



UNIVERSITI PUTRA MALAYSIA

**PRECLINICAL EVALUATION OF *ANDROGRAPHIS PANICULATA*
(BURM. F.) WALL. EX NEEES AS HERBAL MEDICINE FOR TREATMENT
OF BREAST AND PROSTATE CANCER**

AUDREY YONG CHEE HUI

FPSK(p) 2011 19

**PRECLINICAL EVALUATION OF
ANDROGRAPHIS PANICULATA (BURM. F.)
WALL. EX NEEES AS HERBAL MEDICINE FOR
TREATMENT OF BREAST AND PROSTATE
CANCER**

The logo of Universiti Putra Malaysia (UPM) is a shield-shaped emblem. It features a red and white stylized 'Y' shape in the center, with a book icon above it. The letters 'UPM' are written in white on a red background in the top left corner of the shield.

AUDREY YONG CHEE HUI

**DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA**

2011

IMPORTANT

The following manuscript "Preclinical Evaluation of *Andrographis paniculata* as an Herbal Medicine for the Treatment of Breast and Prostate Cancers" is submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the requirements for the degree of Doctor of Philosophy by Audrey Yong Chee Hui. This manuscript can only be used for personal viewing and no part of this manuscript may be reprinted, linked to, or otherwise redistributed, in any form or by any means, without first obtaining the prior written consent of the author.

If you wish to reprint or reproduce this work, or if you have any enquiries, please email Audrey Yong Chee Hui (audreyyong@yahoo.com)



**PRECLINICAL EVALUATION OF *ANDROGRAPHIS PANICULATA*
(BURM. F.) WALL. EX NEES AS HERBAL MEDICINE FOR TREATMENT
OF BREAST AND PROSTATE CANCER**

By

AUDREY YONG CHEE HUI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

August 2011

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment
of the requirement for the degree of Doctor of Philosophy

**PRECLINICAL EVALUATION OF *ANDROGRAPHIS PANICULATA*
(BURM. F.) WALL. EX NEES AS HERBAL MEDICINE FOR TREATMENT
OF BREAST AND PROSTATE CANCER**

By

AUDREY YONG CHEE HUI

August 2011

Chairman : Associate Professor Johnson Stanslas, PhD

Faculty : Medicine and Health Sciences

Most Malaysians use some forms of herbal remedy to manage their diseases and ailments. Unfortunately, most of the time, the science behind the perceived benefits of this alternative therapy has not grown in proportion to its popular usage. The Malaysian National Pharmaceutical Control Bureau (NPCB) requires all herbal medicines to meet acceptable standards for quality, safety and efficacy for the purpose of product registration. Quality control in the phytopharmaceutical products is a multistep process that covers all stages from 'seed to pill'. It must be correctly authenticated, assessed for relative free from contamination and assayed for active

principles. In this study, *Andrographis paniculata* (Burm. F.) Nees (AP) or locally known as Hempedu bumi was evaluated preclinically for its quality, safety and efficacy towards development into a herbal medicine for the treatment of cancer. It is also a promising herb for the treatment of various diseases. There are three main diterpenoid lactones (DL) found in the aerial parts of AP, namely andrographolide (AGP, cytotoxic), 14-deoxy-11, 12-didehydroandrographolide (DDAG, non-cytotoxic) and neoandrographolide (NAG, non-cytotoxic). These three DL were used as marker compounds in assessing the quality of the plant materials and for subsequent *in vitro* and *in vivo* pharmacological studies. The stability and quality of dried AP material and extracts were evaluated in various storage temperatures ($4 \pm 2^\circ\text{C}$, $25 \pm 2^\circ\text{C}$ and $35 \pm 2^\circ\text{C}$) based on of the content of the marker compounds by high performance liquid chromatography (HPLC). In a preliminary study to select the best extraction solvent (ethanol, ethanol: water (1:1), dichloromethane and water), these dried extracts were tested for their cytotoxicity against cancer cell lines using the MTT cell proliferation assay. Dichloromethane extract was found to be most active. Dichloromethane extract was then standardised to contain 15-20% w/w of AGP, 1-5%w/w of DDAG and 1-3% w/w of NAG of the extract weight. This extract was used throughout the *in vitro* and *in vivo* studies. It was found that the extract had improved cytotoxicity when the amount of AGP is equivalent to the pure AGP. This prompted an investigation of whether the DLs have combination cytotoxic effect in cancer cell lines. AGP-NAG and AGP-DDAG combinations produced profound synergistic cytotoxic effect in MCF-7 (breast) and DU 145 (prostate) cancer cells. The acute toxicity of this extract was determined in mice and rats, with escalating dosages from 100 mg/kg to 2 g/kg by oral administration, and no reduction of weight in the animals was observed. The plasma DL levels were determined in mice and rats

