COMPUTATIONAL AND EXPERIMENTAL APPROACHES IN UNDERSTANDING THE THERAPEUTIC POTENTIAL OF *Ficus deltoidea* Jack LEAF EXTRACT

AMIRUL RIDZUAN BIN ABU BAKAR

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Amirul Ridzuan Abu Bakar

Matric No: SHC 140061

Name of Degree: Doctor of Philosophy

Title of Thesis ("this Work"): Computational and Experimental approaches in understanding the therapeutic potential of *Ficus deltoidea* Jack leaf extract

Field of Study: Bioinformatics

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name: Dr. Saharuddin Bin Mohamad

Designation: Supervisor

COMPUTATIONAL AND EXPERIMENTAL APPROACHES IN UNDERSTANDING THE THERAPEUTIC POTENTIAL OF *Ficus deltoidea* Jack LEAF EXTRACT

ABSTRACT

Ficus deltoidea Jack of family Moraceae (FD) is a native shrub in Malaysia that are well known for its ethno-pharmaceutical and therapeutic properties. In this study, computational and experimental approaches were adopted to unravel the therapeutic properties of the FD leaf. Initially, quality control and safety assessment on the FD leaf were undertaken. The findings from this analysis revealed that the FD leaf of high quality, purity and abide by the safety requirements set by the regulatory body. FD leaf was solvent extracted as 50% (v/v) ethanol water extract. Standardization using high performance liquid chromatography (HPLC) revealed vitexin and isovitexin as the major chemical compounds in the FD leaf 50% (v/v) ethanol water extract. FD leaf 50% (v/v) ethanol water extract and its major chemical compounds (vitexin and isovitexin) were subjected to a selected panel of enzymatic inhibition assay, namely, α-amylase and matrix metalloproteinases (MMP-2, MMP-8 and MMP-9). The results showed that FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin exhibited inhibition property against the selected enzyme panels. Then, computational analyses (molecular docking and molecular dynamic simulation) revealed the molecular interaction and ligand-protein complex stability between vitexin and isovitexin against the selected enzyme panels. FD leaf 50% (v/v) ethanol water extract also showed antioxidant activity based on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and *in vitro* cytotoxicity based on 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay revealed the IC_{50} of the extract was at 221 µg/ml on EA.hy926 cells. FD leaf 50% (v/v) ethanol water extract also demonstrated cell migration inhibitory effect on EA.hy926 cells in a dose and time-dependent manner with the highest activity tested at 300 µg/ml after 24

hours treatment. The biological activity of FD leaf 50% (v/v) ethanol water extract was further studied using 2 types of *in vivo* animal models (healthy and streptozotocin-induced diabetic Sprague-Dawley rats). Toxicology analysis was conducted at 1000 mg/kg of FD leaf 50% (v/v) ethanol water extract. Visual inspection, blood analysis and histology analysis revealed no acute toxicity of the leaf extract at the tested concentration. Wound healing activity of FD leaf 50% (v/v) ethanol water extract was studied on both animal models. FD leaf 50% (v/v) ethanol water extract showed highest wound healing property at 100 mg/ml and the diabetic rat model showed faster wound closure rate compared to the healthy rat model. Finally, this study provided comprehensive and valuable information on the potential of FD leaf extract for therapeutic application especially for the wound healing in diabetic cases.

Keywords: *Ficus deltoidea*, Pharmacognosy, CADDs, *in vitro, in vivo*

PENDEKATAN PENGKOMPUTERAN DAN EKSPERIMENTAL UNTUK MEMAHAMI POTENSI THERAPEUTIK EKSTRAK DAUN *Ficus deltoidea* Jack

ABSTRAK

Ficus deltoidea Jack dari keluarga Moraceae (FD) adalah tumbuhan yang terdapat di Malaysia yang terkenal dengan ciri-ciri etno-farmaseutikal dan terapeutiknya. Dalam kajian ini, pendekatan pengkomputeran dan eksperimen telah digunakan untuk mengenalpasti sifat terapeutik daun FD. Pada mulanya, penilaian kualiti dan penilaian keselamatan daun FD telah dilaksanakan. Penemuan dari analisis ini mendedahkan bahawa daun FD berkualiti tinggi, bersih dan mematuhi keperluan keselamatan yang ditetapkan oleh badan kawal selia. Daun FD telak diekstrak dalam larutan air etanol 50% (v/v). Pemiawaian menggunakan kromatografi cecair prestasi tinggi (HPLC) mendedahkan bahawa viteksin dan isoviteksin sebagai bahan kimia utama dalam daun FD 50% (v/v) ekstrak air etanol. Ekstrak daun FD (v/v) air etanol 50% dan sebatian kimia utama (viteksin dan isoviteksin) telah diuji pada satu panel asai perencatan enzim yang disyorkan iaitu α-amilase dan matriks metalloproteinases (MMP-2, MMP-8 dan MMP-9). Keputusan dari ujian ini menunjukkan bahawa ekstrak daun FD 50% (v/v) air etanol, viteksin dan isoviteksin mempamerkan sifat penrencatan terhadap panel enzim yang dipilih. Kemudian, analisis pengkomputeran (molekul dok dan simulasi dinamik molekul) mendedahkan interaksi molekul dan kestabilan kompleks protein ligan antara vitexin dan isoviteksin terhadap panel enzim yang dipilih. Ekstrak daun FD air etanol 50% (v/v) juga menunjukkan aktiviti antioksidan berdasarkan ujian 2,2-diphenyl-1-picrylhydrazyl (DPPH) dan sitotoksisiti *in vitr*o berasaskan ujian 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) yang mempamerkan IC_{50} ekstrak adalah pada 221 μg / ml terhadap sel EA.hy926. Ekstrak FD air ethanol 50% (v/v) juga menunjukkan kesan perencatan penghijrahan sel EA.hy926 pada dos

dan masa bergantung pada cara dengan aktiviti tertinggi direkodkan pada 300 μg/ml selepas menjalani 24 jam rawatan. Aktiviti biologi daun FD 50% (v/v) ekstrak air etanol dikaji dengan lebih lanjut menggunakan 2 jenis dalam model haiwan in vivo (tikus Sprague-Dawley yang sihat dan yang diberikan streptozocin). Analisis toksikologi dilakukan pada ekstrak daun FD 50% (v/v) air ethanol pada kepekatan 1000 mg/kg. Pemeriksaan visual, analisis darah dan analisis histologi menunjukkan tiada ketoksikan akut dihasilkan oleh ekstrak daun pada kepekatan yang diuji. Aktiviti penyembuhan daun FD 50% (v/v) ekstrak air etanol dikaji pada kedua-dua model haiwan. Ekstrak FD daun 50% (v/v) air etanol menunjukkan sifat penyembuhan luka tertinggi pada 100 mg/ml dengan model tikus diabetik menunjukkan kadar penyembuhan lebih cepat berbanding dengan model tikus yang sihat. Konklusinya, kajian ini memberikan maklumat yang komprehensif dan berharga tentang potensi ekstrak daun FD untuk aplikasi terapeutik terutama berkaitan penyembuhan luka dalam kes diabetes.

Kata-kunci: *Ficus deltoidea*, Farmakognosi, CADDs, *in vitro, in vivo*

ACKNOWLEDGEMENTS

Firstly, I would like to praise to Allah the most merciful for giving me the opportunity to further study into PhD level.

Many thanks to my family, my dad, mum, siblings, in-laws, and my wife, Nurul Husna Khairuddin for their support throughout my PhD study. To my dedicated supervisors, Dr. Saharuddin Mohamad and Prof. Dr. Amir Feisal Merican, thanks for your guidance and knowledge that you provided me with.

The RAs, Bioinformatics department, CRYSTAL, UniMAP and UM staff, thank to you guys also.

TABLE OF CONTENTS

Page

ABSTRACT ..... **iii**

ABSTRAK ..... **v**

ACKNOWLEDGEMENTS ..... **vii**

TABLE OF CONTENTS ..... **viii**

LIST OF FIGURES ..... **xiii**

LIST OF TABLES ..... **xvi**

LIST OF SYMBOLS AND ABBREVIATIONS ..... **xvii**

LIST OF APPENDICES ..... **xix**

CHAPTER 1: INTRODUCTION **1**

1.1 Background 1

1.2 Research Questions 4

1.3 Research Objectives 4

1.4 Thesis Organization 5

CHAPTER 2: LITERATURE REVIEW **6**

2.1 Medicinal plants 6

2.2 Drug discovery and development from medicinal plants 7

2.3 Bioinformatics and computational approaches in drug discovery process 11

2.4 *Ficus deltoidea* Jack (Mas cotek) - a promising medicinal plant for wound healing 16

2.4.1 Phytochemicals in FD 19

2.5 Wound healing physiology 21

2.5.1 Angiogenesis 21

2.5.2 Matrix metalloproteinases (MMPs) 21

2.5.3 Normal and impaired wound healing process 23

2.5.4 Wound healing disorder in diabetes 25

CHAPTER 3: MATERIALS AND METHODOLOGY **28**

3.1 Flowchart of the research methodology 28

3.2 Chemicals and reagents 30

3.3 Preparation of plant samples 31

3.4 Quality control of FD raw material 31
3.4.1 Total Foreign Matter 31
3.4.2 Total Ash 31
3.4.3 Total Acid Insoluble Ash 31
3.4.4 Moisture content of raw material 32
3.4.5 Extractive Value 32
3.5 Safety assessment of FD raw material 33
3.5.1 Heavy Metal Content 33
3.5.2 Microbial Limit Test of FD 33
3.5.3 *Pseudomonas aeruginosa* and *Staphylococcus aureus* 34
3.5.4 Detection of *Salmonella sp.* 34
3.5.5 *Escherichia coli* 34
3.5.6 Detection of aflatoxins 34
3.6 Standardization of FD leaf extracts against vitexin and isovitexin 35
3.7 Computational analysis of vitexin and isovitexin 37
3.7.1 Ligand preparation 37
3.7.2 Molecular docking 37
3.7.3 Molecular dynamic simulations 38
3.8 *In vitro* assay of FD leaf 50 % v/v ethanol water extract 40
3.8.1 The α-amylase inhibition assay 40
3.8.2 DPPH scavenging assay 41
3.8.3 MMPs inhibition assay 41
3.8.4 Cytotoxicity study of FD leaf extract 42
3.8.5 Cell migration activity assay 43
3.9 *In vivo* study of FD leaf extract 44
3.9.1 Experimental animals 44
3.9.2 Mortality and behavioural observation 44
3.9.3 Wound healing study of FD leaf extract 45
3.10 Blood and biochemical analysis of SD and STZ-SD rats 46
3.10.1 Full blood counts 46
3.10.2 Biochemical analysis of the blood sample of the SD rats 47
3.10.3 Alkaline phosphatase (ALP) test 48
3.10.4 Aspartate Aminotransferase (AST) and alanine transaminase (ALT) test 48
3.10.5 Total protein 48
3.10.6 Total bilirubin 49

3.10.7 Urea 49
3.10.8 Creatinine 50
3.11 Histology analysis 50
3.11.1 Preparation of hematoxylin and eosin reagent 50
3.11.2 Preparation of histology slide 50
3.11.3 Histology slide staining process 51
3.11.4 Histology analysis 52
3.12 Statistical analysis 52

CHAPTER 4: PHARMACOGNOSTICAL STANDARDIZATION OF *Ficus deltoidea* Jack LEAF 53
4.1 Introduction 53
4.2 Botanical identification 53
4.3 Quality control of FD leaf raw material 54
4.3.1 Physico-chemical analysis of FD leaf 54
4.3.2 Total extractive value 57
4.4 Safety assessment of FD leaf 58
4.4.1 Heavy metal analysis 59
4.4.2 Microbial limit test for the presence of specific pathogen in FD leaf 61
4.4.3 Aflatoxin assessment of FD leaf 63
4.5 Standardization of FD extracts using the HPLC technique 65
4.6 Conclusion 67

CHAPTER 5: ENZYMATIC INHIBITION PROPERTIES OF FD LEAF EXTRACT: COMPUTATIONAL AND EXPERIMENTAL APPROACHES 69
5.1 Introduction 69
5.2 Enzymatic assay of FD leaf 50% (v/v) ethanol water extract 69
5.2.1 The α-amylase inhibition assay 70
5.2.2 Inhibition activity of FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin on MMP-2, 8 and 9 71
5.3 Computational analysis of vitexin and isovitexin towards α-amylase enzyme 74
5.3.1 Molecular dynamic analysis of vitexin and isovitexin with α-amylase enzyme 77
5.3.2 The RMSD analysis of vitexin and isovitexin with with α-amylase enzyme 77
5.3.3 The radius of gyration analysis of vitexin or isovitexin interactions with with α-amylase enzyme 79

5.3.4 The RMSF analysis of vitexin and isovitexin interactions with α-amylase enzyme 81
5.3.5 The structural changes of vitexin-α-amylase complex and isovitexin-α-amylase complex during 20 ns simulation 83
5.4 Computational analysis of vitexin and isovitexin towards MMP-2, MMP-8 and MMP-9 enzyme 85
5.4.1 Simulations of vitexin and isovitexin toward MMP-2, MMP-8 and MMP-9 enzyme 89
5.4.2 Molecular dynamic analysis of vitexin–MMP-2 and isovitexin-MMP-2 complex 89
5.4.3 Molecular dynamic analysis of vitexin–MMP-8 and isovitexin-MMP-8 complex 95
5.4.4 Molecular dynamic analysis of vitexin–MMP-9 and isovitexin-MMP-9 complex 100
5.5 Discussion 105

CHAPTER 6: *IN VITRO* AND *IN VIVO* BIOACTIVITY OF STANDARDIZED FD LEAF 50 % (V/V) ETHANOL WATER EXTRACT 107
6.1 Introduction 107
6.1.1 DPPH inhibition assay 107
6.2 Cell culture study on FD leaf 50 % (v/v) ethanol water extract 109
6.2.1 Cytotoxicity activity of FD leaf 50 % (v/v) ethanol water extract 109
6.2.2 FD leaf 50 % (v/v) ethanol water extract inhibits migration of EA.hy926 cells. 112
6.3 *In vivo* study of FD leaf 50% (v/v) ethanol water extract 117
6.4 Toxicology study of FD leaf 50% (v/v) ethanol water extract 117
6.5 Behavioural observation of the SD rats 118
6.5.1 Biochemical analysis of SD rat blood for toxicity testing 120
6.5.2 Histology observation on the selected organs for toxicity evaluation 121
6.6 Wound healing properties of FD leaf 50 % (v/v) ethanol water extract 122
6.6.1 SD rat acclimatization 123
6.6.2 Preparation of STZ induced diabetic SD rats 123
6.6.3 Wound induction procedure for the SD rats 124
6.7 FD leaf 50 % (v/v) ethanol water extract wound healing properties using a normal non-diabetic and diabetic SD rats 126
6.7.1 Wound closure rate of treated SD rats in normal non-diabetic and diabetic SD rats 129

6.7.2 Hematology profile of treated SD rats in normal non-diabetic and diabetic….. SD rats.....135
6.7.3 Blood biochemical analysis of SD rats after wound healing treatment.....140
6.7.4 Histology study on the selected organs of the normal non-diabetic and diabetic SD rats in the wound healing study.....143
6.8 Conclusion.....150

CHAPTER 7: GENERAL DISCUSION AND CONCLUSION.....156
REFERENCES.....163
LIST OF PUBLICATIONS AND SEMINAR PRESENTED.....178
APPENDIX 181

LIST OF FIGURES

		Page
Figure 2.1:	Drug discovery process from medicinal plants source	8
Figure 2.2:	The leaf of *Ficus deltoidea* Jack	16
Figure 2.3:	FD chemical constituents	20
Figure 2.4:	The wound healing process	24
Figure 3.1:	The overall flow chart of the study	29
Figure 3.2:	The protocol for molecular simulation using GROMACS software	39
Figure 4.1:	The botanical specimen of *Ficus deltoidea* Jack variety Kunstleri	53
Figure 4.2:	HPLC chromatograms of three different extracts of FD leaf at the wavelength of 335 nm	66
Figure 5.1:	Graphical representation of α-amylase inhibition assay of FD leaf 50% (v/v) ethanol-water extract, vitexin and isovitexin. The symbol * represent the significant differences ($p < 0.05$).	71
Figure 5.2:	The results of MMPs inhibition assay for FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin	73
Figure 5.3:	Clustering analysis of vitexin and isovitexin on the α-amylase binding site after 100 runs	75
Figure 5.4:	Visualization of (A) vitexin and (B) isovitexin binding site on the α-amylase enzyme	76
Figure 5.5:	The RMSD analysis of (A) vitexin-α-amylase complex and (B) isovitexin-α-amylase enzyme complex	78
Figure 5.6:	The gyration analysis of (A) vitexin-α-amylase complex and (B) isovitexin-α-amylase enzyme complex	80
Figure 5.7:	The RMSF analysis of vitexin and isovitexin interactions with α-amylase enzyme	82
Figure 5.8:	The conformational changes of the secondary structure of α-amylase throughout the simulation.	84
Figure 5.9:	Autodock results for vitexin and isovitexin interactions against the MMP enzymes (A: MMP-2, B: MMP-8 and C: MMP-9) for 1000 runs	86
Figure 5.10:	Vitexin- and isovitexin-MMPs complexes obtained from the docking analysis.	88
Figure 5.11:	The RMSD graph for vitexin-MMP-2 and isovitexin-MMP-2 complexes	91

Figure 5.12:	The radius of gyration graph for vitexin-MMP-2 and isovitexin-MMP-2 complexes	92
Figure 5.13:	The RMSF graph for vitexin-MMP-2 and isovitexin-MMP-2 complexes	93
Figure 5.14:	The conformational changes vitexin-MMP-2 and isovitexin-MMP-2 complexes	94
Figure 5.15:	The RMSD graph for vitexin-MMP-8 and isovitexin-MMP-8 complexes	96
Figure 5.16:	The radius of gyration graph for vitexin-MMP-8 and isovitexin-MMP-8 complexes	97
Figure 5.17:	The RMSF graph for vitexin-MMP-8 and isovitexin-MMP-8 complexes	98
Figure 5.18:	The conformational changes vitexin-MMP-2 and isovitexin-MMP-2 complexes	99
Figure 5.19:	The RMSD graph for vitexin-MMP-9 and isovitexin-MMP-9 complexes	101
Figure 5.20:	The radius of gyration graph for vitexin-MMP-9 and isovitexin-MMP-9 complexes	102
Figure 5.21:	The RMSF graph for vitexin-MMP-9 and isovitexin-MMP-8 complexes	103
Figure 5.22:	The conformational changes vitexin-MMP-9 and isovitexin-MMP-9 complexes	104
Figure 6.1:	The DPPH scavenging activities graphical result of FD leaf 50% (v/v) ethanol-water extract, vitexin and isovitexin. The asterisk symbol (*) represent the significant values ($p < 0.05$)	108
Figure 6.2:	Photographs of Human endothelial hybrid (EA.hy926)	110
Figure 6.3:	Graphical representation of the dose dependent cytotoxic effect of the compounds tested	111
Figure 6.4:	Dose and time dependent inhibitory effect of FD leaf 50 % (v/v) ethanol water extract, vitexin and isovitexin on migration of EA.hy926 cells	114
Figure 6.5:	Graphical representation of the inhibitory effect of FD leaf 50 % (v/v) ethanol water extract, vitexin and isovitexin on migration of Ea.Hy926 cells.	115
Figure 6.6:	Dose Respone Curve, illustrating the effect of FD leaf 50 % (v/v) ethanol water extract, vitexin and isovitexin on migration of Ea.Hy926 cells in comparison to Vitexin and Isovitexin	116

Figure 6.7: The body weight trends of SD rats in the control and treatment groups 119

Figure 6.8: The food consumption percentage of SD rats in control and treatment groups 119

Figure 6.9: The water measurement percentage of SD rats in control and treatment groups 120

Figure 6.10: The kidney histology stained with Hematoxylin and Eosin for the control and treatment groups 122

Figure 6.11: Liver histology stained with Hematoxylin and Eosin for the control and treatment groups. 122

Figure 6.12: The blood glucose measurement level 124

Figure 6.13: Wound induction procedure 125

Figure 6.14: The body weight changes of the SD rats (average ± SEM) in the normal non-diabetic SD rats throughout the treatment period 127

Figure 6.15: The body weight changes of the SD rats (average ± SEM) in the diabetic SD rats throughout the treatment period 128

Figure 6.16: The wound closure rate (average ± SEM) for each group of the normal non-diabetic SD rats 132

Figure 6.17: The wound closure picture of the treated normal non-diabetic SD rats after wound treatment 133

Figure 6.18: The wound closure rate (average ± SEM) for each group of the diabetic SD rats 134

Figure 6.19: The wound closure picture of the treated SD rats of the diabetic SD rats after wound treatment 135

Figure 6.20: Histology slides (skins) of normal non-diabetic and diabetic SD rats for the wound healing study taken at 20 × magnifications. 144

Figure 6.21: Histology slides (liver) of normal non-diabetic and diabetic SD rats for the wound healing study under 20 × magnifications 146

Figure 6.22: Histology slides (kidney) of normal non-diabetic and diabetic SD rats for the wound healing study under 20× magnifications 147

Figure 6.23: Histology slides (pancreas) of normal non-diabetic and diabetic SD rats for the wound healing study under 20 × magnifications 149

LIST OF TABLES

		Page
Table 3.1:	Chemicals and reagents used for this study	30
Table 4.1:	Physico-chemical analysis of FD leaf	54
Table 4.2:	Yield of three different extractions of FD leaf	57
Table 4.3:	Heavy metal assessment of FD leaf	59
Table 4.4:	Microbial limit test for specific pathogen in FD leaf	62
Table 4.5:	Aflatoxin assessment of FD leaf	64
Table 4.6:	Quantification of vitexin and isovitexin in FD leaf extracts	65
Table 5.1:	The α-amylase inhibition assay of FD leaf 50% (v/v) ethanol-water extract, vitexin, isovitexin and acarbose	70
Table 5.2:	Interacting amino acid residues in ligand-protein complex	87
Table 6.1:	The DPPH scavenging activities of FD leaf 50% (v/v) ethanol-water extract, vitexin and isovitexin	108
Table 6.2:	Observations of Acute toxicity study (Treatment related changes)	118
Table 6.3:	The average ± (SEM) biochemical parameters value from the blood plasma of control and FD leaf 50 % (v/v) ethanol water extract group	121
Table 6.4:	SD rats in each group for the normal non-diabetic and diabetic SD rats	123
Table 6.5:	Full blood count analysis of normal non-diabetic SD rats in the wound healing study	138
Table 6.6:	Full blood count analysis of diabetic SD rats in the wound healing study	139
Table 6.7:	The average ± (SEM) biochemical parameter value from the blood plasma of treated group of normal non-diabetic SD rats	141
Table 6.8:	The average ± (SEM) biochemical parameter value from the blood plasma of treated group of diabetic SD rats	142

LIST OF SYMBOLS AND ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine transaminase
As	Arsenics
AST	Aspartate transaminase
BSA	Bovine serum albumin
CADDs	Computer-aided drug discovery techniques
Cd	Cadmium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	DL-dithiothreitol
ECM	Extracellular matrix
FD	*Ficus deltoidea*
GPUs	Graphical processing units
h	hour
Hg	Mercury
HPLC	High performance liquid chromatography
HTS	High throughput screening
IC_{50}	Median inhibition concentration
kPa	Kilopascal
MD	Molecular dynamics
ml	milliliter
mM	millimolar
MMP	matrix metalloproteinase
µg/ml	microgram per milliliter
µm	micrometer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

nm	nanometer
NNGH	N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid
ns	nanosecond
Pb	Plumbum (Lead)
PBS	Phosphate buffered saline
PDB	Protein data bank
QSAR	Quantitative structure–activity relationship
RMSD	root mean square deviation
RMSF	root mean square fluctuation
rpm	Rotation per minute
RVSEB	Rappaport Vassiliadis Salmonella
SCD	Soybean-Casein digest agar
SD	Sprague Dawley
SDA	Saboroud dextrose agar
STZ	Streptozotocin
UV	Ultraviolet
v/v	volume per volume
2D	2 Dimensions
3D	3 Dimensions
Å	Angstrom

LIST OF APPENDICES

	Page
APPENDIX A	181
APPENDIX B	182
APPENDIX C	189
APPENDIX D	195

CHAPTER 1: INTRODUCTION

1.1 Background

Medicinal plants provide valuable source of pharmacologically active compounds. Many plants have been employed as natural remedies for certain diseases with evidences on its therapeutic activities proven through scientific studies. In Malaysia, there are many promising medicinal plants that can be develop as herbal drugs and *Ficus deltoidea* (FD) is one of the examples.

Native to Southeast Asian countries such as Malaysia (Corner, 1969), *Ficus deltoidea* (FD) is a shrub that is gaining interest for its therapeutic properties. Furthermore, FD is one of the 10 spotlight plants proposed by the Ministry of Agriculture of Malaysia under the high value herbal products plan. FD contains interesting therapeutic potentials and is reputable as traditional medicines and supplements. In an ethno-pharmacological manner, FD leaf is widely used to treat labor pain, toothache, pulmonary edema and blood pressure. Through scientific evidences, FD was found to delivered various pharmacological effects such as anti-nociceptive activity (Sulaiman *et al.,* 2008), antidiabetic activity (Adam *et al.,* 2010; Misbah *et al.,* 2013), anti-melanogenic (Oh *et al*., 2011), antioxidant (Misbah *et al.*, 2013), anti-ulcerogenic (Zahra *et al*., 2009), anti-inflammatory (Abdullah *et al.,* 2009), antimicrobial, anti-cancer (Wei *et al*., 2011) and anti-hypertensive properties (Abdullah *et al*., 2008; Razali *et al*., 2013). From the phytochemical aspects, FD was reported to have various polyphenolics, tannins, and flavonoids such as flavan-3-ol monomers, proanthocyanidins, and C-linked flavone glycosides (Abdullah *et al*., 2009; Omar *et al*., 2011), rutin, quercetin, naringenin (Ong *et al.,* 2011) and lupeol (Suryati *et al*., 2011).

Although the numbers of scientific evidences on FD therapeutic potential are increasing, the understanding of how the active compounds in FD act at molecular level

seems to be lacking. Furthermore, every reported research regarding FD leaf extraction applying different extraction methods that extracted different phytochemicals leading to variation in FD leaf extracts bioactivity. In this study, a standardized procedure to extract therapeutic potential phytochemical based on 50% (v/v) ethanol water was organized according to the safety and guideline by World Health Organization (WHO, 2016) to ensure the safety, quality and repeatability of the extract.

In order to study the bioactivity of the standardized FD leaf extract, a panel of *in vitro* and *in vivo* experiments was conducted. *In vitro* antioxidant activity was studied using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, while the cytotoxicity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on EA.hy926 cells. The inhibitory effect of FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin was also evaluated on EA.hy926 cells. To further analyse the pharmaceutical potential of FD leaf 50% (v/v) ethanol water extract and its major phytochemical compounds (vitexin and isovitexin), enzymatic inhibition activity was studied against a selected panel of enzymes namely, α-amylase and matrix metalloproteinases (MMPs: MMP2, MMP8 and MMP9). The α-amylase enzyme was selected as a known target in anti-diabetic treatment. While, MMPs especially MMP2, MM8 and MMP9 are known to involve in pathological conditions such as diabetes, obesity, cancer and poor nutritional condition (Caley *et al.,* 2015). Computational analysis such as molecular docking was conducted to the extract major phytochemical compounds (vitexin and isovitexin) against the selected enzymes complex to analyse the molecular interaction between the ligands to the enzymes. Additionally, molecular dynamics simulation was conducted to the ligand-protein complexes up to 20 ns to analyse the stability of the ligand-protein complexes. *In vivo* study was conducted using 2 types of animal models. The healthy Sprague-Dawley rats was used as one of the model animal in this study, while streptozotocin-induced diabetic Sprague-Dawley rats

was used to simulated diabetic condition. Acute toxicology test was conducted at 1000 mg/kg in the model animals and visual inspection was conducted for 14 days. At day 14 the animals were scarified for blood analysis and histological study. To analyse the wound healing capability of the FD leaf 50% (v/v) ethanol water extract, both animal models were induced with wound and were treated with the extract as well as the major phytochemical compounds of the extract (vitexin and isovitexin) for 14 days and the rate of wound closure was measured.

This thesis reports a comprehensive analysis combining both experimental and computational techniques to explore the therapeutic properties of FD leaf extract. The results of this study would help to unravel the therapeutic potential of FD leaf extract especially for the development of drugs targeting wound healing in diabetic patients.

1.2 Research Questions

The main research question in this study is to unravel the biological activities and therapeutic potential of FD leaf extract using computational and experimental approaches.

1.3 Research Objectives

To answer the questions, three objectives were addressed:

1. To obtain pharmacognostical standardize FD leaf extracts.
2. To evaluate the enzymatic inhibition properties of FD leaf extract using computational and experimental approaches.
3. To investigate the biological properties of FD leaf 50 % (v/v) ethanol water extract using *in vitro* and *in vivo* methods.

1.4 Thesis Organization

The content of the thesis is divided into several chapters: Chapter 1: Introduction, Chapter 2: Literature review, Chapter 3: Methodology, Chapters 4, 5 and 6: Results and discussion and Chapter 7: General Discussion and Conclusions. Chapter 1 described the introduction, research questions and objectives of this research. Chapter 2 elaborated on the literature reviews of the standardization, bioinformatics technique in drug discovery, the potential of FD plant and MMPs related disease. Chapter 3 explained the techniques used for plant preparation, standardization, computational and experimental approaches to unravel the therapeutic potential of FD. Results chapters 4, 5 and 6 discussed the results and findings of the standardization, computational and experimental procedures of understanding the therapeutic activities that FD delivers. Chapter 7 described the general discussions and conclusion from results that were obtained throughout this study.

CHAPTER 2: LITERATURE REVIEW

2.1 Medicinal plants

Medicinal plants are plants with bioactive compounds that promote therapeutic activities. The field that studies the use of plant as traditional remedies is known as ethno-pharmacology (Mahmudur & Rafieian-Kopaei, 2017). Traditional used of plants can be traced back since the Sumerian civilization in which hundreds of plants are inscribed on clay tablets (Petrosvska, 2012). Ancient Egyptians had described more than 850 medicinal plants in the Eber papyrus (Petrosvska, 2012). Pedanius, an ancient Greek herbalist listed more than 1000 recipes of over 600 plants in his book, De materia medica that are the basis of medicinal pharmacopeia (Anne, 2014).

Herbal medicines have been preferred by consumers worldwide as a source of remedies (Wachtel-Galor & Benzie, 2011). Preparations of plant for medicinal purposes have been widely utilized for centuries, providing primary health care in most of the Asia regions (Chaudhury & Rafei, 2001). In developed countries, medicinal plants incorporated with modern medical science are prepared as a part of complimentary medical treatment (Pan *et al.,* 2014) and being used as treatments for chronic diseases (Roy, 2010). Due to its chemical diversity, medicinal plants provide limitless opportunities in drug discovery and development. Although plants may bear potential as therapeutic medicine, the bioactive compounds within them vary (Ekor, 2013). Several factors affect production of these active compounds such as environmental factors, climate, the soil used and others (WHO, 2005). Therefore, a guideline and standardized procedure of plant extraction must be prepared to bring the full potential of the plant medicinal values and to ensure the safety of the plant extract.

The term ‘standardization’ can be defined as collections of information obtained from various testing that involved various applications of quality control and assessment protocols (Garg *et al.,* 2012). In other word, standardization of extraction procedure promised chemical consistency, therapeutic efficacy and reproducible pharmacological activity of the plant extract (Willard, 1996; Bauer, 1998; Page, 2001). Standardization data are associated with the pre- and post-harvesting data, such as how the plant is being planted, harvested and stored (Bauer, 1998, WHO, 2016). The quality assessment and control are closely monitored to produce reliable and functioning extracts (Hildreth *et al.,* 2007). Extraction procedure standardization is a first step to rationalize a phytopharmaceutical product for consideration as drugs (Garg *et al*., 2012). Nowadays, regulatory authorities emphasize the importance of medicinal plants being standardized, requiring the drug measurability and active ingredient being to be stated on the label as stated by the World Health Organization guideline for pharmaceutical research (WHO, 2006). The guidelines involve evaluations of raw material, herbal preparation, safety assessments, end products and stability of the finished products. As for the safety assessment, plants are introduced to tests that detect the content of microorganisms such as food pathogens and opportunistic fungi. The end results of the standardization process will give valuables information on proper techniques in handling delicate herbal materials as well as provided reproducible result for further phytopharmaceutical analysis (Evans, 1996; Kokate *et al.*, 1999; Hyland, 2002; Garg *et al.*, 2012).

2.2 Drug discovery and development from medicinal plants

Currently, the hunt for new drug derived from medicinal plant is expanding. In general, the process of drug discovery from the medicinal plants (Figure 2.1) can be divided mainly into 3 groups which are the pre-drug, quasi-drug and full drug stage (Pan *et al.,* 2013).

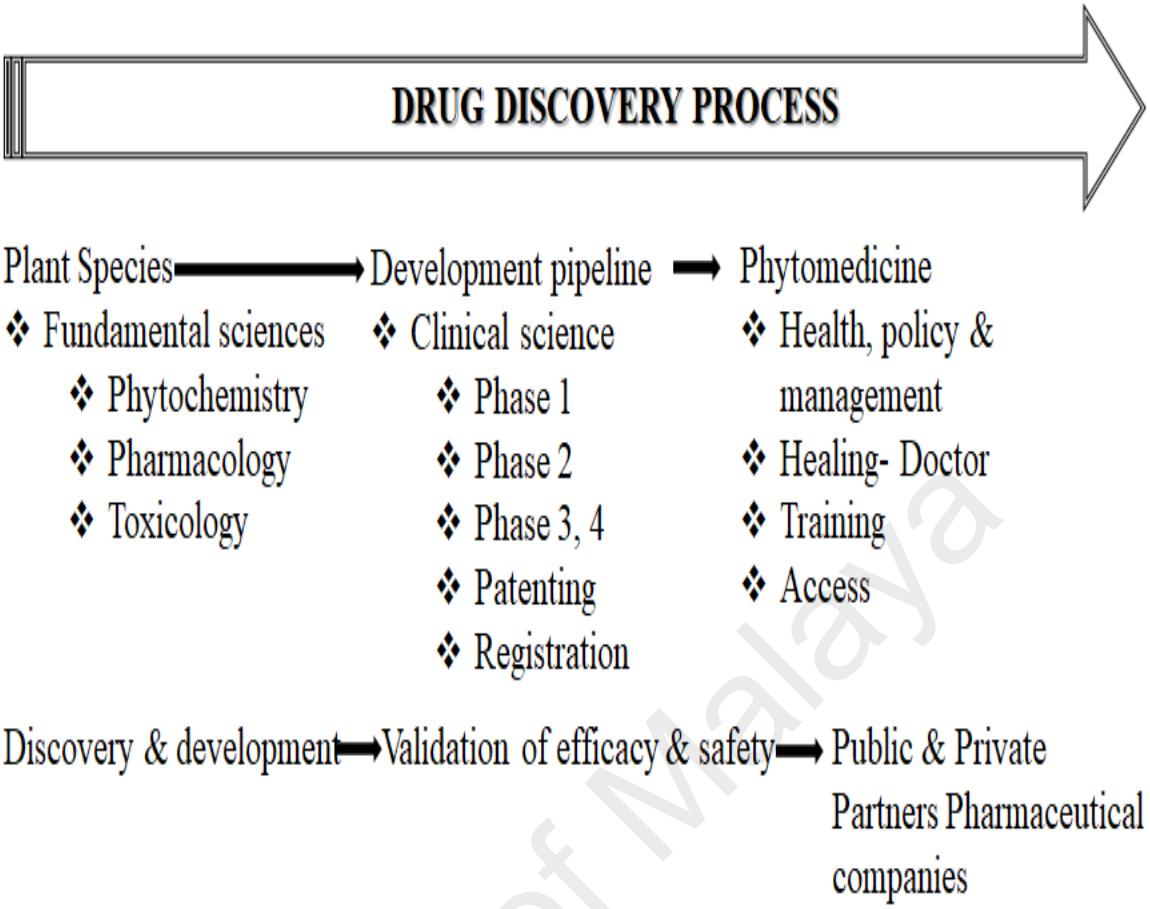


Figure 2.1: Drug discovery process from medicinal plants source adopted from Pan et al. (2013). The steps involved are discovery and development of potential phytopharmaceutical compounds. The development pipeline involved validation of plant efficacy and safety which are important in the process of developing phytomedicines. Clinical sciences determine whether the phytomedicines are safe or hazardous for treatment of diseases in human.

Pre-drug phase is the initial stage of drug discovery in which selection of medicinal plants based on the pool and compilation of information available through scientific evidences (Pan *et al.,* 2012). The medicinal potential of plants as a source for drug development is attaining interest and sought out by researchers leading to a diverse compilation of over 139000 natural products (Boopathy & Kathiresan, 2010). For instance, from 3000 traditional herbal medicines (THMs), a collection of 3563 extracts and 5000 single compounds have been documented in China by 2007 (Deng, 2007), while in USA, a collection of more than 114000 extracts from 35000 plant samples that was documented before the 1990s (Boopathy & Kathiresan, 2010). These compounds that were discovered and isolated are potential candidates for drug discovery. Clinical evidences from the practice of THM eventually engage in the development of modern medicine in which in China alone, an estimated around 140 new drugs such as bicycol, indirubin and anisodamine depends on the compounds either directly isolated from the extracts or chemically synthesized compounds during the clinical trials (Puopko *et al.*, 2007; Xiang & Sheng, 2009).

The quasi-drug stage involves herbal preparation and efficacy testing based on the phytochemicals available in plants (Sasidharan *et al.,* 2011). In this phase, the use of modern and state-of-the art technology provided an in-depth understanding of the compounds extracted out from the plants. These metabolites hold vast potential as therapeutic agents, such example includes the polysaccharides either mono or disaccharides accompanied together by glycosidic bonds delivers immune suppressing effects (Ramberg *et al.,* 2010). Plants containing immunomodulatory polysaccharides are the *Ganoderma lucidum* (Xu *et al.,* 2011), Acai berries (Holderness *et al.,* 2011) and *Cordyceps* sp. (Xu *et al.,* 2011). Another example is flavonoids, which available in almost all plants bear the potentiality in producing therapeutic effects to human. Research findings have proven that flavonoids such as flavones, isoflavone and

xanthone contribute significant outcomes as anti-oxidant, anti-proliferative, anti-diabetic, anti-inflammatory and anti-hypertensive activities (Leornarduzzi *et al.,* 2010; Holderness *et al.,* 2011; Im *et al.,* 2012; Prochazkova *et al.,* 2011; Jäger & Saaby, 2011). Plants collected over the harvesting period are dried either using oven or air-dried technique. The dried raw materials will be transformed into fine powder and extracted following the desired solvents. Normally, water or organic solvents are employed to make the extract. The extracted form of the plants will be introduced to spectrophotometric and chromatographic analysis to understand and unravel the compounds available in the plant extract. Following that is the isolation methods in which the potential compounds will be isolated and purified for biological testing. To summarize, this phase of drug discovery relies on the search of newly therapeutic potential of active ingredients that are available in plants for further drug development.

The full drug stage will take place after the quasi drug phase has been accomplished. In this phase, active ingredients of plants are being investigated through a series of biological testing. Designing a pre- and clinical research is important to unravel the therapeutic properties of the desired plants' active ingredient. In general, there are several phases in the full drug stage. The preclinical phase utilized the testing of drug in non-human subject to understand the efficacy, kinetic and toxicity information (Umscheid *et al.,* 2011). Phase 0 is the second step of the full drug stage with the pharmacokinetics particularly the half-life of drug and oral bioavailability sets as the main goals (Editors, 2009).

This phase enables the go/no-go decisions based on relevant human models instead of relying on the *in vivo* data which sometimes can be misleading. The next stage is the phase I, involving the testing of drugs on healthy volunteers to determine the dose ranging (Norfleet & Gad, 2009). Usually, around 20 up to 100 people are needed in this stage with approximately 70% of success rate. The doses are sub

therapeutic, but ascending throughout the research period. Phase II of drug research incorporated the patients as the main subjects to evaluate the efficacy of the drug used. Therapeutic doses are given to the patients (around 100-300 patients with specific diseases) are the success rate reported was found to be approximately 33 % (Friedman *et al.,* 2015). Phase III involved a larger number of participants up to 3000 patients with specific diseases. The main goal of this phase is to understand the efficacy, effectiveness and safety of the drug given for treatment (FDA, 2017). The patients will be monitored by clinical physician and researchers with success rate up to 30 % were reported. The last stage of full drug research is the phase IV, the post-marketing surveillance period. During this phase, anyone seeking for the drug will be closely monitored to watch the drug's long-term effects. If the drug brings harmful effects to human during this phase, the drug will be withdrawn from the market and may result in the drug no longer be sold or restricted for certain uses (FDA, 2017).

2.3 Bioinformatics and computational approaches in drug discovery process

Traditional approaches in finding new drug are time consuming and costly (Taylor, 2015). There are many challenges that researchers face when trying to come out with compounds that can potentially be developed as drugs. The step-by-step process is highly competitive where the "winner takes it all" and the "loser loses it all" (Garnier, 2008; Iskar et al., 2012). The first to patent their findings for specialized treatment will eminently benefits term of the gross revenue that they make from marketing the drug where the rest need to wait until the patent expired for them to partake in the largesse (Iskar *et al.,* 2012). The increase demand and pressure in the race to discover new drugs are making the big companies to invest heavily in the research and development phase (Garnier, 2008). Most of these companies rely on potential accelerated phase in drug discovery (Whittaker, 2003). For example, the bioinformatics approaches are gaining interest due to the low risk involved and faster outcome

compared to the conventional procedures (Ortega et al., 2012). Prediction and identification of active compound candidates are among the major core of bioinformatics approaches. In fact, the computer-aided drug design was existed due to the high demand for the sophisticated and faster drug discovery tool (Song *et al.,* 2009).

Data mining and database can be used to determine the drug target identification (Chen & Chen 2008; Katara *et al.,* 2011). Currently, the numbers of potential drug targets have increased exponentially. Such example is the process of mining and warehousing data from the human genome sequence, which really help researchers in classifying the gene responsible to code a targeted protein (Yamanishi *et al.,* 2010). Furthermore, this technique helps researchers identify and validate new targets that allow potential for new drugs (Ratti & Trist 2001; Gilbert *et al.,* 2003; Whittaker, 2003). Researchers are blessed with the luxury of choices as new genes are identified and becoming more data-intensive, allowing identification and analysis through bioinformatics pipeline that greatly improve the pharmaceutical companies to determine drug candidates (Whittaker 2003; Ortega *et al.,* 2012).

The processes of drug validation through bioinformatics approaches provided strategies based on algorithms so that prediction of a new drug target can be achieved, stored, and manage (Cheng et al., 2011). When potential candidates are identified, establish, and produces significant outcomes with targeted enzymes based on the disease of interest (Barabasi *et al.,* 2011; Billur *et al.,* 2014). Such technique that can be used in determining, identify and validate potential candidate for drug discovery is the virtual screening technique (Katsila *et al.,* 2016).

Molecular docking in general is a prediction of a stable complex between the two molecules bonding at a preferred orientation (Yuriev *et al.,* 2009). This method is used to predict and verify potential active compounds candidate with a specific targeted protein binding orientation. Available docking libraries and software such as Autodock

(Morris *et al.,* 2009), AutoDock Vina (Trott & Olson, 2010) and Dock (Moustakas *et al.,* 2006) enable the prediction of binding site, binding affinity of the ligand to the target protein leading to the discovery of new drug candidates (Kumar *et al.,* 2012). Ligand obtained from database are in 3D configurations that can be manually generated using open source bioinformatics tool such as Vegazz (Pedretti *et al.,* 2004) and Avogrado (Hanweel *et al.,* 2012). Molecular docking provides references to researchers on how two or more molecular structures such as macromolecules and potential drug or catalyst (ligand) match along to be a perfect fit (Gane & Dean, 2000). The most commonly used molecular docking technique is the protein-ligand based docking. This docking technique predicts the biding orientation of potential drug candidates towards the selected macromolecules by giving information on the affinity and molecular interactions (Hakes *et al.,* 2007).

AutoDock is a molecular docking suite consisting of automated docking tools. AutoDock is considered one of the best programs when it comes to docking and virtual screening (Park *et al.,* 2006). Consists of two main programs, AutoDock and AutoGrid, AutoDock docks the two molecules according to the grid, which is pre-calculated and set by AutoGrid. Various possible problems must be resolved which includes missing atoms, chain breaks, and alternate locations before a protein can be used for AutoDock. These grids represent the energy calculations, and in their most basic form, the grid stashes two types of potentials: the electrostatic and the van der Waals force. The grid was formulated so that the information about the receptor's energy contributions could be stored on grid points. This allowed the necessity of it being read only during ligand scoring. More options can be explored in AutoDock, and the options may vary depending on the complexes that are being docked and the complexity of the problem in hand. The application of docking in a targeted drug-delivery system brings benefit especially when it comes to the study of protein interactions. One can study the size,

shape, charge distribution, polarity, hydrogen bonding, and hydrophobic interactions of both ligand (drug) and receptor (target site). The benefits include identification of the target sites of the ligand in the selected protein, understanding the mechanism of different enzymes, and selecting the best fitted ligand based on the scoring feature provided by AutoDock. Traditional experimental methods for drug discovery is time consuming whereas the molecular docking helps in moving the process of computer-aided drug designing faster and provides every conformation possible based on the receptor and ligand molecule.

Another useful computational approach that can be use in drug discovery is the molecular dynamic simulations. In concept, molecular dynamic simulations stimulated the existing natural molecular forces of protein structure (Hospital *et al.*, 2015) within a time according to the Newton's law of physics (DeVivo *et al.*, 2016). Potentially, the molecular dynamics promise fast processing of data using current technologies in graphical processing units (GPUs), using software codes and are surprisingly affordable. Through utilizing the GPUs (Liu *et al.*, 2008; Shan *et al.*, 2011), researchers can observe the behavior of a compound with targeted enzyme during a period that can be up to a few milliseconds. This provided opportunities to investigate and perform a thorough sampling of a conformational space, including large enzyme. In example, the complex of ligand and enzyme through molecular dynamics can describe the complete pathway of the binding site (Gordon *et al.*, 2016). Additional, the trajectory that the molecular dynamics made can be coupled with free energy which in return, will provide a free-energy profile of protein-ligand complex, with the kinetic and thermodynamic data being critically important for the process of drug discovery (Swope *et al.*, 2004).

GROMACS is one of the most widely used open-source and free software codes in chemistry, used primarily for dynamical simulations of biomolecules. It provides a rich set of calculation types, preparation and analysis tools. GROMACS provided

information on the stability and movement rotation of the protein based on the root-mean-square deviation (RMSD) and fluctuation (RMSF), as well as the radius of gyration. The RMSD described the deviation of an atom superimposed with the selected proteins. At a defined average position, when a dynamical system fluctuates from the RMSD, it can be referred as the RMSF. The fluctuation size can be measured and analyzed to describe the physical information of the protein ligand complex. Radius of gyration is the length that represents the distance in the protein complex between the point about which it is rotating and the point to or from which a transfer of energy has the maximum effect. On simpler term, the rotation of the protein-ligand complex can also be measured using the GROMACS software. Altogether, these are among the key data that GROMACS provides to describe the stability of the protein complex at a given time. These findings will benefits researcher in understanding the molecular behavior of the drug candidates with selected protein under molecular dynamic simulations technique.

Integrating molecular dynamic simulations right after the docking procedure allows a concise and better understanding of the 'after effect' of ligand docking and the induced fit effect towards targeted proteins. It is beyond doubt that with the rise of technology, the computational methods provided a powerful insight for identification, discovery and optimization of the target drug molecules. The information coupled with statistic and bioinformatics tool tremendously shed light to the pathophysiology of certain disease as well as revealing the potential target to be further validated through high throughput screening technologies.

2.4 *Ficus deltoidea* Jack (Mas cotek) - a promising medicinal plant for wound healing

Ficus deltoidea is a shrub from the family of Moraceae that are native to South East Asia regions including Malaysia. It is commonly known as Mas cotek in the Malay language and this mistletoe fig can reach up to 2 m in height with the leaf shaped characterized as a broadly spoon to obovate shape with 4-8 cm in diameter (Figure 2.2).

Figure 2.2: The leaf of *Ficus deltoidea* Jack

For many years, the FD plant was ethno-pharmacologically used as remedies to treat various diseases such as rheumatism, sores, and wound (Ong *et al.*, 2011). It is also known to provide benefits for those who are dealing with afterbirth as it is believed to contract the vaginal and uterus muscle (Bunawan *et al.*, 2014). Apart from that, the boiled leaf was used to traditional treats disorders involving diabetes, menstrual cycle and leucorrhoea (Burkill & Haniff, 1930). The fruits itself are also traditionally used as cold, headache and toothache relief (Bunawan *et al.*, 2014).

Evaluation of the aqueous FD leaf extract on ferric reducing antioxidant potential (FRAP) assay and the total phenolic content by Omar *et al.* (2011) suggested that the compounds in FD extract which is the proanthocyanidins and flavan-3-ol monomers provided 85 % of the antioxidant activities. FD leaf was also reported to have anticancer properties. Akhir *et al.* (2011) studied the activity of ethanol and aqueous extract of FD on the ovarian carcinoma cell line A2780. It was described through microscopic examination that both extracts showed different outcomes with the ethanol extract abate the proliferation activity and the aqueous stimulate the cell detachment. Another study by Oh *et al.* (2011) on B16F1 melanoma cells reported that FD possessed anti-melanogenic effects. The FD extract when applied on the Microphthalmia-associated transcription factor (MITF) expression showed decreasing activity, suggesting that the FD extract having good potential to be a cosmeceutical agent. Hasham et al. (2013) described that FD extract can significantly obstruct the COX-2, IL-1α, IL-6, and TNF-α expression. FD extract was also reported to have antimicrobial activities. The FD leaf extract of methanol, chloroform and petroleum ether inhibits the growth of *Helicobacter pylori* (Uyub *et al.,* 2010). The methanol extract was also found to hinder the growth of *Bacillus subtilis* (Jamal *et al.,* 2011), *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* (Samah *et al.,* 2012).

