.66 mg/kg body weight in both acute and sub-acute studies does not result in significant toxicity or death in rats. The present study provides useful information on the safe use of ACA on normal rat model.

Keywords: *Alpinia conchigera,* 1'-S-1′-acetoxychavicol acetate, acute toxicity, sub-acute toxicity, rats

KAJIAN KETOKSIKAN 1'-S-1 '-ACETOXYCHAVICOL ACETATE DALAM MODEL TIKUS

ABSTRAK

Sebatian semulajadi adalah sumber utama molekul terapeutik untuk pelbagai jenis penyakit pada era ini dan kebanyakan ubat-ubatan yang ada pada masa kini adalah berasal dari tumbuh-tumbuhan, mikroorganisma atau haiwan. Walau bagaimanapun, profil keselamatan sebatian semulajadi ini perlu disahkan sebelum digunakan sebagai ubat. 1'-S-1'-acetoxychavicol acetate (ACA), sejenis phenylpropanoid yang diekstrak dari *Alpinia conchigera* Griff., adalah salah satu sebatian semulajadi yang disiasat untuk tujuan potensi terapinya. ACA sebelum ini dilaporkan mengurangkan jumlah tumour dalam tikus tanpa timus pada dos efektif iaitu 1.56 mg/kg secara dalam peritoneal, dan menyebabkan kematian sel tumor melalui apoptosis dengan aktivasi kaspase 3, 9, dan 8 dalam laluan isyarat mitokondria dan Fas-bergantung serta penyahaktifan laluan isyarat NF-κB dengan mengurangkan kesan sampingan fisiologi pada sel-sel normal. Namun begitu, profil toksikologi yang terperinci untuk ACA masih belum dijalankan. Oleh itu, analisis toksik yang melibatkan rawatan ACA yang diberi secara dalam vena pada tikus jantan/betina Sprague-Dawley bagi kajian akut 2, 4 dan 6.66 mg kg selama 14 hari telah dijalankan. Di samping itu, kajian sub-akut dengan suntikan mingguan sebanyak 0.66, 1.33, dan 2.22 mg/kg juga dijalankan selama 28 hari. Dalam kajian toksik ini, ACA tidak memberi kesan kepada tingkah laku, pengambilan makanan/minuman atau berat badan, serta tidak mengakibatkan sebarang perubahan yang signifikan dalam hematologi dan parameter biokimia ataupun kematian tikus. Maka, nilai LD_{50} untuk ACA adalah lebih tinggi daripada 6.66 mg/ kg tanpa mengira jantina tikus. Walaubagaimanapun, dalam kajian sub-akut, terdapat keradangan ringan pada buah pinggang dan hepatitis lobular, namun ini tidak dikaitkan dengan kesan buruk kepada

fungsi organ-organ yang ketara apabila dibandingkan dengan tikus kawalan. Tahap kesan buruk (NOAEL) untuk rawatan ACA secara dalam vena dalam kajian sub-akut 28 hari adalah 2.22 mg/kg untuk tikus jantan dan betina. Penemuan ini menunjukkan bahawa rawatan ACA secara dalam vena pada dos yang diuji antara 0.66 dan 6.66 mg/kg dalam kedua-dua kajian akut dan sub-akut, tidak mengakibatkan kematian atau ketoksikan yang ketara pada tikus. Kajian ini menyediakan maklumat yang berguna tentang penggunaan ACA yang selamat dalam model tikus yang normal.

Kata kunci: *Alpinia conchigera,* 1'-S-1′-acetoxychavicol acetate, ketoksikan akut, ketoksikan sub-akut, tikus

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
(C=O)	:	Keto group
(–CHO)	:	Aldehyde group
µM	:	Micromolar
5-LOX	:	5-Lipoxygenase
A/G	:	Albumin/Globulin
$C_{13}H_{14}O_4$	:	Chemical Formula of ACA
Ca^{2+}	:	Calcium
CaSki	:	Human cervix carcinoma, epidermoid
Cl^-	:	Chloride
COX-2	:	Cyclooxygenase-2
ft	:	Feet
g	:	Gram
g/mol	:	Gram per ml mol
G_0/G_1	:	Cell cycle phases
Hb	:	Haemoglobin
HepG2	:	Hepatoma G2 (hepatocellular carcinoma cell line)
i.v	:	Intravenous
IgE	:	Immunoglobulin E
IL-4	:	Interleukin 4
K^+	:	Potassium
LD_{50}	:	Lethal Dose, 50%
MCF-7	:	Michigan Cancer Foundation-7 (breast cancer cell line)
mg/kg	:	Miligram per kilogram

min	:	Minute
ml/kg	:	Millilitre per kilogram
mm	:	Millimetre
MMP-9	:	Matrix Metalloproteinase-9
Na^+	:	Sodium
NF-κB	:	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
p	:	*P*-value
PC3	:	Prostate Cancer Cell Line
rhAFP	:	Recombinant Human Alpha Fetoprotein
SW480	:	Adenocarcinoma cancer cell line
TNF-α	:	Tumour Necrosis Factor Alpha
v/v	:	Volume/Volume
β	:	Beta
µm	:	Micrometre
AAALAC	:	Association for Assessment and Accreditation of Laboratory Animal Care
AEA	:	1'S-1'-acetoxyeugenol acetate
ALP	:	Alkaline Phosphatase
ALT	:	Alanine Transaminase
ANOVA	:	Analysis of Variance
AST	:	Aspartate Aminotransferase
CDK4	:	Cyclin-Dependent Kinase 4
CEA	:	Carcinoembryonic Antigen
CNS	:	Central Nervous System
CYP	:	Cytochromes P450
DILI	:	Drug-induced liver injury
DNA	:	Deoxyribonucleic acid

EDTA	:	Ethylenediaminetetraacetic acid
EMA	:	European Medicine Agency
FDA	:	Food and Drug Administration
HDAC2	:	Histone Deacetylase 2
HIV	:	Human Immunodeficiency Virus
HPLC	:	High-Performance Liquid Chromatography
IACUC	:	Institutional Animal Care and Use Committee
ICH	:	International Conference on Harmonisation
NOS	:	Nitric Oxide Synthase
IRF1	:	Interferon Regulatory Factor 1
ISO	:	International Organization for Standardization
IUPAC	:	International Union of Pure and Applied Chemistry
MCH	:	Mean Corpuscular Haemoglobin
MCHC	:	Mean Corpuscular Haemoglobin Concentration
MCV	:	Mean Corpuscular Volume
MTD	:	Maximum Tolerated Dose
NCE	:	New Chemical Entities
NME	:	New Molecular Entities
NMR	:	Nuclear Magnetic Resonance
NO	:	Nitric Oxide
NOAEL	:	The No-Observed-Adverse-Effect Level
NSCLC	:	Non-Small-Cell Lung Carcinoma
OECD	:	Organisation for Economic Co-Operation and Development
PCV	:	Packed Cell Volume
PG	:	Propylene Glycol
PG	:	Propylene Glycol

PPARγ	:	Peroxisome Proliferator-Activated Receptor
RANKL	:	Receptor Activator of Nuclear Factor Kappa-B Ligand
RBC	:	Red Blood Cells
RDW	:	Red Cell Distribution Width
RNA	:	Ribonucleic Acid
SD	:	Sprague-Dawley
SEM	:	Standard Error Mean
SMC	:	Swissmedic
TPR	:	Total Protein
TRAIL	:	TNF-Related Apoptosis-Inducing Ligand
VEGF	:	Vascular Endothelial Growth Factor
WBC	:	White Blood Cells

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Overview of study

Natural compounds have been a major source of therapeutic drugs for various ailments over the years, and most drugs available today are derived either from plants, microorganisms or animals (Nobili et al., 2009). Recent findings revealed that 40% of modern therapeutic agents approved from 1981 to 2010 were either natural products and their derivatives or synthetic mimetics, which were found to be both effective and safe (Newman & Cragg, 2012). Plant-derived agents exhibited less side effects and were favoured more than synthetic ones (Ghosh & Rangan, 2013). It is worth mentioning here the international regulatory agencies organizing drugs entering the markets for clinical uses. These bodies include the Food and Drug Administration (FDA), the European Medicine Agency (EMA) and Swissmedic (SMC). From 2007 to 2016, 134 new drugs have gained approval from all of these regulatory agencies (Zeukeng et al., 2018).

1'-S-1'-acetoxychavicol acetate (ACA), a phenylpropanoid isolated from the rhizomes of *Alpinia conchigera* of the Zingiberaceae family, was reported to possess anti-ulcer (Mitsui et al., 1976) and antimicrobial activity against several bacteria (Janssen & Scheffer, 1985). Also investigations on ACA as a potential anti-tumour agent has been extensively carried out on various cancer cell lines such as breast (MCF-7), liver (HepG2), cervical (CaSki), and prostate carcinoma (PC-3) (Awang et al., 2010; In et al., 2012; Arshad et al., 2015; Liew et al., 2017; Sok et al., 2017; Subramaniam et al., 2018). ACA has been previously reported to reduce tumour volume in nude mice at an effective dose of 1.56 mg/kg body weight intraperitoneally and induce apoptotic death of tumour cells through deactivation of the NF-κB signaling pathway with reduced physiological side effects on normal cells (Arshad et al., 2015).

Toxicity assessment is a major challenge in drug development as the undesirable side effects of these potential drugs must be assessed at the preclinical study phases. Toxicity effect leads to a wide range of adverse events such as decreased production of blood cells, suppression of the normal immune system, hair loss and bleeding (Luo & Cisler, 2016). Many chemotherapeutic agents can produce severe toxicities in vital organs of the gastrointestinal, respiratory and cardiovascular systems. A large number of approved drugs have demonstrated various level of toxicities during the preclinical studies, such as myelosuppression resulting from gemcitabine treatment in animal models (Abbruzzese et al., 1991).

From a historical overview, vinca alkaloids were the first compounds for cancer treatment to be discovered, as a result of screening plant-derivatives as potential therapeutic agents designed by USFDA. Vinblastine and vincristine, isolated from the Madagascar periwinkle, *Catharanthus roseus,* were observed to cause a reduction in white blood cell count and depression of bone marrow in preclinical studies (Gueritte & Fahy, 2005). Bevacizumab, one of the anticancer drugs for advanced-stage squamous and non-small cell lung cancers treatment, was found to demonstrate haemorrhage and proteinuria as adverse events (Chari, 2007).

The morphological changes such as body weight serve as a sensitive indicator of the general health status of animals during treatment. It is useful in addressing dose related toxicity which is reflected by an increase or decrease of body weight (Arshad et al., 2015).

Hematopoietic components are considered as one of the most sensitive targets of toxic substances. Analysis of blood parameters is required in such cases to identify potential toxicity alteration in the hematopoietic system (Ben Ayed et al., 2015). Cytotoxic agents predominantly cause low production of neutrophils in the bone

marrow resulting in neutropenia (Chari, 2007). Also, hepatotoxicity of toxic materials can result in altered liver weight and enzymes levels including morphological changes in hepatocytes.

Early-stage toxicology study program is very crucial for toxicity assessment of a compound. One of the oldest and most common approaches to describe the toxicity is by using the rodent median lethal dose concept (LD_{50}). The LD_{50} is explained as the dose of a new substance tested on a group of animals that causes 50% death. This method is used to minimize the number of animal death cases and establish a starting dose level (Faqi, 2012). Acute and sub-acute toxicity studies are carried out to determine the effect of single and multiple doses respectively in a short period of time.

Another way to describe toxicity at early preclinical stages is through the no-observed-adverse-effect level (NOAEL) approach. NOAEL represents the highest experimental point without any adverse effects (Dorato & Engelhardt, 2005). Also, NOAEL is useful to extrapolate dose from animal to human during clinical trials and drug development (Nair & Jacob, 2016).

1.2 Problem statement

Natural products are affordable materials that yield a variety of bioactive compounds in modern drug discovery. Some of the existing approved drugs are not attainable to some patients, as these drugs are either expensive or there is a shortage in availability.

Natural products such as plant derivatives are seen to produce less undesirable side effects. Since numerous approved drugs currently in clinical use produce side effects, such as occurence of liver and renal toxicity, the search for safer natural drugs is

warranted. As such, a complete toxicity study in animal is required to assess potential adverse effects before initiating clinical trial in human.

1.3 Justification of the study

Secondary metabolites produced by various organisms play a central role in and highly contribute to the discovery of new drugs. These metabolites have diverse chemical structures and bioactivities (Guo, 2017). ACA, a natural compound with potential anti-tumour and other bioactive properties, was selected for the study. ACA is easily available and has an ideal molecular structure. One major and crucial step in early drug development is the assessment of toxicity profile for a new drug candidate. Since ACA has the potential to be developed as an anticancer drug and has limited toxicity profile, this study is therefore carried out to assess and evaluate the safety profile of ACA.

1.4 Scientific hypothesis

As per the literature ACA reduces tumour volume at an effective dose of 1.56 mg/kg b. w. and induces apoptotic death of tumour cells. However, ACA at high doses such as 2.6 mg/kg causes lung inflammation leading to interstitial pneumonitis in nude mice. The correlation between ACA and its toxicity in the lungs and other vital organs is unknown. Further, the toxicity of ACA on other vital organs and its implications on their functions, underlying mechanisms is lacking. We hypothesise that the inflammation induced in vital organs and milder signs of systematic toxicity, could be either due to a pre-existing inflammation among tested animals as it is common or due to an immune response via macrophages of reticuloendothelial system. If the later has any role in inflammation it should be noticeable in other vital organs.

1.5 Objective

The objective of this study is to investigate the safety profile of ACA by performing acute and sub-acute toxicity studies as required for development by all drug regulatory agencies.

1.6 Specific objectives

(1) To evaluate acute toxicity with single dose of ACA for 14-days in normal Sprague-Dawley (SD) male/female rats by analysing biochemical, haematological and histopathological parameters.

(2) To evaluate sub-acute repeated dose toxicity of ACA for 28-days in normal SD male/female rats by analysing biochemical, haematological and histopathological parameters.

(3) To assess the toxicity level in urine following acute and sub-acute studies.

CHAPTER 2: LITERATURE REVIEW

Natural products are being increasingly studied to investigate its diverse phytoconstituents for clinical use, as they serve as a good source of drug lead molecules. ACA is an example of such phytoconstituent that has the potential to be developed as a drug candidate. The current chapter is focused on the role of natural products in drug development, the source of ACA, its physical properties, and the phytochemical and pharmacological profiles of the plant, *Alpinia conchigera*. Since toxicity is the core of the proposed study, a review on toxicity is also presented. This includes an overview on the role of toxicity studies in drug development, types of toxicity methods required to be carried out in animal models, toxicity assessment on vital organs and current toxicity studies conducted on ACA. Further, a brief discussion on the Food and Drug Administration of the United States (USFDA) and other regulatory agency guidelines with respect to the screening of new chemical entities for toxicity is also appended at the end.

2.1 Natural products as sources of new drugs

For many centuries, plants have been the main source of crude drugs used to cure or alleviate human sickness (Saklani & Kutty, 2008). The ancient people of different civilizations throughout the world used plant-based materials to cure their pain and ailments. The generosity of nature as a source of new molecular entities (NMEs) for curing various diseases is exceptional throughout history. Through various drug discovery approaches, researchers have been successfully able to identify naturally occurring constituents to cure several diseases. USFDA revealed that mammals, plants, and microorganisms contribute 50%, 25% and 25% of natural products, respectively (Patridge et al., 2016).

Plants contain secondary metabolites such as alkaloids, flavonoids, lignans phenylpropanoids and terpenoids that possess wide pharmacological activities (Table 2.1). These metabolites could trigger, promote and modulate the enzymes and receptors in signal transduction pathways related to cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis and metastasis in cancer cells. One important secondary metabolite, paclitaxel, was discovered in 1962 as a result of screening natural products for treatment of diseases. It is commercially marketed in the name of Taxol® and has become one of the most effective drugs against breast and ovarian cancer. It has been approved worldwide for the clinical treatment of cancer patients (Saklani & Kutty, 2008).

Despite enormous contribution of natural products in drug discovery by virtue of their structural diversity and physiological activity over the years, the poor solubility, chemical instability, inappropriate molecular size and toxicity of the lead compounds have posed major challenges in the formulation development (Von Nussbaum et al., 2006). Apart from that, in recent years, there has been an increasing number of reports on the undesirable side-effects of approved drugs (Table 2.1). Therefore, the need for new safer alternatives is urgently necessary (Shi et al., 2017).

Table 2.1: Adverse drug reactions and mechanisms of approved natural products in the literature (Tewari et al., 2019)

Secondary metabolite	Compound	Source	Mechanisms of action	Adverse drug reactions
Indole alkaloids	Vinblastine sulfate	*Catharanthus roseus*	Microtubule inhibition	Nausea, diarrhoea, vomiting, leukopenia, skin rash, and aggravation of a peptic ulcer (MacDonald Jr & Lacher, 1966)
	Vincristine sulfate	*Catharanthus roseus*	Inhibition of microtubule formation after binding of vincristine to tubulin	Optic neuropathy (Weisfeld Adams et al., 2007)
	Vinorelbine	*Catharanthus roseus*	Inhibition of tubulin dimers polymerization into microtubules	In combination with neutropenia, thrombocytopenia, and vomiting leukocytopenia (7%), and anaemia (Faller & Pandit, 2011)
	Vinflunine	*Catharanthus roseus*	Microtubule inhibitory effect	Bone marrow suppression, dose limiting toxicity, constipation,(Tournoux-Facon et al., 2011; Gerullis et al., 2017)

Table 2.1, Continued.

Secondary metabolite	Compound	Source	Mechanisms of action	Adverse drug reactions
Alkaloids	Etoposide phosphate	*Podophyllum sps*	Breakdown of DNA strand and DNA synthesis inhibitor	Bronchospasm, chest discomfort, dyspnoea, hypersensitivity reactions and hypotension (Siderov et al., 2002)
	Topotecan	*Camptotheca acuminata*	Topoisomerase I inhibitor	Alopecia, hematologic toxicity, thrombocytopenia and neutropenia (Anastasia, 2001)
	Elliptinium acetate	*Bleekeria vitiensis*	Inhibition of topoisomerase II	Diarrhoea, nausea, vomiting, xerostomia (Buzdar et al., 1990), and immune-mediated haemolytic reactions (Rouesse et al., 1993)
	Docetaxel	*Taxus brevifolia*	Microtubule depolymerization inhibitor	Anaphylactoid reactions, shock, oedema, scleroderma-like skin changes (Heike et al., 2005)
	Paclitaxel	*Taxus brevifolia*	Increase tubulin polymerization to stabilize microtubules	Alopecia, bradycardia and hypotension, neurotoxicity, peripheral neurological symptoms, hypersensitivity reactions, hepatotoxicity, and mucositis (Walker, 1993)

Table 2.1, Continued.

Secondary metabolite	Compound	Source	Mechanisms of action	Adverse drug reactions
Diterpene	Ingenol mebutate	*Euphorbia peplus*	Acts as an agonist for the intracellular protein kinase C	Moderate or severe skin reactions and produce local short-duration side effects which are well tolerated (Conde-Taboada et al., 2017)
Dicatechol	Masoprocol	*Larrea divaricata*	Inhibition of lipoxygenase and cyclin D1 expression	Hepatotoxicity and nephrotoxicity (Lambert et al., 2002; Rahman et al., 2011)

2.2 *Alpinia conchigera* Griff

Alpinia conchigera Griff. of family Zingiberaceae is a perennial herbaceous plant indigenous to South-East Asia, commonly found in Malaysia, Vietnam, Myanmar and Thailand. The *Alpinia* genus is reported to have 250 species worldwide (Xu et al., 2013). It is locally referred as "lengkuas genting" in Malaysia (Ong & Nordiana, 1999), "khaa ling" in Thailand, "pade-gaw" in Myanmar and "rieng rung" in Vietnam (Lim, 2012).

2.2.1 Taxonomy of *Alpinia conchigera*

The taxonomical classification of *Alpinia conchigera* is as shown in Figure 2.1 (Kress et al., 2005)

Kingdom: Plantae
Subkingdom: Viridiplantae
Superdivision: Embryophyta
Division: Tracheophyta
Subdivision: Spermatophytina
Class: Magnoliopsida
Order: Zingiberales
Family: *Zingiberaceae*
Genus: *Alpinia*
Species: *conchigera*

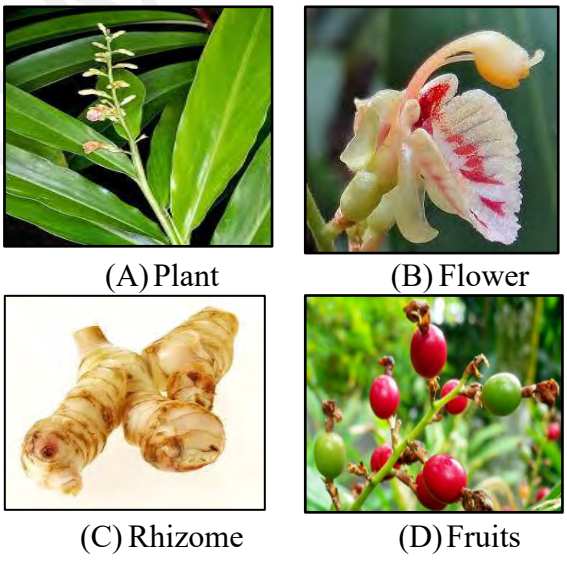


Figure 2.1: Taxonomy and morphological parts of *Alpinia conchigera*

Alpinia sp. grows to a height of 2-5 ft in open wetlands especially on the edges of rice fields and streams. The plant is semi-wild and usually grown under the rubber and palm oil trees utilising the shades. Leaves are glabrous except along leaf margin and midvein (Kress et al., 2005). The flowers are small with short sepal and petal tube. Normally, the flowers

have a yellowish or pinkish-white colour with red stripes on each side. The fruit is fleshy, pink or red colour, round when fresh, oblong when dry, and 8-10 mm wide. Each fruit contains 3-5 seeds that are strongly aromatic (Kress et al., 2005). The rhizomes are slender in their shape and edible as an additive for food flavour and vegetable dishes (Ibrahim et al., 2000).

2.2.2 Ethnobotanical and pharmacological features of *Alpinia conchigera*

The local folk in Malaysia apply the paste of its rhizomes on the skin to treat rheumatic pains and cure fungal infections. The rhizomes are also used as a condiment, and their edible young shoots are cooked in Peninsular Malaysia (Ibrahim et al., 2000). In Thailand, the rhizomes are an essential remedy in traditional medicine to relieve gastrointestinal disorders and hence incorporated in food (Ando et al., 2005).

In Malaysia, some *Alpinia* species such *Alpinia conchigera* and the closely related *Alpinia galanga* are grown in cultivation farms to produce raw materials for herbal medicine and spices (Talip et al., 2003).

Various published research reported different application and consumption of the plant parts such as drinking of the water extract, eating some plant parts and using in the bath, according to the types of diseases. *Alpinia conchigera* leaves were used to treat swelling on the stomach, rhizome for bone pain and rhizome water extract for stomach ache (Hanum & Hamzah, 1999). Also, the plant rhizomes were traditionally used in the treatment of skin infection (Aziz et al., 2013).

