ACUTE ORAL TOXICITY EVALUATION OF BAICALEIN ACTIVE COMPOUND EXTRACTED FROM *OROXYLUM INDICUM* IN SPRAGUE-DAWLEY RATS

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by

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TABLE OF CONTENTS

ACKNOWLEDGEMENT ..... **ii**

TABLE OF CONTENTS ..... **iv**

LIST OF TABLES ..... **viii**

LIST OF FIGURES ..... **ix**

LIST OF ABBREVIATIONS ..... **xi**

LIST OF UNITS AND SYMBOLS ..... **xvi**

LIST OF APPENDICES ..... **xviii**

ABSTRAK ..... **xix**

ABSTRACT ..... **xxi**

CHAPTER 1 INTRODUCTION **1**

1.1 Research background 1

1.2 Rationale of the study 4

1.3 Research objectives 5

1.3.1 Main objective 5

1.3.2 Specific objectives 5

1.4 Significance of study 5

1.5 Summary outline of thesis 6

CHAPTER 2 LITERATURE REVIEW **8**

2.1 Natural products in medicinal history 8

2.2 Botanical description of *Oroxylum indicum* 10

2.3 Ethnopharmacological uses of *O. indicum* 13

2.4 Phytoconstituents of *O. indicum* 14

2.5 Baicalein – an abundant active compound of *O. indicum* 16

2.6 Therapeutic potentials of baicalein active compound 17

2.6.1 Anti-metastatic 18

2.6.2 Neuroprotective 18

2.6.3 Anti-bacterial 19

2.6.4 Anti-obesity 20

2.6.5 Cardioprotective 21

2.6.6 Anti-diabetic 21

2.6.7 Anti-inflammatory 22

2.6.8 Wound healing 23

2.7 Extraction methods for natural products 24

2.8 Solvents used in natural product extraction 26

2.9 *In vivo* toxicity evaluation on natural products 28

2.9.1 Toxic mechanism of flavonoids 28

2.9.2 Types of toxicity testing 30

2.9.3 Animal toxicity testing regulation 31

CHAPTER 3 METHODOLOGY 33

3.1 Materials 33

3.1.1 General chemicals and reagents 33

3.1.2 General consumables 33

3.1.3 General instruments 33

3.2 Preparation of chemical reagents, solutions, media, and buffers 33

3.2.1 Preparation of phosphate buffer saline (PBS) solution 33

3.3 Methods 37

3.3.1 Collection and identification of plant material 37

3.3.2 Crude extraction of plant material 37

3.3.3 Fractionation of baicalein active compound 38

3.3.4 Thin-Layer Chromatography (TLC) analysis to detect baicalein active compound 39

3.3.5 Experimental animal 39

3.3.6 Toxicity studies 40

3.3.6(a) Sighting study 40

3.3.6(b) Acute oral toxicity study 41

3.3.7 Behavioural toxicity evaluation 42

3.3.8 Assessment Parameters 42

3.3.8(a) Blood haematological assay 42

3.3.8(b) Serum biochemical assay 43

3.3.9 Histopathological examination 43

3.3.9(a) Tissue slicing 44

3.3.9(b) Haematoxylin and eosin (H&E) staining 45

3.3.10 Histopathological scoring 46

3.4 Statistical Analysis 47

CHAPTER 4 RESULTS 48

4.1 Extraction and fractionation of baicalein active compound 48

4.2 TLC analysis 48

4.3 Toxicity study 49

4.3.1 Sighting study 49

4.3.2 Acute toxicity study 52

4.4 Behavioural toxicity evaluation 54

4.5 Assessment Parameters 56

4.5.1 Haematological parameters 56

4.5.2 Biochemical parameters 58

4.6 Relative organ weight (ROW) 60

4.7 Histopathological scoring..... 62

CHAPTER 5 DISCUSSION 72

5.1 Extraction and fractionation of *O. indicum* leaves yielded baicalein active compound in F5 72

5.2 Toxicity study 73

5.3 Behavioural toxicity evaluation 74

5.4 Assessment parameters 76

5.5 Body weight and relative organ weight (ROW) 79

5.6 Histopathological scoring..... 81

5.7 Limitations and suggestions for future studies..... 84

CHAPTER 6 CONCLUSION..... 87

REFERENCES..... 88

APPENDICES 103

LIST OF TABLES

Page

Table 3.1 List of general chemicals and reagents 34

Table 3.2 List of general consumables..... 35

Table 3.3 List of general instruments..... 36

Table 4.1 Body weight and its respective changes of control and treated rats in sighting test 51

Table 4.2 Body weight and its respective changes of control and treated rats in acute oral toxicity test 53

Table 4.3 Clinical toxicity signs and animal behaviour in acute oral toxicity study (n=5 for both sexes in control and treated group)..... 55

Table 4.4 Haematological indices of control and treated rats in acute oral toxicity study..... 57

Table 4.5 Biochemical indices of control and treated rats in acute oral toxicity study..... 59

Table 4.6 Relative organ weight (ROW) of control and treated rats in acute oral toxicity study 61

Table 4.7 Histopathological scoring chart 70

LIST OF FIGURES

Page

Figure 2.1 On-site photographs taken of *O. indicum* tree and its various parts. From left: (A) Whole tree; (B) Stem bark; (C) Leaf; (D) Leaf stalks; (E) Flower; (F) Fruit with seeds; (G) Seed 12

Figure 2.2 Chemical structure of baicalein (5,6,7-trihydroxyflavone) 17

Figure 4.1 *O. indicum* MeOH extract fractions (F1-F5) 48

Figure 4.2 TLC analysis plate using (A) short UV wavelength (254 nm) and (B) long UV wavelength (365 nm). Each sample was submitted at an equal concentration of 1 mg/ml 49

Figure 4.3 Schematic diagram for dose selection of F5 in sighting study for acute toxicity study with mortality results (n=1, 1 male and 1 female rat per dose) 50

Figure 4.4 Photomicrographs of treated and control group male and female kidneys (100x magnification) 63

Figure 4.5 Photomicrographs of treated and control group male and female livers (100x magnification) 64

Figure 4.6 Photomicrographs of treated and control group male and female lungs (100x magnification) 65

Figure 4.7 Photomicrographs of treated and control group male and female hearts (100x magnification) 66

Figure 4.8 Photomicrographs of treated and control group male and female brains (100x magnification) 67

Figure 4.9 Photomicrographs of male treated and control group testes (100x magnification)..... 68

Figure 4.10 Photomicrographs of female treated and control group ovaries (100x magnification)..... 69

LIST OF ABBREVIATIONS

Akt	Protein kinase B
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ARASC	Animal Research and Service Centre
AST	Aspartate aminotransferase
ASVCP	American Society for Veterinary Clinical Pathology
AUC	Area under the curve
b.w.	Body weight
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma 2
CAD	Coronary heart disease
CKD	Chronic kidney disease
CLSI	Clinical & Laboratory Standards Institute
CNS	Central nervous system
CRC	Colorectal carcinoma cells
CYP450	Cytochrome P450
DMBA	9,10-dimethylbenz[a]anthracene
DMSO	Dimethyl sulfoxide
DPX	Distyrene, plasticizer and xylene mounting medium

ECG	Electrocardiogram
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EPA	Environmental Protection Agency
ERK	Extracellular signal regulated kinases
FDA	United States Food and Drug Administration
FGF	Fibroblast growth factor
FPG	Fasting plasma glucose
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GHS	Globally Harmonized System
GSK3β	Glycogen synthase kinase-3 beta
H&E	Haematoxylin and eosin
Hb	Haemoglobin
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HFD	High-fat diet
HPLC	High performance liquid chromatography
i.p.v.	Intraportal-venous
IFG	Impaired fasting glucose
IgE	Immunoglobulin E
IGT	Impaired glucose tolerance
IL-13	Interleukin-13
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4

IL-6	Interleukin-6
IRS1	Insulin receptor substrate 1
ISO	Isoproterenol
JNK	c-Jun N-terminal kinases
LC_{50}	Median lethal concentration
LC-MS	Liquid chromatography-mass spectrometry
LD_{50}	Median lethal dose
$LD_{chronic}$	Chronic lethal dose
LH	Luteinizing hormone
LPS	Lipopolysaccharide
MAE	Microwave-assisted extraction
MAPK	Mitogen-activated protein kinase signalling pathways
MARCH-5	Membrane-associated RING-CH-5
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCP-1	Monocyte chemoattractant protein 1
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MeOH	Methanol
MEOI	Methanolic extract of *O. indicum*
NF-κB	Nuclear factor-kappa B
NPRA	National Pharmaceutical Regulatory Agency
OECD	Organization for Economic Co-operation and Development
OGD	Oxygen and glucose deprivation
OGTT	Oral glucose tolerance test

OPPTS	The Office of Prevention, Pesticides and Toxic Substances
P13K	Phosphatidylinositol 3-kinase
PCV	Packed cell volume
PDGF	Platelet-derived growth factor
PET	Petroleum ether
PLE	Pressurized liquid extraction
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
QRS complex	Q wave, R wave and S wave complex
RA	Rheumatoid arthritis
RBC	Red blood cells
ROS	Reactive oxygen species
ROW	Relative organ weight
SASA	Solvent accessible surface area
SEM	Standard Error of Mean
SFE	Supercritical fluid extraction
Smad4	Mothers against decapentaplegic homolog 4
SOCS3	Suppressors of cytokine signalling 3
SPSS®	Statistical Package for Social Sciences
SST™	Serum separating tube
SULT	Sulfotransferase
TBIL	Total bilirubin
TGF-β	Transforming growth factor-beta
TLC	Thin-layer chromatography
TNF-α	Tumour necrosis factor- alpha
TP	Serum total protein

$t_{1/2}$	Half-life (of drug)
UAE	Ultrasound-assisted extraction
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UUO	Unilateral ureteral obstruction
UV light	Ultraviolet light
WBC	White blood cells
Wnt	Wingless-related integration site
β-catenin	Beta-catenin

LIST OF UNITS AND SYMBOLS

%	Percent
°C	Degree Celsius
µg/mL	Microgram per milliliter
µmol/L	Micromole per liter
cm	Centimeter
fL	Femtolitre
g	Gram
g/dL	Grams per deciliter
g/L	Gram per liter
GHz	Gigahertz
GPS	Global Positioning System
IU/L	International units per liter
kHz	Kilohertz
m	Meter
mg	Milligrams
mg/kg	Milligram per kilogram
MHz	Megahertz
mL	Milliliter
ml/kg	Milliliter per kilogram
mmol/L	Millimole per liter
nm	Nanometer
pg	Picogram
pH	Potential of hydrogen

v/v	Volume per volume

LIST OF APPENDICES

Page

Appendix A Voucher specimen of *Oroxylum indicum* (L.) Benth. Ex Kurz plant (USM Herbarium 11751) which was verified by Dr. Rahmad Zakaria and deposited in the Universiti Sains Malaysia (USM) herbarium by Mr. V. Shunmugam 103

Appendix B Animal ethics approval letter 104

PENILAIAN KETOKSIKAN ORAL SEBATIAN AKTIF BAICALEIN YANG DIEKSTRAK DARIPADA *OROXYLUM INDICUM* DALAM TIKUS SPRAGUE-DAWLEY

ABSTRAK

Oroxylum indicum adalah tumbuhan unik yang boleh ditemui di seluruh Asia Tenggara dan semakin menerima perhatian dalam bidang onkologi. Secara umumnya, baicalein ialah sebatian aktif yang dominan dalam tumbuhan *O. indicum*. Ia dilaporkan mempunyai kesan anti-metastatik, anti-bakteria, anti-obesiti, anti-diabetes, anti-keradangan, neuroprotektif, dan kardioprotektif, serta berpotensi untuk penyembuhan luka. Walau bagaimanapun, terdapat jurang dalam pengetahuan saintifik tentang profil toksikologi sebatian aktif baicalein yang diekstrak daripada *O. indicum*. Kajian ini bertujuan untuk menilai kesan ketoksikan akut ekstrak baicalein daripada daun *O. indicum* dengan menjalankan ujian ketoksikan oral akut ke atas model haiwan Sprague-Dawley. Proses pengekstrakan sistem binari Soxhlet menggunakan bahan daun *O. indicum* yang ditumbuk halus telah menghasilkan 3.94 g (16%) serbuk ekstrak mentah. Seterusnya, proses pemecahan serbuk ekstrak mentah dengan menggunakan metanol kepekatan 100% telah menghasilkan F5 tulen yang mengandungi baicalein. Analisis TLC telah mengesan kehadiran baicalein dalam F5 di bawah cahaya UV gelombang pendek (254 nm) dan UV gelombang panjang (365 nm). Kajian ketoksikan awal mendedahkan bahawa F5 tidak menyebabkan sebarang kematian di kalangan model haiwan sepanjang tempoh percubaan apabila dirawat dengan dos tetap 5 mg/kg, 50 mg/kg, 300 mg/kg, dan 2000 mg/kg yang ditentukan oleh garis panduan

Organisation for Economic Coorperation and Development (OECD). Oleh yang demikian, dos sub-maut baicalein adalah lebih daripada 2000 mg/kg. Keputusan kajian ketoksikan akut menunjukkan bahawa berat badan semua haiwan tidak menunjukkan sebarang kenaikan atau pengurangan lebih daripada 20% daripada berat badan awal mereka selepas 14 hari. Namun, perbezaan yang ketara (p <0.05) boleh dilihat dalam berat relatif otak, paru-paru, dan organ reproduktif kumpulan jantan dan betina yang dirawat. Walau bagaimanapun, keputusan indeks hematologi dan biokimia untuk kumpulan kedua-dua jantina yang dirawat semua didapati berada dalam julat normal mengikut garis panduan Clinical and Laboratory Standards Institute (CLSI) dan American Society for Veterinary Clinical Pathology (ASVCP), sama seperti kumpulan kawalan. Selain itu, semua kumpulan model haiwan tidak menunjukkan tanda-tanda klinikal ketoksikan dan perubahan dalam tingkah laku yang berkaitan dengan rawatan. Pemeriksaan histopatologi juga telah mendedahkan bahawa struktur selular semua organ dalaman penting model haiwan adalah normal tanpa kesan ketoksikan yang berkaitan dengan rawatan. Oleh itu, keputusan kajian ini menunjukkan bahawa baicalein yang diekstrak daripada *O. Indicum* tidak menghasilkan kesan toksik yang buruk terhadap tingkah laku dan morfologi tisu organ tikus Sprague-Dawley pada dos tertinggi 2000 mg/kg.

ACUTE ORAL TOXICITY EVALUATION OF BAICALEIN ACTIVE COMPOUND EXTRACTED FROM *OROXYLUM INDICUM* IN SPRAGUE-DAWLEY RATS

ABSTRACT

Oroxylum indicum is a unique plant which can be found throughout Southeast Asia and has gained increasing attention in the field of oncology. Baicalein is the most abundantly found and dominant active compound of the *O. indicum* plant in general. It has been reported to exert anti-metastatic, anti-bacterial, anti-obesity, anti-diabetic, anti-inflammatory, neuroprotective, and cardioprotective, as well as wound healing potentials. However, there is a gap in scientific knowledge on the toxicological profile of baicalein active compound extracted from *O. indicum*. This study aims to evaluate the acute toxicity effect of baicalein extracted from *O. indicum* leaves by conducting acute oral toxicity testing on Sprague-Dawley rats. The Soxhlet binary extraction process using finely crushed *O. indicum* leaf material yielded 3.94 g (16%) of crude extract powder. Next, fractionation of the crude extract powder using 100% methanol concentration generated fraction 5 (F5) with enriched baicalein compound. TLC analysis detected the presence of baicalein in F5 under short-waved (254 nm) and long-waved (365 nm) UV light. The initial sighting study revealed that F5 did not cause any mortality among the rat models throughout the experimental period, at fixed doses of 5 mg/kg, 50 mg/kg, 300 mg/kg, and 2000 mg/kg set by Organisation for Economic Cooperation and Development (OECD) guidelines. Thus, the sub-lethal dose of baicalein was more than 2000 mg/kg. The results of the acute toxicity study revealed

that body weight of all animals did not show any increment or reduction of more than 20% of their initial body weight after 14 days. Although, statistically significant differences ($p < 0.05$) were seen in the relative weight of the brain, lung, and reproductive organs among male and female treated groups. Nevertheless, the haematological and biochemical indices for treated groups of both sexes were all within normal range according to Clinical and Laboratory Standards Institute (CLSI) and American Society for Veterinary Clinical Pathology (ASVCP) guidelines, similar to the non-treated groups. Moreover, the animals showed no clinical signs of toxicity and changes in behaviours related to the treatment. Histopathological examination also revealed normal cellular architecture with absence of treatment related toxicity on all vital internal organs of the treated group. Therefore, the results of this study demonstrate that baicalein extracted from *O. indicum* produced no adverse toxic effects on Sprague-Dawley rat behaviour and organ tissue morphology at highest dosage of 2000 mg/kg.

CHAPTER 1

INTRODUCTION

1.1 Research background

Toxicology is a vast field combining disciplines such as chemistry, biology, pharmacology, and medicine with a focus on the effects of toxins, poisons, and treatment. In the field of pharmacology and medicine, drug toxicity deals with the adverse effects caused by therapeutic or non-therapeutic doses of drugs. In reality, more than 90% of drug candidates do not even make it pass the preclinical stage of development (Sun *et al.*, 2022). An analysis conducted by Harrison (2016), claimed that two major reasons for drug clinical failures were due to insufficient efficacy (52%) and unmanageable toxicity (24%), while the rest were due to commercial, operational and strategic reasons. One of the important aspects of drug development from preclinical stage up until phase 3 clinical trial is the toxicological assessment with the main objective of identifying the potential adverse effects, target, or non-target organ toxicity of a particular drug. Systemic toxicology studies such as single-dose and repeated-dose studies aim to evaluate the adverse effects of a drug on one or few organs as it is absorbed and distributed throughout the whole body. Overall, data generated from toxicology testing with either cell lines or animal models will aid researchers in translating the results into clinical studies involving humans. These data also contribute valuable input for drug regulatory development by governmental agencies. In Malaysia, the findings of toxicological assessments and relevant reports must be submitted together with the application in order for drug candidates to be registered under the National Pharmaceutical Regulatory Agency (NPRA). The NPRA reported that between the years 2017 and 2021, they have received a total of 661 applications

for registration of natural products which was the highest number of applications; the second was for veterinary products, prescription drugs came in third, followed by health supplements and non-prescription products. According to a Technavio (2022) market research report, it is estimated that the global natural product market will increase up to USD 15.89 billion by the year 2026. With the significant growth of natural products in the global market, it is vital to identify their potential toxicity or adverse effects to ensure safe usage.

Plants are a significant resource for therapeutic interventions due to vast species diversity. Humans have used plants to treat ailments for millennia. At present, 10% of half a million vascular plant species in the world are used in medicine to date (Salmerón-Manzano *et al.*, 2020). A group of important compounds used in medicine are plant secondary metabolites. Secondary metabolites are products of the plants' secondary metabolism which are considered non-essential to their growth or development. These compounds serve as the plant's defence mechanism against abiotic and biotic stressors (Ifeoma & Oluwakanyinsol, 2013). Plant-derived natural products are known to be more biologically friendly and less toxic to normal cells (Mishra & Tiwari, 2011) compared to synthetic drugs. In spite of known beneficial properties and relatively fewer side effects than synthetically produced drugs, studies have shown that certain medicinal plants have toxic effects and may cause acute poisoning, particularly in vulnerable groups like children and pregnant women (Ghorani-Azam *et al.*, 2018; Illamola *et al.*, 2020). In 2021, a total of 1,097 registered natural product samples were under surveillance of the NPRA (2021). The assumption that natural products are safe and harmless is not entirely true as there have been numerous reported cases of mild to severe acute adverse drug reactions (ADR) induced by the use of plant-derived medicines (Nur Azra *et al.*, 2021). The NPRA National

Centre for Adverse Drug Reactions Monitoring Report (2019) revealed that a total of 274 natural products were involved in adverse drug reaction reports in that year alone which was an increase from the total of 248 reports in the previous year. Drug toxicity in medicinal plants may be attributed to a few reasons. The first is due to the innate toxicity of phytochemicals (Piasecka *et al.*, 2015). The second is contamination of toxic elements such as cadmium, mercury, lead, arsenic found in the plants depending on environmental origin (Brima, 2017). Herb-drug interactions may also be another source of drug toxicity (Wang *et al.*, 2021). Even so, natural products are subject to less stringent safety assessments compared to synthetically developed drugs (Nur Azra *et al.*, 2021).