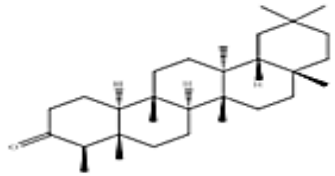
As anti-diabetic agent, a study conducted by Adam *et al.* (2009) evaluates five different extracts and the fraction of FD to boost the basal and the insulin stimulated glucose uptake using Chang liver cell line. The finding suggested that apart from the petroleum ether, FD holds promising activity to promote the stimulation of basal and insulin stimulate glucose uptake and ethanol extract was mentioned to benefit the most in mimicking the insulin activity. Farsi *et al.* (2011) conducted an experiment using the FD leaf extracted in a fraction of n-butanol and evaluated it using the α-amylase and α-glucosidase enzymes. It was shown that this fraction inhibits both enzymes that could benefit postprandial hyperglycemia. However, the compound that exhibited the inhibition property was not clarified in this study. Darman *et al.* (2012) reported that no significant changes in fasting blood glucose, renal and lipid profile of type 2 diabetic patients upon treatment with FD leaf extract, while, Kalman *et al.* (2013) reported FD extract significantly reduced the lipid and glucose level in pre-diabetic adults.

FD was also reported to be a potent anti-inflammatory agent. Zakaria *et al.* (2011) carried out a study using the aqueous extract of FD leaf on the formalin, granuloma, carrageenan-induced paw edema and cotton pellet-induced granuloma test. A dose dependent activity of the FD leaf extract was observed on all the tests studied, suggesting the potential of FD to treat various pain-associated inflammatory responses. Abdullah *et al.* (2009) conducted an experiment to study the inflammatory responses triggered by the introduction of standardized extracts of different varieties of FD. The tests were TPA-induced edema, hyaluronidase and lipoxygnase, all are in-vitro assays in which the FD showed significant anti-inflammatory activities. As a pain reliever, FD leaf of aqueous extract was reported to have anti-nociceptive activity (Sulaiman *et al.,* 2008). In this study, the stimuli were thermal and chemical nociception model and three different methods were used which is the formalin, hot plate and acetic acid-induced abdominal writhing test. The significant anti-nociceptive effect can be observed after 30

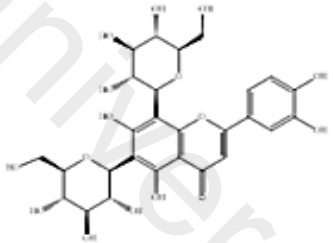
minutes prior to pain induction suggesting that FD could be a useful agent to treat pain. As anti-ulcerogenic agent, Zahra *et al.* (2009) evaluated the FD whole plant extract using the Sprague Dawley rats with gastric ulcer induced by ethanol. The observation showed that The FD extract showed dose dependent manner as anti-ulcerogenic agent proposing that FD has benefits in protection against ulcer. As a wound healing agent, Abdulla *et al.* (2010) evaluated the extract of FD on wounds in the Sprague Dawley rats. Treatments were with placebo with 5% and 10% of the FD extract was shown to contribute faster compared to the blank placebo and negative control treatments. The outcome of this research showed that the wound treated with FD has decreased scar formation alongside with more proliferation of the fibroblast and more collagen fiber in the granulation tissue.

2.4.1 Phytochemicals in FD

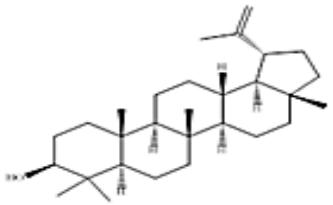
It has been reported that FD contains various phytochemicals and these compounds had been isolated and characterized from the leaf and the fruits (Figure 2.3). For the leaf, Ong *et al.* (2011) described naringenin, quercetin and rutin as among the flavonoids group available in the FD leaf. Moretenol was also shown to be available in the leaf as per isolated and identified by Mohd *et al.* (2009). Lupeol was also mentioned among the active triterpenoids available in the FD leaf (Surayti *et al.,* 2009). Using HPLC-MS methods, a throughout study on the FD leaf compounds revealed that numerous flavonoids are available in the aqueous extract (Omar *et al.,* 2011). This report elaborated more than 25 flavonoids are existed and identified. The group of compounds existed in the aqueous extract were the Shikimic acid, monoterpenes, Sesquiterpenes, for the fig. For the leaf it consists of flavonoids, terpenes and triterpenes. As for the cell culture, rutin, naringenin and quercetin were the flavonoids available.



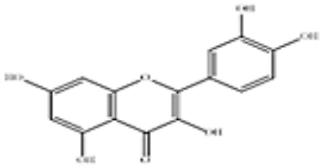
Friedelin (Zunoliza et al., 2009)



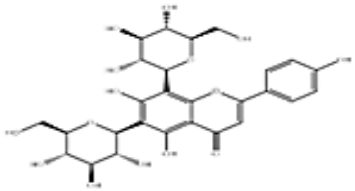
Lucenin-2 (Omar et al., 2011)



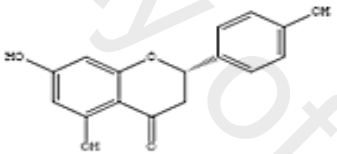
Lupeol (Suryati et al., 2011)



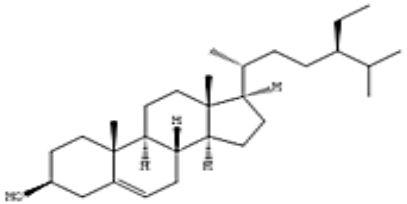
Quercetin (Ling et al., 2010)



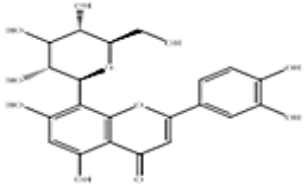
Vicenin-2 (Omar et al., 2011)



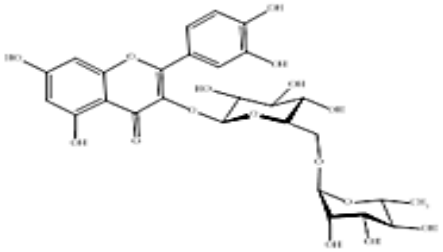
Naringenin (Ling et al., 2010)



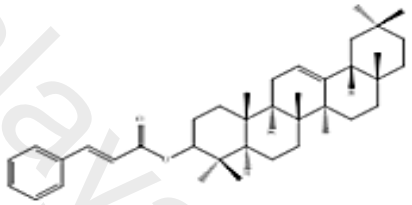
β-sitosterol (Zunoliza et al., 2009)



Orientin (Zunoliza et al., 2009)



Rutin (Ling et al., 2010)



β-amyrin cinnamate (Zunoliza et al., 2009)

Figure 2.3: FD chemical constituents

2.5 Wound healing physiology

2.5.1 Angiogenesis

Blood is an important component in human physiology. All living tissues rely on oxygen and nutrients that are supplied by blood through a massive circulation pathway called the blood vessel. The physiological process involved in forming blood vessels from the existing vascular is known as angiogenesis. Angiogenesis are formed throughout the human life cycle starting from the embryonic stage and continuing through old age. The process of forming new blood vessel through angiogenesis is regulated in a subtle manner involving the angiogenic factors, endothelial cell and the extracellular components. These capillaries formed are vital for the diffusion exchange of metabolites and nutrients to the tissue.

Comprehensively, angiogenesis plays an important role in human physiology such as in wound healing process, reproduction, human growth and development. However, if the tightly regulated angiogenesis is disrupted, it may lead to several disorders such as vascular overgrowth and vascular insufficiency. Therefore, the control of angiogenesis is recognized to have potential as therapeutic approaches in the treatment of vascular related disease. For instance, when angiogenesis is stimulated, it helps in the treatment of heart disease and wound healing disorder. By decreasing or inhibiting angiogenesis, it can be a therapeutic value in treating disease such as cancer, tumor, and rheumatoid arthritis.

2.5.2 Matrix metalloproteinases (MMPs)

The initiation of angiogenesis is influenced by the pro-angiogenic factors such as the VEGF and bFGF triggered from the existing disease cell such as tumor or inflammatory cells. This process then leads to the activation the Matrix metalloproteinases (MMPs) from the endothelial cells. MMPs are a group of Zn-dependent endopeptidases (Verma & Hansch, 2007) with more than 25 members. The

members of this large family can be divided into collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and 9), stromelysins (MMP-3 and 10), matrilysins (MMP-7 and 26), and the membrane-type MMPs (MMP-14 to 17 and 24). In general, the enzymes degrade numerous extracellular matrix (ECM) proteins (Nagase *et al.,* 2006) leading to the facilitation of cell migration. Furthermore, MMPs activities would release the membrane bound signaling molecules such as chemokines, cytokines and growth factors (Sternlicht & Werb, 2001). Physiologically, MMPs involve in many biological processes such as embryogenesis, reproduction, angiogenesis, tissue remodeling and in various aspects of immunity (Balbin *et al.,* 1998; Nguyen *et al.,* 2001; Klein & Birschoff, 2011). Under normal physiological conditions, the regulation of the MMPs activities is precisely regulated by the level of transcription, interaction with specific ECM components and inhibition of endogenous inhibitors (Fanjul-Fernandez *et al.*, 2010).

Although the MMPs are tightly regulated in physiological condition, under certain pathological condition these regulations can be disrupted. Elevated activity of MMPs such as MMP-1, MMP-2, MMP-3, MMP-9, MMP-8 and MMP-10 was reported with the presence of chronic inflammation in diabetes, obesity, cancer and poor nutritional condition (Nwomeh *et al.,* 1999; Krampert *et al.,* 2004; Mulholland *et al.,* 2005; Pirila *et al.,* 2007; Danielsen *et al.,* 2011). These MMPs activities were reported to cause problems such as slower healing rate, cancer metastasis and diabetic ulcer (Nwomeh *et al.,* 1999; Krampert *et al.,* 2004; Mulholland *et al.,* 2005; Pirila *et al.,* 2007, Danielsen *et al.,* 2011). Inflammation process stimulates angiogenesis and tissue remodeling, which contribute to cell migration (Wu *et al.,* 2014). Many studies have shown that chronic inflammation stimulates proliferation and migration of cells due to the release of matrix metalloproteinases (MMPs) from the inflammatory cells (Garg *et al.,* 2010).

2.5.3 Normal and impaired wound healing process

In the wound healing process (Figure 2.4), the matrix metalloproteinases (MMPs) are express rapidly. Activation of the MMPs within the skin is due to the responses of cytokines, hormones and other type of cells within the extracellular matrix. This triggered cell such as keratinocytes, fibroblast and anti-inflammatory cells like the monocytes, lymphocytes and macrophage to act upon being called. The first step is rapid hemostasis where our blood vessel constricts (vasoconstriction) to prevent further blood loss. Next, inflammation occurs in which it signals our body to regenerate in which it releases healthy cells to migrate and proliferates to heal the wounded area. Then, angiogenesis occurs where once the bleeding is under control, the process of building tissues take place. The affected veins and arteries are replaced with new blood vessel with the signaling of chemical activating cascades in our body. Re-epithelization happens once the veins reformed in which the epithelial cells (keratinocytes) will reproduce in the damages skin. Lastly, certain proteins produce blood clots to help further in preventing bleeding.

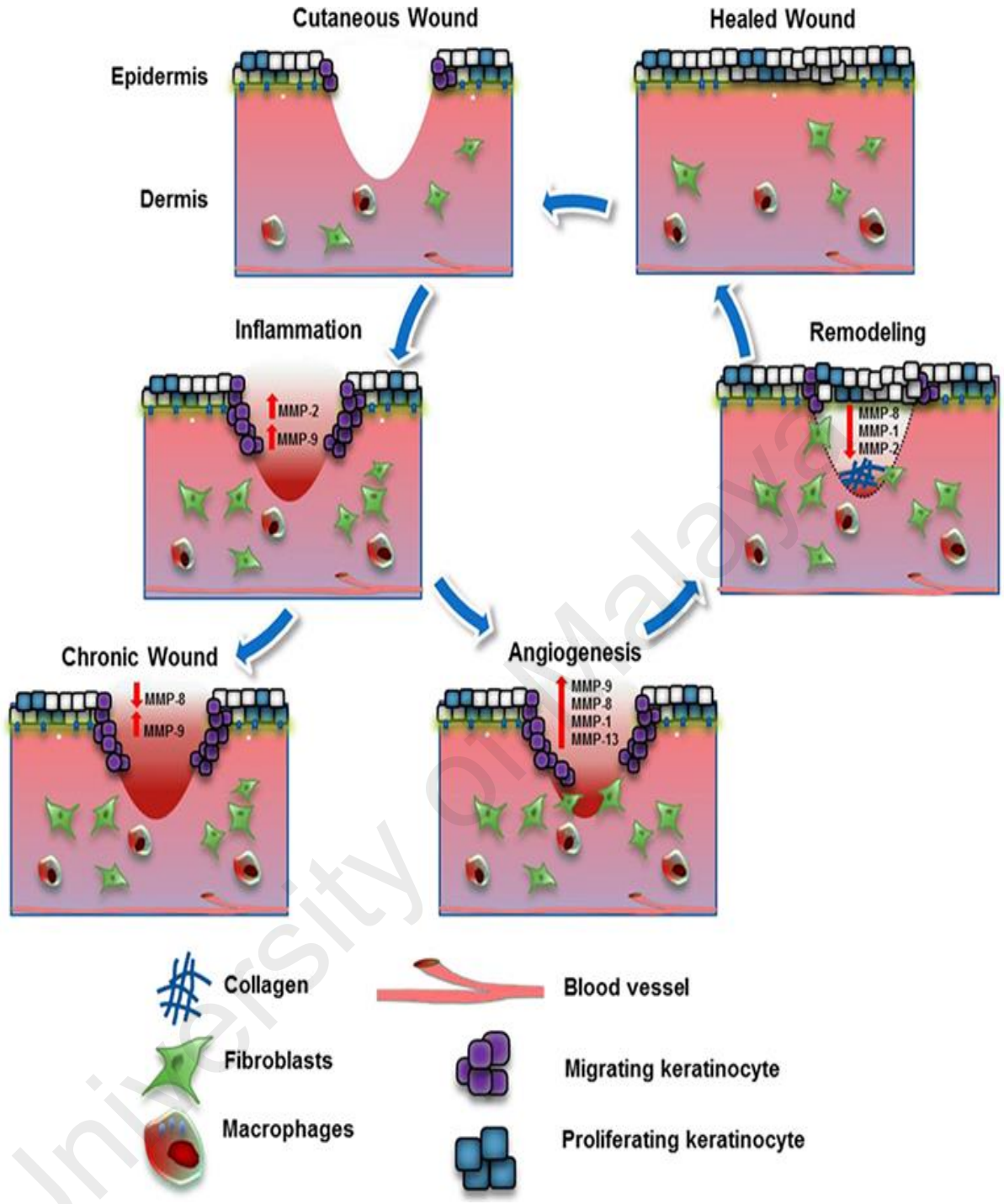


Figure 2.4: The wound healing process (Nguyen *et al.*, 2016). The MMP enzymes involved heavily in the process of wound healing. MMP-2 and MMP-9 increased following the inflammatory phase of wound healing. For chronic wound, the production of MMP-8 decreased and MMP-9 increased.MMP-1, MMP-8, MMP-9 and MMP-13 increased production during the angiogenesis phase. In the remodeling phase, the production of MMP-1, MMP-2 and MMP-8 drops, leading to healed wound

In impaired healing process, the level of the tissue inhibitor of metalloproteinase (TIMPs) decreased, causing the excess MMPs cannot be inhibited (McCarty & Percival, 2013), thus promoting activities such as uncontrolled angiogenesis (Ikenaka *et al.*, 2003; Seo *et al.*, 2003), cell proliferative and prolonged inflammations. These disruptions will cause the normal physiology of wound healing leading to pathological wound healing disorder. For that reason, the importance of MMPs in the healing process such as chronic wound is undeniable. Given that the impaired wound is associated with the rise of MMPs level, inhibition of the enzymes it is necessary to stabilize the condition inside the extracellular matrix (ECM) so that normal wound healing process can take place.

2.5.4 Wound healing disorder in diabetes

Diabetes mellitus is a metabolic disorder that involved high blood sugar. Divided into three types, type 1, type 2 and gestational diabetes, diabetes is a common problem in developed countries. Commonly, type 1 diabetes (Insulin-dependent Diabetes mellitus) is the inability of the pancreas to produce insulin which the main caused is still unknown. For the type 2 diabetes (Non-insulin dependent Diabetes mellitus), it is caused by insulin resistance in which the cells failed to respond to insulin. Thirdly, gestational diabetes generally affected pregnant women with a history of diabetes. As reported in 2014, almost 34 million people are affected by this problem with type 2 leading the percentage with a figure as big as 90%. Death related to diabetes also contributed to frightening figures up to 4.9 million per year with losses in economic globally up to $ 612 million USD in 2014 alone. This figure is expected to rise to 532 million by 2035 and the rate agonizingly high. Prevalence of diabetes in Malaysia is also surprisingly high. The ministry of health releases a report in which out of 30 million citizens in Malaysia, 3.2 million cases of diabetes are found in 2014.

In diabetes, the regulations of MMPs are disrupted and lead to wound healing disorder. Patients with diabetes are prone to wound healing disorder, where the wound heals slower compared to normal healthy people. When the level of glucose in blood increased, it will lead to micro and macrovascular compilations caused by the increase production of MMPs through oxidative stress. In this state, the MMPs activity increased, causing imbalance in the extracellular matrix and tendencies to develop physiological abnormalities. These conditions led to tissue damaged and affected the wound healing process. Diabetic foot ulcer is among the example of side effects of this metabolic imbalance that are common in all ages in diabetic patient. Alteration of MMPs happened in hyperglycemic condition increased the proteolytic environment. Wound healing disorder in diabetes is suggested due to the increase proteolytic condition in which MMP-1, 2 ,8 and 9 were highly expressed leading to slower healing rate of wounds. In comparison to normal wounds, chronic inflammation may produce 50-100 times higher neutrophil derived MMP-8. The imbalance condition of MMPs provokes the white blood cell to continuously cause inflammation leading to wound healing disorder. Other evidences also point out that MMP-1, 8 and 9 are over expressed in venous wound. For instance, the MMP-9 gene expression is altered causing the non-healing diabetic ulcer. The MMPs 2 and 9 play a crucial role in the mechanism of cell migration during the wound healing process (Caley *et al.*, 2015). For that reason, the importance of MMPs in the healing process such as chronic wound is undeniable. Given that the impaired wound is associated with the rise of MMPs level, inhibition of the enzymes it is necessary to stabilize the condition inside the extracellular matrix (ECM) so that normal wound healing process can take place. In severe cases, the wound becomes necrosis and amputation is necessary to prevent further damage. The scenario of wound healing in diabetic patient is different compared to the normal ones. It is a slow process and tends to worsen faster. Elevation of glucose

level in the bloodstream narrows the blood vessel and causes the blood to perform poorly in delivering oxygen and nutrients to affected tissues. Lack of oxygen and nutrients to the affected area will worsen and slower healing process as it is an essential need for the affected tissue to heal.

Nowadays, researchers all over the world are racing to find the solution to fight diabetes and its complication. Prevention is the best ways to fight diabetes. Controlling the sugar in the bloodstream is also a way to fight diabetes. Anti-diabetic drugs such as insulin, alpha glucosidase inhibitors, peptide analogs and other goals are to lower the glucose level in blood. Since MMPs involved in the disorder of wound healing in diabetes condition, controlling the MMPs production is essential as a goal in treatment of diabetic complications

.

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Flowchart of the research methodology

The Figure 3.1 illustrated the flow chart of this research. Standardization of FD leaf involved gravimetric, safety and chromatographic analysis. Computational analyses were the molecular docking and dynamic simulations. The computational analysis software used in this study was AutoDock and GROMACS. Validation of the therapeutic activities was performed using biological approaches, the *in vitro* and *in vivo* methods. The *in vitro* methods were the enzymatic and cell culture assay, while the *in vivo* methods were the toxicology and wound healing study.

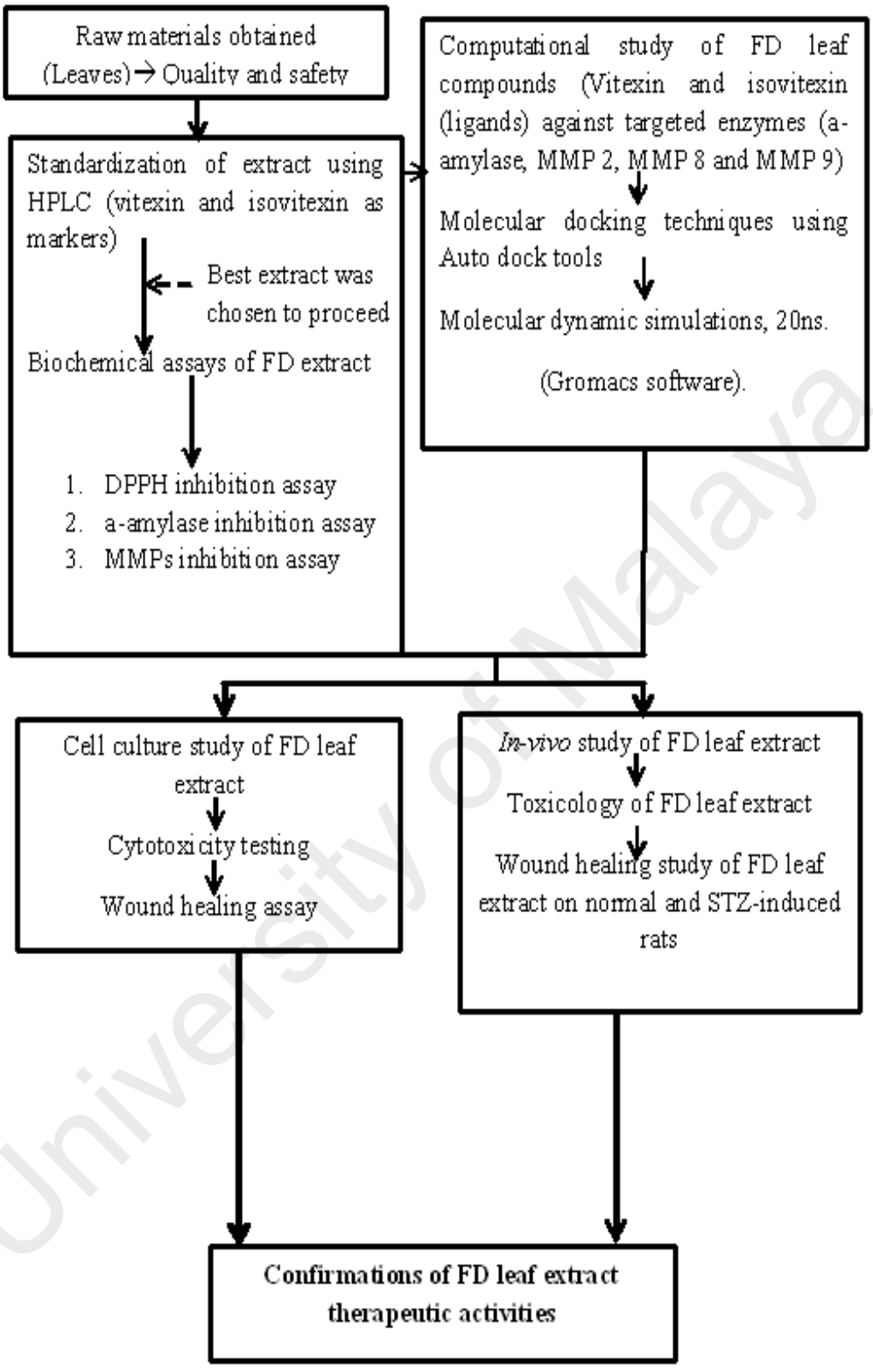


Figure 3.1: The overall flow chart of the study

3.2 Chemicals and reagents

Table 3.1: Chemicals and reagents used for this study

Chemicals and reagents	**Purchased from**
1. Alpha amylase (porcine pancreatic Type IV-B), 2. DL-dithiothreitol (DTT), 3. Sodium potassium tartrate dehydrate, sodium hydroxide (NaOH), 4. Formic acid 5. 3,5-Dinitrosalicylic acid (DNS) 6. Trypsin, 7. Heat inactivated fetal bovine serum (HIFBS) 8. Penicillin/streptomycin (PS) 9. Phosphate buffered saline (PBS) 10. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 11. Vitexin 12. Isovitexin, 13. Acarbose, 14. DPPH (free radicals) 15. Soluble starch 16. Quercetin	Sigma Aldrich (CO, USA).
1. Phosphate buffer saline (PBS) 2. Bovine serum albumin (BSA)	Fluka Biochemika (Buchs, Switzerland)
1. Ethanol (absolute and denatured) 2. Methanol 3. HPLC grade solvent	Scharlau Chemicals (Darmstadt, Germany)
1. Human endothelial (EA.hy 926) cell lines	ATCC, Rockville (MD, USA)
1. Dimethyl sulfoxide (DMSO)	Fisher Scientific (Loughborough, Leicestershire UK)
1. Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen, Gibco (Waltham, MA, USA)
1. Plastic centrifuge tubes (15 and 50 mL)	Corning Life Sciences (Lowell, Massachusetts, USA)
1. Tissue culture flasks (25 cm^3 and 75 cm^3) 2. 0.2 µm polysulphone filter cap 3. 96-well flat bottom tissue culture plates 4. 10 mL serological pipettes, 5. vacuum filtration system 6. PES membrane, syringe filters (0, 22 µm),	Techno Plastic Products (Trasadingen, Switzerland)
1. Matrix metalloproteinase ezymes and inhibitor, 2. N-Isobutyl-N-(4-ethoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH)	Abcam (Cambridge, UK).

3.3 Preparation of plant samples

FD leaves were harvested and collected from a plantation in Batu Pahat, Malaysia from Feb. to May 2015. The plant was authenticated at the Herbarium of the Forest Research Institute of Malaysia (FRIM) in Kepong, Malaysia with given a voucher number: P-ID: 040113-02. Approximately 1kg of cleaned plants were dried at 60^{o}C in the oven before being powdered using a dry miller (Fritsch Pulverisette 15, Germany). The powdered raw material was then kept in a tightly closed container under room temperature until further use.

3.4 Quality control of FD raw material

3.4.1 Total Foreign Matter

The process of determining foreign matters in FD raw material was based on the Malaysian Herbal Monograph Volume 2 (2010). The leaves were randomly collected, weigh at approximately 100 g and disperse on a clean, uncontaminated white sheet. Then, by using a magnifying glass or naked eyesight, materials that are not classified as FD leaves were separated, weighted and calculated as percentage of foreign matter. The formula used to calculate was:

(Total foreign matter ÷ total leaves' weight)×100%

3.4.2 Total Ash

For total ash, roughly a gram of the FD raw material was weighed and put inside a crucible. Then, the crucible was introduced to an incinerator and incinerated with gradual increase in temperature until not more than 450 ^{o}C for 4 hours.

The ash was then let cool and calculated as: (Total ash ÷ total leaves' weight)×100%

3.4.3 Total Acid Insoluble Ash

Following the total ash experiment (section 3.4.3), the ash was added with 25ml of 2N hydrochloric acid. A glass was then placed on top of the crucible. The content in

the crucible was boiled for 5 minutes. Following that, 5 ml of hot water was rinsed onto the watch glass into the crucible. Ashless filter paper was used to collect the insoluble material. Next, the filter paper with insoluble matters was placed in the crucible and incinerated for 4 hours. Lastly, the crucible was placed inside a desiccator for drying purposes and to obtain constant weight.

$$\text{Acid insoluble ash: - (Filtrate} \div \text{ash weight)} \times 100\%$$

3.4.4 Moisture content of raw material

The powdered FD raw material was accurately weight (5 grams) and transferred into a flat open container. Then, the sample was dried in an oven with temperature set at 100-105 ºC for 5 hours. The dried sample was weighed and calculated using the formula:

$$\text{Moisture content \%:} \left(\frac{\text{Weight before} - \text{weight after}}{5}\right) \times 100\%$$

3.4.5 Extractive Value

The process of determining the extractive value of FD raw material was performed using maceration techniques (Malaysian Herbal Monograph, 2010). Relatively 5 g of powdered FD raw material was added to a 100 ml of solvent (water, ethanol and 50% v/v ethanol: water) in a conical flask. The samples were macerated using orbital shaker continuously for 48 hours with the temperature set at 60 ºC.

Then, using a filter paper, the filtrate was separated from the samples. Approximately 20 ml of filtrate was transferred to a watch glass. For drying process, the watch glass containing the filtrate was kept inside an oven with the temperature set at 105ºC. The sample was placed inside a desiccator for cooling purposes until the weight becomes constant. The extractive value is calculated as:

$$\text{Extractive value (\%):} \left(\frac{\text{extract weight}}{\text{sample weight}}\right) \times 100\%$$

3.5 Safety assessment of FD raw material

3.5.1 Heavy Metal Content

The content of heavy metals analysis of the FD leaves was done using the Atomic Absorption Spectroscopy (Perkin Elmer Analyst 800 system, USA). The heavy metals analysed were cadmium (Cd), mercury (Hg), arsenics (As) and lead (Pb) (British Pharmacopoeia commission, 2008). Preparation of the sample was as follows; approximately 0.5 g of the oven dried leaves were weighed and moved to a Teflon vessel and the microwave digestion sets to be 1200 W of max power, 100% of power, 15 min of ramp, pressure set at 120 kPa, temperature: 120ºC and hold for 15 min. Then, 50 ml of distilled water was added into a disposable tube and filtered using 2 µm filters. Then, the filtrate was evaluated using the atomic absorption spectroscopy (AAS). Reference lead, cadmium, arsenic and mercury solutions were separately prepared using 2 % nitric acid (v/v).

3.5.2 Microbial Limit Test of FD

This foundation of these methods followed the procedures proposed by the United States Pharmacopoeia (2009) to analyze for opportunistic food contaminants. The tests established for this safety assessment are the total aerobic count, microbial count and test for specific pathogens namely; *Staphylococcus aureus, Pseudomonas aeruginosa*, *Salmonella spp* and *Escherichia coli* as well as combined mold and yeast counts. To prepare the test solution, 10 g of dried fine powder of FD was added to 90 ml of phosphate buffer (pH 7.2). Then, 1 ml of the test solution was introduced to a petri dish containing sterilized Soybean-Casein digest agar (SCD) for aerobic organism and Saboroud dextrose agar (SDA) for yeast and mold. The incubation period was for 5-7 days at 20-25ºC. Lastly, after the incubation period completed, the dish was removed, and the resulting colonies will be counted.

3.5.3 *Pseudomonas aeruginosa* and *Staphylococcus aureus*

The test solution (1 ml) was incubated at 30-35ºC for 18 – 24 hours. After that, the test solution was streaked onto mannitol salt agar containing cetrimide for detection of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Then, after the streaking process, the plates were incubated for 18-72 hours at 30-35ºC. The plates were later observed for microbial growth.

3.5.4 Detection of *Salmonella sp*.

For *Salmonella sp*., the test solution (10 ml) was incubated for 18-24 hours at 30-35ºC. After that, 0.1 m of the test solution was introduced into 10 ml of Rappaport Vassiliadis Salmonella enrichment broth (RVSEB) for 18-24 hours at 30-35ºC. Then, xylose lysine deoxycholate (XLD) agar was used for the streaking process. After the streaking process, XLD agar was incubated for 18-48 hours at 30-35ºC and later was observed the microbial growth.

3.5.5 *Escherichia coli*

For *Escherichia coli*, 1 ml of the test solution was incubated for 18-24 hours at 30-35ºC. Then, the incubated test solution was added to 100 ml of Mac Conkey broth and incubated at 42-44ºC for 24-48 hours. Then, the streaking process was performed on Mac Conkey agar, plate incubated for 18-27 hours at 30-35ºC. The microbial growth was observed on the plate after the incubation period completed.

3.5.6 Detection of aflatoxins

The procedure to detect aflatoxins followed that of the United State Pharmacopeia (2009). Roughly 50 g of dried FD leaf powder was added to 200 ml of methanol and water (17:3 v/v) in a glass-stoppered flask. The mixture was vortexed for 30 min, filtered and 50 ml of the filtrate was discarded into a separator funnel containing mixture of 25 ml of hexane and 40 ml of sodium chloride solution. Then, the

mixture was vortexes for 1 min before the aqueous layer inside the separator funnel was extracted out through the shaking process with 25 ml of methylene chloride. After the separation process, the combined organic layer was collected in a conical flask and the organic solvent was let to evaporate until dry on a water bath and the residue was then cooled for a few minutes. Aflatoxins standard solutions; aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2 was prepared as 0.5 μg/ml in a mixture of chloroform and acetonitrile (9.8:0.2 v/v). Then, the aflatoxins solution was applied on a thin-layer chromatographic plate coated with a 0.25 mm layer silica gel. The spots were left to dry before transferring into an unsaturated chamber containing a mixture of chloroform, acetone and isopropyl alcohol (85:10:5, v/v/v). The plate was later removed from the developing chamber; solvent was marked and allowed to dry. The spots were located on the chromatographic plate under UV light at 365 nm. The four aflatoxins standard solutions will appear as four clearly separated blue fluorescent spots. If any spot of aflatoxins were obtained in the test solution, the positions of each fluorescent spot of the test solution were matched with those of the aflatoxins standard solutions to identify the type of aflatoxins present.

3.6 Standardization of FD leaf extracts against vitexin and isovitexin

Three different solutions (pure water, 50% (v/v) ethanol-water, and absolute ethanol) were used to prepare the plant extracts. The extraction was carried out at room temperature for 48 hours in an orbital shaker (300 rpm). The suspension obtained was filtered using a 114 Whatman filter paper and filtrate collected. The filtrates were concentrated using a rotary evaporator, and then oven dried (60^{o}C) to obtain a solvent free extract. Then, 10 mg of each extract was prepared with 1ml of their respective solvents, sonicated till fully dissolved. The extracts were kept refrigerated until further use.

High performance liquid chromatography (HPLC) standardization was performed on a Dionex UltiMate 3000 HPLC system equipped with auto-sampler, UV detector, quaternary pump, online degasser, and column incubator. Chromatographic separation was achieved on a Dionex Acclaim Polar Advantage II C-18 column (150 mm × 4.6 mm × 5 µm) using an isocratic mobile phase consisting of 0.1% formic acid in water and methanol (60%: 40%). The detection wavelength was 335 nm with a flow rate of 1 ml/min, an injection volume of 10 µl, and the column temperature was maintained at 30°C throughout the separation for 30 min. The data acquisition was carried out by Chromeleon software version 6.8.

3.7 Computational analysis of vitexin and isovitexin

3.7.1 Ligand preparation

Vitexin and isovitexin molecular structures were separately downloaded from the Pub Chem database (https://pubchem.ncbi.nlm.nih.gov/compound) in sdf file. The compounds were then optimized using UCSF Chimera (Pettersen *et al.,* 2004) to obtain the most stable 3D conformation. For the molecular docking analysis, the non-polar hydrogens were merged and rotatable bonds were fixed to the respective structures.

3.7.2 Molecular docking

3.7.2.1 Alpha-amylase

The crystallized structure of α-amylase [PDB IDs: 3OLD (Qin *et al.,* 2011), 2.0 Å resolution] was downloaded from the Protein Data Bank. Polar hydrogen along with the Kollman partial atomic charge were assigned to the protein by ADT 4.2 (Autodock) (Morris *et al.,* 2009) and saved as Autodock readable file. The ligands (vitexin or isovitexin) were made flexible, torsion root was set free and protein kept rigid. The protein binding site was defined as 80Å × 80Å × 80Å in grid size with 0.375Å spacing and the dimension was x = 8.458, y = -5.795, z = 15.737. Lamarckian genetic algorithm (Fuhrmann *et al.,* 2010) was used for the docking with the following setting; energy evaluation of 250000 and a total of 100 runs inside the binding site. The results were later analyzed using Autodock tools (Morris *et al.,* 2009).

3.7.2.2 MMP enzymes

The crystallized structures of the MMPs [PDB IDs: 1HOV.pdb was MMP-2 (Giangreco *et al.,* 2011), 2OY4.pdb was MMP-8 (Giangreco *et al.,* 2010), and 2OW1.pdb was MMP-9 (Giangreco *et al.,* 2010)] were downloaded from the Protein Data Bank. The files were modified where the crystallographic water molecules and associated ligands were removed. Later, polar hydrogen along with the Kollman partial atomic charge was assigned to the protein by ADT 4.2 (Autodock) (Morris *et al.,* 2009)

and was saved to Autodock readable file. The ligands were made flexible where the torsion root of ligand was set free and the protein was kept rigid. The setting of AutoDock software for MMP-2 (1HOV.pdb) was 40Å × 40Å × 40Å in grid size with 0.486Å spacing and grid center set as x=4.731, y=7.252, and z= 26.648. For MMP-8 (2OY4.pdb), the setting was 40Å × 40Å × 40Å in grid size with 0.375Å spacing and x=-21.056, y= 21.586, and z= -6.03 as grid center. Lastly, for MMP-9 (2OW1.pdb), the setting was 40Å × 40Å ×40Å in grid size and spacing set at 0.486Å with the grid center of x=4.731, y= 7.252, and z= 26.648. A total of 1000 runs were set for the docking analysis of vitexin and isovitexin with the MMPs enzyme.

3.7.3 Molecular dynamic simulations

Simulations of the enzyme-ligand complex were done using GROMACS 5.0.4 software (Pronk *et al.*, 2013, Figure 3.2). Continuity of the docking results, the best complexes in pdbqt were extracted from the ADT and converted to pdb file. The complex coordinates were separated and saved as the ligands (vitexin and isovitexin) and enzymes (α-amylase and MMPs) files. GROMOS 43a1 force field was assigned for the enzymes while the ligands force fields were set up using the PRODRG 2.5 server with minimized energy. The enzyme-ligand complex was then simulated in a cubic box with the SPC/E type of water soaking the complex. Later, the box was added with ions to neutralize the condition inside the system and energy minimization was executed using the steepest descent method. After that, the ligands were restrained and equilibration of NVT (temperature: 300K) and NPT (pressure: 1atm) was performed. Lastly, molecular dynamic simulations were carried out for 20 ns and were analyzed using GROMACS package tools and XMGRACE for graph visualization.

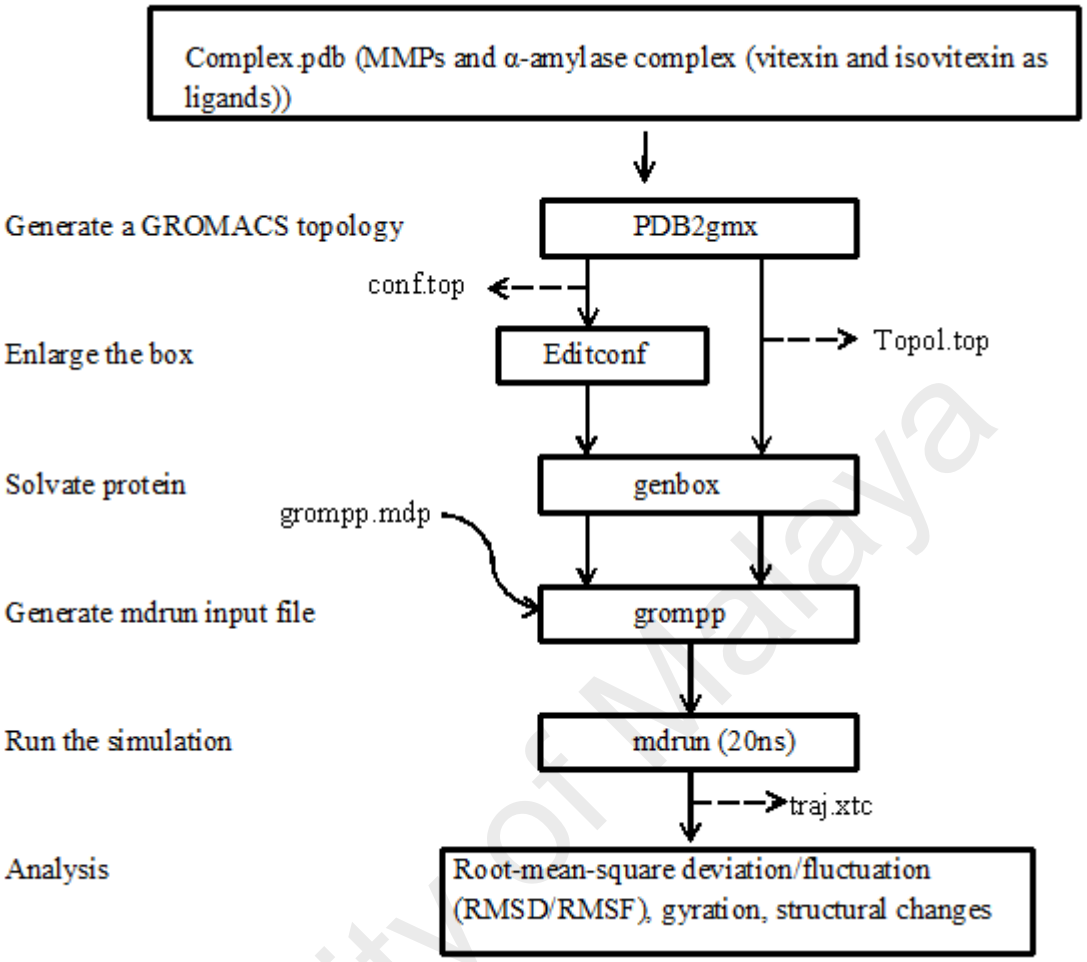


Figure 3.2: The protocol for molecular simulation using GROMACS software

3.8 *In vitro* assay of FD leaf 50 % v/v ethanol water extract

3.8.1 The α-amylase inhibition assay

The α-amylase inhibition assay was carried out using 96 well plates as described by Manaharan *et al.* (2012). Porcine pancreatic α-amylase (Sigma Type IV-B) was dissolved in ice-cold distilled water to a concentration of 2 U/ml. Potato soluble starch solution (1%) was prepared in 20 mM phosphate buffer pH 6.9. DNS solution was prepared with 1g DNS, with 30g sodium potassium tartrate dehydrate and 2M NaOH in 100 ml solution. In the assay, 80 µl of various dilutions of vitexin, isovitexin and FD leaf extract, along with 40 µl of α-amylase enzyme were added and incubated at room temperature for 10 min. Then, 40 µl of starch was added and incubated at 37°C for 10 min. After which 80 µl of DNS solution was added and incubated in a water bath at 95°C for 10 min, to detect the reducing sugar, which will change the DNS from orange to brick red colour. The absorbance was measured at 540 nm with a Cary 50 Bio UV–visible spectrophotometer (Varian, Inc., Palo Alto, CA). Acarbose (12 µg/ml) was used as the positive control. Appropriate blanks were used to exclude background absorbance. The percentage inhibition and IC_{50} were determined as described below:

$$\%\ \text{inhibition} = \frac{(\text{A neg control} - \text{A blank}) - (\text{A sample} - \text{A sample blank})}{(\text{A neg control} - \text{A blank})} \times 100$$

A= absorbance

Inhibitory activity was assessed by plotting percentage inhibition against a range of concentrations (0.01-1 mg/ml) and determining the IC_{50} (Concentration with 50% inhibition) value). Results are expressed as means ± SD of triplicate measurements.

3.8.2 DPPH scavenging assay

The DPPH assay (Sharma & Bhat, 2009) was carried out with stock solution for DPPH prepared as 200 µM. The FD extract was serially diluted with methanol to obtain a concentration of 200, 100, 50, 25, and 12.5 µg/ml. Later, the DPPH solution was mixed well with vitexin, isovitexin or FD leaf extract at the respective concentration and kept in the dark place, at 30ºC for 30 min. Absorbency of the mixture of DPPH and vitexin, isovitexin or FD extract was then measured using UV at 517 nm. Positive and negative control for this study was quercetin and ethanol. Results are expressed as means ± SD of triplicate measurements.

The percentage of DPPH scavenging activity and IC_{50} values were calculated as below:

$$\%\ \text{inhibition} = (1 - \{((\text{A neg control} - \text{A blank}) - (\text{A sample} - \text{A sample blank}))/((\text{A neg control} - \text{A blank}))\}) \times 100$$

A= absorbance

3.8.3 MMPs inhibition assay

The MMPs inhibition study was performed using a colorimetric method with kit purchased from Abcam (Cambridge, United Kingdom). Initially, the MMPs substrate was briefly warm before the procedures started. MMP inhibitor was diluted to 1/200 in the assay buffer. Then, 1 µL of the inhibitor was added to 200 µL of assay buffer in a separate tube (37ºC). Next, the MMPs substrate (2, 8 and 9) was diluted to 1/25 in the assay buffer (37ºC). The MMPs enzyme was also diluted to 1/60 buffer. Later, the blank, control, N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH) in 10 µg/ml (0.032mM) and test inhibitor (FD leaf extract, vitexin and isovitexin) was pipetted into the 96-well microplate at room temperature. An addition of 20 µl of the MMPs was introduced to the control, MMP inhibitor and test inhibitors. For the inhibitor, 20 µl of MMP inhibitor was added. Incubation was for 30-60 minutes

under room temperature. The reaction was started with the introduction of 10 µl of the MMPs substrate. Finally, the microplate reader (Applied Biosystem BTS 310, Applied Biosystem, USA) was used to measure the absorbance reading at 412nm. Percentage inhibition (IC_{50}) of MMPs by the inhibitors was calculated as below:

$$\text{Inhibitors \% activity remaining: } \left(\frac{\text{A inhibitor} - \text{A blank}}{\text{A control} - \text{A blank}}\right) \times 100$$

A= absorbance

3.8.4 Cytotoxicity study of FD leaf extract

Cell proliferation activity of FD leaf extract, vitexin and isovitexin were conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against EA.hy926 cells using the method described by Al-Salahi *et al*. (2013) with slight modifications. The cells were seeded at a density of 1×10^5 mL^{-1} in 96-wells flat-bottomed plates. Then, after 24 h, the samples were introduced to an individual well containing 100 µL media with different concentrations (6.125, 12.5, 25, 50, 100 and 200, µg/ml). The blank was the free culture media and the negative control was 0.1% DMSO. Vitexin and isovitexin were used as reference compounds in EA.hy926 cells. All the test samples were prepared from serially diluted solution giving concentrations of: 6.125, 12.5, 25, 50, 100 and 200, µg/ml. Incubation of the plate was at 37 °C in a controlled humidified atmosphere containing 5% CO_2. After an incubation period of 48 h, the MTT reagent solution (5 $mg \cdot mL^{-1}$ in PBS) was added (20 µl) to each well and incubated again for 4 h. Then, the excessive MTT and medium were removed. After that, 100 µL of DMSO was added into the wells to dissolve the dark blue formazan crystals generated by viable cells. The plates were further incubated for 5 min in a CO_2 incubator.

Lastly, the colorimetric assay was analyzed using a microplate reader, Infinite pro200 (Tecan, Männedorf, Switzerland) at a wavelength of 570 and the inhibition percentage was calculated as:

$$\%\ \text{inhibition} = 1 - \{\frac{(\text{A neg control} - \text{A blank}) - (\text{A sample} - \text{A sample blank})}{(\text{A neg control} - \text{A blank})}\}) \times 100\%$$

A= absorbance

3.8.5 Cell migration activity assay

The EA.hy 926 cell lines were preserved in ECM till the confluent monolayer growth was 100 %. The monolayer was then scratched using a sterile 200 µl micropipette tip (Liang *et al.,* 2007). Following that, 2 ml of ECM with low serum concentration (2.0%) were introduced to individual well with addition of TAF273 at 2.5, 5 and 10 µg/ml final concentrations. Vitexin and isovitexin were chosen as the positive controls and the vehicle (0.1% DMSO) as the negative control. Afterwards, the photographed of 6 - 8 microscopic fields per well were taken at 0, 6, 18 and 24 h using AMG EVOS fI inverted microscope. Measurements of the width of the cell free area were obtained and analysed using Leica Quin software. The result of cell migration activity assay was displayed as average ± SD, using the equation:

$$\text{Cell migration activity}\ \% = 1 - \left(\frac{\text{the width at the indicated times (h)}}{\text{the width at zero time}}\right) \times 100\%$$

A= absorbance

3.9 *In vivo* study of FD leaf extract

3.9.1 Experimental animals

The female Sprague-Dawley (SD) rats were obtained from (Comparative Medicine and Technology Unit (COMeT), University Putra Malaysia, (UPM) with ethical approval number given as UPM/IACUC/AUP-R069/2016.

3.9.2 Acute oral toxicity of FD leaf extract

A number of 8 female Sprague-Dawley (SD) rats were obtained from (Comparative Medicine and Technology Unit (COMeT), University Putra Malaysia, (UPM). For selection of acute toxicity dose, there is a "fix dose" method of Organization for Economic Co-operation and Development, Guideline No.420 (OECD, 2002). The fixed dose (test procedure with a starting dose of 100 mg/kg body weight) was adopted. For the assessment of any biological activities, two dose levels were chosen in such a way that low dose was approximately one-tenth of the maximum dose (1000 mg/kg) during acute toxicity studies and the other high dose was twice that of one-tenth dose (2000 mg/kg). The SD rats was then assigned evenly into 2 groups designated as group G1 (4 female rats received distilled water as vehicle) and G2 (4 female rats given 1000 mg/kg of FD leaf 50% (v/v) ethanol water extract, once). The rats will be fasting overnight (free access to water) prior to FD leaf 50% (v/v) ethanol water extract, dosing.

3.9.2 Mortality and behavioural observation

The dose was administered to the animals by oral gavage. The animals were carefully observed for 30 min and 2, 4, 24 and 48 h following the administration for detecting any toxicological symptoms. Any mortality and behavioral changes were recorded throughout the experimental period of 7 days. The rats were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) intraperitoneally followed by taking blood

samples via cardiac puncture and then by increasing the anesthetic dose (combination of ketamine (150 mg/kg) and xylazine (15 mg/kg), intra-peritoneally. After sacrificing the rats, serum biochemical and histopathological parameters (liver and kidney) were collected. The veterinarian in charge for the acute toxicity was Dr. Mohd Hafidz b. Mohd Izhar of Universiti Putra Malaysia (UPM).

3.9.3 Wound healing study of FD leaf extract

3.9.3.1 Induction of Type 2 diabetes in the SD rats

For induction of diabetes, the rats were subjected to fasting for 14 h. Streptozotocin (STZ) was prepared in 0.1 M citrate buffer saline, pH 4.5. A dosage of 30 mg/kg STZ was injected intra-peritoneally. The hyperglycemic condition was measured using blood glucose levels. The blood was drawn by a tail puncture method using clean and sterile lancet and the blood glucose levels were measured using glucometer (Accu-Check®, USA). Rats with blood glucose levels higher than 11 mmol/l were considered as diabetic rats and were used throughout the experiments (Wu *et al.,* 2016).