Aziz and colleagues reported that the dichloromethane extract of the *Alpinia conchigera* rhizome had potent anticandidal and antifungal activity against *Candida albicans, Microsporum canis* and *Trycophyton rubrum* (Aziz et al., 2013).

Alpinia species extract was reported to have antimicrobial activity against a range of bacteria and parasites. The ethanol extract of the rhizomes was found to cause changes in the cell membrane of *Staphylococcus aureus* (Oonmetta-aree et al., 2006). Meanwhile, the methanol extract of the rhizomes was reported to possess platelet-activating factor antagonistic and hepatoprotective activities. The extract was also able to reduce the necrotic cell count in rat liver (Kaushik et al., 2011).

2.2.3 Phytochemical profile of *Alpinia conchigera*

Natural derivatives with therapeutic activities against various diseases like cancer, diabetes, ulcer and many neural disorders were discovered from *Alpinia* species. Different parts of plants especially the rhizomes are known to produce biologically active ingredients.

The essential oils of *Alpinia conchigera* rhizomes are rich in monoterpenoids and sesquiterpenoids like β-sesquiphellandrene, β-bisabolene, 1,8-cineole, β-pinene, chavicol, β-elemene and β-caryophyllene (Ibrahim et al., 2009).

Chemical investigations have identified phytoconstituents such as neolignans, diarylheptanoids, flavonoids and phenylpropanoids (Athamaprasangsa et al., 1994).

This genus has complex chemical profiles and possesses diverse flavonoids responsible for the yellow pigmentation. Flavonoids are a potential source of antioxidants, and many have anticancer activities due to the presence of functional keto (C=O) or aldehyde (–CHO) groups (Williams et al., 2004; Ghosh & Rangan, 2013).

The rhizome of *Alpinia* species contains various metabolites like sesquiterpenes, monoterpenoids and phenylpropanoids. The phenylpropanoid is formed from the amino

acid phenylalanine and tyrosine. Notably, the phenylpropanoid ACA is present in small yield and mostly confined to rhizomes and root parts of the plant (Baradwaj et al., 2017).

The *n*-hexane extracts of *Alpinia conchigera* rhizomes were reported to have stigmasterol, β-sitosterol, and six phenylpropanoids (Figure 2.2), which are chavicol acetate, *p*-hydroxy cinnamaldehyde, 1'-S-1'-acetoxychavicol acetate (ACA), *trans-p*-coumaryl diacetate, 1'S-1'-acetoxyeugenol acetate (AEA), 1'-hydroxychavicol acetate and p-hydroxycinnamyl acetate (Awang et al., 2010; Aziz et al., 2013). Among these compounds, ACA was studied extensively for its wide pharmacological and therapeutic uses. It has also been isolated from other species of *Alpinia* such as *Alpinia galanga* (Janssen & Scheffer, 1985).

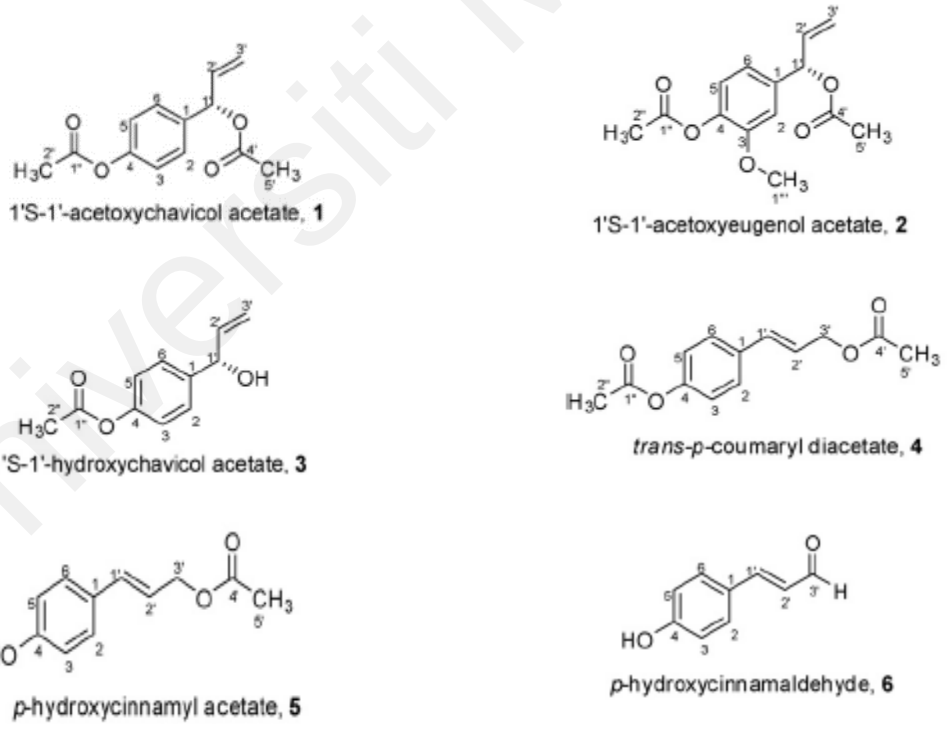


Figure 2.2: Major phytochemical compounds isolated from *Alpinia conchigera* (Awang et al., 2010; Aziz et al., 2013).

2.3 Extraction and Isolation of ACA

Dried *Alpinia conchigera* rhizomes were extracted with dichloromethane for 72 hrs at room temperature using soxhlet apparatus. The solvent removed from the extract by rotary evaporator under vacuum, was subjected to column chromatography on silica gel (Merck Kiesegel 60) eluting with gradient of hexane-ethyl acetate (100:0 → 50:50). The fractions of compounds were isolated and concentrated separately under vaccum at 40 °C. Fractions that have same profile in TLC were pooled to give six sub-fractions, which yielded 1'-S-1'-acetoxychavicol acetate as the major constituent. The structure of this compound was determined based on comparison of its spectral data with those reported in the literatures (Awang et al., 2010).

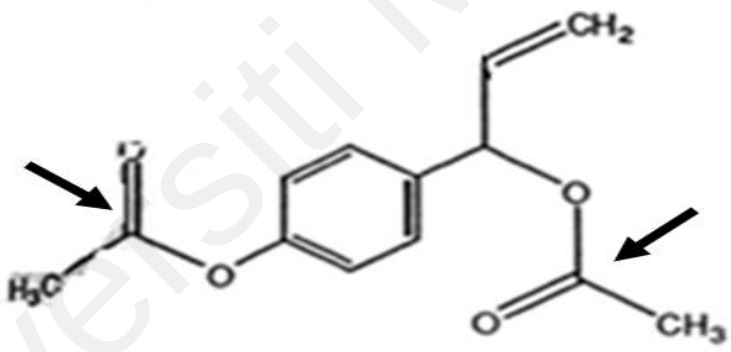


Figure 2.3 Chemical structure of 1'-S-1′-acetoxychavicol acetate.

Awang et al. (2010) reported that ACA is yellowish oil, with an empirical formula of $C_{13}H_{14}O_4$, 234.2479; Found 234, 192, 150, 149, 132, 104, 77. FTIR (CCl_4) Wavenumber, cm^{-1}: 1761, 1645, 1234. UV λmax, nm: 304.5. 1H-NMR ($CDCl_3$), δ: 2.08 (3H, s), 2.27 (3H, s), 5.22 (2H, dd, J = 10.0 Hz), 5.98 (1H, m), 6.23 (1H, d, J = 5.8 Hz), 7.03 (2H, d, J = 8.8 Hz), 7.33 (2H, d, J = 8.8 Hz). 13C-NMR (CDCl3), δ: 21.2 (CH3), 21.3 (CH3), 75.6 (CH),

117.2 (CH2), 121.7 (2CH), 128.5 (2CH), 136.1 (CH), 136.5 (C), 150.5 (C), 169.4 (C), 169.7 (C)

2.3.1 Liquid chromatography–mass spectrometry analysis

The LCMS analysis was done using Shimadzu LCMS-IT-TOF instrument (Columbia, MD, USA) equipped with a binary pump, an automatic injector and a photodiode array detector (SPD-M20A). Waters Xbridge C18 column (50 × 2.1 mm, 2.5 μm) was used to carry out separation on a binary gradient as the A solvent was a double-distilled water, together with solvent B, which was acetonitrile as follows: 100% A and 0% B (0.0 min), 0% A and 100% B (6.0 min), 100% A and 0% B (8.5–10.0 min). rat flow was set as 0.5 mL/min. The solution was filtered through Whatman 13 mm, 0.2 μm nylon membrane syringe filters before use. The LC-MS Analysis revealed that ACA gives a chromatogram at 254 nm and the strongest peak at tR = 4.1 min (Awang et al., 2010).

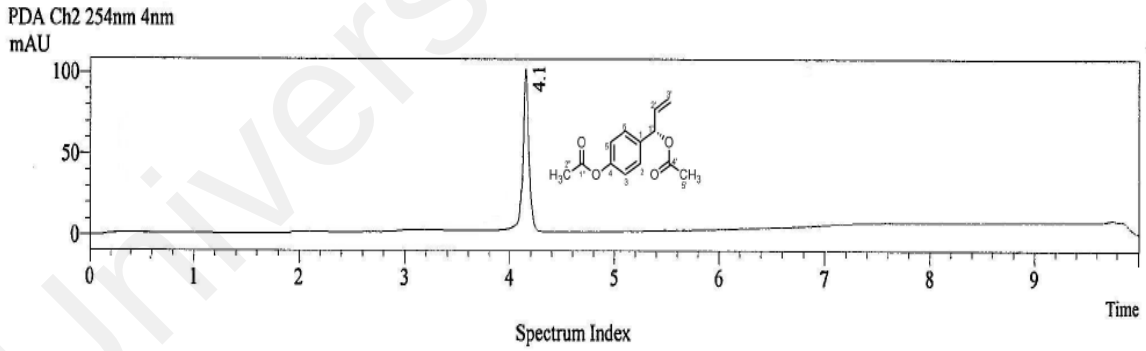


Figure 2.4 Chromatogram of 1'S-1'-acetoxychavicol acetate at 254 nm.

2.3.2 Physico-chemical properties of ACA

ACA is a yellowish oil with $C_{13}H_{14}O_4$ chemical formula and a molecular weight of 234.251 g/mol (Awang et al, 2010). It is a chavicol structure derivative. Chemically, it is an

acetate ester and a phenylpropanoid. The chavicol acetate allows substitution by an acetoxy group at position 1 (Figure 2.3). The compound has other physical and chemical properties summarized in Table 2.2 **(**Kim et al., 2019).

Table 2.2: Physical and chemical properties of ACA

Property name	Property value
IUPAC name	[4-[(1S)-1-acetyloxyprop-2-enyl] phenyl] acetate
Molecular weight	234.251 g/mol
Hydrogen bond donor count	0
Hydrogen bond acceptor count	4
Rotatable bond count	6
Exact mass	234.089 g/mol
Monoisotopic mass	234.089 g/mol
Topological polar surface area	52.6 A^2
Heavy atom count	17
Formal charge	0
Complexity	290
Isotope atom count	0

Solubility is defined as the maximum amount of a substance that will dissolve in a specified volume of solvent at a stated temperature. Also, it can be defined as the capability of a substance to dissolve in a solvent to produce a homogenous solution (Mittal, 2016). Low soluble drugs require dissolution enhancement prior to use in further drug development (Azuma et al., 2011). Known limitations of ACA are low yield production from plant sources, poor solubility in water, instability in aqueous solution by undergoing hydrolysis and formation of isomers (Azuma et al., 2011; Baradwaj et al., 2017).

2.3.2.1 Propylene glycol to enhance the solubility of ACA

Propylene glycol (PG) is nontoxic and used as a co-solvent in various drugs approved by FDA for oral solution up to 92% and topical drugs up to 98.09%. PG, which is soluble in water, is a clear solvent with no odour and has less toxicity than other solvents like ethylene, although it has a similar structure to ethylene (Gad et al., 2006). Furthermore, PG is considered as a non-carcinogenic substance (Fiume et al., 2012). It is well tolerated in rats when administered orally at a dose of 5 ml/kg for four weeks and intravenously at a dose of 100 mg/kg (Gad et al., 2006). PG can dissolve in normal saline when administered intravenously to rabbits at an infusion rate of 2.8 mg/min per kg to assess its pharmacokinetics (Dale & Sawchuk, 1987).

2.4 Pharmacological activities of ACA

ACA has multiple pharmacological effects and has been studied for several decades. Recently an evidence for its anti-viral activity against the human immunodeficiency virus (HIV) was identified. ACA at low concentration can inhibit Rev transport by binding to chromosomal region and accumulate HIV-1 RNA in the nucleus, resulting in a block in HIV-1 replication in peripheral blood mononuclear cells. Thus, this ability of ACA is similar to anti-HIV agents (Ye & Li, 2006).

The antiallergic function of ACA was also reported by Yasuhara and co-workers to inhibit antigen-IgE-mediated TNF-α and IL-4 cytokine production, which plays an important role in the late phase of allergic reactions (Yasuhara et al., 2009).

An *in vivo* study conducted by Li and co-workers demonstrated the anti-inflammatory effect of ACA-β-1, 3-glucan polysaccharide soluble complex *via* suppression

of TNF-α. Since ACA has low solubility in water, its solubility was increased by forming a conjugate with polysaccharide β-1, 3-glucan. They further emphasised ACA's role in the treatment for dermatitis (Li et al., 2015)

Antiulcer activity of ACA investigated by Matsuda and co-workers showed gastrointestinal protective effect *via* modifying the levels of glutathione, endogenous prostaglandins and sulfhydryl groups against *Helicobacter pylori* induced ulcer (Matsuda et al., 2003).

The most studied pharmacological activity of ACA was its ability to exhibit anti-tumour activity. Cytotoxic agents naturally lack differentiation between cancerous and normal cells. As such, the two main features that characterises any successful anti-tumour drug are cytotoxicity and selectivity towards cancerous cells (Blagosklonny, 2004).

Numerous studies have attempted to explain the antiproliferative activity of ACA. The studies conducted thus far provide evidence that ACA significantly inhibited cellular growth of multiple myeloma cells by inducing apoptosis along with activation of caspase 3, inhibition of NF-κB activity, upregulation of the expression of both TNF-related apoptosis-inducing ligand/Apo2 ligand (TRAIL/Apo2L) and TRAIL death receptor 5 (DR5). This confirms the involvement of death signalling of TRAIL by ACA-induced apoptosis of myeloma cells (Ito et al., 2005).

According to Ando and co-workers, ACA has the inhibitory effect on nitric oxide (NO) production. They suggested that ACA inhibited IFN-β mRNA expression as well as NF-κB activation. Activated NF-κB promotes transcription of the IRF1 gene to increase expression of the iNOS gene and alter production of NO as shown in Figure 2.5 (Ando et al., 2005).

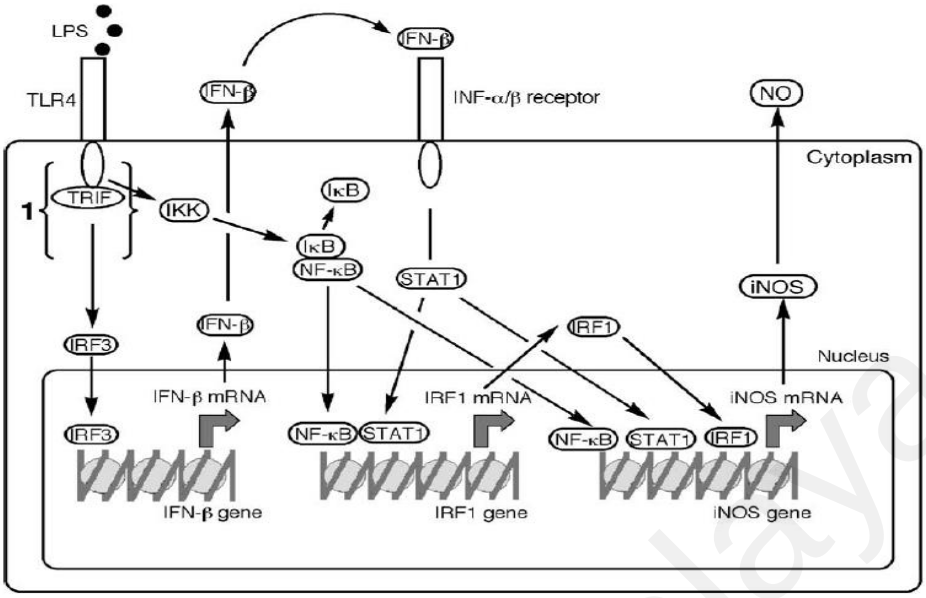


Figure 2.5: Effect of ACA on nitric oxide (NO) production and potential action site of ACA (Ando et al., 2005)

ACA was also reported to alleviate bone-related complications by causing osteoclastogenesis via blocking of RANKL-induced NF-κB activation in cancer-associated bone loss (Ichikawa et al., 2006).

Evidence suggests that the anti-tumour effect of ACA relies on its role in inducing apoptosis by increasing protein expression of the activated form of caspase-3 (Campbell et al., 2007). In this study, the cell viability decreased in a time- and dose-dependent manner. Another study concluded and confirmed the apoptotic effects of ACA via the DNA fragmentation assay and induced cell cycle arrest at the G_0/G_1 phase (Awang et al., 2010).

The inhibitory effects of ACA on oral, lung and prostate cancer cell lines were investigated, and significant apoptotic effect was found to take place due to down-regulation of NF-κB and its regulated genes. Reduction in the expression of proinflammatory cyclooxygenase-2 (COX-2) and proliferative cyclin D1 in tumour tissues

were observed, which is further potentiated in the presence of the chemotherapeutic agent, cisplatin (Awang et al., 2010; In et al., 2012). Arshad and co-workers investigated the role of ACA and recombinant human alpha fetoprotein (rhAFP) as a combination to overcome the non-specific targeting of tumour cells of ACA. Additionally, they also included *in vivo* experiments on ACA standalone treatment. As in the previous study, ACA was able to down-regulate NF-κB activation and reduced the expression of NF-κB regulated genes and inflammatory biomarkers, such as vascular endothelial growth factor (VEGF), histone deacetylase 2 (HDAC2), 5-lipoxygenase (5-LOX), cyclin-dependent kinase 4 (CDK4), matrix metalloproteinase-9 (MMP-9) and COX-2. Furthermore, the expression of tumour antigens, carcinoembryonic antigen (CEA) in lung tumours and prostate specific antigen (PSA) in prostate tumours, was significantly reduced during treatment with ACA (Arshad et al., 2015).

In previous studies, cisplatin was used as a positive control. ACA was found to have better tumour volume reduction compared to cisplatin (Arshad et al. 2015). In addition, ACA was reported to enhance the cytotoxic effects of cisplatin in a synergistic manner (In et al., 2012).

A comprehensive anti-proliferative effect of ACA on colorectal adenocarcinoma cell line (SW480) was recently revealed by Bharadwaj et al. (2017). They reported that ACA significantly suppressed the growth of cancer cells by arresting cell cycle at the G_0/G_1 phase *via* DNA damage and mitochondrial depolarisation. Additionally, significant downregulation of cyclin D and overexpression of p21 were seen in comparison to untreated control.

Autophagy is a catabolic cellular mechanism that allows the cell to maintain a balance between the synthesis, degradation, and recycling of cellular products (Mroz et al., 2011). Reduction in Beclin-1 protein expression results in autophagy. ACA induces pro-survival autophagy through Beclin-1-independent pathway in NSCLC cell line (Sok et al., 2017).

Therefore, ACA possesses significant anti-tumour activity against a wide range of carcinoma cells and is an important potential anti-tumour agent.

2.5 Significance of toxicity study in drug development

The main goal of a drug development process is to ensure that the new drug is safe, potent and effective. Preclinical development of a drug is the phase before the drug enters human clinical trials. Purposes of carrying out preclinical studies is to achieve goals such as to understand the toxicological profile of the agent towards target organs and exposure /toxicity relationship, identify pharmacologic properties of the agent and determine a safe starting dose for the first human studies.

Whenever a new drug is being developed, the crucial steps must be the identification of side effects that resulted from treatment and implementation of ways to decrease these adverse events. Gastrointestinal, cardiovascular and nephrotoxicity are examples of adverse events that result from anticancer treatment in many cases. The undesirable side effects lead to a wide range of physiological abnormalities such as decreased production of blood cells, suppression of the immune system, hair loss and bleeding (Luo & Cisler, 2016). Therefore, a comprehensive toxicity study is a prerequisite to drug development.

2.5.1 Stages of drug development

Developing a new drug from its source to the launch of a finished product is an extremely costly and complex process which can take 12–15 years (Hughes et al., 2011). Several preclinical and clinical phases are involved in the overall drug development process (Figure 2.6). Prior to the clinical trial phase, preclinical tests must be carried out on the new chemical entities (NCEs).

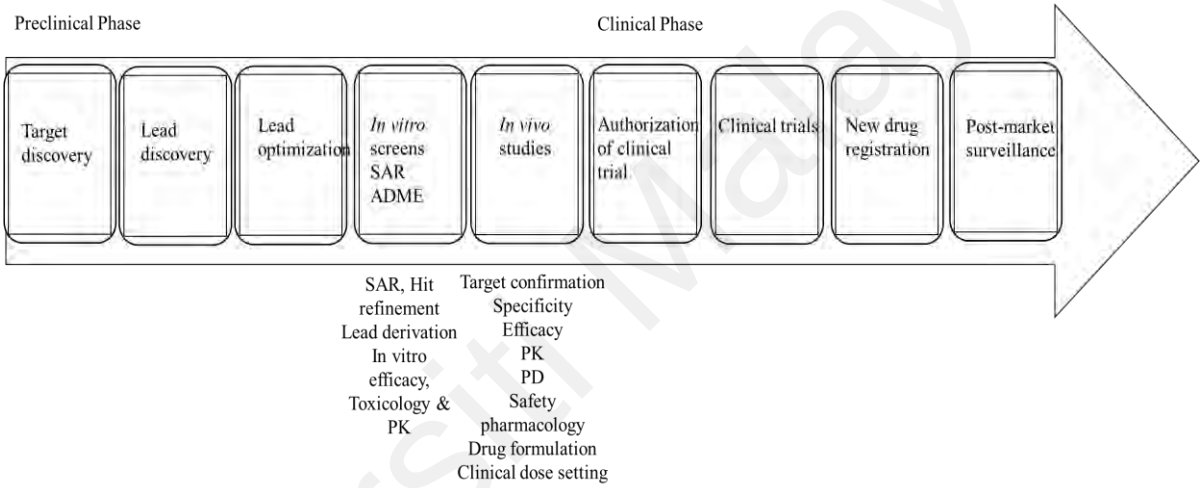


Figure 2.6: The key stages of drug discovery and development (Gad, 2008).

Toxicity study in animal models is one of the important steps in preclinical drug development to assess the safety of the test compound. The aim of this step is to predict any potential adverse events that may result after a treatment. The target organ toxicity, relationship between the dose and response, relevant human effects and any complications arising during treatment should be established through preclinical evaluations (Parasuraman, 2011).

Further, toxicological studies may intend to elucidate vital information regarding single or repeated doses of the test compound to assess the effect on genetic material and vital organs (King, 2002).

Therefore, international regulations and agencies oversee toxicity studies for short and long-term duration, role of dosage regime, animal selection and other protocols.