The plant in focus for this current study is the *Oroxylum indicum* which is a consumable plant found sparsely throughout the South and Southeast Asian regions. Most parts of the plant such as the bark, pendulous-sword-like fruits, night-blooming flowers, thin-papery seeds, and compound leaves are consumed for its various medicinal qualities. Flavonoids are considered the most abundant group of secondary metabolites which can be derived from the plant. Not only do flavonoids serve as the plant's ultraviolet-B (UV-B) radiation and stress protection (Falcone Ferreyra *et al.*, 2012), but it also produces the pigment for its flowers (Panche *et al.*, 2016). Notably, baicalein active compound which is a form of flavonoid found in *O. indicum* gained recent popularity, particularly in cancer, diabetes, and cardiovascular disease research. Baicalein has been the subject of many *in vitro* and *in vivo* studies. Through these studies, it has proven to exhibit many potentials such as antioxidant, anti-cancer, and anti-inflammatory properties. Despite its numerous benefits and potentials, its safety and toxicological profile is not well documented. The main mechanism behind the therapeutic benefits is its cytotoxicity on targeted cells and how the active compound

potentiates its chemo-preventive effects. Although there have been no reports of adverse effects caused by baicalein in studies, it was reported that potential toxicity of flavonoids, in general, may be caused by metabolic activation and subsequent inhibition of cytochrome P450 enzymes which may interfere with the metabolism and clearance of other drugs taken concomitantly (Galati & O'Brien, 2004). Nevertheless, the systemic toxicity of baicalein active compound remains elusive. It is dangerous to assume that baicalein by itself or in synergy with other active compounds derived from *O. indicum* is non-toxic and safe without proper scientific investigation focusing on its potential toxicity. Therefore, a careful assessment of the acute oral toxicity of baicalein active compound extracted from *O. indicum* was performed on Sprague-Dawley rats based on the Organization for Economic Cooperation and Development (OECD) guidelines test no. 420 (Acute Oral Toxicity – Fixed Dosed Procedure). These guidelines were developed with the international consensus of various government, industrial, and academic bodies to ensure chemical safety testing on animals were done according to a consistent set of specifications and methods.

1.2 Rationale of the study

Despite numerous studies on the use of baicalein compound extracted from *O. indicum* in clinical and therapeutic studies, there is limited literature on the toxicological profile and safety of baicalein active compound extracted from *O. indicum* on higher order animal models such as rodents. Therefore, it is anticipated that this study could add to the extensive current literature on baicalein compound extracted from *O. indicum*.

1.3 Research objectives

1.3.1 Main objective

To evaluate the acute toxicity effect of baicalein extract from *O. indicum* on animal model by conducting acute oral toxicity testing.

1.3.2 Specific objectives

1. To perform extraction and fractionation of baicalein from *O. indicum* leaves.
2. To determine the sub-lethal dose of baicalein extracted from *O. indicum* leaves on Sprague-Dawley rats.
3. To determine the acute toxicity effect of baicalein extracted from *O. indicum* leaves on Sprague-Dawley rats' behaviour and organ tissue morphology.

1.4 Significance of study

This study was carried out to address the gap in information regarding the acute toxicity effects of baicalein active compound on Sprague-Dawley rats, mainly on their behaviour and organ morphology. The results of this study may further support the current literature available on the therapeutic potentials of baicalein active compound extracted from the *O. indicum* plant especially in cancer research. Thus, this study may provide more evidence for the scientific community on the possible acute systemic toxicity effects of baicalein and confront the current assumption that the compound is safe to be used as a drug in clinical settings or for commercial purposes. Most importantly, this study could also benefit the local communities which have been consuming the *O. indicum* plant for generations by providing systematic and scientific data in ensuring its safety. The awareness of its safety will drive the economic trade of

O. indicum plant and may generate economic opportunities for marginalised areas. However, this opportunity needs to be carefully regulated by governmental agencies through legislation in order to prevent overharvesting among local communities or by opportunistic research companies and to protect indigenous intellectual property rights, i.e., enactment of Malaysian Access to Biological Resources and Benefit Sharing Act 2017.

1.5 Summary outline of thesis

In *Chapter One*, the research background has been introduced followed by the rationale of the study. The main and specific objectives have also been identified. The significance of the study has also been discussed.

In *Chapter Two*, the existing literature on the use of *O. indicum* plant, its ethnopharmacological uses, phytoconstituents, and the potentials of baicalein active compound will be reviewed. The importance of toxicity evaluation on the plant will provide a clear picture on the objective of this research.

In *Chapter Three*, the methodology for the collection, extraction, and fractionation of the *O. indicum* plant material will be presented followed by the method of detection of baicalein active compound. The toxicity evaluation procedures will also be outlined, along with behavioural observations and histopathological examination procedures.

In *Chapter Four*, the overall results from the data collected will be presented which includes the extraction and fractionation product, TLC analysis results, toxicity studies and histopathological scoring data.

In *Chapter Five*, a summary and discussion of the results obtained will be presented. The limitations of the current study will also be highlighted along with the suggestions for future research.

In *Chapter Six*, a conclusion will be provided to summarise the key findings of the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural products in medicinal history

Natural products have been known to be widely used as a source of medicine since recorded history of early mankind. It is assumed that early humans discovered the therapeutic properties of natural products by happenstance through foraging following their innate behaviour to self-medicate (Hardy, 2021). This theory was proved by the discovery of a fossilised Neanderthal (archaic humans emerged at least 200,000 years ago) body buried together with diagenesis-resistant pollen grains of several species of medicinal plants in an archaeological site found in Shanidar Cave, Iraq (Lietava, 1992). Since pollen grains consists of mainly sporopollenin on its exterior layer, which is an inert biopolymer, it is very resistant to diagenesis and can remain in deposits for millions of years (Yamada *et al.*, 2021). The knowledge of natural products was passed down to subsequent generations and soon more systematic healing systems were formed. In the earliest Indian civilization of 5000 BC, the Indians developed Ayurveda which is a medical system utilizing natural products such as herbs, minerals and animal-based products to treat ailments (Sharma *et al.*, 2021). On the other hand, ancient Chinese medicine began with a medical theory which places an emphasis on ‘qì’ and ‘xuè’ (Hsu, 2018), followed by the Zhou Dynasty in 1046 BC when Chinese medical practitioners started to develop a more advanced form of medicine which uses herbal medicine containing fruits, vegetables, grains and animal meat to treat different kinds of diseases (Mortlock, 2020). In ancient Greek history, Hippocrates, the ‘Father of Modern Medicine’, along with other Greek medical

practitioners suggested the use of herbal remedies to treat patients in his collection of works in the Hippocratic Corpus. Soon, the establishment of the Islamic empire in the 7th Century by the Prophet Muhammad resulted in a rise in science and translation of Greek literature and knowledge which included the use of natural products in treatment of diseases or ailments (Majeed, 2005). Natural products became more systematically categorized and catalogued into literature such as the illustrious 'Kitab al-Qanun fi al-Tibb' (The Canon of Medicine) written by Abu Ali Al-Hussein Ibn Abdullah Ibn Sina (Amr & Tbakhi, 2007) or 'Species Plantarum' by Carl Linnaeus.

Fast forward to the present, these medicinal plants and their isolated bioactive compounds build a bridge between ancient and modern pharmacology. Plant products make up almost 25% of modern global pharmaceutical sales which amounts to roughly US$ 75 billion (Kate & Laird, 2019). In Asia, India and China are collectively responsible for the significant global trade of medicinal plants (Vasisht *et al.*, 2016). Some plants used in ancient times are still used today such as ginseng (Park *et al.*, 2012), camphor, nutmeg (Gupta *et al.*, 2020), turmeric (Kumar *et al.*, 2017), aloe vera (Foster *et al.*, 2011), and saffron (Mzabri *et al.*, 2019). Even garlic (Muhammad *et al.*, 2020), *Ginkgo biloba* (More *et al.*, 2021), and *Andrographis paniculate* (Okhuarobo *et al.*, 2014) are widely used as either health supplements or traditional medicine. Through trading and exploration, these knowledge of natural products in the originating community have been shared with the rest of the world. Malaysia provides a prime example of biodiversity in plant-based medicine due to the rich variety in flora in the region and practices from Malay, Indian, Chinese, Indonesian, and Thai cultures among many others which are often assimilated into the indigenous traditional medicine system.

Out of the myriad of plants used in traditional medicine or consumed as food in Malaysia, *O. indicum* is a unique plant which can be found sparsely throughout the Southeast Asian region and has gained increasing interest in the field of oncology. Unfortunately, its popularity in traditional medicine in India has reached its limit, as the plant was categorised as a threatened species in various states of India due to climate change, poor regeneration, and low germination rate (Kumar *et al.*, 2021). However, other countries such as China, and Southeast Asian countries including Malaysia continue to benefit from it ethnopharmacologically and scientifically.

2.2 Botanical description of *Oroxylum indicum*

Oroxylum indicum (L.) Benth ex Kurz plant belongs to the Bignoniaceae family which are native to India. It is also found in other South Asia countries like Nepal, and Southeast Asia that includes countries like Malaysia, Indonesia, tropical parts of China such as Yunnan or Guangdong provinces, Myanmar, Thailand and Vietnam (Tran *et al.*, 2015). The term 'oros' means mountain in Greek and 'xylos' means wood. The term 'indicum' in Latin refers to the country of origin which is India where the tree is naturally found (Wiart, 2006). It is commonly known as the tree of Damocles, Midnight Horror, Broken Bones, and Indian Trumpet in English while in Malaysia, it is locally known as "beko" or "bonglai". The scientific description of this plant was first published in the 'Forest Flora of British Burma' by S. Kurz in 1877 from the Royal Botanic Gardens of Calcutta.

It is an angiosperm and dicotyledon plant which is autotrophic in nature. It is characterised as a semi-deciduous tree, which can reach up to heights of 27 meters (Figure 2.1A). The tree itself has a greyish-brown bark as a trunk which is covered in fissures (Figure 2.1B). The leaves are compound in shape and the leaflets are oval or

oblong in shape (Figure 2.1C) which grow on stalks that mimic broken bones once they wither and fall to the ground (Figure 2.1D). The flowers of the *O. indicum* are large brownish yellow to dirty violet in colour (Figure 2.1E). Individual flowers are comprised of 5 reflexed lobes, 5 stamens, 1 style and a superior ovary. The flowers are night-blooming and gives out a foul odour. The fruits of the *O. indicum* are woody pendulous capsules or pods that are sword-shaped (Figure 2.1F). The pod itself turns black when ripe. The seeds are round and large in shape (Figure 2.1G). The seeds have thin, transparent papery wings. Each branch of the plant blooms independently of other branches (National Parks of Singapore, 2022; Wiart, 2006).

Despite its unique and eerie appearance especially when seen during the night, *O. indicum* is not merely a decorative tree. Each part of the tree can be consumed and possesses a number of therapeutic uses which have been sourced skilfully by the people of Southeast Asia particularly by the native Indian population.

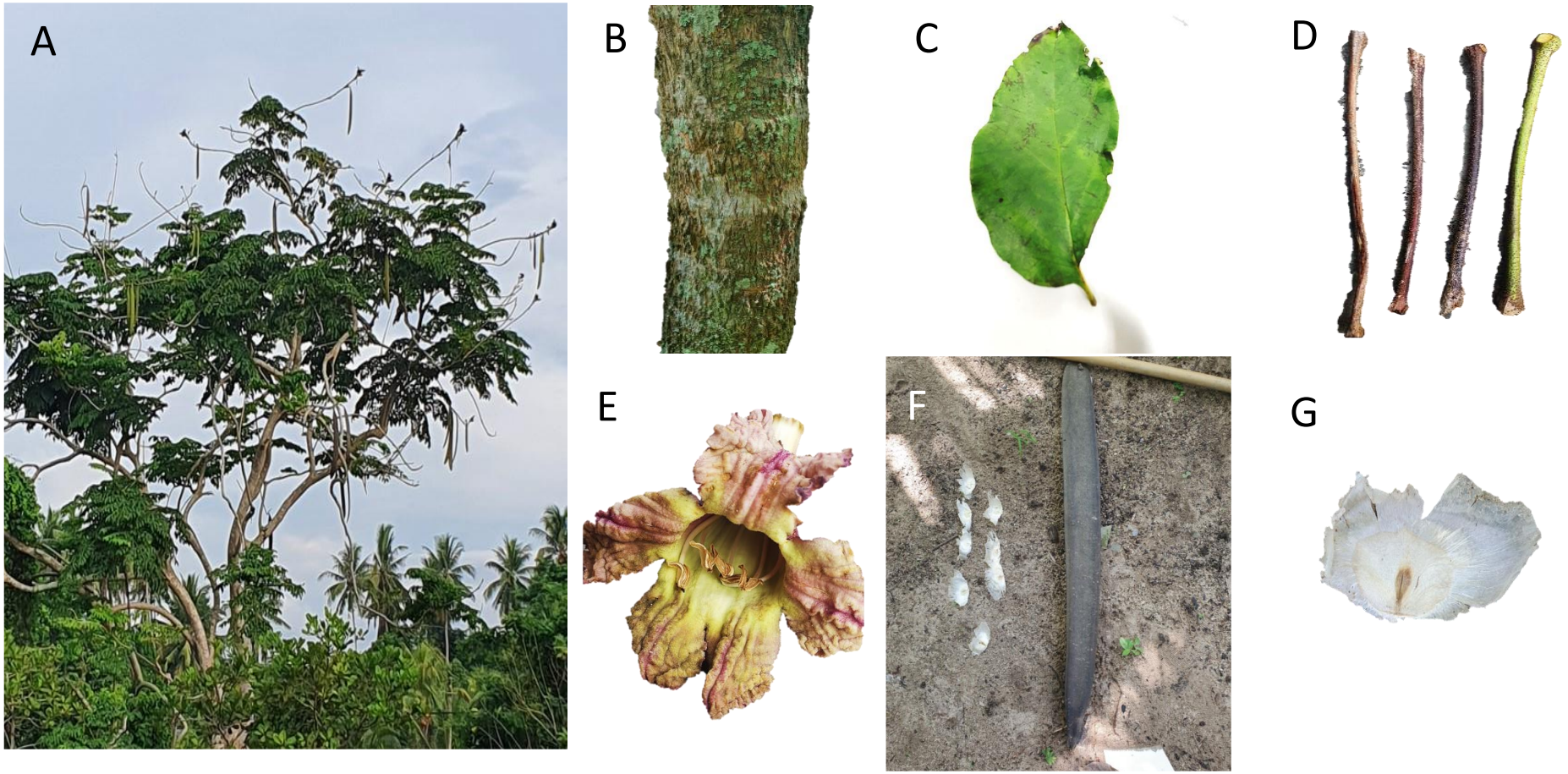


Figure 2.1 On-site photographs taken of *O. indicum* tree and its various parts. From left: (A) Whole tree; (B) Stem bark; (C) Leaf; (D) Leaf stalks; (E) Flower; (F) Fruit with seeds; (G) Seed

2.3 Ethnopharmacological uses of *O. indicum*

Ethnopharmacology is considered 'the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man'(Mukherjee *et al.*, 2010). The discovery of new drugs from medicinal plants traditionally used by the indigenous people is an important contribution to the scientific world through the ethnopharmacological approach. In the case of *O. indicum* plant, the identification of its traditional uses by the Southeast Asian population may provide an avenue for further scientific research. In India, *O. indicum* stem bark is traditionally used to treat gastritis, jaundice, and dysentery (Ahad *et al.*, 2012; Bhushan & Kumar, 2013; Sharma *et al.*, 2012). In Malaysia where it is commonly known as "beko" or "bonglai", the stem bark of the plant is used topically to treat allergic dermatitis, burns and wounds (Gaur *et al.*, 2011; Rasadah, 2001). The root bark is used to treat sore throat and diarrhea (Prakash, 2005). On the other hand, the leaves and fruits of the plant are sold in markets and consumed as a raw salad or "ulam" by locals in Malaysia (Nik Nur Hakimah *et al.*, 2020). The flowers and fruits are also consumed as a vegetable in Thailand (Jagetia, 2021). A decoction of the leaves of the plant is drunk to treat stomach-ache and rheumatism (National Parks of Singapore, 2022). Furthermore, the fruits of *O. indicum* are used to treat bronchitis and intestinal worms (Bhushan & Kumar, 2013; Drury, 2006). Lastly, the seeds of *O. indicum* are consumed for the treatment of throat infections, fever, and hypertension (Rai *et al.*, 2022).

The many therapeutic uses of *O. indicum* is garnered from the phytoconstituents extracted from the plant. Phytoconstituents or secondary metabolites are compounds which are unique to a plant and are products of biosynthesis that are not essential for physiological growth. Evidently, autotrophic plants such as the *O. indicum* are unable to escape from predation and migrate from harsh environments in

which they grow in. They have evolved mechanisms and innate chemical defenses to shield themselves from danger, successfully adapt to these harsh environments and co-exist with herbivores. Thus, phytoconstituents are basically active plant chemical compounds that serve as a protection against biotic and abiotic stressors (Ifeoma & Oluwakanyinsol, 2013). On the other hand, some of these compounds, particularly phenolic compounds contribute to the plant's colour, odour, taste and other properties pertaining to the senses (Dias *et al.*, 2021). As phytoconstituents offer a range of benefits to the plants themselves, humans have evidently learned to harvest its protective and healing potentials. Interestingly, a single plant may be considered a treasure trove of phytochemicals, often yielding various chemical compounds in a single segment or different segments of the plant.

2.4 Phytoconstituents of *O. indicum*

Many phytoconstituents can be derived from different parts of the *O. indicum* plant. The phytoconstituents found in the plant's stem bark are flavonoids, isoflavonoids, naphthalenes, steroids, and other phenolic compounds. The flavonoids present in the stem bark include baicalein, chrysin, oroxylin A, baicalin, scutellarein, scutellarein 7-O-rutinoside, and baicalein 7-O-glucoside. Isoflavonoids found in the stem bark are methyl oroxylopterocarpan, hexyl oroxylopterocarpan, and heptyl oroxylopterocarpan. Naphthalenes extracted from the stem bark are lapachol and dehydro-iso-α-lapachone. Besides that, the steroids found in the stem bark are β-sitosterol and β-sitosterol glucoside which is essential for plant growth and resistance to biotic or abiotic stressors (Vriet *et al.*, 2012). Another constituent found in the plant's stem bark is p-coumaric acid (Dev *et al.*, 2010).

On top of that, the phytoconstituents present in the roots are also flavonoids, isoflavonoid, naphthalenes, steroids, and phenolics. The flavonoids include baicalein, chrysin, oroxylin A, and baicalein 6-O-glucoside. The only isoflavonoid present in the roots of the plant are biochanin A. The naphthalenes found in the roots are also lapachol, dehydro-iso-α-lapachone, and faramol among many others. The steroids found in the roots are similar to those found in the stem bark with an additional chemical constituent of stigmasterol glucoside (Dinda *et al.*, 2015). Ellagic acid is another phenolic compound found in the roots of *O. indicum* (Dev *et al.*, 2010) which is a product of ellagitannin hydrolysis that confers chemical defence against pathogenic infections and predation for the plant (Landete, 2011).

The leaf extracts of *O. indicum* have been shown to produce phenolic compounds such as flavonoids, tannins, and phlobatannins (SatyaEswari *et al.*, 2018). Flavonoids serve as ultraviolet-B (UV-B) radiation and stress protection for the plant (Falcone Ferreyra *et al.*, 2012). Phlobatannins are natural antioxidants and anti-inflammatories which aid in wound healing and acts as analgesics. The leaf extracts were also shown to produce cardiac steroidal glycosides that serve as the plant's chemical defence system (Morsy, 2017) which can be used to regulate heart rate (SatyaEswari *et al.*, 2018).