administered with AP extract at the doses of 250 mg/kg. The 250 mg/kg and 500 mg/kg doses were applied in the therapeutic studies using nude mice xenografted with breast and prostate tumours. The results from the stability study showed that the suitable storage temperature for dry AP material was at 35°C up to 12 months to maintain content of AGP \geq 70% of the initial content, and for the crude ethanol extract was at 35°C for up to 6 months. For pharmacokinetic study in rodents (BALB/c, NU/Nu mice and rats), when 250 mg/kg dose of AP extract was administered orally, the maximum plasma concentrations (C_{max}) of DLs were found to be in the following ranges: AGP (2.1 – 5.3 μ M), NAG (2.3 – 6 μ M) and DDAG (3.1 – 4.7 μ M). Preclinical therapeutic study revealed antitumor activity of AP extract against DU 145 prostate but not against MDA-MB-231 breast tumour xenografts. It is highly suggestive that the synergistic cytotoxic effect of DLs may have contributed to the *in vivo* antitumour activity of AP extract. This study also established suitable storage conditions for AP dry materials to preserve the quality in order to obtain reliable and consistent amounts of the DLs in extracts for *in vitro* and *in vivo* studies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan untuk ijazah Doktor Falsafah

**PENILAIAN PRAKLINIKAL *ANDROGRAPHIS PANICULATA* (BURM. F.)
WALL. EX NEES SEBAGAI UBATAN HERBA UNTUK RAWATAN
KANSER PAYUDARA DAN PROSTAT**

Oleh

AUDREY YONG CHEE HUI

Ogos 2011

Pengerusi : Profesor Madya Johnson Stanslas, PhD

Fakulti : Perubatan dan Sains Kesihatan

Kebanyakan rakyat Malaysia menggunakan pelbagai ubatan herba untuk menguruskan penyakit dan kesihatan mereka. Malangnya, sebahagian besar ilmu di sebalik manfaat yang dirasakan daripada terapi alternatif sumber tumbuhan in belum berkembang setanding dengan penggunaan popularnya. Biro Pengawasan Farmaseutikal Kebangsaan Malaysia (BPFK) menghendaki semua ubatan herba untuk mencapai tahap kepiawaian kualiti, keselamatan dan efikasi (keberkesanan) untuk tujuan pendaftaran produk. Kawalan kualiti dalam penghasilan produk fitofarmaseutikal adalah proses yang merangkumi semua peringkat pengembangan dari 'benih sampai pil'. Ubatan herba harus disahkan tulen dan benar, bebas daripada pencemaran dan diuji mengandungi prinsip-prinsip yang aktif. Dalam kajian ini, *Andrographis paniculata* (Burm. F.) Nees (AP) atau lebih dikenali sebagai Hempedu bumi dinilai secara praklinikal dari aspek kualiti, keselamatan dan efikasi (keberkesanan) terhadap pengembangan menjadi ubatan herba untuk rawatan kanser.