2.5.2 Types of toxicity studies

The types of toxicity studies differ based on the duration of study, use of animals, drug route of administration, and dosage regimen. The history of toxicity studies begins with Paracelsus (1493–1541), who determined specific chemicals responsible for the observed toxicity of plants and animals. He demonstrated the harmless and beneficial effects of toxins and proved dose-response relationships (Parasuraman, 2011). There are several toxicity assessment methods that are focused in detail in the following sections.

2.5.2.1 Acute toxicity study

The goal of acute toxicity study is to recognize the major adverse events resulting from a single dose on the experimental animal. Pharmaceutical industry prefers to obtain an early toxicology profile through toxicity study. The oral LD_{50} test, which identifies lethal dosage that causes death in 50% of the treated animals used to be carried out in order to identify the therapeutic dose. The Organisation for Economic Co-Operation and Development (OECD) withdrew the oral LD_{50} test and replaced with the acute toxicity study instead (Walum, 1998). The International Conference on Harmonisation (ICH) has also provided guidelines to refine the acute toxicity test (Robinson et al., 2008).

Acute toxicity testing is a short and important study to reveal the fate of any NECs being developed as a successful drug. The study is carried out to determine the effect of a single dose on animal models where the test agent is administered at different dose levels and the effect is observed for 14 days. Route of drug administration can vary according to the properties of the test compound although the oral route is preferred according to the OECD guidelines for both animal sexes. Targeted undesirable effects include any change in function or biochemical lesions that lead to impairment or reduced ability of any organs (Walum, 1998).

2.5.2.2 Sub-acute toxicity study

Sub-acute repeated dose toxicity study must be undertaken to evaluate the toxic characteristics of a drug/chemical after obtaining the initial toxicity profile from acute toxicity study. The repeated dose toxicity testing is administered in specified regimens for a minimum of 28 days. This study is intended to reveal the toxic effects on the nervous, immune and endocrine systems that arise from the repeated doses (OECD, 2008).

2.5.2.3 Sub-chronic and chronic toxicity studies

A sub-chronic study is carried out for less than 90 days and a chronic for 6 months or more. These toxicology studies provide information about the long-term effect of a test compound in animal models before these doses can be extrapolated to the human trials. Using both sexes for rodents, at least 20 animals per sex per group should be used at each dose level, while for non-rodents, a minimum of 4 per sex per group is recommended. At least three dose levels should be used in addition to the concurrent control group (OECD, 2008).

2.5.2.4 Teratogenetic toxicity (Prenatal Developmental Toxicity Study)

This toxicity testing is designed to provide general information concerning the effects of prenatal exposure on the pregnant test animal and on the developing organism (OECD, 2001).

2.5.3 Common signs of test compound toxicity

In the toxicological evaluation of NCEs, the changes in parameters of interest are measured, such as body weight, food /water consumption, clinical signs and clinical pathology involving biochemical and haematological evaluations. All the findings and deviations from normal (Tables 2.3 and Table 2.4) on these parameters due to the biological effect of the administered test compound can provide information on the adverse events (Gad, 2008)

Toxicity is assessed on various organs such as liver, central nerve system, kidneys, lungs and heart. The toxic test compound may impair the physiological functions of the vital organs and be fatal. The toxicity arises due to the interaction of the test compound with an unintended target in addition to the desired therapeutic effect. Alternately, toxicity may also be produced by hypersensitivity and immune responses such as allergic reactions. In addition, the toxic test compound may result in modification or bioactivation of proteins and convert to reactive metabolites (Guengerich, 2010).

Table 2.3: Association of changes in biochemical parameters with actions on target organs (Gad, 2008)

	Organs								
Parameters	**Blood**	**Heart**	**Lung**	**Kidney**	**Liver**	**Bone**	**Intestine**	**Pancreas**	**Significance**
Albumin					↓				Produced by the liver. Very significant reductions indicate extensive liver damage.
ALP (alkaline phosphatase)					↑	↑	↑		Elevations usually associated with cholestasis. Bone alkaline phosphatase tends to be higher in young animals
BUN (blood urea nitrogen)				↑	↓				Estimates blood - filtering capacity of the kidneys. Is not significantly elevated until kidney function is reduced 60 – 75%
Calcium				↑					Can be life threatening and result in acute death
Cholinesterase				↑	↓				Found in plasma, brain, and RBCs.
CPK (creatine phosphokinase)		↑							Most often elevated due to skeletal muscle damage but can also be produced by cardiac muscle damage. Can give a more sensitive evaluation than that of histopathology

Table 2.3, Continued.

Parameters	Organs								Significance
	Blood	Heart	Lung	Kidney	Liver	Bone	Intestine	Pancreas	
Creatine				↑					Estimate blood - filtering capacity of kidney, more specific than BUN
Glucose								↑	Alterations, other than those associated with stress, are uncommon and reflect an effect on the pancreatic islets or anorexia
GGT (gamma-glutamyltransferase)					↑				Elevated in cholestasis. The enzyme level often increases in response to microsomal enzyme induction
HBDH (hydroxybutyric dehydrogenase)		↑			↑				Most prominent in cardiac muscle tissue
LDH (lactic dehydrogenase)		↑	↑	↑	↑				Increase usually due to skeletal and cardiac muscles and liver damage. Not very specific unless isozymes are evaluated
Protein (total)				↑	↑				Absolute alterations are usually associated with decreased production in liver or increased loss in kidney

Table 2.3, Continued.

	Organs								
Parameters	**Blood**	**Heart**	**Lung**	**Kidney**	**Liver**	**Bone**	**Intestine**	**Pancreas**	**Significance**
Bilirubin (total)	↑				↑				Usually elevated due to cholestasis either due to obstruction or hepatopathy
SGOT (serum glutamic-oxaloacetic transaminase); also called AST (asparate aminotransferase)		↑		↑	↑			↑	Present in skeletal muscle and heart and most commonly associated with damage to the two organs
SGPT (serum glutamic-pyruvic transaminase); also called ALT (alanine aminotransferase)					↑				Evaluations usually associated with hepatic damage or disease
SDH (sorbitol dehydrogenase)					↑ or ↓				Liver enzyme that can be quite sensitive but is fairly unstable.

Table 2.4: Association of changes in haematological parameters with actions on target organs (Gad, 2008)

Parameter	Elevation	Reduction
Red blood cells	1. vascular shock 2. excessive diuresis 3. chronic hypoxia 4. hyperadrenocorticism	1. anaemias (a) blood loss (b) haemolysis (c) low RBC production
Hematocrit	1. increased RBCs 2. stress 3. polycythemia 4. shock (a) trauma (b) surgery	1. anaemias 2. pregnancy 3. excessive hydration
Hemoglobin	1. polycythemia (increase in production of RBCs)	1. anaemias 2. lead poisoning
Mean cell volume	1. anaemias 2. vitamin B_{12} deficiency	1. iron deficiency
Mean corpuscular hemoglobin	1. reticulocytosis	1. iron deficiency
White blood cells	1. bacterial infections 2. one marrow stimulation	1. bone marrow depression 2. cancer chemotherapy 3. chemical intoxication 4. splenic disorders
Platelets		1. bone marrow depression 2. immune disorder
Neutrophils	1. acute bacterial infections 2. tissue necrosis 3. strenuous exercise 4. convulsions 5. tachycardia 6. acute hemorrhage	1. viral infections
Lymphocytes	1. leukemia 2. malnutrition 3. viral infections	
Monocytes	1. protozoal infections	
Eosinophils	1. allergy 2. irradiation 3. pernicious anemia 4. parasitism	
Basophils	1. lead poisoning	

2.5.4 Hepatotoxicity

The liver is a large organ consisting of a right and left lobe. It works together with pancreas to digest, absorb and process nutrition. Its main role is to filter blood coming from the digestive tract to detoxify chemicals and metabolise drugs before passing it to the rest of the body.

Drug-induced liver injury (DILI) is an adverse event which has a considerable impact on the drug development process, and there is an insufficient way for its prevention. DILI may result due to the treatment of herbal agents, drugs and toxic substances (Ghabril et al., 2010).

Hepatotoxicity leads to either increase or decrease of enzymes, weight and morphological changes in the liver. Hepatocyte hypertrophy is often due to stimulation of drug metabolism and is non-adverse, but this change could lead to potentially severe toxicity at higher doses or on prolonged treatment.

Importantly, liver injury is one of the main causes of drug withdrawal at preclinical and clinical stages and even after a drug goes to markets (Kotsampasakou et al., 2017).

In recent years, there has been an increasing interest in determining hepatotoxicity at preclinical stages in animal models. However, findings revealed that the preclinical animal study outcomes share low concordance with human hepatotoxicity (Chen et al., 2011).

2.5.5 Neurotoxicity

The central nervous system (CNS) consists of the brain, spinal cord, sensory organs and the nerves that connect these organs. The CNS is responsible for the control of the body and the communication among its parts. The toxicity in the nerve system includes the

change in neuronal pigment and damage. CNS toxicity generally presents signs such as tremors, increased or decreased activity/sedation, recumbency, loss of balance/ataxia, hypothermia, seizures/convulsions and death. Brain section can be made for further investigation to notice vascular injury as a result of direct neurotic action of the test compound (Faqi, 2012).

2.5.6 Cardiotoxicity

The heart and blood vessels constitute the cardiovascular system. The heart pumps the blood to the lungs to allow the red blood cells to carry oxygen and then pumps oxygen-rich blood to the body. Toxicity of heart can lead to myocardial injury. Cardiotoxicity is caused by alterations in biochemical pathways, energy metabolism, cellular structures, electrophysiology and contractility leading to decreased cardiac output and peripheral tissue hypoperfusion (Faqi, 2012). Between 1994 and 2006, cardiotoxicity was the main reason for the withdrawal of 45% of all total drugs that have been withdrawn for various reasons.

Cardiovascular dysfunction can occur due to mitochondrial death in myocardia. Toxic agents or chemical compounds such as cisplatin, trastuzumab (Herceptin®), arsenic trioxide (Trisenox®), mitoxantrone (Noantrone®), imatinib (Gleevec®), and bevacizumab (Avastin®) were reported to have a direct effect on mitochondrial deterioration. Cardiotoxicity is known to be in high prevalence in cardiovascular diseases (Varga et al., 2015).

2.5.7 Nephrotoxicity

The nephron is the functional component of the kidney, which produces urine in the process of removing waste and excess substances from the blood. Nephrotoxicity is one of the most well-known adverse events of drugs treatment. It occurs during chemotherapy-

induced renal dysfunction. Damage to vasculature or structures of the kidneys, haemolytic-uraemic syndrome and prerenal perfusion deficits are the main causes of kidney failure.

In cancer treatment, signs of streptozocin-induced nephrotoxicity include hypophosphataemia, hypokalaemia, hypouricaemia, renal tubular acidosis, glucosuria, aceturia and aminoaciduria (Kintzel, 2001). Cisplatin is another example of drugs that cause dose-dependent renal toxicity. It causes tubular damage and dysfunction due to intracellular effects of drug involving regulatory genes causing direct cytotoxicity with reactive oxygen species (Kintzel, 2001; Yao et al., 2007).

2.5.8 Pulmonary toxicity

The lungs are a pair of spongy, air-filled organs. As a result of their large surface area, the lungs become a target to a variety of toxic compounds. The link between NCEs and pulmonary toxic side effects is well established. Mechanisms of harm by these drugs include direct pulmonary toxicity and indirect effects through the augmentation of inflammatory responses, which include interstitial inflammation and fibrosis, bronchospasm, pulmonary edema, and pleural effusions.

Drug-induced interstitial lung disease (DILD) can be caused by chemotherapeutic agents, antibiotics, antiarrhythmic drugs and immunosuppressive agents. The most common form of drug-induced lung toxicity is drug-induced interstitial lung disease (Schwaiblmair et al., 2012).

2.6 Routes of drug administration and toxicity

Route of drug administration is the mode of delivery into the body for the treatment of various diseases and disorders. Various routes of administration play a crucial role in determining the bioavailability of the active drug in the body. The systemic route consists of enteral route such as oral, sublingual and rectum while parenteral route consists of intravenous, subcutaneous and intramuscular (Verma et al., 2010).

The bioavailability of the active drug depends on several factors such as first pass effect in the liver and gastrointestinal tract, which is a fraction of the dose administered that escapes metabolism that is usually small and variable. This phenomenon is clear in drugs administered orally, and hence large oral doses are administered through oral route than intravenous to achieve equivalent plasma concentrations (Pond & Tozer, 1984).

2.7 Role of regulatory bodies

A clear statement of the objectives of the intended toxicology study is the first requirement that regulatory agencies seek from an investigator prior to approval or registration (Gad, 2008). The intention of regulatory toxicology is to control production, usage and deposition of unsafe test compounds to avoid adverse effects on human well-being and the environment. (Reichl & Schwenk, 2014).

The design of toxicological studies has always been governed by numerous guidelines to ensure maximum benefits of the intended research, animal welfare and cost-effective study plan. These bodies produce and update guidelines focusing on regulations to conduct toxicology studies to produce high-quality toxicology information. The regulatory organisations include The United States Food and Drug Administration (USFDA),

European Medicines Agency (EMA) and The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) (Persad, 2009).

Most of the international guidelines emphasise on implementing rules and law for product development, data reporting, quality of data, product registration as well as animal welfare. Also, these guidelines encourage the use of the 3 Rs concept (Replacement, Reduction and Refinement) when it comes to the planning of toxicology studies as toxic test compounds can cause severe pain, injury and even mortality to animals (Stokes, 2002).

2.7.1 USFDA guidelines for nonclinical safety studies

The USFDA requires conducting of toxicology studies before planning any future clinical trial. The guideline of nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals made the role of acute and repeated toxicity studies essential. The goals of the toxicity study as per the guideline include the description of toxic effects with respect to target organs, dose dependence, relationship to exposure and the chance of potential reversibility. The vital information obtained is used to estimate an initial safe starting dose and dose range for the human trials. Another important aspect is to identify parameters for clinical monitoring for potential adverse effects.

In acute toxicity, the outcome information can be limited to the clinical route of administration used in the study. Also, the information is useful to determine the consequences of human overdose situations which is crucial data to support clinical trial phase 3. For the repeated dose study, the duration of the study and therapeutic indication must be related to the proposed clinical study as stated in the guideline. The duration should be equal or exceed the intended clinical trial duration.

2.7.2 EMA guideline for nonclinical safety studies

The EMA guideline specifies the important early toxicology information of NECs obtained in acute toxicity study. In this study, the observations must be recorded regularly for at least 14 days after a single administration of a high dose. An autopsy with macroscopic observation and histological examination of relevant findings involving haematology, clinical chemistry, necropsy and histopathology data after a single administration must be carried out, with a further evaluation conducted 2 weeks later to assess delayed toxicity and/or recovery.

As stated in the guideline, the repeated dose study contributes to the important development of safe medicinal products. Repeated dose toxicity studies should be carried out in accordance with good laboratory practice for a period equal to the proposed therapeutic use in human. Equal numbers of male and female animals should be used, and the size of the treatment group should be sufficient to allow meaningful scientific interpretation of the data generated.

2.7.3 ICH guidelines for nonclinical safety studies

The ICH S7A guideline recommended safety pharmacological studies for human pharmaceuticals to help protect clinical trial participants and patients receiving marketed products from potential adverse effects of pharmaceuticals, while avoiding unnecessary use of animals and other resources. This guideline generally applies to new chemical entities and biotechnology-derived products for human use. It is important to adopt a rational approach when selecting and conducting safety pharmacology studies. The specific studies that should be conducted and their design will vary based on the individual properties and

intended uses of the pharmaceuticals. Scientifically valid methods should be used and when there are internationally recognized methods that are applicable to pharmaceuticals, these are preferable. Moreover, the use of new technologies and methodologies in accordance with sound scientific principles is encouraged. Pharmacology studies can be divided into three categories: safety pharmacology, primary pharmacodynamic, and secondary pharmacodynamic studies.

The S9 guideline of ICH gave recommendations for nonclinical evaluation for anticancer test drugs. As malignant tumours are life-threatening, the death rate from these diseases are high, and existing therapies have limited effectiveness, it is therefore desirable to provide new, effective anticancer drugs to patients. This guideline aims to facilitate and accelerate the development of anticancer pharmaceuticals and to protect patients from unnecessary adverse effects, while avoiding excessive use of animals, in accordance with the 3R principles (reduce/refine/replace), and other resources. Nonclinical evaluations are conducted to identify the pharmacologic properties of a test drug, establish a safe initial dose and understand the toxicological profile. An assessment of the test drug effect on vital organ functions should be available before the initiation of clinical studies. The timing of the toxicity assessment in the non-clinical studies should be scientifically justified based on the anticipated toxicity profile and the clinical schedule. A common approach for many small molecules is to set a start dose at 1/10 of the severely toxic dose in 10% of the animals (STD 10) in rodents. If the non-rodent is the most appropriate species, then 1/6 the highest non-severely toxic dose (HNSTD) is considered an appropriate starting dose.

The ICH S4 guideline discussed the duration for the chronic toxicity studies. For rodents and non-rodents, study duration of 6 and 9 months respectively is acceptable, sufficient and study duration of 12 months is usually not necessary. The above conclusion

was drawn based on the findings and scientific consensus to develop a hormonised chronic toxicity testing to ensure a more economical use of materials, animal and human resources, while at the same time safeguarding the public health.

2.8 Toxicity studies of ACA

Despite abundant reports of pharmacological activities on ACA, its toxicity profile is not well established. In preclinical drug development, every NEC must go under several assessments through *in vitro* studies such as potential interactions with other drugs as assessed with the liver cytochrome P450 superfamily as per USFDA guidelines, since the interaction with other co-administered drugs arises from inhibition of the metabolising cytochrome P450s enzyme. Haque and his colleagues showed that ACA exhibited drug-drug interactions when co-administered with other drugs that are metabolised specifically by CYP1A2, CYP2D6 or CYP3A4 enzymes (Haque et al., 2017). A brief *in vivo* oral toxicity study of ACA was reported in the literature (Kale & Namdeo, 2014), which showed that an oral dose of 20 mg/kg in mice was non-toxic and caused no lethality. Arshad and co-workers administered ACA for 8 weeks in nude mice xenograft tumour model *via* sub-cutaneous route on a twice-a-week regimen and identified an effective dose of 1.56 mg/kg to reduce tumour volume significantly. At a higher dose of 2.6 mg/kg, ACA was found to cause lung inflammation.

In view of insufficient toxicity data in the literature on ACA and based on the most probable route of cancer chemotherapy in clinical medicine, it was therefore necessary to conduct a detailed intravenous (IV) toxicity evaluation of this potential natural anti-cancer compound (Arshad et al., 2015).

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant materials, chemicals and reagents

Rhizomes of *Alpinia conchigera* Griff were collected from Jeli province of Kelantan, east-coast of Peninsular Malaysia. The sample was identified by Prof. Dr. Halijah Ibrahim from the Institute of Biological Science, Division of Ecology and Biodiversity, Faculty of Science, University of Malaya. Extraction of the natural compound involved solvent extractions, chromatographic methods, HPLC profiling and NMR structure verification, all of which were conducted by Ms. Haslinda Mohd Salleh from the Department of Chemistry, Faculty of Science, University Malaya. A voucher specimen (KL5049) was deposited in the Herbarium of Chemistry Department, Faculty of Science, University of Malaya. Healthy Sprague-Dawley (SD) rats were obtained from the Faculty of Veterinary Medicine, University Putra Malaysia, Malaysia. Propylene glycol was purchased from R & M Chemicals, Malaysia. Metabolic cages were purchased from Techniplast, Italy. Ketamine (80 mg/kg) – xylazine (8 mg/kg) cocktail was supplied by veterinary doctors at Animal Experimental Unit, University of Malaya. Decon 90 was purchased from Decon Laboratories Ltd, United Kingdom. Alcohol swabs, vacutainer Spray-Coated K2EDTA and vacutainer SST II Advance (serum with gel) tubes were purchased from Becton, Dickinson, American Medical Technology, United States of America. Disposable syringes 1 and 5 mL, needle size 26 G x 1/2 inch and needle size 25G x 1 inch were purchased from Terumo company. Nylon syringe filter (0.22 μm) purchased from Chromfilter company, Hong Kong. Polypropylene rat housing cages and rodent food (standard dry pellet). A 1, 2, and 10 mL volumetric flasks. A 10, 50, 250, 500, and 1000 mL glassware tubes. Ear-puncher and dissection kits purchased from Roboz Surgical Instrument Co, United States of America. Phosphate buffered saline (PBS), ethanol absolute 99.8% AR, formalin, alcohol,

paraffin, hematoxylin and eosin dyes, plastic cubes for mounting organ sections, containers. Urine collection tubes, dichloromethane, C fold towels, cotton, tissues, micropipette tips, pipettes, centrifuge system.

3.2 Source of 1'-S-1'-acetoxychavicol acetate (ACA)

Briefly, 2.1 kg of rhizomes of *A. conchigera* were washed, dried and pulverised into powder. The constituents of the powder were extracted using hexane for seventy-two hours at room temperature. The solvent was evaporated under vacuum and the resulting extract was subjected to silica gel column chromatography (Merck Kiesegel 60).

3.3 Sample preparation

ACA, with 98 % of purity, was dissolved in 2% propylene glycol and 98% sterile phosphate buffered saline (v/v) mixture and filtered through 0.22 μm syringe filter (Nylon) to ensure sterility of the sample. The solutions were prepared freshly on the day of use under aseptic condition. Prior to administration of the sample, each rat was anesthetised by intraperitoneal administration of ketamine (80 mg/kg) – xylazine (8 mg/kg) cocktail.

3.4 Experimental animals and study design

Healthy adult SD rats of both sexes aged between 6-8 weeks and weighing 200-300 g were purchased from the Faculty of Veterinary Medicine, University Putra Malaysia, Malaysia. The study adopted the methods based on the OECD guideline 407 for the Testing of Chemicals (OECD, 2008) with slight modification and a published *in vivo* study of De Jong et al. (2013). The IV route was chosen in continuation to our previous intraperitoneal *in vivo* studies and it is also the recommended route of anticancer medicinal products for human use to give 100% bioavailability. The SD rats were quarantined for at least a week

prior to the initiation of dosing to acclimatise them to the environment and were assigned randomly into groups. The rats were ear-punched for identification and housed in polypropylene cages under controlled environmental conditions at 22-25°C, 55 ± 15% relative humidity with light/dark cycles of 12 hours. They were provided food (standard dry pellet) and water *ad libitum.* All experimental handling procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, University Malaya, Malaysia (Ethics approval no: 2015-181103/IBS/R/YOAA). The study was conducted in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal house facility available within the University.

3.4.1 Sighting toxicity study

The doses for the acute toxicity study were determined based on a preliminary sighting study. Briefly, three intravenous doses of minimum (5), median (10) and maximum (20) mg/kg of ACA were administered through the lateral tail vein in a single rat (female), one after another with a gap of 24 hours. One third, one fifth and one tenth of this maximum tolerated dose (MTD) was used in the acute study.