The main phytoconstituents found in the *O. indicum* flowers are flavonoids, particularly baicalein and chrysin (Rojsanga *et al.*, 2017). High amounts of both constituents are found in young buds of the plant, however as the flowers enter the blooming stage, the amounts of baicalein and chrysin are at its lowest (Rojsanga *et al.*, 2017). Flavonoids have been attributed to the colour and fragrance of flowers (Panche *et al.*, 2016). Besides that, it serves as chemical attractants for pollinators and repellents against pathogens and herbivores (Falcone Ferreyra *et al.*, 2012). On the other hand,

the phytoconstituents found in the fruits of the plant are also baicalein and chrysin (Sithisarn *et al.*, 2019). Another phytoconstituent which may be isolated from the plant's fruit includes a triterpenoid, namely ursolic acid (Jiwajinda *et al.*, 2002).

The phytoconstituents isolated from the seeds of the *O. indicum* are also flavonoids, triterpenoids, steroids, cyclohexylethanoids, glycosides and fatty acids. Major flavonoids isolated from the seeds are also baicalein, baicalin, chrysin, oroxin B, oroxin A, and chrysin 7-O-diglucoside (Krüger & Ganzera, 2012; Wu *et al.*, 2019). Methanolic extract of *O. indicum* (MEOI) seeds contains a high amount of total phenolic content (Samatha *et al.*, 2012).

Among the major phytoconstituents extracted from the plant, flavonoids are identified as the biggest group of secondary metabolites found in most parts of the *O. indicum* plant. Active chemicals such as oroxylin-A, chrysine, scutellarin, and baicalein have been isolated from flavonoids of the *O. indicum*. Among all these bioactive compounds, baicalein is the most abundantly found and dominant active compound of the *O. indicum* plant in general. Therefore, the toxicity of baicalein was evaluated in this study.

2.5 Baicalein – an abundant active compound of *O. indicum*

Baicalein is chemically known as 5,6,7-trihydroxy-2-phenyl-4H-chromen-4-one with chemical formula of $C_{15}H_{10}O_5$ (Figure 2.2) and molecular weight of 270.24 g/mol (Verma *et al.*, 2021). The hydroxy groups are located at positions C-5, -6 and -7 (National Center for Biotechnology Information, 2022). It is the aglycone of baicalin. It is a flavone found in the flavonoid class of substances that is often present in natural products. Baicalein can be well-absorbed in the stomach and small intestine (Tuli *et al.*, 2020). Many previous studies have established the potential of baicalein to exhibit

anti-cancer (Epelle *et al.*, 2022; Liu *et al.*, 2016), anti-inflammatory (Shukla *et al.*, 2019) and antioxidant benefits (Kang *et al.*, 2012) which gives cause to its increasing popularity in cancer research (Epelle *et al.*, 2022). Baicalein has also been found to target multiple vital pathways such as phosphatidylinositol 3-kinase (P13K)/protein kinase B (Akt)/nuclear factor-kappa B (NF-κB) (Yu *et al.*, 2017), Wnt/β-catenin (Yaylagül and Ülger, 2020), Smad4, AMPK, and ROS signalling (Liu *et al.*, 2016). Despite being present in only few plants such as the *O. indicum*, *S. baicalensis*, and *S. lateriflora*, baicalein active compound has been extensively discussed in various literatures which involve *in vitro* and *in vivo* studies on cell lines, animal models and even human subjects.

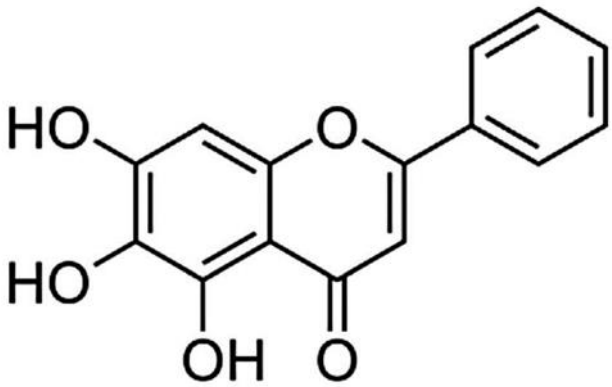


Figure 2.2 Chemical structure of baicalein (5,6,7-trihydroxyflavone)

2.6 Therapeutic potentials of baicalein active compound

Baicalein active compound, be it derived from crude extract or synthetically produced has been reported to exert many beneficial therapeutic potentials to either prevent or alleviate the symptoms of human diseases. Some of these potentials will be discussed further below such as anti-metastatic, anti-bacterial, anti-obesity, anti-diabetic, anti-inflammatory, neuroprotective, cardioprotective, as well as the potential for wound healing.

2.6.1 Anti-metastatic

Metastasis is a process whereby tumour cells leave the primary tumour and travel to a distant site by way of the circulatory system, thus establishing a secondary tumour. The metastasis of tumour cells involves multiple stages, starting with invasion or migration, followed by angiogenesis, attachment and finally tumour proliferation. Numerous studies have proved the anti-metastatic potential of baicalein on tumour cells. A study conducted by Lalou *et al.*, (2013) found that baicalein extracted from *O. indicum* demonstrated highest inhibitory effect on proliferation and migration on colon carcinoma (CT-26) cell line as compared with other phytoconstituents of the plant. A study conducted found the potential therapeutic effects of baicalein for hepatocellular carcinoma (HCC) as it inhibited tumour cell invasion and metastasis by reducing cell motility and migration via the suppression of the extracellular signal regulated kinases (ERK) pathway (Chen *et al.*, 2013). Another study done to investigate the anti-metastatic effects of baicalein on colorectal carcinoma cells (CRC) found that baicalein reduced the phosphorylation of extracellular signal regulated kinases (ERK1/2) signalling pathway which in turn suppressed matrix metalloproteinases -2/-9 and reduced CRC cell invasion (Chai *et al.*, 2017). In addition, Lin *et al.*, (2020) found that 50, 75, and 100 μM baicalein treatment inhibited cell migration and invasion in human osteosarcoma (MG-63) cells when tested using wound healing and Transwell assay.

2.6.2 Neuroprotective

Brain damage is often caused by inflammation due to glial activation or cytokine production. A study investigating the effects of the compound on the nervous system, specially involving Parkinson's disease had found that rats with rotenone-

induced motor dysfunction successfully achieved a significant improvement from motor impairment and brain damage after treated with baicalein (Zhang *et al.*, 2017). The baicalein treated rats showed improved scores in prehensile traction and catalepsy tests compared to the non-treated group. It was found through this study that baicalein was able to suppress the production of proinflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), inhibit astrocytosis and microglia activation and block the NF-κB and mitogen-activated protein kinase (MAPK) signalling pathways, thus demonstrating the neuroprotective effects of baicalein (Zhang *et al.*, 2017).

2.6.3 Anti-bacterial

The increase prevalence of antibiotic drug-resistance has become a major concern in healthcare that has driven new research on alternative and combination therapies. A recent study had also shown the antibacterial properties of baicalein when tested against two gram-positive bacteria, *Staphylococcus aureus* and *Micrococcus luteus*. After 24 hours, clear zones of inhibition were seen in the petri dishes containing both bacterial species when ethanolic extract of baicalein from *O. indicum* callus was applied onto the agar plates (Faraz *et al.*, 2020). The results of this study were further supported by newer evidence that a combination of baicalein with ampicillin was able to improve infection caused by *Streptococcus suis* serotype 2 by binding, inhibiting, and destroying the molecular structure of an important virulence factor, suilysin that promotes the pathogenesis of the bacterial strain (Lu *et al.*, 2021). Therefore, it can be surmised that baicalein has the potential to be used to fight infection by pathogenic bacteria and extend antibiotic resistance.

2.6.4 Anti-obesity

Furthermore, in a 2019 survey conducted by the Ministry of Health Malaysia, it was reported that a staggering 50.1% of Malaysian adults are either overweight or obese (Institute for Public Health (IPH), 2020). As Malaysia was ranked the top country in Southeast Asia with the highest obesity prevalence among adults, it remains a national effort to control the overweight and obese population. Baicalein may provide a solution to the problem. Obesity stems from energy imbalance which may be caused by genetics, behavioural and environmental factors (de Ferranti & Mozaffarian, 2008). These factors often result in the storage of excess energy in adipocytes which manifests as an increase in intracellular lipids and larger adipocyte size (adipose hypertrophy) and increased number of adipocytes (adipose hyperplasia) (de Ferranti & Mozaffarian, 2008). The process of adipose hyperplasia occurs when there is an increase in preadipocyte numbers and when the preadipocytes differentiate into mature adipocytes (Avram *et al.*, 2007). The differentiation of preadipocytes is mediated by various hormones and transcription factors such as glucocorticoid, insulin, tumour necrosis factor- α (TNF-α) (Avram *et al.*, 2007), and peroxisome proliferator-activated receptor-γ (PPAR-γ) (Drolet *et al.*, 2008). Therefore, the potential for baicalein to target either the process of differentiation of preadipocytes into mature adipocytes or the factors involved in the process may prove beneficial in counteracting obesity. It was revealed in a previous study that a concentration of 10-20 μM of baicalein was able to prevent the differentiation of preadipocyte to adipocyte within 4 days of induction when tested on 3T3-L1 preadipocytes (Madsen *et al.*, 2003). It was suggested that baicalein was able to suppress the mTOR signalling pathway of treated 3T3-L1 preadipocytes resulting in the inhibition of adipogenic factors like PPAR-γ (Seo *et al.*, 2014).

2.6.5 Cardioprotective

Cardiovascular diseases such as coronary heart disease (CAD), stroke, and heart failure are common risks among obese and diabetic patients. One of the key factors promoting the pathogenesis of these cardiovascular diseases are abnormal mitochondrial fission and mitophagy. Mitochondrial fission and mitophagy is regulated by membrane-associated RING-CH-5 (MARCH-5) protein located in the outer membrane of the mitochondria. Down-regulation of MARCH-5 may cause an increase in mitochondrial fission activity and cell apoptosis. A study conducted by Li *et al.*, (2020), revealed that baicalein was able to upregulate MARCH-5 expression, promote cardiomyocyte mitophagy, inhibit cell apoptosis, thus reducing the effects of oxidative damage on cardiomyocytes caused by hydrogen peroxide (H_2O_2)-induced cardiotoxicity. The results demonstrate the cardioprotective potential of baicalein in cardiovascular dysfunction. Cardiotoxicity caused by medication is also quite common, an example being a chemotherapy antibiotic called doxorubicin. A 2019 study conducted by Menon *et al.*, provided evidence that 70% MEOI was able to protect doxorubicin-treated Sprague-Dawley rats from potential cardiotoxicity, which was proven by the electrocardiogram (ECG) and Q wave, R wave and S wave (QRS) complex normalization in the animals' hearts. Thus, baicalein may be used as a potential supplementary therapy without interfering with doxorubicin efficacy in cancer treatment and cause any myocardial damage (Menon *et al.*, 2019).

2.6.6 Anti-diabetic

As diabetes is one of the factors which contribute to the increased risk of cardiovascular disease, it is important to nip the disease at the bud and prevent prediabetes condition from progressing into type 2 diabetes mellitus. Prediabetes is

characterized by an impaired glucose tolerance and impaired fasting glucose when tested with an oral glucose tolerance test (OGTT). Insulin resistance is a factor involved in the progression of prediabetes to diabetes. Patients with an impaired fasting glucose (IFG) will usually have a fasting plasma glucose (FPG) level between ≥ 6.1 and < 7.0 mmol/L, without impaired glucose tolerance (IGT). On the other hand, patients with IGT are found to have a FPG concentration of < 7.0 mmol/L and a 2-hour post-load plasma glucose level between ≥ 7.8 and < 11.1 mmol/L (Dong *et al.*, 2019). An *in vivo* study involving mice fed with high-fat diet (HFD) conducted by Sun, *et al*. (2017) found evidence that a combination of 40 and 160mg/kg of baicalein and 4 mg/kg of acarbose reduced the chances of progression from prediabetes to diabetes by 83.3%. Suppressors of cytokine signaling 3 (SOCS3) is an insulin-induced regulator involved in the signaling of insulin which has insulin receptor substrate 1 (IRS1), protein kinase B-1 (Akt1) and Glycogen synthase kinase-3β (GSK3β) as downstream proteins. It was discovered that baicalein was able to improve insulin resistance by interacting with SOCS3, decreasing its expression while increasing the insulin-stimulated phosphorylation of IRS1 and Akt1, at the same time decreasing the GSK3β phosphorylation (Sun *et al.*, 2017).

2.6.7 Anti-inflammatory

In addition, inflammation is known to be a common factor in the progression of many diseases such as cancer, bowel and kidney disease, rheumatoid arthritis (RA) and also diabetes (Tsalamandris *et al.*, 2019). In chronic kidney disease (CKD), chronic inflammation involving many types of kidney and immune cells causes renal fibrosis, which occurs during the last stages of the disease is characterized by loss of renal function and eventual kidney failure (Eddy, 2014). The progression of renal

fibrosis involves the release of fibrogenic inflammatory cytokines such as interleukin-1β (IL-1β), TNF-α and monocyte chemoattractant protein 1 (MCP-1) by NF-κB through mitogen-activated protein kinase signaling pathways (MAPK) which occurs when p38 MAP kinase, c-Jun N-terminal kinases (JNK) and ERK are phosphorylated (Stambe *et al.*, 2004). A study was conducted to investigate the anti-inflammatory effects of baicalein on unilateral ureteral obstruction (UUO)-induced mice which supposed to model the pathological changes observed in human chronic renal fibrosis (Wang *et al.*, 2015). UUO in animal models are observed to have interstitial inflammation, accumulation of collagen and fibronectin in kidney scars, presence of myofibroblasts and inflammatory cytokines (Eddy, 2014). It was found that UUO-induced mice which were given 50 and 100 mg/kg dose of baicalein showed significant reduction in fibronectin accumulation and reduction in expression of fibrogenic pro-inflammatory cytokines such as IL-1β, TNF-α and MCP-1 by the inactivation of NF-κB and MAPK signaling pathways (Wang *et al.*, 2015). On top of that, baicalein was also able to prevent the intrusion of macrophages and lymphocytes in kidney tissue scars (Wang *et al.*, 2015).

2.6.8 Wound healing

Natural wound healing is a dynamic process involving different phases that occur coinciding with each other which are haemostasis, inflammation, proliferation, matrix formation and remodelling. Wound healing is mediated by cytokines or growth factors secreted by platelets such as platelet-derived growth factor (PDGF), transforming growth factor- β (TGF-β), fibroblast growth factor (FGF), and epidermal growth factor (EGF) which are required in all the phases of inflammation, proliferation and matrix formation to induce fibroblast proliferation, keratinocyte and collagen

synthesis (Witte & Barbul, 1997). The deposition of collagen in wounds are vital in determining the strength of scar formation (Witte & Barbul, 1997). In a study conducted by Lalrinzuali *et al.* (2018), baicalein along with other major phytoconstituents found in *O. indicum* were also found to promote wound healing. The topical application of 10% concentration *O. indicum* enhanced wound contraction and shorten wound healing time in mice with deep dermal excision wounds. It was postulated that 10% concentration of *O. indicum* extract was able to stimulate maximum DNA and collagen syntheses which increased the proliferation of fibroblasts and keratinocytes (Lalrinzuali *et al.*, 2018). These findings further strengthen evidence on the traditional use of *O. indicum* stem bark used in Malaysia to treat wounds and burns (Gaur *et al.*, 2011; Rasadah, 2001).

2.7 Extraction methods for natural products

As evidenced by the numerous therapeutic potentials of baicalein, the extraction process is an important step in obtaining this active compound from *O. indicum*. Extraction is the separation of bioactive compounds from plant tissues using solvents through different techniques (Abubakar & Haque, 2020). The main challenge in working with natural products such as the *O. indicum* plant lies with the low yield of active compounds which can be obtained from the plant. Often, the process is time-consuming and labour intensive. There is a myriad of extraction methods used for natural products ranging from conventional to more novel or greener methods. Conventional extraction methods include maceration, percolation, decoction and Soxhlet extraction. Alternatively, more sophisticated, or greener extraction methods may be utilized to extract plant material such as pressurised liquid, supercritical fluid, ultrasound-assisted, and microwave-assisted extraction. Each of these extraction

methods have its strength and limitations depending on the type of natural product sample, final yield, appropriate solvent, extraction temperature and duration (Zhang *et al.*, 2018).

In this study, dual-phase Soxhlet extraction using petroleum ether and methanol solvents was used to extract crude compounds from the leaves of *O. indicum*. Soxhlet extraction method is a popular method to extract *O. Indicum* in few studies (Bhusari *et al.*, 2019; Kang *et al.*, 2019; Saha *et al.*, 2017; Khaizil Emylia *et al.*, 2013). This method involves continuous hot extraction. The apparatus used for this method is called the Soxhlet extractor which consists of few parts assembled together, such as the round bottom flask, extraction chamber, siphon tube, and condenser. Dried, finely powdered plant material is placed into a thimble (a porous filter paper or cloth bag) and placed into the extraction chamber. The solvent used for extraction is poured into the round bottom flask and heated using a heating mantle. The solvent evaporates, passes through the condenser where it condenses and drips down into the extraction chamber. When the level of solvent in the extraction chamber reaches the top of the siphon tube, the solvent and plant material mix flows back into the flask. This process is continuous until the evaporated solvent flowing from the extraction chamber does not leave any residue. This method does not require large amounts of solvent and suitable for partially soluble plant material in chosen solvent. It also utilizes shorter extraction time and not as labour intensive as compared to maceration, percolation, and decoction methods (Azwanida, 2015; Handa, 2008). It does not require the use of high heat which may destroy the molecular structure of active compounds available in the sample. More novel extraction methods such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) have a disadvantage when extracting plant material as they must be operated

under high temperature and pressure which require more complex system configurations and optimization procedures. In addition, CO_2 which is often used as a solvent in SFE method is not suitable to extract polar compounds due to its non-polar nature (Liew *et al.*, 2020). A co-solvent such as methanol (MeOH) may be added into the procedure to improve the extraction efficiency of SFE method for polar compounds (Liew *et al.*, 2020). According to Geow *et al.*, (2021) MAE and UAE methods also require precision in operating the systems and more optimization on the settings and parameters such as microwave power and frequency level to obtain appropriate quality of yield. Techniques such as maceration, decoction and percolation require longer extraction time and more solvent as compared to Soxhlet extraction method. Therefore, Soxhlet extraction method was chosen due to the polar nature of *O. indicum* extract and its relatively straightforward technique.

2.8 Solvents used in natural product extraction

Solvent selection is another important factor when performing the extraction process. Selecting the appropriate solvent depends on the type of plant material, part of the plant to be extracted, nature of the bioactive compounds, and availability of the solvent. Polarity and miscibility of solvent and solute are also other important factors to consider depending on the choice of extraction method. A polar solvent such as MeOH is used in extraction of polar compound, whereas a non-polar solvent such as petroleum ether (PET) is used in extraction of non-polar compounds due to its miscibility as a result of having similar polarity. Nonetheless, organic solvents have high polarity and are more efficient for extraction of phenolic compounds such as flavonoids (Chaves *et al.*, 2020).

Water is the most common solvent used in extraction and is considered a "green" solvent. It is also the most polar solvent as it dissolves a wide range of plant material. Kang *et al.* (2019) reported that based on their extraction, aqueous extract of *O. indicum* leaf material resulted in the highest yield (66%) compared to MeOH (14%) and a combination of PET-MeOH (27%) which was assumed to be attributed to the miscibility of polysaccharides and glycosides (SatyaEswari *et al.*, 2018), which are present in the leaf of the plant, in water. Major disadvantages of water as a solvent are that it promotes bacterial growth, may cause hydrolysis and large amount of heat is required to concentrate the extract (Tiwari *et al.*, 2011).