Tumbuhan herba ini adalah ramuan yang menjanjikan rawatan pelbagai penyakit. Terdapat tiga lakton diterpenoid (DL) utama yang diasingkan dari bahagian hijau, di atas tanah (batang and daun) AP, iaitu andrografolid (AGP, sitotoksik), 14-deoksi-11, 12-didehydroandrographolide (DDAG, tidak sitotoksik) dan neoandrographolide (NAG, tidak sitotoksik). Ketiga-tiga DL digunakan sebagai sebatian penunjuk dalam penilaian kualiti bahan mentah tumbuhan AP kering dan untuk selanjutnya, dalam kajian farmakologi pra-klinikal *in vitro* dan *in vivo*. Kestabilan dan kualiti bahan mentah tumbuhan kontang dan ekstrak AP dinilai dalam pelbagai suhu simpanan ($4 \pm 2^\circ\text{C}$, $25 \pm 2^\circ\text{C}$ dan $35 \pm 2^\circ\text{C}$) dengan penentuan kandungan sebatian penanda dengan kromatografi cecair prestasi tinggi (HPLC). Dalam satu kajian pendahuluan untuk memilih pelarut ekstraksi terbaik (etanol, etanol: air (1:1), diklorometana dan air), ekstrak kontang diuji kesitotoksikan terhadap sel-sel kanser dengan menggunakan asai proliferasi sel MTT. Ekstrak diklorometana didapati paling aktif. Dalam kajian ini, ekstrak piawai diklorometana AP mengandungi AGP dalam julat 15-20%, DDAG dalam julat 1-5% dan 1-3% adalah NAG. Ekstrak ini digunakan dalam kajian farmakokinetik dan terapeutik *in vivo*. Didapati bahawa kesitotoksikan ekstrak AP lebih tinggi dibandingkan dengan jumlah setara AGP tulen. Perkara ini mendorong kita untuk menyiasat aakah ketiga-tiga DL ini akan memberi kesan kombinasi sitotoksik terhadap se-sel kanser. Kombinasi AGP-NAG dan AGP-DDAG didapati menghasilkan kesan sinergistik ke atas sel-sel kanser MCF-7 (payudara) dan DU 145 (prostat). Untuk menentukan ketoksikan akut ekstrak ini dalam mencit dan tikus, dos meningkat dari 100 mg/kg sehingga 2 g/kg diberikan secara oral dan tidak berlaku pengurangan berat badan haiwan tersebut diamati. Paras plasma DL ditentukan dalam plasma mencit dan tikus yang diberikan ekstrak AP pada dos 250 mg/kg. Dos yang sama dan 500 mg/kg diterapkan dalam kajian kesan terapeutik

terhadap xenograft tikus telanjang yang membawa tumor payudara dan prostat manusia. Hasil dari kajian kestabilan menunjukkan bahawa suhu simpanan yang sesuai untuk bahan AP kering adalah pada 35°C hingga 3 bulan untuk mempertahankan kandungan AGP $70\% \geq$ dari kandungan awal dan suhu simpanan yang sesuai untuk etanol ekstrak kasar di 35° C hingga 6 bulan. Dos untuk kajian farmakokinetik pada haiwan roden (mencit-mencit BALB/c dan NU/NU serta tikus), apabila 250 mg/kg ekstrak AP yang diberikan secara oral kepada mencit dan tikus menunjukkan kepekatan maksimum plasma-DL (C_{max}) ditemui dalam julat berikut: AGP (2.1-5.3 μ M), NAG (2.3-6 μ M) dan DDAG (3.1-4.7 μ M). Dalam kajian terapeutik *in vivo*, ekstrak AP telah menunjukkan kesan merencatkan pertumbuhan tumor terhadap DU 145 tumor prostat tetapi tidak terhadap MDA-MB-231 tumor xenografts mencit telanjang. ini mencadangkan bahawa kesan sitotoksik sinergistik DL mungkin telah menyumbang dalam aktiviti antitumor *in vivo* AP ekstrak. Penyelidikan ini turut juga mengenalpasti keadaan simpanan yang sesuai untuk bahan AP kontang untuk menjamin kualiti dan seterusnya memberi jumlah DL yang kukuh dan konsisten dalam ekstrak untuk menjalankan kajian *in vitro* dan *in vivo*.