3.9.3.2 Wound healing excision on SD and STZ-SD rats

In wound healing experiment, 24 normal and diabetic rats (STZ-SD) were used. The rats were randomly divided into four groups; positive control, negative control and two experimental groups which received dosage of 100 and 200 mg/kg of the FD extract each. The same grouping technique was also applied to the diabetic rats. For induction of wound healing, the rats were anesthetized with proper dosage of Ketamine (50 mg/kg) and xylazine (5 mg/kg), intra-peritoneal). Under sterile condition, the skin was shaved and exposing the scalp. An area of uniform wound of 1.8 cm in diameter was excised from the nape of the dorsal neck of all rats using a circular stamp. Treatments were applied twice a day, for 14 days, by topical application on site of wound/infection. Normal and diabetic control rats were given Tween 20 as control

treatment, whereas the intrasite gel was applied to the positive control groups. Every two days, the wounds were assessed and recorded in terms of its macroscopically healing progress. On day 15th, the rats were anesthetized with Ketamine (50 mg/kg) and xylazine (5mg/kg), intra-peritoneal) for blood collection. Cardiac puncture was performed to collect the blood serum for biochemical analysis. The rats were sacrificed by overdosing with Ketamine (150 mg/kg) and xylazine (15 mg/kg), followed by harvesting of organs (kidney, skin, pancreas and liver) for histopathology analysis. The veterinarian in charge for the wound healing study was Dr. Mohd Hafidz b. Mohd Izhar of Universiti Putra Malaysia (UPM).

3.10 Blood and biochemical analysis of SD and STZ-SD rats

3.10.1 Full blood counts

The full blood count analysis for the SD rats was performed using Cell-Dyn® (3700) Hematology analyzer, USA. The blood samples were aspirated using the automated sample loader to the machine at a volume of 355 µl ± 5%. After that, the sample went through the shear valve that then rotated to isolate the whole blood sample into three segments, which is 32 µl for the WOC (white blood cell / WBC Optical Count) dilution, 20 µl for the WIC/HGB (WBC Impedance Count) dilution and 0.74 µl for the RBC/PLT dilution. WOC measurement was performed with the syringe dispensing 1.6 ml of Sheath reagent through the shear valve, 32 µl of the WOC sample segment aspirated. Then, the sample segment and sheath were routed to the WOC Mixing Chamber, in which the dilution was bubble-mixed to obtain a final dilution of 1:51. After that, the WOC peristaltic pump was transferred from the mixing chamber to the sample feed nozzle in the WOC Flow Cell. A stream of WOC Sheath Reagent was directed through the Flow Cell followed with the WOC metering syringe injecting 78 µl of the WOC dilution into the Flow Cell sheath stream. The dilution was then hydrodynamically focused into a narrow stream in which a laser beam will also be

focused upon the Flow Cell. As the sample stream intersects the laser beam, the light scattered by the cells was measured at four different angular intervals. For the WIC measurement, 5.25 ml of the diluent was dispensed from the WIC/HGB Diluent Syringe through a shear salve, where an approximately 20 µl WIC/HGB injected in the sample segment. The segment and diluent were routed to the mixing chamber located in the von Behrens WIC transducer. Then, the WIC/HGB lyse syringe simultaneously delivered 0.75 ml of WIC/HGB lyse to the mixing chamber where the dilution was bubble-mixed at a dilution of 1:301. The dilution was pulled through the aperture by vacuum and a process known as volumetric metering was applied, ensuring that 200 µl of the dilution was used for the measurement. Electrical Impedance was used to count the WBCs as they traverse the aperture. Lastly, when the count portion of the cycle completed, the aperture cleaning circuit automatically cleaned the aperture. Measurement of the red blood cell (RBC) and platelet was performed with the dispensed of 7.2 ml diluent using RDC diluent syringe through the shear valve and 0.74µL of RBC/PLT was introduced to the sample segment. The sample segment and diluent were routed to the mixing chamber of the von Behrens RBC Transducer, where the dilution was bubble-mixed. The final dilution was1: 9,760. The dilution was then pulled through the aperture by vacuum. The volumetric metering process ensures that 100 µl of the dilution was used for the measurement. Electrical Impedance is used to count the RBCs as they traverse the aperture.

3.10.2 Biochemical analysis of the blood sample of the SD rats

The analysis of the biochemical components in the blood sample of the SD rats was performed using BioLis 24i Premium Chemistry analyzer from BIOREX MANNHEIM *Diagnostics* (Germany). The system composed of the sample, sample delivery, reagent tray, reaction tray, mixing units, cuvette washing unit and spectrophotometer. The sample tube containing the serum was set in the sample and the

reagent bottle was set inside the reagent tray. The main principle of the measurement involved a combined movement of the mechanical parts and the optical measurement feature.

3.10.3 Alkaline phosphatase (ALP) test

Determination of the ALP level inside the blood serum was conducted by mixing 1000 µl of reagent 1 [containing 2-amino-2-methyl-1-propanol (1.1 mol/l at pH 7.4), Magnesium acetate (2mmol/l), Zinc sulfate (2.5 mmol/l) and HEDTA (N-{2-[Bis(carboxymethyl)amino]ethyl}-N-(2-hydroxyethyl)glycine at 80 mmol/l)] and 20 µl of sample. The first mixture was incubated for 1 min before 250 µl of reagent 2 which [containing p-Nitrophenylphosphate (80 mmol/l)] was added. The mixture of reagents 1 and 2 were then incubated for 1 min before the absorbance reading at wavelength of 405 nm.

3.10.4 Aspartate Aminotransferase (AST) and alanine transaminase (ALT) test

The measurement of AST was conducted by mixing 100 µl of sample and 1000 µl reagent 1 (Tris buffer (110 mmol/l at pH 7.65), L-aspartate (320 mmol/l), malate dehydrogenase (800 u/l) and lactate dehydrogenase (1200 u/l)). In the ALT test, malate dehydrogenase was not included in reagent 1. Then, the mixture was incubated for approximately 5 min after which 250 µl of reagent 2 (2-Oxoglutarate (65 mmol/l) and NADH (1 mmol/l)) were added. The mixture of reagent 1 and reagent 2 was let to sit on the bench for 1 min before the absorbance reading was done at 340 nm wavelength.

3.10.5 Total protein

The total protein (TP) analysis of the blood serum was conducted by mixing 20 µl of the sample and 1000 µl of reagent 1 (Sodium hydroxide (100 mmol/l) and Potassium sodium tartrate (17 mmol/l). The mixture absorbance was read at 540 nm and later incubated for 5 min at 20-25 ^{o}C. After that, the first mixture was added with 250 µl

of reagent 2 [(Sodium hydroxide (100 mmol/l), potassium sodium tartrate (80 mmol/l), potassium iodide (75 mmol/l and copper sulfate (30 mmol/l))]. The mixture was incubated for another 5 min at 20-25°C. The second mixture reading was taken at 540 nm wavelength. For calibration, the method was the same as above with the sample was replaced by distilled water. The calculation of total protein was:

$$\mathrm{TP(g/l)} = \mathrm{conc.\,calibrator} \times \frac{\mathrm{conc.\,sample}}{\mathrm{conc.\,calibrator}}$$

3.10.6 Total bilirubin

The total bilirubin test was done by adding the sample (25 µl) to 1000 µl of reagent 1 [Phosphate buffer (50 mmol/l) and sodium chloride (150 mmol/l)]. The mixture was incubated for 5 min at 20-25°C. Then, after incubation process, the reading of the wavelength was obtained at 546 nm. After that, the first mixture was added with 250 µl of the second reagent [(2,4- Dichlorophenyl diazonium salt (5 mmol/l) and hydrochloric acid (130 mmol/l)]. The secondary mixture was incubated for exactly 5 min at 37°C. The absorbance of the second mixture was taken at 546 nm wavelength. For calibration, the sample was substitute with distilled water. The concentration of total bilirubin was calculated as:

$$\mathrm{Total\ bilirubin\ (mg/dl)} = \mathrm{conc.\,calibrator} \times \frac{\mathrm{conc.\,sample}}{\mathrm{conc.\,calibrator}}$$

3.10.7 Urea

For urea testing, 10 µl of the serum sample was added with 1000 µl of reagent 1 [Tris buffer pH 7.8 (150 mmol/l), 2-oxoglutarate (9 mmol/l), ADP (0.75 mmol/l), urease (7 kU/l) and glutamate dehydrogenase (1 kU/l)]. Then, the mixture was incubated for 5 min at 37°C. After that, the mixture was added with 250 µl of reagent 2 [NADH (1.3 mmol/l)]. Following that, the mixture was incubated for 40 seconds at 37°C and the readings of the absorbance was taken at wavelength of 340 nm.

3.10.8 Creatinine

The creatinine analysis was performed with 50 µl of the serum sample added with 1000 µl of sodium hydroxide (0.2 mol/l). The mixture was incubated for 5 min. Then, 250 µl of picric acid (20 mmol/l) was added. After mixing for 60 seconds, the readings of the absorbance were taken at 492 nm wavelength. For the calibration, the sample was substituted with distilled water. The calculation of the creatinine in the blood serum is as below:

$$\text{Creatinine (mg/dl)} = \text{conc. calibrator} \times \frac{\text{conc. sample}}{\text{conc. calibrator}}$$

3.11 Histology analysis

3.11.1 Preparation of hematoxylin and eosin reagent

The preparation of histology slides was based on the hematoxylin and eosin protocol (Kiernan, 2008). Mayer's hematoxylin solution was prepared by dissolving 50 g of aluminum potassium sulfate in 1000 ml of distilled water. After the mixture was completely dissolved, 1 mg of hematoxylin was added and vortexed until completely dissolved. Then approximately 0.2 g of sodium iodate was added together with 20 ml of acetic acid and was left to boil, cool and filtered.

For eosin, the reagent was prepared with 1 g of eosin added to 100 ml of distilled water. The mixture was vortexed until fully dissolved. Then, 1 g of phloxine was added with distilled water and mixed until totally dissolved. After that, 100 ml of eosin stock solution was added with 10 ml of phloxine solution, 780 ml of 95% (v/v) ethanol and 4 ml of glacial acetic acid. The mixture was vortexed until totally dissolved.

3.11.2 Preparation of histology slide

The organ specimens collected from the rat were soaked with formaldehyde at a ratio of 10:1 volume to specimen ratio for 48 hours. Then, the specimen was transferred

to a cassette and was trimmed to fully fit into the area using a scalpel. After that, the tissue processing step was conducted. The first step involved dehydrating the specimens in which an increasing concentration of alcohol was used to remove water and formalin from the specimens. Then, the clearing step was performed to remove alcohol by adding xylene. After that, the embedding process was conducted by adding molten paraffin wax and let to rest until the block solidified. After the block was solidified, microtome was used for sectioning process and the layer of the tissue was cut approximately 5 µm in thickness. Next, the tissue ribbons were transferred to a warm bath where it was allowed to float for fishing process in which tissue was scooped up by a clear slide. Then, the slide containing the tissue was allowed to dry for a few hours at 37°C. Lastly, excess paraffin wax was gently melt and leaving the tissue section intact.

3.11.3 Histology slide staining process

The staining process first involved the deparaffinize section in which 2 changes of xylene were performed at an interval of 10 minutes each. Then, the slide was rehydrated with absolute alcohol for 5 minutes each. After that, the slide was soaked with 95% of alcohol for 2 minutes and 70% of alcohol for another 2 minutes. Next, the slide was stained with hematoxylin solution for 8 min before washing with running tap water for another 5 minutes. Then, the slide was differentiated in 1% (v/v) of acid-alcohol for approximately 30 seconds before washing with running tap water for 1 minute. The next process involved bluing in 0.2% of ammonia water for 30 seconds followed by washing for 5 minutes using running tap water. The slide was then rinsed in 95% alcohol for 10 dips before counter staining in eosin-phloxine solution for 1 minute. The slide was then dehydrated with 95% (v/v) alcohol for 5 minutes and clearing in 2 changes of xylene at 5 minutes interval before it was mounted with xylene based mounting medium.

3.11.4 Histology analysis

The histological slides were analyzed and reported by Dr. Adiratna Mat Ripen, a certified immunologist from the Institute of Medical Research (IMR), Kuala Lumpur.

3.12 Statistical analysis

All laboratory experiments were performed and analyzed at every time point from each experiment. Means and standard deviations were calculated from replicates within the experiments and analyses were done using SPSS version 20 (IBM SPSS Statistics, 2013).

CHAPTER 4: PHARMACOGNOSTICAL STANDARDIZATION OF *Ficus deltoidea* Jack LEAF

4.1 Introduction

Ficus deltoidea (FD) leaf was introduced to the standardization process. Throughout this process, the FD leaf was evaluated and assessed on the quality and safety parameters. After that, the FD leaf prepared in extracts was standardized against vitexin and isovitexin using HPLC method.

4.2 Botanical identification

The FD leaf was prepared as a botanical specimen and was sent to Forest Research Institute of Malaysia (FRIM) to be identified. The specimen was authenticated by Dr. Richard Chung Cheng Kong and classified as *Ficus deltoidea* variety Kunstleri. The specimen was deposited into the FRIM herbarium and given a voucher specimen number of **P-ID: 040113-02.**

Figure 4.1: The botanical specimen of *Ficus deltoidea* Jack variety Kunstleri**.** The specimen was kept at FRIM herbarium (P-ID: 040113-02)

4.3 Quality control of FD leaf raw material

In the practice of drug discovery, ensuring the quality of the raw materials are essential as initial steps of the drug development process (Fouad, 2017). The same principles applied to the herbal medicines that are being developed into nutraceutical or cosmeceutical products. Herbal medicines are herbal products that fall in the national drug regulatory framework as herbal preparations and herbal finished/medicinal products (Ekor, 2013). In some regions, these herbal products are being consumed as functional foods (US FDA, 2011). Therefore, the regulatory boards have addressed some requirements so that these medicinal plants are safe enough to be consumed by consumers (WHO, 1993; WHO, 1996; WHO, 2002). Maintaining the cleanliness of the raw materials are among the requirements stated by the regulatory board to ensure the plants used for drug development are in good quality and shape (WHO, 1999; WHO, 2003; WHO 2004). To determine the cleanliness of raw materials during handling process, a series of physico-chemical analysis such as total foreign matter, total ash, acid insoluble ash and total moisture content was conducted (British Pharmacopeia Commission, 2008; Malaysian Herbal Monograph, 2010).

4.3.1 Physico-chemical analysis of FD leaf

The parameters analyzed were total foreign matter, total ash, acid insoluble ash and total moisture content. Physico-chemical analysis results of FD leaf were presented as Table 4.1.

Table 4.1: Physico-chemical analysis of FD leaf. The results (mean ± SD) for total foreign matter, total ash and total moisture content test

	Test (%)			
FD leaves	Total foreign matters	Total ash	Total moisture	Acid insoluble ash
	1.35 ± 0.3	7.8 ± 0.1	7.3 ± 0.3	1.62 ± 0.3

The total foreign matters test evaluates the contaminants resides in the FD raw material apart from the leaf using organoleptic techniques (WHO, 2011). As the first step of FD raw material quality control, determining the total foreign matters are essential and required to be performed at optimum care as unwanted materials such as poison, harmful things, mold, insect contamination or animal excreta could affect the quality of the raw materials (Kunle *et al.,* 2012). Using organoleptic aid such as a magnifier could be beneficial in detecting small contaminants that are hard to be visualized by naked eyes (Garg *et al.,* 2012). From this analysis, the FD leaf raw materials showed acceptable total foreign matters as most of the contaminants were long twigs and the fruits.

The next step of the physicochemical parameter testing is determining the total ash value. The value of total ash provides information on the ash content the FD leaf raw material. The cleanliness of the raw material used for the drug development is indicated by the total ash values (Goyal & Kumar, 2011). Briefly, the ash value can be divided in two, the physiological and non-physiological ash (Janoti & Kumar, 2013). The physiological ash is the naturally occurred inorganic salts which derived from the plant tissue itself, whereas the non-physiological ash is the adulteration purposes such as the environmental contaminations (Rao & Xiang, 2008). A high concentration of ash value indicates the raw materials having adulteration, contamination and carelessness during the harvesting period (Shivatare *et al.,* 2013). Therefore, the total ash value should be at low percentage to ensure a proper handling of the plant materials. The total ash for FD leaf was found to be in acceptable result as reading higher than 14 % are considered above limit (Vaikosen *et al.,* 2011).

To represent a clearer index in describing the quality of FD raw material, acid insoluble ash analysis was conducted. Generally, acid insoluble ash is a treatment on the ash remains using diluted acid such as hydrochloric acid (Rao & Xiang, 2008;

Malaysian Herbal Monograph, 2010). The remains of this acid treatment ash are silica, which presence in soils, sand and siliceous earth (Rao & Xiang, 2008). The purity of the FD raw material is indicated by the low acid insoluble ash value (Table 4.1) suggested that the FD raw material handling process complied with the cleanliness requirements proposed by regulatory guidelines.

The total moisture test was performed to determine the moisture contents of FD leaf. High moisture content is among the major factors that deteriorates herbal drugs (Chandel *et al.,* 2011). The high moisture contents of herbal raw material will favor the growth of microbes and fungi (Sharma *et al.,* 2014). Considering that opportunistic microbes will contaminate plants with such favorable conditions, having low moisture content is desirable in herbal raw materials preparation. Maintaining the quality of raw materials by preventing favorable conditions for microbes to growth is considered one of the main goals for standardization process (Chandel *et al.*, 2011). This study recorded moisture content below than 14%, which is deemed to be good as water content in herbal drugs should not be greater than 14% (Abdu *et al.,* 2015). The total moisture content of the FD leaf obtained from the test showed that the FD raw material was careful handled during storage period and is acceptable for further drug development process.

4.3.2 Total extractive value

The extractive value represents the estimation mixture of active phyto-constituents in a plant material that was extracted with specific solvents (WHO, 1996). The phyto-constituents' nature was based on the polarity of the solvent used for extraction and the technique used (Sasidaran *et al.*, 2010). It also indicates either the process of developing herbal drug is exhausted or not by providing the extracted yield data obtained through specific extraction technique.

In this analysis, the FD leaves were extracted using three different solvents which were ethanol, water and 50 % (v/v) ethanol water through maceration technique. The results are presented in Table 4.2. Overall, the 50 % (v/v) ethanol-water extract exhibited the highest yield compared to other solvents with percentage yield of 25.2 ± 0.1 %. The extracted yield for water extract and ethanol was 6.7 ± 0.4 %, and 13.2 ± 0.2 %, respectively.

Table 4.2: Yield of three different extractions of FD leaf

FD extracts	**% Yield of extractions**	**Amount in (mg FD extract/g FD raw material)**
Water extract	6.7 ± 0.4	33.7 ± 0.6 mg/g
50% (v/v) ethanol-water extract	25.2 ± 0.1	126.2 ± 0.6 mg/g
Ethanol extract	13.2 ± 0.2	66.3 ± 0.2 mg/g

4.4 Safety assessment of FD leaf

Nowadays, the benefits of herbs as therapeutic agents are increasing. There are lots of herbal drugs and formulation available in the markets. However, there are still concerns regarding their safety and toxicity that the herbal products may contain. Believing natural herbs to be safe is a widespread misconception with many had been reported producing adverse effects and toxicity incidents (Ekor, 2013). The toxic effects of herbal preparations are related to contaminants such as microbes, heavy metals and toxins (Efferth & Kaina, 2011). This eventually will lead to adulterations of the raw materials, making the herbs becoming unsuitable for drug discovery process. Soil geochemical characteristic, water, air, contaminants, transportation, storage facilities and conditions typically are the factors that contribute to adulteration of raw herbal materials (Dghaim *et al.*, 2015).

Therefore, FD leaf was subjected to a series of safety assessment test to evaluate the quality of the raw materials before the standardization process takes place. The main objective of this analysis is to observe the quality of the pre- and post-harvesting process since there are possibilities of hazardous contaminants and residues affecting the plant. Safety measurements of the raw material were based on recommendations by regulatory boards such as the WHO, British Pharmacopeia and the Malaysian herbal monograph (British Pharmacopoeia, 2008; WHO, 2010; Malaysian herbal Monograph, 2010).

4.4.1 Heavy metal analysis

The FD leaf was tested for the content heavy metals namely the lead, cadmium, arsenic and mercury since these elements are known to be contaminants available in the soil (Tchounwou, *et al.*, 2012). The results are presented in Table 4.3 showed no serious contaminants of heavy metal observed on the FD raw material indicating that the leaf was treated with care during the pre- and post-harvesting period.

Table 4.3: Heavy metal assessment of FD leaf

Heavy metal	**Heavy metal concentration**	**Acceptable concentration (British Pharmacopoeia, 2008)**
Lead	0.01 ppm	Not more than 10 ppm
Cadmium	< 0.001 ppm	Not more than 0.3 ppm
Arsenic	< 0.001 ppm	Not more than 5.0 ppm
Mercury	< 0.001 ppm	Not more than 0.5 ppm

From this analysis, the FD leaf was found to contain very little heavy metals which were well below the acceptable limits provided by the British pharmacopeia (2008) suggesting that the FD plant was planted in suitable environment and handled immaculately. Recently, attention has arisen regarding the toxicity of environments and heavy metals in human (Zhuang *et al.,* 2014). Contaminated soil with traces of unsafe heavy metals can be transferred through plants to human and have the tendency to accumulate in the food chain (Kurz, 1999; Arao *et al.,* 2010). The appearance of heavy metals such as cadmium in human affected the human physiology by damaging the kidneys as it's having a low excretion rate (Järup *et al.,* 2003). Lead, a known environmental pollutant can affect human health by delivering adverse effect on the blood, nervous, renal, skeletal and brain physiology. Arsenic on other hand disrupted the production of adenosine triphosphate (ATP), leading to metabolic dysfunctions (Mazumder, 2008). Mercury, a well-known toxic contaminant can affect the liver and renal function of the human health leading to diseases such as liver and renal failure (Tchounwou *et al.,* 2012). The summary of the hazardous potentials that these heavy metals deliver established an important guide for researchers to conduct a heavy metals test before proceeding with drug discovery. Since the excessive uptakes of the dietary heavy metals were responsible for numerous health problems, assessing the heavy metals could provide a deep insight on the safety and efficacy of the raw materials used in herbal preparations.

4.4.2 Microbial limit test for the presence of specific pathogen in FD leaf

Herbal plants can host ample spectrum of microorganisms (Berg *et al.,* 2009). In perception, microbial contaminations in plants are the results of animate and inanimate origin and can be easily transferred by air or soil borne vectors (Kneifel *et al.,* 2002). Intrinsic and extrinsic factors such as environments and agriculture influence the persistence and resistance attribution of plant microorganisms (Upadhyay *et al.,* 2014). Bacterial and fungal endospores dominate the contaminant group within the herbal plants as broad varieties of bacteria and fungi can be found in or on the plant materials (Frey-Klett *et al.,* 2011). Microbial contaminants can occur during the pre-, post-harvesting and storage period. The raw materials if exposed to microbial favorable conditions could result microbial contaminations and opportunistic food pathogen infestation such as *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Salmonella spp* (British Pharmacopoeia, 2008, WHO 2004**)**. Microbial limit test for specific pathogen result for the FD leaf assessment is presented in Table 4.4.

Through this analysis, the results showed that the total viable aerobic count and total yeast and mold count were within the acceptable range. The analysis also *Enterobacteria* and Gram-negative bacteria were not present in the 1 g of raw material. For the food pathogen, the raw material of FD was shown to be free of *Pseudomonas aeruginosa Escherichia coli, Staphylococcus aureus* and *Salmonella spp.* To conclude, the findings of this analysis justified that the pre-, post-harvesting and storage were carefully handled and safe enough to proceed with the drug development process.

Table 4.4: Microbial limit test (MLT) for specific pathogens in FD leaf

Test	**MLT values for FD leaf raw material**	**Acceptable value (British Pharmacopoeia, 2008)**
Total viable aerobic count	2.1×10^4 Cfu/g	10^7 Cfu/g
Total yeast and mold count	1.9×10^3 Cfu/g	10^7 Cfu/g
Enterobacteria and Gram negative bacteria	Absent in 1g	Absent in 1g
Pseudomonas aeruginosa	Absent in 1g	Absent in 1g
Escherichia coli	Absent in 1g	Absent in 10g
Staphylococcus aureus	Absent in 1g	Absent in 1g
Salmonella spp.	Absent in 1g	Absent in 25g

Cfu/g= colony forming units/gram

4.4.3 Aflatoxin assessment of FD leaf

Aflatoxins are a family of toxin produced by molds (*Aspergillus flavus* and *Aspergillus parasiticus*) which can be found in agricultural crops (U.S. Department of Agriculture Grain Inspection, 2009; FDA, 2012). Aflatoxins B1, B2, G1 and G2 are the four major types of aflatoxins and exposures of it is hazardous to human health as it's caused an array of acute and chronic diseases (Kumar *et al.*, 2016). Contamination of these toxins usually occurs during the storage period in which the soils and decay vegetation usually plays an important role in promoting contamination by the aflatoxins (Jacobsen, 2008; Kader & Hussein, 2009). Assessments of aflatoxins are important to produce quality and safe herbal products. Table 4.5 showed the aflatoxin assessment results for the FD leaf. The results showed that there were no signs of the toxins in the FD raw material, complied with the requirements proposed by British Pharmacopeia (British Pharmacopoeia, 2008), suggesting that the handling of the raw material was during the storing period were immaculate.

Table 4.5: Aflatoxin assessment of FD leaf

Aflatoxin	**Aflatoxins detection in FD leaf**
Aflatoxin B1	Not detected
Aflatoxin B2	Not detected
Aflatoxin G1	Not detected
Aflatoxin G2	Not detected

4.5 Standardization of FD extracts using the HPLC technique

HPLC is a technique to separate a mixture into its component based on their polarity by applying solvent system and introduced it through a specific column. Then, it will be analyzed by a detector in which a specific wavelength is being input. The result will come out as a chromatogram and the compounds in the chromatogram can be identified by applying the HPLC markers. In this study, vitexin and isovitexin were used as the markers to standardize the FD leaf extract. Reproducibility of the chromatogram is an important aspect to produce a reliable HPLC result. Additionally, through mathematical calculation, the quantified value of each compound present in the extract can be determined. Three different FD leaf extracts (water, ethanol and 50 % (v/v) ethanol water) were prepared for the analysis. From the FD leaf extract, the quantified value of each compound (vitexin and isovitexin) is presented in Table 4.6. The chromatograms of the HPLC analysis were depicted as Figure 4.2.

Table 4.6: Quantification of vitexin and isovitexin in FD leaf extracts (mean ± SD)

FD extracts	**Content of vitexin (mg/g dry weight)**	**Content of isovitexin (mg/g dry weight)**	**Vitexin to isovitexin ratio in each extract**
Water extract	0.42 ± 0.05	0.20 ± 0.02	**2:1**
50% (v/v) ethanol-water extract	0.62 ± 0.01	0.26 ± 0.04	**2.4:1**
Ethanol extract	0.51 ± 0.01	0.22 ± 0.03	**2.3:1**

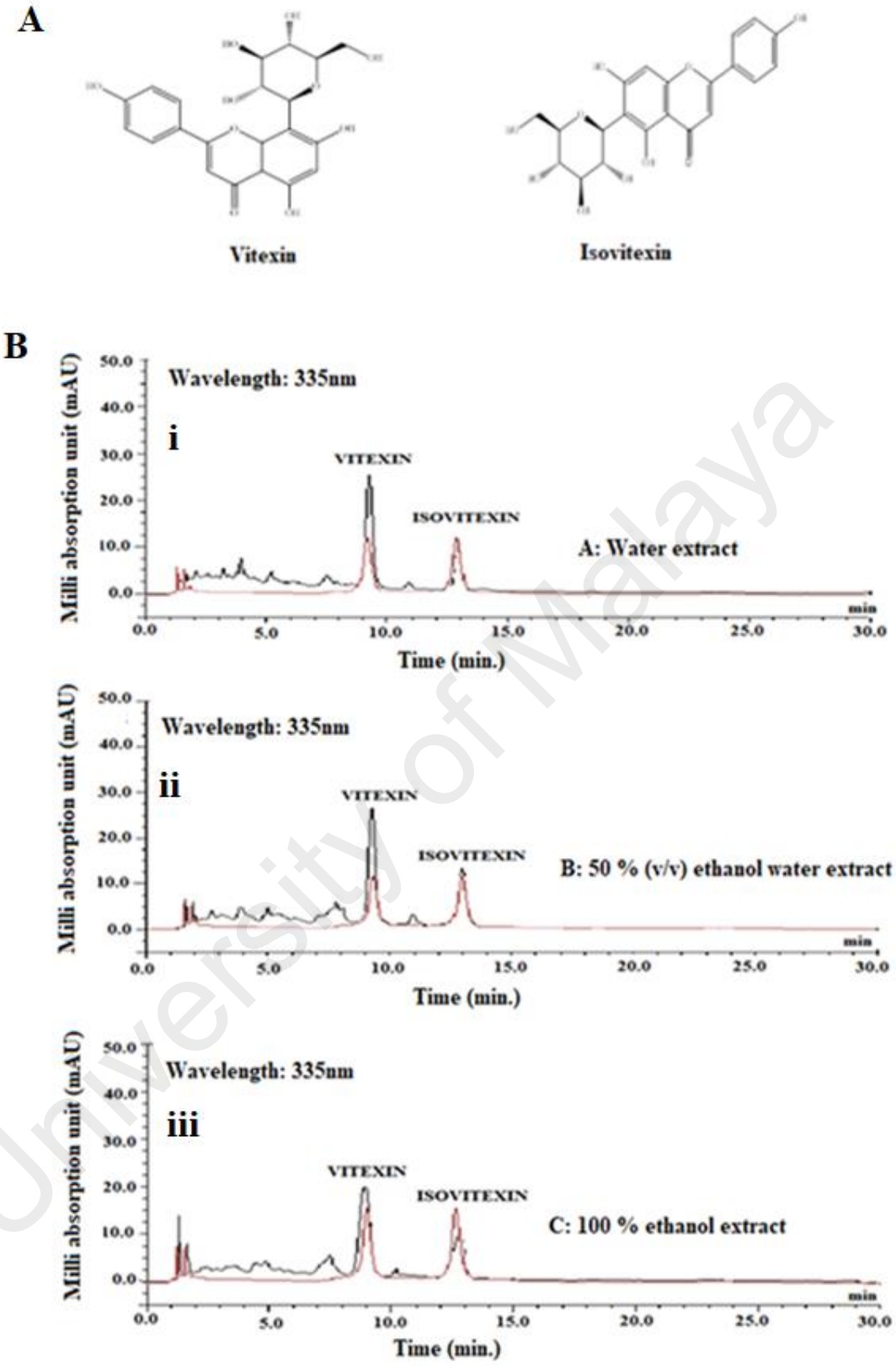


Figure 4.2: A) Vitexin and isovitexin chemical structure. B) HPLC chromatograms of three different extracts of FD leaf at the wavelength of 335 nm. (i) Water extract, (ii) 50% (v/v) ethanol-water extract, and (iii) 100% ethanol extract. The red line in the chromatogram indicates the peaks of standard markers (vitexin and isovitexin)

The analysis of the FD leaf extract using HPLC showed that vitexin and isovitexin were the main compounds in the extract with the highest concentration of these compounds was in the 50% (v/v) ethanol-water extract with vitexin comprising 0.62 ± 0.01% and isovitexin 0.26 ± 0.04% (percentage dry weight). For the water extract, the value for was vitexin was 0.42 ± 0.05% and isovitexin was 0.20 ± 0.02% (percentage dry weight). Lastly, the ethanol extract quantified data for vitexin was 0.57 ± 0.01% and isovitexin was 0.22 ± 0.03% (percentage dry weight).

4.6 Discussion

The standardization results of the FD leaf described the quality, safety and efficacy of the raw material used before proceeding with the herbal drug development process (Kumar, 2013; Patwekar *et al.,* 2015; Garg & Sardana 2016). Preparing good FD leaf raw material at the early stage is important as adulterations and contaminations of the raw materials are influenced by the way the leaf was handled and stored (WHO, 2016). Plants with adulterations and contaminations could heavily affect the drug development process, potentially provides a safety hazard to consumers upon consumption of the materials (Avigan *et al.,* 2016). Therefore, standardization is necessary to be conducted on herbal plants so that a proper guideline on the quality, safety and efficacy can be monitored and checked.

Overall, the standardized FD leaf data used in this study demonstrated that proper handling was conducted on the raw material. The post-harvesting process of the FD raw material was shown to be excellence with very low foreign material and ash detected. The storing of the FD leaf was also in preferable conditions as low moisture contents were detected in the FD leaf. The safety assessments on the FD leaf was found to be in a safe range with the microbial, aflatoxins and heavy metals results far below the limits proposed by regulatory boards.

Through HPLC methods, the phytochemical constituents of the FD leaf extracted in all three solvents illustrated that vitexin and isovitexin are two prominent compounds with the FD 50 % (v/v) ethanol water extract producing the highest concentration of both compounds. Vitexin and isovitexin are flavonoids with therapeutically potential activities such as anti-inflammatory, anti-oxidant (Prabhakar *et al.,* 2007; Ragone *et al.,* 2007), and α−glucosidase inhibitory effect (Choo *et al.,* 2012). Through HPLC method, the vitexin and isovitexin can be standards for standardizing FD extract. A range of accepted vitexin and isovitexin concentrations can be established as a guideline for researchers when dealing with the FD plant materials. Producing reliable and reproducible results are the main goal of herbal standardization. Since the constituents of the plant are generally consistently inconsistent, having a standardization data could eminently benefits researchers in producing high quality plant extracts for drug discovery. To conclude, the findings through these experiments fulfilled the first objective of this study by revealing that the FD leaf used are in good shape, complying with all parameters measurement proposed by regulatory boards, and efficacy was standardized through HPLC technique.

CHAPTER 5: ENZYMATIC INHIBITION PROPERTIES OF FD LEAF EXTRACT: COMPUTATIONAL AND EXPERIMENTAL APPROACHES

5.1 Introduction

The results from chapter 4 showed that vitexin and isovitexin were the major bioactive constituents in the FD leaf extract. In general, vitexin and isovitexin are both isomeric flavonoids with differences on its glycoside attachment. Vitexin glycoside attachment is on the 8th carbon, whereas the isovitexin glycoside attached to the 6th carbon of the flavonoid backbone. In this chapter, vitexin, isovitexin and FD leaf extract potentials as therapeutic agent were investigate using enzymatic assay on two different types of enzyme namely the α-amylase and MMPs. Following that, computational analyses were conducted to study the molecular behavior of both the bioactive constituents of the FD leaf as potential therapeutic agents. Therefore, these studies consist of molecular docking analysis and molecular dynamic simulations (Wang *et al.,* 2001; Hansson *et al.,* 2002; Karplus & Kuriyan, 2005) were conducted to unravel vitexin and isovitexin interaction with specific enzymes as well as to observe their molecular behavior during simulations.

5.2 Enzymatic assay of FD leaf 50% (v/v) ethanol water extract

The FD leaf 50% (v/v) ethanol water extract was chosen as the tested FD leaf extract due to the nature of its having the highest concentration of vitexin and isovitexin per proven by HPLC analysis (Chapter 4). For the enzymes, α-amylase and MMPs enzyme were selected due their involvement in metabolic related diseases such as diabetes and it complications namely wound healing disorders, prolonged inflammations and necrosis.

5.2.1 The α-amylase inhibition assay

The α-amylase inhibition assay was conducted on the FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin (Figure 5.1). Acarbose was used as a positive control for this assay (Manaharan *et al.*, 2012). The result of α-amylase inhibition activity is shown as Table 5.1. The IC_{50} values for each sample were presented as mean ± SD.

Vitexin showed the most potent inhibitory activity with IC_{50} value of 0.02 ± 0.1 µg/ml, followed by isovitexin (IC_{50}: 0.06 ± 0.1 µg/ml) and the FD leaf 50% (v/v) ethanol water extract (IC_{50}: 3.8 ± 0.1 µg/ml). The positive control, acarbose on the other hand showed an inhibition IC_{50} of 10.4 ± 5.2 µg/ml, which was lower when compared to the three samples.

Table 5.1: The α-amylase inhibition assay of FD leaf 50% (v/v) ethanol-water extract, vitexin, isovitexin and acarbose. The symbol * represent the significant differences ($p < 0.05$) when compared to acarbose.

Samples	**IC_{50} (µg/ml) of α-amylase inhibition activity**
FD leaf 50% (v/v) ethanol-water extract	3.8 ± 0.2
Vitexin	0.02 ± 0.1*
Isovitexin	0.06 ± 0.1*
Acarbose (Positive control)	10.4 ± 5.2

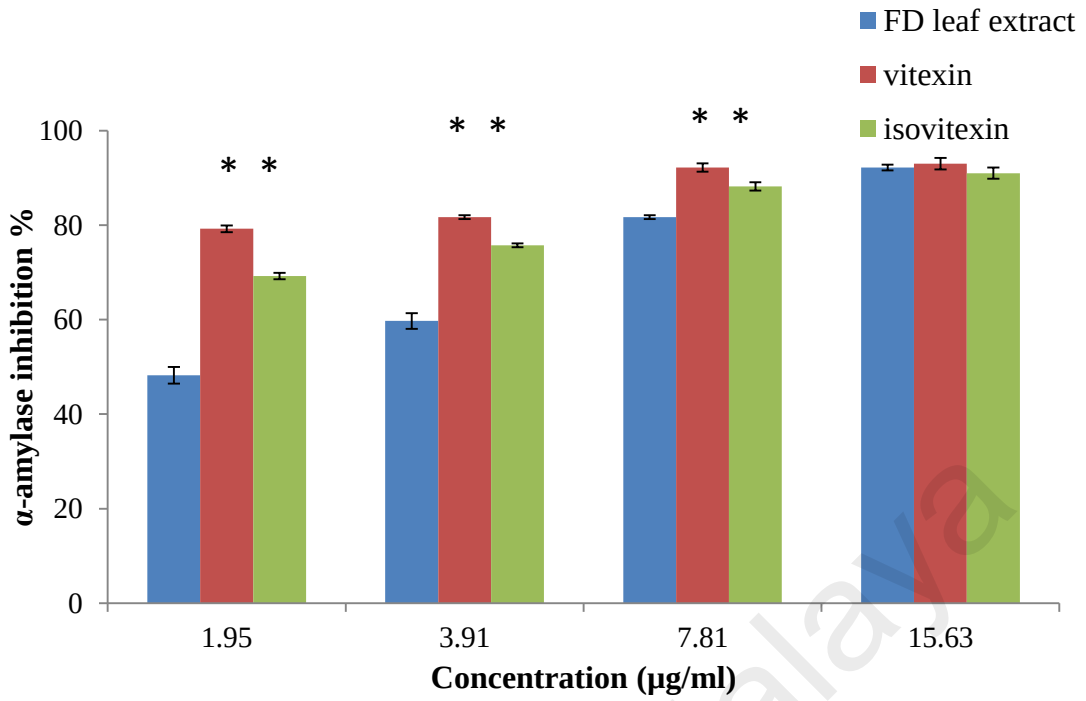


Figure 5.1: Graphical representation of α-amylase inhibition assay of FD leaf 50% (v/v) ethanol-water extract, vitexin and isovitexin. The symbol * represent the significant differences ($p < 0.05$)

5.2.2 Inhibition activity of FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin on MMP-2, 8 and 9

Enzymatic inhibition activity of FD leaf 50% (v/v) ethanol water extract was tested on selected MMPs. MMP-2, MMP-8 and MMP-9 were used in this study because of their potential as therapeutic targets in various diseases (Solan *et al.*, 2012). The inhibition activity of FD leaf 50% (v/v) ethanol water extract and its bioactive constituents (vitexin and isovitexin) is depicted in Figure 5.2. Overall, vitexin, isovitexin and FD leaf 50% (v/v) ethanol water extract demonstrated significant inhibition activity ($p < 0.05$) for the MMP enzymes.

The half maximal inhibitory concentration (IC_{50}) value for FD leaf 50% (v/v) ethanol water extract was 90 ± 0.01 µg/ml for MMP-2, 290 ± 0.01 µg/ml for MMP-8 and MMP-9 was 320 ± 0.02 µg/ml. Vitexin IC_{50} value was found to be 210 ± 0.02 µg/ml (0.49 mM) for MMP-2, 360 ± 0.003 µg/ml (0.83 mM) for MMP-8 and 320 ±

0.02 µg/ml (0.74 mM) for MMP-9. Lastly, isovitexin demonstrated an IC_{50} value of 110 ± 0.01 µg/ml (0.25 mM) for MMP-2, 170 ± 0.01 µg/ml (0.39 mM) for MMP-8 and 240 ± 0.01 µg/ml (0.56 mM) for MMP-9.

The results showed that FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin are having enzymatic inhibitory effect on MMP-2, 8 and 9. For FD leaf extract and isovitexin the strongest inhibitory effect was detected in MMP-2 followed by MMP-8 and MMP-9. Meanwhile in vitexin, the highest inhibitory effect was identified in MMP2 followed by MMP-9 and MMP-8.

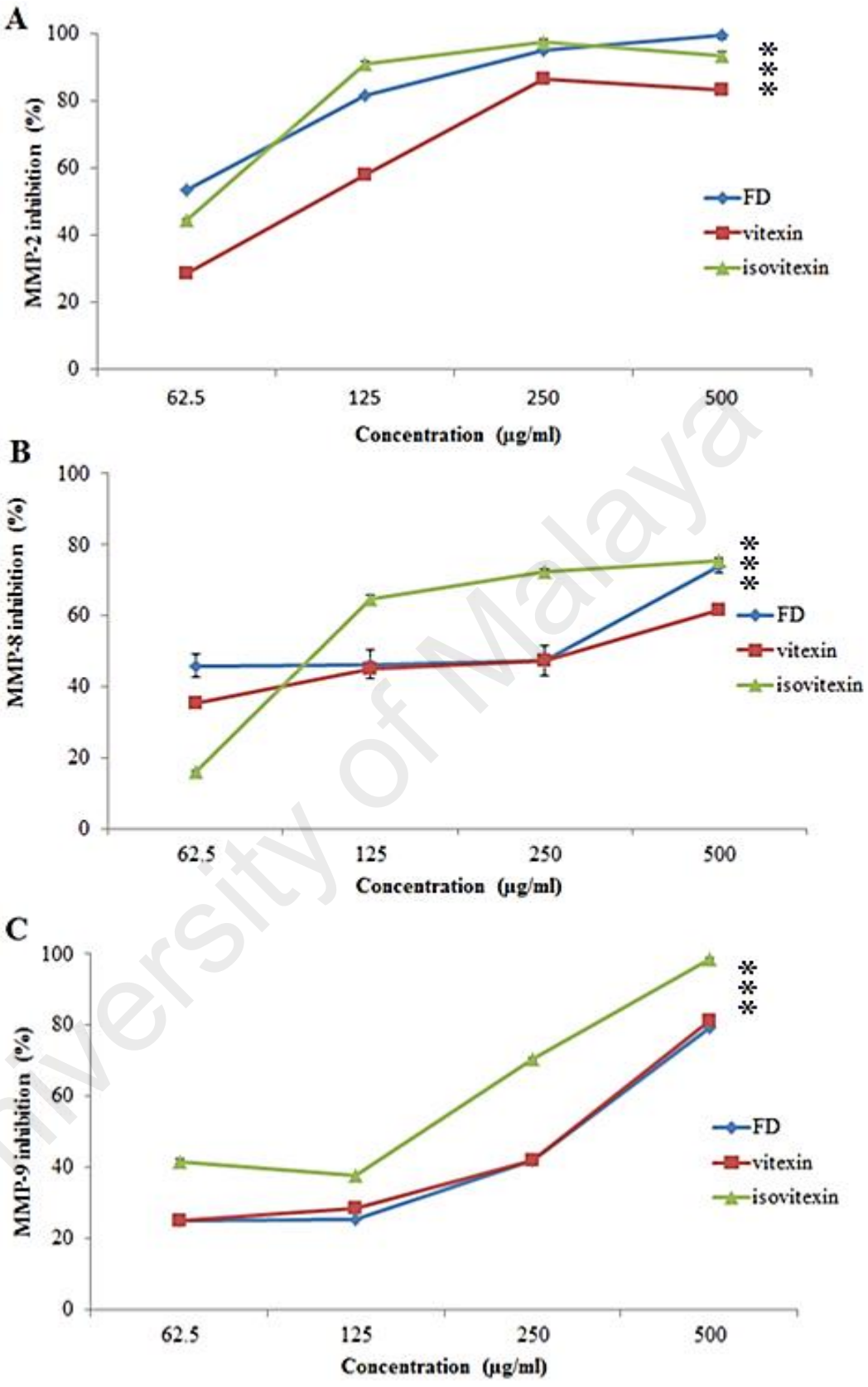


Figure 5.2: The results of MMPs inhibition assay for FD leaf 50% (v/v) ethanol water extract. The symbol * represent the significant differences ($p < 0.05$)

5.3 Computational analysis of vitexin and isovitexin towards α-amylase enzyme

Binding affinity of vitexin and isovitexin towards α-amylase enzyme was analyzed using Autodock (Morris *et al.,* 2009). Initially, blind docking analysis was introduced to determine the possible binding site of vitexin and isovitexin towards α-amylase enzyme. The objective was to un-biasedly map the binding patterns of vitexin and isovitexin by scanning the entire surface of α-amylase enzyme. The result defined the location of active site residue and was used throughout the docking analysis. Docking results for vitexin and isovitexin were clustered with root mean square deviation (RMSD) tolerance of 2.0 Å (Figure 5.3). Visualization of the binding sites on the α-amylase enzyme is shown in Figure 5.4. The analysis revealed that vitexin has 16 multi-member conformation clusters with the lowest binding energy (LBE: -7.64 kcal/mol) and exhibited the highest populated conformations (22 out of 100 conformations). For isovitexin, 11 multi-member clusters were identified with the LBE was - 6.07 kcal/mol and the highest number of conformations was in rank 3 (LBE: -5.26 kcal/mol, 29 out of 100 runs).

From the docking analysis of vitexin to α-amylase enzyme, it was revealed that the interacting residues were HIS 305, HIS299, GLU 233, LYS 200, HIS 201, ILE 235, GLY 306, LEU 162, ALA 198, TYR 62, HIS 101, ASP 197, ASP 300 and TRP 58. Meanwhile, isovitexin interacting residues were GLY 306, LEU 237, GLU 240, LYS 200, LEU 162, ALA 307, ASP 236, ILE 235 GLY 238, TYR 151 and HIS 201.

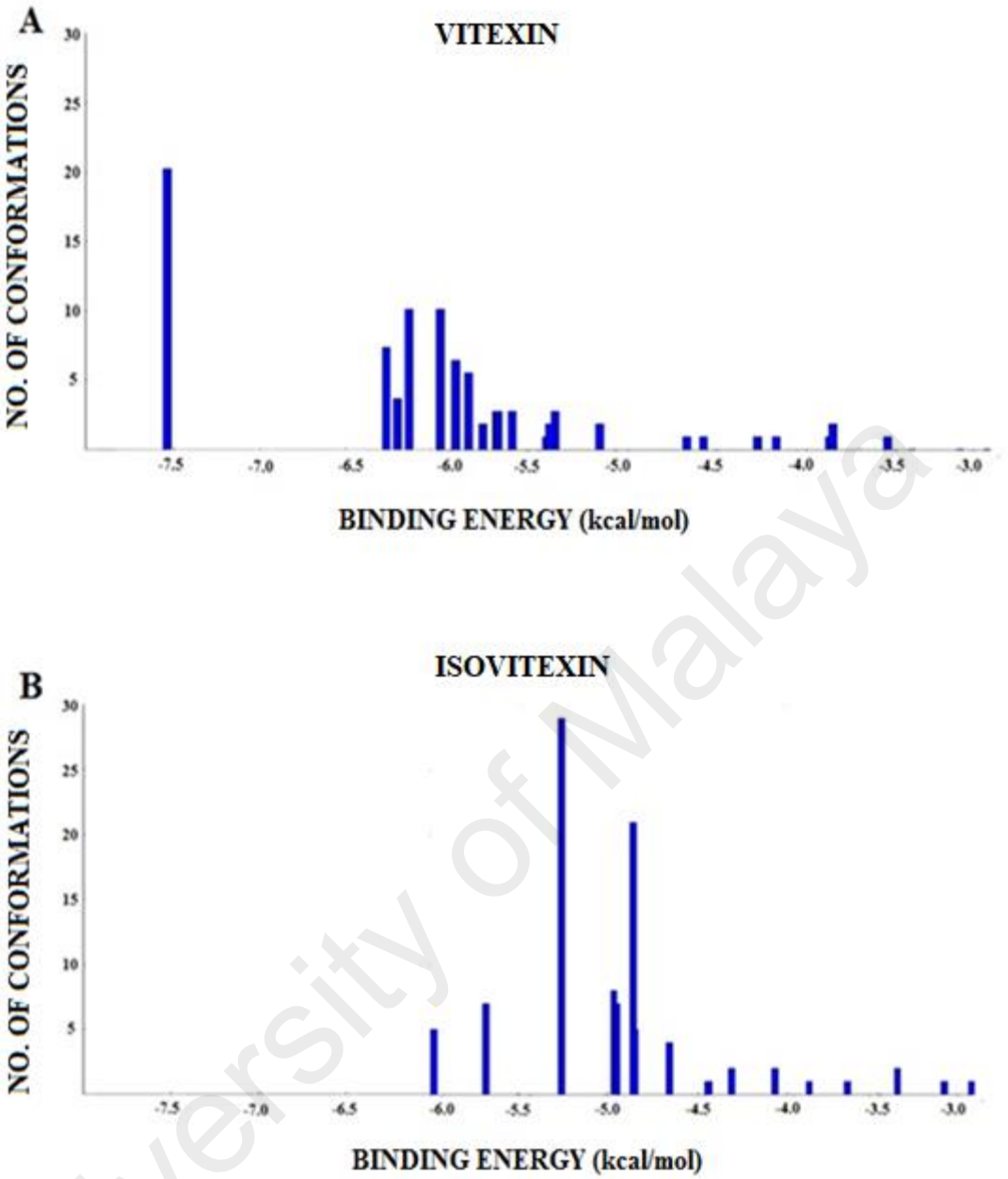


Figure 5.3: Clustering analysis of vitexin and isovitexin on the α-amylase binding site after 100 runs**.** Vitexin produced the lowest binding energy of -7.64 kcal/mol while isovitexin produced the lowest binding energy value at - 6.07 kcal/mol

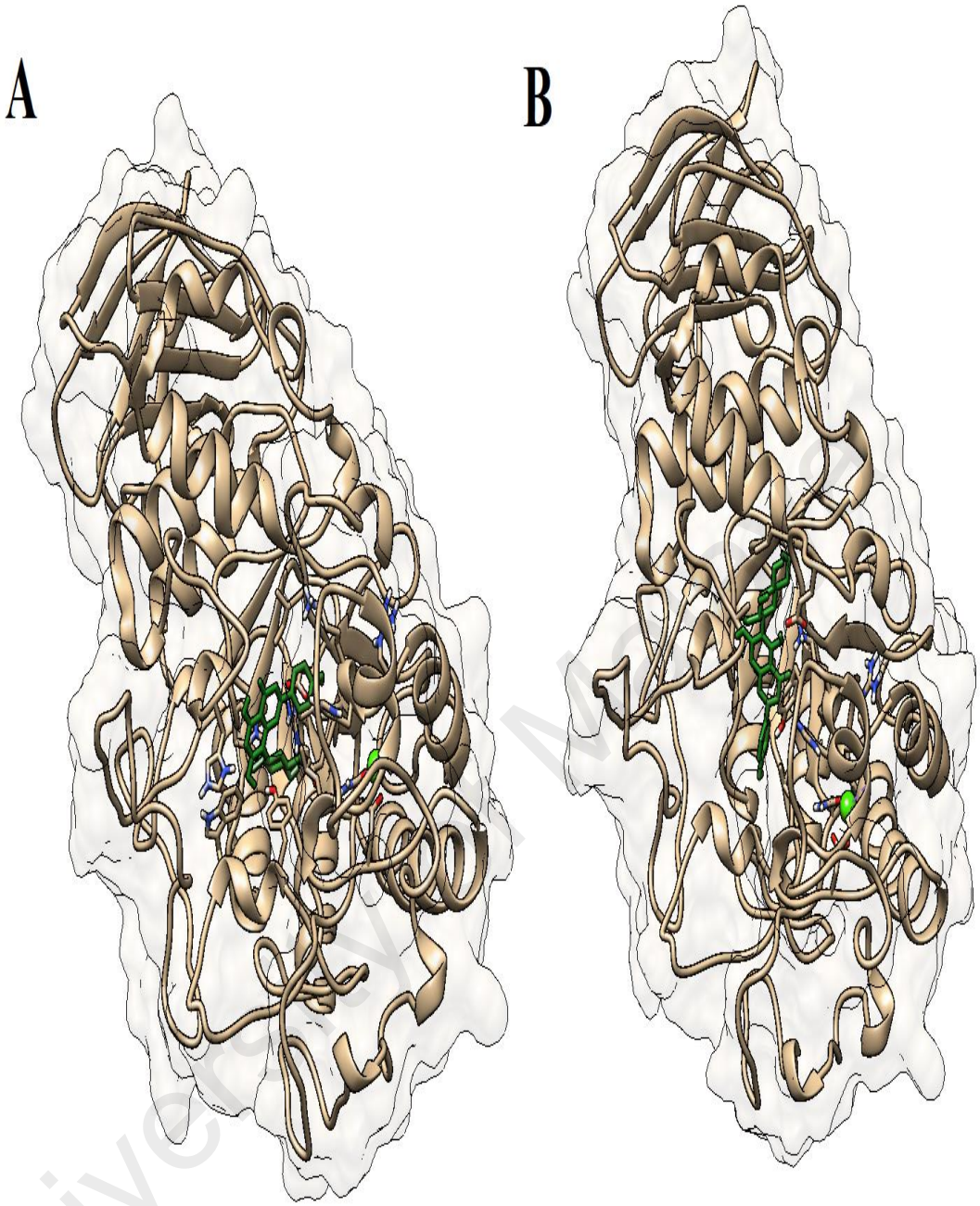


Figure 5.4: Visualization of (A) vitexin and (B) isovitexin binding site on the α-amylase enzyme. The compounds fit into the binding pocket of the α-amylase enzyme

5.3.1 Molecular dynamic analysis of vitexin and isovitexin with α-amylase enzyme

Molecular dynamic simulations of vitexin or isovitexin with the α-amylase enzyme were conducted using GROMACS software (Pronk *et al.,* 20013; Abraham *et al.*, 2015). A simulation of 20 ns was used to analyze the biding patterns of vitexin and isovitexin with the α-amylase enzyme. The analysis involved the root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration and conformational changes to the structures.