Furthermore, alcohol is a polar organic solvent which is slightly less polar when compared to water. Standard alcohol solvents used in extraction of plant material are ethanol or MeOH, and 1-butanol. Alcohols have different boiling points, in which ethanol has a boiling point of 78°C, MeOH has a boiling point of 64.7°C, acetone's boiling point is 56.05°C, while 1-butanol has the highest boiling point of 117.7°C. It is non-toxic at low concentrations. Few major disadvantages of alcohol are that it does not dissolve fats, gums, and wax, its cytotoxic, combustible, and volatile. However, it can be used to extract polar secondary metabolites (Abubakar & Haque, 2020; Tiwari *et al.*, 2011) which is considered suitable for flavones such as baicalein.

Ethers are also organic solvents which include petroleum ether (PET) or diethyl ether. Ethers are non-polar solvents and useful in extraction of alkaloids, terpenoids, coumarins and fatty acids (Tiwari *et al.*, 2011). It has a low boiling point of 60-80°C and is able to mix with water. It is a very stable compound and does not react with acids, bases, and metals. However, it is highly volatile and combustible (Abubakar & Haque, 2020).

In conclusion, the choice of solvent for natural product extraction is an important step towards effectively obtaining a target compound. Water, MeOH, and PET have been used in previous studies to successfully extract *O. indicum* phytoconstituents (Lalrinzuali *et al.*, 2015; Rai *et al.*, 2020; Tenpe *et al.*, 2009). MeOH especially was found to be able to extract the highest flavonoid content from *O. indicum* as compared to chloroform, benzene, and PET (Moirangthem *et al.*, 2013; Samatha *et al.*, 2012). On the other hand, PET was found to produce higher alkaloid and flavonoid content using Soxhlet extraction compared to chloroform and ethanol (Panda *et al.*, 2018; Samarath & Panda, 2018). Since baicalein is known as a flavonoid active compound, therefore, MeOH and PET are considered suitable choice of solvents to be used in this study due to their consistent successful flavonoid extraction yield from previous studies.

2.9 *In vivo* toxicity evaluation on natural products

2.9.1 Toxic mechanism of flavonoids

Toxicity studies and testing is an important step in developing new drugs for the global market and can be conducted from the start of drug discovery to later in clinical development. Pertaining to the use of natural products, there is a common presumption that "it is natural, therefore safe". However, that may not always be the case. As mentioned, plants produce many active chemicals and secondary metabolites to serve as an innate protective mechanism, however secondary metabolites such as flavonoids may induce toxic effects that can be both beneficial and detrimental to the human body. Flavonoids may be toxic when consumed concomitantly with other drugs due to metabolic activation that could irreversibly modify an active site and cause inactivation of cytochrome P450 enzymes or through metabolic activation by

intestinal bacteria which can form reactive metabolites (Galati & O'Brien, 2004). Thus, it may result in potentially toxic herb-drug interactions or decline in efficacy (Wang *et al.,* 2021). Additionally, the inhibition of CYPs may also impair the metabolism and elimination of nontherapeutic agents causing hepatotoxicity or overall toxic accumulation in the body (Galati & O'Brien, 2004). However, few studies have investigated the effects of baicalein on pharmacokinetics of certain drugs. In a study conducted by Li *et al.* (2011), it was found that co-administration of baicalein with tamoxifen significantly increased the oral bioavailability and area under the curve (AUC) for plasma concentration-time of tamoxifen in rats compared to the control group which was given only tamoxifen. This result was attributed to baicalein's ability to inhibit activity of CYP3A4 in metabolising tamoxifen. Similarly, baicalein was found to increase the AUC and half-life ($t_{1/2}$) of simvastatin through possible inhibition of CYP3A4 when administered concomitantly in a study conducted by Meng *et al.* (2021) on Sprague-Dawley rats. These two studies have demonstrated that baicalein may have a positive effect on drugs taken concomitantly and its ability to inhibit CYP450 enzymes, however further investigations are required to understand the consequences or end-results of these effects on the whole body.

A previous study conducted by Luitel *et al.*, (2010), found that baicalein extracted from the bark of *O. indicum* were active against brine-shrimp with LC_{50} (median lethal concentration) of 10.0 µg/ml. On the flip side, *in silico* analysis performed by Pondugula *et al.* (2021) using QikProp filter (Schrödinger, LLC, New York, NY) found that baicalein extracted from *O. indicum* showed favourable absorption, distribution, metabolism and elimination (ADME) profile for safe and effective use as a drug in humans exhibiting solvent accessible surface area (SASA) of 483.69 and an acceptable human oral absorption percentage of 76.82% in which

percentages of more than 80% is considered high. Although these toxicity evaluations, including *in vitro* studies contribute valuable data regarding the safety of baicalein, there is a lack of information on the systemic toxic effects of this active compound in a whole organism which can only be achieved through *in vivo* studies. Therefore, it is vital to determine the potential adverse effects of baicalein active compound extracted from *O. indicum* and limits of exposure level *in vivo* using an animal model in response to a single or short-term exposure to the extract, thus ensuring its safety for human consumption.

2.9.2 Types of toxicity testing

Generally, toxicity testing is devised to generate data regarding the adverse effects of a substance on human or animal health, and the environment in order to identify a potential hazard and conduct appropriate dose-response assessment. This data is extremely useful for regulation development by the government and other agencies for the efficient management of chemicals, public safety, and environment protection. Toxicity testing on animal models can be divided into 3 forms, namely acute, sub-acute or sub-chronic and chronic. Acute systemic toxicity evaluates the adverse effects that occur following exposure of organisms to a single dose of a test substance within 24 hours to 2 weeks by oral, dermal or inhalation route (Saganuwan, 2016). It is an assessment of the adverse effects which might arise due to either accidental or deliberate short-term exposure to the substance (Erhirhie *et al.*, 2018). Traditionally, LD_{50} (median lethal dose) is used to estimate the dose of a substance that causes 50% death in a specific animal species tested. The LD_{50} test was introduced by J. W. Trevan in 1927 (Erhirhie *et al.*, 2018). Conventional methods such as the Karber method, Reed - Muench method, or Miller - Tainter method, for determining

LD_{50} involved the use of larger number of animals ranging between 30 – 50 (Erhirhie *et al.*, 2018; Zhang *et al.*, 2022). In later decades, alternative methods using fewer animals were implemented inspired by the recommendation by William Russell and Rex Burch who introduced the 3Rs (reduce, refine, and replace) principle. Additionally, subacute toxicity studies are performed to evaluate the relatively long-term acute toxicity effect of a test substance for a period of 4 weeks to 3 months. Subacute chronicity indicates the cumulative effects of the test substance in the body's tissues and organs, although it may exhibit low acute toxicity (Saganuwan, 2016). It is usually conducted in the same manner as the acute toxicity test; however multiple doses or a single dose is administered every day. On the other hand, chronic toxicity studies evaluate the long-term effect of a test substance and are carried out between 12 months. Chronicity of a test substance usually indicates the overall cumulative effects of the substance (Saganuwan, 2016). $LD_{chronic}$ is used to calculate the chronic lethal dose (Chinedu *et al.*, 2015).

2.9.3 Animal toxicity testing regulation

As *in vivo* toxicity testing is always conducted on animal models such as rodents, rabbits, primates, or zebrafish prior to clinical trials (Arome & Chinedu, 2014), there is a need for stringent regulation in the process to ensure that animal welfare and the environment are protected. In Malaysia, the main legislations governing the care and use of laboratory animals is the Animal Welfare Act 2015 and the Animal (Amendment) Act 2013. The NPRA (2021) which is the main regulatory agency for drugs in Malaysia specifically requires that all potential drug applicants undergo non-clinical studies conducted in OECD Good Laboratory Practice (GLP) compliant facilities. Additionally, MyCode developed by the Laboratory Animal

Science Association of Malaysia (LASAM) is a Malaysian code of practice for the care and use of animals for scientific purposes which outlines the responsibilities of Institutional Animal Care and Use Committees (IACUCs), and investigators involved covering aspects such as veterinary care, husbandry procedures, and construction guidelines. However, MyCode does not propose specific guidelines for each type of toxicity testing done on specific laboratory animals. In the global context, the United States Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) have developed a set of guidelines for the testing of toxic substances in rodents which are accepted in the country such as The Office of Prevention, Pesticides and Toxic Substances (OPPTS) guidelines. Nevertheless, these guidelines were sourced from the OECD guidelines. The OECD guidelines determined by the Organization for Economic Co-operation and Development with international consensus is a set of specifications and methods for regulatory chemical safety testing on animals. The guidelines are based on a global collective input from scientists in the government, industry and academic laboratories which are regularly updated to maintain relevancy. Additionally, the guidelines for *in vivo* studies such as the OECD Test Guidelines 420, 423, 407, and 452 are developed to specifically reduce the number of animals used in toxicity testing which is an effort to adhere to Russell and Burch's 3Rs principle. In this study, OECD Test Guideline 420 (Acute Oral Toxicity – Fixed Dose Procedure) was used. By adhering to internationally agreed guidelines for chemical testing, the methods used in this study will be standardised, and it will create wider acceptance of the data or results collected.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 General chemicals and reagents

List of chemicals and reagents used in this study are tabulated in Table 3.1.

3.1.2 General consumables

List of consumables used in this study are tabulated in Table 3.2.

3.1.3 General instruments

List of laboratory instruments used in this study are tabulated in Table 3.3.

3.2 Preparation of chemical reagents, solutions, media, and buffers

3.2.1 Preparation of phosphate buffer saline (PBS) solution

1 L of PBS (containing NaCl: 137 mM; KCl: 2.7 mM; Na2HPO4: 10 mM; KH2PO4: 1.8 mM) was prepared by adding 100 mL of 10X PBS to 900 mL of water.

Table 3.1 List of general chemicals and reagents

Chemicals (abbreviation)	**Brand/Manufacturer**
0.2% Ammonia (NH_3)	Bendosen, Progressive Scientific Sdn. Bhd. (Selangor, Malaysia)
10% Dimethyl sulfoxide (DMSO)	Bio Basic Canada Inc. (Ontaria, Canada)
10% Neutral buffered formalin (NBF)	ThermoFisher Scientific (Massachusetts, USA)
37% Hydrochloric acid	Qrec (Asia) Sdn. Bhd. (Selangor, Malaysia)
Chloroform ($CHCl_3$)	Qrec (Asia) Sdn. Bhd. (Selangor, Malaysia)
DPX Mountant	R&M Chemicals, Kumpulan Saintifik KSFE (Selangor, Malaysia)
Eosin	Merck KGaA (Darmstadt, Germany)
Ethanol (EtOH)	HmbG Chemicals, Progressive Scientific Sdn. Bhd. (Selangor, Malaysia)
Hematoxylin	Merck KGaA (Darmstadt, Germany)
Methanol (MeOH)	HmbG Chemicals, Progressive Scientific Sdn. Bhd. (Selangor, Malaysia)
Paraffin wax	ThermoFisher Scientific (Massachusetts, USA)
Petroleum ether (PET)	Bendosen, Progressive Scientific Sdn. Bhd. (Selangor, Malaysia)
Phosphate buffer saline (PBS)	Merck KGaA (Darmstadt, Germany)
Resin	Merck KGaA (Darmstadt, Germany)
Xylene (C_8H_{10})	Merck KGaA (Darmstadt, Germany)

Table 3.2 List of general consumables

Consumables (abbreviation)	**Brand/Manufacturer**
101 Series tissue embedding cassettes	Sakura Finetek (Nagano, Japan)
Ciringe syringes (1, 3, and 5 mL)	Muzamal Industries Sdn. Bhd. (Selangor, Malaysia)
Cover slips	ThermoFisher Scientific (Massachusetts, USA)
Diamond aluminum foil	Reynolds Consumer Products (Illinois, USA)
Ethylenediaminetetraacetic acid (EDTA) tubes	Vacutest Kima (Arzergrande, Italy)
High performance cellulose extraction thimble	Whatman (Maidstone, United Kingdom)
High-profile disposable microtome blades 818	Leica Biosystems (Nussloch, Germany)
Hypodermic needles	Terumo Corporation (Tokyo, Japan)
Sail brand frosted microscope slides	Sail brand (Hangzhou, China)
Serum separator tubes (SST)	Vacutest Kima (Arzergrande, Italy)
TaniSegar cotton balls	Jaga Marketing Sdn. Bhd. (Johor, Malaysia)
TLC plate, silica gel coated with fluorescent indicator F254	Merck KGaA (Darmstadt, Germany)
Universal specimen container	Muzamal Industries Sdn. Bhd. (Selangor, Malaysia)
Watson’s facial cotton	Watson’s Personal Care Stores Sdn. Bhd. (Kuala Lumpur, Malaysia)

Table 3.3 List of general instruments

Instruments (model)	**Brand/Manufacturer**
Automated tissue processor (TP 1020)	Leica Biosystems (Nussloch, Germany)
Automated tissue stainer (PRIMSA-E25)	Sakura Finetek (Nagano, Japan)
Electrical powder grinder DE-250g	Golden Bull
Heating mantle	Favorit, PLT Scientific Sdn. Bhd. (Selangor, Malaysia)
Incubator	BINDER GmbH (Tuttlingen, Germany)
Rotary evaporator	Heidolph Instruments (Schwabach, Germany)
Rotary microtome (RM 2135)	Leica Biosystems (Nussloch, Germany)
Soxhlet extractor (50/42)	Favorit, PLT Scientific Sdn. Bhd. (Selangor, Malaysia)
Water bath (HI 1210)	Leica Biosystems (Nussloch, Germany)
Wax embedder (EG 1160)	Leica Biosystems (Nussloch, Germany)

3.3 Methods

3.3.1 Collection and identification of plant material

O. indicum leaves were collected from a village known as Kampung Pasir Parit, Pasir Mas, Kelantan (GPS coordinates: latitude 5.905471, longitude 102.1884469). The authentication of the plant material was performed by Dr. Rahmad Zakaria, and voucher specimen (USM Herbarium 11751) (Appendix A) was deposited by the officer-in-charge, Mr. V. Shunmugam in the herbarium of Universiti Sains Malaysia (The step was assisted by Dr. Kang In Nee).

3.3.2 Crude extraction of plant material

The leaves were cleaned with distilled water and oven-dried before it was crushed into powder form (109 g) using an electrical powder grinder. A total of 25 g of the *O. indicum* leaf powder was weighed and transferred into the Soxhlet thimble. A plug of facial cotton was placed on top of the thimble covering the powder to prevent spillage during the extraction process. The *O. indicum* leaf powder was subject to a binary solvent system extraction process. The baicalein extraction process was performed in the same manner as described by Kang *et al.*, (2019). In brief, 300 ML petroleum ether was added into a distillation flask and placed on top of the heating mantle. The thimble containing the powder was loaded into the Soxhlet extractor and placed on top of the distillation flask. The heating mantle was turned on and remained at the boiling point of petroleum ether of 42-62°C, while the whole procedure was monitored to prevent rolling boil until the solvent was observed to be turned into dark green in colour. The solvent was discarded and replaced with new 300 mL of petroleum ether. The gentle boil was maintained until the colour of the solvent was observed to be clear in colour. The solvent was discarded again, and the thimble was

air dried to remove petroleum ether residues. The organic non-polar petroleum ether (polarity index: 0.1) solvent was used to remove undesired lipid components from the *O. indicum* leaves to eliminate low polarity fatty constituents which can produce a purer natural product. Then the leaves were subjected to a second phase extraction using 500 mL MeOH (polarity index: 5.1) and repeated with the same extraction procedure at MeOH boiling point of 62-65°C. The solvents were collected into a same glass bottle after each extraction process and later dried using a rotary evaporator.

3.3.3 Fractionation of baicalein active compound

In the first step, Diaion HP-20 resin (Mitsubishi Chemical Corporation, Tokyo, Japan) was prepared by following the wetting procedure as outlined by the manufacturer. The dry resin was filled into a 500 mL beaker and sufficient MeOH was added into the beaker to cover the resin bed by 5 cm. The resin was stirred gently for a minute to ensure complete mixing. The mixture was allowed to stand for 15 minutes. The MeOH was later decanted to remove most of the solvent and was replaced with distilled water. The mixture was stirred and allowed to stand for 10 minutes. The column was prepared by pouring the mixture of distilled water and resin slowly. As the column was filled, excess water was drained through the bottom of the column. However, the liquid level was not allowed to fall below 2.5 cm of the top of the resin bed. The column was then rinsed with 400 mL mobile phase (10% MeOH) in order to remove any resin residue remaining on surface to equilibrate the column. Then, 5g of *O. indicum* crude extract powder obtained from the extraction procedure was dissolved in 5 mL of MeOH to form slurry suspension before being loaded onto the top of the resin column without disturbing the surface. The column was eluted with 300 mL of increasing concentrations of MeOH in a stepwise manner from 10%, 30%, 50%, 70%,

and 100%. The final fraction (F5) using 100% MeOH was collected and dried using rotary evaporator.

3.3.4 Thin-Layer Chromatography (TLC) analysis to detect baicalein active compound

TLC analysis on F5 was performed using aluminium sheets coated with silica gel 60 F254 (Merck KGaA, Darmstadt, Germany) to detect the baicalein active compound. *O. indicum* F5 with a final concentration of 1 mg/mL was prepared and spotted on the TLC plate. Synthetic baicalein compound of the same concentration was also spotted on the same TLC plate as standard marker in this assay. The spotted TLC plate was dried and placed in a developing chamber with a mobile phase consisting of chloroform and MeOH with a ratio of 20:1 (v/v). Once the solvent front almost reached the edge, the TLC plate was removed, subsequently dried, and immediately examined under short wavelength UV (254 nm) and long wavelength UV (365 nm) light.

3.3.5 Experimental animal

The experiment was performed using healthy male and female Sprague-Dawley rats of 7-8 weeks old with body weight ranges between 200-300 g. The animals were obtained from Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Health Campus and maintained in the inhouse facilities. The rats were fed with standard laboratory diets and given water *ad libitum.* They were kept under laboratory conditions of 22°C temperature with artificial lighting of 12 hours light and 12 hours dark cycle. All animals were acclimatised to laboratory conditions for 7 days in their respective cages and were uniquely tagged before

commencement of toxicity studies. The experimental procedures involving care, handling and treatment of animals were reviewed and approved by University Sains Malaysia (USM) Institutional Animal Care and Use Committee (IACUC) [No. of Animal Ethics Approval: USM/IACUC/2019/(120)(1019)] (Appendix B).

3.3.6 Toxicity studies

3.3.6(a) Sighting study

The toxicity testing for this study was conducted on Sprague-Dawley rats adhering to the OECD Test Guideline 420 (Acute Oral Toxicity – Fixed Dose Procedure). The sighting study was conducted on a group of 5 male rats and 5 female rats in total. One pair of male and female rats (n=1 for each sex) were weighed and dosed with 5 mg/kg F5 by oral gavage. An observation period of 24 hours was allowed after administration of F5. In the event where the lowest fixed dose of 5 mg/kg was seen to cause death in one of the rats, F5 will be assigned under GHS category 1 and the study will be terminated according to the OECD guidelines (2002). On the other hand, if there was no mortality observed, subsequent dose of 50 mg/kg of F5 was administered to another pair of male and female rats (n=1 for each sex) and observed for another 24 hours. If still no mortality was observed, the sighting study was continued for 300 mg/kg and 2000 mg/kg of F5 based on OECD guidelines (2002). One pair of male and female rats were given 10% DMSO and saline as a control. All animals were observed for 14 days. The highest dose which did not cause mortality after 14 days was selected as the sub-lethal dose for subsequent acute toxicity test.

The dilution of F5 in vehicle in appropriate dosage for oral administration was done according to OECD guidelines (2000) which states that dosage of drug (mg)

should not exceed 10 ml/kg body weight of experimental animals for non-aqueous solvent via oral administration.