3.4.2 Acute toxicity study of ACA

Twenty-four healthy SD rats of both sexes were assigned randomly into four groups; each group consisted of six rats (n = 6). Doses for single-dose acute study ($1/10^{th}$, $1/5^{th}$ and $1/3^{rd}$ of 20 mg/kg, respectively) were selected based on the maximum tolerable dose (MTD) from preliminary sighting study. Animals were fasted overnight prior to the administration of ACA and were given a single dose via the intravenous route in lateral tail vein with doses of 0 (control), 2 (low dose), 4 (medium dose) and 6.66 mg/kg (high dose) of ACA. The volume of administration was 1 mL/kg body weight. All animals were

observed closely for 24 hours and up to 14 days for any mortality or presence of behavioural changes and clinical symptoms of toxicity, such as changes in skin, lacrimation and fur/hair loss.

3.4.3 Sub-acute toxicity study of ACA

Sixty-four healthy male and female SD rats were divided into four experimental groups, comprising of three treatment groups and one vehicle control group. During the experiment, rats were assigned into groups of 16 (8 males and 8 females), housed at 3 rats/cage. The rats in the control group were injected with the vehicle through the caudal vein, and the rats in treatment groups were injected with a low dose (0.66 mg/kg), medium dose (1.33 mg/kg) and high dose (2.22 mg/kg) at an administration volume of 1 mL/kg body weight once a week for 28 days through the same route. At the end of the 28 days study, the recovery groups, which consisted of three rats per sex per treatment were monitored for an additional two weeks. No treatment was given during the recovery period. All animals were observed closely and daily for any mortality or presence of behavioural changes and clinical symptoms of toxicity. Body weights of individual rat and food/water consumptions were measured on a weekly basis.

3.5 Determination of haematological parameters

The night before the day of dissection, all rats were fasted for 12 hours and provided only with water. Rats were placed in metabolic cages for urine collection. The rats were observed closely. At the time of sacrifice, rats were given a dose of ketamine-xylazine. 3 mL blood samples were withdrawn via cardiac puncture method. The blood samples were collected in K_2EDTA tubes for whole blood haematological determinations. The blood samples were kept immediately in ice until analysis. The parameters such as haemoglobin

(Hb), total red blood cells (RBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular Hb (MCH), mean corpuscular Hb concentration (MCHC), red cell distribution width (RDW), total white blood cells (WBC), neutrophils, lymphocytes, eosinophils, monocytes and platelet count were assessed. All blood samples were sent to an ISO-certified pathology lab (MS ISO 15189:2006) in Kuala Lumpur, Malaysia for analysis. Gribbles pathology lab is well-equipped with basic and advanced analytical instruments for various samples of human and animal origin. The company provides diagnostic and analytical services to research groups, government, and pharmaceutical industries as well as veterinary practitioners.

3.6 Determination of biochemical parameters

5 mL blood samples were withdrawn from rat heart for biochemical blood analysis. After blood collection, the collected fresh blood was placed in serum separation gel tube to separate serum. The tubes were inverted 5 to 6 times and kept for 45 min in stand position at room temperature. The collected samples were centrifuged at 1500 x g for 15 mins and kept in ice until analysis. The following parameters were assessed and examined: total protein (TPR), albumin, globulin, albumin/globulin (A/G) ratio, alkaline phosphatase (ALP), total bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT); electrolytes, such as, K^+, Na^+, Cl^- and Ca^{2+} (Gribbles® Pathology lab, Malaysia).

3.7 Histopathological examination

After the blood collection, rats were euthanised and sacrificed via anaesthetic overdose of ketamine (80 mg/kg) and xylazine (7 mg/kg) mixture and vital organs, such as brain, lungs, heart, liver, kidneys, spleen, ovaries and testes, were harvested. Histopathological examination was carried out following the routine haematoxylin and

eosin staining protocol. The organs were then weighed by a digital balance (Mettler Toledo PB5001) and preserved in 10% neutral buffered formaldehyde solution. The organs were sent for analysis to Assoc. Prof. Dr. Mun Kein Seong, Histopathology Laboratory, Faculty of Medicine, University of Malaya. Trimmed specimens are placed into plastic cassettes and put through the Leica TP 1020 Tissue Processor, then embedded in paraffin wax using Leica EG 1160 Paraffin Embedding Center. Thin (4 - 6 μm) sections of paraffin-embedded tissues were sectioned using a microtome (Rotary Microtome Leica RM2245) and stained with hematoxylin and eosin (Leica ST 5010 Autostainer). Stained sections were then visualized for pathological changes using the microscope Olympus BX51 and captured using a Olympus LC30 digital camera.

Relative organ weight (%) was determined for all organs (Sabetghadam et al., 2013), using the equation given below:

$$\text{Relative organ weight (\%)} = \frac{\text{Weight of the organ (g)}}{\text{Weight of the body (g)}} \text{ x } 100$$

3.8 Urinalysis

During the last day of the experiment in both acute and sub-acute toxicity studies, rats were kept individually in metabolic cage for 12 hours to collect fresh urine samples. Urine samples were collected in sterile containers and kept in ice until analysis. Urine parameters assessed in the study were pH, protein, ketones, glucose, presence of epithelial cells and blood cells. All the analyses were carried out by Gribbles® Pathology lab.

3.9 Statistical analysis

The data were analysed using the Statistical Package for Social Sciences software version 24 (IBM Inc. USA). Results were expressed as mean ± standard error mean (SEM).

One-way and two-way analysis of variance (ANOVA) followed by Dunnett's post-hoc tests were carried out to analyse the statistical significance of treatments on gender and their interaction, as well as the differences between ACA treated groups and the control. Statistical significance was set at $p < 0.05$.

CHAPTER 4: RESULTS

The test compound, ACA, is evaluated for acute and sub-acute toxicity profile in rodents and the results of the study are presented in this chapter. This chapter also includes the body weight, water/food consumption, behavioural aberrations, biochemical, haematological, histopathological and urine analysis data. The variations in the results of the vital parameters were statistically analysed to find clinical significance and relevance to extrapolate the findings for further studies.

4.1 Sighting study

The sighting study was carried out to allow selection of the appropriate starting dose for the acute toxicity investigation. Intravenous (i.v) doses of 5, 10 and 20 mg/kg were used and there were no deaths in the rats even at the highest dose during the sighting study as described in section 3.4. Hence, the 20 mg/kg of ACA was considered as the maximum tolerated dose (MTD). Based on the results of the sighting study, the dose range for acute toxicity investigation was fixed at $1/10^{th}$, $1/5^{th}$ and $1/3^{rd}$ of MTD, which are 2, 4 and 6.66 mg/kg.

4.2 Acute toxicity study of ACA

Toxicity assessment of test compounds is a regulatory requirement prior to drug development. As a part of this development process, such test compounds have to be evaluated primarily for the acute toxicity effects. The acute study was performed using SD rats of both sexes as mentioned in section 3.4.2. The animals were treated with a single i.v dose of ACA at 2, 4 and 6.66 mg/kg body weight for the experimental period of 14 days.

The physical, haematological, biochemical, histopathological and urinary parameters were recorded and analysed.

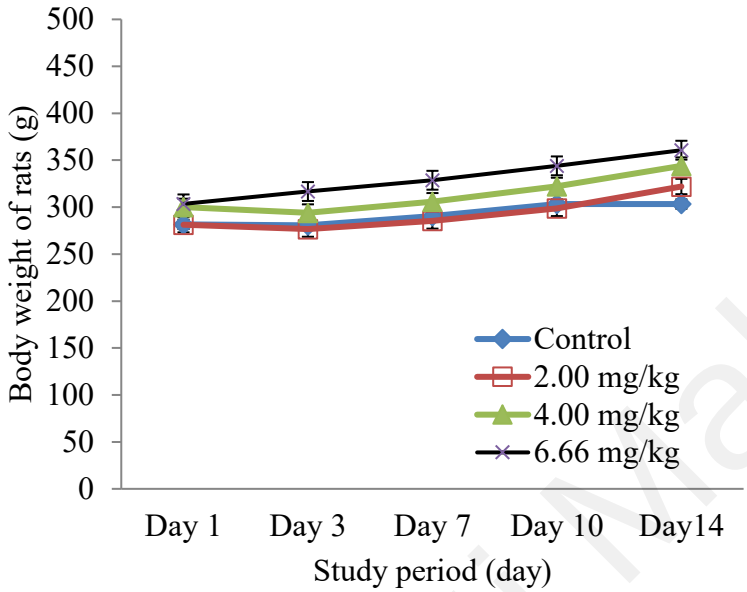
4.2.1 Body weight and food/water consumption in acute toxicity study

The i.v administration of single doses at 2, 4 and 6.66 mg/kg treatment resulted in no death of the treated male and female SD rats. The respective figures showing the body weight changes are presented in Figure 4.1A and B. Physical conditions of rats such as behaviour, gait, appearance, hair loss and general activity were observed after treatment of ACA each day.

The body weights were taken regularly every three days. There was a normal weight gain observed during the 14 days of study. Therefore, body weight was not a parameter affected by ACA treatment in both genders. No significant difference was recorded when comparing treated animals with the control. No toxicity signs were observed on animals following the 14 days of treatment with the three doses of ACA. All animals survived until the end of the acute toxicity study.

The food and water consumptions were recorded at intervals of twice a week as shown in Figure 4.2A and B. There were no significant changes in the food and water consumption in the SD female and male rats when compared with the control.

(A)



(B)

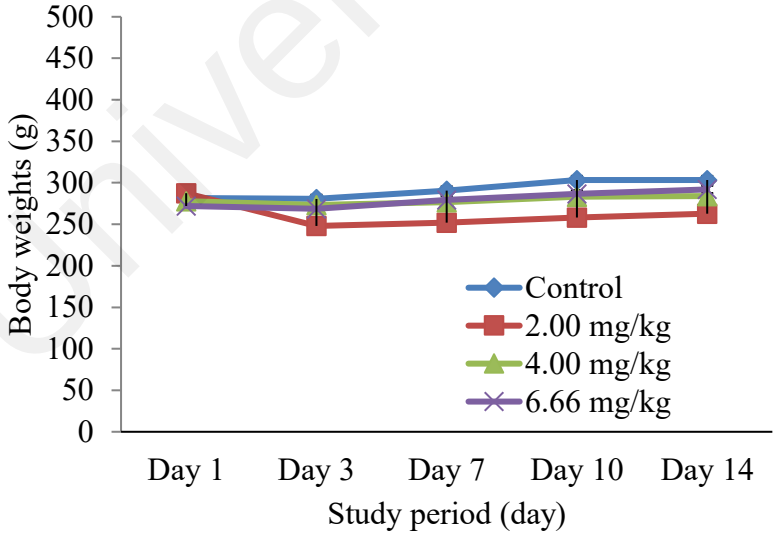
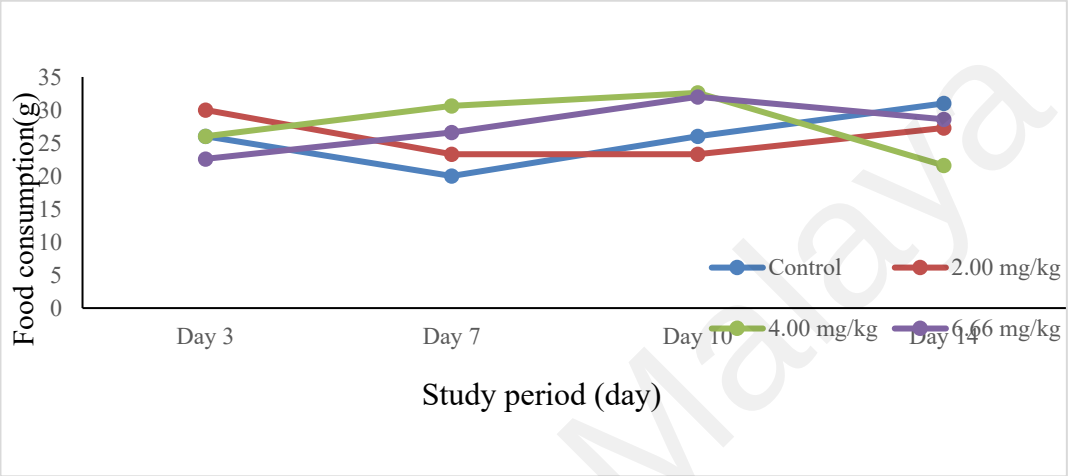


Figure 4.1: Body weight measurements (g) in the male (A) and female (B) SD rats

(A)



(B)

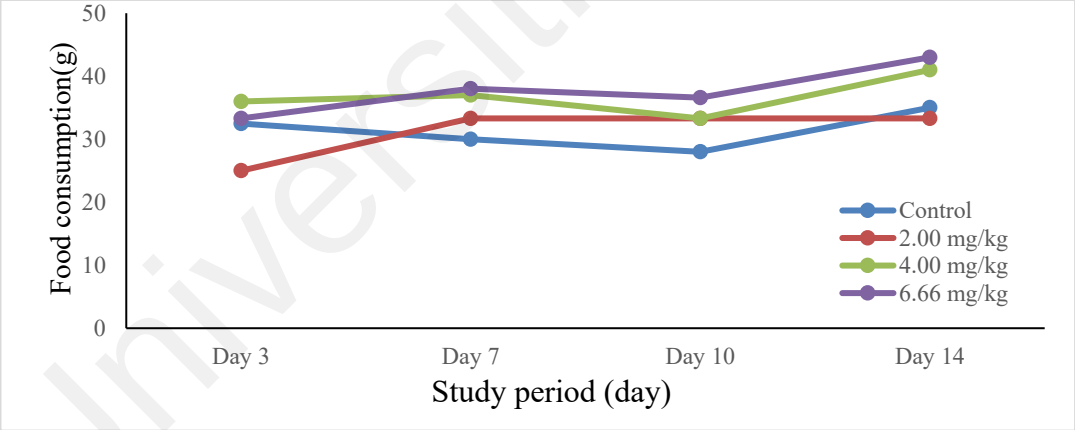
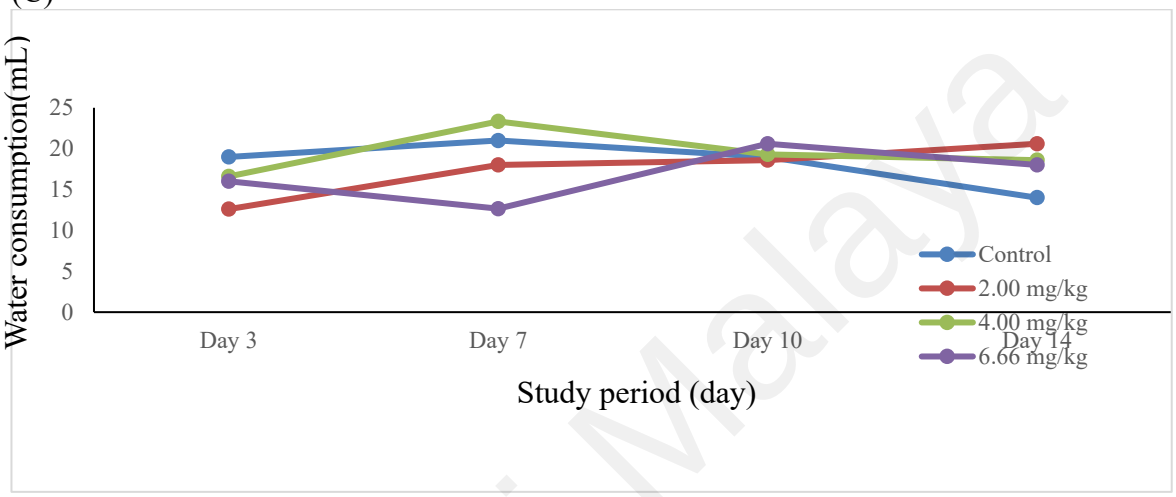


Figure 4.2A: Measurements of food consumption (g) in male (A) and female (B) SD rats, respectively.

(C)



(D)

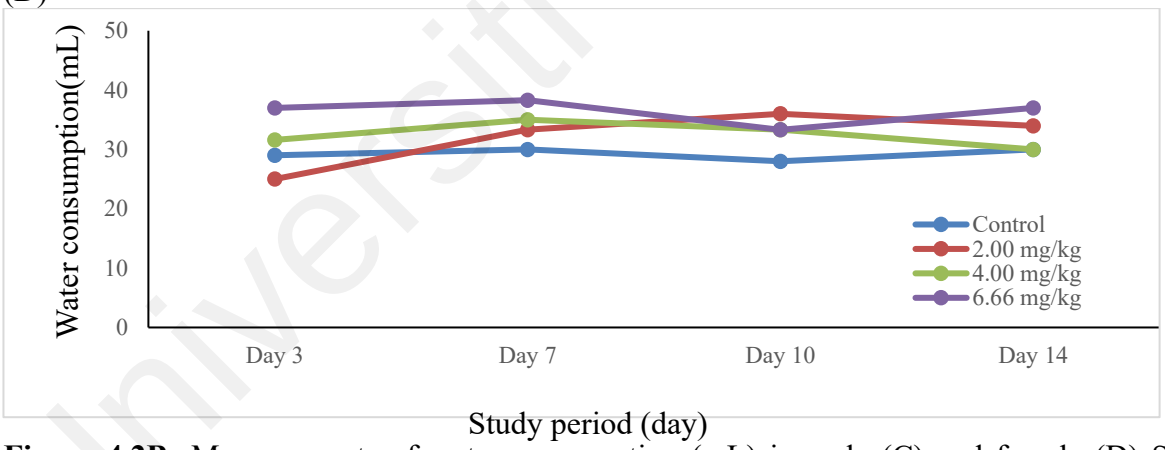


Figure 4.2B: Measurements of water consumption (mL) in male (C) and female (D) SD rats, respectively.

4.2.2 Haematological parameters in acute toxicity study

The blood samples from experimental animals were collected through cardiac puncture under ketamine-xylazine anaesthesia at the end of the 14 days study and were analysed for various haematological parameters. 3 mL of blood was collected into EDTA tubes, mixed gently and sent to Gribbles® for the complete blood count analyses. The results were presented in Table 4.1. The haematological parameters evaluated were red blood cells (RBC), white blood cells (WBC), packed cell volume (PCV), haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), neutrophils (NEU), lymphocytes (LYM) and platelets (PLT). Majority of the haematological parameters were not significantly affected, except for WBC, MCHC and neutrophils. WBC was reduced significantly ($p < 0.05$) in the groups treated with the 4 and 6.66 mg/kg dose of ACA as compared to the respective controls. MCHC was significantly high ($p < 0.05$) in males treated with the 4 and 6.66 mg/kg doses in comparison to the controls. Neutrophils were significantly ($p < 0.05$) increased in male rats at the 2 and 4 mg/kg doses. Despite the significant differences in levels of WBC, MCHC and neutrophils in the treated groups, their levels were all within the normal range.

Table 4.1: Haematological parameters of male and female SD rats treated with a single dose of ACA for 14-days acute toxicity study.

Parameters	Male (n = 3)				Female (n = 3)			
	Control	2 mg/kg	4 mg/kg	6.66 mg/kg	Control	2 mg/kg	4 mg/kg	6.66 mg/kg
RBC ($\times 10^{12}$/L)	7.10 ± 0.69	8.62 ± 0.14	8.28 ± 0.12	8.52 ± 0.13	8.02 ± 0.11	7.51 ± 0.21	7.79 ± 0.04	7.30 ± 0.30
Hb (g/L)	143.67 ± 7.27	154.00 ± 1.00	155.50 ± 6.5	156.33 ± 1.67	148.67 ± 2.91	136.67 ± 2.96	138.50 ± 6.50	137.00 ± 2.00
MCV (fL)	61.33 ± 0.88	56.50 ± 1.50	57.33 ± 2.73	54.00 ± 0.58	57.33 ± 1.20	57.00 ± 0.58	55.00 ± 2.00	59.33 ± 1.67
MCH (pg)	19.00 ± 0.00	17.50 ± 0.50	18.67 ± 0.67	18.33 ± 0.33	18.67 ± 0.33	18.00 ± 0.00	18.00 ± 1.00	18.67 ± 0.33
MCHC (g/L)	312.67 ± 5.21	316.50 ± 1.50	339.00 ± 2.00*	340.33 ± 2.85*	323.33 ± 1.86	319.67 ± 0.88	323.00 ± 2.00	320.50 ± 2.5
WBC ($\times 10^{9}$/L)	9.43 ± 1.03	6.05 ± 0.15	4.60 ± 0.84*	5.00 ± 0.42*	9.13 ± 0.94	5.13 ± 0.52	4.55 ± 2.05*	4.37 ± 0.85*
NEU (%)	14.33 ± 1.45	24.50 ± 4.50*	23.00 ± 1.00*	15.33 ± 0.88	17.66 ± 1.60	19.33 ± 2.20	20.66 ± 3.90	23.66 ± 1.20
LYM (%)	81.00 ± 1.73	73.50 ± 5.50	74.50 ± 2.50	77.67 ± 0.88	77.66 ± 2.72	75.33 ± 2.72	64.50 ± 14.50	70.00 ± 6.90
PLT ($\times 10^{9}$/L	569.33 ± 91.40	813.00± 167.00	655.33 ± 147.58	761.33 ± 45.81	665.33 ± 83.43	767.67 ± 15.32	690.00 ± 197.00	774.67 ± 102.44

RBC: total red blood cell, Hb: haemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular Hb, MCHC: mean corpuscular Hb concentration, WBC: total white blood cell, NEU: neutrophils, LYM: lymphocytes, PLT: platelets count.
Values are means ± SEM. Significantly different from the control, * $p < 0.05$

4.2.3 Biochemical parameters in acute toxicity study

Blood samples were collected from the animals *via* cardiac puncture into collection tubes and allowed to stand for 30-45 min at room temperature before being centrifuged to separate the serum and sent to Gribbles® for biochemical analyses. The biochemical parameters evaluated from blood serum samples were the total protein (TPR), albumin (ALB), globulin (GLO), albumin/globulin ratio (A/G ratio), cholesterol, blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), potassium, chloride, calcium, sodium and creatinine. TPR, ALB and GLO levels were significantly increased ($p < 0.05$) in male rats treated with 6.66 mg/kg. Calcium and ALT were significantly reduced ($p < 0.001$) in female rats treated with 4 mg/kg. Total cholesterol was significantly elevated ($p < 0.05$) at 6.66 mg/kg. Other parameters such as A/G ratio, potassium, chloride, sodium, BUN, creatinine and AST were not significantly changed in male and female after treatment with ACA for all three doses (Table 4.2).

Table 4.2: Biochemical parameters of male and female SD rats treated with a single dose of ACA for 14-days for acute toxicity study.