ACKNOWLEDGEMENTS

Throughout the course of this study, many individuals have given their fullest support in making the completion of this study possible. I would like to gratefully acknowledge the support of each and every individual, who contributed in some way to this thesis. Without their constant source of support and advices, this study would not have been possible.

First and foremost, I would like to express my sincere heartfelt gratitude to my supervisor, Associate Professor Dr. Johnson Stanslas for the continuous support of my Ph.D study and research, for his advices, patience, motivation, enthusiasm, and immense knowledge. His tireless encouragement and guidance throughout the course of this study have helped me in completing this research and thesis. The one thing I most appreciate is his effort in making me see how ‘simple’ and logical science REALLY is. I could not have imagined having anyone else as supervisor and mentor for my doctoral research

I wish to express my warm and sincere thanks to the rest of my thesis supervisory committee: Prof. Dr. Nordin Lajis, Assoc. Prof. Dr. Khozirah Shaari, Assoc. Prof. Dr. Said Saad and Assoc. Prof. Dr. Nashiru Billa for their valuable guidance and help throughout the course of this study.

My sincere thanks also go to Professor Mike Bibby, Dr. Paul Loadman, Dr Steve Shnyder; Ms Patricia Cooper for hosting me the summer placement in the Tom

Connors Cancer Research Centre, University of Bradford, showing and leading me onto a path of diversity in cancer research.

Not forgetting all the Cancer Research and Drug Discovery group members, Alex Christopher, Jebril Ali Abdalla, Dr Noor Wijayahadi, Lim Siang Hui, Riyadh Saif Ali, Sreenivasa Rao Sagineedu, Vikneswaran Selvarajan, Velan Suppaiah, Fatma Sri Wahyuni, and Sandra Maniam, with whom I shared unforgettable moments of laughter and frustration during the many years I worked in the lab.

Many thanks as well for the generous support of the staff of Faculty of Medicine and Health Sciences, UPM especially Mr Rijalana, Kak Siti Muskinah, Mrs Noor Hamzani, and Mr Ramli Suhaimi for assisting me in carrying out my project in this faculty. I would like to extend my special thanks to Dr. Naseem Malik, Ms. Sockalingam Rabinchamala and Mr. Zulkifli Ahmad for their assistance in conducting the *in vivo* animal studies in the Institute of Medical Research, Kuala Lumpur.

Last but not least, I am eternally grateful to my family for their understanding, patience and encouragement when it was most required. Without which, this work would not have been possible.

I certify that an Examination Committee has met on 15th August 2011 to conduct the final examination of Audrey Yong Chee Hui on her PhD thesis entitled “Preclinical Evaluation of *Andrographis paniculata* as an Herbal Medicine for the Treatment of Breast and Prostate Cancer” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the Doctor of Philosophy degree. Members of the Thesis Examination Committee were as follows:

Fauziah Othman, PhD

Professor
Department of Human Anatomy,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia
(Chairman)

Dr. Muhammad Nazrul Hakim Abdullah, PhD

Professor
Department of Biomedical Sciences
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Latifah Saiful Yazan, PhD

Lecturer
Department of Biomedical Sciences
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mohd Zaini Asmawi, PhD

Professor
School of Pharmaceutical Sciences
Universiti Sains Malaysia
(External Examiner)

SEOW HENG FONG, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This was thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy
The members of the Supervisory Committee were as follows:

Johnson Stanslas, PhD

Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Md Nordin Lajis, PhD

Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

Khozirah Shaari, PhD

Associate Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

Mohd Said Saad, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Nashiru Billa, PhD

Associate Professor
School of Pharmacy
Faculty of Health and Biological Sciences
The University of Nottingham Malaysia Campus
(Member)