Dosage calculation as follows:

$$\text{Dosage in mg} = \frac{\text{Body weight of animal (g)}}{1000\ \text{g}} \times \text{dose (mg)}$$

Dissolution of dose in a suitable vehicle for oral administration was calculated as follows:

$$\text{Amount of solution required for dilution} = \frac{\text{Body weight of animal (g)}}{100\ \text{mL}}$$

3.3.6(b) Acute oral toxicity study

In the main oral acute toxicity study, a total of 5 males and 5 females (8 weeks of age) were used to test the sub-lethal dose of F5 baicalein extract selected in the sighting study. The animals were weighed and F5 was administered to them with the respective dosage selected in the sighting study and of volume not more than 10 ml/kg b.w. using oral gavage technique. Another 5 males and 5 females were weighed and fed 10% DMSO and saline to act as a control. All the rats were observed for 14 days daily at the same time, and all observations were systematically recorded. Obvious signs of toxic reactions were observed such as changes in skin and fur, eye colour, mucous membranes with special attention to tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. At the end of the 14 days observation period, blood parameters were collected using cardiac puncture method after the animals were anaesthetized using cocktail dosage of ketamine (100 mg/kg) and xylazine (5 mg/kg) b.w. The blood samples were separated into ethylene diamine tetra acetic acid (EDTA)

tubes for haematological assay and serum separating tubes (SST) for biochemical assay. Subsequently the animals were sacrificed by cervical dislocation.

3.3.7 Behavioural toxicity evaluation

The behavioural toxicity evaluation was done according to OECD guidelines test No. 420 (Acute Oral Toxicity – Fixed Dose Procedure). The animals were observed individually after dosing at least once during first 30 minutes, periodically during the first 24 hours, with special attention given during first 4 hours. The animals were observed daily thereafter, for a total of 14 days. The duration of observation was determined by the presence of toxic reactions, time of onset and length of recovery period. All observations of each animal were systematically recorded and maintained individually. Observations of changes in skin, coat texture, eyes and mucous membranes were made. Observations of changes in behaviour to indicate signs of toxicity or severe pain and distress which are outlined in the OECD Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000) were also observed such as convulsions, salivation, diarrhoea, coma, abnormal vocalisation, and self-mutilation to name a few. Changes in body weight, food and water consumption were also monitored daily.

3.3.8 Assessment Parameters

3.3.8(a) Blood haematological assay

A standard automatic blood cell analyser (Pathlab, Malaysia) was used to test for blood haematological parameters. The whole blood samples were analysed for red

blood cell count (RBC), white blood cell count (WBC), WBC differentials (neutrophil, eosinophil, basophil, lymphocyte, and monocyte counts), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelet count. The values of blood haematological parameters in F5 treated groups were compared to the standard reference values in healthy Sprague-Dawley rats established by He *et al.* (2017) complying with internationally accepted American Society for Veterinary Clinical Pathology [ASVCP] (2012) and The Clinical and Laboratory Standards Institute [CLSI] (2010) guidelines.

3.3.8(b) Serum biochemical assay

The whole blood was collected and centrifuged at 3500 rpm for 10 minutes for serum separation and the serum collected was subjected to biochemical analysis using standard automatic biochemical analyser (Pathlab, Malaysia). The biochemical parameters analysed included serum total protein (TP), albumin (ALB), total bilirubin (TBIL), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The values of serum biochemical indices in F5 treated groups were compared to the standard CLSI (2010) and ASVCP (2012) reference values in the same manner as described for the haematological assay.

3.3.9 Histopathological examination

After the animals in the acute toxicity study were sacrificed, the following organs were harvested through careful dissection: liver, kidney, reproductive organs (testis and ovary), lungs, heart, and brain. The organs were put into separate Petri

dishes that contained 10% normal saline. A comprehensive macroscopy observation was carried out on each of the organs to observe for any signs of abnormalities or lesions. The isolated organs were then dried with cotton wools and weighed on a sensitive balance. The relative weight of each organ (ROW) was standardised to 100 g body weight of each rat at the day of sacrifice using the following formula as below:

$$\text{ROW} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100$$

3.3.9(a) Tissue slicing

Cross sections and lateral sections of the organs were placed in embedding cassettes and fixed in 10% neutral buffered formalin (pH 7.4) for 72 hours. The tissues were subsequently dehydrated using graded concentrations of ethanol in increasing order using automated tissue processor (Leica TP 1020, Germany) as below:

80% ethanol for 1 hour

95% ethanol for 1 hour (twice)

100% ethanol for 1 hour (thrice)

The dehydrated tissues were cleared in three changes of xylene for 1 hour each cycle in the automated tissue processor. The wax impregnated tissues were then embedded in paraffin blocks using the same grade of wax by embedder (Leica EG 1160, Germany). The tissue blocks were cut into ribbon of 4 m thick using a rotary microtome (Leica RM 2135, Germany) and the ribbons were floated on warm water of 41°C, inside a water bath (Leica HI 1210, Germany) to allow for proper straightening and then placed onto a glass side. The sections were then dried in an oven at 60°C for 5 minutes and then were allowed to cool.

3.3.9(b) Haematoxylin and eosin (H&E) staining

H&E staining was conducted using an automated tissue stainer (Sakura PRISMA-E25, Japan) and the steps involved are listed as follows:

The sections were deparaffinised by immersing them in a horizontal staining jar containing xylene for a period of 2 minutes twice. The sections were then hydrated in a series of descending concentration of ethanol as follows:

Absolute ethanol twice for 2 minutes

95% ethanol for 2 minutes

80% ethanol for 2 minutes

70% ethanol for 2 minutes

After the hydration process, the sections were washed with tap water for 2 minutes and stained with haematoxylin for 20 minutes. The sections were then washed again with tap water for 2 minutes and subsequently dipped in acid alcohol (1% HCl in 70% alcohol) to remove excess stain. The sections were washed with tap water for 2 minutes and then dipped in weak ammonia of 0.2%.

The sections were rinsed with running tap water for 2 minutes to slow the alkalisation process and was counter stained in 1% aqueous eosin for 7 minutes. The excess stain was washed again with tap water for 1 minute and was allowed to dry. The complete dehydration and clearing process of stained sections were conducted using ascending concentration of alcohol and xylene as described below:

70% ethanol for 15 seconds

80% ethanol for 15 seconds

90% ethanol for 15 seconds

Absolute ethanol for 15 seconds

Xylene for 15 seconds, thrice

Once cooled, the sections were mounted in distyrene, plasticizer and xylene (DPX) mounting medium which has the optical index of glass. The sections were wetted in xylene and inverted onto the mount and covered with coverslip. The architecture of the tissues was observed using low power objective lens of the Olympus microscope. Photomicrographs were taken for every specimen slide using Dino-Eye microscope eyepiece camera with DinoCapture 2.0 imaging software Version 1.5.45 (AnMo Electronics Corporation, New Taipei City, Taiwan).

3.3.10 Histopathological scoring

A binomial scoring chart was used for histopathological scoring for all selected organs. The binomial scoring chart that was used in this study was developed by a consultant pathologist Dr. Anani Aila Mat Zin (School of Medical Sciences, Universiti Sains Malaysia, Health Campus) based on validated histological scoring literature by previous researchers. The aspects which were examined were such as necrosis, changes in nuclear and cytoplasmic features, inflammation, fibrosis, steatosis, and ballooning for the liver (Kleiner *et al.*, 2005). Glomeruli and tubular abnormalities were examined along with oedema, necrosis, and inflammation in the kidney tissues (Ibrahim *et al.*, 2018). As for the lungs, epithelial thickening and degeneration were examined including oedema, haemorrhage, and inflammation (Chu *et al.*, 2019; Kleiner *et al.*, 2005). Besides, myocardial necrosis, fibrosis and infiltration of

inflammatory cells were examined in the heart (Klopfleisch, 2013). Neuronal degradation characterized by triangulated pyknotic nuclei, inflammation, angiogenesis, ischemic neuron and infarction areas were examined in the brain (Janke *et al.*, 2019; Mohamed *et al.*, 2016; Silva *et al.*, 2015). Histopathological scoring for the ovary included stages of follicle development, presence of corpus luteum, oedema, haemorrhage, and inflammation (Hu *et al.*, 2020; Sayar *et al.*, 2016). Finally, the male reproductive organs were examined for testicular tissue architecture distortion, necrosis of tubular lining epithelium, vacuolisation, interstitial tissue oedema, vascular dilation and stages of spermatogenic cell (Adamkovicova *et al.*, 2014; Fouad *et al.*, 2020).

3.4 Statistical Analysis

All results were expressed as mean ± standard error of mean (SEM). The differences in all parameters for treated and control rats were analysed using a One-way Analysis of Variance (ANOVA), followed by post-hoc Duncan's Multiple Range Test to measure specific differences between pairs of means using IBM SPSS® Version 26.0 for Windows. Data were considered as significant where $p < 0.05$.

CHAPTER 4

RESULTS

4.1 Extraction and fractionation of baicalein active compound

The binary extraction process using 25 g of finely crushed *O. indicum* leaf material, yielded 3.94 g (16%) of crude extract powder. Besides that, fractionation of the crude extract using five different concentrations of MeOH starting from 10% (400 mL), 30% (300 mL), 50% (300 mL), 70% (300 mL), and 100% (300 mL) yielded five MeOH fractions (Figure 4.1). Thin-layer chromatography (TLC) analysis was performed on the last fraction (F5) to detect presence of baicalein.

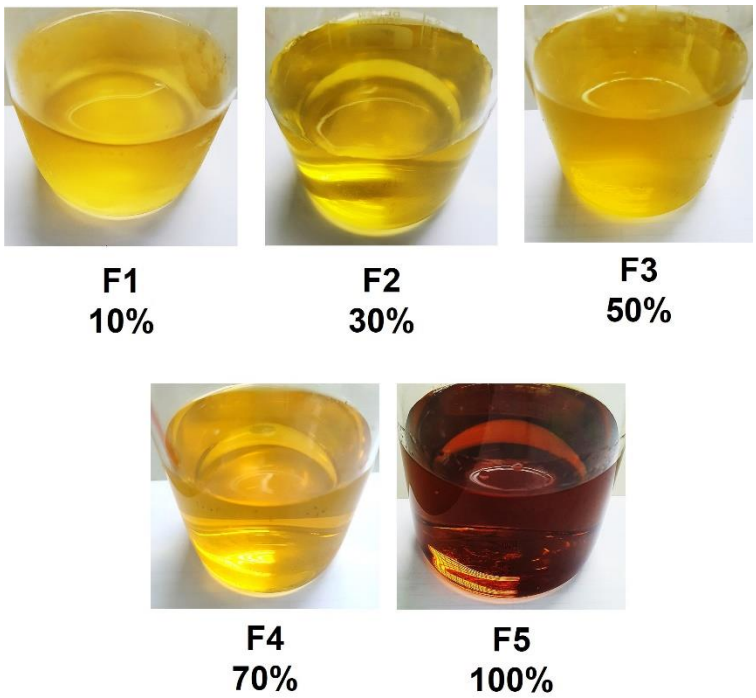


Figure 4.1 *O. indicum* MeOH extract fractions (F1-F5)

4.2 TLC analysis

The TLC analysis detected the presence of baicalein in F5 under short-waved (254 nm) (Figure 4.2A) and long-waved UV light (365 nm) (Figure 4.2B). It was

evidently shown in the TLC analysis result that baicalein active compound was successfully extracted and fractioned from the plant material which is indicated by the red boxes in Figure 4.2A and Figure 4.2B.

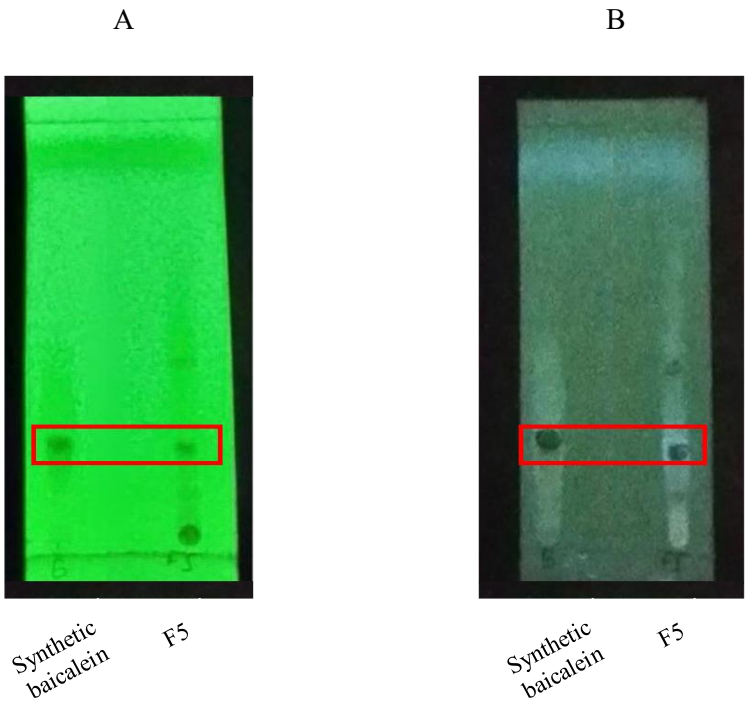


Figure 4.2 TLC analysis plate using (A) short UV wavelength (254 nm) and (B) long UV wavelength (365 nm). Each sample was submitted at an equal concentration of 1 mg/ml

4.3 Toxicity study

4.3.1 Sighting study

The sighting study conducted for a period of 14 days, revealed that F5 did not cause any mortality among all the treated groups (5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg) throughout the experimental period (Figure 4.3). The male treated group showed weekly increment of body weight changes between the lowest to highest range of 1.9% and 13.8%. The female treated group showed a range between a minimum 2.6% and maximum 8.7% of weekly body weight changes (Table 4.1). However, all

the weekly body weight changes recorded for both the treated and control groups were within normal increment rate of less than 20% deviation from their initial body weights. None of the groups exhibited a reduction of body weight over the 14 days (Table 4.1). On top of that, none of the tested rats showed signs of toxicity in their external appearance and behaviour upon consistent daily observation. Based on these results, the dose of 2000 mg/kg was selected as the test dose for the main acute toxicity study.

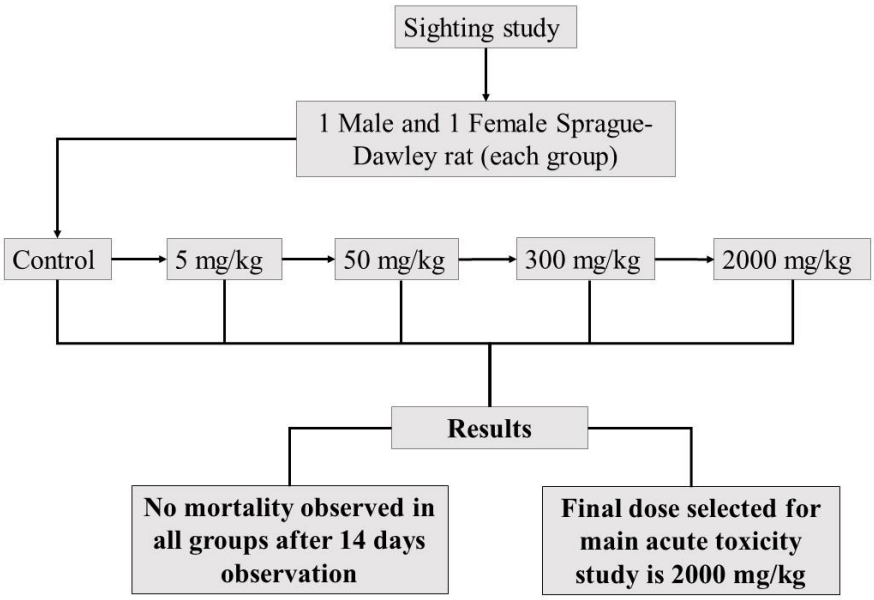


Figure 4.3 Schematic diagram for dose selection of F5 in sighting study for acute toxicity study with mortality results (n=1, 1 male and 1 female rat per dose)

Table 4.1 Body weight and its respective changes of control and treated rats in sighting test

Groups (n=1)	Body weight (g) at Day 0	Body weight (g) at Day 7	Body weight (g) at Day 14	Body weight changes between Day 0 and Day 7*	Body weight changes between Day 7 and Day 14*
Male					
Control	301	309	335	+8g (+2.7%)	+26g (+8.4%)
5 mg/kg	312	318	345	+6g (+1.9%)	+27g (+8.5%)
50 mg/kg	295	303	345	+8g (+2.7%)	+42g (+13.8%)
300 mg/kg	316	344	360	+28g (+8.8%)	+16g (+4.7%)
2000 mg/kg	270	295	320	+25g (+9.2%)	+25g (+8.5%)
Female					
Control	191	200	215	+9g (+4.7%)	+15g (+7.5%)
5 mg/kg	184	200	210	+16g (+8.7%)	+10g (+5.0%)
50 mg/kg	225	235	240	+10g (+4.4%)	+15g (+6.4%)
300 mg/kg	219	229	235	+10g (+4.5%)	+6g (+2.6%)
2000 mg/kg	203	212	230	+9g (+4.4%)	+18g (+8.5%)

*All weekly body weight changes recorded were within normal increment rate (< 20% deviation from initial weight).

4.3.2 Acute toxicity study

No mortality was observed in the acute toxicity study male (n = 5) and female (n = 5) rats after administration of 2000 mg/kg dose. In both the male and female groups, there were no statistically significant differences in weekly body weight between the period of first dose administration and day 7, subsequently between the period of day 7 and day 14. Also, there was no reduction of body weight in the rats recorded across the groups. In addition, all the body weight changes recorded were less than 20% from the initial weight before first dose (Table 4.2), which was considered within normal range indicating absence of clinical toxicity signs as detailed in the OECD Guidance Document (2000).

Table 4.2 Body weight and its respective changes of control and treated rats in acute oral toxicity test

Groups (n=5)	Body weight (g) at Day 0	Body weight (g) at Day 7	Body weight (g) at Day 14	Body weight changes between Day 0 and Day 7*	Body weight changes between Day 7 and Day 14*
Male					
Control	286.80 ± 8.07	303.60 ± 5.81	310.60 ± 6.35	4 to 31 g (+1.35 to +12.15%)	2 to 11 g (+0.70 to +3.67%)
Treated	313.60 ± 8.63	335.60 ± 9.09	354.00 ± 10.11	16 to 28 g (+5.42 to +9.55%)	13 to 22 g (+4.04 to +6.58%)
Female					
Control	218.80 ± 2.65	213.40 ± 4.30	233.20 ± 1.39	-14 to 5 g (-6.51 to +2.28%)	8 to 30 g (+3.57 to +14.56%)
Treated	200.80 ± 3.13	212.40 ± 3.80	224.00 ± 2.00	9 to 14 g (+4.68 to +6.67%)	6 to 18 g (+2.78 to +8.95%)

* All the weekly body weight changes recorded were within normal increment or reduction range (< 20% deviation from initial weight). Each value represents the mean ± SEM (n = 5).

4.4 Behavioural toxicity evaluation

The behaviours of each animal were observed throughout the 14 days acute oral toxicity study period at approximately the same time daily. It was observed that even with single oral administration of F5 at 2000 mg/kg, the rats showed no clinical signs of toxicity and changes in behaviours related to the treatment (Table 4.3). The clinical toxicity signs which were outlined in the OECD Guidance Document (2000) such as changes in skin and fur, occurrence of secretions and excretions, diarrhoea, tremors, and self-mutilation to name a few, were absent in all groups of treated (n=5) and control rats (n=5). Furthermore, all the rats exhibited normal grooming, burrowing, and play fighting behaviours upon daily observation.

Table 4.3 Clinical toxicity signs and animal behaviour in acute oral toxicity study (n=5 for both sexes in control and treated group)

Clinical Toxicity Signs	0.5h	1h	2h	3h	4h	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
Mortality	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Changes in skin, fur, eyes	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Changes in mucous membranes	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Occurrence of secretions and excretions	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Diarrhoea	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Tremors/convulsions	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Bizarre behaviour (Self-mutilation, walking backwards)	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Salivation	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Behaviour																		
Grooming	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Coprophagia	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Climbing	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Bruxing	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Investigation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Burrowing	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Play fighting	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Vocalization	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

A: Absence; N: normal

4.5 Assessment Parameters

4.5.1 Haematological parameters

In the male treated group, it was recorded that the values of MCHC, platelet, leukocytes and lymphocytes showed statistically significant difference compared with the control as determined by one-way ANOVA. On the other hand, the RBC, Hb, PCV, MCHC, leukocytes, and lymphocytes values in the female treated group showed statistically significant differences compared with the control group of the same sex as determined by one-way ANOVA. However, all the values were within normal range according to CLSI (2010) and ASVCP (2012) guidelines (Table 4.4).