Parameters	Male (n = 3)				Female (n = 3)			
	Control	2 mg/kg	4 mg/kg	6.66 mg/kg	Control	2 mg/kg	4 mg/kg	6.66 mg/kg
TPR (g/L)	61.67 ± 0.88	61.67 ± 2.03	62.33 ± 0.88	73.00 ±1.00*	65.33 ± 0.88	63.00 ± 3.79	63.67 ± 2.91	60.67 ± 1.76
ALB (g/L)	34.67 ± 0.33	33.67 ± 0.67	32.67 ± 0.67	37.67 ± 0.88*	35.33 ± 0.33	33.50 ± 2.5	35.33 ± 1.76	34.00 ± 0.00
GLO (g/L)	27.00 ± 0.58	28.00 ± 1.53	29.67 ± 0.33	35.33 ± 1.2*	30.00 ± 1.00	27.00 ± 1.00	28.33 ± 1.33	26.67 ± 1.76
A/G ratio	1.29 ± 0.02	1.21 ± 0.06	1.10 ± 0.02*	1.07 ± 0.06	1.23 ± 0.02	1.33 ± 0.07	1.25 ± 0.04	1.29 ± 0.08
Cholesterol (μmol/L)	1.63 ± 0.09	1.53 ± 0.03	1.37.00 ±0.13	2.07 ± 0.12*	1.67 ± 0.13	2.07 ± 0.43	2.10 ± 0.25	1.91 ± 0.21
Potassium (mmol/L)	4.43 ± 0.12	5.17 ± 0.32	5.10 ± 0.31	5.43 ± 0.23	4.53 ± 0.23	4.53 ± 0.19	5.00 ± 0.42	4.97 ± 0.13
Chloride (mmol/L)	102.00 ± 1.16	105.67 ± 1.76	104.67 ± 0.33	101.67 ± 0.33	104.00 ± 0.00	103.00 ± 0.58	103.33 ± 0.88	104.67 ± 1.2
Calcium (mmol/L)	2.38 ± 0.02	2.39 ± 0.08	2.37 ± 0.05	2.51 ± 0.05	2.52 ± 0.04	2.46 ± 0.04	2.39 ± 0.02**	2.40 ± 0.01
Sodium (mmol/L)	141.00 ± 0.00	142.33 ± 0.67	144.00 ± 0.58	143.67 ± 0.33	141.33 ± 0.67	141.50 ± 1.5	143.67 ± 1.33	140.67 ± 0.88
BUN (mmol/L)	5.57 ± 1.02	6.37 ± 0.67	7.97 ± 0.33	7.57 ± 0.27	5.03 ± 0.26	4.93 ± 0.49	6.47 ± 1.22	7.83 ± 0.68
Creatinine (μmol/L)	25.33 ± 2.73	27.33 ± 2.85	26.33 ± 1.76	24.00 ± 1.73	34.00 ± 3.46	30.00 ± 4.04	35.00 ± 0.58	32.00 ± 3.00
AST (U/L)	123.33 ±2.91	133.00 ± 17.21	115.00 ± 10.39	101.00 ± 0.00	132.00 ± 19	110.50 ± 7.5	141.00 ± 8.74	119.00 ± 11.79
ALT (U/L)	34.67 ± 0.88	37.00 ± 2.52	45.67 ± 2.85	46.00 ± 2.65	40.67 ± 2.73	41.50 ± 1.50	27.33 ± 1.76**	38.67 ± 1.76

TPR: Total protein ALB: Albumin, GLO: Globulin, A/G ratio: Albumin/Globulin ratio, BUN: Blood urea nitrogen, AST: aspartate aminotransferase, ALT: alanine aminotransferase. Values are means ± S.E.M. Significantly different from the control, * $p < 0.05$, ** $p < 0.001$

4.2.4 Histopathological analysis in acute toxicity study

For histopathological analysis, tissue samples from major organs, such as brain, liver, spleen, lungs, adrenal glands, heart, testes, ovaries and kidneys were harvested. Morphological observations of the organs showed no significant changes in their respective shapes, sizes and colours. The organs were then weighed and preserved in 10% neutral buffered formaldehyde solution. Trimmed specimens were processed and embedded in paraffin wax. Thin (4 - 6 μm) sections were stained with haematoxylin and eosin.

Stained sections were then visualized for pathological changes using the microscope Olympus BX51. Findings are captured using a Olympus LC30 digital camera and shown in Figures 4.3A, B and C. Histopathological examination revealed interstitial pneumonitis of moderate severity in the lungs, scattered multinucleated giant cells in the spleen and mild lobular hepatitis in the liver in all treated and control rats. No necrosis or significant pathology were observed in tissue sections from other organs. There were no significant differences in the absolute and relative organs weight measurements between the treated and control groups as shown in Table 4.4.

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Table 4.4: Absolute and relative organ weights of male and female SD rats treated with a single dose of ACA in acute toxicity study

Organ	Male (n=3)				Female (n=3)			
	Control	2.00 mg/kg	4.00 mg/kg	6.66 mg/kg	Control	2.00 mg/kg	4.00 mg/kg	6.66 mg/kg
Brain (g)	1.75 ± 0.05	1.70 ± 0.05	1.73 ± 0.06	1.93 ± 0.03	1.80 ± 0.05	1.93 ± 0.12	1.93 ± 0.06	1.86 ± 0.14
Brain (% g)	0.62 ± 0 .05	0.54 ± 0.01	0.54 ± 0.03	0.55 ± 0.02	0.62 ± 0.05	0.73 ± 0.03	0.71 ± 0.04	0.68 ± 0.05
Heart (g)	1.03 ± 0.08	1.10 ± 0.00	1.20 ± 0.00	1.10 ± 0.00	1.03 ± 0.08	1.06 ±0.03	1.10 ±0.05	0.96 ± 0.08
Heart (% g)	0.35 ± 0.01	0.48 ± 0.07	0.34 ± 0.02	0.35 ± 0.01	0.35 ± 0.01	0.40 ± 0.03	0.40 ± 0.02	0.35 ± 0.01
Lung (g)	1.85 ± 0.15	1.93 ± 0.17	1.90 ± 0.10	2.10 ± 0.10	1.66 ± 0.20	1.433 ± 0.08	1.50 ± 0.10	1.66 ± 0.16
Lung (% g)	0.57 ± 0.05	0.62 ± 0.10	0.59 ± 0.02	0.66 ± 0.02	0.57 ± 0.05	0.55 ± 0.06	0.55 ± 0.03	0.64 ± 0.16
Liver (g)	11.85 ± 0.75	9.13 ± 0.08	11.16 ± 0.89	11.35 ± 1.25	9.60 ± 1.50	10.73 ±0.74	8.33 ± 0.57	8.70 ± 0.63
Liver (% g)	3.64 ± 0.41	3.02 ± 0.01	3.47 ± 0.23	3.59 ± 0.24	3.24 ± 0.16	3.93 ± 0.30	3.06 ± 0.16	3.19 ± 0.29
Kidney (g)	0.95 ± 0.05	1.11 ± 0.04	1.12 ± 0.12	1.28 ± 0.10	1.03 ± 0.08	1.00 ± 0.13	0.90 ± 0.00	0.88 ± 0.06
Kidney (% g)	0.35 ± 0.01	0.46 ± 0.11	0.37 ± 0.02	0.36 ± 0.01	0.35 ± 0.01	0.37 ± 0.02	0.33 ± 0.03	0.32 ± 0.03
Spleen (g)	0.75 ± 0.05	0.76 ± 0.03	0.70 ± 0.00	0.90 ± 0.10	0.66 ± 0.08	0.73± 0.06	0.66 ± 0.03	0.66 ± 0.03
Spleen (% g)	0.22 ± 0.02	0.31 ± 0.07	0.21 ± 0.01	0.25 ± 0.02	0.22 ± 0.02	0.27 ± 0.01	0.24 ± 0.03	0.24 ± 0.02

Values are means and standard error of the means (n= 3/sex /group). Significantly different from the control, * $p < 0.05$.

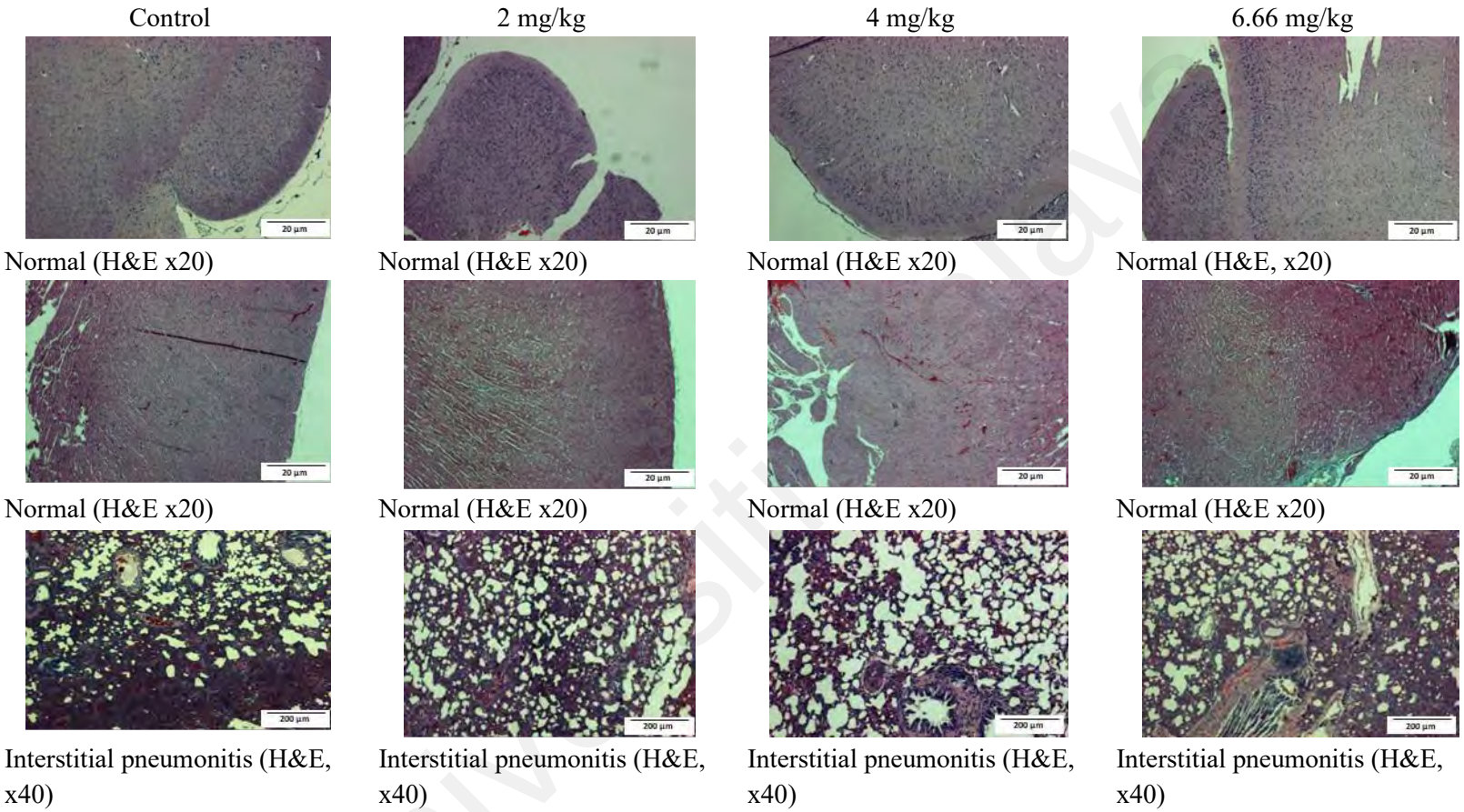


Figure 4.3A: Histopathological observations of the brain, heart and lung tissue sections in SD rats treated with ACA for 14 days of acute toxicity study. The photomicrographs are from representative SD rat of the respective groups. Sections from the heart and brain showed no significant abnormalities. The lung sections showed interstitial pneumonitis in rats from all the groups.

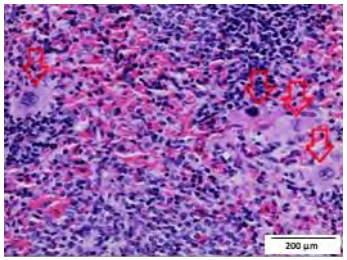
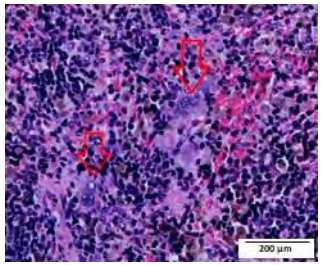
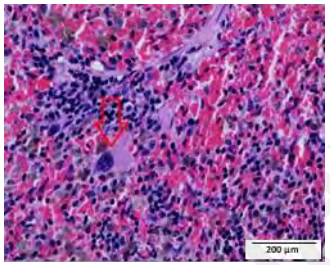
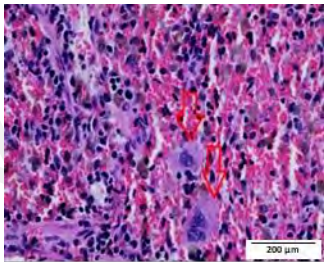
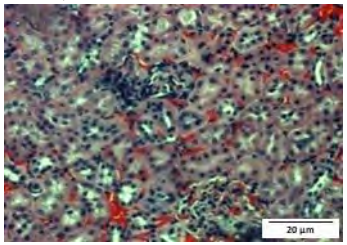
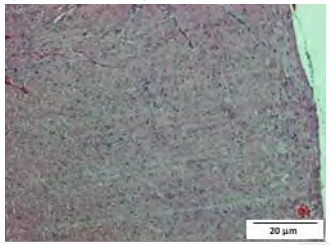
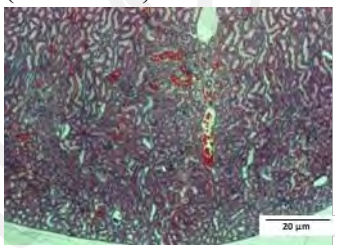
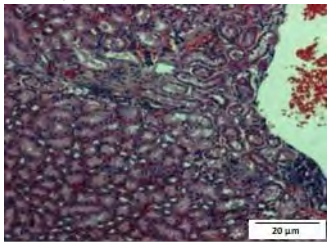
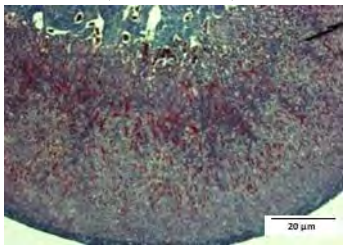
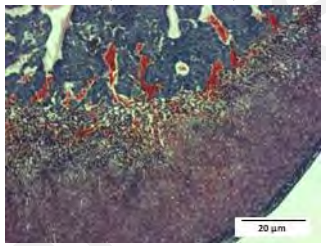
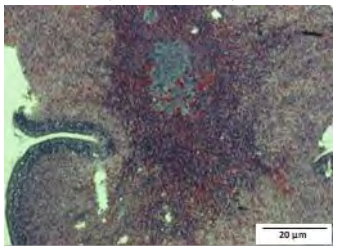
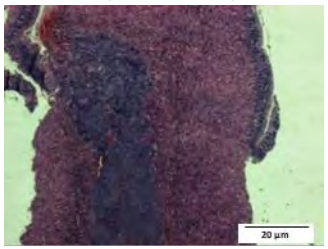
	Control	2 mg/kg	4 mg/kg	6.66 mg/kg
Spleen				
	Multinucleated giant cells (H&E x200)	Multinucleated giant cells (H&E x200)	Multinucleated giant cells (H&E x200)	Multinucleated giant cells (H&E x200)
Kidney				
	Normal (H&E, x20)	Normal (H&E, x20)	Normal (H&E, x20)	Normal (H&E, x20)
Adrenal gland				
	Normal (H&E, x20)	Normal (H&E, x20)	Normal (H&E, x20)	No abnormalities (H&E, x20)

Figure 4.3B: Histopathological observations of the spleen, kidney and adrenal gland tissue sections in SD rats treated with ACA for 14 days of acute toxicity study. The photomicrographs are from representative SD rat of the respective groups. Scattered multinucleated giant cells are seen in the spleen.

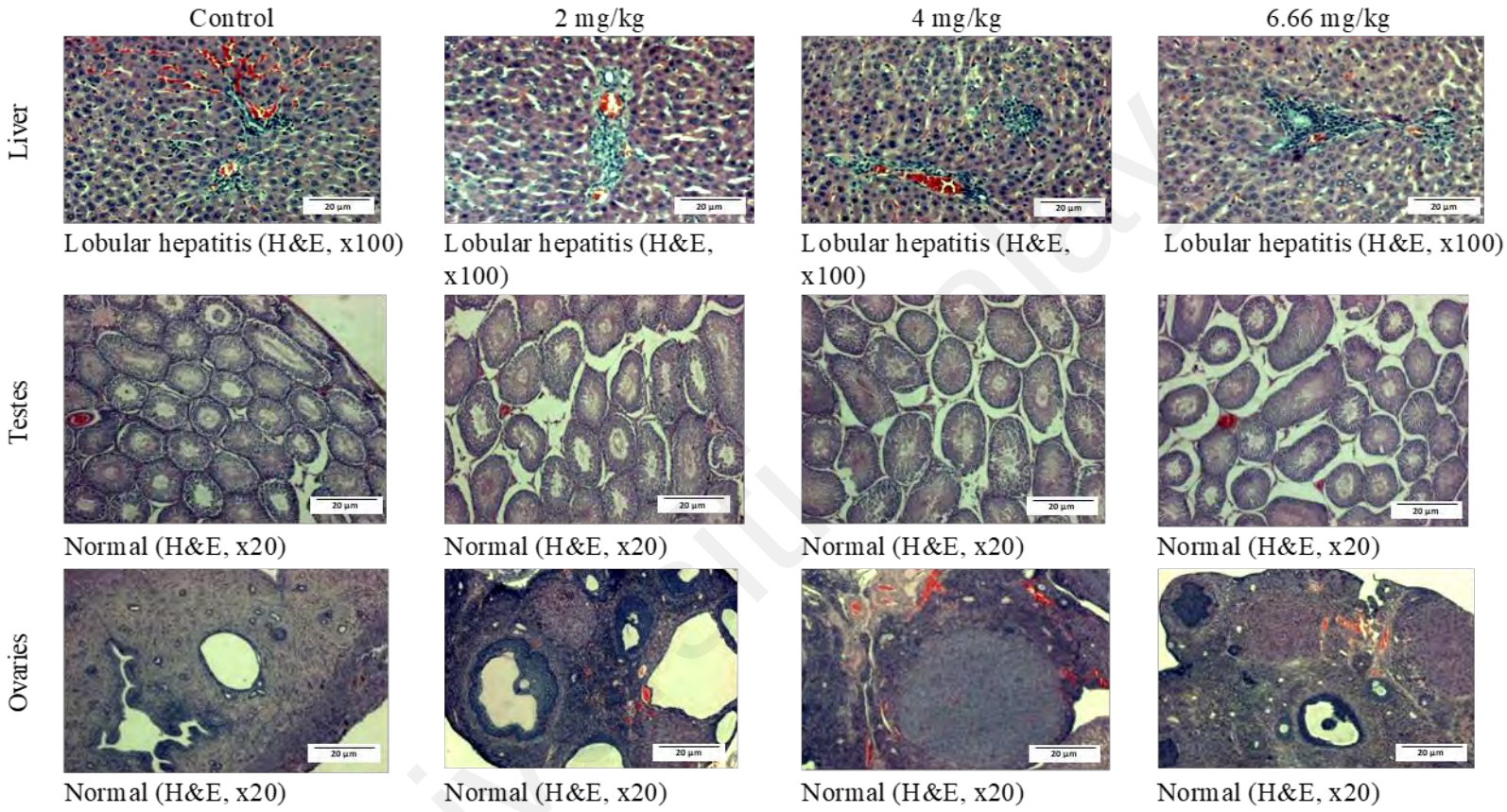


Figure 4.3C: Histopathological observations of the liver, testes and ovary tissue sections in SD rats treated with ACA for 14 days of acute toxicity study. The photomicrographs are from representative SD rat of the respective groups. The liver sections showed mild lobular hepatitis. The testes and ovaries are unremarkable and with normal limits.

4.2.5 Urine analysis in acute toxicity study

After placing individual animals in metabolic cages (Techniplast, Italy), urine samples were collected for urinalysis from all animals prior to the scheduled necropsy. The results of the urinalysis are presented in Table 4.3. The findings showed presence of protein and glucose in the 2 mg/kg treated group but not in the 4 and 6.66 mg/kg treated groups. However, traces of ketones were observed in the 2 and 6.66 mg/kg treated groups. The other urine parameters were normal at the end of 14-days acute toxicity study.

Table 4.3: Urinalysis findings of male rats treated with ACA for 14-days of acute toxicity study.

Parameters		Treatment doses (mg/kg)							
		Male (n=3)				Female (n=3)			
		Control	2	4	6.66	Control	2	4	6.66
Protein	N	2	0	2	0	0	0	0	1
	1+	0	0	1	3	1	2	1	2
	2+	1	3	0	0	0	1	2	0
	3+	0	0	0	0	2	0	0	0
Glucose	N	3	1	3	3	3	3	3	3
	T	0	2	0	0	0	0	0	0
pH	7	0	0	0	0	0	0	0	1
	7.5	2	2	3	3	0	1	1	2
	8	0	0	0	0	1	2	0	0
	8.5	1	1	0	0	2	0	2	0
Ketone	N	0	0	0	0	0	0	0	0
	T	2	0	2	0	3	1	0	3
	1+	0	1	1	1	0	2	3	0
	2+	1	2	0	2	0	0	0	0
Blood cells	N	3	2	3	3	3	3	3	3
	T	0	1	0	0	0	0	0	0
Epithelial cells	N	3	3	3	3	3	2	3	3
	T	0	0	0	0	0	1	0	0
Crystals	N	2	3	2	3	0	1	0	1
	T	1	0	1	0	3	2	3	2
Casts	N	3	3	3	3	3	3	3	3
	T	0	0	0	0	0	0	0	0

Urinalysis of treated rats with ACA for 14-days (n= 3/sex /group).
N = Nil; T = Traces.

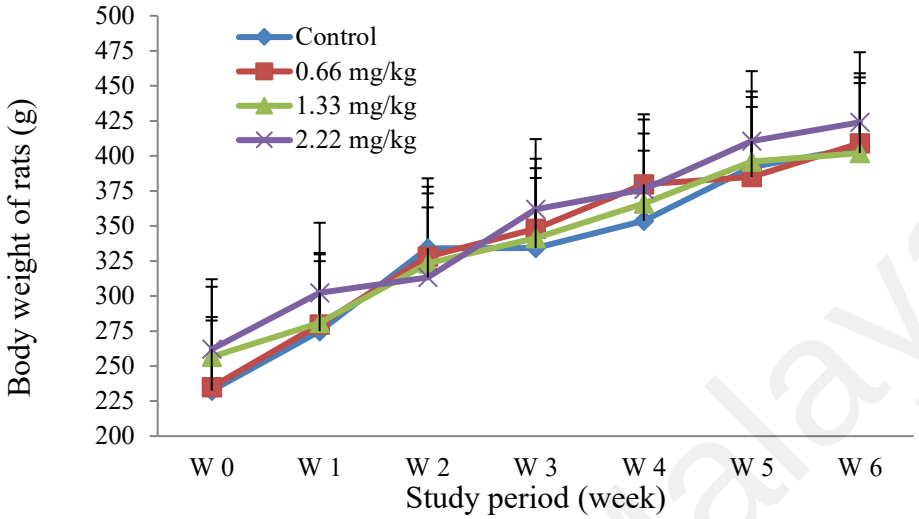
4.3 Sub-acute toxicity study of ACA

Sub-acute toxicity assessment of test compound is a regulatory requirement to see the effect of repeated doses during drug development. The sub-acute toxicity study was performed using SD rats of both sexes as mentioned in the section 3.4.3. The animals were treated with repeated i.v doses of ACA at 0.66, 1.33 and 2.22 mg/kg body weight for the experimental period of 28 days. The physical, haematological, biochemical, histopathological and urinary parameters were recorded and analysed.