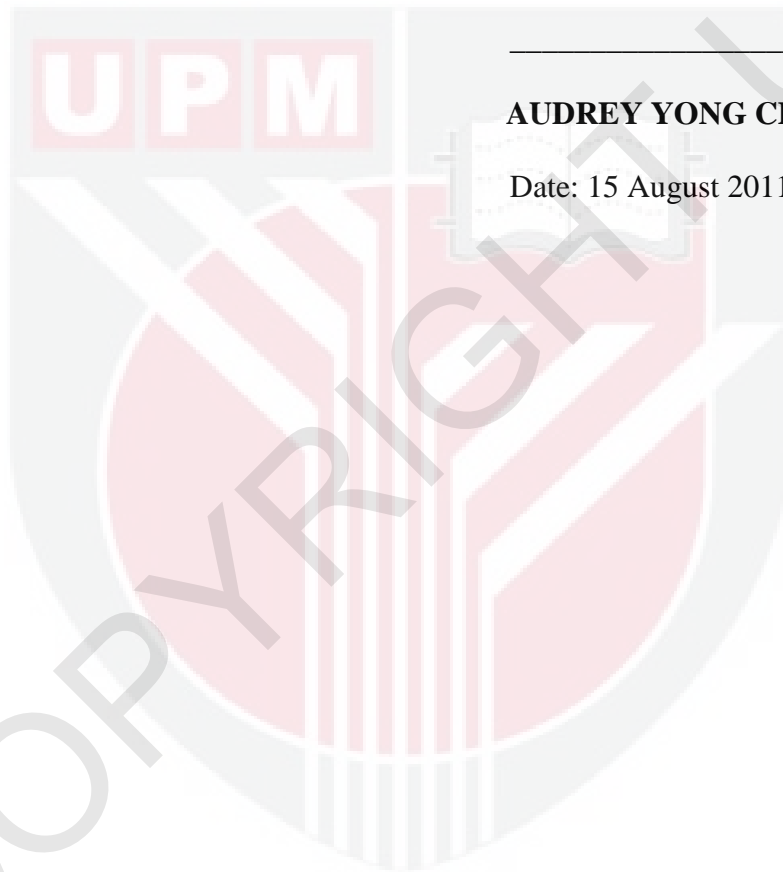
BUJANG BIN KIM HUAT, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or other institution.



AUDREY YONG CHEE HUI

Date: 15 August 2011

TABLE OF CONTENTS

	Page
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	xii
DECLARATION	xiii
LIST OF TABLES	xviii
LIST OF FIGURES	xx
LIST OF ABBREVIATIONS	xxiii
CHAPTER	
1 INTRODUCTION	1
1.1 Background	1
1.2 Hypothesis	3
1.3 Objectives	3
2 LITERATURE REVIEW	5
2.1 Herbal medicine	5
2.2 The Worldwide Phenomenon Of Increased Use Of Herbal Medicinal Products - Opportunities And Threats	6
2.3 Regulatory Background of Herbal Medicines	14
2.3.1 European countries	15
2.3.2 United States of America	18
2.4 Cancer	20
2.4.1 Carcinogenesis	22
2.4.1.1 Self-sufficiency in Growth Signals	23
2.4.1.2 Insensitivity to Antigrowth Signals	26
2.4.1.3 Evading Apoptosis	27
2.4.1.4 Limitless Replicative Potential	28
2.4.1.5 Sustained Angiogenesis	29
2.4.1.6 Tissue Invasion and Metastasis	30
2.5 Problems in development of herbal medicinal product	30
2.6 Approaches in discovery of natural product-based anticancer drugs	33
2.6.1 Development of Anticancer Herbal Medicine	39
2.6.2 Relevance of Preclinical Pharmacology	41
2.7 <i>Andrographis paniculata</i>	41
2.7.1 Medicinal Effects of <i>Andrographis paniculata</i>	44
2.7.1.1 Cardiovascular Effects	44
2.7.1.2 Immunomodulatory and antiviral effects	45
2.7.1.3 Immunological Benefits: Cancer	46
2.7.1.4 Fertility Effects	49
2.8 Standardisation Of Chemical Aspects Using Marker Analysis	50
2.9 Stability Of Pharmaceutical Products	53