5.3.2 The RMSD analysis of vitexin and isovitexin with with α-amylase enzyme

The RMSD analysis of vitexin o isovitexin with α-amylase enzyme showed that the vitexin-α-amylase and isovitexin-α-amylase complexes were stable throughout the 20 ns simulation. Both complexes showed fluctuation within 0.1 nm (10 Å). The graphs of both complexes are illustrated in Figure 5.5.

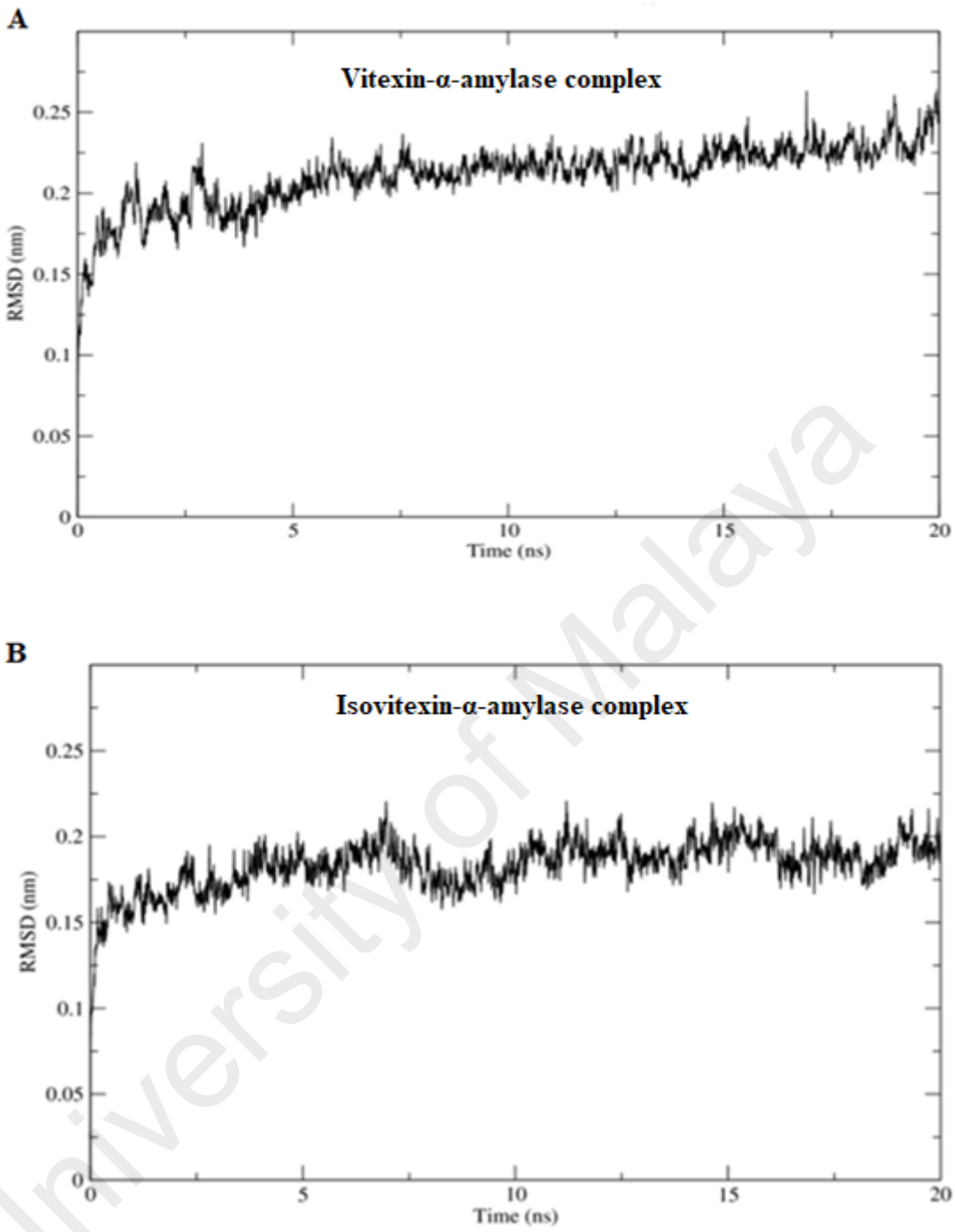


Figure 5.5: The RMSD analysis of (A) vitexin-α-amylase complex and (B) isovitexin-α-amylase enzyme complex

5.3.3 The radius of gyration analysis of vitexin or isovitexin interactions with with α-amylase enzyme

The gyration analysis of vitexin-α-amylase and isovitexin-α-amylase enzyme complexes showed that the rotation of ligand-enzyme complex is sufficiently stable within the 20 ns simulation. The vitexin-amylase complex showed a steady trend compared to isovitexin-amylase complex. However, the radius of gyration for both complexes fluctuates within 0.1 nm (Figure 5.6).

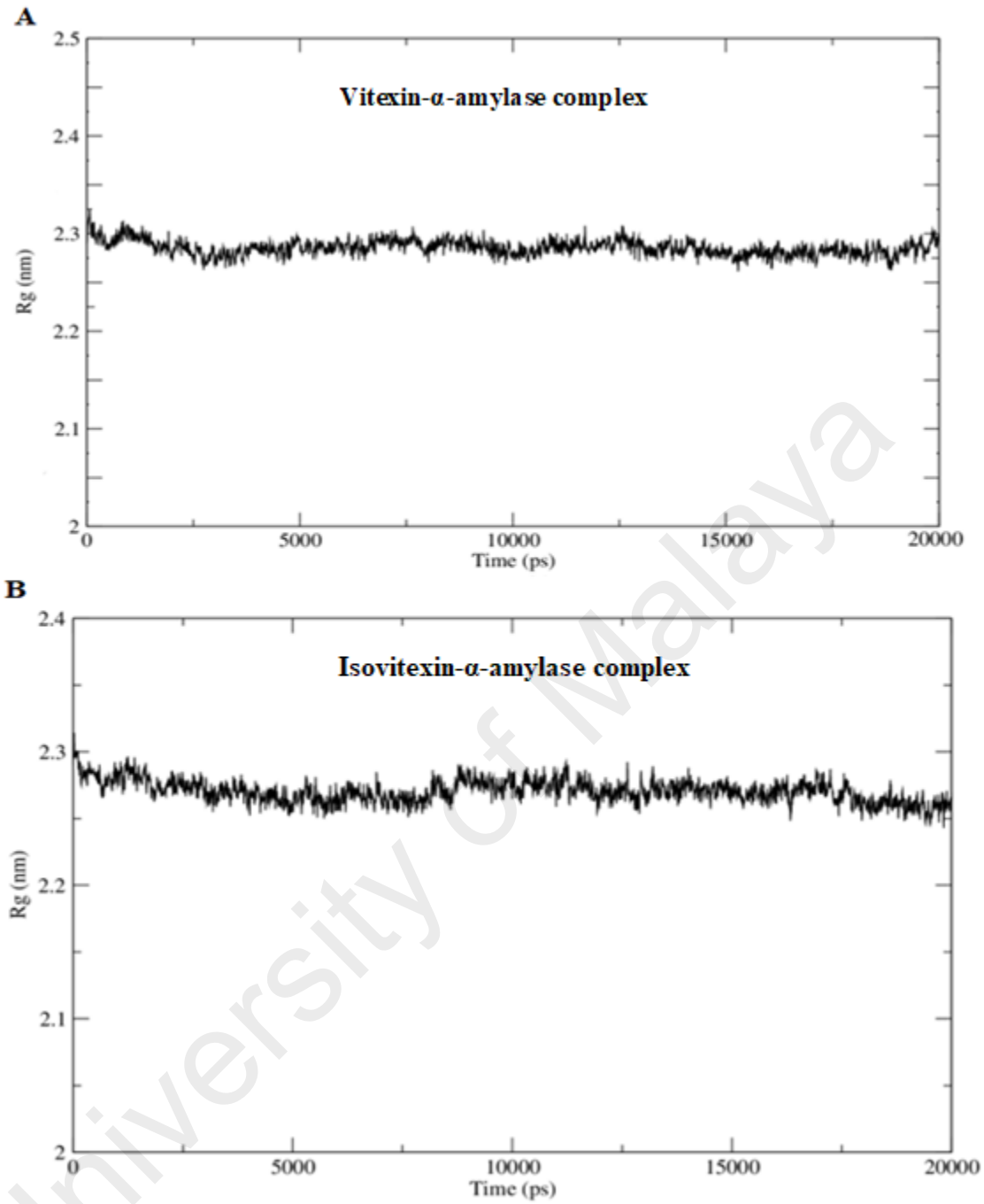


Figure 5.6: The gyration analysis of (A) vitexin-α-amylase complex and (B) isovitexin-α-amylase enzyme complex

5.3.4 The RMSF analysis of vitexin and isovitexin interactions with α-amylase enzyme

The RMSF analysis revealed that throughout the 20 ns molecular dynamic simulation, vitexin-α-amylase and isovitexin-α-amylase complexes exhibited similar fluctuation pattern within the first 300 residues. However, at residue 350 and above, isovitexin-α-amylase complex showed a slightly higher fluctuation compared to vitexin-α-amylase complex. The trajectories of the atomic position from the RMSF are shown in Figure 5.7.

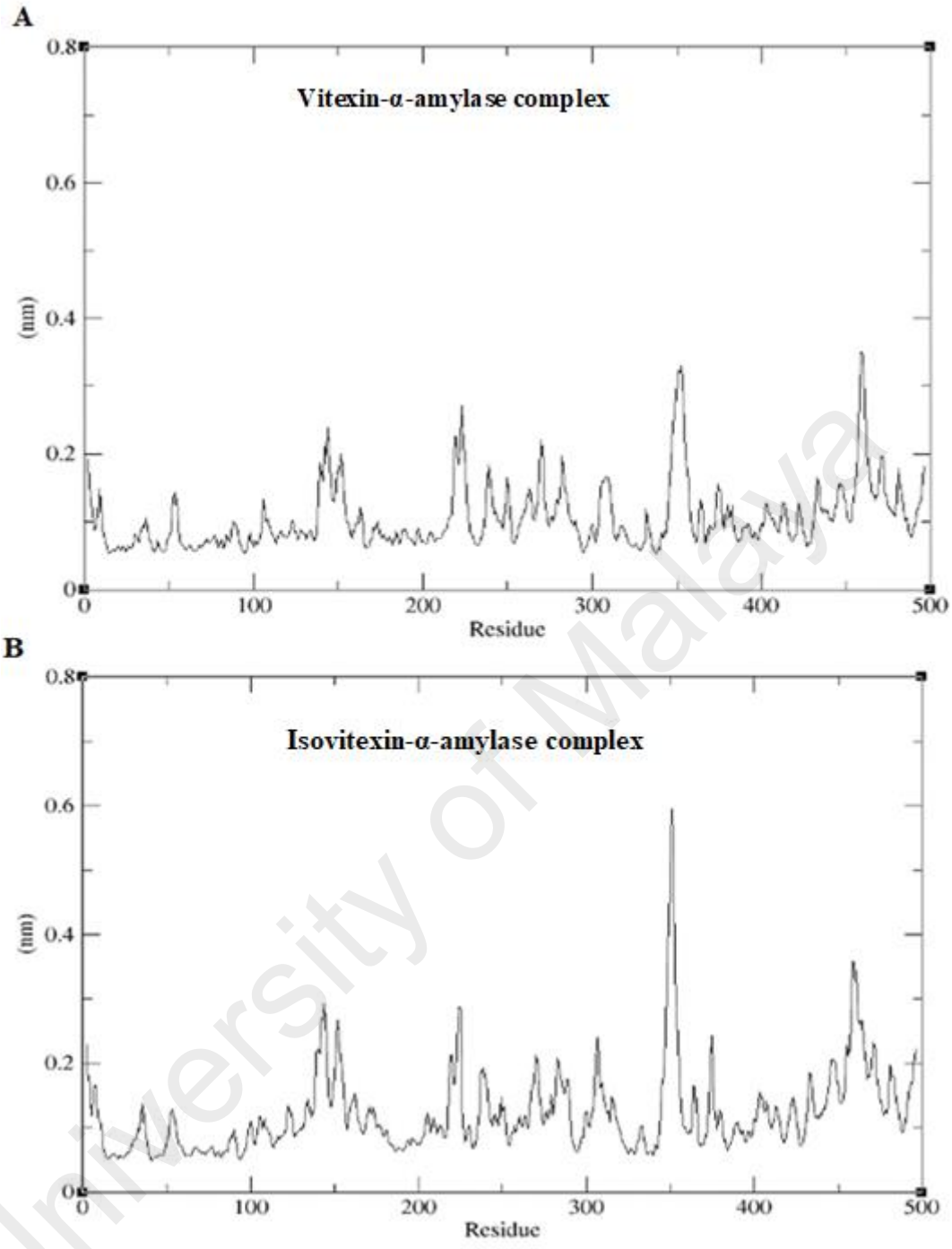


Figure 5.7: The RMSF analysis of vitexin and isovitexin interactions with α-amylase enzyme

5.3.5 The structural changes of vitexin-α-amylase complex and isovitexin-α-amylase complex during 20 ns simulation

To have a better understanding on the interactions of both the compounds α-amylase, we analyzed the conformational changes for all residues throughout the 20 ns simulation. The conformational changes on the β-sheet, coil, β-bridge, bend, turn, α-helix, 3-helix and 5-helix (Zacharias & Knapp, 2014) were shown as Figure 5.8. From the analysis, different conformational changes can be found at residues 35, 150, 310 and 350 for vitexin-α-amylase while 450 for isovitexin-α-amylase complexes. At residue 35, vitexin-α-amylase complex was in turn elements then folded to β-sheet from 10ns and onwards whereas the isovitexin-α-amylase complex geometrically favors the turn and bend elements of the secondary protein structure. Residue 150 revealed that vitexin-α-amylase complex showed stable folding pattern of α-helix and turn elements for almost 15 ns before reversing completely the polypeptide chain as turn element, whereas the isovitexin-α-amylase complex showed consistently geometric formation of α-helix. At residue 310, the vitexin-amylase complex favors turn and bend elements of the secondary protein structure while the isovitexin-amylase complex was in stable folding state of α-helix during the simulation. At residue 350, strong differences can be seen for both protein-ligands complexes. Vitexin-α-amylase complexes favored the bend, a slight turn and β-sheet folding of the secondary protein structure. For isovitexin-α-amylase complex, bend and coil elements were geometrically pronounced in this state. This may explain the fluctuations in the RMSF graph as shown in Figure 5.7, suggesting a greater deviation in terms of atom drifting. For residue 450, the secondary structure in vitexin-α-amylase complex showed pronounced turn elements. The isovitexin-α-amylase complex on other hand was revealed to be in turn direction before curving to bend regions of the secondary protein structure.

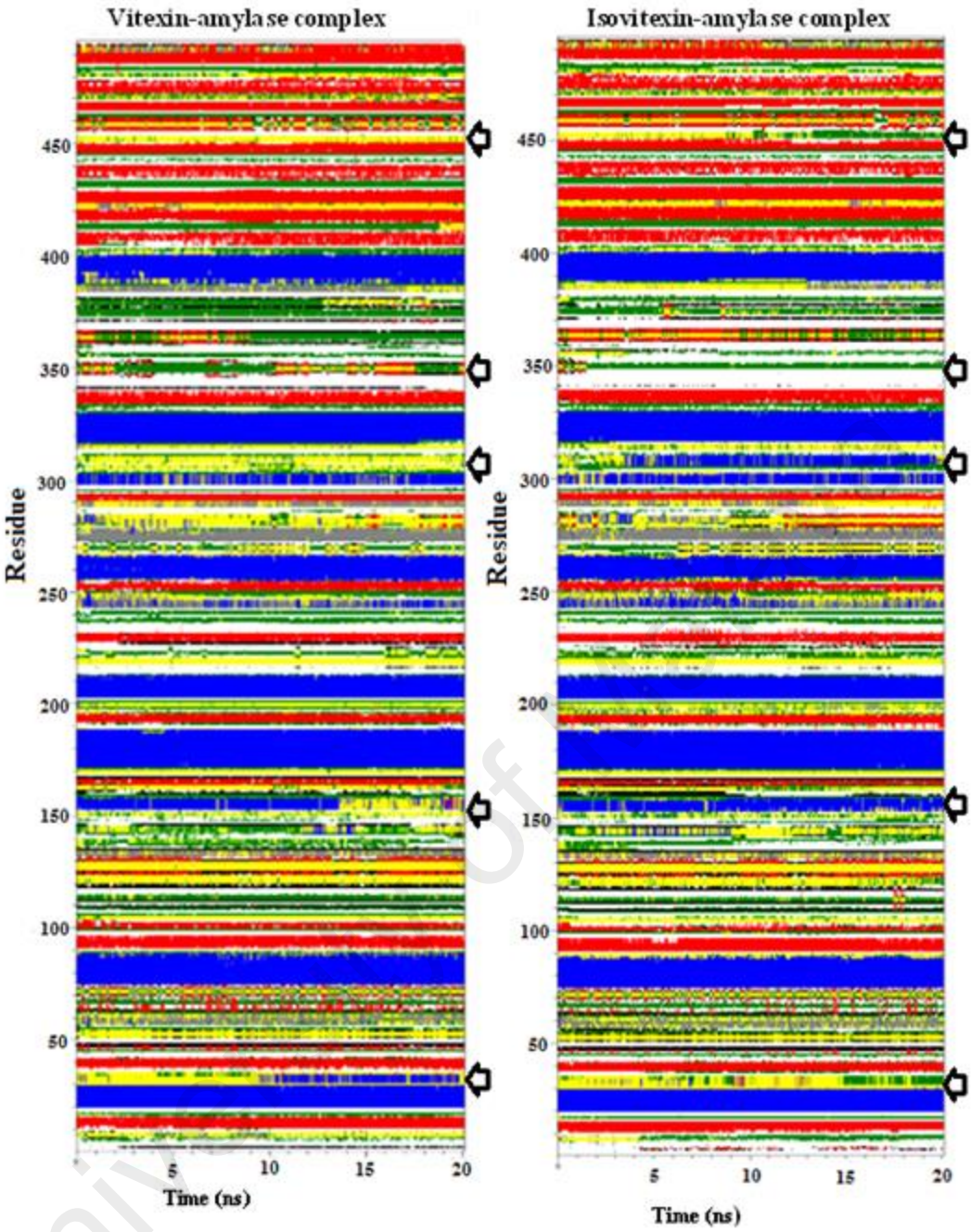


Figure 5.8: The conformational changes (denoted by left pointing arrowheads) of the secondary structure of α-amylase throughout the simulation. White: Coil, Red: β-sheet, Black: β-bridge, Green: Bend, Yellow: Turn, Blue: α-helix, Purple: 5-helix and Grey: 3-helix

5.4 Computational analysis of vitexin and isovitexin towards MMP-2, MMP-8 and MMP-9 enzyme

The molecular study of vitexin and isovitexin towards the MMPs enzyme (MMP-2, MMP-8 and MMP-9) were conducted using Autodock to evaluate the molecular interactions between the ligands and the enzymes (Klein & Bischoff, 2011). MMPs involved in normal physiology and pathological activities in human. Molecular docking analysis (Figure 5.9) showed positive results on the ligands (vitexin and isovitexin) interactions with the MMP enzymes (MMP-2, MMP-8 and MMP-9). For MMP-2, the binding affinity for vitexin was -7.31 kcal/mol and isovitexin was -9.59 kcal/mol. In MMP-8, the binding affinity for vitexin was -7.42 kcal/mol and isovitexin was -7.47 kcal/mol. As for the MMP-9, the binding affinity for vitexin and isovitexin was -9.35 kcal/mol and -11.65 kcal/mol, respectively.

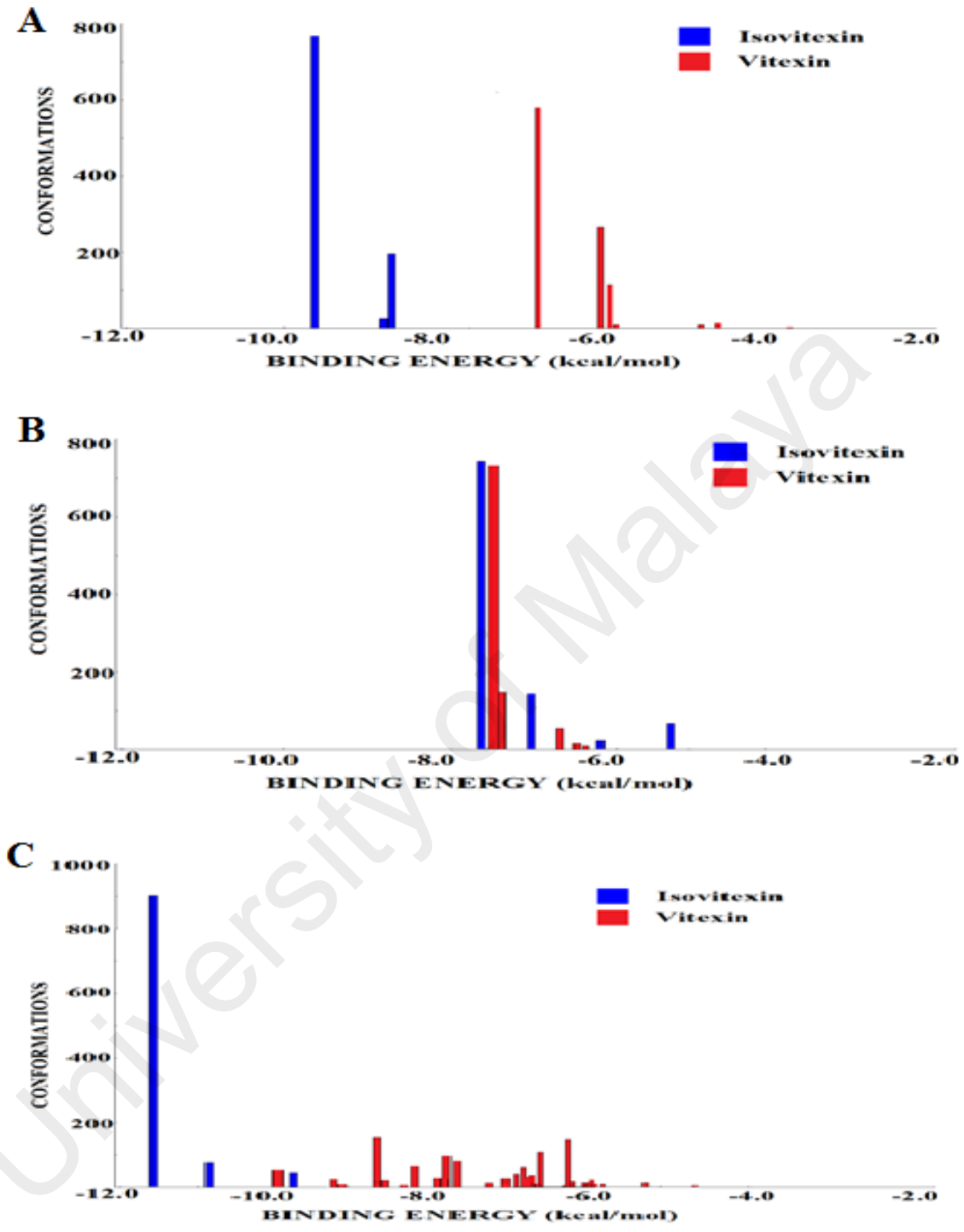


Figure 5.9: Autodock results for vitexin and isovitexin interactions against the MMP enzymes (A: MMP-2, B: MMP-8 and C: MMP-9) for 1000 runs

The positioning of both vitexin and isovitexin inside the catalytic region of the MMP enzymes was analyzed and illustrated in Figure 5.10. This study also identified that in all the ligand-protein complexes, the ligand bound to the active site proximity with the interacting amino acids as stated in Table 5.2.

Table 5.2: Interacting amino acid residues in ligand-protein complexes

Protein	Ligand	Interacting amino acid residues
MMP-2	Vitexin	LEU83, ALA84, HIS130, PRO140, ALA139, MET138, ALA136, LEU137, THR143, TYR142, VAL117, HIS120 and ILE141.
	Isovitexin	GLU121, LEU83, ALA84, PRO140, ILE141, HIS120, ALA136, LEU116, ALA139 and LEU137.
MMP-8	Vitexin	LEU193, TYR219, ALA161, ASN218, HIS207, GLU198, GLY158, PRO217, HIS201 and VAL194.
	Isovitexin	VAL112, SER218, LEU119, GLU118, ASN226, SER225, THR224, ASN184, PHE192 and ASP115.
MMP-9	Vitexin	GLY186, PRO246, HIS236, MET247, TYR248, VAL223 and LEU188.
	Isovitexin	GLU241, ALA242, PRO255, PRO254, THR251, LEU222, TYR248, ARG241, MET247, HIS226 and GLU227

.

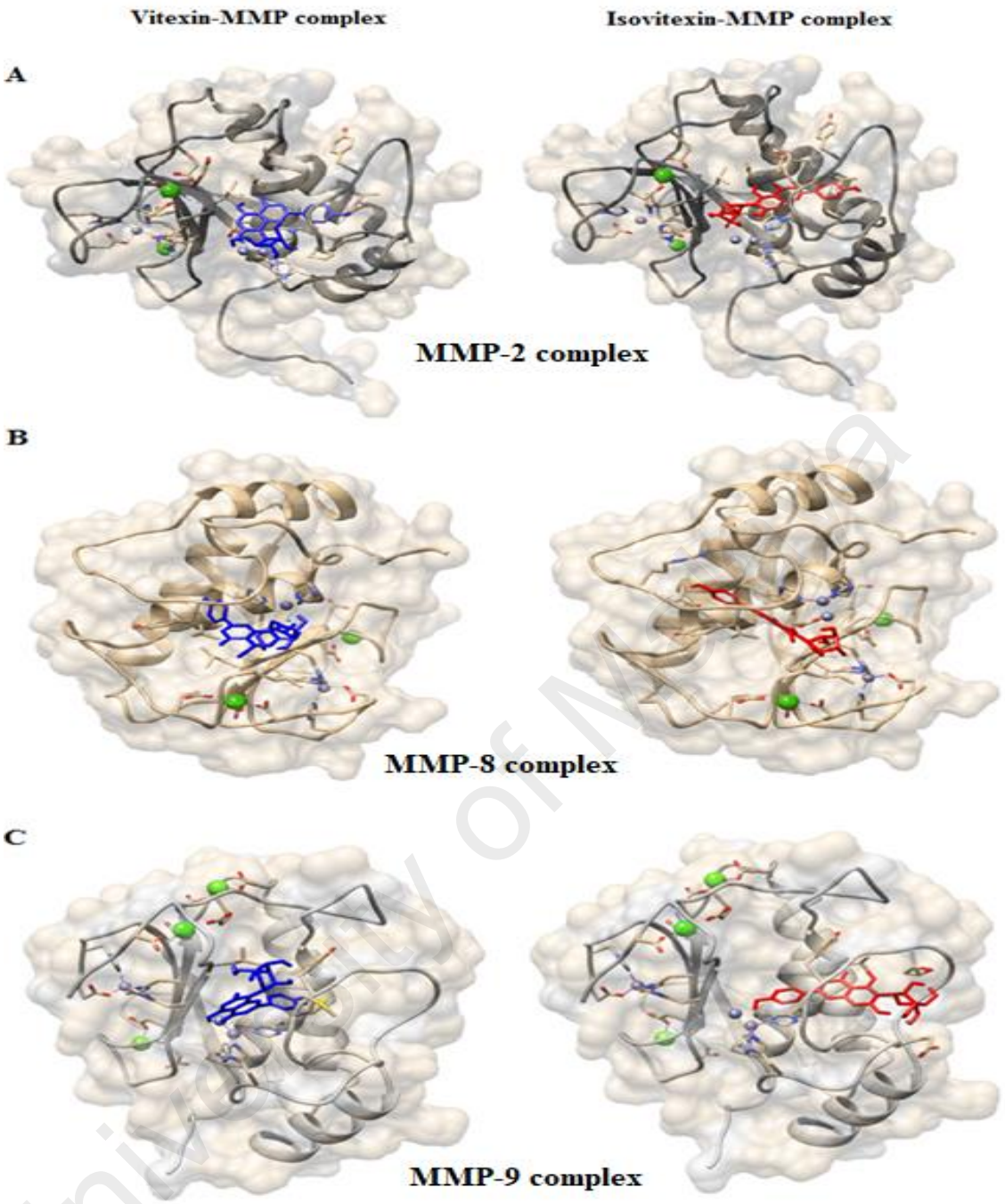


Figure 5.10: Vitexin- and isovitexin-MMPs complexes obtained from the docking analysis. A: Vitexin- and isovitexin-MMP-2 complexes, B: Vitexin- and isovitexin-MMP-8 complexes and C: Vitexin- and isovitexin-MMP-9 complexes

5.4.1 Simulations of vitexin and isovitexin toward MMP-2, MMP-8 and MMP-9 enzyme

As presented in section 5.4, the best binding affinity for vitexin and isovitexin interactions with the MMPs formed stabled complex based on the NPT and NVT equilibration in term of the temperature, pressure and density throughout the simulation.

The results of the RMSD, RMSF and radius of gyration for each complex were further analyzed as these factors contribute towards stability throughout the simulations. Analysis of the root mean square deviation (RMSD) calculates the trajectory of the complexes throughout the simulation processes (Maiorov & Crippen, 1995). Radius of gyration computes the atomic position of the trajectory and the flexibility of the complexes (Fiorin *et al.*, 2013). The fluctuation of the atomic residue can be a way of confirming the stability of the enzyme-ligand complex during the simulation study (Mutt & Sowdhamimi, 2016). The RMSF value for each complex showed little variation between the complexes during the simulation period. The findings suggested that the interaction between the ligands (vitexin and isovitexin) and the MMPs (MMP-2, 8 and 9) are strong enough to form stable complexes formation during the 20ns simulation.

5.4.2 Molecular dynamic analysis of vitexin–MMP-2 and isovitexin-MMP-2 complex

The RMSD analysis (Figure 5.11) showed that the complex formation between the ligands and enzymes were stable during the 20 ns simulations. For the radius of gyration (Figure 5.12), the complex formed between the ligands (vitexin and isovitexin) and MMP-2 showed stable motion of the complex during the 20 ns with isovitexin complex showed slightly steady movement compared to the vitexin. The RMSF analysis (Figure 5.13) of the complex revealed that isovitexin complex showed a prominent fluctuation at residue 75 and above. However, the measurement of distance

between the residues fluctuation is still within 1 Å suggesting that the complex formed are stable during 20 ns simulations. The secondary structural changes (Figure 5.14) within the complexes were analyzed through GROMACs software. The changes were prominently seen at residue 10 in which the vitexin-MMP-2 complex secondary protein structure was in geometric bend state, whereas the isovitexin favors the bend element from 0 till 7.5 ns before structurally reversed as turn element for the rest of the simulation. The residue 30 also showed different behavior when compared for both complexes. The first 5 ns of vitexin-MMP-2 complex showed secondary protein structure with turn and bend elements throughout the simulations whereas the isovitexin-MMP-2 complex is geometrically in turn element within the first 5 ns before curved to bend regions throughout the rest of simulations. The residue 85 of vitexin-MMP-2 complex showed stable formation of β-sheet while the isovitexin-MMP-2 complex favors the coils element state throughout the 20 ns simulations. Different residual state condition was also seen at residue 150. The vitexin-MMP-2 complex favors the helix formation throughout the 20 ns simulations. The isovitexin-MMP-2 complex on the other hand formed α-helix structure for the first 5 ns and changed to turn element for the rest of the simulations.

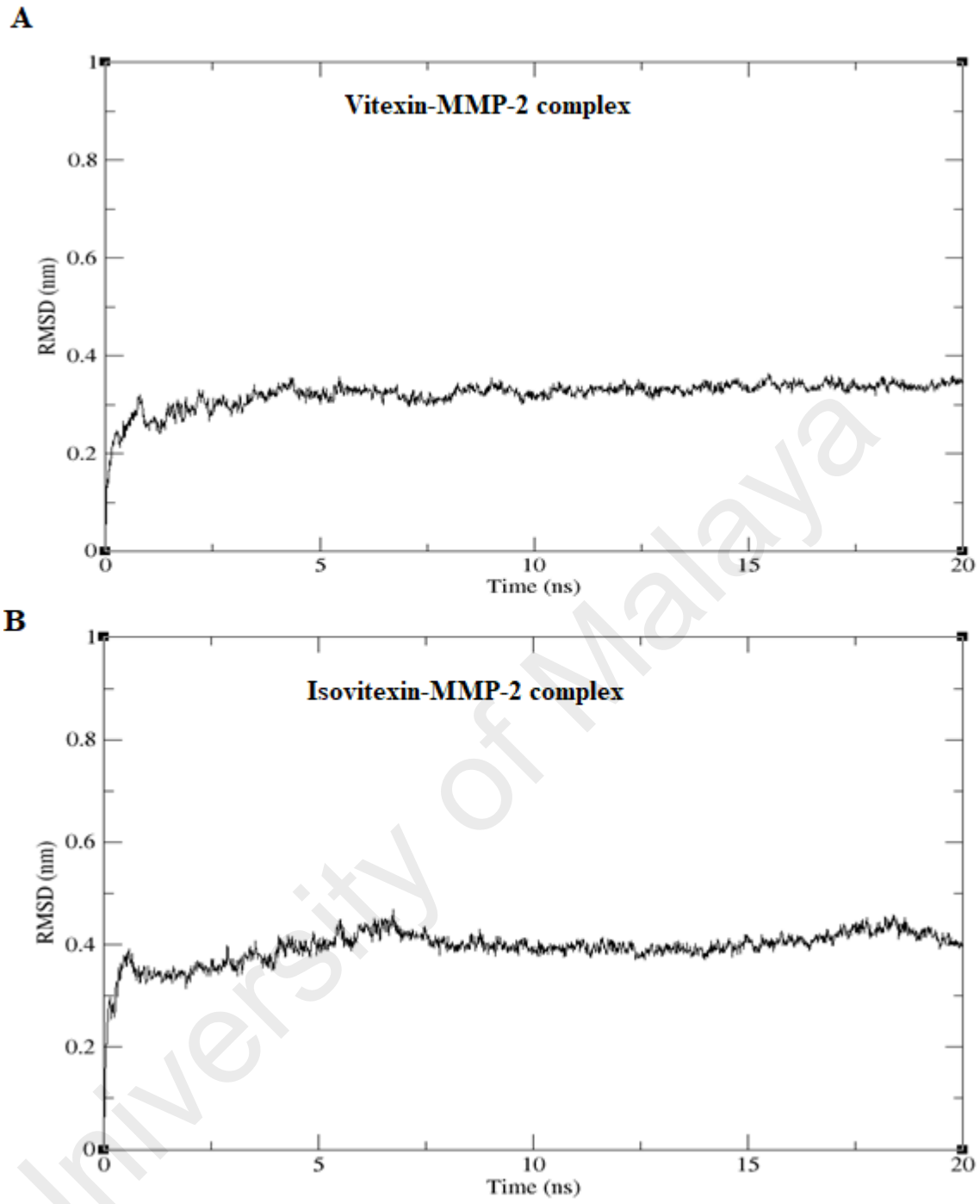


Figure 5.11: The RMSD graph of the modelled structure for vitexin-MMP-2 and isovitexin-MMP-2 complexes during 20 ns simulations

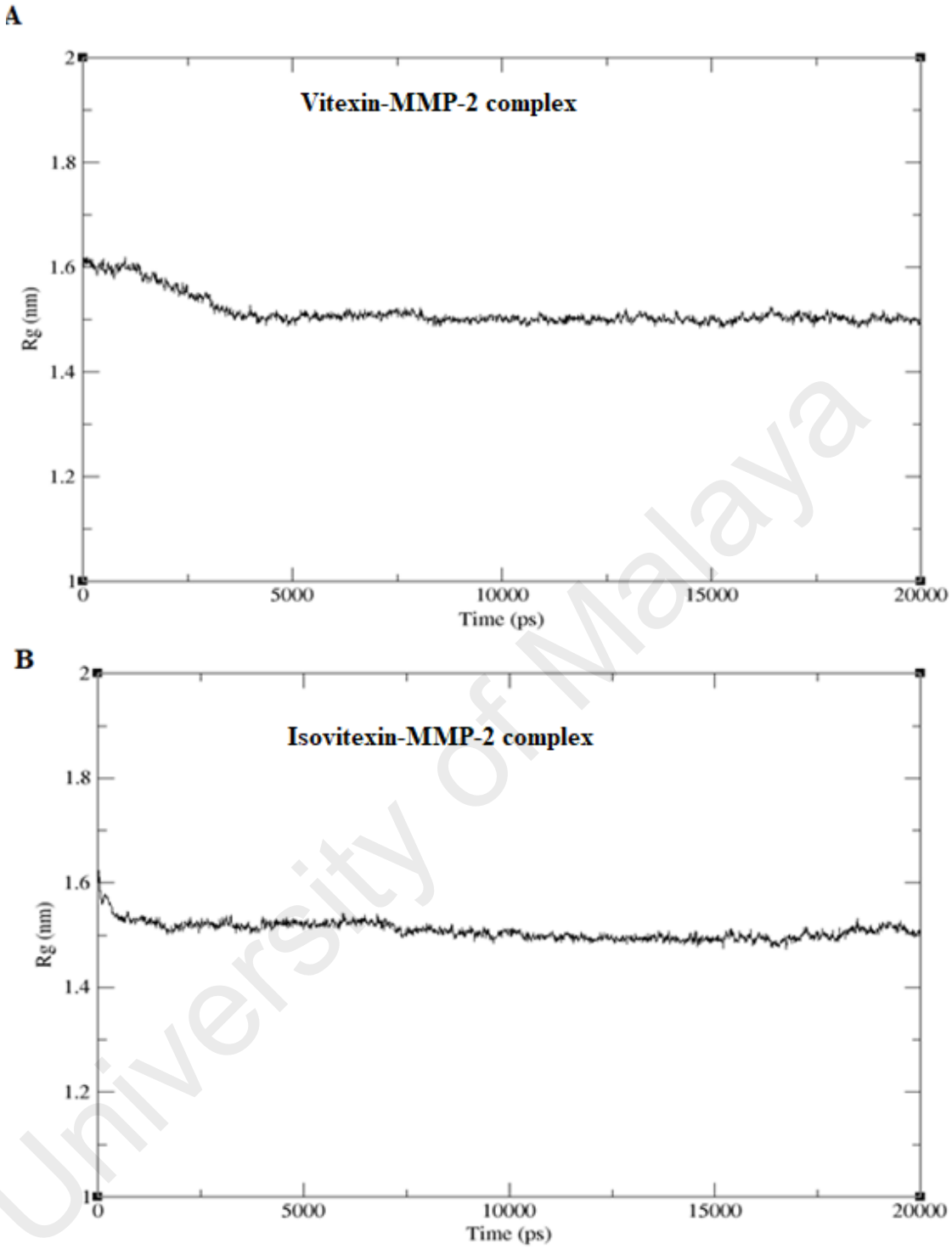


Figure 5.12: The radius of gyration graph of the modelled structure for vitexin-MMP-2 and isovitexin-MMP-2 complexes during 20 ns simulations

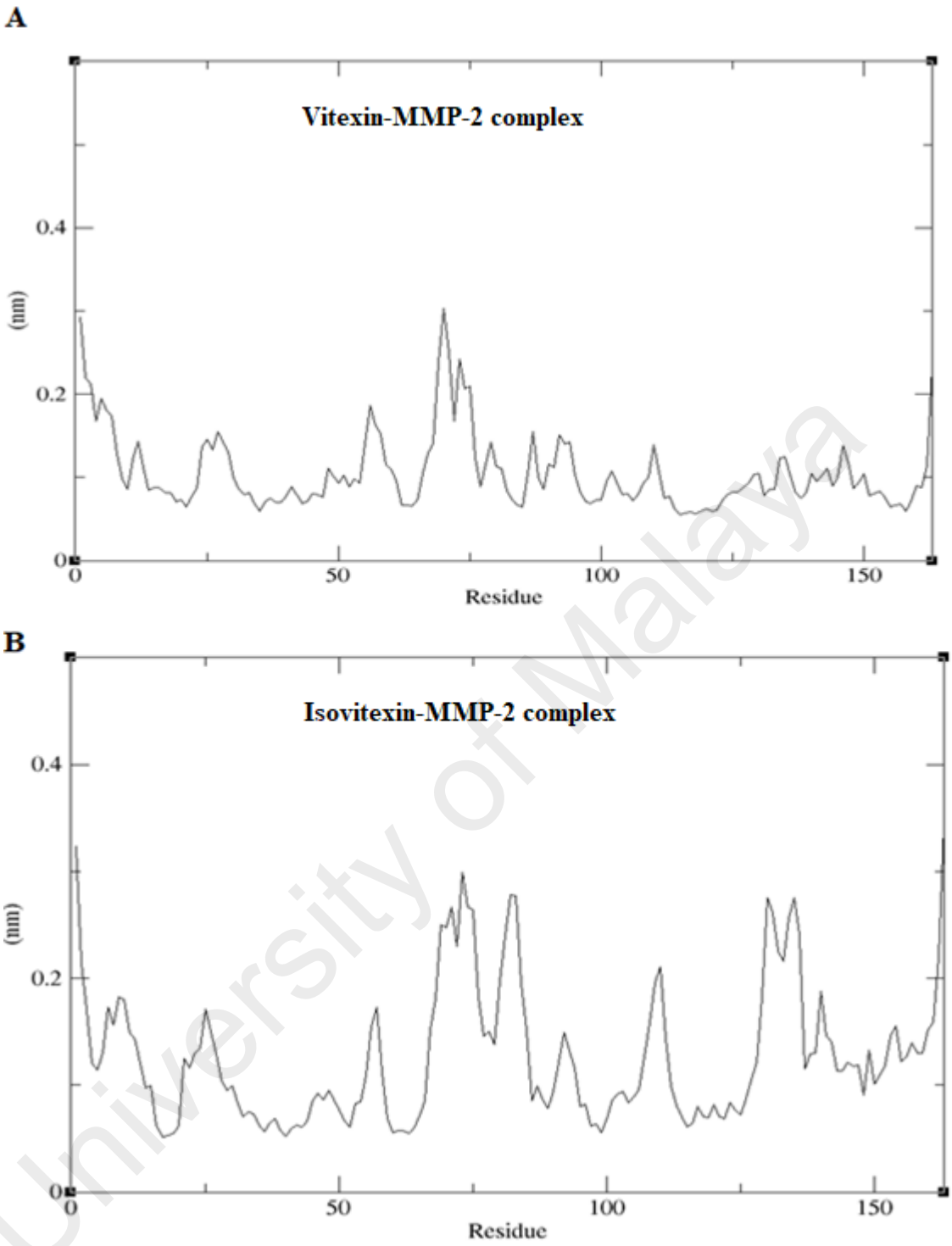


Figure 5.13: The RMSF graph of the modelled structure for vitexin-MMP-2 and isovitexin-MMP-2 complexes during 20 ns simulations

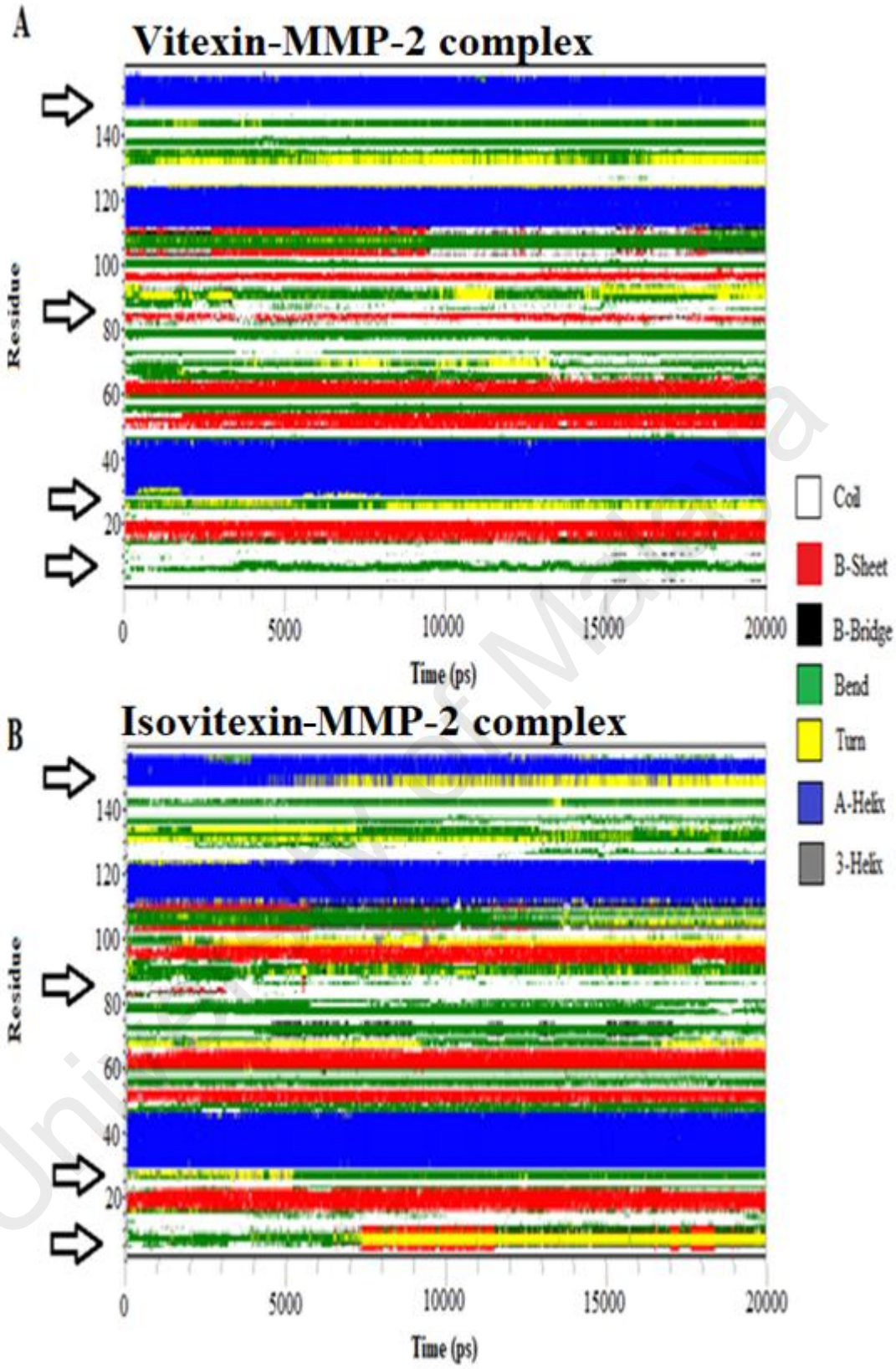


Figure 5.14: The conformational changes (denoted by right pointing arrowheads) vitexin-MMP-2 and isovitexin-MMP-2 complexes. A) Vitexin-MMP-2 complex; B) Isovitexin-MMP-2 complex.