4.3.1 Body weight and food/water consumption in sub-acute toxicity study

For the sub-acute toxicity study, the animals were administered with ACA at weekly doses of 0.66 mg/kg, 1.33 mg/kg and 2.22 mg/kg with the untreated placebo group as control for a period of 28 days**.** The animals were then observed closely for mortality or presence of any physical changes after the ACA administration. During the study, the animals in each group were healthy, consumed food/water normally and active. There was no death or any abnormal discharge from the nose, eyes or mouth. The rats gained weight regularly with time. The body weights revealed no significant differences ($p < 0.05$) between the treated and control groups as shown in Figure 4.4A and B. There were no statistically significant differences ($p < 0.05$) in total food or water intake as presented in Figure 4.5C, D, E and F in both female and male SD rats.

(A)



(B)

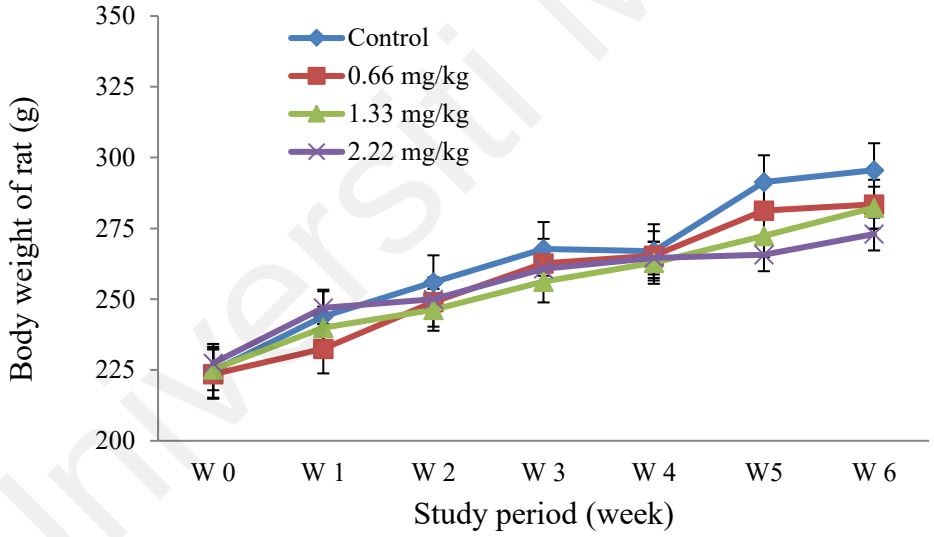
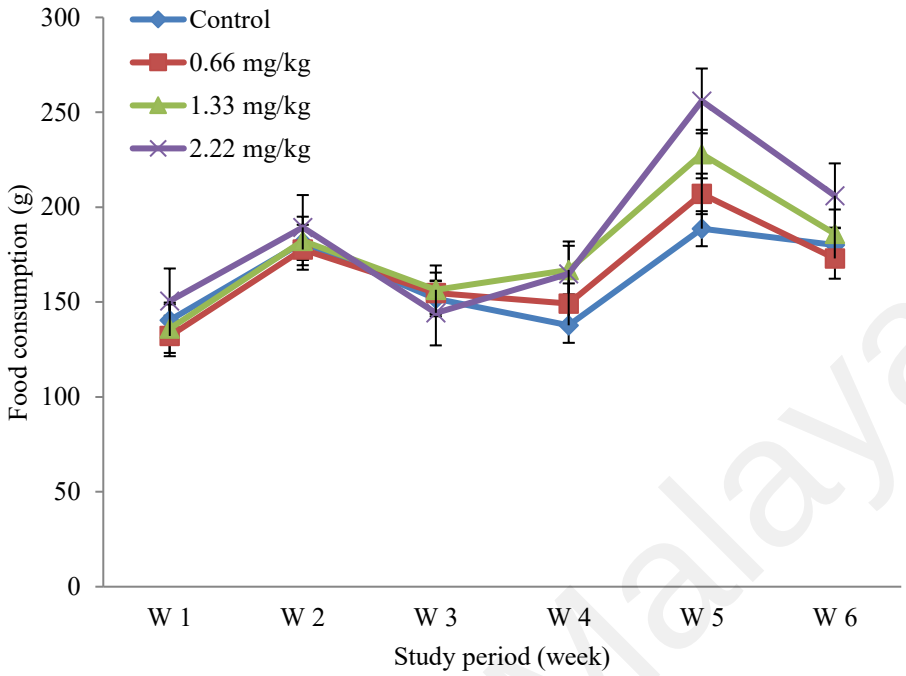


Figure 4.4: Effects of intravenous administration of ACA in sub-acute toxicity study on mean of body weight in SD male (A) and female rats (B). The error bars represent SEM and the sample size, n=8 until treatment period, n=3 during recovery period. No significant difference ($p < 0.05$) from the control.

(C)



(D)

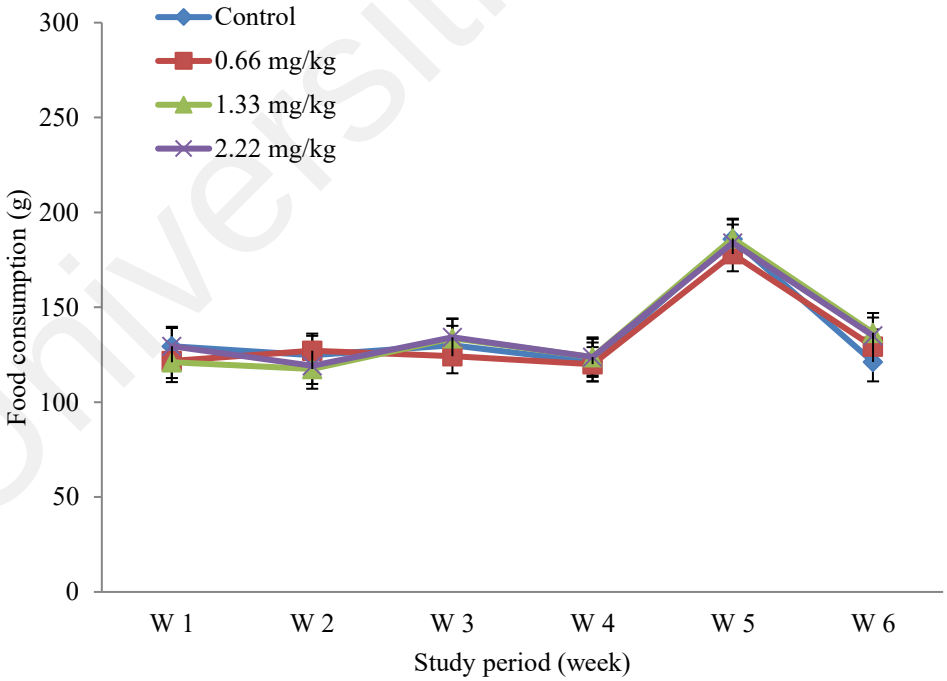
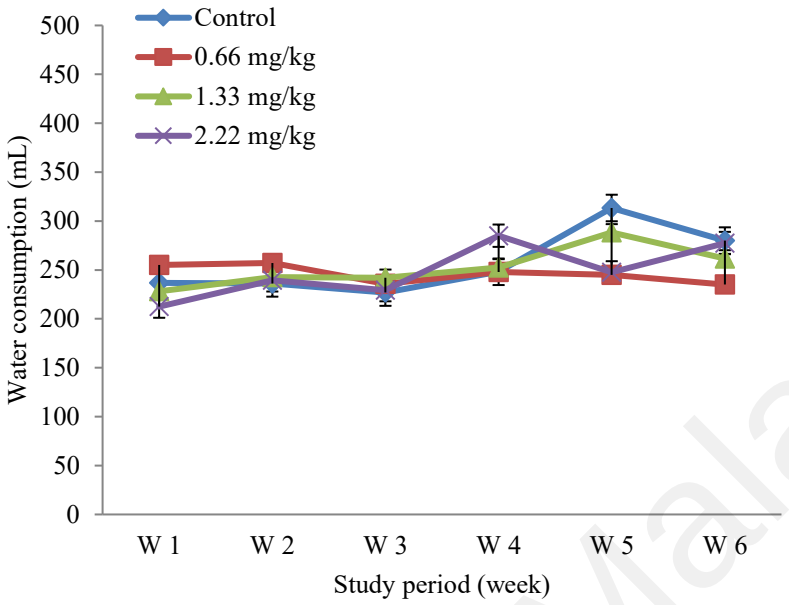


Figure 4.5: Effects of intravenous administration of ACA in sub-acute toxicity study on mean food consumptions in SD male (C) and female rats (D). The error bars represent SEM and the sample size, n=8 during treatment period, n=3 during recovery period. No significant difference ($p < 0.05$) from the control.

(E)



(F)

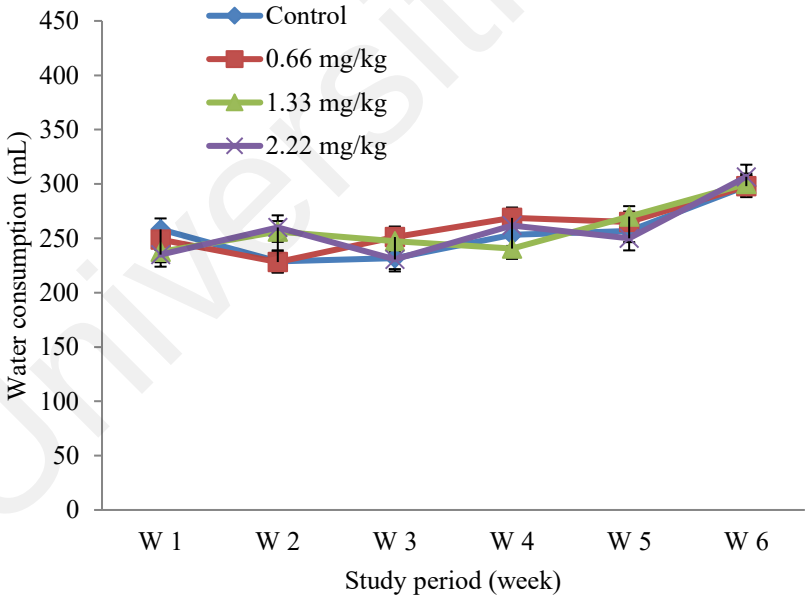


Figure 4.5: Effects of intravenous administration of ACA in sub-acute toxicity study on mean water consumptions in SD male (E) and female rats (F). The error bars represent SEM and the sample size, n=8 during treatment period, n=3 during recovery period. No significant difference ($p < 0.05$) from the control.

4.3.2 Haematological parameters in sub-acute toxicity study

The blood samples from experimental animals were collected through cardiac puncture under ketamine-xylazine anaesthesia at the end of the 28 days study and were analysed for various haematological parameters. 3 mL of blood was collected into EDTA tubes, mixed gently and sent to Gribbles® for the complete blood count analyses. The results were presented in Table 4.5 and 4.6. The haematological parameters evaluated were red blood cells (RBC), white blood cells (WBC), packed cell volume (PCV), haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), neutrophils (NEU), lymphocytes (LYM) and platelets (PLT). Majority of the haematological parameters were not significantly affected. However, the MCHC level that should be corresponding to the level of MCV was found to be significantly increased ($p < 0.001$) in 1.33 and 2.22 mg/kg dose female treated groups. This finding was not observed in male rats.

4.3.3 Biochemical parameters in sub-acute toxicity study

Blood samples were collected from the animals *via* cardiac puncture into collection tubes and allowed to stand for 30-45 min at room temperature before being centrifuged to separate the serum and sent to Gribbles® for biochemical analyses. The biochemical parameters evaluated from serum samples were total protein (TPR), albumin (ALB), globulin (GLO), albumin/globulin ratio (A/G ratio), cholesterol, blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), potassium, chloride, calcium, sodium and creatinine. The majority of the biochemical parameters in the male rats were unaffected insignificantly ($p > 0.05$) with treatment by all three doses, except there was significant increase ($p < 0.05$) in the levels of some liver parameters were

found in blood serum samples, such as, TPR, albumin and globulin treated with 1.33 and 2.22 mg/kg of ACA in comparison to the control group (Table 4.7 and Table 4.8). However, the increase in the levels of these parameters does not implicate significant liver toxicity. Notably, the increased level of these liver parameters was not observed in the female rats.

Similarly, majority of renal parameters did not show significant toxicity, except a significant reduction was seen in the potassium levels of male rats treated with 1.33 and 2.22 mg/kg of ACA. Another renal marker, creatinine was significantly reduced ($p < 0.05$) in the recovery group of female rats treated with 2.22 mg/kg of ACA.

Table 4.5: Hematological parameters of male and female SD rats after 28 days treatment of ACA in sub-acute toxicity study.

Parameters	Treatment (n = 5/sex)			
	Control	0.66 mg/kg	1.33 mg/kg	2.22 mg/kg
Male				
RBC ($\times 10^{12}$/L)	8.10 ± 0.08	7.54 ± 0.33	7.58 ±0.38	7.76 ± 0.21
Hb (g/L)	147.00 ± 0.58	137.00 ± 6.76	134.80 ± 6.63	141.00 ± 4.26
PCV (L/L)	0.43 ± 0.02	0.40 ± 0.01	0.40 ± 0.02	0.43 ± 0.02
MCV (fL)	56.00 ± 0.95	56.00 ± 1.52	53.00 ± 0.32	54.50 ± 0.87
MCH (pg)	18.00 ± 0.00	18.67 ± 0.33	17.80 ± 0.20	18.00 ± 0.00
MCHC (g/L)	324.60 ± 4.68	330.50 ± 4.71	335.80 ± 2.65	332.51 ± 2.40
RDW (%)	13.88 ± 0.35	13.86 ± 0.63	13.82 ± 0.35	13.55 ± 0.30
WBC ($\times 10^{9}$/L)	6.67 ± 0.54	5.60 ± 0.79	5.57 ± 0.79	6.43 ± 0.77
NEU (%)	18.75 ± 1.11	19.00 ± 2.08	16.25 ± 0.63	16.67 ± 2.73
LYM (%)	75.41 ± 3.03	75.60 ± 2.02	78.00 ± 1.73	76.33 ± 3.18
MONO (%)	2.50 ± 0.50	2.40 ± 0.68	3.00 ± 0.82	3.00 ± 0.71
PLT ($\times 10^{9}$/L)	631.00 ± 86.12	649.50 ± 125.24	732.67 ± 32.77	673.33 ± 118.97
Female				
RBC ($\times 10^{12}$/L)	7.53 ± 0.51	7.28 ± 0.40	7.66 ± 0.14	7.86 ± 0.25
Hb (g/L)	143.20 ± 8.51	135.40 ± 8.06	145.20 ± 3.17	147.40 ± 3.93
PCV (L/L)	0.43 ± 0.03	0.43 ± 0.02	0.44 ± 0.01	0.45 ± 0.01
MCV (fL)	59.00 ± 0.89	58.80 ± 0.37	57.40 ± 0.51	57.60 ± 0.68
MCH (pg)	18.20 ± 0.2	18.40 ± 0.25	19.00 ± 0.32	18.60 ± 0.40
MCHC (g/L)	309.60 ± 2.36	315.00 ± 3.07	330.0 ± 3.42**	325.40 ± 0.87**
RDW (%)	13.53 ± 0.39	13.10 ± 0.27	13.22 ± 0.2	13.16 ± 0.23
WBC ($\times 10^{9}$/L)	5.90 ± 0.6	7.60 ± 0.90	6.65 ± 1.00	5.82 ± 0.70
NEU (%)	19.40 ± 2.84	14.00 ± 0.71	16.75 ± 1.03	21.00 ± 2.70
LYM (%)	77.00 ± 2.88	81.60 ± 2.04	74.6 ± 3.33	73.60 ± 2.96
MONO (%)	4.00 ± 0.68	4.10 ± 1.11	4.67 ± 0.98	4.75 ± 0.66
PLT ($\times 10^{9}$/L)	705.20 ± 51.1	691.25 ± 44.8	752.00 ± 17.69	609.6 ± 46.01

RBC: total red blood cell, Hb: haemoglobin, PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular Hb, MCHC: mean corpuscular Hb concentration, RDW: red cell distribution width, WBC: total white blood cell, NEU: neutrophils, LYM: lymphocytes, MONO: monocytes, PLT: platelets count. Values are means ± SEM. Significantly different from the control, ** $p < 0.001$

Table 4.6: Hematological parameters of male and female SD rats after 14 days recovery of ACA treatment in sub-acute toxicity study.

Parameters	Recovery period (n = 3/sex)			
	Control	0.66 mg/kg	1.33 mg/kg	2.22 mg/kg
Male				
RBC ($\times 10^{12}$/L)	8.00 ± 0.23	7.82 ± 0.38	7.93 ± 0.4	8.20 ± 0.82
Hb (g/L)	145.00 ± 3.22	143.00 ± 6.00	139.33 ± 8.99	143.50 ± 11.5
PCV (L/L)	0.46 ± 0.01	0.45 ± 0.01	0.42 ± 0.02	0.48 ± 0.06
MCV (fL)	57.33 ± 1.20	57.50 ± 1.50	56.00 ± 1.16	58.00 ± 1.00
MCH (pg)	18.33 ± 0.33	18.00 ± 0.00	17.67 ± 0.33	17.50 ± 0.50
MCHC (g/L)	316.67 ± 3.76	323.00 ± 2.00	312.00 ± 2.31	302.5 ± 11.50
RDW (%)	15.73 ± 0.52	14.95 ± 1.15	16.13 ± 0.67	16.40 ± 0.70
WBC ($\times 10^{9}$/L)	7.20 ± 1.59	8.30 ± 1.37	8.40 ± 1.50	7.60 ± 1.80
NEU (%)	26.00 ± 5.03	24.50 ± 2.50	26.67 ± 4.84	28.50 ± 0.50
LYM (%)	68.33 ± 4.41	69.00 ± 3.00	63.33 ± 6.01	63.50 ± 0.50
MONO (%)	5.33 ± 1.33	6.00 ± 0.00	6.33 ± 1.50	5.12 ± 0.00
PLT ($\times 10^{9}$/L)	533.33 ± 124.98	580.00 ±81.00	634.00 ± 85.2	774.0 ± 58.00
Female				
RBC ($\times 10^{12}$/L)	7.34 ± 0.78	8.14 ± 0.25	7.14 ± 0.37	7.32 ± 0.34
Hb (g/L)	136.67 ± 13.93	132.00 ± 4.00	135.33 ± 6.17	134 ± 3.79
PCV (L/L)	0.42 ± 0.04	0.46 ± 0.01	0.42 ± 0.02	0.41 ± 0.01
MCV (fL)	57.33 ± 0.88	56.33 ± 0.67	58.33 ± 0.88	56.33 ± 1.33
MCH (pg)	18.67 ± 0.33	19.00 ± 0.00	19.00 ± 0.58	18.33 ± 0.33
MCHC (g/L)	325.00 ± 4.73	331.67 ± 2.67	324.67 ± 3.93	325.67 ± 2.33
RDW (%)	12.40 ± 0.15	12.60 ± 0.26	13.13 ± 0.50	13.57 ± 0.03
WBC ($\times 10^{9}$/L)	7.83 ± 0.29	6.80 ± 1.32	4.03 ± 1.39	5.47 ± 1.19
NEU (%)	14.67 ± 1.33	16.67 ± 1.76	22.67 ± 2.03	19.00 ± 3.22
LYM (%)	82.33 ± 2.03	78.33 ± 2.33	75.50 ± 3.5	76.00 ± 2.08
MONO (%)	2.33 ± 0.33	3.67 ± 1.86	3.50 ± 1.45	3.60 ± 1.2
PLT ($\times 10^{9}$/L)	671.33 ± 52.41	690.67 ± 51.8	613.67± 51.02	570.3 ± 66.46

RBC: total red blood cell, Hb: haemoglobin, PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular Hb, MCHC: mean corpuscular Hb concentration, RDW: red cell distribution width, WBC: total white blood cell, NEU: neutrophils, LYM: lymphocytes, MONO: monocytes, PLT: platelets count. Values are means ± SEM. Significantly different from the control, ** $p < 0.001$

Table 4.7: Biochemical parameters of male and female SD rats after 28 days treatment of ACA in sub-acute toxicity study.

Parameters	Treatment (n = 5/sex)			
	Control	0.66 mg/kg	1.33 mg/kg	2.22 mg/kg
Male				
Sodium (mmol/L)	142.25 ± 0.95	142.2 ± 0.49	143.21 ± 0.37	142.00 ± 0.32
Potassium (mmol/L)	5.77 ± 0.30	5.08 ± 0.31	4.66 ± 0.17*	4.84 ± 0.10*
Chloride (mmol/L)	102.60 ± 0.87	102 ± 1.10	102.40 ± 0.93	103.60 ± 0.68
BUN (mmol/L)	5.03 ± 0.33	6.43 ± 0.47	6.38 ± 0.46	5.80 ± 0.2
Creatinine (μmol/L)	25.75 ± 1.89	25.60 ± 0.81	25.00 ± 1.87	23.60 ± 1.12
Calcium (mmol/L)	2.42 ± 0.03	2.43 ± 0.03	2.42 ± 0.02	2.46 ± 0.03
Calcium corrected(mmol/L)	2.51 ± 0.04	2.5 ± 0.03	2.51 ± 0.03	2.55 ± 0.03
Phosphate (mmol/L)	2.33 ± 0.16	2.48 ± 0.11	2.52 ± 0.13	2.62 ± 0.08
TPR(g/L)	62.00 ± 0.81	65.00 ± 0.58	65.00 ± 1.54	65.60 ± 0.40*
ALB (g/L)	34.41 ± 0.68	36.20 ± 0.37	36.80 ± 0.97*	35.60 ± 0.40
GLO (g/L)	27.20 ± 0.49	28.80 ± 0.86	29.40 ± 0.68	30.00 ± 0.45*
A/G ratio	1.27 ± 0.03	1.26 ± 0.03	1.25 ± 0.02	1.19 ± 0.03
ALP (U/L)	146.75 ± 5.56	140.2 ± 8.41	150.33 ± 36.8	136.8± 9.23
AST (U/L)	169.8±13.27	158.8± 9.09	145.67±2.6	142.75 ± 7.4
ALT (U/L)	41.75±0.75	36.33±0.88	39.33±0.88	38.50 ± 2.99
Female				
Sodium (mmol/L)	141.25 ± 0.25	141.25 ± 0.25	141.00 ± 0.41	142.00 ± 0.71
Potassium (mmol/L)	4.58 ± 0.18	4.34 ± 0.33	4.80 ± 0.78	4.46 ± 0.29
Chloride (mmol/L)	104.80 ± 0.58	103.75 ± 0.48	104.75 ± 0.95	106.40 ± 1.08
BUN (mmol/L)	5.68 ± 0.38	5.78 ± 0.17	4.68 ± 0.62	5.60 ± 0.55
Creatinine (μmol/L)	30.40 ± 1.94	30.00 ± 4.22	31.80 ± 2.8	33.40 ± 2.50
Calcium (mmol/L)	2.40 ± 0.02	2.44 ± 0.02	2.45 ± 0.01	2.44 ± 0.06
Calcium corrected(mmol/L)	2.46 ± 0.02	2.52 ± 0.01	2.51 ± 0.03	2.53 ± 0.04
Phosphate (mmol/L)	1.99 ± 0.14	1.89 ± 0.11	1.88 ± 0.04	2.03 ± 0.15
TPR(g/L)	64.40 ± 1.63	64.25 ± 0.95	65.8 ± 0.97	63.60 ± 0.93
ALB (g/L)	35.00 ± 0.95	34.60 ± 1.50	36.20 ± 0.49	35.00 ± 0.45
GLO (g/L)	29.40 ± 1.08	28.25 ± 0.25	29.00 ± 1.00	28.60 ± 0.60
A/G ratio	1.23 ± 0.05	1.25 ± 0.02	1.22 ± 0.03	1.22 ± 0.02
ALP (U/L)	149.80 ± 13.4	143.0 ± 18.35	140.20 ± 2.8	136.00 ±14.25
AST (U/L)	159.4± 18.78	135.60±15.26	165.8± 14.29	143.00 ± 12.3
ALT (U/L)	35.00 ± 3.03	41.00 ± 3.61	42.80 ± 5.14	42.20 ± 5.19

BUN: Blood urea nitrogen, TPR: Total protein ALB: Albumin, GLO: Globulin, A/G ratio: Albumin/globulin ratio ALP: Alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase. Values are means ± SEM. Significantly different from the control, * $p < 0.05$, ** $p < 0.001$

Table 4.8: Biochemical parameters of male and female SD rats after 14 days recovery of ACA treatment in sub-acute toxicity study.