Table 4.4 Haematological indices of control and treated rats in acute oral toxicity study

Parameters (n=5)	*Male*			*Female*		
	Control group	**Treated group**	***Normal range values***	**Control group**	**Treated group**	***Normal range values***
RBC (10^{12}/L)	7.62 ± 0.29	7.72 ± 0.09	*6.39 – 8.01*	7.04 ± 0.11	7.90 ± 0.09 *	*6.39 – 8.01*
PCV (%)	41.60 ± 1.56	42.20 ± 0.66	*18.00 – 48.00*	38.80 ± 0.66	44.00 ± 0.54 *	*10.00 – 47.00*
Hb (g/dL)	14.64 ± 0.52	14.36 ± 0.22	*10.40 – 16.50*	13.76 ± 0.26	14.88 ± 0.23 *	*8.60 – 15.38*
MCH (pg)	19.00 ± 0.00	18.40 ± 0.24	*18.37 – 36.98*	19.20 ± 0.20	18.60 ± 0.24	*13.07 – 41.57*
MCHC (g/dL)	35.00 ± 0.00	34.00 ± 0.00 *	*25.41 – 80.55*	35.40 ± 0.24	34.20 ± 0.20 *	*21.16 – 95.00*
MCV (fL)	54.40 ± 0.24	54.40 ± 0.24	*29.41 – 123.07*	54.60 ± 0.24	54.40 ± 0.60	*15.15 – 119.44*
Platelet (10^9/L)	504.00 ± 56.72	650.60 ± 22.61 *	*423.00 – 1580.00*	547.40 ± 46.61	632.00 ± 34.35	*423.00 – 1580.00*
WBC (10^9/L)	6.78 ± 0.64	8.30 ± 0.60	*3.00 – 9.22*	6.00 ± 0.76	6.74 ± 0.45	*2.58 – 7.34*
WBC differential:						
Eosinophil (%)	1.34 ± 0.34	0.30 ± 0.06 *	*0.54-3.39%*	0.36 ± 0.21	0.60 ± 0.30	*0.30-4.29%*
Basophil (%)	2.42 ± 0.44	1.46 ± 0.18	*0.00-5.00%*	2.78 ± 0.81	1.62 ± 0.32	*0.00-3.50%*
Lymphocytes (%)	75.60 ± 1.33	81.80 ± 0.89 *	*69.68-86.89%*	88.74 ± 2.64	82.52 ± 1.59 *	*71.77-89.94%*
Monocytes (%)	0.94 ± 0.12	0.86 ± 0.09	*0.80-3.80%*	1.90 ± 0.25	0.88 ± 0.13 *	*0.80-3.90%*
Neutrophil (%)	19.70 ± 0.80	15.38 ± 0.60 *	*6.14-22.95%*	6.22 ± 1.72	14.38 ± 1.20 *	*4.27-18.48%*

Each value represents the mean ± SEM (n = 5). * Indicates statistically significant difference compared to control group ($p < 0.05$). RBC: Red blood cells; Hb: Haemoglobin; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; WBC: White blood cells. Normal range values indicate normal haematological conditions in rats according to the CLSI (2010) and ASVCP (2012) guidelines.

4.5.2 Biochemical parameters

As seen in Table 4.5, the ALB and ALT levels in the female and male treated groups showed statistically significant differences among the treated and control groups ($p < 0.05$) as determined by one-way ANOVA. Specifically, the mean ALB levels were significantly higher in the female treated group as compared with the control group after administration of 2000 mg/kg F5 containing baicalein active compound. The mean ALT levels were significantly lower in the male treated group when compared with the control group. However, no statistically significant differences were recorded for TP, TBIL, ALP, and AST levels in both male and female groups of rats. Overall, the values of biochemical indices tested were within the normal range for both sexes according to CLSI and ASVCP guidelines.

Table 4.5 Biochemical indices of control and treated rats in acute oral toxicity study

	Male			***Female***		
Parameters (n=5)	**Control group**	**Treated group**	***Normal range values***	**Control group**	**Treated group**	***Normal range values***
TP (g/L)	52.60 ± 1.57	55.60 ± 0.68	*51.10 – 64.55*	55.40 ± 0.93	56.80 ± 1.39	*51.10 – 64.55*
ALB (g/L)	32.60 ± 0.81	34.60 ± 0.40	*26.88 – 38.55*	33.80 ± 0.73	37.00 ± 1.00 *	*26.88 – 38.55*
TBIL (µmol/L)	1.00 ± 0.00	1.00 ± 0.00	*1.00 – 2.00*	0.60 ± 0.40	1.00 ± 0.00	*1.00 – 2.00*
ALP (IU/L)	223.00 ± 7.99	237.00 ± 7.44	*160.80 – 838.30*	152.60 ± 10.23	164.60 ± 8.99	*150.00 – 724.20*
AST (IU/L)	185.80 ± 41.34	115.00 ± 4.09	*65.00 – 203.00*	127.60 ± 24.98	84.40 ± 3.71	*74.00 – 143.00*
ALT (IU/L)	72.40 ± 6.82	59.60 ± 1.63 *	*19.00 – 100.70*	59.60 ± 3.33	51.20 ± 1.28	*14.00 – 84.40*

Each value represents the mean ± SEM (n = 5). * Indicates statistically significant difference compared to control group ($p < 0.05$). TP: Total protein; ALB: Albumin; TBIL: Total bilirubin; ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase. Normal range values indicate normal biochemical conditions in rats according to the CLSI and ASVCP guidelines.

4.6 Relative organ weight (ROW)

After the 14-days acute toxicity study period, all animals were sacrificed, and their organs were harvested for measurement of relative organ weight (ROW). Statistically significant differences ($p < 0.05$) were observed in the ROW of brain, lung, and reproductive organs of both male (with relative difference of -0.14 g, -0.28 g, -0.13 g respectively) and female treated (with relative difference of 0.19 g, -0.31 g, -0.17 g respectively) group as determined by one-way ANOVA (Table 4.6). The brain, lung, and testes weighed less in the male treated group compared to the control after treatment with F5. However, a marked increase was observed in brain ROW of female treated group, while the lung and ovaries had similar pattern of reduction in ROW when compared to the control group. However, no statistically significant differences between the treated and control group were observed in the heart, liver, and kidney ROW of both sexes.

Table 4.6 Relative organ weight (ROW) of control and treated rats in acute oral toxicity study

Organ (g)	Control group	Treated group	ROW relative difference
Male			
Brain	0.63 ± 0.04	0.54 ± 0.03 *	-0.14
Heart	0.39 ± 0.02	0.38 ± 0.01	-0.03
Lung	0.69 ± 0.04	0.49 ± 0.01 *	-0.28
Liver	3.97 ± 0.26	3.83 ± 0.19	-0.04
Kidney	0.73 ± 0.03	0.66 ± 0.03	-0.09
Testis	1.16 ± 0.04	1.00 ± 0.04 *	-0.13
Female			
Brain	0.67 ± 0.02	0.80 ± 0.02 *	0.19
Heart	0.41 ± 0.01	0.44 ± 0.01	0.07
Lung	0.83 ± 0.07	0.57 ± 0.01 *	-0.31
Liver	4.05 ± 0.13	4.18 ± 0.12	0.03
Kidney	0.74 ± 0.02	0.70 ± 0.02	-0.05
Ovary	0.06 ± 0.01	0.05 ± 0.01 *	-0.17

Each value represents the mean ± SEM (n = 5). * Indicating a statistically significant difference compared to control group ($p < 0.05$).

4.7 Histopathological scoring

H&E staining revealed normal cellular architecture with absence of treatment related toxicity on all vital organs of the treated group which were given 2000 mg/kg of F5 containing baicalein active compound. Similar results were seen in the control group of both sexes (Figure 4.4 - 4.10). Histopathological examination of H&E-stained organs of each male and female Sprague-Dawley rat in the control and test group was performed using a binomial scoring chart (Table 4.7).

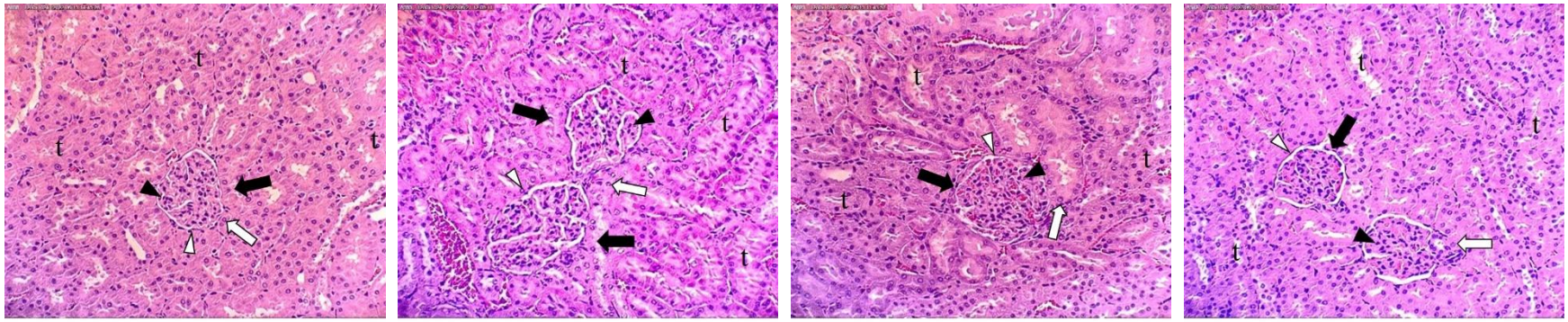
Organ	Male		Female	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Kidney				

Figure 4.4 Photomicrographs of treated and control group male and female kidneys (100x magnification)

Normal structure of the cortex and medulla was observed in the kidney of control and treated rats. Renal glomeruli in cortex showed normal structure (arrow) with presence of juxtaglomerular cells located near the glomerulus (white arrow). Normal presence of podocytes (black arrowhead) and parietal epithelium (white arrowhead) were noted. The proximal and distal convoluted tubules (t) appeared normal upon examination but were not easily distinguishable in H&E staining.

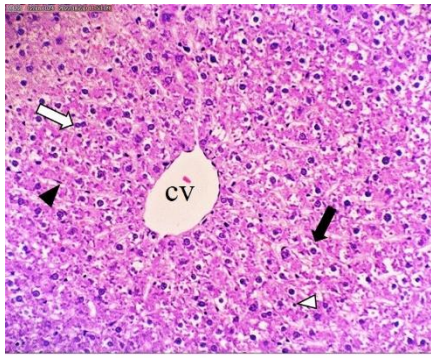
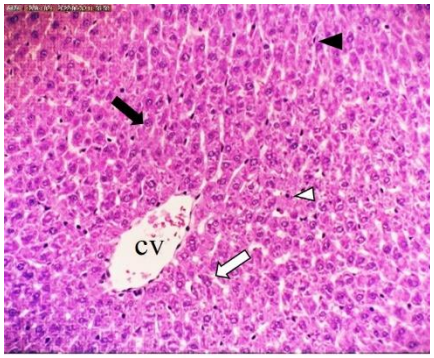
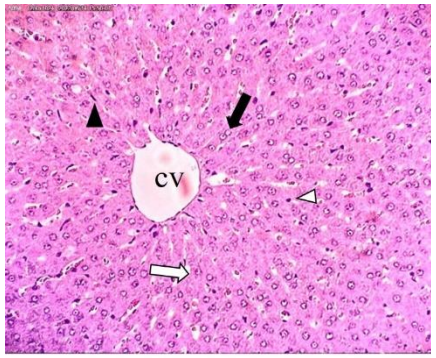
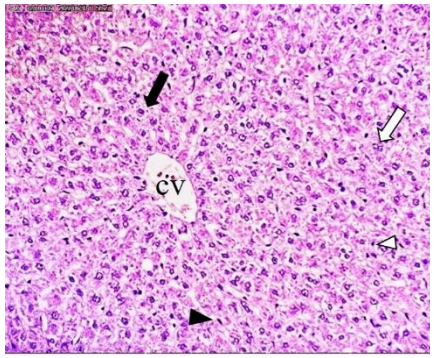
Organ	Male		Female	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Liver				

Figure 4.5 Photomicrographs of treated and control group male and female livers (100x magnification)

The liver of control and treated rats showed a normal structure. The nuclear and cytoplasmic features of hepatocytes (black arrow) appeared normal upon examination. The well-preserved sinusoids were identified as the lighter coloured spaces surrounding the hepatocytes. The walls of some of the sinusoids showed normal presence of liver macrophages known as Kupffer cells (black arrowhead). The presence of inflammatory cells especially leukocytes (white arrowhead) were noted. Besides, a number of binucleate hepatocytes were also observed (white arrow). Glycogen accumulations were observed in a number of control and treated rats (as pictured in male control and female treated photomicrographs).

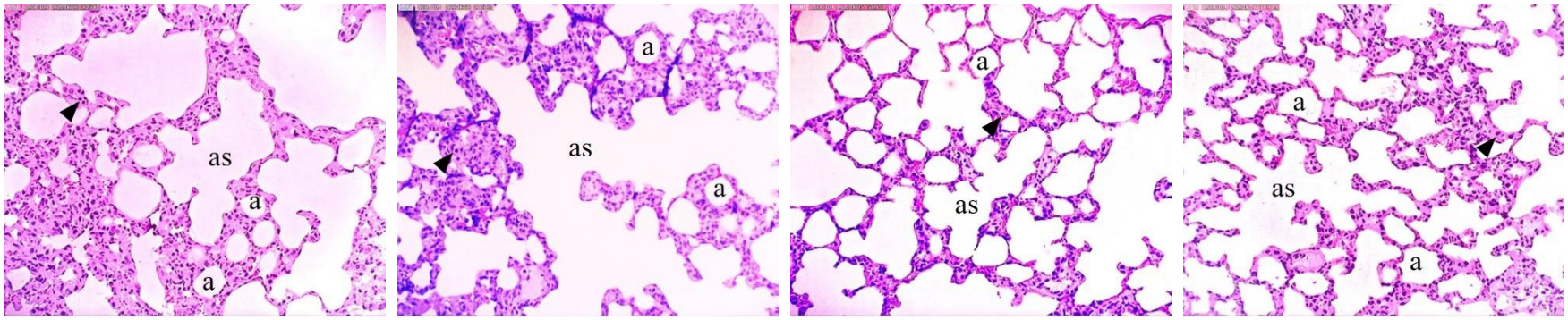
Organ	Male		Female	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Lung				

Figure 4.6 Photomicrographs of treated and control group male and female lungs (100x magnification)

Control and treated rats of both sexes showed normal lung architecture without alteration in the structure of alveolar sacs (as) and alveoli (a). The lungs of both male and female groups showed no significant inflammatory cell infiltration. The alveoli lined by thin squamous epithelium did not appear to be thickened or degenerated. Presence of type I and II pneumocytes located close to the alveoli were also noted, however they were not easily distinguished in H&E staining. Normal presence of neutrophils among the interalveolar septa were observed (black arrowhead).

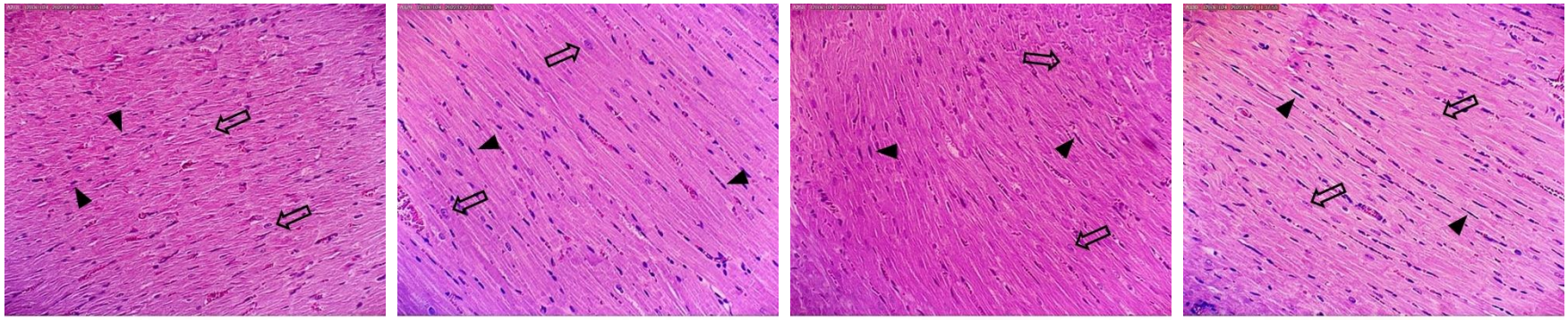
Organ	Male		Female	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Heart				

Figure 4.7 Photomicrographs of treated and control group male and female hearts (100x magnification)

The treated group showed close to normal cellular morphology as in the control group with absence of cardiomyocyte damage. The cardiomyocyte nuclei appeared normal (arrows), the connective tissue was normal, the nuclei and cardiac muscle fibres were well arranged in intercalating discs characteristic of cardiac muscle. Normal presence of fibroblast nuclei (black arrowheads) was also notable in the tissues examined.

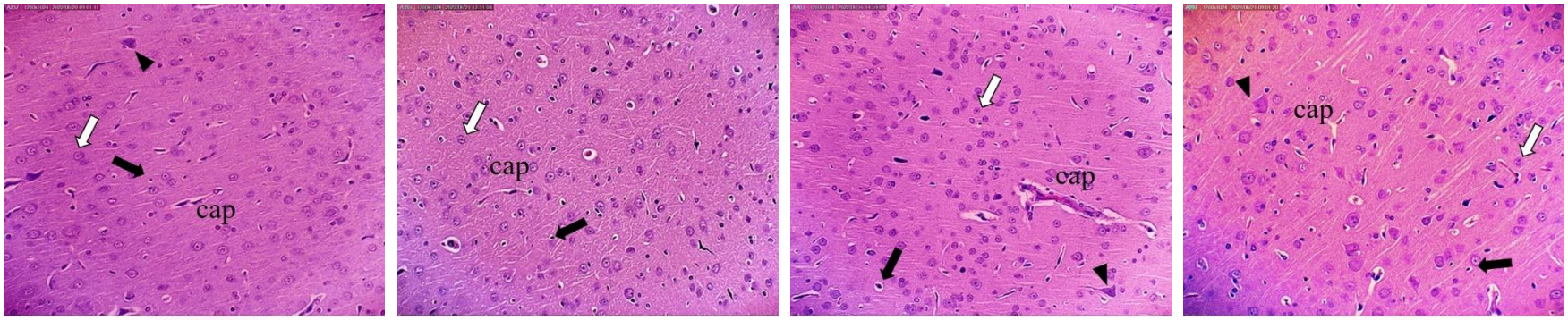
Organ	Male		Female	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Brain				

Figure 4.8 Photomicrographs of treated and control group male and female brains (100x magnification)

Examination of the centre brain cross-section of both male and female treated, and control groups showed normal histological structure of the cerebral cortex, thalamus, hypothalamus, amygdala, and hippocampus (pictured). There was a normal presence of neural capillaries (cap), intact pyramidal or stellate neurons (black arrowhead) with absence of degradation, oligodendrocytes (black arrow), and Nissl bodies (white arrow) which were surrounded by the brains' neuropil.