5.4.3 Molecular dynamic analysis of vitexin–MMP-8 and isovitexin-MMP-8 complex

The RMSD analysis of vitexin- and isovitexin-MMP-8 complexes (Figure 5.15) showed similar patterns for both complexes; vitexin-MMP-8 complex and isovitexin-MMP-8 complex. The first 6 ns of the simulations showed an increased drifting pattern that slowly steadied at 7 till 18 ns and slightly decrease for the last 2 ns. The drifting of residue is within 1 Å for both complexes suggesting that the complexes formed were stable during the 20 ns simulations. The movement of the atom can be seen from the radius of gyration graph (Figure 5.16) in which the compactness of the vitexin- and isovitexin-MMP-8 complexes were found relatively similar to each other during the 20 ns simulations. The RMSF graph (Figure 5.17) on the other hand, provides a depth understanding of the fluctuation behavior of each individual's residues in each complex. Overall, the fluctuation of vitexin-MMP-8 complex and isovitexin-MMP-8 complex showed similar style with exception of the first 40 residues of isovitexin-MMP-8 complex that showed slightly higher drifting compared to vitexin-MMP-8 complex.

The structural changes (Figure 5.18) of the vitexin- and isovitexin-MMP-8 complexes were found to be noticeable at residue 10, 90 and 150. At residue 10, the vitexin-MMP-8 complex showed bending element of the secondary protein structure while the isovitexin-MMP-8 complex was geometrically in bend element for the first 3 ns before coiled throughout the rest of the simulation. The residue 90 for vitexin-MMP-8 complex was in β-sheet and bridge formations whereas isovitexin-MMP-8 complex structurally motif in coil element. Lastly, for residue 150, the residue showed mostly turn element for vitexin-MMP-8 complex while the isovitexin-MMP-8 complex structural motif changes direction to favours the bend element.

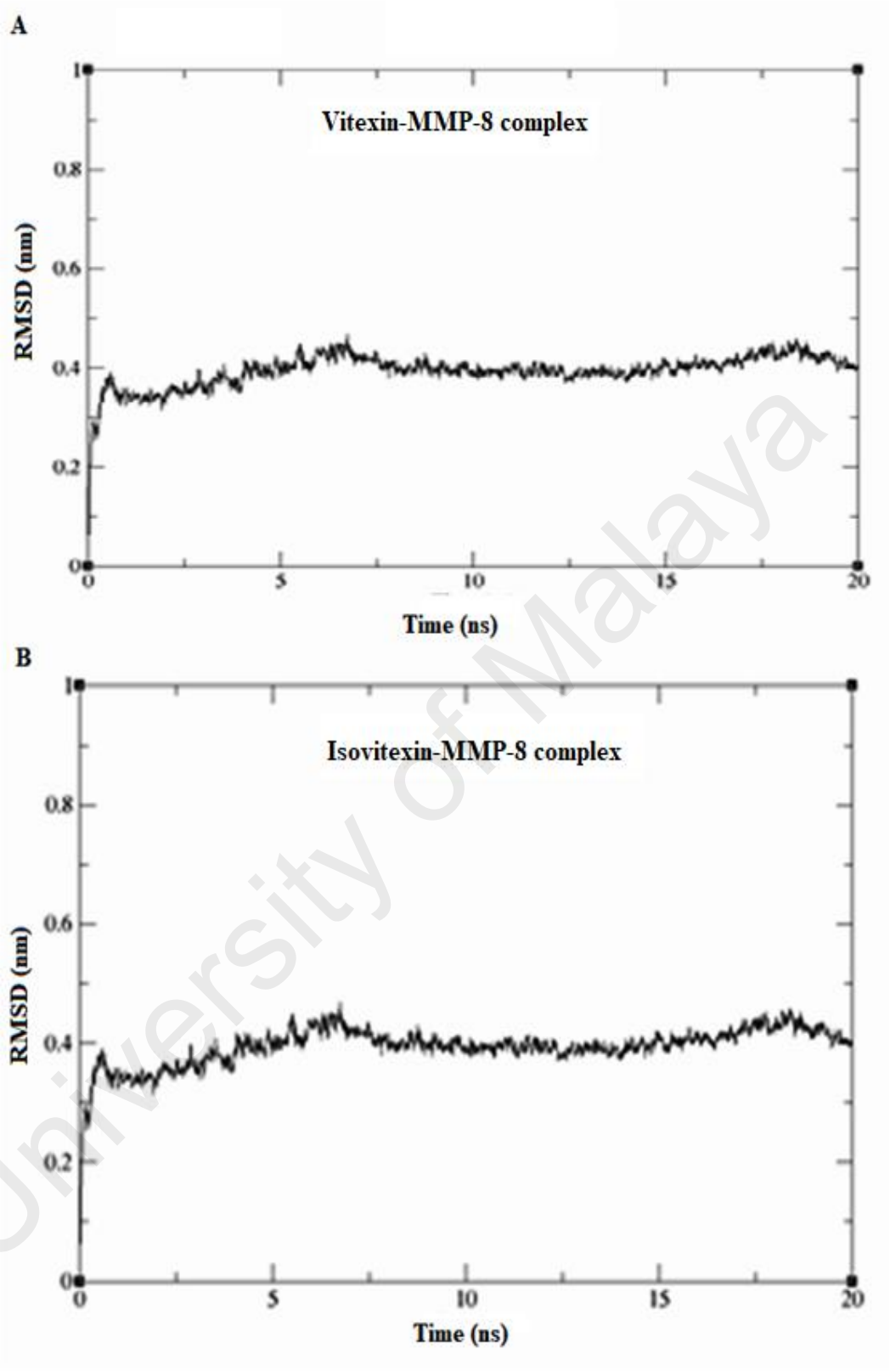


Figure 5.15: The RMSD graph of the modelled structure for vitexin-MMP-8 and isovitexin-MMP-8 complexes during 20 ns simulations

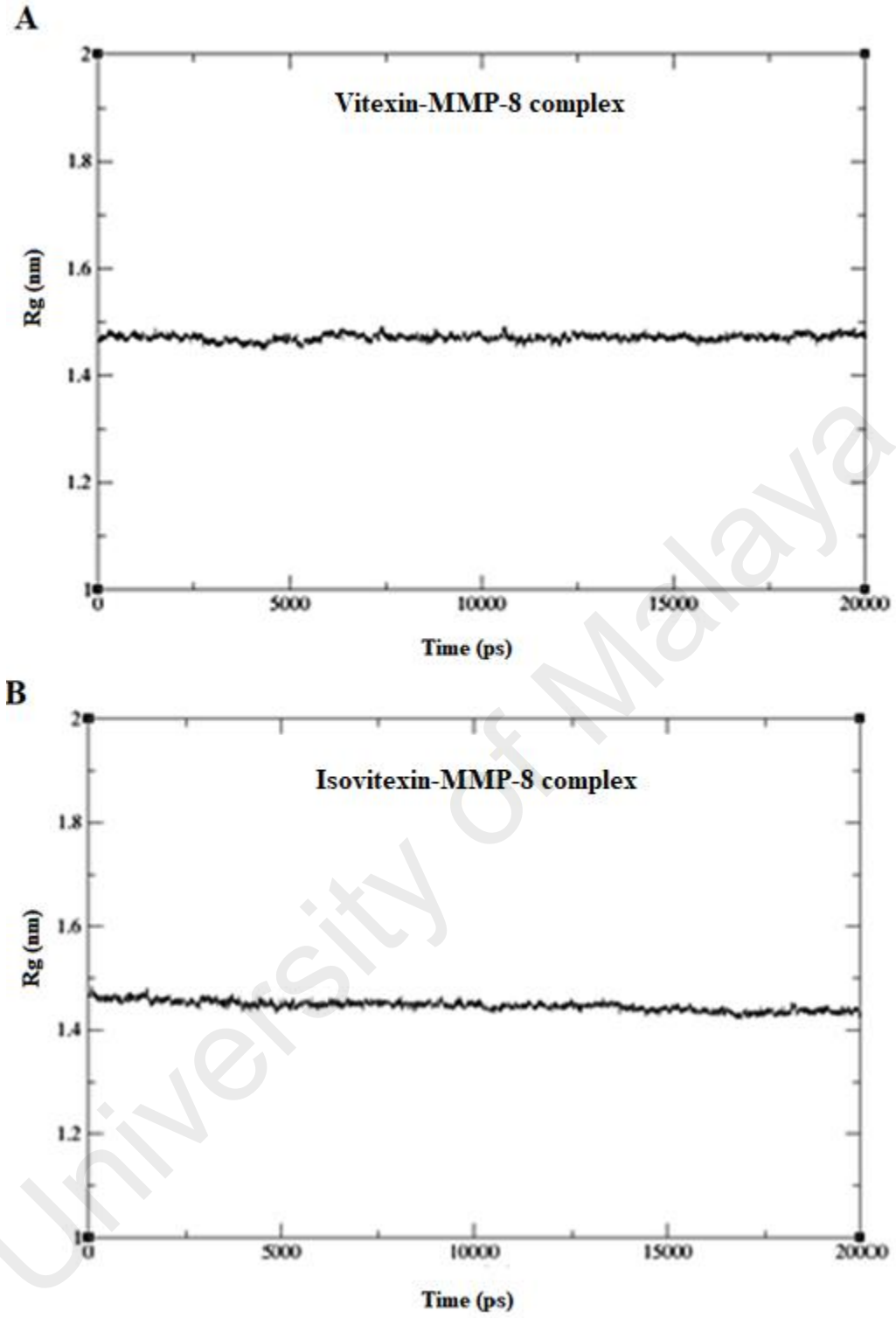


Figure 5.16: The radius of gyration graph of the modelled structure for vitexin-MMP-8 and isovitexin-MMP-8 complexes during 20 ns simulations

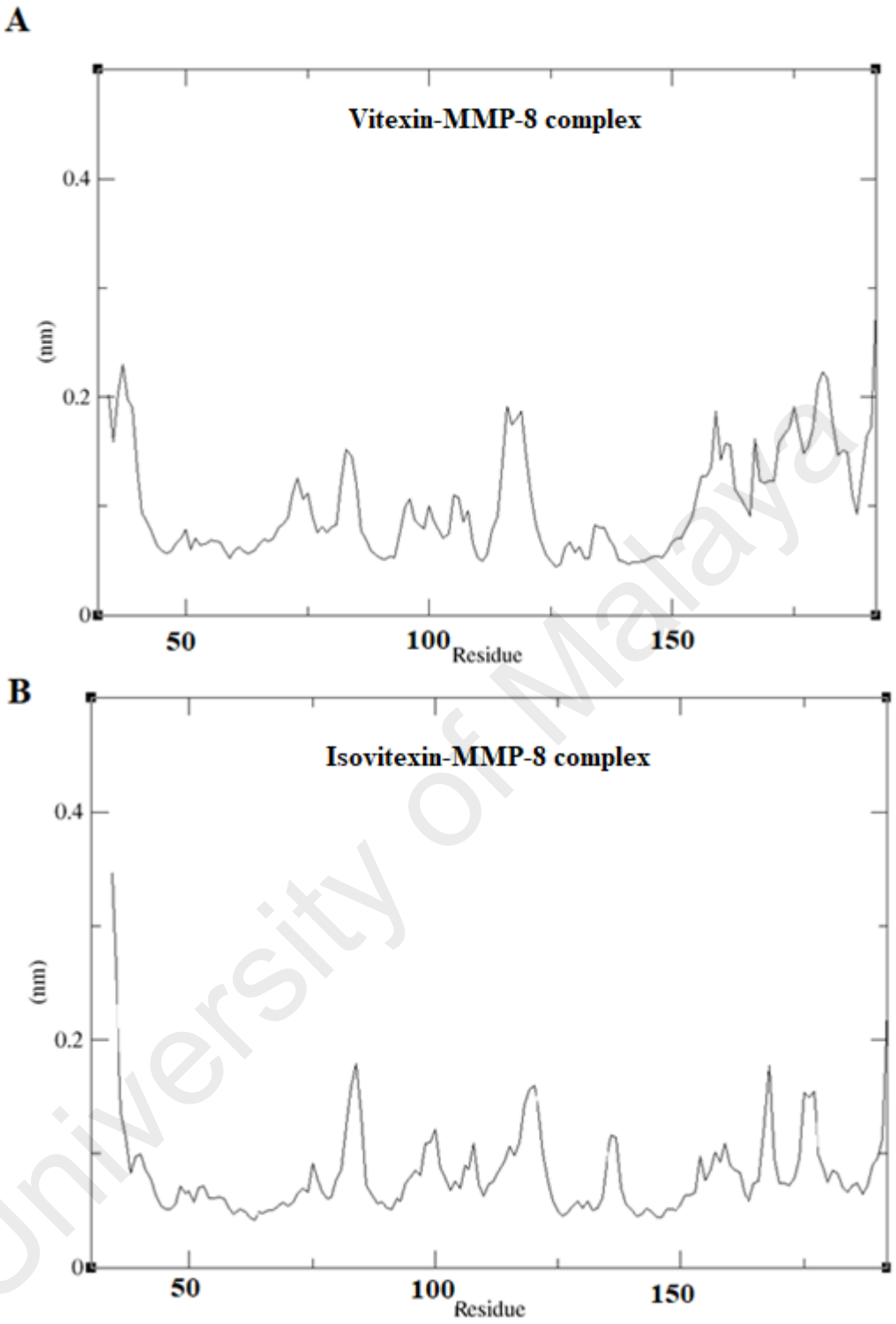


Figure 5.17: The RMSF graph of the modelled structure for vitexin-MMP-8 and isovitexin-MMP-8 complexes during 20 ns simulations

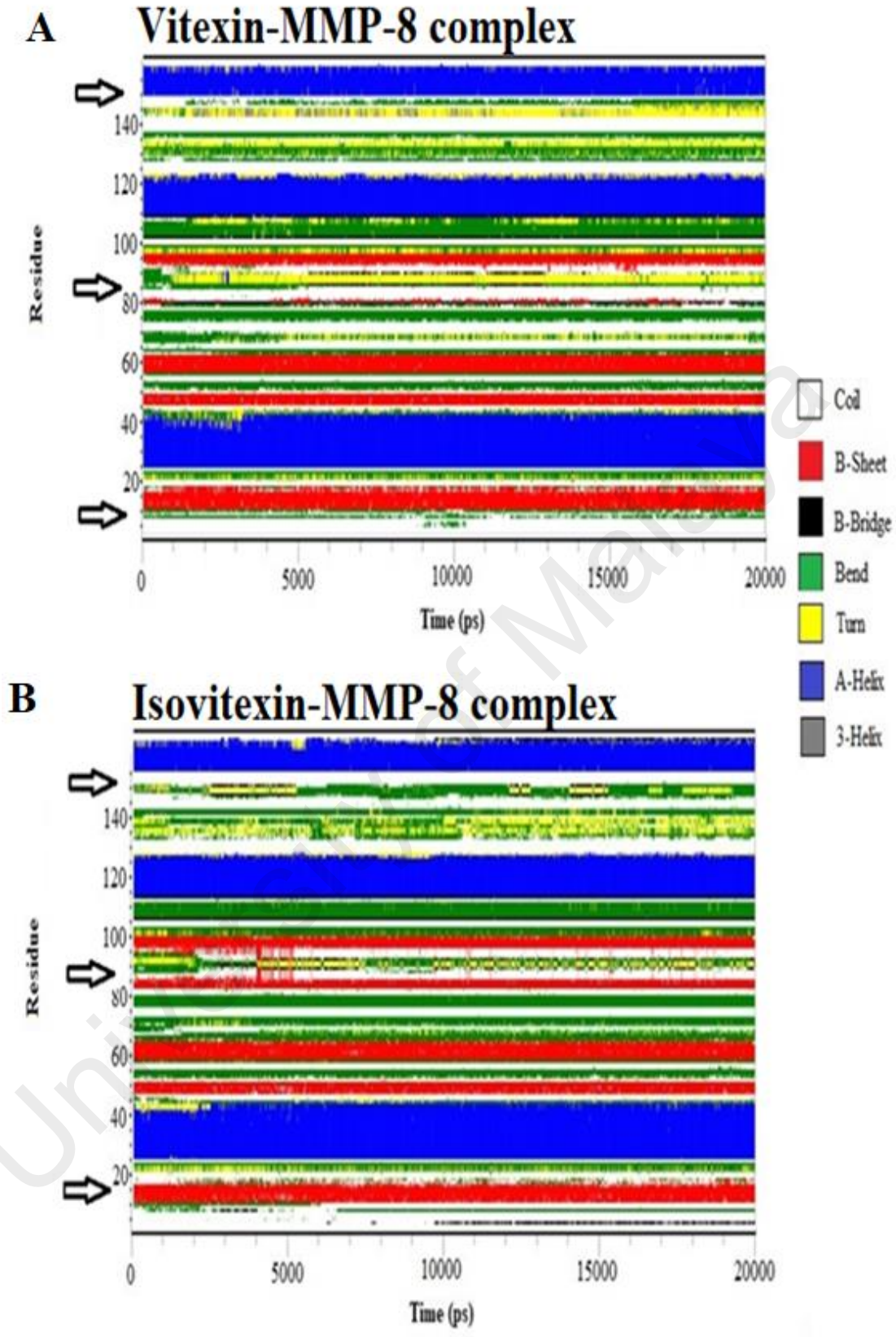


Figure 5.18: The conformational changes (denoted by right pointing arrowheads) vitexin-MMP-8 and isovitexin-MMP-8 complexes. A) Vitexin-MMP-8 complex; B) Isovitexin-MMP-8 complex

5.4.4 Molecular dynamic analysis of vitexin–MMP-9 and isovitexin-MMP-9 complex

The RMSD analysis (Figure 5.19) for vitexin- and isovitexin-MMP-9 complexes revealed that the atom in both complexes drifts at a steady range within 2 Å throughout the 20 ns simulations. Both vitexin and isovitexin-MMP-9 complex showed similar pattern of atomic drifting. The radius of gyration analysis (Figure 5.20) on the vitexin- and isovitexin-MMP-9 complexes was found to be quite similar with vitexin-MMP-9 complex showed a more constant protein gyration movement compared to the isovitexin-MMP-9 complex. The RMSF analysis (Figure 5.21) on vitexin-MMP-9 and isovitexin-MMP-9 complex showed that the residue fluctuates at identical pattern with isovitexin-MMP-9 complex showed higher fluctuation for the early residues.

Significant structural changes of the complex (Figure 5.22) during the 20 ns simulations can be seen at residue 20, 70 and 130. The residue 20 for vitexin-MMP-9 complex showed bend element in the secondary protein structure, whereas the isovitexin-MMP-9 complex conditions were interchangeable between bend and turn. For residue 70, the structural changes that can be observed are coil and β-sheet formation for vitexin-MMP-9 complex and coiled coil for the isovitexin-MMP-9 complex. The residue 130 for vitexin-MMP-9 complex favours the turn and bend elements while the isovitexin-MMP-9 complex favours the turn element of the secondary protein structure.

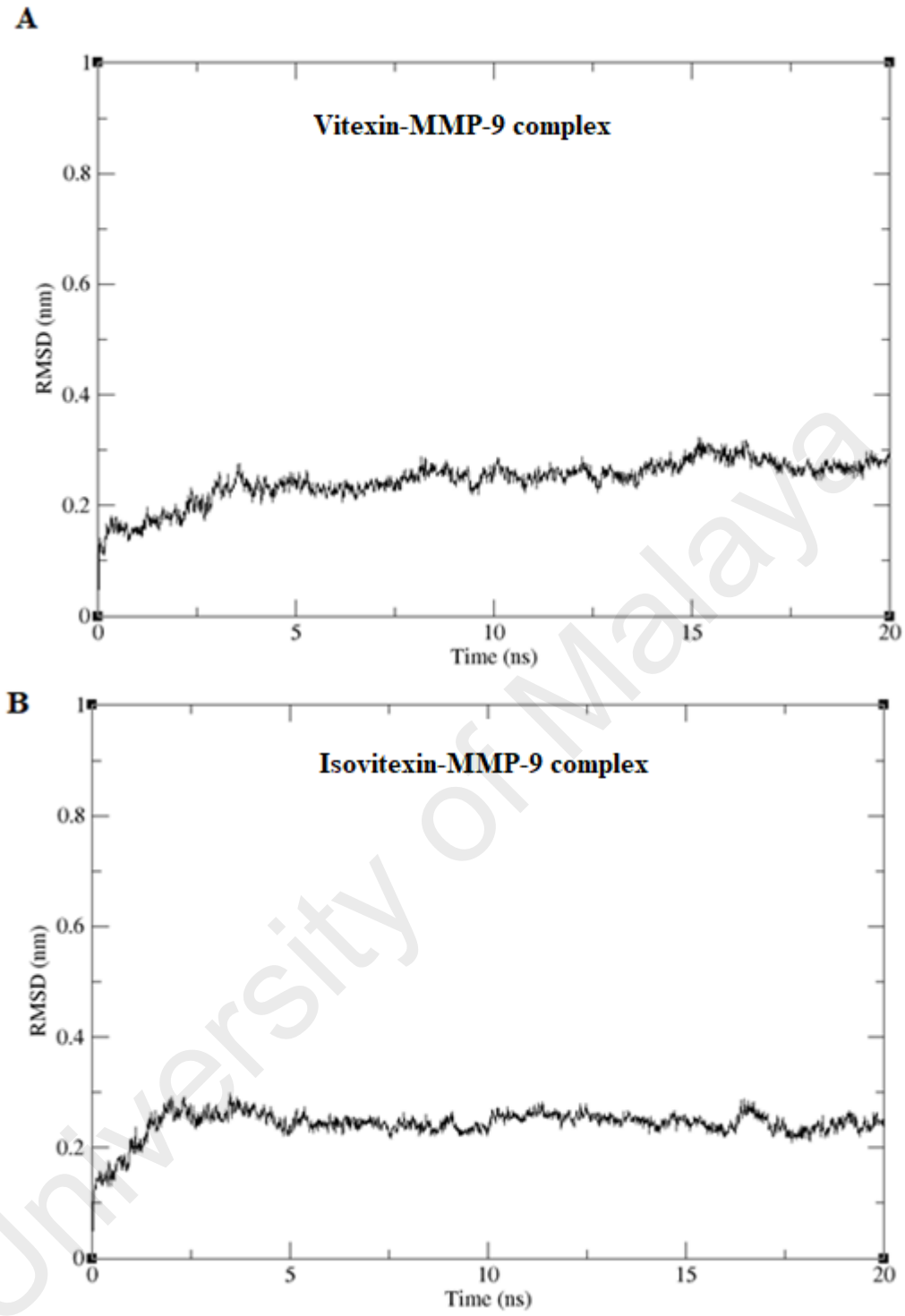


Figure 5.19: The RMSD graph of the modelled structure for vitexin-MMP-9 and isovitexin-MMP-9 complexes during 20 ns simulations

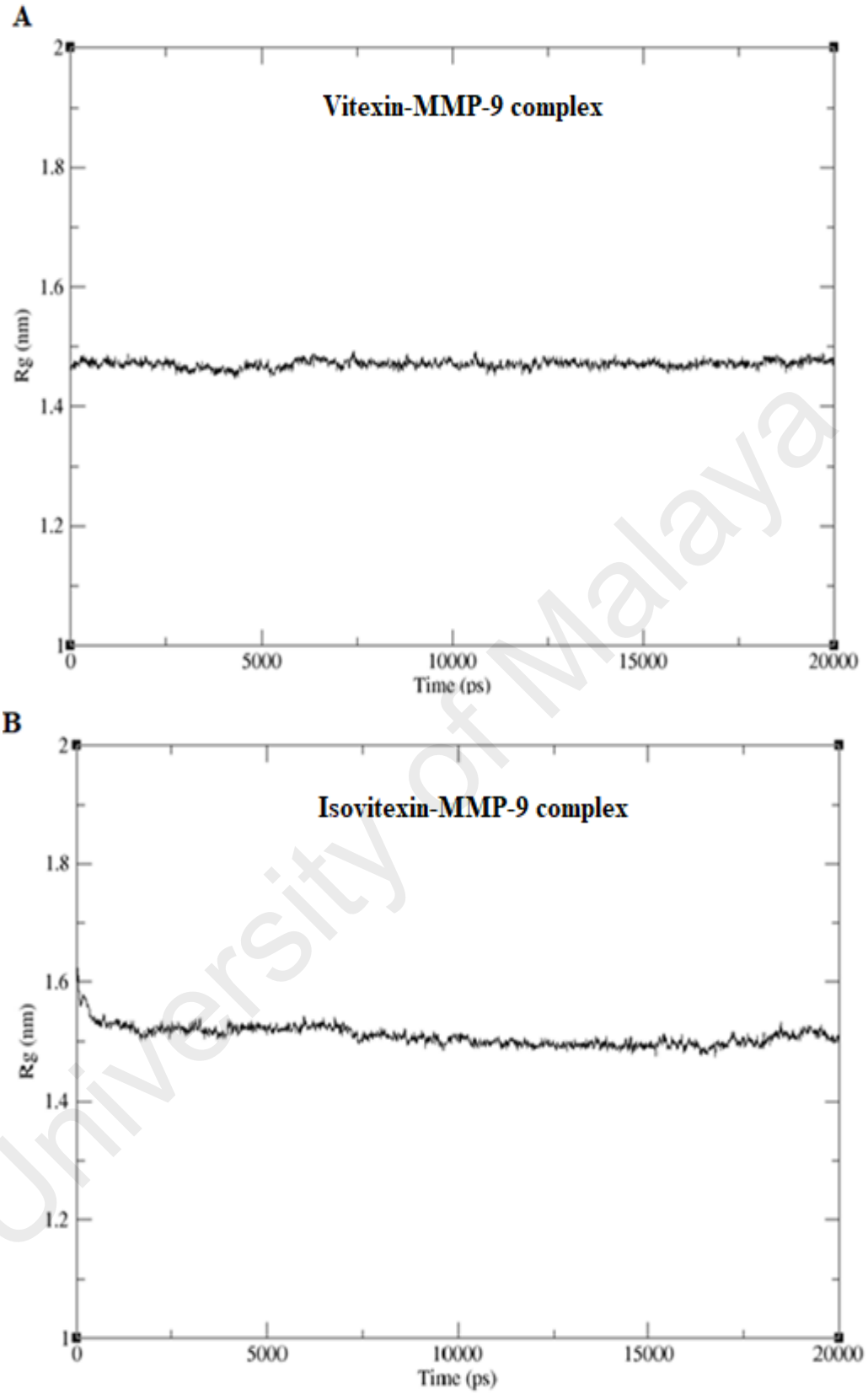


Figure 5.20: The radius of gyration graph of the modelled structure for vitexin-MMP-9 and isovitexin-MMP-9 complexes during 20 ns simulations

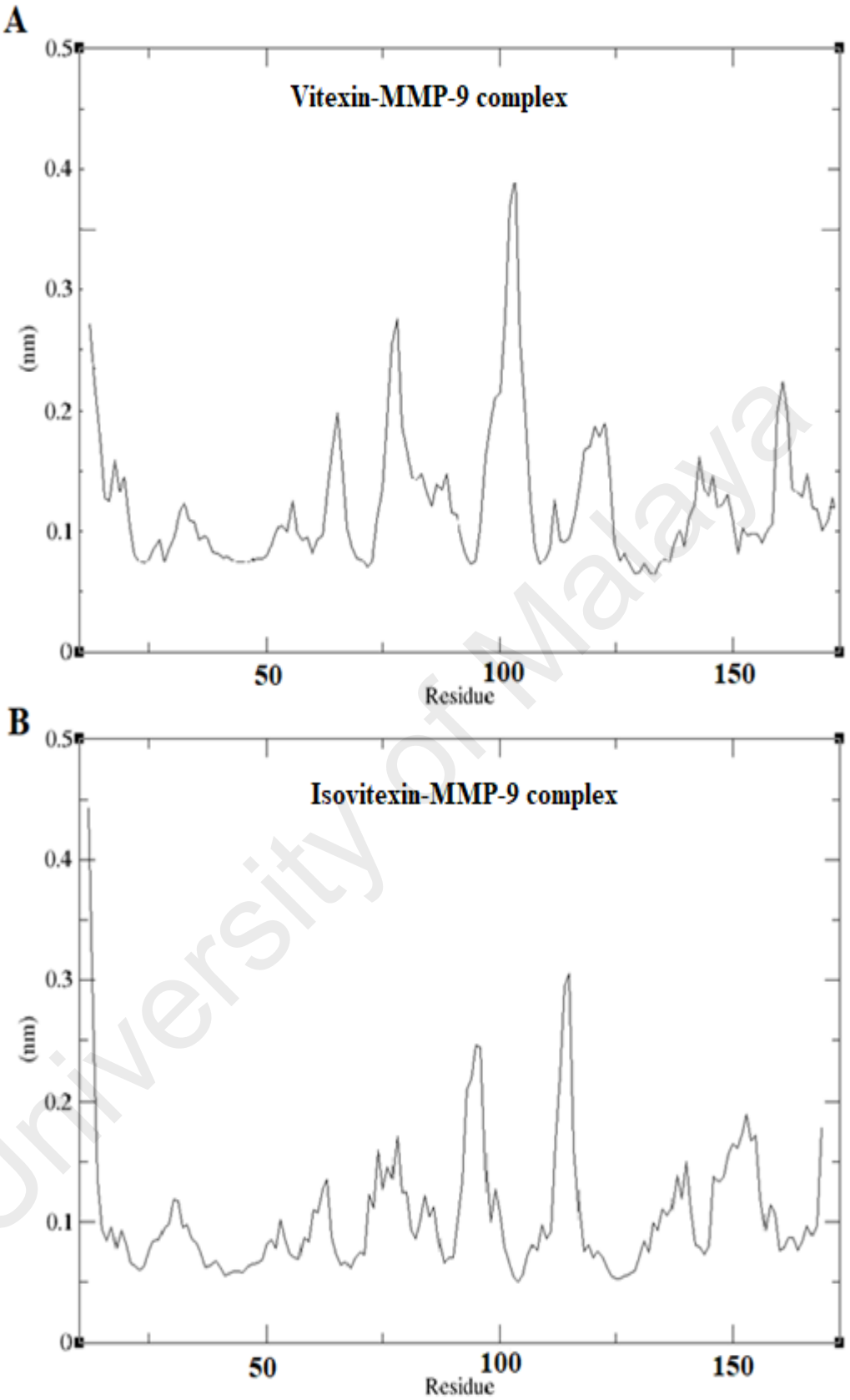


Figure 5.21: The RMSF graph of the modelled structure for vitexin-MMP-9 and isovitexin-MMP-9 complexes during 20 ns simulations

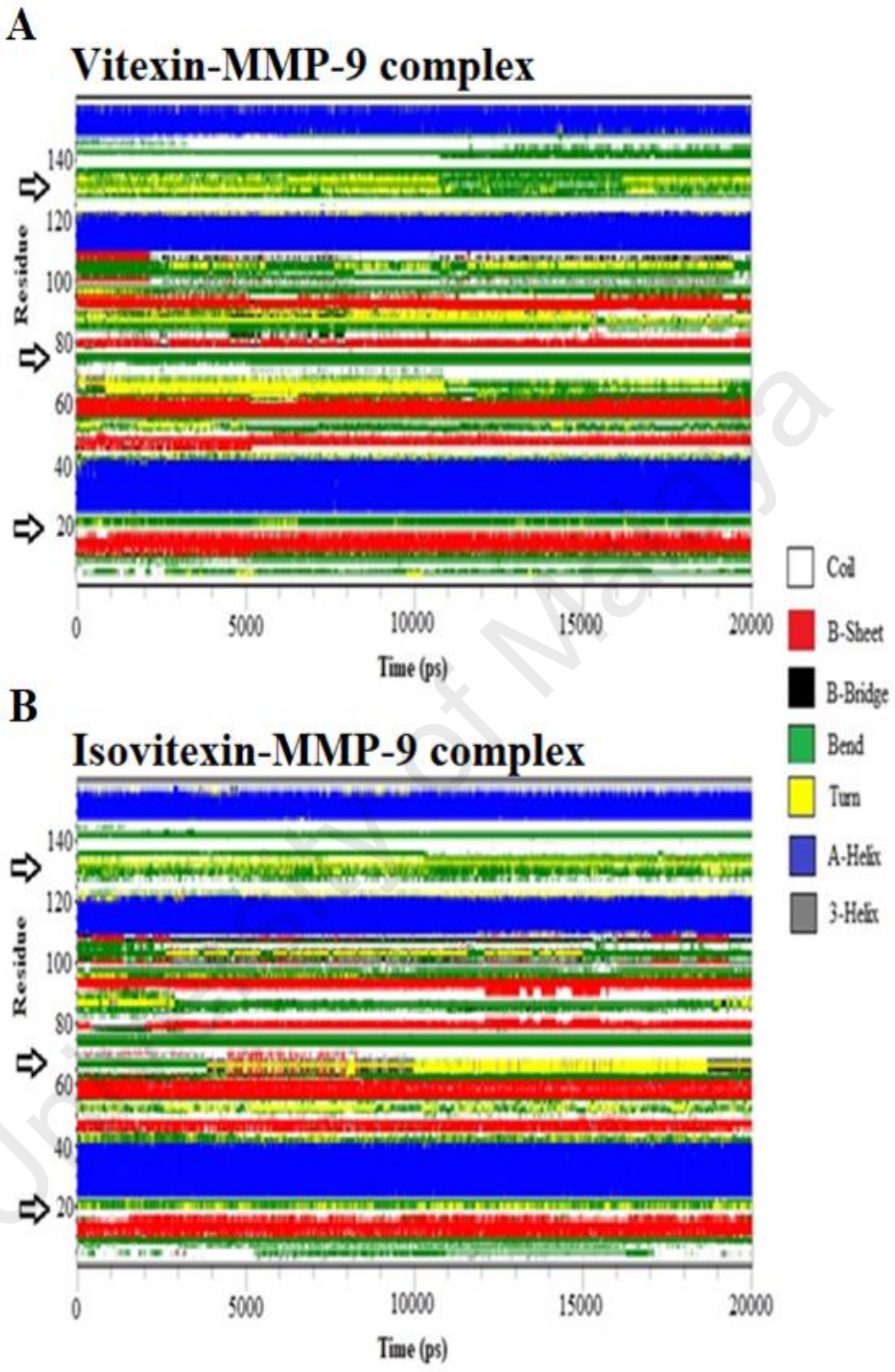


Figure 5.22: The conformational changes (denoted by right pointing arrowheads) vitexin-MMP-9 and isovitexin-MMP-9 complexes. A) Vitexin-MMP-9 complex; B) Isovitexin-MMP-9 complex

5.5 Discussion

Vitexin, isovitexin and FD leaf 50% (v/v) ethanol water extract enzymatic activities were investigated using the α-amylase and MMPs (MMP-2, MMP-8 and MMP-9) inhibition assay. For the α-amylase inhibition assay, FD leaf 50% (v/v) ethanol water extract was revealed as potent inhibitor of this enzyme. Convincingly, due to the present of vitexin and isovitexin, the FD leaf 50% (v/v) ethanol water extract showed potent inhibitory effect on the α-amylase enzyme. Subsequently, FD leaf 50% (v/v) ethanol water extract was also introduced to the MMPs (MMP-2, MMP-8 and MMP-9) inhibition assay. It was revealed that FD leaf 50% (v/v) ethanol water extract and its compounds, vitexin and isovitexin produced pronounced inhibition of the MMPs enzyme.

Afterwards, computational studies were conducted for vitexin and isovitexin towards α-amylase and MMPs enzyme. Nowadays, computational approaches to unravel the potentials of plants bioactive compounds are gaining interest. Utilizing the sophisticated computational power to describe the interactions between the active constituents and selected protein are a straightforward, economical and rapid technique (Liao *et al.,* 2011). Through docking and molecular dynamics programs such as Autodock and GROMACS, the behavior of an active compound, binding affinity as well as the residues interaction with a targeted enzyme can be analyzed and visualized. In this study, vitexin showed high binding affinity towards the α-amylase enzyme, whereas the isovitexin showed interesting attraction with the MMP enzymes. During molecular dynamics simulations, vitexin and isovitexin formed stable complex with the targeted enzyme (α-amylase and MMPs) throughout the 20 ns time length. These findings provided valuable additional information on therapeutic properties of FD leaf extract. Unravelling the therapeutic potential and the nature of a compound behavior

with targeted enzyme enhanced the understanding of how the FD leaf extract delivers its biological activities.

To conclude, the second objective of this research was achieved throughout this chapter. The enzymatic assay and computational approaches in evaluating the FD leaf extract compounds (vitexin and isovitexin) provided valuable in-depth preliminary information for researchers to plan and carry out experiments in validating the sought-out therapeutic activities.

CHAPTER 6: *IN VITRO* AND *IN VIVO* BIOACTIVITY OF STANDARDIZED FD LEAF 50% (V/V) ETHANOL WATER EXTRACT

6.1 Introduction

To evaluate the bioactivity of FD leaf 50% (v/v) ethanol water extract and its major phytochemical compounds, several *in vitro* and *in vivo* studies were conducted. This chapter will describe the *in vitro* and *in vivo* evaluations of the FD leaf 50% (v/v) ethanol water extract and its major phytochemical compounds.

6.1.1 DPPH inhibition assay

The FD leaf 50 % (v/v) ethanol water extract was tested in the DPPH inhibition assay (Figure 6.1) to investigate the antioxidant properties that the standardized FD leaf extract may have. The extract was compared to the positive control compound, the quercetin (Sharma & Bhat, 2010). The result of the DPPH scavenging assay is tabulated as Table 6.1, where the IC_{50} values for each sample were presented as mean ± SD. From the DPPH scavenging assay, the FD leaf extract showed low inhibition of the DPPH substrate with IC_{50} value of 15.7 ± 3.0 µg/ml. When compared to the positive control, both vitexin and isovitexin showed significant inhibition of DPPH with IC_{50} of 0.4 ± 0.1 µg/ml and 0.5 ± 0.1 µg/ml, respectively.

Table 6.1: The DPPH scavenging activities of FD leaf 50% (v/v) ethanol-water extract, vitexin and isovitexin. Quercetin was the positive control. The asterisk symbol (*) represent the significant values ($p < 0.05$)

Samples	IC_{50} (µg/ml)
FD leaf 50% (v/v) ethanol-water extract	15.7 ± 3.0
Vitexin	0.4 ± 0.1*
Isovitexin	0.5 ± 0.1*
Quercetin (Positive control)	2.2 ± 0.1

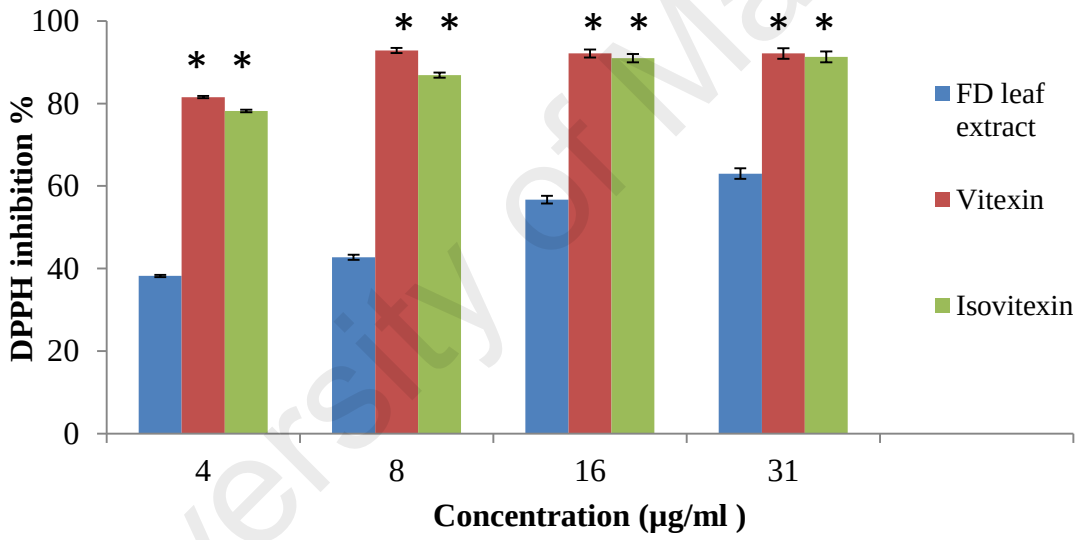


Figure 6.1: The DPPH scavenging activities graphical result of FD leaf 50% (v/v) ethanol-water extract, vitexin and isovitexin. The asterisk symbol (*) represent the significant values ($p < 0.05$)

6.2 Cell culture study on FD leaf 50% (v/v) ethanol water extract

6.2.1 Cytotoxicity activity of FD leaf 50% (v/v) ethanol water extract

Cytotoxicity activity of the FD leaf 50% (v/v) ethanol water extract and its major phytochemical compounds (vitexin and isovitexin) on EA.hy926 cells was evaluated by the MTT assay as described in Chapter 3. The result of the cytotoxicity activity revealed that vitexin produced the highest cytotoxic effect with IC_{50} value of 97.6 µg/ml. For the FD leaf 50% (v/v) ethanol water extract, the cytotoxic activity IC_{50} value was 189 µg/ml. Isovitexin on the other hand exhibited the lowest cytotoxic effect compared to vitexin and FD leaf 50% (v/v) ethanol water extract with IC_{50} value of 221 µg/ml.

Figure 6.2 shows the morphological studies on the treated cells. Figure 6.3 illustrated the comparative effects of the FD leaf 50% (v/v) ethanol water extract in comparison with vitexin and isovitexin in the cytotoxic activity assay.

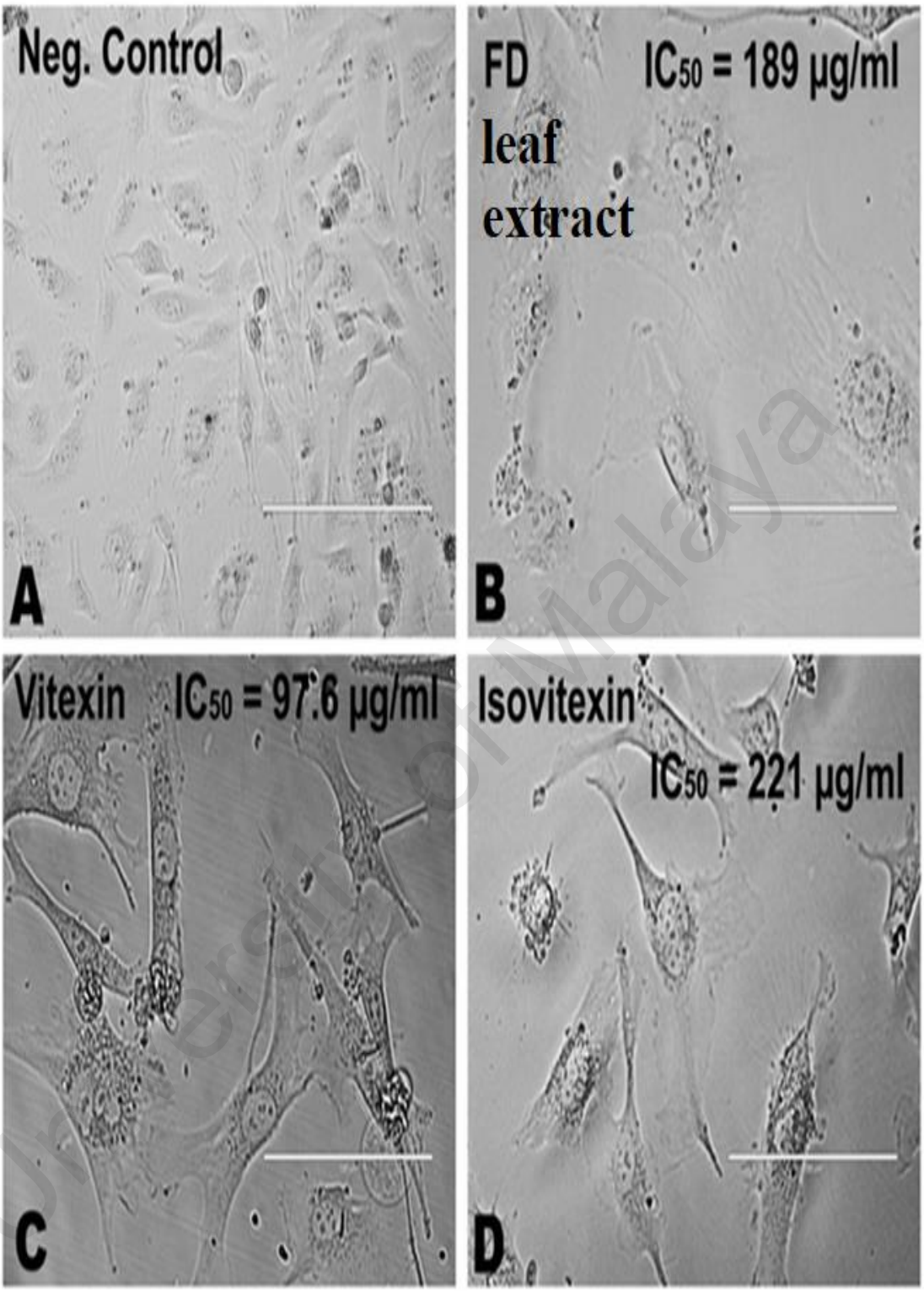


Figure 6.2: Photographs of Human endothelial hybrid (EA.hy926). The photographs were taken using an inverted microscope attached with a digital camera (100 × magnification) after 48 hours treatment with FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin

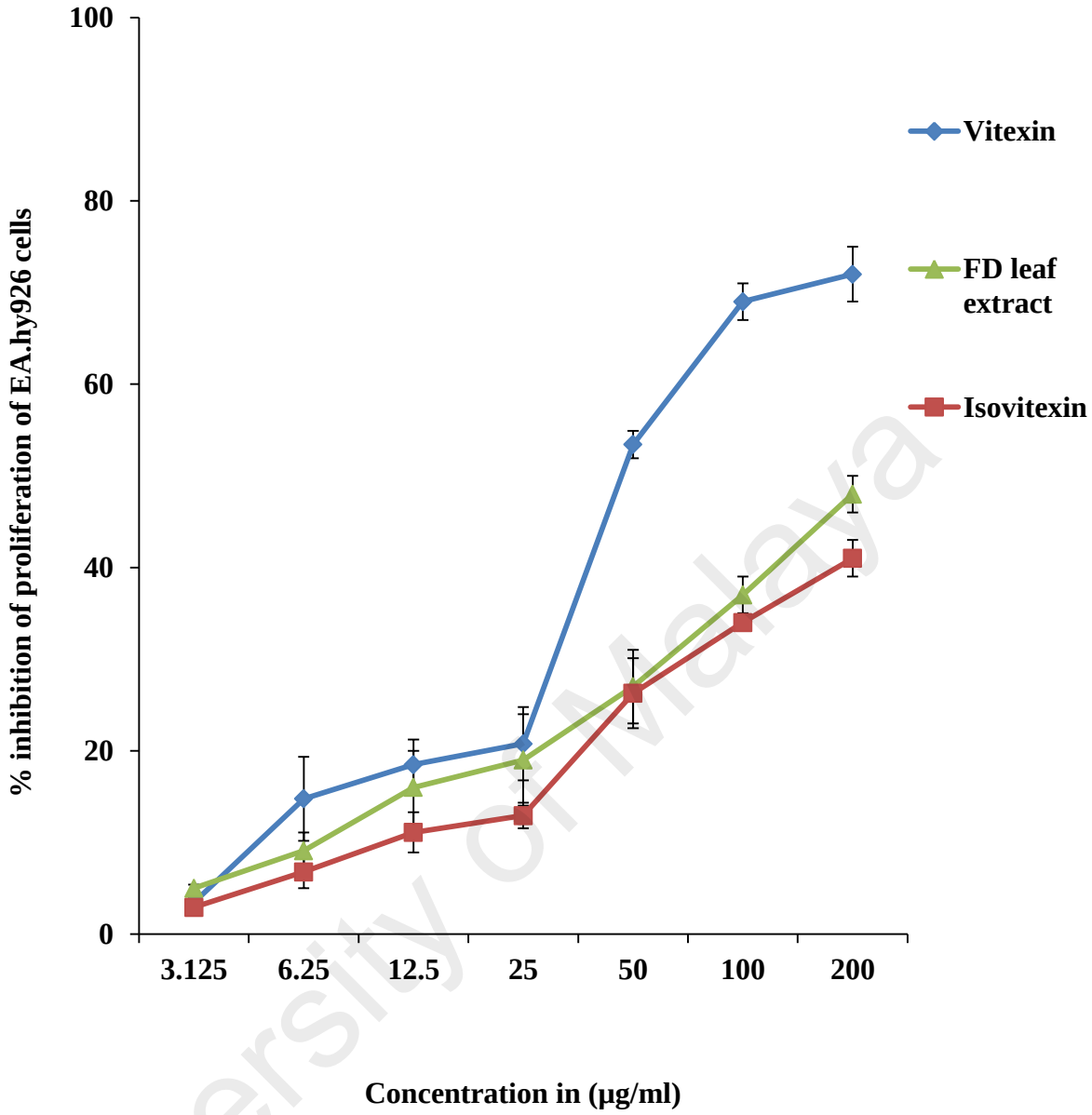


Figure 6.3: Graphical representation of the dose dependent cytotoxic effect of FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin. The data are presented as mean ± standard deviation (S.D), with n=3

Cells representing the negative control group (treated with 0.1% DMSO) displayed fully confluent compact monolayer of proliferating EA.hy926 cells (Figure 6.2A). However, cells treated with the three samples showed cytotoxic effect on growth of the EA.hy926 cells. The FD leaf 50% (v/v) ethanol water extract produced noticeable cytotoxic effect on the endothelial cells with IC_{50} = 189 µg/ml (Figure 6.2B). The FD leaf 50% (v/v) ethanol water extract reduced the population doubling time of the cells as it is evident from the reduced number of proliferating cells after 48 hour treatment (Figure 6.3). Cellular morphological analysis from the photomicrographic images of the cells revealed that FD leaf 50% (v/v) ethanol water extract caused severe modifications in the morphology as the normal pseudopodial like cellular projections were affected in the treated cells. The cells become round and several vacuoles can be seen in the cytoplasm of the cells. Similarly, vitexin also exhibited pronounced cytotoxic effect against the endothelial cells with IC_{50} = 97.6 µg/ml (Figure 6.2C). The vitexin treated cells showed autophagic changes in the cellular morphology. Isovitexin on the other hand demonstrated moderate cytotoxic effect with IC_{50} = 221 µg/ml (Figure 6.2D).