Parameters	Treatment (n = 5/sex)			
	Control	0.66 mg/kg	1.33 mg/kg	2.22 mg/kg
Male				
Sodium (mmol/L)	142.33 ± 0.88	142.50 ± 0.5	140.67 ± 0.88	141.00 ± 0.00
Potassium (mmol/L)	4.87 ± 0.12	4.75 ± 0.05	4.90 ± 0.25	5.25 ± 0.25
Chloride (mmol/L)	102.67 ± 0.88	105.00 ± 1.00	103.67 ± 1.2	105.00 ± 0.00
BUN (mmol/L)	6.47 ± 0.52	7.45 ± 0.05	6.50 ± 0.40	7.60 ± 0.10
Creatinine (μmol/L)	33.00 ± 0.58	29.50 ± 0.50	30.33 ± 1.20	31.50 ± 4.50
Calcium (mmol/L)	2.46 ± 0.03	2.43 ± 0.07	2.33 ± 0.02	2.38 ± 0.05
Calcium corrected(mmol/L)	2.56 ± 0.05	2.53 ± 0.05	2.46 ± 0.02	2.49 ± 0.04
Phosphate (mmol/L)	2.32 ± 0.17	2.05 ± 0.14	2.29 ± 0.06	2.44 ± 0.27
TPR(g/L)	65.67 ± 1.67	62.50 ± 4.5	62.00 ± 1.00	63.50 ± 0.5
ALB (g/L)	35.00 ± 0.58	35.00 ± 1.00	33.67 ± 0.67	34.50 ± 0.5
GLO (g/L)	30.67 ± 1.2	27.50 ± 3.5	28.33 ± 0.33	29.00 ± 0.00
A/G ratio	1.14 ± 0.04	1.29 ± 0.13	1.19 ± 0.01	1.19 ± 0.02
ALP (U/L)	139.00 ± 12.29	134.50 ± 18.5	121.00 ± 1.00	153.50 ± 8.50
AST (U/L)	145.33 ± 17.13	143.50 ± 10.50	147.67 ± 9.91	146.00 ± 6.50
ALT (U/L)	38.33 ± 3.28	37.00 ± 1.00	38.00 ± 0.58	38.00 ±3.00
Female				
Sodium (mmol/L)	141.33 ± 0.88	141.67 ± 0.88	141 ± 0.58	139.67 ± 0.33
Potassium (mmol/L)	4.07 ± 0.03	4.00 ± 0.1	4.13 ± 0.15	3.95 ± 0.25
Chloride (mmol/L)	103.67 ± 1.76	104.67 ± 0.88	105.33 ± 0.88	104.00 ± 0.58
BUN (mmol/L)	5.10 ± 0.29	6.00 ± 0.60	5.43 ± 0.46	5.70 ± 0.25
Creatinine (μmol/L)	38.67 ± 2.4	37.33 ± 2.03	31 ± 0.58	27.3 ± 2.19**
Calcium (mmol/L)	2.39 ± 0.05	2.38 ± 0.01	2.49 ± 0.05	2.48 ± 0.03
Calcium corrected(mmol/L)	2.46 ± 0.06	2.42 ± 0.01	2.51± 0.03	2.54 ± 0.02
Phosphate (mmol/L)	1.96± 0.2	1.83 ± 0.13	1.66 ± 0.2	2.14 ± 0.08
TPR(g/L)	65.33 ± 3.76	68.33 ± 1.20	67.67 ± 1.33	64.00 ± 0.58
ALB (g/L)	34.67 ± 1.45	36.00 ± 0.00	38.67 ± 1.33	37.00 ± 0.58
GLO (g/L)	30.67 ± 3.18	32.33 ± 1.20	29.00 ± 0	27.00 ± 0.00
A/G ratio	1.15 ± 0.11	1.12 ± 0.04	1.33 ± 0.05	1.37± 0.02
ALP (U/L)	146.33 ± 7.8	142.67 ± 5.21	142 ± 25.66	131± 14.5
AST (U/L)	128.33 ± 10.9	134.00 ± 2.00	132.67± 12.73	115.67 ± 6.01
ALT (U/L)	44.33 ± 5.18	45.33 ± 6.96	38.67± 1.20	33.00 ± 2.65

BUN: Blood urea nitrogen, TPR: Total protein ALB: Albumin, GLO: Globulin, A/G ratio: Albumin/globulin ratio ALP: Alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase.
Values are means ± SEM. Significantly different from the control, * $p < 0.05$, ** $p < 0.001$

4.3.4 Histopathological analysis in sub-acute toxicity study

For histopathological analysis, tissue samples from major organs, such as, brain, liver, spleen, lungs, adrenal glands, heart, testes, ovaries and kidneys were harvested and preserved in 10% neutral buffered formaldehyde solution. The organs were weighed by sensitive digital balance. They were then examined for any degeneration, necrosis, discoloration or haemorrhage and trimmed. Then the specimens were processed and embedded in paraffin wax.

Thin sections of paraffin-embedded tissues were sliced using a microtome and stained with hematoxylin and eosin. Stained sections were visualized for pathological changes using the Olympus microscope and camera as shown in Figures 4.6 and 4.7.

The treated animals did not produce significant changes in the absolute and relative organ weights as compared to control, except in kidney and liver of female rats, but these were not in a dose-dependent manner (Table 4.9 and Table 4.10). The two-way ANOVA test confirmed there were no gender-dependent variations on organ weights.

Histopathological examination revealed that 3 out of 10 rats in the 0.66 and 1.33 mg/kg groups and 5 out of 10 rats in the 2.22 mg/kg group, exhibited mild focal lobular hepatitis and interstitial inflammation in the kidney (Figure 4.6), which were persistent till the end of the recovery period. However, the glomeruli and tubules in the kidneys did not show any significant pathological signs in all treated groups. Mild interstitial pneumonitis in lungs was observed in the controls as well as in all ACA-treated rats. All other organs such as heart, brain, testes and ovaries were found to be normal as shown in Figures 4.6 and 4.7.

Table 4.9: Absolute organs weight of male SD rats after 28 days treatment of ACA in the sub-acute toxicity study.

Organs	Treatment (n = 5/sex)				Recovery period (n = 3/sex)			
	Control	0.66 mg/kg	1.33 mg/kg	2.22 mg/kg	Control	0.66 mg/kg	1.33 mg/kg	2.22 mg/kg
Brain (g)	1.72 ± 0.04	1.90 ± 0.06	1.70 ± 0.09	1.78 ± 0.04	1.83 ± 0.03	1.75 ± 0.15	1.83 ± 0.12	1.85 ± 0.05
Brain (%)	0.51 ± 0.01	0.52 ± 0.02	0.47 ± 0.01	0.47 ± 0.02	0.46 ± 0.02	0.44 ± 0.01	0.46 ± 0.05	0.40 ± 0.04
Heart (g)	1.30 ± 0.06	1.36 ± 0.07	1.28 ± 0.04	1.30 ± 0.04	1.30 ± 0.10	1.35 ± 0.05	1.40 ± 0.10	1.20 ± 0.20
Heart (%)	0.39 ± 0.03	0.36 ± 0.02	0.35 ± 0.02	0.34 ± 0.00	0.33 ± 0.02	0.34 ± 0.04	0.35 ± 0.03	0.26 ± 0.03
Lungs (g)	1.62 ± 0.07	1.68 ± 0.03	1.86 ± 0.13	1.83 ± 0.10	1.83 ±0.09	1.70 ± 0.00	1.50 ± 0.06	1.70 ± 0.30
Lungs (%)	0.48 ± 0.01	0.49 ± 0.04	0.52 ± 0.05	0.52 ± 0.03	0.46 ± 0.02	0.43 ± 0.03	0.35 ± 0.01	0.36 ± 0.04
Liver (g)	13.12 ± 0.83	13.72 ± 0.49	14.00 ± 0.87	14.20 ± 0.88	12.97 ±0.56	14.65 ± 0.85	14.10 ± 0.42	13.40 ± 0.00
Liver (%)	4.18 ± 0.3	3.58 ± 0.02	3.84 ± 0.21	3.75 ± 0.20	3.25 ± 0.07	3.70 ± 0.03	3.49 ± 0.05	2.90 ± 0.19
Spleen (g)	0.78 ± 0.03	0.90 ± 0.07	0.84 ± 0.04	0.86 ± 0.02	0.70 ± 0.10	0.85 ± 0.05	0.80 ± 0.10	0.95 ± 0.05
Spleen (%)	0.22 ± 0.02	0.24 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	0.15 ± 0.01	0.22 ± 0.03	0.18 ± 0.04	0.18 ± 0.02
Kidney (g)	1.21 ± 0.03	1.39 ± 0.07	1.27 ± 0.06	1.44 ± 0.10	1.27 ± 0.03	1.30 ± 0.10	1.30 ± 0.15	1.45 ± 0.25
Kidney (%)	0.36 ± 0.01	0.36 ± 0.02	0.37 ± 0.00	0.39 ± 0.02	0.32 ± 0.01	0.33 ± 0.00	0.32 ± 0.02	0.31 ± 0.03
Testes (g)	1.68 ± 0.05	1.68 ± 0.11	1.58 ± 0.07	1.77 ± 0.04	1.60 ± 0.17	1.50 ± 0.00	1.53 ± 0.17	1.90 ± 0.40
Testes (%)	0.50 ± 0.02	0.44 ± 0.02	0.46 ± 0.02	0.47 ± 0.02	0.40 ± 0.03	0.38 ± 0.02	0.38 ± 0.03	0.41 ± 0.06

Values are means ± SEM. Significantly different from the control.

Table 4.10: Absolute organs weight of female SD rats after 28 days treatment of ACA in the sub-acute toxicity study.

Organs	Treatment (n = 5/sex)				Recovery period (n = 3/sex)			
	Control	0.66 mg/kg	1.33 mg/kg	2.22 mg/kg	Control	0.66 mg/kg	1.33 mg/kg	2.22 mg/kg
Brain (g)	1.82 ± 0.10	1.84 ± 0.08	1.82 ± 0.06	1.90 ± 0.04	1.80 ± 0.06	1.90 ± 0.06	1.75 ± 0.05	1.83 ± 0.09
Brain (%)	0.70 ± 0.03	0.71 ± 0.03	0.71 ± 0.02	0.71 ± 0.02	0.61 ± 0.02	0.69 ± 0.04	0.68 ± 0.04	0.69 ± 0.03
Heart (g)	0.96 ± 0.05	0.94 ± 0.08	0.90 ± 0.04	1.08 ± 0.06	1.20 ± 0.06	1.07 ± 0.09	1.33 ± 0.03	1.10 ± 0.15
Heart (%)	0.37 ± 0.02	0.36 ± 0.03	0.35 ± 0.01	0.40 ± 0.03	0.41 ± 0.01	0.38 ± 0.02	0.47 ± 0.03	0.42 ± 0.05
Lungs (g)	1.80 ± 0.17	1.60 ± 0.09	1.58 ± 0.09	1.60 ± 0.10	1.50 ± 0.10	1.43 ± 0.24	1.50 ± 0.06	1.30 ± 0.06
Lungs (%)	0.67 ± 0.07	0.61 ± 0.02	0.61 ± 0.03	0.59 ± 0.03	0.50 ± 0.02	0.51 ± 0.07	0.53 ± 0.04	0.49 ± 0.01
Liver (g)	10.8 ± 0.17	9.08 ± 0.51	9.42 ± 0.20	10.3 ± 0.65	10.8 ± 0.87	10.07 ±0.15	11.2 ± 0.17	10.50 ± 0.42
Liver (%)	4.14 ± 0.13	3.46 ± 0.11*	3.66 ± 0.15	3.81 ± 0.18	3.65 ± 0.25	3.63 ± 0.12	3.98 ± 0.11	3.98 ± 0.08
Spleen (g)	0.74 ± 0.05	0.72 ± 0.06	0.66 ± 0.04	0.84 ± 0.06	0.70 ± 0.10	0.70 ± 0.10	0.87 ± 0.03	0.93 ± 0.03
Spleen (%)	0.29 ± 0.02	0.27 ± 0.02	0.26 ± 0.02	0.29 ± 0.02	0.23 ± 0.03	0.25 ± 0.03	0.31 ± 0.01	0.35 ± 0.01*
Kidney (g)	1.03 ± 0.06	1.10 ± 0.06	0.96 ± 0.05	1.04 ± 0.05	0.90 ± 0.00	0.80 ± 0.00	1.17 ± 0.03**	0.97 ± 0.07
Kidney (%)	0.40 ± 0.02	0.42 ± 0.02	0.37 ± 0.02	0.39 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.42 ± 0.03**	0.37 ± 0.02

Values are means ± SEM. Significantly different from the control, ** $p < 0.001$

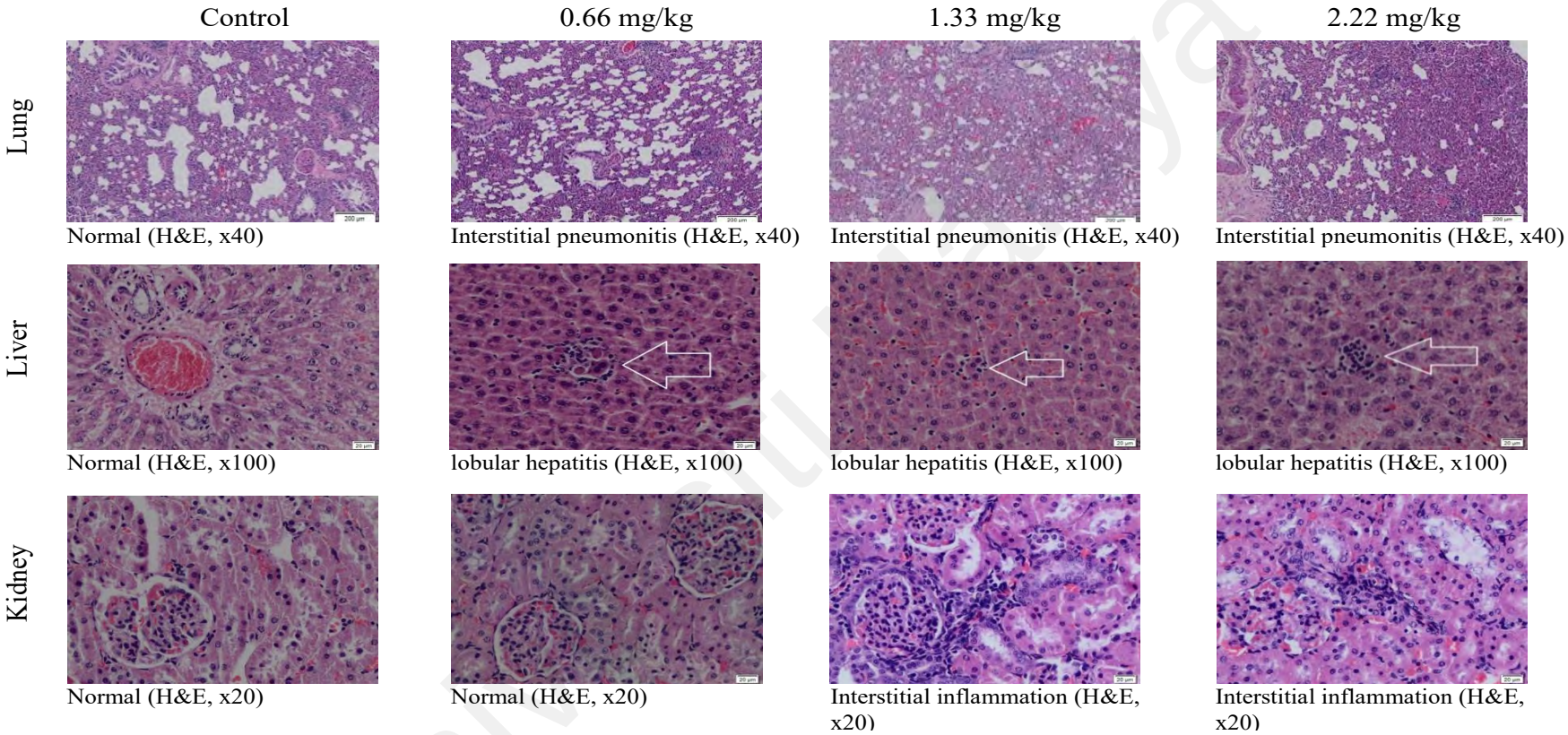


Figure 4.6: Effects of ACA on vital organs for 28 days sub-acute toxicity study of female SD rats. Photomicrographs show histological features of selected organs from representative SD rats from respective groups. Lungs showed mild to moderate interstitial pneumonitis in all the treated groups in both sexes. Liver showed lobular hepatitis (white arrow) in a dose dependent manner, with mild lobular hepatitis in lower dose but moderate lobular hepatitis in higher dose. Mild focal interstitial inflammation (white arrow) was observed in kidneys of rats in groups treated with 1.33 and 2.22 mg/kg

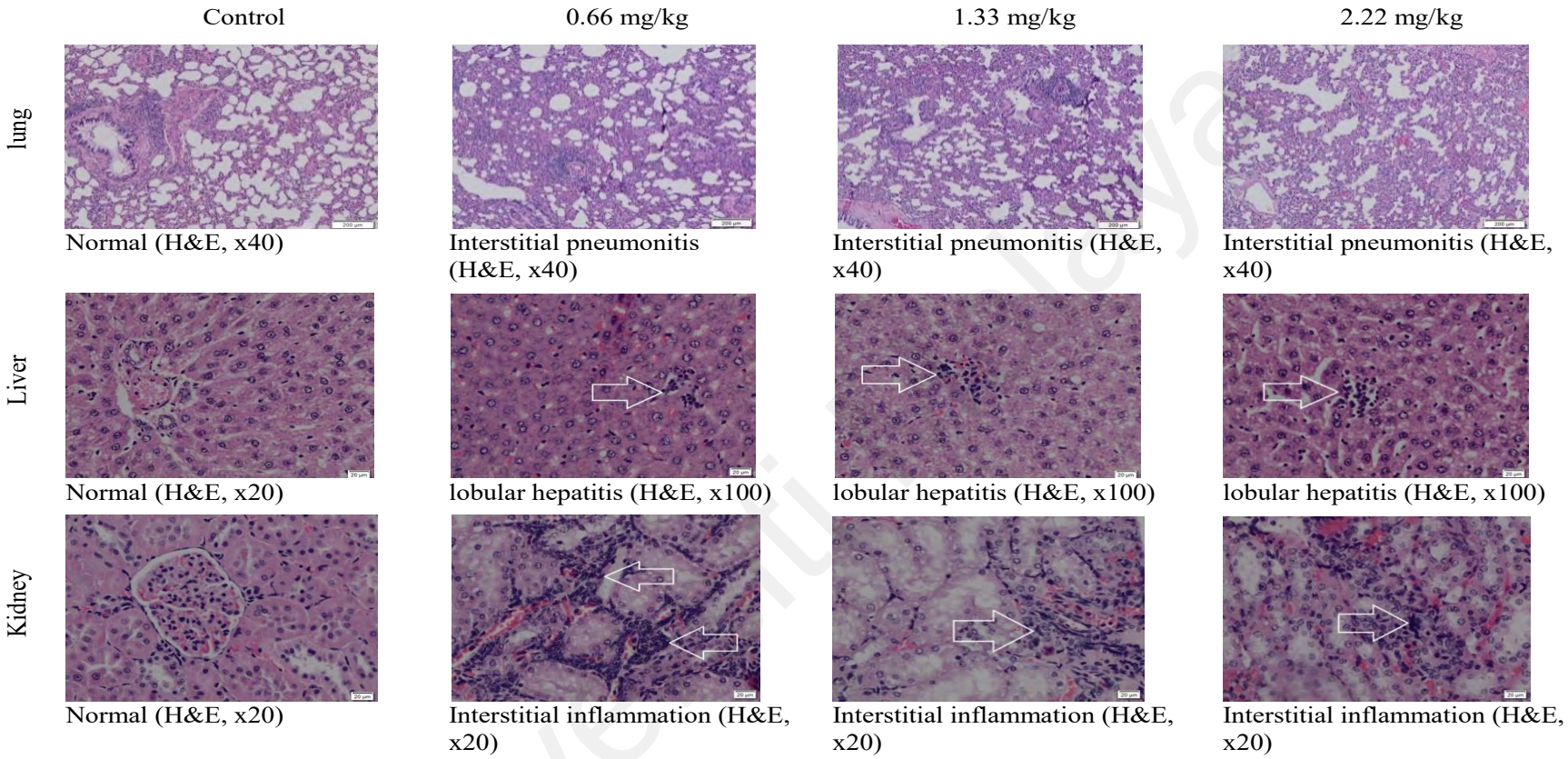


Figure 4.7: Effects of ACA on vital organs after 14 days recovery period in sub-acute toxicity study of female SD rats. Photomicrographs show histological features of selected organs from representative SD rats from respective groups. There was no recovery from interstitial pneumonitis in the lungs of the treated groups in both sexes. In the liver, there was incomplete recovery from lobular hepatitis (white arrow) which are shown here. Mild focal interstitial inflammation in kidneys (white arrow) was observed in recovery groups suggesting irreversible damage.

4.3.5 Urine analysis in sub-acute toxicity study

After placing individual animals in metabolic cages (Techniplast, Italy), urine samples were collected for urinalysis from all animals prior to the scheduled necropsy. The results of the urinalysis are presented in Table 4.11 and 4.12. The urinalysis results showed minor changes in the urine parameters with a slight increase in protein excretion and ketones at 0.66 mg/kg in male rats compared to control. The minor changes did not occur in a dose-dependent manner and were seen to be incidental and unrelated to the treatments.