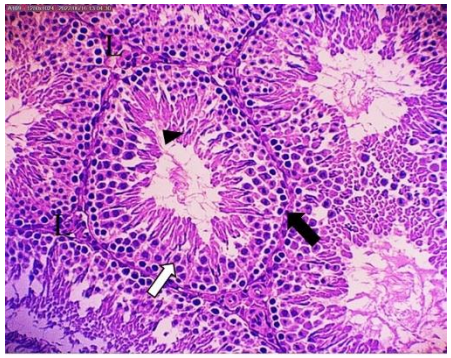
Organ	Male		Female	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Testis			N/A	N/A

Figure 4.9 Photomicrographs of male treated and control group testes (100x magnification)

Normal histology of seminiferous tubules with normal shape and arrangement of their cellular components were observed in both male control and treated groups. The seminiferous tubules were surrounded by interstitial cells or Leydig cells (L). Spermatogonia (black arrow) rest on intact basement membranes. Large primary spermatocytes (white arrow) with characteristic large-rounded nuclei and normal spermatozoa (black arrowhead) were also observed during examination.

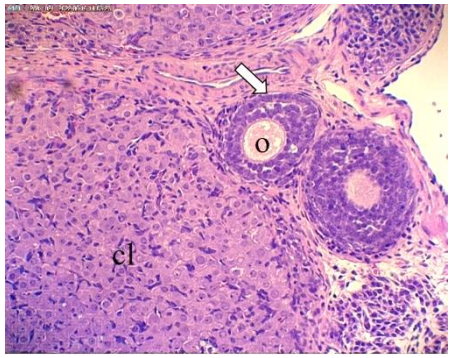
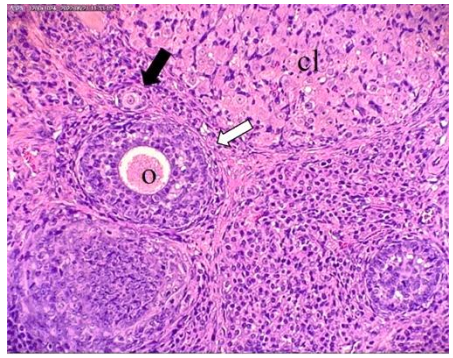
Organ	Male		Female	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Ovary	N/A	N/A		

Figure 4.10 Photomicrographs of female treated and control group ovaries (100x magnification)

Histological examination on female ovaries showed normal histology in both control and treated groups. As ovulation number is usually relatively large in rats, there was a presence of normal large number of follicles such as primary (black arrow), secondary (white arrow) follicles with oocytes (o) at different stages of maturation and corpora lutea (cl) observed in the cortex. There was also a mixture of old and newly formed corpora lutea.

Table 4.7 Histopathological scoring chart

	Male (n=5)		*Female (n=5)*	
Lesions in organs	**Control**	**Treated**	**Control**	**Treated**
Kidney				
Glomeruli abnormalities	0	0	0	0
Tubule abnormalities	0	0	0	0
Oedema	0	0	0	0
Necrosis	0	0	0	0
Inflammation/infiltration of inflammatory cells	0	0	0	0
Liver				
Changes in nuclear and cytoplasmic features	0	0	0	0
Inflammation/infiltration of inflammatory cells	0	0	0	0
Fibrosis	0	0	0	0
Steatosis	0	0	0	0
Ballooning	0	0	0	0
Lung				
Epithelial thickening and degeneration	0	0	0	0
Fibrosis	0	0	0	0
Oedema	0	0	0	0
Inflammation/infiltration of inflammatory cells	0	0	0	0
Haemorrhage	0	0	0	0
Necrosis	0	0	0	0

Table 4.7 Continued

Heart				
Myocardial necrosis	0	0	0	0
Inflammation/infiltration of inflammatory cells	0	0	0	0
Fibrosis	0	0	0	0
Brain				
Neuronal degradation/Ischemic neuron	0	0	0	0
Inflammation/infiltration of inflammatory cells	0	0	0	0
Angiogenesis	0	0	0	0
Infarction	0	0	0	0
Testis				
Distortion of testicular tissue architecture	0	0	0	0
Necrosis of tubular lining epithelium	0	0	0	0
Vacuolization	0	0	0	0
Interstitial tissue oedema	0	0	0	0
Vascular dilation and congestion	0	0	0	0
Spermatogenesis	0	0	0	0
Ovary				
Follicle development	0	0	0	0
Presence of corpus luteum	0	0	0	0
Congestion	0	0	0	0
Oedema	0	0	0	0
Inflammation/infiltration of inflammatory cells	0	0	0	0
Haemorrhage	0	0	0	0

0: No abnormality detected, 1: Abnormality detected

CHAPTER 5

DISCUSSION

A plant found in Southeast Asia, *O. indicum* is one of the many plants utilised by the Asian population as a popular medicinal plant due to its many potentials such as anti-inflammatory, anti-cancer, and antioxidant benefits among many others (Epelle *et al.*, 2022; Kang *et al.*, 2012; Liu *et al.*, 2016; Shukla *et al.*, 2019). *O. indicum* possess many phytochemicals which contribute to its medicinal potential. The focus of this study was directed to the therapeutic potential of one of the most dominant active compounds derived from *O. indicum* which is baicalein. Despite an extensive literature on the therapeutic potentials of baicalein extracted from *O. indicum* in treating cancer, diabetes, and obesity to name a few, very little information is known about its potential adverse toxic effects and limits of exposure level.

5.1 Extraction and fractionation of *O. indicum* leaves yielded baicalein active compound in F5

Although baicalein active compound can be manufactured synthetically (J. Li *et al.*, 2019), it was found that the proposed synergistic effects of baicalein with other phenolic compounds of similar polarity such as its aglycone baicalin and baicalein-7-O-glucoside extracted from the *O. indicum* plant itself demonstrated significantly more effective inhibitory effects than synthetic baicalein pure compound against glioblastoma multiforme (GBM) cell line (Kang *et al.*, 2019). This finding provides a sound rationale behind the first specific objective of the present study in extracting and performing fractionation to obtain F5 from the leaf material of *O. indicum* rather than

employing pure synthetic baicalein to conduct the toxicity studies. Based on the results obtained, the binary solvent system using PET, followed by MeOH was found to be an optimized method to extract baicalein active compound from *O. indicum* using the Soxhlet extractor (Kang *et al.*, 2019) as almost similar percentage of yield was obtained with that of Kang *et al*. (2019). The TLC analysis done on F5 further confirmed that the binary solvent system was successful in purifying the crude extract which will produce higher accuracy in determining the toxicity effects of baicalein in this study. The PET acted as a solvent to remove unsought solute containing low-polarity lipid constituents, while MeOH acted as a solvent to extract the polar constituents which includes baicalein (Kang *et al.*, 2019).

5.2 Toxicity study

Secondary metabolites specifically flavonoids play a dual role in drug discovery due to their numerous biological functions which may be beneficial to certain organisms but have cytotoxic properties. Baicalein is known to have cytotoxic effect on tumours through induction of apoptosis via Ca^{+2} influx (Lin *et al.*, 2007) and autophagy via endoplasmic reticulum (ER) stress and suppression of ERK pathway (Chen *et al.*, 2013; Lin *et al.*, 2020; Wang *et al.*, 2014). In spite of that, knowledge on the cytotoxic effect of baicalein on the body's normal cells remains limited. One of the specific objectives of this study was to determine the sub-lethal dose of baicalein. This was successfully determined through the sighting study which was conducted prior to the main acute toxicity study. Sub-lethal dose of baicalein active compound in F5 determined from the sighting study was greater than 2000 mg/kg as no mortality was observed, together with absence of any behavioural toxicity signs in the group of rats administered with that dose. Therefore, it was predicted that baicalein active

compound is safe to be used in clinical studies. This finding is consistent with results from a clinical study conducted by Li *et al.*, (2014) which demonstrated that 100-2800 mg single oral doses of baicalein extracted from the root of *Scutellaria baicalensis* Georgi manufactured into chewable tablets was safe on healthy human subjects of both sexes as it produces no signs of liver and kidney toxicity upon haematological and biochemical analysis.

Additionally, the common use of outbred rats such as the Sprague-Dawley in toxicity studies are due to the fact that this strain exhibit favourable characteristics such as high disease resistance, long life spans, early fertility, and rapid growth (Parker *et al.*, 2014) which can provide more consistent results that can later be translated and replicated in clinical studies. Sprague-Dawley rats have been considered a reliable animal model demonstrated by its use in numerous studies involving baicalein (Li *et al.*, 2021; Meng *et al.*, 2021; Menon *et al.*, 2019; Shi *et al.*, 2018), one of which was mentioned earlier in this thesis. Generally, rodents represent a good animal model in toxicological studies as most physiological and biochemical functions of their organs are similar to that of the human body. However, appropriate validation and careful translation of rodent data to human systems is vital when interpreting results (Treuting *et al.*, 2018).

5.3 Behavioural toxicity evaluation

It is long recognised that animals do experience pain and distress, however this experience can only be determined by observations of the animal's appearance and behaviours (Carstens & Moberg, 2000). Behavioural responses due to toxicity observed in this study was mainly to aid the researcher in evaluating humane endpoints

with less emphasis on studying the effects of baicalein on central nervous system (CNS) activity and neurological function. The main purpose of applying humane endpoints in this study was to accurately predict severe pain, distress, suffering or impending death which is in accordance with Russell and Burch's refinement principle. The behavioural changes and clinical toxicity signs were outlined in the OECD Guidance Document (2000) which serves as indicators of a compromised well-being of individual animals. In general, there were no obvious signs of toxicity and severe pain, or distress observed in the sighting and acute study groups. Changes in physical appearance such as skin, coat texture, eyes and mucous membranes including changes of behaviour such as self-mutilation, abnormal vocalisation or aggressiveness among other behaviours were not observed in the rats of the sighting and acute study groups. Food and water intake were also consistent daily. These findings indicate that F5 containing baicalein active compound did not compromise the overall well-being of the rats in not only the sighting group but the treated groups of the main acute toxicity study as well.

The behavioural observations gathered in this study further supports the findings of Yu *et al.* (2012) as it is postulated that F5 did not induce any cognitive side-effects which includes autonomic (salivation, appetite) and somatic functions (tremors, convulsions) of all the rats in the treated groups of both sexes. This may provide support to other research in the development of baicalein active compound for applications other than CNS-targeted drugs such as anti-obesity drugs and heart medication.

5.4 Assessment parameters

Haematological and biochemical parameters serve as an indicator of internal exposure in the rats to any toxic substances. The whole blood specimens of the rats were collected and sent for analysis. Values of MCHC, platelet, neutrophil, eosinophil, and lymphocytes in the male treated group and the RBC, Hb, PCV, MCHC, neutrophil, lymphocytes, and monocytes in the female treated group showed statistically significant differences after the 14-day study period. The increase in RBC and Hb count seen in the female treated group might be attributed to the increase in the rate of erythrocyte production and may suggest that the extract exhibited erythropoietin potential. Surprisingly, the MCHC counts in the treated group of both sexes (34.0 g/dL in males, 34.2 g/dL in females) showed statistically significant difference compared to the control groups (35.0 g/dL in males, 35.4 g/dL in females). MCHC is a measure of average concentration of haemoglobin in a given volume of packed red blood cell or simply a measure of average oxygen-carrying capacity of RBCs circulating in the body of an organism. The results of the MCHC counts gathered in this study may indicate that the oxygen-carrying capacity of the blood and amount of oxygen delivered to the tissues was not necessarily enhanced by the single dose of F5 administration despite increase of RBC and Hb count. Besides that, PCV is the measurement of percentage of RBCs in circulating blood which may indicate blood loss, failure of bone marrow production and cell destruction if the percentage is abnormally low or dehydration if the percentage is abnormally high. Although the PCV percentage in the female treated group (44.0%) was higher than the control group (38.8%), it was not out of normal range to indicate any clinical pathology. Platelets also known as thrombocytes originate from the bone marrow and spleen of rodents and function similarly to human platelets, that is to form clots to control excessive bleeding through myeloid stem cell

stimulation by thrombopoietin. It was found that the platelet number in the male treated group was higher (650.60 10^9/L) than the control group (504.00 10^9/L) which may indicate that F5 has a stimulatory effect on thrombopoietin.

In the case of WBC differentials, leukocytes which include granular neutrophils and eosinophils and agranular lymphocytes and monocytes play a vital part in the body's innate defence system. Neutrophils function in response to tissue and cell injury especially in inflammation and pathogen infections (Treuting *et al.*, 2018) and are usually associated with stress and excitement response (O'Connell *et al.*, 2015). On the other hand, lymphocytes are an abundant leukocyte in most rodent strains comprising 70% to 80% of the WBC differential count and are involved in immune response against viral infections and inflammation (O'Connell *et al.*, 2015). In this study, neutrophil percentage in the male treated rats (15.38 %) were found to be lower than the control group (19.70%), however the results were reversed for the female treated group (14.38%) compared to the control group (6.22%). The opposite effect is seen in lymphocyte percentage in which the female treated group (82.52%) had lower percentage than the control (88.74%), while the male treated group (81.80%) had higher lymphocyte percentage than the rats in the control group (75.60%). This could be evidence of sexual dimorphism in rodent immune response due to sex hormone regulation of leukocytes which was reported in previous studies (Kosyreva *et al.*, 2018; Spitzer & Zhang, 1996). Moreover, eosinophils are known to function in response to parasitic and allergic reactions. As the percentage of eosinophils were found to be lower in the male treated group (0.30%) than the control group (1.34%), this result is in agreement with the findings of a previous study conducted by Mabalirajan *et al.* (2013) in which baicalein was able to suppress infiltration of eosinophils in the respiratory tract of mouse model of allergy by reducing IL-4 and IL-13 levels.

Monocytes which produce cytokines in the blood including TNF-α and IL-6 that function in response to bacterial infection or chronic inflammation (O'Connell *et al.*, 2015) exhibited significant lower percentage in the female treated group (0.88%) than the control group (1.90%). Since the percentage of neutrophils, eosinophils, monocytes, and lymphocytes for the treatment group in both sexes were not out of the normal range, the data suggested that there was no inflammation or abnormalities in the rats' health. Overall, the haematological indices in both the male and female treated and control groups were within the normal range according to CLSI and ASVCP guidelines.

Serum enzymes ALT, AST, and ALP are useful indicators of liver and kidney injury as they are mainly found in those two organs. These enzyme levels are usually raised signifying tissue damage. However, the ALT levels in the male treated group showed statistically significant difference after F5 treatment, as it was documented that the levels were lower than the control group with a mean value of 59.60 IU/L compared with the 72.40 IU/L mean value recorded in the control group. Thus, it can be surmised that F5 produced hepatoprotective effect on the rats. This finding is supported by a similar result observed in a study conducted by Zhou *et al.* (2018) as baicalein was able to ameliorate acetaminophen (APAP)-induced acute liver damage and histological hepatocyte changes in mice by preventing MAPK pathway activation. However, longer study periods are required to confirm this theory. On the other hand, the ALB levels in the female treated group showed statistically significant difference at the end of the 14-day study period with a mean of 37.00 g/L ($p < 0.05$) which is higher compared to the 33.80 g/L seen in the control group. Higher than normal ALB levels may indicate dehydration, decreased glomerular permeability and decreased renal tubular reabsorption of ALB which may lead to renal injury (Chapman *et al.*,

2020). However, all the values for TP, ALB, TBIL, ALT, AST, and ALP were within the normal range for both sexes according to CLSI and ASVCP guidelines suggesting that F5 containing baicalein did not cause any liver or kidney damage to the rats.

5.5 Body weight and relative organ weight (ROW)

Body weight changes are a valuable assessment into the rats' overall health and well-being (Ghasemi *et al.*, 2021). Changes in body weight of all animals in the acute toxicity study did not show any increment or reduction of more than 20% from their initial body weight after 14 days. Maximum body weight changes between Day 0 and Day 7 in male control and treated group were 12.15% and 9.55% respectively. However, between Day 7 and Day 14, there was a reduction in maximum body weight changes in the control and treated group which were 3.67% and 6.58% respectively. On the other hand, the female rats exhibited maximum body weight change of 2.28% in the control and 6.67% in the treated group between Day 0 and Day 7. During the period of Day 7 and Day 14, the females in the control and treated group exhibited a significant maximum body weight gain of 14.56% and 8.95% respectively. According to Slob and van der Werff Ten Bosch (1975), sex differences in body weight can be attributed to the post-natal effects of testicular and ovarian hormones. Initial rapid growth may still be observed in both sexes of Sprague-Dawley rats until 168 post-natal days (Ghasemi *et al.*, 2021). Besides, body weight of the rats may also exhibit variability even between individual animals of the same age and from the same colony (McCutcheon & Marinelli, 2009). Thus, it can be inferred that F5 containing baicalein active compound does not produce any toxic effects on the rats' overall health and did not cause any signs of pain and discomfort to the animal.

Furthermore, organ weight changes are commonly accepted as a sensitive indicator of chemically induced toxic effects on organs as statistically significant differences in weight may occur despite lack of any morphological or physical changes in the organs themselves. The results of this study revealed a clear pattern as both the brain, lungs, and main reproductive organs of both sexes between the control and treated groups had statistically significant differences in ROW. The statistically significant differences were seen in the relative weight of the brain, lung, and testes of the male treated group in which there was a lower overall relative decrease of -0.14 g, -0.28 g, and -0.13 g respectively in comparison with the control group. Statistically significant differences were also seen in the ROW of the brain, lung and ovaries of female treated group when compared with the control group. There was a relative increase of 0.19 g in brain ROW between the female treated and control group. However, there was a relative decrease of -0.31 g in the lungs and -0.17 g in the ovaries between the two groups. According to Bailey *et al.* (2004), ROW differences may be influenced by secondary means such as overall developmental growth in the rats' life span during the toxicity study or through environmental stress. The rats used in this study were of 8-12 weeks of age which is by definition an adult purely based on sexual maturity without consideration on the development of the selected organs in question (Ghasemi *et al.*, 2021; Jackson *et al.*, 2017). It is suggested that these differences may be an indication of development or possible stress in response to laboratory conditions (Bailey *et al.*, 2004; Ghasemi *et al.*, 2021). According to van der Schoot and Uilenbroek (1983), a difference in ovarian weight, which is evident during the oestrous cycle is perhaps linked to development and regression of corpora lutea. It is noteworthy, that the two main organs often used as a sensitive predictor of toxicity which are the liver and kidney (Michael *et al.*, 2007) did not exhibit any statistically

significant differences in ROW between the control and treated group in this study further strengthening the assumption that F5 is not toxic to the rats. These results are further corroborated with histopathological examination and scoring done on the H&E-stained tissues of each organ.

5.6 Histopathological scoring

An important stage of drug discovery involves the safety assessment of drugs through histopathological evaluation on toxic-induced changes in laboratory animals (Greaves, 2011). Comparative histological toxic manifestations seen in animal models may provide a basis for future research in clinical studies involving humans. At the level of the light microscope, the tissues between the Sprague-Dawley rat and human may look similar but critical differences must be recognized as described by Treuting *et al.* (2018). Thus, the scoring system used in this study was developed through validated methods published in previous studies (Adamkovicova *et al.*, 2014; Chu *et al.*, 2019; Fouad *et al.*, 2020; Hu *et al.*, 2020; Ibrahim *et al.*, 2018; Janke *et al.*, 2019; Kleiner *et al.*, 2005; Klopfleisch, 2013; Mohamed *et al.*, 2016; Sayar *et al.*, 2016; Silva *et al.*, 2015). The main organs involved in predicting toxicity is the liver and kidney. Nevertheless, other susceptible vital organs such as the heart, brain, lungs, and reproductive organs are equally useful predictors of toxicity as these organs are well-perfused and receive a rich supply of blood in which toxins are extensively distributed via oral route. Therefore, examining the histopathological changes seen in these organs may provide a compelling insight into its pharmacodynamic effect.