6.2.2 FD leaf 50% (v/v) ethanol water extract inhibits migration of EA.hy926 cells.

The endothelial cells-based migration assay represents an important step in the formation of new blood vessels and is a straightforward and economical method to study the cell migration phenomenon (Goodwin, 2007). A scratch wound model was created on the monolayer of EA.hy926 cells (Liang *et al.,* 2010). The wound healing properties of FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin to cause closure of the wound was observed. As shown in the Figure 6.4, the cell migratory property was much faster in the negative control group with the percentage of cell migration recorded at 6, 18 and 24 h was 37.4 ± 3, 72.7 ± 5 and 95.0 ± 2%, respectively. Whereas, FD leaf 50% (v/v) ethanol water extract suppressed endothelial migration significantly ($p < 0.01$) in a dose and time-dependent manner. Upon treatment of

endothelial cells with the FD leaf 50% (v/v) ethanol water extract (200 µg/ml), the percentage cell migration at 6, 18 and 24 h was 20.6 ± 2, 48.9 ± 2 and 71.3 ± 6%, respectively. However, the higher concentration (300 µg/ml) of FD leaf 50% (v/v) ethanol water extract showed a more pronounced inhibitory effect on the migration with the cell migratory property at 6, 18 and 24 h was 9 ± 1, 36.8 ± 4 and 51 ± 1%, respectively. For isovitexin, the result showed that at lower dose (110 µg/ml), endothelial migration is noticeable where the wound is fully closed on the 18th hour. On the other hand, vitexin demonstrated potent inhibitory effect on migration of the EA.hy926 cells. At a concentration of 150 µg/ml, vitexin displayed significant activity at 6, 18 and 24 h with 4.7 ± 1, 10.0 ± 1 and 14 ± 2%, respectively. Figures 6.5 and 6.6 show the dose and time dependent inhibitory effect of FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin on the migratory property of endothelial cells.

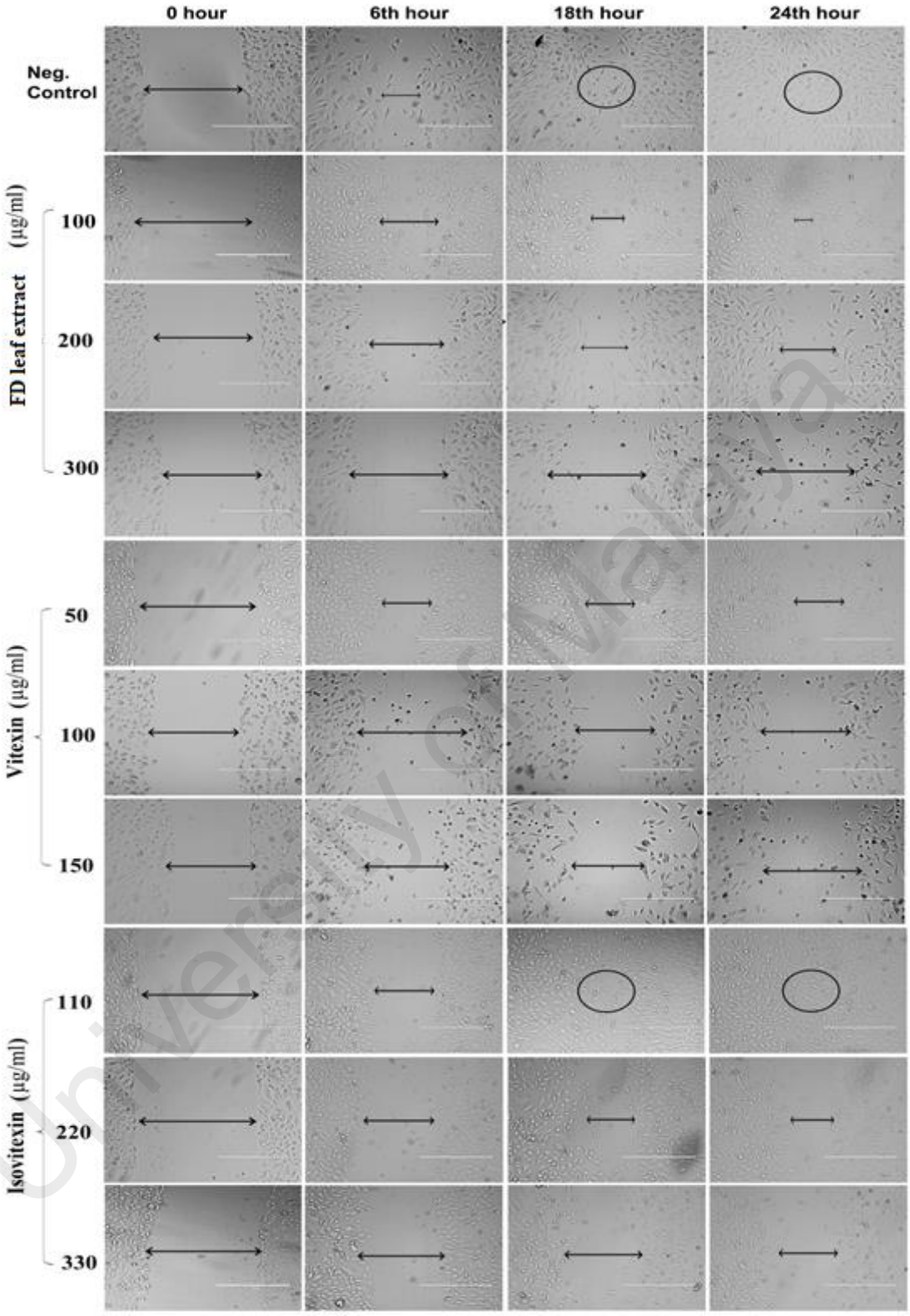


Figure 6.4: Dose and time dependent inhibitory effect of FD leaf 50 % (v/v) ethanol water extract, vitexin and isovitexin on migration of EA.hy926 cells

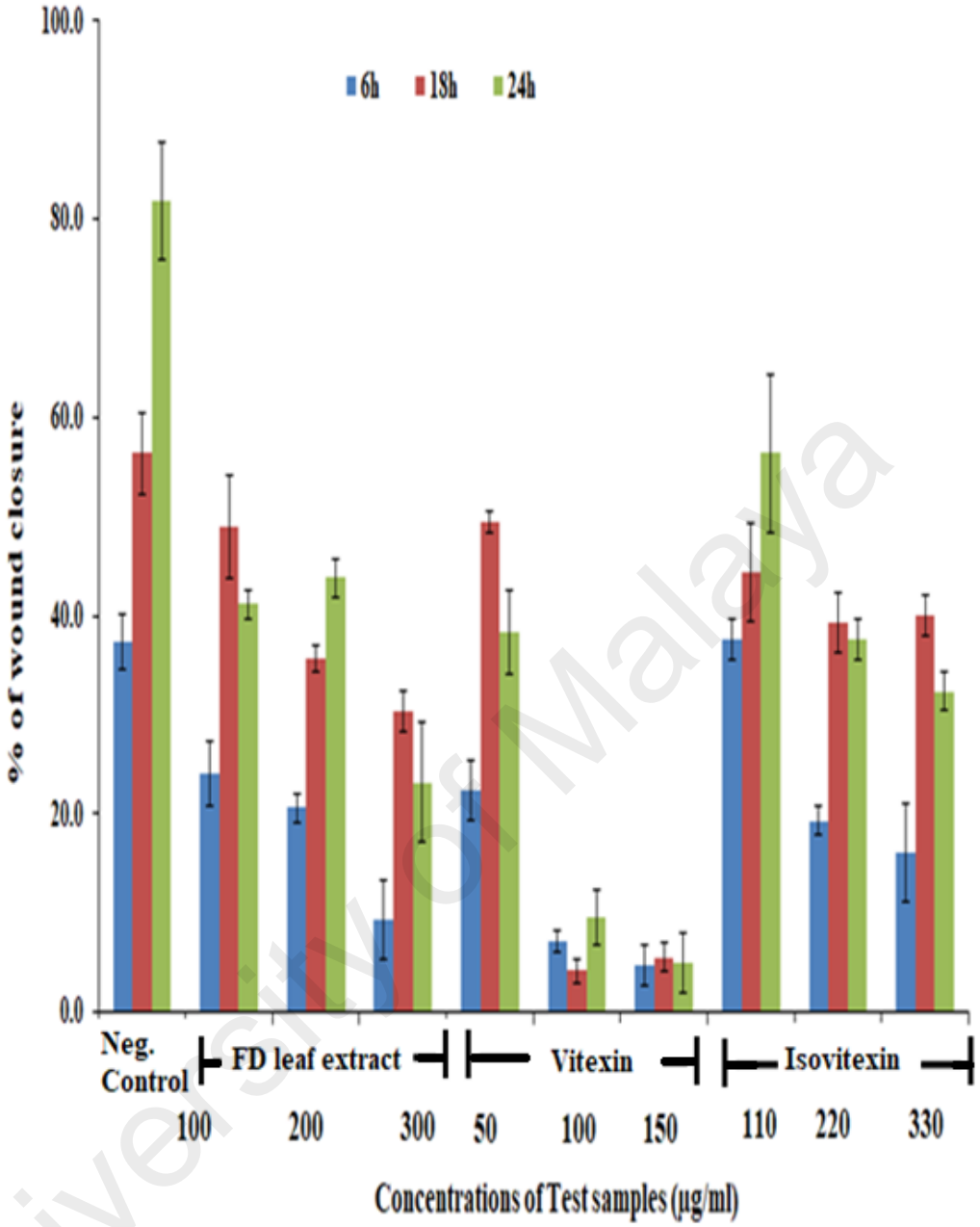


Figure 6.5: Graphical representation of the inhibitory effect of FD leaf 50 % (v/v) ethanol water extract, vitexin and isovitexin on migration of EA.hy926 cells. Results are presented in mean ± SD, n = 6)

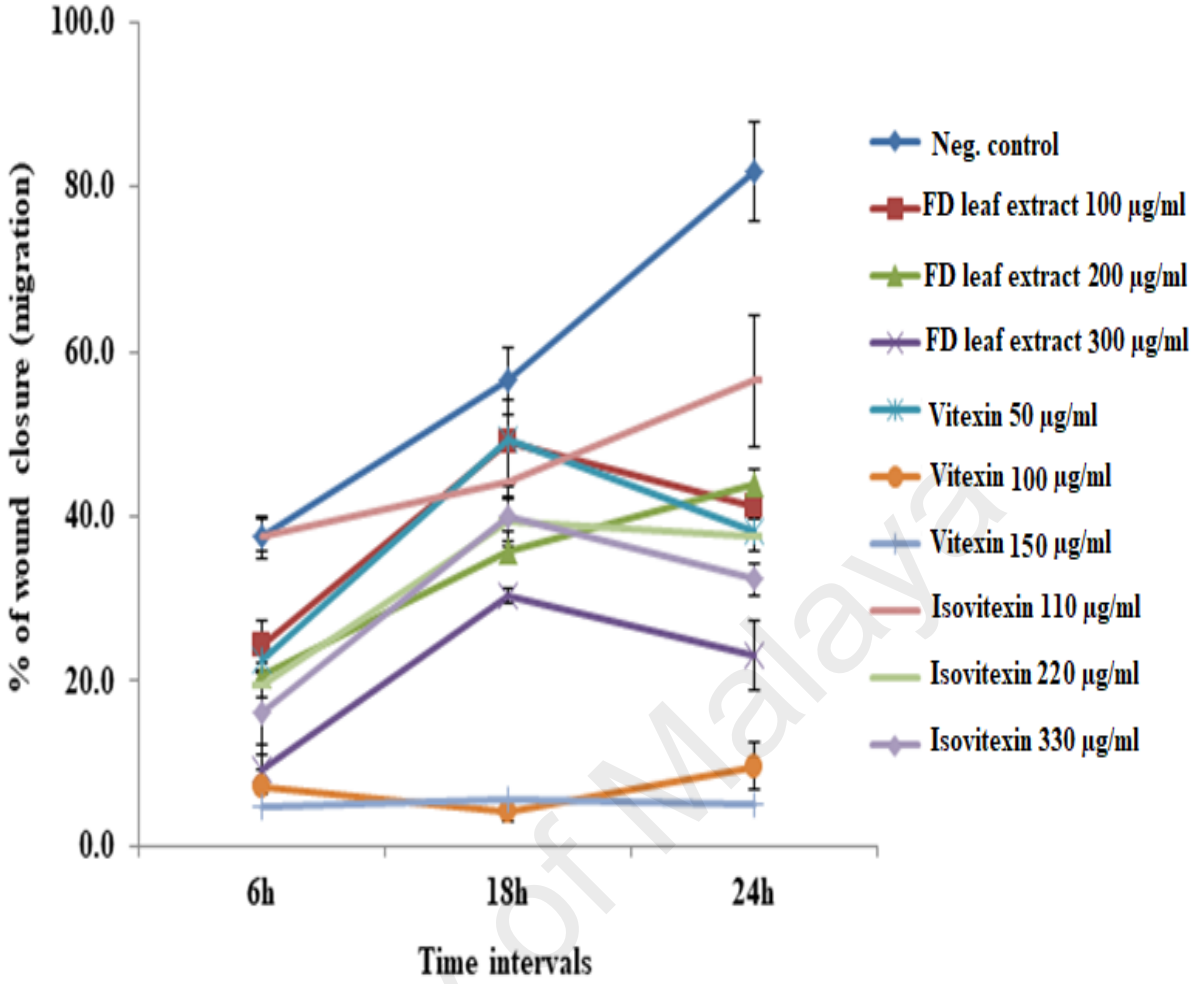


Figure 6.6: Dose Response Curve illustrating the effect of FD leaf 50 % (v/v) ethanol water extract, vitexin and isovitexin on migration of EA.hy926 cells

6.3 *In vivo* study of FD leaf 50% (v/v) ethanol water extract

Validation of the FD leaf 50% (v/v) ethanol water extract therapeutic activity was conducted through *in vivo* study using Sprague-Dawley (SD) rats. Initially, the toxicology study was performed to evaluate the toxicity of the FD leaf 50% (v/v) ethanol water extract. Following that, a concise study on the wound healing activities of the FD leaf 50% (v/v) ethanol water extract was performed on the diabetic and non-diabetic SD rat model. The animal ethic was approved by the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM) with reference number: UPM/IACUC/AUP-R069/2016. The UPM animal resource unit supplied all the SD rats used in this study. The SD rats were kept at the Comparative Medicine and Technology unit (COMet), UPM.

6.4 Toxicology study of FD leaf 50% (v/v) ethanol water extract

The toxicity evaluation of the FD leaf 50% (v/v) ethanol water extract was performed on 8 female SD rats. Two groups of SD rat were formed, the treatment and control groups. A single dose of 1000 mg/kg of the FD 50% (v/v) ethanol water extract was given to the treatment group while the control group was given tap water. The method of administrating the extract and tap water for both groups was through oral gavage. After that, the SD rats for both groups were observed for any abnormal changes in the behavior throughout the period of the acute toxicology study.

6.5 Behavioural observation of the SD rats

The food and water were given daily (150 g of food pellet and 250 ml of water). The SD rat weight, water and food consumption for both groups were recorded daily (see Figure 6.7 to 6.9). From the observation, there was no significant ($p > 0.05$) changes on weight, water and food consumption were found in the SD rats in the treatment groups, indicating that the FD leaf 50% (v/v) ethanol water extract does not cause any side effects such as apathy, stomach discomfort, vomiting and loss of appetite (Table 6.2). After that, the SD rats in both groups were sacrificed. The blood was drawn out and organs were harvested for biochemical and histology analysis to further evaluate the toxicity of the FD leaf 50% (v/v) ethanol water extract.

Table 6.2: Observations of Acute toxicity study (Treatment related changes)

Animal no.	**FD leaf extract dose (mg/kg)**	**Apathy**	**Circling**	**Loss of appetite**	**Respiratory rate**	**Diarrhea**	**Reflexes**	**Paralysis**	**Vomiting**
T1	1000	N	N	N	N	N	N	N	N
T2	1000	N	N	N	N	N	N	N	N
T3	1000	N	N	N	N	N	N	N	N
T4	1000	N	N	N	N	N	N	N	N
C1	C	N	N	N	N	N	N	N	N
C2	C	N	N	N	N	N	N	N	N
C3	C	N	N	N	N	N	N	N	N
C4	C	N	N	N	N	N	N	N	N

T-Treatment group C- Control group N- Normal A-Abnormal

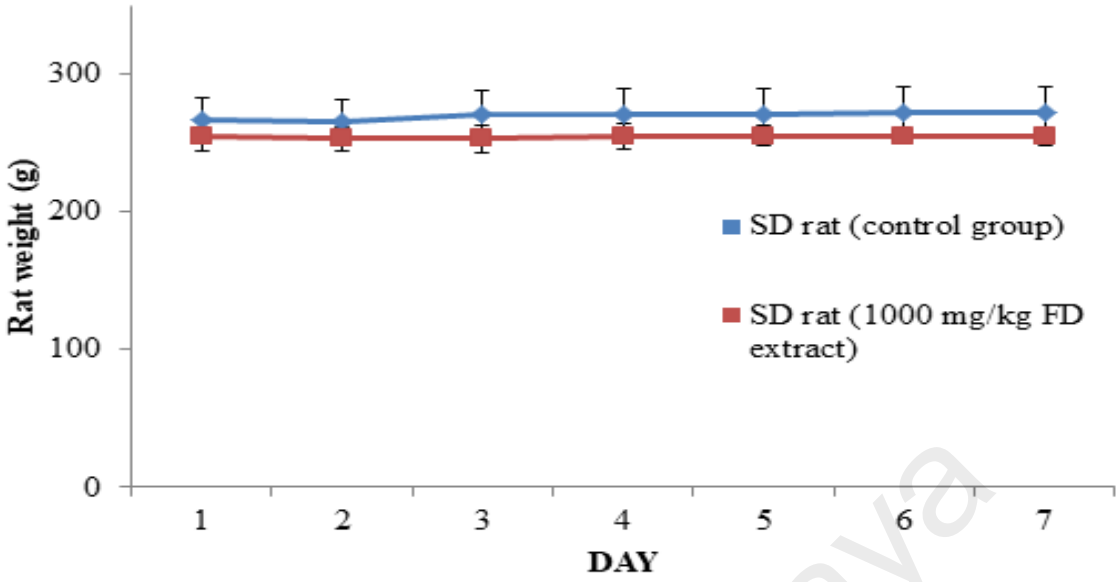


Figure 6.7: The body weight trends of SD rats in the control and treatment groups. No significant changes on the rat weight were found for both groups throughout the observation period ($p > 0.05$)

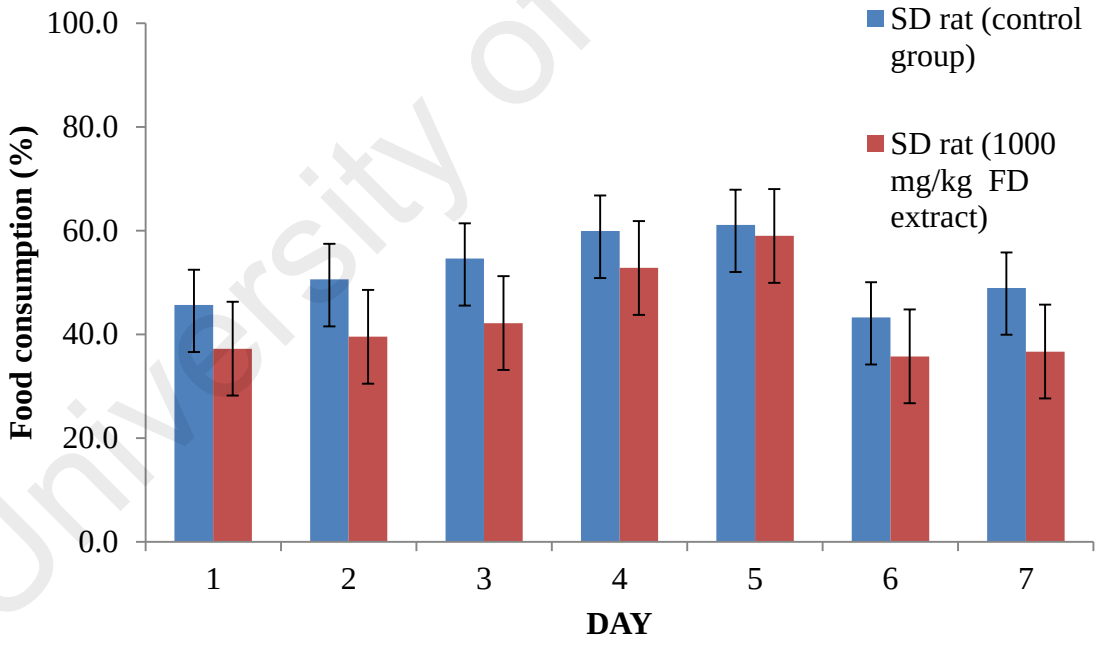


Figure 6.8: The food consumption percentage of SD rats in control and treatment groups. No significant changes on the food consumption were found for both groups throughout the observation period ($p > 0.05$)

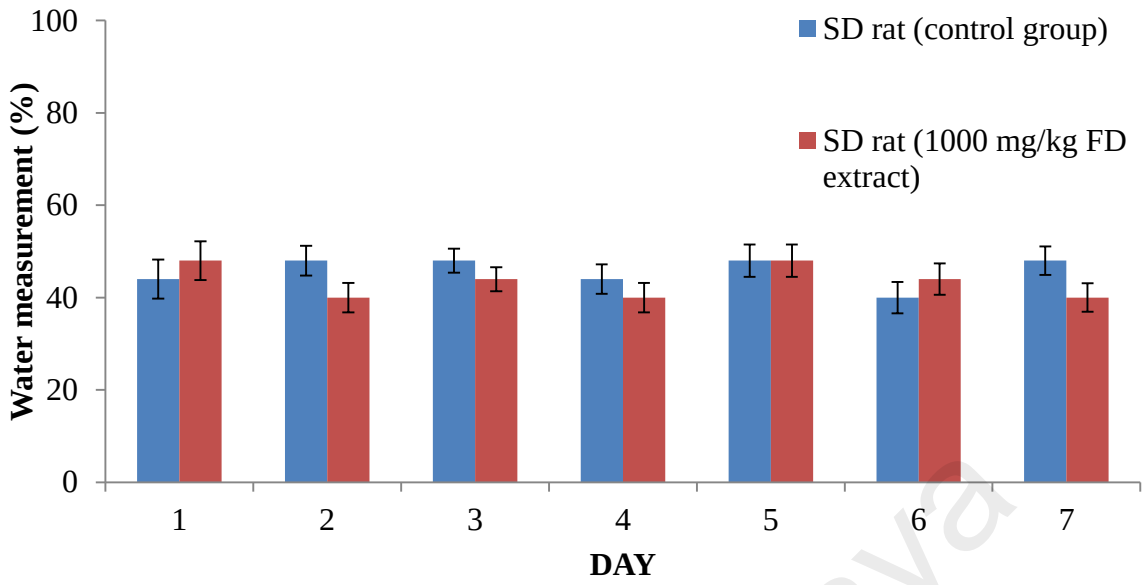


Figure 6.9: The water measurement percentage of SD rats in control and treatment groups. No significant changes on the water measurement were found for both groups throughout the observation period ($p > 0.05$)

6.5.1 Biochemical analysis of SD rat blood for toxicity testing

The SD rats were subjected to several biochemical analyses: alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), total protein, total bilirubin, urea and creatinine concentration in the blood plasma. Table 6.3 represents the average concentration of tested blood components for the control and treatment groups. The ALP, ALT, ASP, total protein and total bilirubin represents the liver function test while urea and creatinine described the kidney function test. The blood biochemical analysis revealed that the treatment group showed no significant differences of each individual parameter when compared to the control group. The blood biochemical profile for both groups was also found within the required range, indicating that there was no sign of toxicity in the blood plasma of the SD rats in treatment group.

Table 6.3: The average ± (SEM) biochemical parameters value from the blood plasma of control and FD leaf 50 % (v/v) ethanol water extract group

	Control group	**Treatment group**	**Range (Petterino & Argentino-Storino, 2006)**
ALP (U/L)	133.7.7 ± 4.9	135.2 ± 6.1	**131.6-459**
AST (U/L)	127.0 ± 17.8	132.7 ± 17.4	**64.1-168.1**
ALT (U/L)	40.0 ± 7.8	44.7 ± 8	**34.9-218.1**
Total protein (g/L)	77.2 ± 2.4	76.3 ± 1.7	**53.5-70.6**
Total bilirubin (umol/L)	5.1 ± 0.2	5.1 ± 0.5	**0-5.1**
Urea (mmol/L)	15.2 ± 0.4	15.0 ± 0.3	**11.1-31.7**
Creatinine (umol/L)	52.0 ± 0.4	47.3 ± 0.3	**35.4-88.4**

ALT= Alanine transaminase AST= Aspartate transaminase
ALP=Alkaline phosphatase

6.5.2 Histology observation on the selected organs for toxicity evaluation

The histology slides were prepared using Hematoxylin and Eosin (H & E) stain and observed microscopically for any abnormalities in the cells. By comparison to the control group, the changes in the cell morphology could provide evidence if there exist any organ toxicity. The organs evaluated were the kidney (Figure 6.10) and liver (Figure 6.11). The histopathological finding for the kidney in the control treatment groups displayed normal glomeruli with no abnormalities found on the kidney tubules and macrophage infiltration. For the liver, the blood vessel for the control and treatment groups exhibited normal condition with the hepatocytes sheet uniformly packed. Conclusively, the toxicity evaluations on the FD leaf 50 % (v/v) ethanol water extract of 1000 mg/kg suggested that the extract is non-toxic and safe to consume by the SD rats.

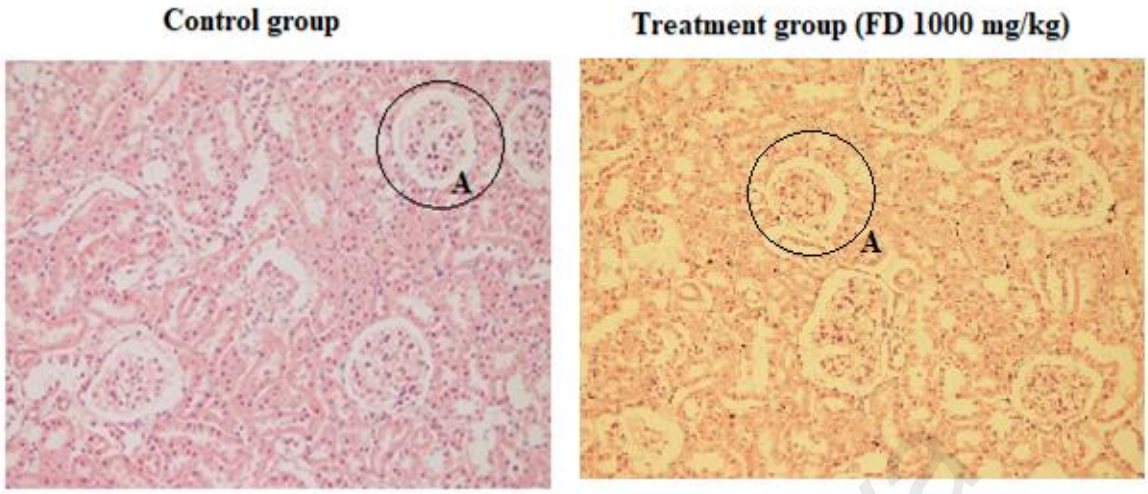


Figure 6.10: The kidney histology stained with Hematoxylin and Eosin for the control and treatment groups (20 × magnifications). A: Indicate the glomerus

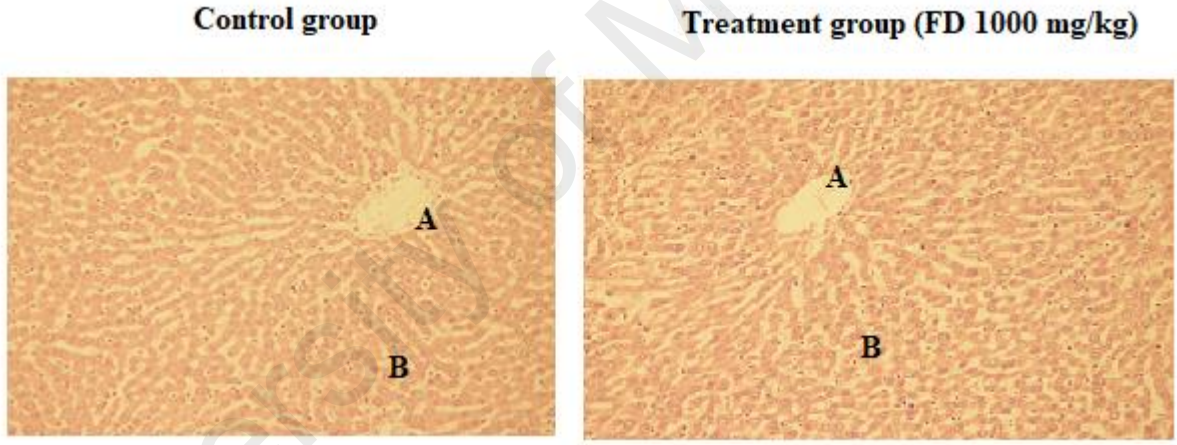


Figure 6.11: Liver histology stained with Hematoxylin and Eosin for the control and treatment groups (20 × magnifications). A: The blood vessel and B: Hepatocytes sheet

6.6 Wound healing properties of FD leaf 50 % (v/v) ethanol water extract

Wound healing properties of FD leaf 50 % (v/v) ethanol water extract was assessed on normal non-diabetic and diabetic SD rats. Normal non-diabetic SD rats are the normal, healthy SD rats while the diabetic condition was the streptozotocin (STZ) induced rats. FD leaf 50 % (v/v) ethanol water extract was prepared in Tween 20 as the vehicle and applied topically. Two different doses were chosen, 100 mg/kg and 200 mg/kg of FD leaf 50 % (v/v) ethanol water extract. The doses were calculated based on

Guideline No.420 (OECD, 2002). For both models, the control and positive control groups were given Tween 20 and intrasite gel group respectively.

6.6.1 SD rat acclimatization

A total of 48 SD rats aged 7-8 weeks weighing roughly 250 g were acclimatized for 7 days. The SD rats were randomly chosen as a group of 6 for each treatment group in both SD rat models (Table 6.4). The food pellet and water were given daily. The main purposed of the acclimatization period is to prepare the SD rat for *in vivo* study.

Table 6.4: SD rats in each group for the normal non-diabetic and diabetic SD rats

SD rat model / **Group**	**Normal non-diabetic SD rats**	**Diabetic SD rats**
Tween 20 (Negative control)	6 rats	6 rats
Intrasite gel (Positive control)	6 rats	6 rats
FD leaf extract (100 mg/kg)	6 rats	6 rats
FD leaf extract (200 mg/kg)	6 rats	6 rats

6.6.2 Preparation of STZ induced diabetic SD rats

For the diabetic SD rats group, the blood glucose level was taken on day 0 using the tail puncture method. After that, the SD rats were injected intra-peritoneal with 30 mg/kg dose of STZ. The blood glucose level of each rat was measured on day 1, day 7 and day 14. The blood glucose measurement level was analyzed and shown in Figure 6.12.

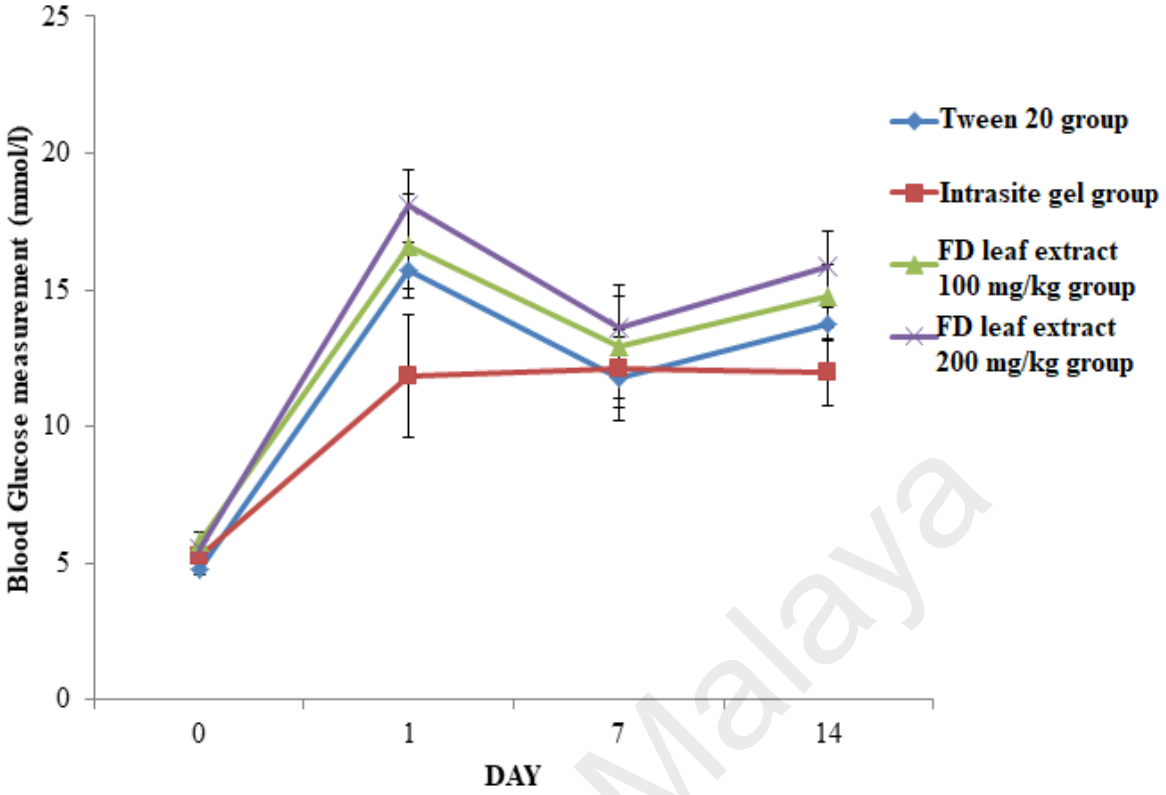


Figure 6.12: The blood glucose measurement level (average mmol/L ± SEM)

The SD rats induced by STZ showed significant increase in the blood glucose level ($p < 0.05$). The blood glucose level was maintained at higher than 11 mmol/L throughout the 14 days experiments after STZ induction indicating that the SD rats is in a diabetic state throughout the wound healing study.

6.6.3 Wound induction procedure for the SD rats

The wound induction for the normal non-diabetic and diabetic SD rats were done on the dorsal region of the SD rats using a circular stamp. The procedure involved anesthetizing the SD rats for a few hours before creating a uniform wound approximately 1.8 cm in diameter (Figure 6.13). Each rat from both groups (normal non-diabetic and diabetic SD rats) was transferred to an individual cage so that the SD rat will feel less cramped and stress that could affect the outcome of this study.

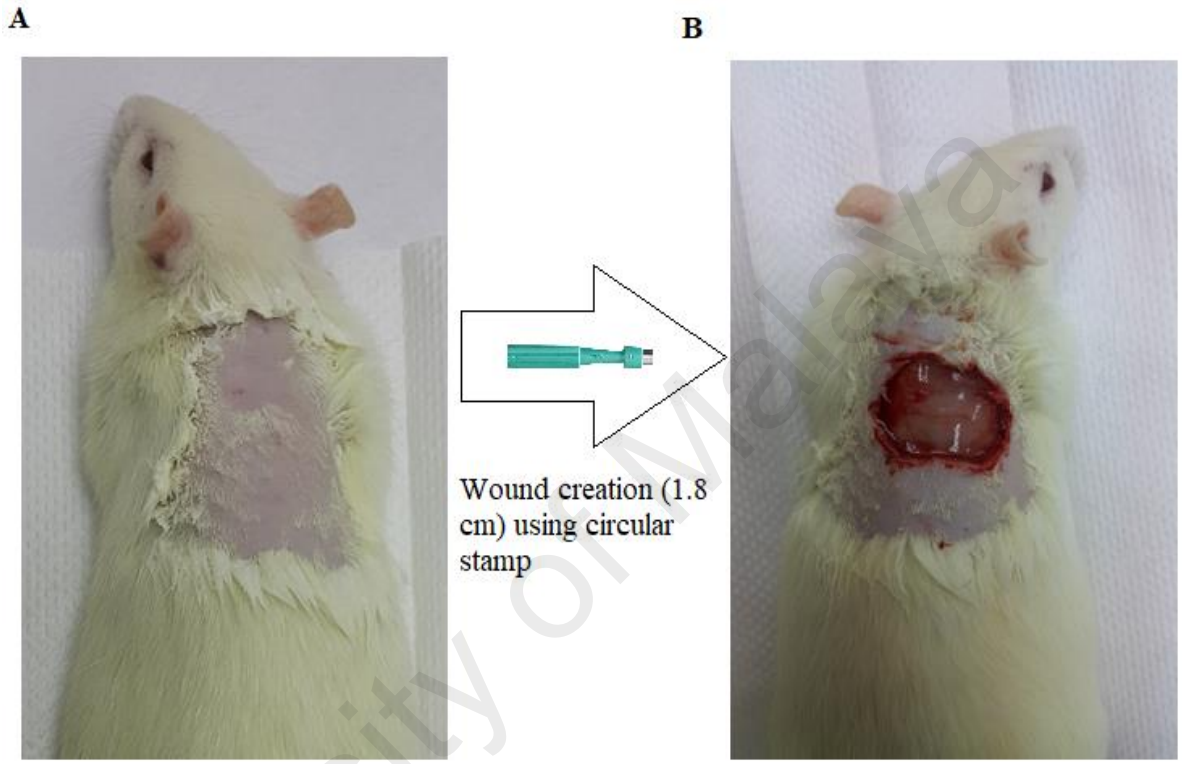


Figure 6.13: Wound induction procedure. A) The dorsal region of the SD rats was clean shaved, B) The wound created using circular stamp at approximately 1.8 cm in diameter

6.7 FD leaf 50 % (v/v) ethanol water extract wound healing properties using a normal non-diabetic and diabetic SD rats

The body weight changes of the SD rats throughout the treatment period for the normal non-diabetic and diabetic SD rats were analyzed (Figure 6.14). Overall, the normal non-diabetic SD rats showed a small fluctuation of the body weight reading. For the Tween 20 group (negative control), significant changes ($p < 0.05$) in the body weight were found to be on day 4, 8, 12 and 14. In the positive control group (Intrasite gel), significant changes of the body weight were found to be on day 2 and 12. The treated rats with 100 mg/kg FD leaf 50 % (v/v) ethanol water extract showed significant changes in body weight on day 14 while the 200 mg/kg FD leaf 50 % (v/v) ethanol water extract body weight significantly changed on day 8 and day 14. The body weight of the treated SD rats in diabetic SD rats (Figure 6.15) showed slight decreased throughout the treatment period. This could be explained due to the high blood glucose level in the diabetic SD rats that prone to cause body weight loss in the SD rats.

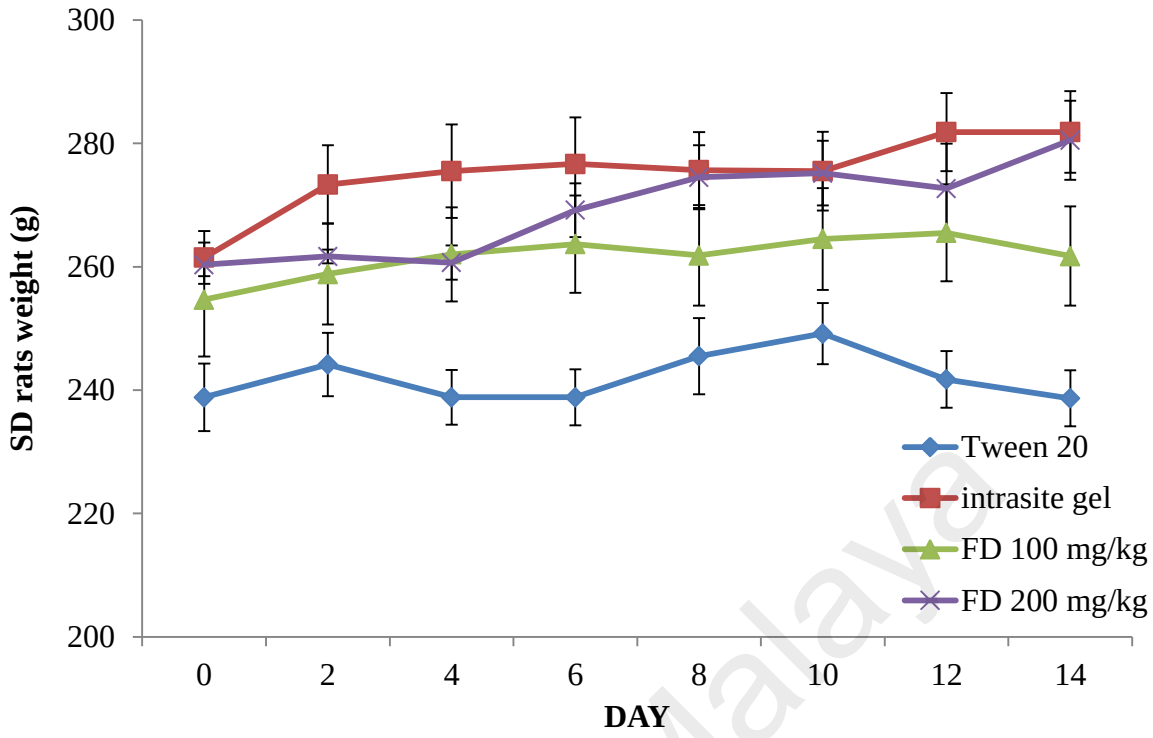


Figure 6.14: The body weight changes of the SD rats (average ± SEM) in the normal non-diabetic SD rats throughout the treatment period. No significant changes were observed ($p > 0.05$) for the weight changes of all groups throughout the treatment period

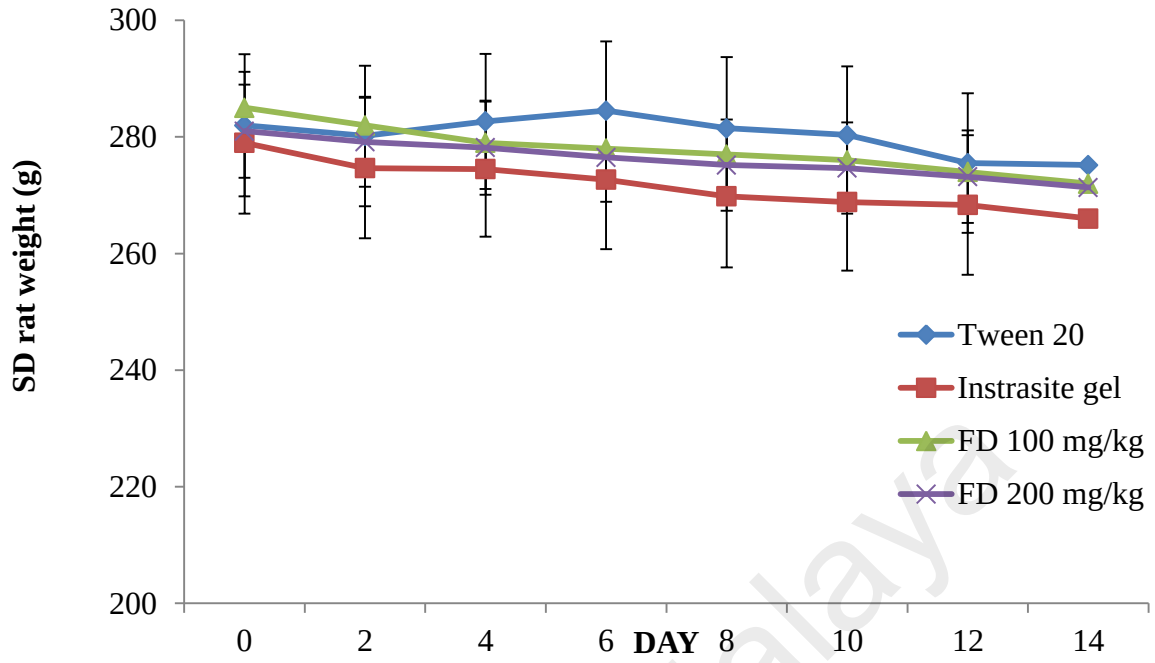


Figure 6.15: The body weight changes of the SD rats (average ± SEM) in the diabetic SD rats throughout the treatment period. No significant changes were observed ($p > 0.05$) for the weight changes of all groups throughout the treatment period

6.7.1 Wound closure rate of treated SD rats in normal non-diabetic and diabetic SD rats

Daily records of the wound closure for normal non-diabetic and diabetic SD rats were taken and analyzed. Figure 6.16 depicts the wound closure rate for each treatment groups in the normal non-diabetic SD rats. The wound closure picture of each treatment groups in normal non-diabetic SD rats was is shown in Figure 6.17. For the diabetic SD rats, Figure 6.18 and 6.19 depicted the wound closure rate for each treatment groups and wound closure picture of the treated SD rats, respectively. In the normal non-diabetic model, the highest percentage of wound closure rate after 14 days was the FD leaf 50 % (v/v) ethanol water extract dose of 100 mg/kg (58.3 ± 0.2%), followed by the FD leaf 50 % (v/v) ethanol water extract dose of 200 mg/kg (50 ± 0.3%), Tween 20 (50 ± 0.2%) and lastly the intrasite group (37 ± 0.2%). Significant changes ($p < 0.05$) on the wound closure rate in the normal non-diabetic SD rats was found in the intrasite gel groups at day 10, FD leaf 50 % (v/v) ethanol water extract dose of 100 mg/kg on day 12 and 14. For the diabetic SD rats, the highest percentage of wound closure rate after 14 days was the FD leaf 50 % (v/v) ethanol water extract dose of 100 mg/kg (82 ± 0.1%), intrasite gel (79 ± 0.2%), FD leaf 50 % (v/v) ethanol water extract dose of 200 mg/kg (69 ± 0.3%) and lastly the Tween 20 (47 ± 0.4%).

The control group (Tween 20-treated wounds) for the normal non-diabetic and diabetic SD rats displayed similar wound healing closure rate. Tween 20 was previously described as the vehicle for testing extract in wound healing study (Somboonwong *et al.*, 2012). Tween 20 is preferred for the wound healing study due to its viscosity and lipophilicity which are excellent for suspension of extract (Choudhury *et al.*, 2017).

The intrasite gel is a hydrogel manufactured by Smith & Nephew. Intrasite gel is an amorphous hydrogel which gently re-hydrates necrotic tissue, facilitating autolytic debridement, while being able to loosen and absorb slough and exudate. It can also be

used to provide the optimum moist wound management environment during the later stages of wound closure (Hess, 2011). In this study, the intrasite gel showed the lowest wound closure rate for the normal non-diabetic SD rats and the second highest for the diabetic SD rats. Intrasite gel is also known as diabetic dressing due to its non-adherent and does not harm viable tissue or the skin surrounding the wound (Hess, 2011). The used of intrasite gel that contains a modified carboxymethylcellulose polymer (2.3%) and propylene glycol (20%) as dressing for diabetic researches have been reported by many studies (Al-Bayaty & Abdulla, 2012). Complications of diabetes such as diabetic ulcer are concerned over the use of highly adherent dressings in the treatment (Cuschieri *et al.,* 2013). It should be avoided to limit trauma to the surrounding skin. Diabetic ulcers have drier wounds and very often required the application of an intrasite gel to maintain a moist wound healing environment (Dumville *et al.,* 2013). This probably explained why the intrasite gel displayed higher activities on the diabetic SD rats. The FD leaf extracts on the other hand, exhibit dose dependent effects on the wound closure at a faster rate on the diabetic SD rats compared to the normal non-diabetic SD rats. This could be explained due to the significant anti-oxidant and MMPs inhibitory properties that FD leaf extract contains. The nature of wound healing process is complex with various factors could affect its normal homeostasis (Eming *et al.,* 2007). Such examples are the MMP enzymes. The MMPs play vital roles during the wound healing process (Caley *et al.,* 2015). During normal wound healing process, the physiological conditions are tightly regulated with normal responses of the MMP enzymes triggering tissue remodeling process (Atala *et al.,* 2010). However, should there be abnormal conditions such as diabetic related, the production of MMPs usually increased, and causing imbalance leading to prolonged wound complications (Caley *et al.,* 2015). Therefore, to control this kind of situation, the MMPs should be stabilized by enhancing the MMPs inhibition activities. In this study, it was proven that FD leaf

extract alongside its compounds (vitexin and isovitexin) potently involved in inhibition of the MMPs (Chapter 5). The cell migratory assay also revealed that FD leaf extracts exhibited dose dependent effect on cell migrations of EA.hy 926 cells. This probably described why the FD leaf extract exhibited dose dependent effects more pronounced in the diabetic SD rats compared to the normal non-diabetic SD rats.