Table 4.11: Urinalysis findings of male rats treated with ACA for 28-days and 14-days recovery period in sub-acute toxicity study

Parameters		Male							
		Doses (mg/kg)				Recovery period			
		Control	0.66	1.33	2.22	Control	0.66	1.33	2.22
Protein	N	1	0	0	0	0	0	0	0
	1+	0	0	0	0	1	0	1	0
	2+	2	4	3	1	2	2	2	1
	3+	2	1	2	4	0	0	0	1
Glucose	N	5	4	5	5	1	1	3	2
	T	0	1	0	0	2	1	0	0
pH	7	0	0	0	0	0	0	0	0
	7.5	0	1	0	0	0	0	1	1
	8	3	3	3	4	1	0	2	1
	8.5	2	1	2	1	2	2	0	0
Ketone	N	0	1	0	0	0	0	0	0
	T	0	1	1	1	1	0	0	0
	1+	1	2	1	0	1	0	3	1
	2+	4	1	3	4	1	2	0	2
Blood cells	N	5	5	5	5	3	3	2	3
	T	0	0	0	0	0	0	1	0
Epithelial cells	N	5	5	5	5	3	2	3	3
	T	0	0	0	0	0	1	0	0
Crystals	N	0	0	0	0	0	0	1	0
	T	5	5	5	5	3	3	2	3
Casts	N	5	5	5	5	3	3	3	3
	T	0	0	0	0	0	0	0	0

Urinalysis of treated rats with ACA for 28-days (n= 5/sex /group) and recovery period (n= 3/sex /group). N = Nil; T = Traces.

Table 4.12: Urinalysis findings of female rats treated with ACA for 28-days and 14-days recovery period in sub-acute toxicity study.

Parameters		Female							
		Doses (mg/kg)				Recovery period			
		Control	0.66	1.33	2.22	Control	0.66	1.33	2.22
Protein	N	1	0	0	0	0	0	0	0
	1+	2	1	2	0	0	1	0	0
	2+	1	2	0	5	3	2	2	2
	3+	1	2	3	0	0	0	1	1
Glucose	N	5	5	5	5	3	3	3	1
	T	0	0	0	0	0	0	0	2
pH	7	0	0	0	0	0	0	0	0
	7.5	2	4	1	0	0	0	0	0
	8	2	0	2	3	3	2	1	0
	8.5	1	1	2	2	0	1	2	3
Ketone	N	3	1	2	1	1	1	0	0
	T	2	2	0	3	2	2	2	1
	1+	0	1	2	1	0	0	1	2
	2+	0	1	1	0	0	0	0	0
Blood cells	N	5	4	5	5	3	3	3	3
	T	0	1(+)	0	0	0	0	0	0
Epithelial cells	N	5	5	5	4	3	3	3	3
	T	0	0	0	1(+)	0	0	0	0
Crystals	N	5	5	5	5	3	3	3	3
	T	0	0	0	0	0	0	0	0
Casts	N	5	5	5	5	3	3	3	3
	T	0	0	0	0	0	0	0	0

Urinalysis of treated rats with ACA for 28-days (n= 5/sex /group) and recovery period (n= 3/sex /group). N = Nil; T = Traces.

4.3.6 Results of two-way ANOVA for haematological and biochemical responses for the sub-acute toxicity study

A two-way ANOVA was conducted to examine the effect of sex and treatment on MCHC, potassium, creatinine, TPR, albumin and globulin levels. There was no statistically significant interaction observed between these two factors ($p > 0.05$) on the above and other haematological and biochemical parameters, except for ALP. In other words, the effect of treatment on these parameters did not differ in the male or female groups. Since the interactions were not significant, the results were discussed in terms of each factor independently. The results of the two-way ANOVA for haematological and biochemical parameters in the recovery groups are shown in Table 4.13 and Table 4.14. The effects of treatment were not altered in relation to sex for all parameters, except in creatinine and calcium levels ($p < 0.05$).

Table 4.13: Results of two-way analysis of variance for hematological/biochemical responses in SD rats treated for 28 days with ACA in sub-acute toxicity study.

Parameters	Sex			Treatment			Treatment * Sex		
	df	F	P	df	F	P	df	F	P
MCH (pg)	1	6.710	0.014*	3	0.554	0.650	3	1.935	0.144
MCHC (g/L)	1	15.694	0.000**	3	8.912	0.000**	3	0.726	0.544
Potassium (mmol/L)	1	6.638	0.015*	3	2.029	0.129	3	2.243	0.102
Chloride (mmol/L)	1	13.636	0.001*	3	2.409	0.085	3	0.467	0.707
Creatinine (μmol/L)	1	10.529	0.003*	3	0.139	0.936	3	0.886	0.459
Phosphate (mmol/L)	1	10.865	0.002*	3	0.434	0.730	3	0.224	0.879
ALP (U/L)	1	1.409	0.245	3	2.994	0.048*	3	4.831	0.008*
ALT (U/L)	1	0.265	0.611	3	3.926	0.018*	3	1.438	0.252

df: degrees of freedom, MCH: mean corpuscular Hb, MCHC: mean corpuscular Hb, ALP: Alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase. Significantly different at * $p < 0.05$, ** $p < 0.001$

Table 4.14: Results of two-way analysis of variance for hematological/biochemical responses in SD rats treated for 28 days with ACA in sub-acute toxicity study after two-week recovery period.

Parameters	Sex			Treatment			Treatment * Sex		
	df	F	P	df	F	P	df	F	P
MCH (pg)	3	1.022	0.413	1	11.065	0.005*	3	0.701	0567
MCHC (g/L)	3	3.017	0.065	1	18.847	0.001*	3	1.211	0.342
RDW (%)	3	2.076	0.149	1	61.578	0.000**	3	0.308	0.819
NEU (%)	3	1.202	0.345	1	12.544	0.003*	3	0.624	0.611
LYM (%)	3	1.275	0.324	1	20.577	0.001*	3	0.139	0.935
Potassium (mmol/L)	3	0.564	0.649	1	58.686	0.000**	3	1.068	0.397
BUN (mmol/L)	3	2.699	0.089	1	24.882	0.000**	3	0.368	0.777
Creatinine (μmol/L)	3	4.493	0.021*	1	3.294	0.091	3	3.542	0.043*
Calcium (mmol/L)	3	0.177	0.910	1	1.297	0.274	3	3.756	0.036*
Phosphate (mmol/L)	3	1.840	0.186	1	10.700	0.006*	3	0.615	0.617
ALB (g/L)	3	0.799	0.515	1	9.577	0.008*	3	3.322	0.051

df: degrees of freedom, MCH: mean corpuscular Hb, MCHC: mean corpuscular Hb concentration, RDW: red cell distribution width, NEU: neutrophils, LYM: lymphocytes, BUN: Blood urea nitrogen. ALB: Albumin
Significantly different at * $p < 0.05$, ** $p < 0.001$

CHAPTER 5: DISCUSSION

Natural products have a reliable and significant role in drug development and therapeutic uses. Traditionally, medicinal plants have been used to treat various ailments of mankind throughout history. They contain active phytoconstituents of diverse chemical nature and are capable of alleviating diseased state. Different natural phytochemicals used for the treatment of numerous illnesses including cancer have become attractive for drug discovery and research. Despite the major contribution of natural products, there are many challenges faced by pharmaceutical companies in drug development. Some of the major challenges encountered are low solubility, functional group reactivity, molecular complexity and instability (Press et al., 2019). In addition, even potential natural compounds in the final stages of drug development can still be rejected due to lack of efficacy and toxicity effects. Hence, preclinical drug safety studies are essential at earlier stages of development to avoid difficulties in later phases (Anadón et al., 2014).

ACA, a natural phenylpropanoid isolated from the rhizomes of *Alpinia* species, has been studied extensively for its pharmacological activities. Seo and co-workers reported that ACA ameliorates ovalbumin-induced asthma in mice by reducing the infiltration of WBCs and IgE in lungs and by suppressing the expression of Th1 and Th2 cytokines and interleukin IL-4 (Seo et al., 2013). Another study conducted by Ohnishi and colleagues found that ACA significantly inhibited adipogenesis in rats when concurrently fed with a high-fat diet. Its anti-obesity effects worked primarily by inhibiting the cellular lipid accumulation via the downregulation of PPARγ and C/EBPα transcription inhibitors (Ohnishi et al., 2012). Similarly, cytotoxic activities of ACA against multi-drug resistant bacteria (Latha et al., 2009) and inhibition of human immunodeficiency virus type 1 replication were also established (Ye & Li, 2006).

Previous investigations confirmed the anti-proliferative activity of ACA against five different human cancer cell lines (Awang et al., 2010). In addition, a significant reduction was observed in human carcinoma xenograft in mice via proinflammatory microenvironment alterations (In et al., 2012). It was revealed that multiple mechanisms are responsible to cause apoptotic actions of ACA, such as caspase-3 activation (Williams et al., 2013), induction of TRAIL (Ito et al., 2005) and inhibition of NF-κB (Ito et al., 2005, In et al., 2011; Arshad et al., 2015). Furthermore, ACA induced apoptotic activity against human hepatocarcinoma cells and reduced azoxymethane induced colon carcinogenesis (Kato et al., 2014).

Therefore, the current study is imperative to find details of ACA acute toxicity with single doses of 2, 4, 6.66 mg/kg and sub-acute toxicity with repeated doses of 0.66, 1.33 and 2.22 mg/kg. The control group received only the solvent used to dissolve ACA, which is a mixture of 2% (v/v) propylene glycol in PBS.

The sighting study was implemented in toxicity evaluation test to identify a safe starting dose in the Sprague Dawley rats. The experimental animals, usually females, received a single dose at a number of fixed concentration levels (Stallard et al., 2011). The sighting study carried out comprised of female SD rats dosed with 5, 10 and 20 mg/kg. The result revealed that ACA resulted in no death even at the highest concentration of 20 mg/kg. However, the internal effects of this dosage on organs were not revealed in the sighting study.

The acute and sub-acute toxicity studies did not show any adverse clinical symptoms or mortality at all tested doses. Body weight changes are often the first sign of toxicity and are an important parameter for the objective evaluation of the effects of a test compound in experimental animals (Sireeratawong et al., 2008). Breast cancer patients demonstrated differences in body weight records due to variations in

metabolism, food intake, reduction in energy expenditure and physical activity (Trestini et al., 2018). No variations in body weight, water and food consumptions between the treated and control groups were observed in the current study.

The correlation of food consumption with body weight can be evaluated to address the toxicity effect of a test compound. Both acute and sub-acute toxicity studies revealed no drastic changes in body weight measurement of treated rats compared to the control group. Conforming to that, there was also no variation in food and water consumptions recorded during the acute and sub-acute toxicity studies between control and treated groups.

Examination of organ weight in toxicity studies is an imperative endpoint for characterisation of the potentially harmful effects of a test compound. Alterations in organ weight between treatment groups are often accompanied by differences in body weight. Thus, the interpretation of organ weight differences should not be taken as an absolute indicator of toxicity (Bailey et al., 2004). In the sub-acute toxicity study, treated rats did not show significant changes in the absolute and relative organ weights, except for the weight of the kidney and liver of female rats that were significantly higher compared to the control group. However, the findings were not observed in a dose-dependent manner, that is, a higher dose did not result in higher toxicity.

Another factor that could give rise to variations in the relative and absolute weight was the age of the rats. Marino found that there were increases in absolute weights and reduction in relative weights of brain, liver, kidney, lung, heart and testis with respect to the increase in age in their rat model study (Marino, 2012).

Toxicity or any alteration in haematopoietic system and biochemical parameters produce undesirable outcomes and are useful to predict possible adverse events prior to clinical trials in human subjects (Wang et al., 2014). Analysis from the two-way

ANOVA indicated that both sex and treatment have no effects on the haematological and biochemical parameters. Therefore, the results of each factor were discussed independently.

Myelosuppression or bone marrow depression represents one of the major side effects of antineoplastic agents. Several approved drugs, such as gemcitabine, currently in clinical use for the treatment of cancer have demonstrated various levels of toxicity during the preclinical animal studies, such as myelosuppression (Abbruzzese et al., 1991). Hajhashemi and co-workers found an anticancer drug, HESA-A from a marine natural product induced myelosuppression (Hajhashemi et al., 2001).

Also, the cytotoxic effects of compounds can lead to decreased production of blood cells and result in suppression of the immune system (Luo & Cisler, 2016). An example is docetaxel, another highly potent anticancer agent used in the treatment of various types of cancer (Lee et al., 2011) that was reported to cause an immediate reduction in WBCs count, which was recoverable after 3 weeks. In another study, Hajhashemi and co-workers observed a decrease in WBCs, RBCs, platelet and other bone marrow components upon anticancer treatment with HESA-A (Hajhashemi et al., 2001).

In the acute toxicity study of ACA, the WBC was reduced significantly ($p < 0.05$) in the groups treated with the 4 and 6.66 mg/kg dose as compared to the respective controls. However, neutrophils were significantly ($p < 0.05$) increased in male rats at the 2 and 4 mg/kg doses. The reduction of WBC is expected as ACA has been shown to possess cytotoxic effect. Neutrophils are a type of WBC that function to repair damaged tissue and to fight off infection. Since the level of WBC is reduced, the elevated level of neutrophils may not be due to infection and could be due to natural responses due to stress or injuries.

In the acute study, MCHC was significantly high ($p < 0.05$) in males treated with the 4 and 6.66 mg/kg doses in comparison to the controls. MCHC is the measurement of of haemoglobin (Hgb) concentration in the RBC and normally is calculated by dividing the haemoglobin (Hgb) with the haematocrit (Hct) value, while MCV is an estimate of the average volume of RBCs. In most cases, the MCV levels will normally change proportionally to that of MCHC (Wentworth et al., 1974). Similar findings were noted in the sub-acute toxicity study, where the MCHC value was significantly higher in the 1.33 and 2.22 mg/kg doses in the female rats with no changes observed in the MCV levels at all doses and between the two sexes. The most common causes of elevated MCHC are macrocytic anaemias, such as Vitamin B_{12} and folate deficiency and haemolytic anaemia. The increase of MCHC in the acute and sub-acute toxicity studies were not accompanied by an increase in MCV, or causing a reduction in RBC count, Hb and hematocrit level. Thus, this finding is not suggestive to the development of anaemia or other haemotological disorders (Wentworth et al., 1974).

The kidneys are one of the important organs that function to excrete waste products and toxins such as urea, creatinine and uric acid, and regulating the extracellular fluid volume, serum osmolality and electrolyte concentrations (Wurochekke et al., 2008). There are a few parameters that are useful in investigating and evaluating kidney function. Creatinine level, for example is an important parameter that can be used to estimate the glomerular function of the kidney. Other parameters that should be considered when assessing the renal function are the level of serum electrolytes such as sodium, potassium, chloride and blood urea nitrogen (BUN). In addition, urinalysis using dipstick method provides qualitative analysis to detect the presence of albuminuria and proteinuria, as well as traces of protein, glucose, RBC, ketones and bilirubin in the urine (Newman et al., 2005).

The most commonly used urinary parameters to estimate the glomerular function is serum creatinine. However, serum creatinine should not be the sole basis of the estimation of renal function as there are many other factors that can affect the creatinine level, such as age, sex, muscle mass and low-protein diets. Other than the creatinine level, the BUN level is often measured concomitantly. BUN is the end product of protein metabolism that is formed in the liver from ammonia and later eliminated by the kidney (Kaid et al., 2019). Simultaneous increase of BUN level with an increase of serum creatinine may imply acute renal injury.

The present study found that the creatinine and BUN levels of animals treated with ACA in the acute and sub-acute investigations were not significantly different from the controls, except in the recovery group of female rats treated with 2.22 mg/kg of ACA, where there was a significant reduction of the creatinine level as compared to the control. The reduction of creatinine level, however, was not observed in a dose-dependent manner. The levels are still within the normal physiologic ranges and thus, was not considered as clinically important. Since both creatinine and the BUN levels were not affected by the ACA treatment, it is suggested that ACA has no toxicity effect on the kidney function.

Assessment of serum electrolytes is commonly paired with the renal function tests for further investigation of clinical conditions or disease state (Gowda et al., 2010). Toxicity effect to the kidney may cause disruption of the serum electrolytes balance as the kidneys are the primary organ responsible for the homeostasis of water and electrolytes (Pazhayattil & Shirali, 2014). The assessment of the serum electrolytes indicated the potassium levels were slightly reduced in the 1.33 and 2.22 mg/kg doses in male rats during the sub-acute study. However, the levels were in the normal physiologic ranges and thus, were not considered to be hypokalemia. Other important

serum electrolytes such as sodium, chloride, calcium and phosphate were not affected at all the tested doses of ACA. The findings paired with the other renal parameters such as creatinine and BUN levels confirmed our hypothesis that ACA has no toxicity effect to the kidneys.

Urinalysis consists of physical observation, chemical, and microscopic analysis is often carried out to aid in the diagnosis of diseases, particularly involving the kidney and urinary tract. In the past, the urine has been used to diagnose renal disorders by observing visual appearance and smell (Rodriguez-Suarez et al., 2014). In modern clinical diagnosis, changes in urinary proteins reflect tissue damage, not only confined to the kidney but also diseases of other systems. Proteinuria is one of the important markers for chronic renal impairment. The urinalysis findings revealed the presence of proteinuria in all treated groups, especially at the highest tested dose of .22 mg/kg. Although there is a good correlation of renal damage to that of proteinuria, their presence in urine could be associated with a high concentration of proteins in plasma and the limited capacity of tubular cells to reabsorb such proteins (Jia et al., 2009). The absolute alterations of protein content are usually associated with decreased production in the liver or increased loss in the kidney. It is evident that proteinuria is variable and assessing the function of kidney solely on this parameter is not a reliable indicator for nephrotoxicity. Instead, overall assessment should include other renal markers such as creatinine, electrolytes and BUN (Jantos-Siwy et al., 2008). Furthermore, there is no significant presence of glucose, RBC, crystals or casts detected in the urine of all treated rats, signifying no tubular damage or glomerulonephritis due to the effect of ACA treatment.

Examination of the kidneys showed mild focal interstitial inflammation at 1.33 and 2.22 mg/kg doses, which persisted in the recovery period. However, the glomeruli

and tubules in the kidneys were unaffected. Since the histopathological changes caused no functional changes of the kidney, confirmed by the renal biochemical results, these changes were considered mild.

The vital function of the liver includes metabolism and detoxification of drugs, thus making the liver a common target of drug-related damage. Biochemical parameters that assesses hepatocellular injury are liver transaminases, such as ALT, AST and ALP. Other parameters that are often evaluated to provide an assessment of liver function include albumin and clotting factors.

Albumin is a major plasma protein that aids in maintaining plasma oncotic pressure and plays an important role in the binding and transport of drugs, hormones and fatty acids. As one of the vital functions of the liver is to produce albumin (Schreiber et al., 1971), this parameter is often used to measure the ability of the liver to synthesize them.

ACA was found to cause mild elevation of total protein (TPR), albumin and globulin in the sub-acute toxicity study, particularly in male rats. TPR measures the total amount of albumin and globulin in the body, indicating that the liver is capable to carry out its function to produce these proteins. Low synthesis of protein by the liver usually is caused by hepatic cirrhosis, which is the scarring of the liver caused by many factors, such as alcohol, viruses or ingested toxin. In addition, high levels of albumin and TPR have been associated with hepatoprotective effects of phytoextracts (Chandekar et al., 2017). The current results support previous studies on crude extracts of Alpinia species and their chemical constituents for their hepatoprotective effects (Ma et al., 2017).

The aminotransferases or transaminases are the enzymes that are primarily located in the hepatocytes that will be released in large quantity following damage to

the hepatocytes. Acute live injury is normally manifested with alanine aminotransferase (ALT), to be highly elevated at more than three times the baseline level. AST is not only located in the hepatocytes but can also be found in the cardiac muscle, kidneys, brains, lungs and intestines. In the acute and sub-acute toxicity studies, the levels of all the liver transaminases, ALT, AST and ALP were not significantly different from the controls in all the tested doses of ACA. However, the levels of these enzymes were at the upper limits of the normal ranges.

To further investigate the toxicity effect of ACA to the liver, the histopathology examination of the liver was carried out. In the acute toxicity study, liver sections of all rats including the controls showed mild lobular hepatitis, while in the sub-acute study, histopathological examination of the liver showed 3 out of 10 rats in the 0.66 and 1.33 mg/kg groups and 5 out of 10 rats in the 2.22 mg/kg group exhibited mild lobular hepatitis with incomplete recovery. The histopathological results complement the levels at the upper limits of the normal ranges for the liver transaminases, signifying possible mild toxicity of ACA to the liver.

In a previous *in vitro* analysis, Haque and co-workers reported the inhibitory effect of ACA on three cytochrome p450 enzymes, CYP1A2, CYP2D6 and CYP3A4. In the current study, mild lobular hepatitis might have been due to the formation of toxic metabolites induced by ACA treatment (Haque et al., 2017). CYP enzymes metabolize the toxic drugs that may induce hepatotoxicity (Villeneuve & Pichette, 2004).

Histopathological examination in the lungs in the sub-acute study revealed mild to moderate interstitial pneumonitis in all the treated groups and did not show any signs of recovery. This finding supports the result of the previous study (Arshad et al., 2015) where ACA-induced lung inflammation in nude mice at a dose of 2.6 mg/kg.

Nevertheless, control groups in this study also showed similar observations indicating that interstitial pneumonitis may be a common effect in research rats (Livingston et al., 2011).

The previous mechanistic anticancer studies reported on ACA inhibition of the inflammatory NF-κB pathway. ACA was found to down-regulate NF-κB activation and reduce the expression of its regulated genes, such as COX-2 and cyclin D1 in mice tumour tissues (In et al., 2012; Arshad et al., 2015). In another previous *in vivo* study, the enhanced levels of inflammatory cytokines, such as IL-6, IL-1β and TNF-α confirmed the induction of lung inflammation by ACA (Subramaniam et al., 2018). This was also seen in the lung histological sections with mild interstitial pneumonitis of some rats in the current study.

CHAPTER 6: CONCLUSION

The international regulatory bodies require a preclinical study and toxicity assessment to provide the necessary information to determine whether the new drug being developed is safe and effective for use in clinical trials.

The undertaken studies were proposed to provide a toxicity profile of ACA, a phenylpropanoid extracted from *Alpinia conchigera.* The studies attempted to achieve its goals through acute and subacute toxicity approaches according to the current guidelines for evaluation the safety of new test compounds in order to fulfil the requirements of the local and international agencies.

In acute toxicity study, rats of both sexes were administered a single dose of ACA via intravenous route at 0, 2, 4 and 6.66 mg/kg body weight for 14 days. In sub-acute toxicity study, the rats received weekly repeated doses of ACA at 0, 0.66, 1.33 and 2.22 mg/kg body weight for 28 days. All parameters related to haematological, biochemical and histopathological sign of toxicity were assessed, statistically analysed and clinically interpreted. Results from the preclinical animal toxicity study may be used to predict the risk of the tested compound in human.

The study showed that ACA caused minimal changes in the liver, kidneys and lungs with no significant functional adverse effects as reflected in the haematological and biochemical examinations. The findings also revealed that exposure to intravenous administration of ACA at doses tested between 0.66 and 6.66 mg/kg body weight in both acute and sub-acute studies did not cause death or significant toxicity.

Thus, the no-observed-adverse-effect level (NOAEL) for IV administration of ACA was established at 2.22 mg/kg body weight from a healthy, non-tumour-bearing rat model.

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