As widely understood, the liver is the main site for drug metabolism and detoxification in which nutrients and macromolecules are freely exchanged between the main functional cells of the liver which are hepatocytes and plasma across the

liver's "capillaries", known as sinusoids. Normal hepatic microscopic architecture is composed of polygonal lobules centred on the central vein. Upon histopathological assessment of hepatotoxicity, the liver of all the treated and control rat groups in both sexes did not exhibit any toxicity. Certain hepatocytes contain two nucleases, however it is considered not unusual to discover binucleated or polyploidy hepatocytes in tissue examined (Greaves, 2011). Glycogen accumulation was observed in four out of five control and three out of five treated rats of both sexes. These changes were considered as normal as it was present in the control groups as well. The degree of glycogen accumulation within all hepatocytes in each lobule is high during the morning hours and will decline throughout the day as rodents are nocturnal feeders (U.S. Department of Health and Human Services, 2014). These findings suggest F5 containing baicalein active compound did not induce inflammatory cytokine release (Jaeschke & Ramachandran, 2020) or hepatocyte damage and obstruction on blood flow throughout the liver (Chebaibi *et al.*, 2019) which was reflected in the normal haematology results in the treated group of both sexes. In summary, the findings in the liver histology coupled with ALT, AST, albumin results may provide further support for the use of baicalein in hepatocellular carcinoma (HCC) treatment as discovered by Chen *et al.*, (2013).

The kidney is also considered one of the most frequent targets for drug-induced toxicity besides the liver. The kidney consists of nephrons which within itself is comprised of the glomerulus, proximal tubules, convoluted distal tubules, descending and ascending loops of Henle (Treuting *et al.*, 2018). In this study, it was found that there were no glomeruli abnormalities marked by distortion and diminished morphology (Ibrahim *et al.*, 2018) with normal presence of podocytes covering the urinary surface. Besides that, there were no dilated tubules observed which suggests

no tubule necrosis undergoing repair indicated by the presence of flattened regenerating epithelium (Treuting *et al.,* 2018). Moreover, excess infiltration of inflammatory cells and oedema were not present in the tissues of the treated rats of both sexes which implies that F5 did not induce any pro-inflammatory response, renal sodium and water retention (Liu *et al.*, 2022) in acute doses. In this study, histopathological scoring on the kidneys of treated group in both sexes suggests that baicalein may be used in CKD treatment as proposed by Wang *et al.,* (2015) due to the compound's anti-inflammatory potential.

Additionally, there were no observable cardiomyocyte necrosis characterized by cell swelling and disintegration of organelles. These results reflect the outcome of a previous study conducted by Li *et al.* (2020) in which baicalein was found to have an inhibitory effect on H_2O_2 induced cardiotoxicity by stabilizing or increasing the expression of MARCH5 by suppressing mitochondrial fission and reduce cell apoptosis. Besides that, examination of the lung tissues in treated and control groups of both sexes revealed presence of preserved normal alveolar architecture with no notable histological changes. This finding supports the results gathered in a study involving lipopolysaccharide (LPS)-induced acute lung injury in mice in which 50, 100, and 200 mg/kg baicalein improved the respiratory function, inhibited inflammatory cell infiltration in the lung, and decreased the levels of IL-1β, and TNF-α in serum (Song *et al.*, 2021). As evidenced by numerous *in vitro* and *in vivo* studies involving the use of baicalein for CNS treatment (Rui *et al.*, 2020; Tsai *et al.*, 2012; Wei *et al.*, 2017; Zhang *et al.*, 2017), the results gathered from the histopathological scoring in this study were validated where there was no excess inflammation, neuronal degradation, angiogenesis, ischemic neuron, and infarction in the brain tissues of both treated groups. On top of that, it was discovered that the testes of the male treated

group and ovaries of the female treated group showed no adverse signs of toxicity at the end of the 14-day treatment with F5. The results may provide further support for previous studies involving the potential of baicalein to be used in ovarian cancer (Chen *et al.*, 2013) and testicular cancer treatment (Wang *et al.*, 2014).

In conclusion, histopathological findings of the organs collected in this acute toxicity study with 2000 mg/kg F5 consisting of baicalein active compound did not provide any evidence of organ toxicity which correlates with the results of the biochemical and haematological indices. The findings of this study provide a promising opportunity for future research on baicalein active compound extracted from *O. indicum* in clinical studies.

5.7 Limitations and suggestions for future studies

Overall, this study has four major limitations. The first limitation is in time constraints as the data collection and analysis of the project was scheduled to run from the month of April to August, which only allowed for acute toxicity study to be conducted on top of the time required for baicalein active compound extraction. This limitation provides an opportunity for further studies to be conducted involving subacute, sub-chronic and chronic toxicity testing on baicalein active compound. As it has been established that *O. indicum* is widely consumed in the South and Southeast Asian population, multiple dose studies will be useful in evaluating the safety profile of baicalein extracted from this plant. A previous study discovered that baicalein underwent rapid clearance in Sprague-Dawley rats' liver through efficient phase II metabolism which includes glucuronidation and sulfation pathways via i.p.v. infusion which is meant to mimic oral administration (Zhang *et al.*, 2011). Therefore, it is plausible that cumulative toxic effects may occur at very low doses. As an example,

upon comparison of doses required to cause 50% loss of primordial follicles in female mice, it was found that those mice exposed to a repeated low-dose of 9,10-dimethylbenz[*a*]anthracene (DMBA) for 15 days (Borman *et al.*, 2000) were found to exhibit ovo-toxicity 250-times greater than mice exposed to a single high-dose of DMBA within 1-2 days (Mattison & Thorgeirsson, 1979). Thus, the outcome of these studies demonstrates the need for multiple low-dosage studies over a longer time period in evaluating the safety of baicalein extracted from *O. indicum*.

The second limitation of the current study is the lack of haematological and biochemical data to support the descriptive findings in histopathological scoring of brain, heart, lung, testis, and ovary. For example, measuring the levels of progesterone hormone, LH and FSH will be useful to further observe potential hormonal changes induced by F5 containing baicalein on the reproductive organs of male and female groups. Besides, glutathione (GSH) levels and malondialdehyde (MDA) levels may provide evidence on presence of oxidative stress in the organs mentioned. Thus, future research may include a more comprehensive haematological and biochemical testing to gain a deeper understanding of the toxicity effects, if any, of baicalein to the biochemistry of animal models.

The third limitation is regarding the relative organ weight calculation. According to Lazic *et al.* (2020), when measuring the direct effects of the drug on organ weight, few causal relationships emerge. The first causal relationship is seen when a drug directly affects an organ weight; the second causal relationship is seen when a drug affects organ weight indirectly by altering overall body weight; the third causal relationship is seen when drug affects organ weight both directly and indirectly; the fourth relationship is seen when the drug affects the organ weight, which causes the rat to become ill, leading to overall reduction in body weight. Therefore, these

confounding factors will have to be ruled out before making causal pharmacodynamic claims about how the drug may affect the organ weight (Lazic *et al.*, 2020). It was suggested that measuring covariance (ANCOVA) of organ weight is a more suitable method (Bailey *et al.*, 2004) which considers body weight as a covariate, however greater understanding and further expertise in analysis of the results is required (Lazic *et al.*, 2020). Future studies may employ the covariance method to measure organ weight in order to accurately evaluate the effect of baicalein on specific organs.

Lastly, the final limitation of this study lies in the lack of characterisation of F5. Baicalein active compound was not isolated from the fraction to be administered on the animal models. Therefore, the possible synergistic effect of baicalein with other phytoconstituents found in F5 may have contributed to the results obtained in this study. The findings of the study conducted by Kang *et al.* (2019) have also previously proposed the same assertion. Future studies may include characterisation and/or quantification of other phytoconstituents besides baicalein in F5 using high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS) analysis to further clarify the effects of F5 for clinical use.

CHAPTER 6

CONCLUSION

This study investigated the acute toxicity effect of baicalein active compound extracted from *O. indicum* in Sprague-Dawley rats. Baicalein active compound was successfully extracted using Soxhlet extraction and fractioned using 100% methanol. It was also successfully characterized in F5 using TLC analysis. Acute toxicity studies using 2000 mg/kg F5 containing baicalein active compound demonstrated no toxic effect on Sprague-Dawley rats' behaviour upon evaluation of clinical toxicity signs and deviation from normal behaviour. The extract also did not cause deviation of more than 20% from initial animal body weight although statistically significant differences were recorded in the brain, lung, and reproductive organs of both sexes. The haematological and biochemical indices of the rats were all within normal range based on ASVCP an CLSI guidelines despite presence of statistically significant differences. Histopathological scoring of each animal organ also revealed no adverse effects caused by F5 containing baicalein treatment. In conclusion, 2000 mg/kg of F5 containing baicalein active compound extracted from *O. indicum* is proposed to be safe for future research focusing on the effects of multiple dosage in subacute and chronic studies.

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APPENDICES

Appendix A Voucher specimen of *Oroxylum indicum* (L.) Benth. Ex Kurz plant (USM Herbarium 11751) which was verified by Dr. Rahmad Zakaria and deposited in the Universiti Sains Malaysia (USM) herbarium by Mr. V. Shunmugam

Bilik Herbarium
Kompleks Rujukan Biodiveristi (D32)
Pusat Pengajian Sains Kajihayat

Herbarium No.: 11751

Picture of Herbarium

Picture of Label

USM Herbarium
Pusat Pengajian Sains Kajihayat
Universiti Sains Malaysia

Family: Bignoniaceae
Genus/Species: *Oroxylum indicum (L.) Benth. Ex Kurz*
Date: 29/01/2018
Locality: Pasir Mas, Kelantan.
Habitat: Small tree up to 12m tall, mostly sighted along the river bank or slopes of the hill and often cultivated as an ornamental plant.
Notes: A evergreen tree or partly deciduous tree up to 15m high sparingly branched with gray-brow bark. Somewhat fissured glabrous throughout twigs pale grey, massive with big scars pimply from lenticels. Leaves 1m-3m long wide, very large, spreading, the main stalk and side stalk arched, swollen and jointed at the attachment of the side stalk and leaflets ¾ m – 1¾ m x ½ m elliptic, shortly tipped, stalk thin with wavy edge, light green and dark along the vein, base narrowed or heart shaped, general asymmetric. Flowers with foxy stink, inflorescences up to 1½ m-3m long, flowering upper part, fruiting below calyx, fleshy, lurid reddish purple to liver brown, outside greenish white and yellow on the inside. Pods ¾ m- 1m, curved at the base with fine edge on each side.
Collector: Kang In Nee
Project supervisor: Dr. Tan Suat Cheng
Identified by: Dr. Rahmad Zakaria

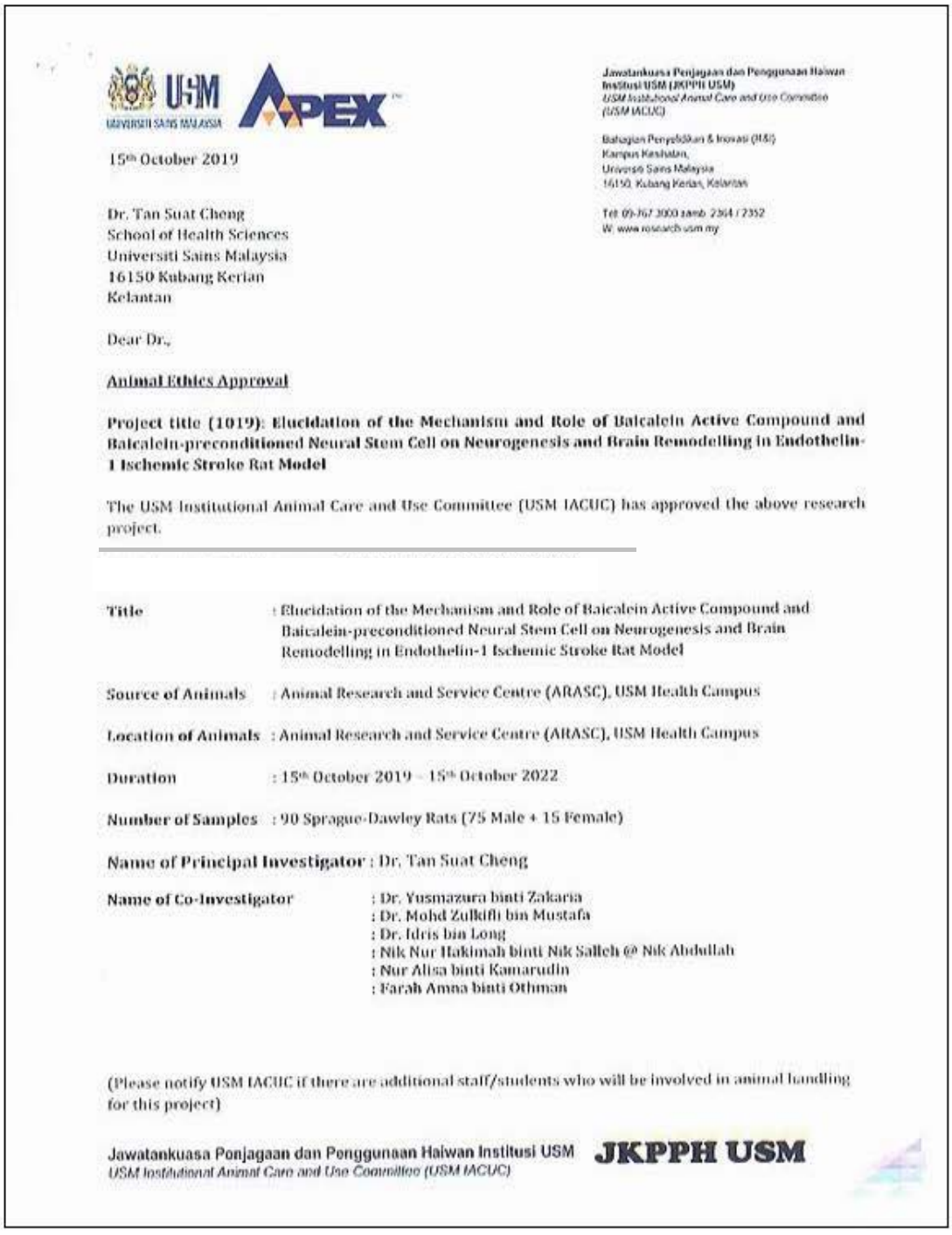
Picture of Plant


Stem and leaves


Flower


Fruit

Appendix B Animal ethics approval letter



Jawatankuasa Penjagaan dan Penggunaan Haiwan Institusi USM (JKPPH USM)
USM Institutional Animal Care and Use Committee (USM IACUC)

Bahagian Penyelidikan & Inovasi (RIS)
Kampus Kesihatan,
Universiti Sains Malaysia
16150, Kubang Kerian, Kelantan

Tel: 09-767 3000 samb. 2364 / 2352
W: www.research.usm.my

15th October 2019

Dr. Tan Suat Cheng
School of Health Sciences
Universiti Sains Malaysia
16150 Kubang Kerian
Kelantan

Dear Dr.,

Animal Ethics Approval

Project title (1019): Elucidation of the Mechanism and Role of Baicalein Active Compound and Baicalein-preconditioned Neural Stem Cell on Neurogenesis and Brain Remodelling in Endothelin-1 Ischemic Stroke Rat Model

The USM Institutional Animal Care and Use Committee (USM IACUC) has approved the above research project.

Title	: Elucidation of the Mechanism and Role of Baicalein Active Compound and Baicalein-preconditioned Neural Stem Cell on Neurogenesis and Brain Remodelling in Endothelin-1 Ischemic Stroke Rat Model
Source of Animals	: Animal Research and Service Centre (ARASC), USM Health Campus
Location of Animals	: Animal Research and Service Centre (ARASC), USM Health Campus
Duration	: 15th October 2019 – 15th October 2022
Number of Samples	: 90 Sprague-Dawley Rats (75 Male + 15 Female)
Name of Principal Investigator	: Dr. Tan Suat Cheng
Name of Co-Investigator	: Dr. Yusmazura binti Zakaria : Dr. Mohd Zulkifli bin Mustafa : Dr. Idris bin Long : Nik Nur Hakimah binti Nik Salleh @ Nik Abdullah : Nur Alisa binti Kamarudin : Farah Amna binti Othman

(Please notify USM IACUC if there are additional staff/students who will be involved in animal handling for this project)

Jawatankuasa Penjagaan dan Penggunaan Haiwan Institusi USM
USM Institutional Animal Care and Use Committee (USM IACUC)

JKPPH USM

Appendix B Continued

The following items (X) were received and reviewed in connection with the above study to be conducted by the investigator.

(X) Copy of Proposal	(Date : 11th September2019)
(X) Animal Ethics Committee Approval Application Form	(Date : 11th September 2019)
(X) Reviewer's Comment Form	(Date : 11th October 2019)
(X) Reply for Clarification Letter	(Date : 14th October 2019)

Members of the Animal Ethics Committee USM who reviewed the documents as follows:

Member (Title and Name)		Occupation (Designation)
Chairperson: Prof. Dr. Aida Hanum Ghulam Rasool		Lecturer, School of Medical Sciences (Pharmacology)
Secretary: Siti Nurshazwani Muhamad Sayuti		Secretary
Members:		
1	Prof. Dato' Dr. Jafri Malin Abdullah	Lecturer, School of Medical Sciences (Surgery/Behaviour Study)
2	Prof. Madya Dr. Badrul Hisham Yahaya	Director, Animal Research and Service Centre, ARASC (Human Genetic Studies)
3	Prof. Madya Dr. Mahaneem Mohamed	Lecturer, School of Medical Sciences (Physiology)
4	Prof. Madya Dr. Zurina binti Hassan	Lecturer, Centre for Drug Research (Neurobehaviour & Electrophysiology)
5	Dr. Saidi Jaafar	Lecturer, School of Dental Sciences (Developmental Biology/Knock-out & Transgenic)
6	Dr. Basaruddin Ahmad	Lecturer, School of Dental Sciences (Medical Statistics/Dental Public Health)
7	Dr. Nor Aini Saidin	Lecturer, Advanced Medical & Dental Institute (Toxicology)
8	Dr. Nor Azlina Khalil	Veterinary Officer, Seksyen Penyelidikan Haiwan, AMDI
9	Dr. Noziah Ghani	Veterinary Officer, ARASC
10	Puan Hajah Seri Dewi Mohd. Ibrahim	Layperson

Thank you.

Yours sincerely,

PROF. DR. AIDA HANUM GHULAM RASOOL
Chairperson
USM Institutional Animal Care and Use Committee (USM IACUC)

Appendix B Continued

Jawatankuasa Penjagaan dan Penggunaan Haiwan
Institusi USM (JKPPH USM)
USM Institutional Animal Care and Use Committee
(USM IACUC)

Kampus Kesihatan,
Universiti Sains Malaysia
16150, Kubang Kerian, Kelantan

Tel: 09-767 3000 samb. 2364 / 2352
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22 JUN 2022

Dr. Tan Suat Cheng
Pusat Pengajian Sains Kesihatan
Universiti Sains Malaysia
16150 Kubang Kerian
Kelantan

Dr.,

KELULUSAN PENAMBAHAN NAMA PENYELIDIK BERSAMA
Tajuk Projek: Elucidation of the Mechanism and Role of Baicalein Active Compound and Baicalein-preconditioned Neural Stem Cell on Neurogenesis and Brain Remodelling in Endothelin-1 Ischemic Stroke Rat Model [USM/IACUC/2019/(120)(1019)]

Perkara di atas adalah dengan hormatnya dirujuk. Jawatankuasa Penjagaan dan Penggunaan Haiwan Institusi USM (JKPPH) telah meluluskan permohonan pihak Dr. sebagaimana berikut:

BIL.	PENAMBAHAN NAMA PENYELIDIK BERSAMA
1.	Nama : Asmaa' binti Mohd Satar Jawatan : Pembantu Penyelidik (RA) No. K/P : 980118-03-6268 No. Telefon : 011-25769396
2.	Nama : Yeap Mei Yan Jawatan : Pelajar (Master by Mixed Mode – Biomedicine Program) No. Matrik : P-SKM0014/21 No. Telefon : 014-3047635
3.	Nama : Ma Chunhui Jawatan : Pelajar (Final Year Project – Biomedicine Program) No. Matrik : 145331 No. Telefon : 017-9638815

Sekian, terima kasih.

"WAWASAN KEMAKMURAN BERSAMA 2030"

"BERKHIDMAT UNTUK NEGARA"

Saya yang menjalankan amanah,

PROF. DR. AIDA HANUM GHULAM RASOOL
Pengerusi
Jawatankuasa Penjagaan dan Penggunaan Haiwan Institusi USM (JKPPH USM)