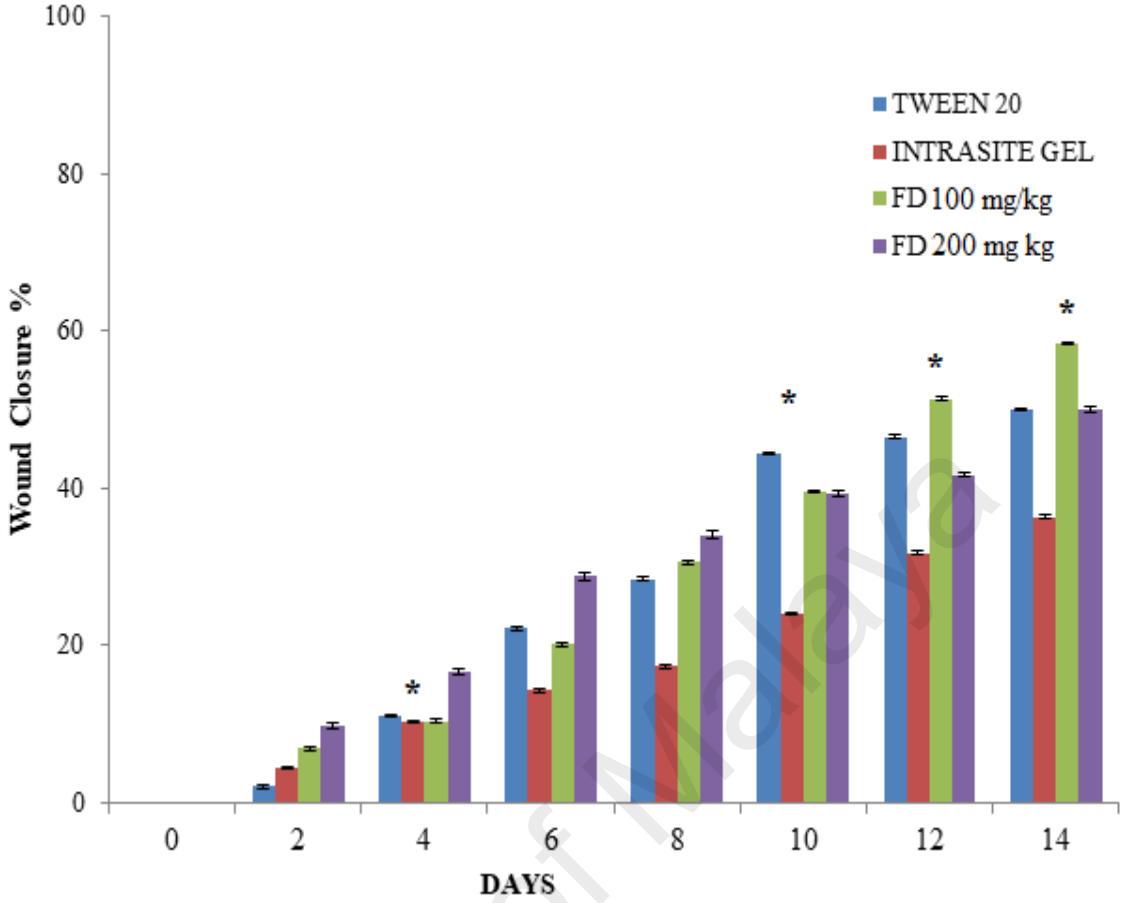


Figure 6.16: The wound closure rate (average ± SEM) for each group of the normal non-diabetic SD rats. The symbol * represented the significant difference ($p < 0.05$) when compared to the control group

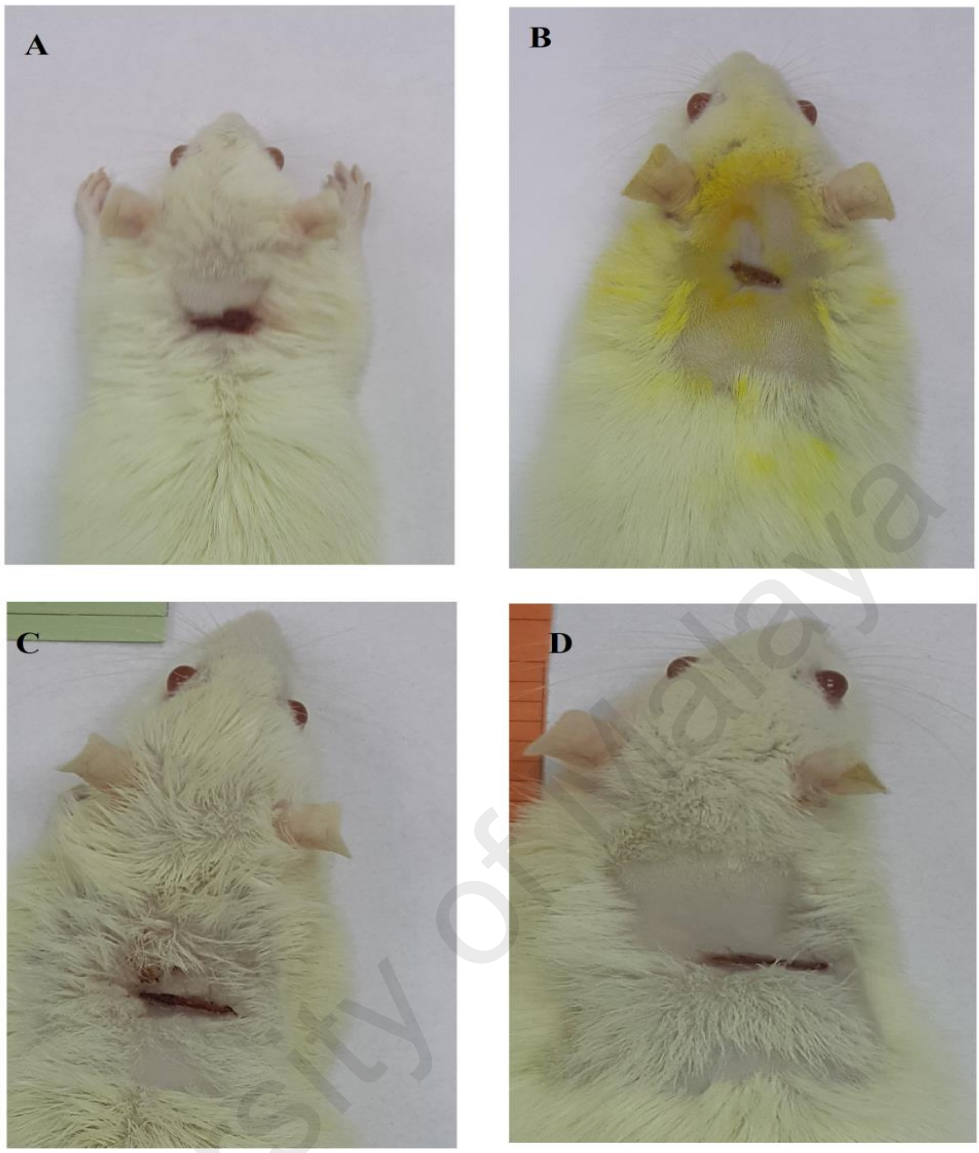


Figure 6.17: Photograph of wound closure of the treated normal non-diabetic SD rats after treatment**.** A) SD rat treated with Tween 20, B) SD rat treated intrasite gel, C) SD rat treated with 200 mg/kg FD leaf 50% (v/v) ethanol water extract and D) SD rat treated with 200 mg/kg FD leaf 50% (v/v) ethanol water extract

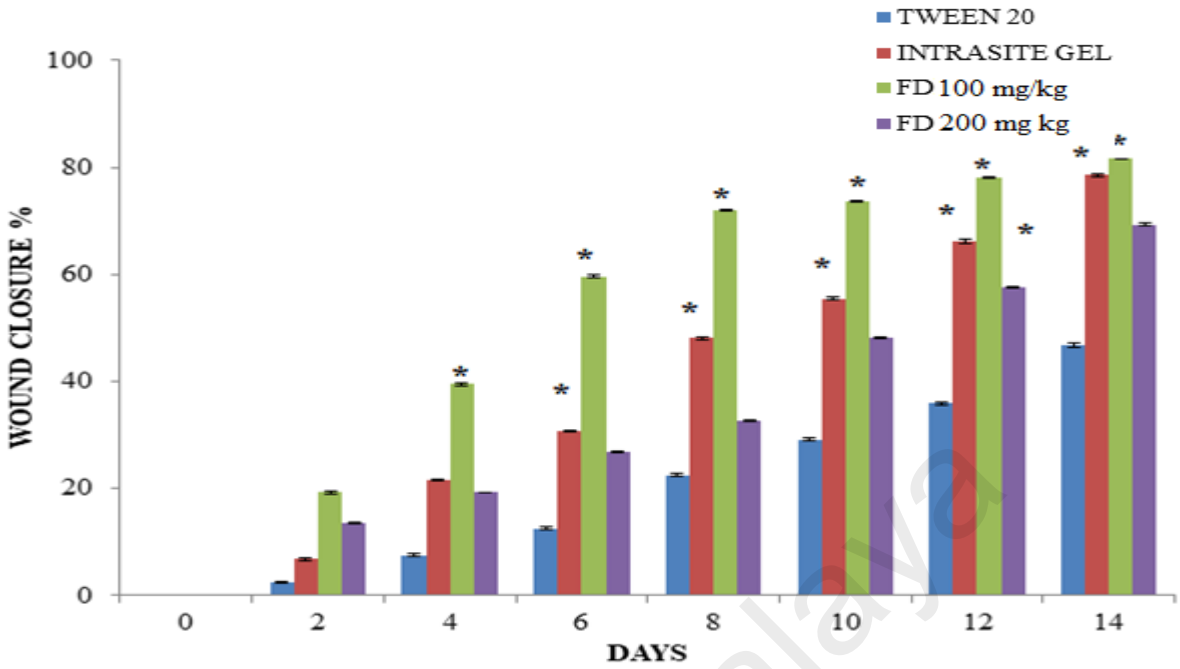


Figure 6.18: The wound closure rate (average ± SEM) for each group of the diabetic SD rats. The symbol * represented the significant difference ($p < 0.05$) when compared to the control group.

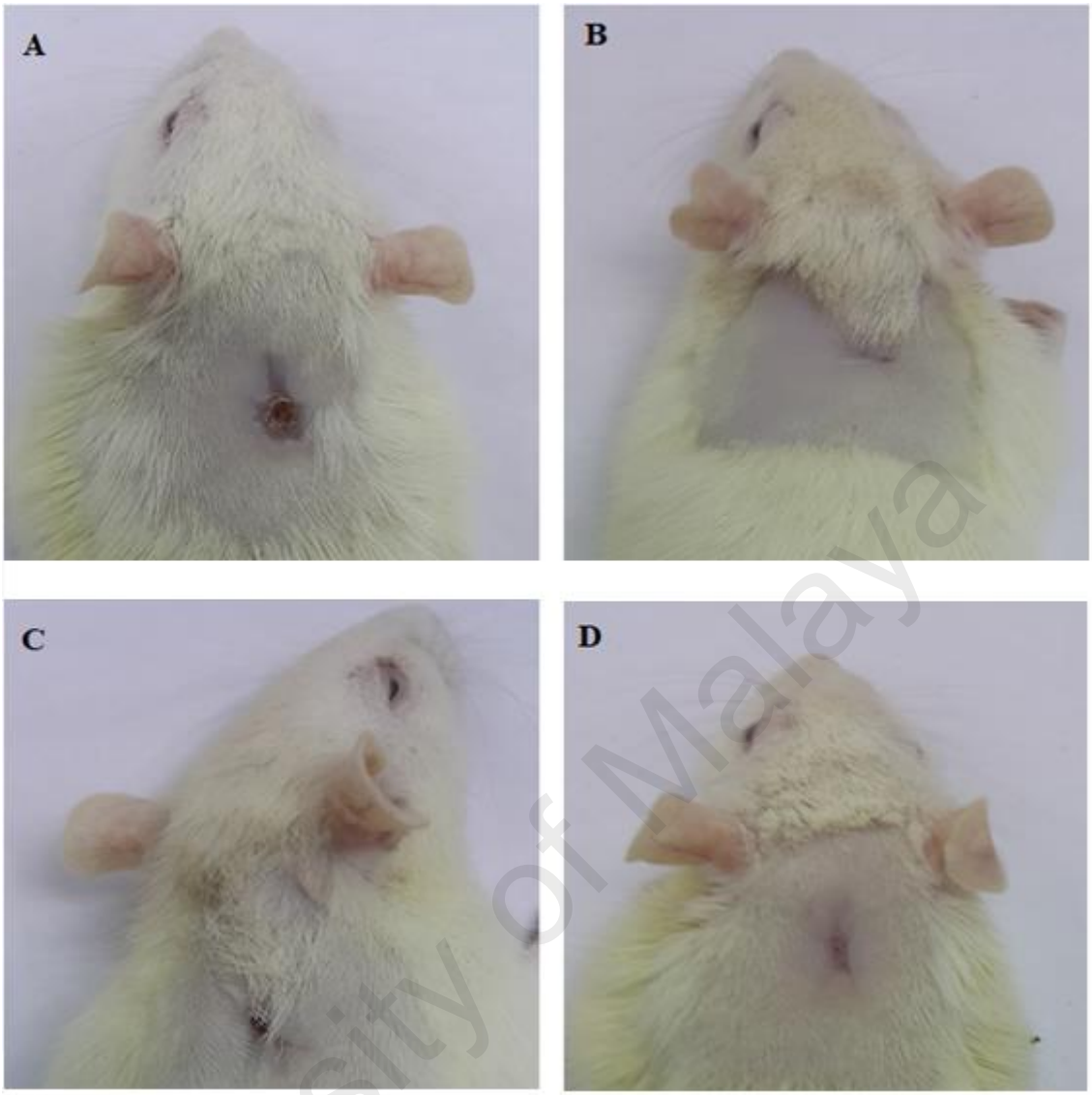


Figure 6.19: Photograph of the treated diabetic SD rats after wound treatment**.** A) SD rat treated with Tween 20 group, B) SD rat treated intrasite gel, C) SD rat treated with 200 mg/kg FD leaf 50% (v/v) ethanol water extract and D) SD rat treated 200 mg/kg FD leaf 50% (v/v) ethanol water extract

6.7.2 Hematology profile of treated SD rats in normal non-diabetic and diabetic SD rats

The full blood count parameters analyses were the red blood cell and white blood cell components. The red blood cell components are red blood cell count (RBC), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). The white blood cell components are white blood cell count (WBC), neutrophil, lymphocytes percent, monocytes percent and eosin. Tables 6.5 and 6.6 described the values of the full blood

count parameters obtained from the blood of the normal non-diabetic and diabetic SD rats, respectively (Han *et al.,* 2010).

The full blood count analysis represents an initial step to determine abnormalities of the blood cells such as infections and inflammations. Should there be signs of prolonged infections and inflammations, the behavior of the SD rats will change, exhibiting feverish and inactive conditions (Inoue *et al.,* 2008). In conditions such as poor circulation or a suppressed immune system, infection slows the wound healing process (Guo *et al.,* 2010). Wound opening is a route for the microorganisms to enter (Janeway *et al.,* 2001). Usually the microorganisms that enter through the wound route may cause harm to the SD rats, especially in the diabetic state as the normal homeostasis is disrupted at this point. The infection may become a chronic infection if it penetrates deep into the body tissues such as bone, or when the infection occurs in tissue that has an inadequate circulation (Rolfes *et al.,* 2007).

For the RBC components, polycythemia represents high value of RBCs (Pearson, 2001) whereas anemia is due to low RBCs (Stedman, 2006). In anemic conditions, the decrease of RBC components lowered the ability of blood to carry the oxygen, leading to prolonged wound healing process (Rodak, 2007). Conditions such as renal failure, chronic inflammations, and infections caused disturbance on the RBCs production and destruction, leading to anemic state (Kumar *et al.,* 2007). Polycythemia on the other hand is a pathological condition due to increase of RBC components concentration. Pathological conditions such as infections, hypoxia and hepatomegaly increase the range of the RBC component (Copstead & Banasik, 2012). The values of RBC for the normal non-diabetic and diabetic SD rats obtained from the hematology analysis were found to be in the normal range, indicating no anemic or polycythemia present in the SD rats. For the WBC components, leucopenia described conditions with decrease WBC components whereas leukocytosis represents condition with high WBC

components. The pathological characteristics of leucopenia are due to infections, viral diseases, kidney failure and blood cell dysfunction (Kumar *et al.,* 2007). Leukocytosis happened due to conditions such as inflammations, viral, bacterial and fungal infections (Copstead & Banasik, 2012). From the hematology profile, the normal non-diabetic and diabetic SD rats showed normal value of WBC components, indicating that there were no sign of infections and inflammations occurred in both groups.

Overall, the results from the full blood count analysis showed that the reading were in normal range for normal non-diabetic and diabetic SD rats, indicating no sign of prolonged inflammations and infections throughout the duration of the study.

Table 6.5: Full blood count analysis of normal non-diabetic SD rats in the wound healing study. The results are represented as average ± SEM

	Tween 20 (Negative control)	**Intrasite gel (Positive control)**	**FD leaf extract 100 mg/kg**	**FD leaf extract 200 mg/kg**	**Range (Han *et al.*, 2010)**
RBC (× 10^{12}/L)	7.34 ± 0.4	7.59 ± 0.2	7.69 ± 0.2	7.86 ± 0.1	**6.7-9.8**
Hb (g/L)	165.5 ± 14.2	163.3 ± 5.0	163.6 ± 4.9	164.6 ± 4.3	**125-178**
PCV (L/L)	0.39 ± 0.04	0.46 ± 0.01	0.45 ± 0.01	0.45 ± 0.01	**0.34-0.57**
MCV (fL)	60.7 ± 0.5	61.1 ± 1.3	58.5 ± 1.2	57.6 ± 1.2	**51.4-63.1**
MCHC (g/L)	350.7 ± 11.1	352.9 ± 8.7	363.8 ± 2.3	363.3 ± 2.4	**301-371**
WBC (× 10^{9}/L)	6.4 ± 2.9	6.7 ± 0.9	7.7 ± 1.9	8.3 ±1.7	**3.0-12**
Neutrophil (%)	41.0 ± 4.1	48.3 ± 1.3	48.0 ± 1.5	46.3 ± 0.3	**4.9-58.1**
Lymphocytes (%)	55.2 ± 1.2	47.3 ± 0.3	47.2 ± 0.3	47.6 ± 0.3	**33.3-91.5**
Monocytes (%)	1.0 ± 0.1	0.67 ± 0.3	1.0 ± 0.6	1.3 ± 0.3	**0.1-7.5**
Eosin (× 10^{9}/L)	0.6 ± 0.03	0.5 ± 0.02	0.10 ± 0.06	0.12 ± 0.05	**0.3-10.0**

RBC= Red blood cells, HB= Hemoglobin, PCV= Packed cell volume, MCV= Mean corpuscular volume, MCHC= Mean corpuscular hemoglobin concentration, WBC= white blood cell count

Table 6.6: Full blood count analysis of diabetic SD rats in the wound healing study. The results are represented as average ± SEM

	Tween 20 (Negative control)	**Intrasite gel (Positive control)**	**FD leaf extract (100 mg/kg)**	**FD leaf extract (200 mg/kg)**	**Range (Han *et al.*, 2010)**
RBC (× 10^{12}/L)	8.6 ± 0.4	8.1 ± 1.4	8.3 ± 0.9	8.4 ± 0.3	**6.7-9.8**
Hb (g/L)	163.0 ± 6.1	169.3 ± 9.2	161.3 ± 2.1	165.0 ± 4.3	**125-178**
PCV (L/L)	0.5 ± 0.02	0.4 ± 0.07	0.4 ± 0.05	0.4 ± 0.01	**0.34-0.57**
MCV (fL)	52.3 ± 0.1	52.3 ± 1.4	51.7 ± 1.5	51.3 ± 0.7	**51.4-63.1**
MCHC (g/L)	352.7 ± 12.0	358.0 ± 16.3	356.3 ± 0.8	353.0 ± 4.8	**301-371**
WBC (× 10^{9}/L)	4.9 ± 0.7	3.9 ± 1.2	5.7 ± 1.4	5.2 ±1.3	**3.0-12**
Neutrophil (%)	49.7 ± 0.1	50.0 ± 4.2	51.7 ± 2.3	52.3 ± 3.2	**4.9-58.1**
Lymphocytes (%)	50.8 ± 0.6	37.6 ± 0.3	47.1 ± 0.3	50.9 ± 0.6	**33.3-91.5**
Monocytes (%)	1.0± 0.1	0.7 ± 0.3	1.3 ± 0.3	1.0 ± 0.05	**0.1-7.5**
Eosin (× 10^{9}/L)	0.5 ± 0.01	0.3 ± 0.02	0.8 ± 0.03	0.5 ± 0.02	**0.3-10.0**

RBC= Red blood cells, HB= Hemoglobin, PCV= Packed cell volume, MCV= Mean corpuscular volume, MCHC= Mean corpuscular hemoglobin concentration, WBC= white blood cell count

6.7.3 Blood biochemical analysis of SD rats after wound healing treatment

The blood from the treated SD rats (normal non-diabetic and diabetic SD rats) were analyzed for each parameter, namely the ALP, AST, total protein, total bilirubin, urea and creatinine. Table 6.7 and 6.8 show values of the biochemical analysis obtained from the blood of the normal non-diabetic and diabetic SD rats, respectively.

The ALP, ASP, total protein and total bilirubin are the liver function test. This test described the condition of the liver, either healthy or in pathological state. Liver in pathological state will produced abnormal values of these enzymes. Conditions such as coagulopathy, hemorrhage and sepsis are the consequences of liver injuries that could lead to mortality (Banihashemi *et al.,* 2017). The kidney function test on the other hand measured the physiological condition of the kidney. The biochemical parameters are urea and creatinine. In pathological state, the kidney produced high values of urea and creatinine. Sepsis, infection, and vasculitis are among the cause of kidney disease (Kumar *et al.,* 2007). Overall, from the blood biochemical analysis, the liver and kidney function test showed that the blood biochemical parameters were within the reference range (Petterino & Argentino-Storino, 2006). This indicates that the liver and kidney functions were normal throughout the duration of the study.

Table 6.7: The average ± (SEM) biochemical parameter value from the blood plasma of treated group of normal non-diabetic SD rats

	Tween 20 (Negative control)	**Intrasite gel (Positive control)**	**FD leaf extract (100 mg/kg)**	**FD leaf extract (200 mg/kg)**	**Range (Petterino & Argentino-Storino, 2006)**
ALP (U/L)	164.7 ± 9.5	162.3 ± 7.6	168.7 ± 2.7	164.0 ± 4.0	**131.6 - 459**
AST (U/L)	86.3 ± 29.7	87.0 ± 25.3	91.3 ± 29.6	91.0 ± 27.5	**64.1 - 168.1**
ALT (U/L)	37.3 ± 8.1	39.7 ± 10.0	36.7 ± 10.0	36.7 ± 9.9	**34.9 - 218.1**
Total protein (g/L)	64.8 ± 1.2	59.3 ± 4.6	60.4 ± 5.1	61.8 ± 5.8	**53.5 - 70.6**
Total bilirubin (umol/L)	5.0 ± 0.1	4.7 ± 0.2	4.7 ± 0.3	4.8 ± 0.3	**0 - 5.1**
Urea (mmol/L)	15.2 ± 0.5	14.2 ± 0.7	14.2 ± 0.7	14.6 ± 0.8	**11.1 - 31.7**
Creatinine (umol/L)	52.0 ± 4.0	47.3 ± 4.4	47.0 ± 4.0	52.0 ± 5.1	**35.4 - 88.4**

ALT= Alanine transaminase AST= Aspartate transaminase ALP=Alkaline phosphatase

Table 6.8: The average ± (SEM) biochemical parameter value from the blood plasma of treated group of diabetic SD rats

	Tween 20 (Negative control)	**Intrasite gel (Positive control)**	**FD leaf extract (100 mg/kg)**	**FD leaf extract (200 mg/kg)**	**Range (Petterino & Argentino-Storino, 2006)**
ALP (U/L)	184.1 ± 1.5	182.3 ± 2.8	188.7 ± 4.9	189.7 ± 3.3	**131.6 - 459**
AST (U/L)	113.7 ± 4.8	125.7 ± 24.8	137.7 ± 4.8	147.0 ± 5.7	**64.1 - 168.1**
ALT (U/L)	50.3 ± 7.25	56.3 ± 3.8	49.7 ± 3.8	46.3 ± 8.5	**34.9 - 218.1**
Total protein (g/L)	56.1 ± 1.7	59.1 ± 1.7	55.2 ± 1.7	62.9 ± 0.4	**53.5 -70.6**
Total bilirubin (umol/L)	4.2 ± 1.4	4.1 ± 1.5	4.4 ± 0.3	4.7 ± 0.5	**0 - 5.1**
Urea (mmol/L)	22.5 ± 0.3	20.4 ± 0.2	12.5 ± 0.2	12.2 ± 0.3	**11.1 - 31.7**
Creatinine (umol/L)	57.7 ± 2.4	56.7 ± 3.4	55.0 ± 2.6	54.3 ± 2.2	**35.4 - 88.4**

ALT= Alanine transaminase AST= Aspartate transaminase ALP=Alkaline phosphatase

6.7.4 Histology study on the selected organs of the normal non-diabetic and diabetic SD rats in the wound healing study

The normal non-diabetic and diabetic SD rats were sacrificed and 4 organs were collected for histology analysis. The organs dissected for analysis were the skin, liver, kidney and pancreas. Observation of any changes on cell morphology and abnormalities was conducted on the organs. Should there be any complications during the treatment such as organ infections or prolonged inflammations, the findings from the histology analysis will indicate the presence of abnormalities in the tissue.

6.7.4.1 Skin

The skin was taken and prepared as histological slides to investigate the wound closure process microscopically. The observations focused on the epidermis, and dermis layer, collagen and the fibroblast cell. Microscopic observations indicated that both groups (non-diabetic and diabetic) showed complete healing with the diabetic SD rats showing more prominent effects. The histological analysis of the skin on the normal non-diabetic and diabetic SD rats showed uniformly packed fibroblast suggesting the beginning of the macromolecular organization of the granulation tissue. The observations also indicated that the reepithelization processes of both groups produced high numbers of epithelial cell as well as differentiation of the nuclear keratinocytes to keratinized cells. There were also no abnormalities for the presence of macrophages found in the histology slide of both groups indicating no prolonged inflammation process. The skin histology slides for both groups are shown in Figure 6.20.

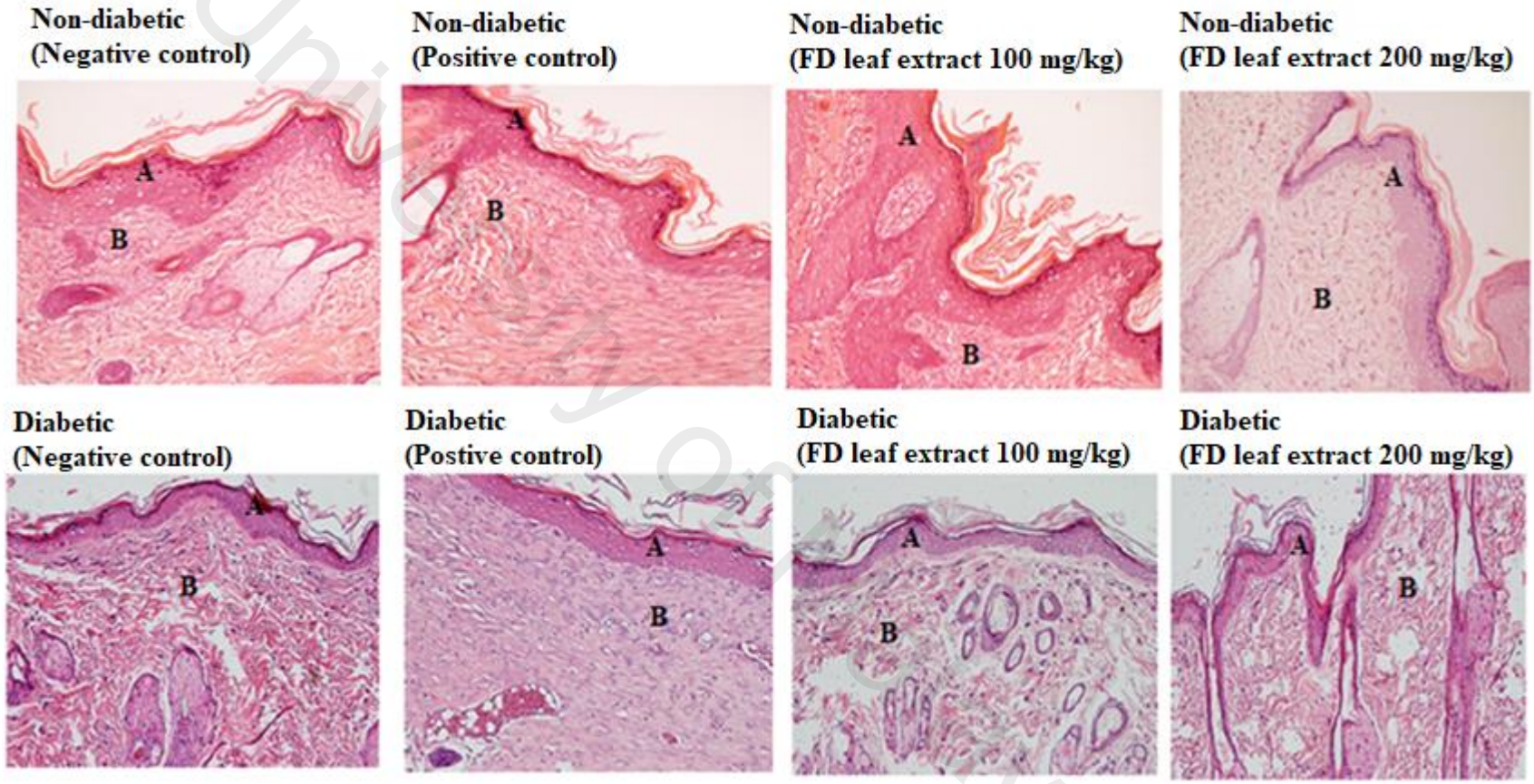


Figure 6.20: Histology slides (skins) of normal non-diabetic and diabetic SD rats for the wound healing study taken at 20 × magnifications using microscope. A: The epidermis region and B: The dermis region

6.7.4.2 Liver and kidney

The liver and kidney organs were analyzed microscopically to unravel any side effects that the treated SD-rats obtained during the treatment process. Generally, the liver and kidney are organs that store, detoxified, metabolized and excreted metabolites. The liver acts by maintaining and controlling the blood glucose through a process known as glycogenesis and glycogenolysis (Ward, 2014). Destruction of the liver cell activates inflammatory response (Kolios *et al.*, 2006). Oxidative stress also plays an important role in the mechanism of liver and kidney destruction (Li *et al.*, 2015). Prolonged inflammations and infections facilitate the production of abnormal cytokine and growth factors leading to alterations in the liver and kidney cells (Markiewski & Lambris, 2007).

The liver tissue on the other hand showed normal structure of densely packed hepatocytes with no abnormal numbers of macrophage indicating that there were no prolonged inflammations and infections during the wound healing study of the diabetic and non-diabetic SD rats (Figure 6.21). Histopathological evaluation of the normal kidney tissue of the non-diabetic and diabetic rats demonstrated the normal structure of glomerulus surrounded by the Bowman's capsule, distal convoluted tubules and proximal without any inflammatory alterations (Figure 6.22).

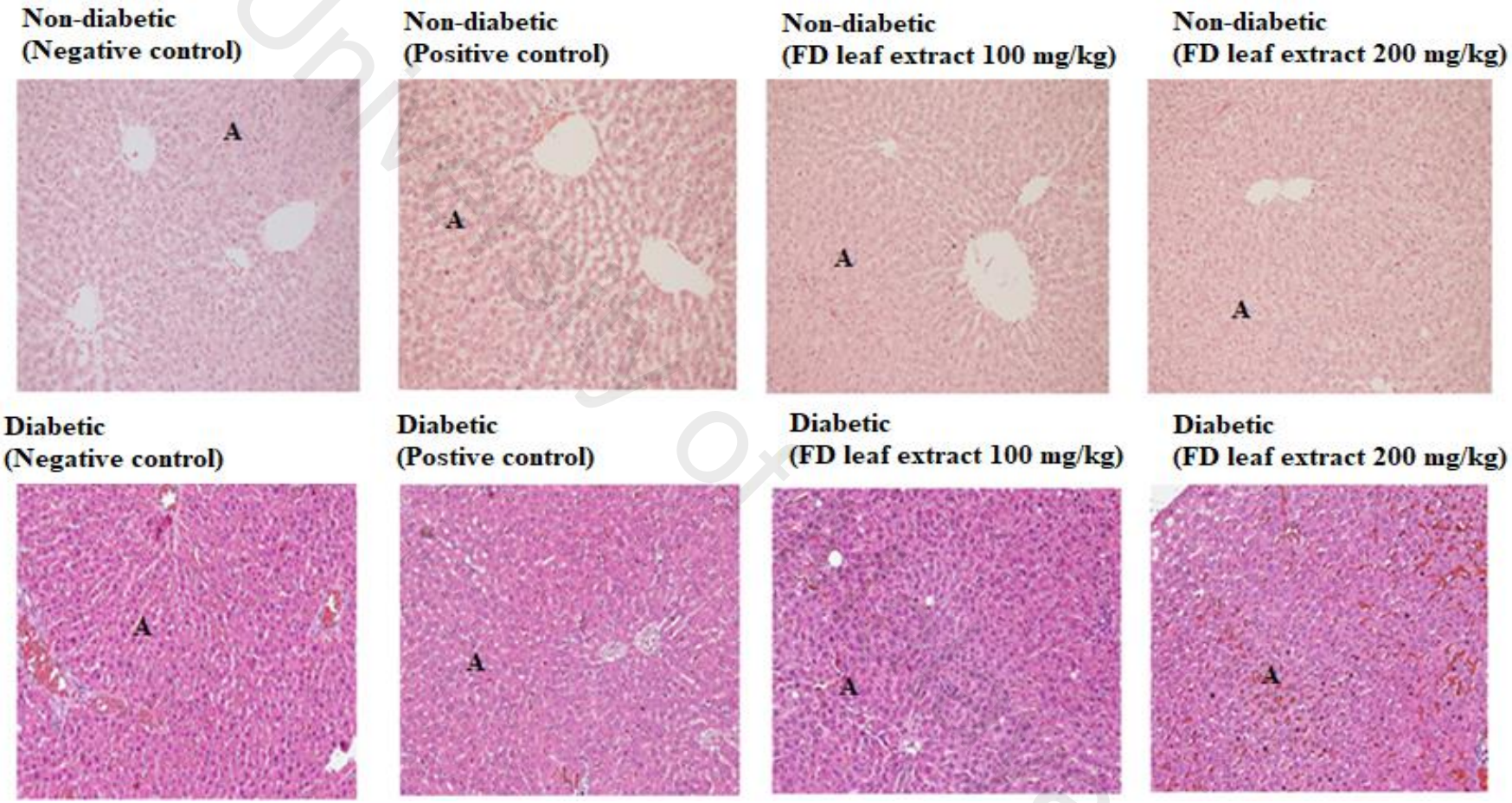


Figure 6.21: Histology slides (liver) of normal non-diabetic and diabetic SD rats for the wound healing study under 20 × magnification using microscope. A: The hepatocytes sheet and sinusoids of the liver

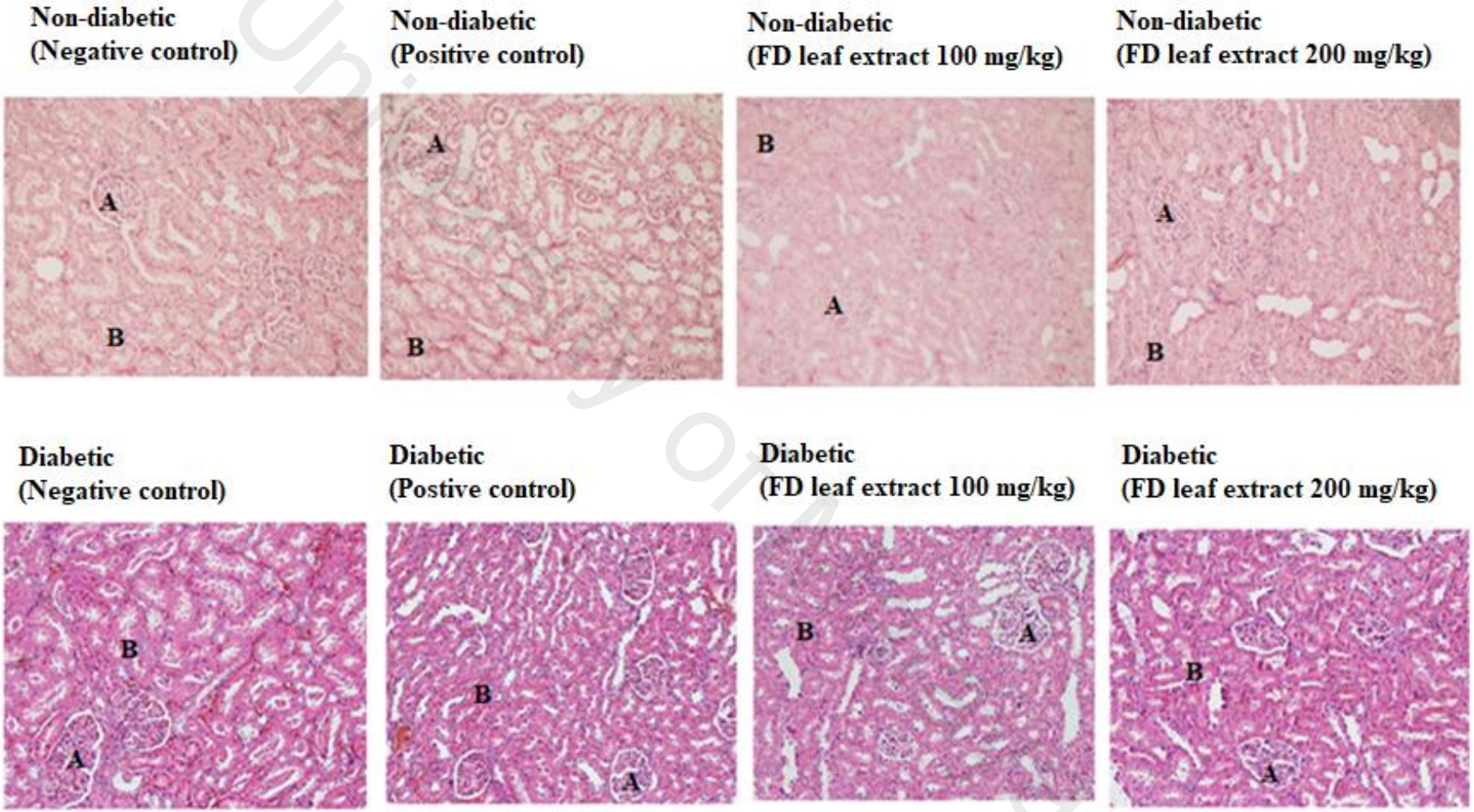


Figure 6.22: Histology slides (kidney) of normal non-diabetic and diabetic SD rats for the wound healing study under 20 × magnifications using microscope. A: The glomerulus and B: Kidney tubules

6.7.4.3 Pancreas

Pancreases were taken for histology study for comparison between the non-diabetic and diabetic rats (Figure 6.23). Since the STZ induced hyperglycemia in rats, it should have affected the pancreas as it is the main organ for glucose regulation. From the histology slide, microscopic investigation of pancreas sections of non-diabetic rats showed the normal appearance of Islets of Langerhans. The islets appeared lightly stained than the surrounding acinar cells. The acinar cells are formed of pyramidal cells with basal nuclei and apical acidophilic cytoplasm.

However, the diabetic rats showed pathological changes of both the exocrine and endocrine components. The acinar cells were swollen and small vacuoles were observed in almost all acinar cells. Interlobular ducts were lined with flattened epithelium. Islet β-cells are almost entirely lost in STZ-treated rats. The increased in the blood glucose level in STZ-induced rats were influenced by the destruction of the islet β-cells that are important for blood glucose regulations.

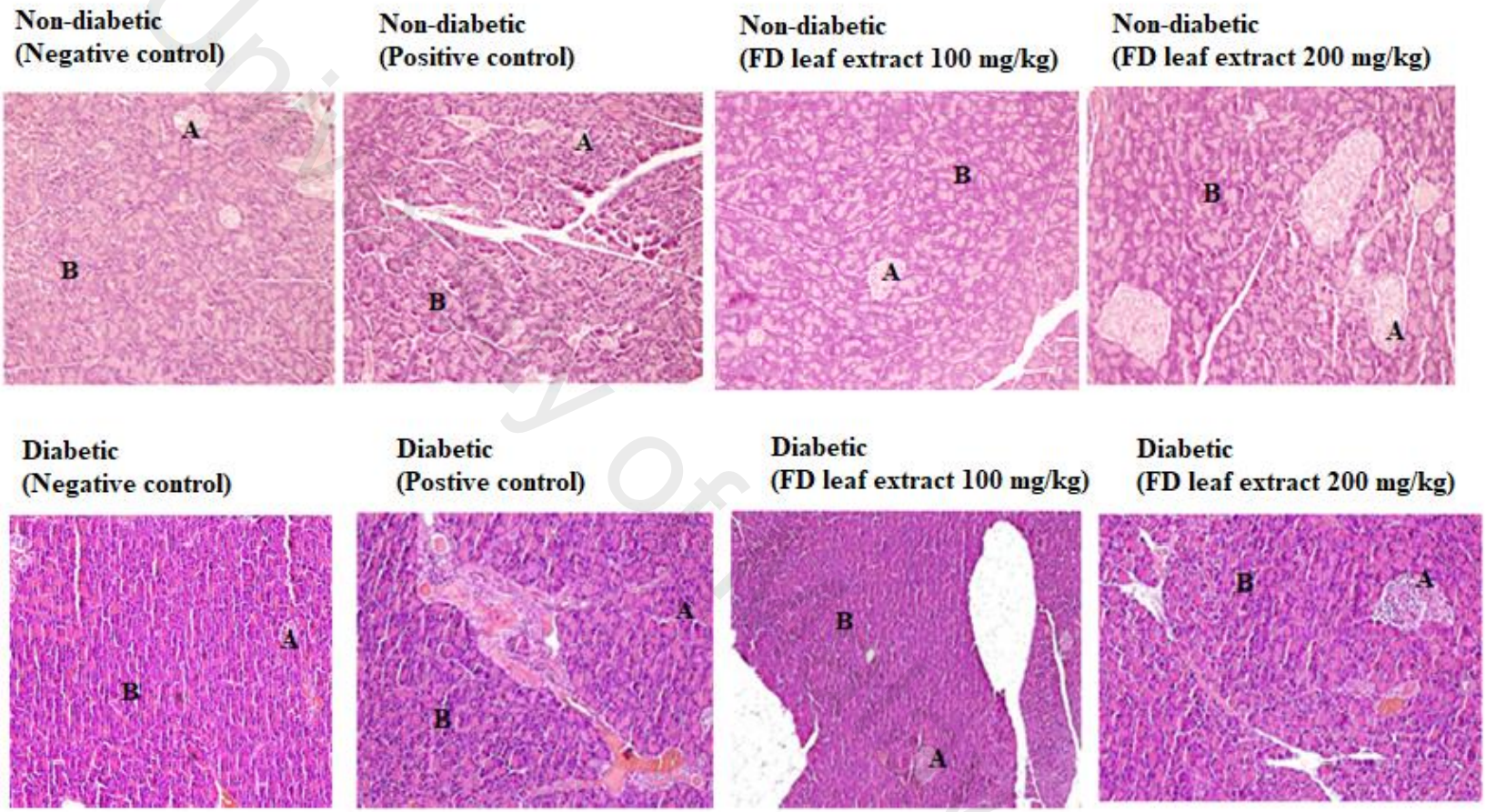


Figure 6.23: Histology slides (pancreas) of normal non-diabetic and diabetic SD rats for the wound healing study under 20 × magnifications using microscope. A: Islet of Langerhans and B: Exocrine secretion acini

6.8 Discussion

From the study conducted using computational approaches (Chapter 5), FD leaf extract, vitexin and isovitexin were shown to have inhibition properties for the α-amylase and MMP enzymes. Therefore, for validation purposes, FD leaf 50 % (v/v) ethanol water extract was subjected to a series of biological activity testing using *in vitro* and *in vivo* techniques.

Initially, the FD leaf 50% (v/v) ethanol water extract anti-oxidant activity was tested using the DPPH scavenging assay. Generally, free radical such as DPPH are inevitably produced in biological systems and encountered exogenously, and are known to cause various degenerative disorders, like wound healing disorder, mutagenesis, carcinogenesis, cardiovascular disturbances and ageing (Kedare & Singh, 2008). Antioxidants are compounds combat the free radicals by intervening at any one of the three major steps of the free radical mediated oxidative process, namely initiation, propagation and termination (Cui *et al.*, 2004). These antioxidants are also produced by biological system and occur naturally in many foods and the balance between oxidants and antioxidants decide ones' health and vigor. The outcome of the DPPH scavenging assay revealed that FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin were potent anti-oxidant agent.

The MMP enzymes are influential enzymes which were involved in many pathogenesis pathways. One of the many involvements of the MMPs in pathological disorder is the wound healing process. Overexpression of MMPs leads to prolong inflammations, angiogenesis and slow healing rate (Caley *et al.*, 2015). From the MMPs inhibition assay, FD leaf 50% (v/v) ethanol water extract was revealed to have potential in the wound healing process. Angiogenesis is a tightly regulated process of blood vessel formation from existing vascular and substantially influenced by the MMPs (Deryugina & Quigley, 2010). FD leaf 50% (v/v) ethanol water extract, vitexin and

isovitexin tested for cytotoxicity showed anti-proliferative activity with vitexin having the most potent activity with IC_{50}: 97.6 µg/ml (0.22 mM), followed by FD leaf extract (IC_{50}: 189 µg/ml), and isovitexin (IC_{50}: 221 µg/ml (0.55 mM)). FD leaf extract, vitexin and isovitexin were then tested on the cell migration assay and showed dose dependent effect on the inhibition of cell migration activity of EA.hy 926 cells. This chapter showed that FD leaf 50% (v/v) ethanol water extract with its compounds, vitexin and isovitexin inhibits the key aspects of angiogenesis, that is the proliferation of cells and migratory of cell through MMPs inhibition. Altogether, the findings obtained from the *in vitro* assay revealed that FD leaf 50% (v/v) ethanol water extract potential in the treatment of wound healing disorder due overexpression of MMPs.

The FD leaf 50% (v/v) ethanol water extract was subjected to acute toxicity evaluation on female SD rats. FD leaf 50% (v/v) ethanol water extract of 1000 mg/kg was administrated into the SD rats using oral gavage technique. The toxicity evaluation of FD leaf 50% (v/v) ethanol water extract revealed that the extract does not show any toxicity sign such as stomach discomfort, loss of appetite and weight. Another aspect of toxicologial index tested was the hematopoietic system, which are sensitive to introductions of toxic compounds that disrupted the pathophysiological aspects of the SD rats (Dekant & Vamvakas, 2004). The blood biochemical profile detected revealed the findings from the FD 50% (v/v) ethanol water extract group of having similar results with the control group and within the normal required range. Throughout the toxicology study, no treatment related changes in the hematopoietic system were observed, indicating that FD leaf 50% (v/v) ethanol water extract of 1000 mg/kg does not affect the hematopoiesis in the SD rats. In other words, the introductions of FD leaf 50% (v/v) ethanol water extract of 1000 mg/kg through oral gavage to the SD rats cause no interference on the blood biochemical parameters. The histology findings revealed that there was no sign of abnormalities in the kidney and liver tissue, indicating that the FD

leaf 50% (v/v) ethanol water extract of 1000 mg/kg does not cause any organ damaged to the SD rats throughout the toxicology evaluations. Should the extract be toxic, the SD rats will show signs of decrease body weight as this parameter are a simple yet sensitive index of toxicity (Kulkarni & Veeranjaneyulu, 2012). The selected dose showed no sign of body weight changes when compared to the control group, suggesting that the FD leaf extract does not obstruct the growth of the SD rats. Altogether, the finding through observation, blood parameters analysis and histology on the organ concluded that FD leaf 50% (v/v) ethanol water extract was safe when tested for acute toxicity.

Subsequently, validation of FD leaf 50% (v/v) ethanol water extract as wound healing agents were conducted on the diabetic and normal non-diabetic SD rats. *In vivo* study to validate the potency of FD leaf 50% (v/v) ethanol water extract as a wound healing agent are essential as it will experiment on living animals to observe the overall effects as well as confirming the findings obtained from the *in vitro* studies. Normal non-diabetic and diabetic SD rats were chosen for this study due to both having different pathophysiology. The non-diabetic and diabetic conditions represent the normal and abnormal physiology of wound healing process, respectively. Briefly, wound healing is a dynamic yet complex process that occurs when the trauma was introduced (Eming *et al.*, 2014). The optimal process of wound healing involved several distinct steps, 1: rapid homeostasis, 2: Inflammation, 3: differentiation, proliferation and migration of cell, 4: angiogengesis, 5: re-epithelization and 6: production of collagen (Guo & DiPietro, 2010). These steps will be activated in a precise and regulated manner. However, interruptions, aberrancies, or prolongation can lead to delayed wound healing or a non-healing chronic wound. There are multiple factors that impaired the wound healing process. These factors are categorized into local and systemic groups (Guo & DiPietro, 2010). The local groups are the factors influenced by the wounds while the systemic groups are the state of health and disease of the individual itself.

Diabetes is a well-known systemic disease that affected the wound healing process by interrupting the physiological homeostasis. Diabetes influenced hypoxia, leading to non-healing wound. Hypoxia is caused by insufficient perfusion and angiogenesis, which are detrimental for wound healing process. In this condition, hypoxia amplified inflammatory responses leading to prolong wound healing process that eventually increased the free radical level (Mathieu *et al.,* 2006; Woo *et al.,* 2007). Increased of free radical exceeding the anti-oxidant production contributes to hyperglycemia induced oxidative stress (Vincent *et al.,* 2004). In addition, diabetic condition also produced high levels of MMPs, which lead to tissue destruction, prolonged wound and inhibition of the normal wound repair mechanism (Woo *et al.,* 2007; Sibbald & Woo, 2008).

The findings from this study revealed that FD leaf 50% (v/v) ethanol water extract exerted a dose dependent effect on the wound closure rate for the normal non-diabetic and diabetic SD rats. The FD leaf 50 % (v/v) ethanol water extract of 100 mg/kg showed the fastest wound closure rate after 14 days for both normal non-diabetic and diabetic SD rats. It turns out that FD leaf 50 % (v/v) ethanol water extract was more potent in the diabetic SD rats compared to the normal non-diabetic SD rats. This could be explained due to the FD leaf 50% (v/v) ethanol water extract having a potent inhibitory effect on the MMPs as in the diabetic SD rats, the MMPs production are imbalanced. As elucidated from the *in vitro* study, FD leaf 50% (v/v) ethanol water extract as well as vitexin and isovitexin demonstrated impressive anti-oxidant and MMPs (MMP-2, MMP-8 and MMP-9) inhibitory properties. Therefore, the compelling effects of the FD leaf 50% (v/v) ethanol water extract on the diabetic wound healing process are due to the inhibition of excess free radicals and MMPs that are dominant in diabetic conditions. Abdulla *et al.,* (2010) reported that flavonoids in FD leaf involved in the wound healing process. Hence, in this study, vitexin and isovitexin were probably

the flavonoids responsible for assisting wound healing process as per described from the enzymatic (Chapter 5), *in vitro* and *in vivo* study findings (Chapter 6). The blood and biochemical profile revealed that the diabetic and normal non-diabetic SD rats are within normal required range, suggesting that there were no chronic infections and prolonged inflammation that could affect the wound healing process. The histology findings (liver and kidney) proved the absent of cell abnormalities, alterations of cell morphology or macrophage infiltration, confirming the results obtained from the blood biochemical analysis. The skin histology analysis revealed that the non-diabetic and diabetic SD rats of having similar conditions. The process of wound healing in diabetic and normal non-diabetic SD rats described that normal skin repair occurred, although the diabetic condition experiences different pathophysiology compared to the normal non-diabetic SD rats. This confirmed the therapeutic potential of FD leaf 50% (v/v) ethanol water extract inhibits the overexpressing MMPs that are dominant in diabetic state. The pancreas tissues were removed and analysed to confirm the hyperglycemia state of the diabetic SD rat models. The findings obtained from the histological analysis acknowledge the destruction of β-cell inside the Islet of Langerhans as seen in the diabetic model. For the non-diabetic group, the β-cell inside the Islet of Langerhans was shown to be in a normal state. STZ is a toxic chemical that affects the insulin producing β-cell of the pancreas. It is widely used to generate hyperglycemia in animal models. The findings from the pancreas histology analysis confirmed that the STZ- induced SD rats of the diabetic SD rat models were hyperglycemic due to the destruction of the β-cell inside the Islet of Langerhans.

Conclusively, FD leaf 50% (v/v) ethanol water extract as wound healing agent was confirmed using experimental approaches (*in vitro* and *in vivo* technique). This study described how FD leaf 50% (v/v) ethanol water extract demonstrated its therapeutic activity as a wound healing agent. Vitexin and isovitexin, the constituents of

FD leaf 50% (v/v) ethanol water extract was found to significantly produced the therapeutic activities of FD leaf 50% (v/v) ethanol water extract. Altogether, the FD leaf 50% (v/v) ethanol water extract inhibits the key aspects of wound healing disorder such as prolonged angiogenesis, excess free radicals and MMP enzymes.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION

7.1 Discussion and conclusion

Medicinal plant benefits assist researchers in exploring the potentials of herbal drugs to treat various diseases (Ekor, 2013). The main goals of herbal drug discoveries are to produce safe and efficacious drugs in various disease treatments (Atanas *et al.*, 2015). When the medicinal plant phytochemical constituents are standardized, it will become an excellent candidate for plant based drug discovery process. Even though there is an increase demand for plant-based medicines, the process of drug discovery is tedious, slow pace and exorbitant in cost (DiMasi *et al.*, 2015). Researchers nowadays are finding solutions to increase the value of medicinal plants at a faster rate with low expenditure. Computational techniques have been a choice for researchers to accelerate the drug discovery process. Through various methods of computational drug discovery approach such as structure prediction and docking methods helped researchers in establishing a valuable integrated pipeline for drug discovery process and readily shown great potential and success (Katsila *et al.*, 2016). The process of prediction and filtering molecular database are cheap and fast that tremendously assist researchers in selecting promising bioactive molecules (Katsila *et al.*, 2016). Eventually, the risk of wasting resources on possibly ineffective compounds could be eliminated by selecting only the molecules that showed promising results during the computational analysis followed by biological activity testing, thus saving a lot of time, expenditure and resources.

A combination of computational and experimental study was conducted on the FD leaf extract to unravel the therapeutic potentials that it contains. Initially, the standardization process of the FD leaf raw material was conducted (Chapter 4). Standardizing the medicinal plants against it phytochemistry compounds is important in order to produce a reliable extract with reproducible effects. The strategies to standardize medicinal plants involved the determination of the quality, safety and

efficacy as well as phytochemical analysis of the medicinal plant used. A well standardized medicinal plant is suitable to produce good quality nutraceutical, cosmeceutical and herbal products. In terms of quality, the FD leaf raw material demonstrated excellent results indicating that the handling process of pre-, post-harvesting and storage followed strictly the requirements set by the respective regulatory body. The safety assessment on the FD leaf was also conducted involving microbial limit test, test for presence of specific pathogens, aflatoxins and heavy metal analysis. Safety assessments play crucial roles in standardization of plant as it will determine whether the plant materials used are safe or not for herbal drug formulations. Safety assessment test guidelines such as the Malaysian herbal Monograph (Malaysian herbal Monograph, 2010), and British Pharmacopeia (British Pharmacopeia, 2008) were published by regulatory bodies for researchers to refer when assessing the quality and safety of the medicinal plant. The results obtained from the safety assessment (Chapter 4) revealed that the FD leaf used is safe without any contaminations from other sources. Subsequently, the FD leaf extracts were prepared in ethanol, water and 50% (v/v) ethanol water. The resultant FD leaf extracts were subjected to HPLC analysis for standardization based on two important chemical markers, vitexin and isovitexin. The chromatograms produced by the HPLC confirmed that the vitexin and isovitexin were among the major constituents in the extracts. The FD leaf 50% (v/v) ethanol water extract produced the highest concentration compared to the rest.

In Chapter 5, the FD leaf extract main compounds (vitexin and isovitexin) were investigated through enzymatic assay and computational approaches. The enzymatic assays were the α-amylase and MMPs inhibition assay. The α-amylase assay was chosen due to the nature of the FD leaf of having good activities as anti-diabetic agent. The MMP enzymes notably MMP-2, MMP-8 and MMP-9 were chosen due to its involvement in various diseases such as wound healing disorder, diabetic complications,

cancer and inflammatory disorders. For the enzymatic assay, the result from this study revealed that FD leaf 50% (v/v) ethanol water extract, vitexin and isovitexin were excellence inhibitors for the α-amylase and MMP enzymes. Convincingly, the compounds present in FD leaf (vitexin and isovitexin) exhibited potent activity against the α-amylase and MMP enzymes (MMP-2, MMP-8 and MMP-9). Afterwards, docking and molecular simulations were used to unravel the molecular behavior and interactions of both compounds (vitexin and isovitexin) towards the α-amylase and MMP enzymes (MMP-2, MMP-8 and MMP-9). Interactions between vitexin and isovitexin with the enzymes (α-amylase and MMP enzymes) were found to be strong. Both ligands occupied the active regions of the enzymes. Molecular dynamic simulations of both compounds and the enzymes showed formations of stable complex throughout the simulation process (20 ns). Comprehensively, the findings obtained through enzymatic assay and computational study described thoroughly the therapeutic properties of FD leaf 50% (v/v) ethanol water extract as α-amylase and MMP enzymes inhibitor.

In Chapter 6, evaluation of the FD leaf 50% (v/v) ethanol water extract activities were performed based on the docking and molecular findings as reported in Chapter 5. *In vitro* studies consisting of anti-oxidant and cell culture assays were conducted to validate the therapeutic activity of vitexin, isovitexin and FD leaf 50% (v/v) ethanol water extract. DPPH scavenging assay described the ability of a tested sample inhibiting the free radical, DPPH. The DPPH scavenging assay revealed that vitexin, isovitexin and FD 50% (v/v) ethanol water extract were potent anti-oxidant agents. The enzymatic assay (Chapter 5) and anti-oxidant assay revealed that FD 50% (v/v) ethanol water extract as well as vitexin and isovitexin are excellent candidate as therapeutic agent. Further evaluation of vitexin, isovitexin and FD leaf 50% (v/v) ethanol water extract as potential therapeutic agents were performed using cell culture techniques. The cytotoxicity of vitexin, isovitexin and FD leaf 50% (v/v) ethanol water extract was

evaluated using MTT assay. The MTT assay revealed the inhibitory activity of cell proliferation with vitexin exhibited the highest inhibitory activity. Additionally, cell migration assay was conducted for vitexin, isovitexin and FD leaf 50 % (v/v) ethanol water extract. This assay revealed dose dependent effects on the cell migration within 24 hour periods with vitexin exhibited the dominant migratory activity compared to FD leaf 50% (v/v) ethanol water extract and isovitexin. Results obtained from the biological assay on vitexin, isovitexin and FD leaf 50% (v/v) ethanol water extract provided valuable information regarding its potential as therapeutic agents. FD leaf 50% (v/v) ethanol water extract was shown to have exceptional inhibitory activities on DPPH, α-amylase and MMPs enzymes. Through cell culture techniques, FD leaf 50% (v/v) ethanol water extract was shown to have cytotoxic effects and produced dose dependent effect on the migratory property of EA.hy926 cells. Validation of FD leaf 50% (v/v) ethanol water extract therapeutic activity was conducted on wound healing activity using two different rat models, normal non-diabetic and diabetic female Sprague-Dawley (SD) rats.

Toxicity testing was initially conducted to assess the safety of the FD leaf 50% (v/v) ethanol water extract on female SD rats. Two different groups were established: control and test groups. The control and test groups were given tap water and extract, respectively. A dose of 1000 mg/kg was chosen for the toxicity testing following the protocol proposed by OECD guideline (OECD, 2002). Through oral gavage, the findings of this study revealed that the extract given was non-toxic to the rats. Observations throughout the period of the toxicity study showed that the test SD rats behave normally like the control group. There was no sign of toxicity such as frequent urine, weakening state and reduced or no eating activity was shown by the test SD rats. The body weight analysis showed that there was no significant loss of body weight throughout the toxicity study. Biochemical analyses of the blood of the SD rats were

also found similar to the control group with no significant differences were observed. Histology findings in the liver and kidney unfolds no cell abnormalities for both groups, indicating that there was no sign of organ toxicity produced by the FD leaf 50% (v/v) ethanol water extract. The findings obtained from the toxicology study indicated that FD leaf 50% (v/v) ethanol water extract at high dose (1000 mg/kg) displayed no sign of toxicity, suggesting that the extract was safe enough to be used on the SD rats.

FD leaf 50% (v/v) ethanol water extract evaluations on the wound healing activity were conducted on normal non-diabetic and diabetic SD rats (STZ-induced). For each model, 4 subgroups were created. The subgroups were the negative control group (treated with Tween 20), positive control group (treated with intrasite gel), and treatment groups (each treated with 100 and 200 mg/kg of FD leaf 50% (v/v) ethanol water extract, respectively). Wound induction was created at a uniform scale of 1.8 cm in the dorsal region of each SD rat using punch biopsy tools. The treatment period was 14 and on day 15, the SD rats for both models were sacrificed for blood and organ collection (kidney, liver, pancreas and skin). The full blood profile and biochemical analyses showed that the rats have no complications regarding the wound creation with no sign of prolonged inflammation and infection. For the histology analysis, the diabetic and non-diabetic groups showed similar histological findings (skin, liver and kidney) with no unusual sightings of cell abnormalities or WBC infiltrations. However, the pancreas tissue on the diabetic model showed destruction of the β-cell in the Islet of Langerhans indicating that the STZ induced hyperglycemia through destruction of pancreatic cells. The findings obtained from the histology study as well as the blood analyses justified that the introduction of FD leaf 50% (v/v) ethanol water extract on the treated rats caused no harm to the SD rat especially on the organs (skin liver and kidney). Altogether, the findings obtained from the in vivo study confirmed the

therapeutic properties of FD leaf 50% (v/v) ethanol water extract as a wound healing agent.

In conclusion, this study utilized both experimental and computational approaches to unravel the therapeutic potential of FD leaf. The results obtained through the experimental and computational approaches had successfully shown the biological activities of FD leaf 50% (v/v) ethanol water extract. Overall, the FD leaf 50% (v/v) ethanol water extract was found to be safe, non-toxic and a reliable extract in the treatment of wound healing either in non-diabetic or diabetic conditions.

7.2 Future study

In this study, the therapeutic potentials of FD leaf extract were discussed through computational and experimental techniques. However, there are some limitations occurred throughout this research. One of the limitations in this research was to pin-point which compounds contribute the most, vitexin or isovitexin when delivering the wound healing activities. Therefore, several future studies can be proposed to further understand the therapeutic potential of FD leaf extract from the overall findings of this research. Pharmacokinetic testing of the FD leaf extract and compounds could reveal the mechanism of action through adsorption, distribution, metabolism and excretion when delivering the therapeutic actions.

Besides pharmacokinetic, applying nanoparticles on the FD leaf extract should be an interesting project. Nanoparticles are widely regarded as the most efficient ways to deliver drugs. By adding nanoparticle technology on the FD leaf extract, the therapeutic potential of the plant could exponentially increase due to the advantage of having smaller size and increase adsorption properties.

Another suggested study is to investigate the wound ulcers in type II diabetic patient using foot. Foot contains lots of blood vessel. Therefore, it is prone towards

exogenous damage leading to amputation, necrosis and prolonging ulcers. By conducting this model, it could provide promising data to treat complication of diabetes in a more clinical ways.

REFERENCES

Abdu, B.A., Adamu, U., Sani S.M., & Joshua O.O. (2015). Physical and phytochemicals study of some local herbal remedies. *Journal of Pharmacy and Biological Sciences, 10*(4), 5-10.

Abdulla, M.A., Ahmed, K.A.-A., Abu-Luhoom, F.M., & Muhanid, M. (2010). Role of *Ficus deltoidea* extract in the enhancement of wound healing in experimental rats. *Biomedical Research, 21*, 241-245.

Abdullah, N., Karsani, S., & Aminudin, N. (2008). Effects of *Ficus deltoidea* Extract on the serum protein profile of simultaneously hypertensive rats (SHR). *Journal of Proteomics & Bioinformatics, 2*, 143-143.

Abdullah, Z., Hussain, K., Zhari, I., Rasadah, M.A., Mazura, P., Jamaludin, F., & Sahdan, R. (2009). Evaluation of extracts of leaf of three *Ficus deltoidea* varieties for antioxidant activities and secondary metabolites. *Pharmacognosy Research, 1*, 216.

Abdullah, Z., Hussain, K., Ismail, Z., & Ali, R.M. (2009). Anti-inflammatory activity of standardised extracts of leaves of three varieties of *Ficus deltoidea*. *International Journal of Pharmaceutical and Clinical Research, 1*, 100-105.

Abraham, M.J., Murtola T., Schulz R., Pall S., Smith J.C., Hess B., & Lindahl, E. (2015). GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX, 1*(2), 19-25

Adam, Z., Hamid, M., Ismail, A., & Khamis, S. (2009). Effect of *Ficus deltoidea* extracts on hepatic basal and insulin-stimulated glucose uptake. *Journal of Biological Sciences, 9*(8), 796–803.

Adam, Z., Hamid, M., Ismail, A., Khamis, S., & Marsidi, N. (2010). Antihyperglycemic and glucose tolerance activity of *Ficus deltoidea* ethanolic extract in diabetic rats. *Jurnal Sains Kesihatan Malaysia, 8*, 25-30.

Akhir, N.A.M., Chua L.S., Majid F.A.A., & Sarmidi, M.R. (2011). Cytotoxicity of aqueous and ethanolic extracts of *Ficus deltoidea* on human ovarian carcinoma cell line. *British Journal of Medicine and Medical Research, 1*(4), 397–409.

Anne, S. (2014). *Critical approaches to the history of western herbal medicine: From classical antiquity to the early modern period.* London: A&C Black.

Al-Bayaty, F., & Abdulla, M.A. (2012). A comparison of wound healing rate following treatment with aftamed and chlorine dioxide gels in streptozotocin-induced diabetic rats. *Evidence-Based Complementary and Alternative Medicine, 2012*, Article ID 468764.

Al-Salahi, O.S.A., Lam, C.K., Majid, A.M.S.A., Al-Suede, F.S.R., Saghir, S.A.M., Abdullah, W.Z., Ahamed, M.K. & Yusoff, N.M. (2013). Anti-angiogenic quassinoid-rich fraction from *Eurycoma longifolia* modulates endothelial cell function. *Microvascular Research, 90*, 30-39.

Arao, T., Ishikawa, S., Murakami, M., Abe, K., Maejima, Y., & Makino, T. (2010). Heavy metal contamination of agricultural soil and countermeasures in Japan. *Paddy and Water Environment, 8*(3), 247–257.

Atala, A., Irvine, D.J., Moses, M., & Shaunak, S. (2010). Wound healing versus regeneration: Role of the tissue environment in regenerative medicine. *MRS Bulletin / Materials Research Society, 35*(8), 10.

Avigan, M. I., Mozersky, R. P., & Seeff, L. B. (2016). Scientific and regulatory perspectives in herbal and dietary supplement associated hepatotoxicity in the United States. *International Journal of Molecular Sciences, 17*(3), 331.

Bakar, A.R.A., Manaharan, T., Merican A.F., & Mohamad, S. (2017). Experimental and computational approaches to reveal the potential of *Ficus deltoidea* leaves extract as α-amylase inhibitor. *Natural Product Research, 32*(4), 473-476.

Balbin, M., Fueyo, A., Knauper, V., Pendas, A.M., Lopez, J.M., Jimenez, M.G., Murphy, G. & Lopez-Otin, C. (1998). Collagenase 2 (MMP-8) expression in murine tissue-remodeling processes. Analysis of its potential role in postpartum involution of the uterus. *Journal of Biological Chemistry, 273*, 23959–23968.

Banihashemi, M., Safari, A., Nezafat, N., Tahamtan, M., Negahdaripour, M., Azarpira, N., & Ghasemi, Y. (2017). Effect of fibrin packing on managing hepatic hemorrhage and liver wound healing in a model of liver stab wound in rat. *Bulletin of Emergency & Trauma, 5*(1), 18–23.

Barabasi, A.-L., Gulbahce, N., & Loscalzo, J. (2011). Network medicine: A network-based approach to human disease. *Nature Review Genetics, 12*, 56–68.

Bauer, R. (1998). Quality criteria and standardization of phytopharmaceuticals: can acceptable drug standards be achieved? *Drug Information Journal, 32*, 101-110.

Berg, G., Rybakova, D., Grube, M., & Köberl, M. (2009). The plant microbiome explored: Implications for experimental botany. *Journal of Experimental Botany, 67*(4), 995–1002.

Billur, E.H., Gursoy, A., Nussinov, R., & Keskin, O. (2014). Network-based strategies can help mono-and poly-pharmacology drug discovery: A systems biology view. *Current Pharmaceutical Design*, 20, 1201–1207.

Boopathy, N.S., & Kathiresan, K. (2010). Anticancer drugs from marine flora: An overview. *Journal of Oncology, 18*, Article ID 214186.

British Pharmacopoeia Commission. (2008). *British pharmacopoeia*. London: Stationery Office.

Bunawan, H., Amin, N.M., Bunawan, S.N., Baharum, S.N. & Mohd Noor, N. (2014). *Ficus deltoidea* Jack: A review on its phytochemical and pharmacological importance. *Evidences Based Complementary Alternative Medicine*, 902734.

Caley, M.P., Martins, V.L.C. & O'Toole EA. (2015). Metalloproteinases and wound healing. *Advance in Wound Care, 4*(4), 225-234.

Chandel, H.S., Pathak, A.K., & Tailang, M. (2011). Standardization of some herbal antidiabetic drugs in polyherbal formulation. *Pharmacognosy Research, 3*(1), 49–56.

Chaudhury, R.R., & Rafei, U.M. (2001). *Traditional medicine in Asia, R.O.f.S.E.A. World Health Organization*. New Delhi: SEARO Regional Publications.

Chen, Y.P., & Chen, F. (2008). Identifying targets for drug discovery using Bioinformatics. *Expert Opinion on Therapeutic Targets, 12*, 383–389.

Cheng, T., Li, Q., Wang, Y., & Bryant, S.H. (2011). Identifying compound-target associations by combining bioactivity profile similarity search and public databases mining. *Journal of Chemical Information and Modeling, 51*, 2440–2448.

Choo, C.Y., Sulong, N.Y., Man, F., & Wong, T.W. (2012). Vitexin and isovitexin from the leaves of *Ficus deltoidea* with *invivo* a-glucosidase inhibition. *Journal of Ethnopharmacology, 142*(3), 776–781.

Choudhury, H., Gorain, B., Pandey, M., Chatterjee, L., Sengupta, P., Das, A., Molugulu, N., & Kesharwani P. (2017). Recent update on nanoemulgel as topical drug delivery system. *Journal of Pharmaceutical Science, 106*(7), 1736-1751.

Corner, E.J.H. (1969). The complex of *Ficus deltoidea*; a recent nvasion of the Sunda shelf. *Philosophical Transactions of the Royal Society B, 256*, 281–317.

Cuschieri, L., Debosz, J., Miiller, P., & Cellis, M. (2013). Autolytic debridement of a large, necrotic, fully occluded foot ulcer using a hydrocolloid dressing in a diabetic patient. *Advance in Skin Wound Care, 26*(7), 300-304.

Copstead, L-E.C., & Banasik J. (2012). *Pathophysiology*. London, United Kingdom: Elsevier Health Sciences.

Cui, K., Luo, X., & Murthy, M.R.V. (2004). Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 28*, 771–799.

Danielsen, P.L., Holst, A.V., Maltesen, H.R., Bassi, M.R.,. Holst, P.J., Heinemeier, K.M., … M.S. Agren. (2011). Matrix metalloproteinase-8 overexpression prevents proper tissue repair. *Surgery, 150*, 897–906.

Dekant, W. & Vamvakas, S. (2004). *Toxicology. Ullmann's encyclopedia of industrial chemistry*. New Jersey: Wiley.

Deng, Z.L. (2007). Application of new techniques in the innovative research of Chinese herbal medicine. *Chinese Pharmaceutical, 16*, 580–589.

Deryugina, E.I., & Quigley, J P. (2010). Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: Contrasting, overlapping and compensatory functions. *Biochimica et Biophysica Acta, 1803*(1), 103–120.

DeVivo, M., Masetti, M., Bottegoni, G., & Cavalli, A. (2016). Role of molecular dynamics and related methods in drug discovery. *Journal of Medicinal Chemistry, 59*(9), 4035-4061.

DiMasi, J.A., Grabowski, H.G., & Hansen, R.W. (2015). The cost of drug development. *New England Journal of Medicine, 372*(20), 1972.

Dumville, J.C., Deshpande, S., O'Meara, S., & Speak, K. (2013). Hydrocolloid dressings for healing diabetic foot ulcers. *The Cochrane Database of Systematic Reviews, 8*, CD009099.

Efferth, T., & Kaina, B. (2011). Toxicities by herbal medicines with emphasis to traditional chinese medicine. *Current Drug Metabolism, 12*(10), 989 – 996.

Ekor, M. (2013). The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology, 4*, 177.

Eming, S.A., Martin, P., & Tomic-Canic, M. (2014). Wound repair and regeneration: mechanisms, signaling, and translation. *Science Translational Medicine, 6*(265), 265sr6.

Evans, W.C. (1996). *Techniques in microscopy: quantitative microscopy. A text book of pharmacognosy.* (14th ed) London: WB Saunders Company Ltd.

Farsi, E., Shafaei, A., Yee Hor, S., Khadeer Ahamed, B. M., Yam, M. F., Attitalla, I. H., Asmawi, M. Z., & Ismail, Z. (2011). Correlation between enzymes inhibitory effects and antioxidant activities of standardized fractions of methanolic extract obtained from *Ficus deltoidea* leaves. *African Journal of Biotechnology, 10*(67), 15184–15194.

Fiorin, G., Klein, M.L., & Hénin, J. (2013). Using collective variables to drive molecular dynamics simulations. *Molecular Physics, 111*, 22-23.

Food and Drug Administration. (2012). *Bad bug book, foodborne pathogenic microorganisms and natural toxins, second edition*. Laurel, MD: Food and Drug Administration.

Food and Drug Administration. (2017). *Development approval process. Drug innovation.* Laurel, MD: Food and Drug Administration.

Fouad, A. (2017). The role of quality standards for biomanufacturing raw materials managing and prioritizing risk is essential to ensuring raw material quality. USP is developing new guidelines to make the work easier. *BioPharm International, 30*(8), 32–36.

Friedman, L.M., Furberg, C.D., DeMets, D., Reboussin, D.M., & Granger, C.B. (2015). *Fundamentals of clinical trials.* Basel, Switzerland: Springer International Publishing.

Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., & Sarniguet, A. (2011). Bacterial-fungal interactions: Hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiology and Molecular Biology Reviews, 75*(4), 583-609.

Garg. P., Sarma. D., Jeppsson. S., Patel. N.R., Gewirtz, A.T., Merlin, D. & Sitaraman, SV. (2010). Matrix metalloproteinase-9 functions as a tumor suppressor in colitis-associated cancer. *Cancer Research, 70*(2), 792-801.

Garg, V., Dhar, V.J., Sharma, A., & Dutt, R. (2012). Facts about standardization of herbal medicine: A review. *Chinese Journal of Integrative Medicine, 10*(10), 1077-1083.

Garg, P., & Sardana, S. (2016). Herbal drug standardization: An Overview. *European Journal of Biomedical and Pharmaceutical sciences, 3*(8), 2016.

Garnier, J.P. (2008). *Rebuilding the R&D Engine in Big Pharma*. Cambridge: Harvard Business Review.

Giangreco, I,, Nicolotti, O., Carotti, A., Carlo, F.D., Gargano, G. & Bellotti R. (2010). Analysis of x-ray structures of matrix metalloproteinases via chaotic map clustering. *BMC Bioinformatics, 11*(1), 1.

Giangreco, I., Lattanzi, G., Nicolotti, O., Catto, M. & Laghezza A. (2011). Insights into the complex formed by matrix metalloproteinase-2 and alloxan inhibitors: molecular dynamics simulations and free energy calculations. *PLoS ONE, 6*(10), e25597.

Gilbert, J., Henske, P., & Singh, A. (2003). Rebuilding big pharma's business model. *In vivo Business and Medicine Report, 21*, 10.

Goodwin, A.M. (2007). *In vitro* assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvascular Research, 74*(2-3), 172–183.

Gordon, SE., Weber, D.K., Downton, M.T., Wagner, J., & Perugini, M.A. (2016). Dynamic modelling reveals "hotspots" on the pathway to enzyme-substrate complex formation. de groot bl, ed. *PLoS Computational Biology, 12*(3), e1004811.

Goyal, S., & Kumar, S. (2011). Anti-anxiety activity studies of various extracts of *Pulsatilla nigricans* Stoerck. *International Journal of Pharmaceutical Sciences and Drug Research, 3*(2), 158-161.

Guo, S., & DiPietro, L.A. (2010). Factors affecting wound healing. *Journal of Dental Research, 89*(3), 219–229.

Han, Z.Z., Xu, H.D., Kim, K.H., Ahn, T.H., Bae, J.S., & Lee, J.Y. (2010). Reference data of the main physiological parameters in control Sprague-Dawley rats from pre-clinical toxicity studies. *Laboratory Animal Research, 26*(2),153-164.

Hansson, T., Oostenbrink, C., & van Gunsteren, W. (2002). Molecular dynamics simulations. *Current Opinion in Structural Biology, 12*, 190–196.

Hasham, R., Choi, H.K., Sarmidi, M.R., & Park, C.S. (2013). Protective effects of a *Ficus deltoidea* (Mas cotek) extract against UVB-induced photoageing in skin cells. *Biotechnology and Bioprocess Engineering, 18*(1), 185–193.

Hanwell, M.D., Curtis, D.E., Lonie, D.C., Vandermeersch, T., Zurek, E., & Hutchison, G.R. (2012). Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. *Journal of Cheminformatics, 4*(1), 17.

Hildreth, J., Hrabeta-Robinson, E., Applequist, W., Betz, J., & Miller, J. (2007). Standard operating procedure for the collection and preparation of voucher plant specimens for use in the nutraceutical industry. *Analytical and Bioanalytical Chemistry, 389*(1), 13-17.

Hess, T. (2011). Checklist for factors affecting wound healing. *Advances in Skin and Wound Care, 24*(4), 192.

Holderness, J., Schepetkin, I. A., Freedman, B., Kirpotina, L. N., Quinn, M. T., Hedges, J. F., & Jutila, M. A (2011). Polysaccharides isolated from açaí fruit induce innate immune responses. *PLoS ONE, 6*(2), 17301.

Hospital, A., Goñi, J.R., Orozco, M., & Gelpí, J. L. (2015). Molecular dynamics simulations: Advances and applications. *Advances and Applications in Bioinformatics and Chemistry : AABC, 8,* 37–47.

Ikenaka, Y., Yoshiji, H., Kuriyama, S., Yoshii, J., Noguchi, R., Tsujinoue, H., … Fukui, H. (2003). Tissue inhibitor of metalloproteinases-1 (TIMP-1) inhibits tumor growth and angiogenesis in the TIMP-1 transgenic mouse model. *International Journal of Cancer, 105*(3), 340–346.

Im, A.R., Kim, Y.H., Uddin, M.R., Lee, H.W., Chae. S.W., Kim, YH., … Lee, M.Y. (2012). *Scutellaria baicalensis* extracts and flavonoids protect rat L6 cells from antimycin a-induced mitochondrial dysfunction. *Evidence Based Complementary and Alternative Medicine,* Article ID 517965.

Inoue, W., Somay, G., Poole, S., & Luheshi, G.N. (2008). Immune-to-brain signaling and central prostaglandin E2 synthesis in fasted rats with altered lipopolysaccharide-induced fever. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 295*(1), 133-143.

Iskar, M., Zeller, G., Zhao, X.M., van Noort, V., & Bork, P. (2012). Drug discovery in the age of systems biology: the rise of computational approaches for data integration. *Current Opinion in Biotechnology, 23,* 609–616.

Jacobsen, B. J. (2008). "Predicting the Incidence of Mycotoxins," in proceeding of 26th annual international symposium on man and his environment in health and disease: molds & mycotoxins, hidden connections for chronic diseases, (Dallas, TX: American Environmental Health Foundation), 13–17.

Janoti, D.S., & Kumar, M. (2013). Pharmacognostic evaluation of leaves of *Meizotropis pellita* wall. Ex hook, f & grew. (Patwa). *International Journal of Pharmacognosy and Phytochemical Research, 5*(4), 282-284.

Jamal, P., Karim, I.A., Abdullah, E., Raus, R.A., & Hashim, Y.Z. (2011). Phytochemical screening for antibacterial activity of potential Malaysian medicinal plants. *African Journal of Biotechnology, 10*(81), 18795–18799.

Janeway, C.A., Jr., Travers, P., Walport, M., & Shlomchik, M.J. (2001). *Immunobiology: The Immune system in health and disease.* (5th Edition). New York: Garland Science

Jäger, A.K., & Saaby, L. (2011). Flavonoids and the CNS. *Molecules, 16*(2), 1471–1485.

Järup, L. (2003). Hazards of heavy metal contamination. *British Medical Bulletin, 68*(1), 167–182.

Kader, A.A., & Hussein, A. M. (2009). *Harvesting and postharvest handling of dates*. Aleppo: ICARDA.

Kalman, D.S., Schwartz, H.I., Feldman, S., & Krieger D.R. (2013). Efficacy and safety of *Elaeis guineensis* and *Ficus deltoidea* leaf extracts in adults with pre-diabetes. *Nutrition Journal, 12*(1), 36.

Karplus, M., & Kuriyan, J. (2005). Molecular dynamics and protein function. Proceedings of the National Academy of Sciences of the United States of America, *102*, 6679–6685.

Katara, P., Grover, A., Kuntal, H., & Sharma, V. (2011). *In silico* prediction of drug targets in *Vibrio cholerae*. *Protoplasma, 248*, 799–804.

Katsila, T., Spyroulias, G.A., Patrinos, G.P., & Matsoukas, M-T. (2016). Computational approaches in target identification and drug discovery. *Computational and Structural Biotechnology Journal, 14*, 177-184.

Kedare, S. B., & Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology, 48*(4), 412–422.

Kiernan, J.A. (2008). *Histological and histochemical methods: Theory and practice.* (4th ed). Bloxham, UK: Scion.

Klein, T. & Bischoff, R. (2011). Physiology and pathophysiology of matrix metalloproteases. Amino Acids, *41*(2), 271–290.

Kneifel, W., Czech, E., & Kopp, B. (2002). Microbial contamination of medical plants - A review. *Planta Medica, 68*, 5-15.

Kokate, C., Purohit, A., & Gokhale, S. (1994). *Text book of pharmacognosy*. (13th ed). Pune: Nirali Prakashan.

Krampert, M., Bloch, W., Sasaki T., Bugnon, P., Rulicke, T., Wolfe, E., … Werner, S. (2004). Activities of the matrix metalloproteinase stromelysin-2 (MMP-10) in matrix degradation and keratinocyte organization in wounded skin. *Molecular Biology of the Cell, 15*, 5242–5254.

Kumar, V., Abbas, A.K., Fausto, N., Mitchell, R., & Sheppard, R. (2007) *Basic Pathology*. Philadelphia: Saunders.

Kulkarni, Y.A., & Veeranjaneyulu, A. (2012). Toxicological evaluation of the methanol extract of *Gmelina arborea roxb*. bark in mice and rats. *Toxicology International, 19*(2), 125–131.

Kumar, A., Voet, A., & Zhang, K.Y.J. (2012). Fragment based drug design: From experimental to computational approaches. *Current Medicinal Chemistry, 19*, 5128–5147.

Kumar, T. (2013). Standardization of herbal drugs – A review. *International Journal of Pharma and Bio Sciences, 2*, 13.

Kumar, P., Mahato, D. K., Kamle, M., Mohanta, T. K., & Kang, S. G. (2016). Aflatoxins: A global concern for food safety, human health and their management. *Frontiers in Microbiology, 7*, 2170.

Kunle, O.F., Egharevba, H.O., & Ahmadu, P.O. (2013). Standardization of herbal medicines: A review. *International Journal of Biodiversity and Conservation, 4*(3), 101-112.

Kurz, H. (1999). Selection of cultivars to reduce the concentration of cadmium and thallium in food and fodder plants. *Journal of Plant Nutrition and Soil Science, 162*(3), 323–328,

Li, S., Tan, H.-Y., Wang, N., Zhang, Z.-J., Lao, L., Wong, C.-W., & Feng, Y. (2015). The role of oxidative stress and antioxidants in liver diseases. *International Journal of Molecular Sciences, 16*(11), 26087–26124.

Liang, C.C., Park, A.Y. & Guan J.L. (2007). *In vitro* scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocol, 2*, 329.

Liao, C., Sitzmann, M., Pugliese, A., & Nicklaus, M. C. (2011). Software and resources for computational medicinal chemistry. *Future Medicinal Chemistry, 3*(8), 1057–1085.

Ling, A., Ong, S., Poospooragi, R., & Moosa, S. (2011). Production of flavonoid compounds in cell cultures of *Ficus deltoidea* as influenced by medium composition. *International Journal of Medicinal and Aromatic Plants, 1*, 62-74.

Liu, W., Schmidt, B., Voss, G., & Müller-Wittig, W. (2008). Accelerating molecular dynamics simulations using graphics processing units with CUDA. *Computer Physics Communications, 179*, 634–641.

Malaysian Herbal Monograph Volume 2. (2010). *Malaysian herbal monograph technical committee malaysian herbal monograph technical committee*. Kuala Lumpur: Forest Research Institute Malaysia.

Maiorov, V.N., & Crippen, G.M. (1995). Size-independent comparison of protein three-dimensional structures. *Proteins: Structure, Function, and Genetics, 22*(3), 273-283.

Manaharan, T., Palanisamy, U.D., & Ming, C.H. (2012). Tropical plant extracts as potential antihyperglycemic agents. *Molecules, 17,* 5915-5923.

Markiewski, M. M., & Lambris, J. D. (2007). The role of complement in inflammatory diseases from behind the scenes into the spotlight. *The American Journal of Pathology*, *171*(3), 715–727.

Mathieu, D., Linke, J-C., & Wattel, F. (2006). Non-healing wounds. *In: Handbook on hyperbaric medicine.* Netherlands: Springer.

Mazumder, G. (2008). Chronic arsenic toxicity & human health. *Indian Journal of Medical Research*, *128*(4), 436–447.

McCarty, S.M., & Percival, S.L. (2013). Proteases and delayed wound healing. *Advances in Wound Care, 2*(8), 438-447.

Misbah, H., Aziz A.A., & Aminudin N. (2013). Antidiabetic and antioxidant properties of *Ficus deltoidea* fruit extracts and fractions. *BMC Complementary and Alternative Medicine, 13,* 118.

Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., & Belew, R.K. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Compututational Chemitry, 30,* 2785–2791.

Moustakas, D., Lang, P.T., Pegg, S., Pettersen, E., & Kuntz, I. (2006). Development and validation of a modular, extensible docking program: DOCK 5. *Journal of Computer-Aided Molecular Design, 20,* 601–619.

Mulholland, B., Tuft, S.J., & Khaw, P.T. (2005). Matrix metalloproteinase distribution during early corneal wound healing. *Eye, 19,* 584–588.

Mutt, S., & Sowdhamini, R. (2016). Molecular dynamics simulations and structural analysis to decipher functional impact of a twenty residue insert in the ternary complex of mus musculus tdt isoform. *PLoS ONE, 11*(6), e0157286.

Nagase, H., Visse, R., & Murphy, G. (2006). Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Research, 69*(3), 562-573.

Norfleet, E., & Gad, S.C. (2009). *Phase I clinical trials: Shayne Cox Gad, clinical trials handbook.* New Jersey: Wiley.

Nwomeh, B.C., Liang, H.X., Cohen, I.K., & Yager, D.R. (1999). MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers. *Journal of Surgical Research, 81,* 189–195.

Nguyen, M. Arkell, J., & Jackson, C.J. (2000). Activated protein C directly activates human endothelial gelatinase A. *Journal of Biological Chemistry, 275,* 9095–9098.

Nguyen, T.T., Mobashery, S., & Chang, M. (2016). *Roles of matrix metalloproteinases in cutaneous wound healing, wound healing - New insights into ancient Challenges*. Vienna: InTech.

Organisation for economic co-operation and development. (2002). *Test No. 420: Acute oral toxicity. Fixed dose procedure-section 4.* Paris: OECD Publishing.

Omar, M.H., Mullen, W., & Crozier, A. (2011). Identification of proanthocyanidin dimers and trimers, flavone C-glycosides, and antioxidants in *Ficus deltoidea*, a malaysian herbal tea. *Journal of Agricultural and Food Chemistry, 59*(4), 1363–1369.

Ong, H.C., Zuki, R.M., & Milow, P. (2011). Traditional knowledge of medicinal plants among the Malay villagers in Kampung Mak Kemas, Terengganu, Malaysia. *Ethno Medicine, 5*, 175-185.

Ortega, S.S., Cara, L.C., & Salvador, M.K. (2012). *In silico* pharmacology for a multidisciplinary drug discovery process. *Drug Metabolism and Drug Interactions, 27*, 199–207

Page, L.R. (2001). Whole herbs or standardized plant constituents? *Total Health*, *23*(4), 20.

Pan, S.Y., S. H., Gao, S.F., Zhou, Tang, M.k., Yu, Z.L., & Ko, K.M. (2012). New perspectives on complementary and alternative medicine: an overview and alternative therapy. *Alternative Therapies in Health and Medicine, 18*(4), 20–36.

Pan, S.Y., Zhou, S.F., Gao, S.H., Yu, *Z.L.*, Zhang, S.F., Tang, M.K., … Ko, K.M. (2013). New perspectives on how to discover drugs from herbal medicines: cam's outstanding contribution to modern therapeutics. *Evidence-Based Complementary and Alternative Medicine*, Article ID 627375, 25

Pan, S.Y., Litscher, G., Gao, S.H, Zhou, S.F., Yu, Z.L., Chen, H.Q., … Ko, K.M. (2014). Historical perspective of traditional indigenous medical practices: the current renaissance and conservation of herbal resources. *Evidence-Based Complementary and Alternative Medicine*, Article ID 525340, 20.

Patwekar, S.L., Suryawanshi, A.B., Gaikwad, M.S., Pedewad, S.R., & Potulwar, A.P. (2015). Standardization of herbal drugs: An overview. *Journal of Pharmaceutical Innovation, 4*, 100-104

Pirila, E., Korpi, J.T., Korkiamaki, T., Jahkola, T., Gutierrez-Fernandez, A., Lopez-Otin, C., … Sorsa T. (2007). Collagenase-2 (MMP-8) and matrilysin-2 (MMP-26) expression in human wounds of different etiologies. *Wound Repair Regeneration, 15*, 47–57.

Pedretti, A., Villa, L., & Vistoli, G. (2004). VEGA - An open platform to develop chemo-bioinformatics applications, using plug-in architecture and script" programming. *Journal of Computer-Aided Molecular Design, 18*, 167–173.

Petterino, C., & Argentino-Storino, A. (2005). Clinical chemistry and haematology historical data in control Sprague-Dawley rats from pre-clinical toxicity studies. *Experimental and Toxicologic Pathology, 57*(3), 213-219.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., & Ferrin, T.E. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of Computational Chemistry, 25*(13), 1605-1612.

Prabhakar, M., Bano, H., Kumar, I., Shamsi, M., & Khan, S. (2007). Pharmacological investigations on vitexin. *Planta Medica, 43*, 396-403.

Pronk, S., Pall S., Schulz, R., Larsson, P., Bjelkmar, P., Apostolov, L., … van der Spoel, D. (2013). GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics, 29*(7), 845–854.

Ragone, M.I., Sella, M., Conforti, P., Volonté, M.G., & Consolini, A.E. (2007). The spasmolytic effect of *Aloysia citriodora* Palau (South American cedrón) is partially due to its vitexin but not isovitexin on rat duodenums. *Journal of Ethnopharmacology, 113*, 258-266.

Ramberg, J.E., Nelson, E.D., & Sinnott, R.A. (2010). Immunomodulatory dietary polysaccharides: A systematic review of the literature. *Nutrition Journal, 9*, 54.

Ratti, E., & Trist, D. (2001). Continuing evolution of the drug discovery process. *Pure Applied Chemistry, 73*, 67–75.

Rao, Y & Xiang, B. (2009). Determination of total ash and acid-insoluble ash of Chinese herbal medicine *Prunellae spica* by near infrared spectroscopy. *YAKUGAKU ZASSHI, 129*(7), 881-886.

Razali, N., Dewa, A., Asmawi, M.Z., Ismail, Z., Manshor, N.M., & Hassan, Z. (2013). Vascular reactivity on aortic rings of spontaneously hypertensive rats treated with methanolic and water extracts of *Ficus deltoidea. Journal of Experimental and Integrative Medicine, 3*, 93-102.

Rodak, B.F. (2007). *Hematology: clinical principles and applications* (3rd ed.). Philadelphia: Saunders.

Rolfe, K.J., Richardson, J., Vigor, C., Irvine, L.M., Grobbelaar, A.O., & Linge, C. (2007). A role for TGF-beta1-induced cellular responses during wound healing of the non-scarring early human fetus? *Journal of Investigative Dermatology, 127*, 2656–2667.

Roy, R. (2010). Integrative medicine to tackle the problem of chronic diseases. *Journal of Ayurveda and Integrative Medicine, 1*(1), 18-21.

Seo, D.W., Li, H. & Guedez, L. (2003). TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. *Cell, 114*(2), 171–180.

Samah, O. A., Zaidi, N.T.A, & Sule, A.B. (2012). Antimicrobial activity of *Ficus deltoidea* Jack (Mas Cotek). *Pakistan Journal of Pharmaceutical Sciences, 25*(3), 675–678.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M., & Yoga, L. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary, and Alternative Medicines, 8*(1), 1–10.

Shan, Y., Kim, E.T., Eastwood, M.P., Dror, R.O., & Seeliger M.A. (2011). How does a drug molecule find its target binding site? *Journal of the American Chemical Society, 133*, 9181–9183.

Sharma, O.P, & Bhat, TK. (2009). DPPH antioxidant assay revisited. *Food Chemistry, 113*, 1202–1205.

Sharma, S., Gupta, M., & Bhadauria, R. (2014). Mycobiota of commercially available triphala powder: A well known dietary supplement of indian system of medicine. *Journal of Mycology, 8*, 836036.

Shivatare, R.S., Pande, A.S., Bhusnar, H.U., Kadam, P.V., Yadav, K.N., Patil, M.J. (2013). Physicochemical and phytochemical standardization of fruit of *Sesbania grandiflora*. *Asian Journal of Biomedical and Pharmaceutical Sciences, 3*(23), 23-27.

Sibbald, R.G., & Woo, K.Y. (2008). The biology of chronic foot ulcers in persons with diabetes. *Diabetes/Metabolism Research and Reviews, 24*(1), 25-33.

Solan, P.D., Dunsmore, K.E., Denenberg, A.G., Odoms, K., Zingarelli, B. & Wong, H.R. (2012). A novel role for matrix metalloproteinase-8 in sepsis. *Critical Care Medicine, 40*(2), 379-387.

Somboonwong, J., Kankaisre, M., Tantisira, B., & Tantisira, M.H. (2012). Wound healing activities of different extracts of *Centella asiatica* in incision and burn wound models: An experimental animal study. *BMC Complementary and Alternative Medicine, 12*, 103.

Song, C.M., Lim, S.J., & Tong, J.C. (2009). Recent advances in computer aided drug design. *Brief Bioinformatics, 10*, 579–591.

Sternlicht, M.D., & Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. *Annual Review of Cell and Developmental Biology, 17*, 463–516.

Sulaiman, M.R., Hussain, M.K., Zakaria, Z., Somchit, M., Moin, S., Mohamad, A., & Israf, D., (2008). Evaluation of the antinociceptive activity of *Ficus deltoidea* aqueous extract. *Fitoterapia, 79*, 557-561.

Suryati, S., Nurdin, H., Dachriyanus, D., & Lajis, M.N.H. (2011). Structure elucidation of antibaterial compound from *Ficus deltoidea* Jack leaves. *Indonesian Journal of Chemistry, 11*, 67-70.

Swope, W.C, Pitera, J.W., Suits, F., Pitman, M., Eleftheriou, M., Fitch, B.G., … Zhou, R. (2004). Describing protein folding kinetics by molecular dynamics simulations. 2. Example applications to alanine dipeptide and a β-hairpin peptide. *Journal of Physical Chemistry B, 108*(21), 6582–6594.

Taylor, D. (2015). *The pharmaceutical industry and the future of drug development.* London: Royal Society of Chemistry.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., & Sutton D.J. (2012). *Heavy metal toxicity and the environment*. Basel: Experientia Supplementum.

Editors. (2009). Phase 0 trials: a platform for drug development? *The Lancet, 374*, 176.

Umscheid, C.A., Margolis, D.J., Grossman, C.E. (2011). Key concepts of clinical trials: a narrative review. *Postgraduate Medicine, 123*(5), 194-204.

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., & Venkitanarayanan, K. (2014). Combating pathogenic microorganisms using plant-derived antimicrobials: A minireview of the mechanistic basis. *BioMed Research International*, Article ID 761741.

U.S. Department of Agriculture Grain Inspection. (2009). *Packers and stockyards administration. Aflatoxin handbook.* Washington, DC: Federal Grain Inspection Service.

Uyub, A.M., Nwachukwu, I.N., Azlan, A.A., & Fariza, S.S. (2010). *Invitro* antibacterial activity and cytotoxicity of selected medicinal plant extracts from Penang island Malaysia on metronidazole-resistant *Helicobacter pylori* and some pathogenic bacteria. *Ethnobotany Research and Applications, 8*, 95–106.

Trott, O., Olson, A.J. (2010). AutoDock vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Compututational Chemitry, 31*, 455–461.

Vaikosen, E.N., & Alade, G.O. (2011). Evaluation of pharmacognostical parameters and heavy metals in some locally manufactured herbal drugs. *Journal of Chemical and Pharmaceutical Research, 3*(2), 88-97.

Verma, R.P., & Hansch, C. (2007). Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. *Bioorganic & Medicinal Chemistry, 15*(6), 2223–2268.

Vincent, A.M., Russell, J.W., Low, P., & Feldman, E.L. (2004). Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocrine Reviews, 25*, 612-628.

Wachtel-Galor, S., & Benzie, I.F.F. (2011). *Herbal medicine: Biomolecular and clinical aspects.* (2nd Edition). Boca Raton (FL): CRC Press/Taylor & Francis.

Wang, W., Donini, O., Reyes, C.M., & Kollman, P.A. (2001). Biomolecular simulations: recent developments in force fields, simulations of enzyme catalysis, protein- ligand, protein-protein, and protein-nucleic acid noncovalent interactions. *Annual Review of Biophysics and Biomolecular Structure, 30*, 211–243.

Ward, C. (2014). Glycogenolysis and glycogenesis. *Diapedia*, *4*, 11-13.

Wei, L.S., Wee, W., Siong, J.Y.F., & Syamsumir, D.F. (2011). Characterization of antioxidant, antimicrobial, anticancer property and chemical composition of *Ficus deltoidea* Jack leaf extract. *Journal of Biologically Active products from Nature, 1*, 1-6.

Willard, T. (1996). *Edible and medicinal plants of the Rocky mountains.* Calgary: Wild Rose College of Natural Healing.

Whittaker, P. (2003). What is the relevance of bioinformatics to pharmacology? *Trends in Pharmacological Sciences, 24*, 434–439.

Woo, K., Ayello, E.A., & Sibbald, R.G. (2007). The edge effect: current therapeutic options to advance the wound edge. *Advance in Skin Wound Care, 20*, 99-117.

WHO. (1993). *Research guidelines for evaluating the safety and efficacy of herbal medicines.* Manila: WHO.

WHO. (1996). *Annex II. Guidelines for the assessment of herbal medicines (WHO technical report series No. 863).* Geneva:WHO.

WHO. (1999). *WHO monographs on selected medicinal plants. Vol. 1*. Geneva: WHO.

WHO. (2002). *The importance of pharmacovigilance - safety monitoring of medicinal products.* Geneva: WHO.

WHO. (2003). *Guidelines on good agricultural and collection practices (GACP) for medical plants.* Geneva: WHO.

WHO. (2004). *WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems.* Geneva: WHO.

WHO. (2005). *National policy on traditional medicine and regulation of herbal medicines. Report of a World Health Organization global survey.* Geneva, Switzerland: WHO.

WHO. (2011). *World Health Organization (WHO) Quality control methods for herbal materials World Health Organization.* Geneva: WHO.

WHO. (2016). *Guidelines on good herbal processing practices (GHPP) for herbal medicines.* Geneva: WHO.

Xiang, D.R., & Sheng, J.F. (2009). The analyse of effectiveness in HBeAg-positive chronic viral hepatitis B treated by adefovir dipivoxil combined with bicyclol. *Chinese Journal of Experimental and Clinical Virology, 23*(4), 299–301.

Xu, Z., Chen, X., Zhong, Z., Chen, L., & Wang, Y. (2011). *Ganoderma lucidum* polysaccharides: immunomodulation and potential anti-tumor activities. *The American Journal of Chinese Medicine, 39*(1), 15–27.

Yamanishi, Y., Kotera, M., Kanehisa, M., & Goto, S. (2010). Drug-target interaction prediction from chemical, genomic and pharmacological data in an integrated framework. *Bioinformatics, 26*, 246–254.

Yuriev, E., Agostino, M., & Ramsland, P.A. (2011). Challenges and advances in computational docking: 2009 in review. *Journal of Molecular Recognition, 24*, 149–164.

Zahra, M.A.S.F., Mahmood, A.A., Hapipah, M.A., Suzita, M.N., & Salmah, I. (2009). Anti-ulcerogenic activity of aqueous extract of *Ficus deltoidea* against ethanol-induced gastric mucosal injury in rats. *Research Journal of Medical Sciences, 3*(2), 42–46.

Zakaria, Z.A., Hussain, M.K., Mohamad, A.S., Abdullah, F.C., & Sulaiman, M.R. (2012). Anti-inflammatory activity of the aqueous extract of *Ficus deltoidea*. *Biological Research for Nursing, 14*(1), 90–97.

Zacharias, J., & Knapp, E.W. (2014). Protein secondary structure classification revisited: Processing DSSP information with PSSC. *Journal of Chemical Information and Modeling, 54*(7), 2166-2179

Zhang, S. & Satyanarayanajois, S.D. (2011). *Drug design and discovery*. New York: Humana Press.

Zhuang, P., Lu, H., Li, Z., Zou, B., & McBride, M. B. (2014). Multiple exposure and effects assessment of heavy metals in the population near mining area in south China. *PLoS ONE, 9*(4), e94484.

LIST OF PUBLICATIONS AND SEMINAR PRESENTED

Manuscript published

1. **Bakar, A.R.A.,** Manaharan, T., Merican A.F., & Mohamad, S. (2017). Experimental and computational approaches to reveal the potential of *Ficus deltoidea* leaves extract as α-amylase inhibitor. *Natural Product Research, 32*(4), 473-476.

2. **Bakar, A.R.A.,** Ripen A.M., Merican A.F., & Mohamad, S. (2018). Enzymatic inhibitory activity of *Ficus deltoidea* leaf extract on matrix metalloproteinase-2, 8 And 9. Doi: 10.1080/14786419.2018.1434631.