3	STABILITY STUDY OF <i>ANDROGRAPHIS PANICULATA</i>	56
3.1	Introduction	56
3.2	Materials And Methods	59
3.2.1	Chemicals and Reagents	59
3.2.2	Plant Materials	60
3.2.3	Post Harvest Processing	62
3.2.4	Extraction AP	62
3.2.4.1	Preparation of dichloromethane AP crude extract	62
3.2.4.2	Preparation of crude AP ethanol extract	63
3.2.5	Storage Studies of Dry Materials and Extract of AP	62
3.2.5.1	Experiment I: Storage of dry AP plant material	64
3.2.5.2	Experiment II: Storage of dry AP plant material in N ₂ gas (nitrogen packed)	65
3.2.5.3	Experiment III: Storage of crude AP extract	65
3.2.6	Determination of Diterpenoid Lactones Using High Performance Liquid Chromatography (HPLC)	65
3.2.6.1	High Performance Liquid Chromatography conditions	65
3.2.6.2	Preparation of Standard solution-Calibration curve	66
3.2.6.3	Data analysis	66
3.3	Results	67
3.3.1	Effects Of Storage Temperature On Diterpenoid Lactones In Dry Materials	67
3.3.2	Effects Of Storage Temperature On Diterpenoid Lactones In Nitrogen Packed AP Dry Materials	71
3.3.3	Effects of Storage Temperature on Diterpenoid Lactones in AP Crude Extracts	73
3.4	Discussion	77
4	CYTOTOXICITY OF EXTRACT AND DITERPENOID LACTONES OF <i>A. PANICULATA</i>	80
4.1	Introduction	83
4.2	Materials	83
4.2.1	Test agents	83
4.2.2	Cell Lines	84
4.2.3	Reagents and Chemicals	84
4.2.4	Tissue Culture Materials	84
4.2.5	Instrumentations	85
4.3	Methods	85
4.3.1	Cell Culturing	85
4.3.2	Plating	86
4.3.3	Cryogenic Preservation and Recovery	87
4.3.4	Microculture Tetrazolium (MTT) Assay	88
4.3.5	AP Extract and Compounds Dilution and Preparation	89
4.3.5.1	Treatment with Crude Extract	90
4.3.5.2	Combination Treatment with 2 Compound	90
4.3.6	Analysis of Combination Effects	92
4.3.7	Statistical Analysis	92
4.4	Results	93
4.4.1	Cytotoxic Effect of AP Extract and Compounds	93

4.4.1.1	Single Agents	93
4.4.2	Combination Treatment	94
4.6	Discussion	105
5	PHARMACOKINETIC STUDIES OF DITERPENOID LACTONES AND EXTRACTS OF <i>ANDROGRAPHIS PANICULATA</i> IN RODENTS	109
5.1	Introduction	109
5.2	Materials	112
5.2.1	Test Sample-AP Dichlorometahane Extract	112
5.2.2	Animals	113
5.2.3	Chemicals and Reagents	113
5.2.4	Laboratory Wares and Consumables	114
5.2.5	Instrumentation	114
5.3	Method	115
5.3.1	Determination Of Maximum Tolerated Dose (MTD) Of AP Extract In Mice	115
5.3.2	Pharmacokinetic Study of AP Extract	116
5.3.2.1	Dosing of Animal with Vehicle and Test Extract	116
5.3.2.2	Dosing solution	116
5.3.2.3	Sample Collection and Handling	116
5.3.2.4	Sample preparation	117
5.3.2.5	Analysis of Plasma AGP, NAG and DDAG Concentrations	118
5.3.2.6	Calibration Curve and Method Validation	118
5.3.2.7	Data Analysis	119
5.4	Results	120
5.4.1	Maximum Tolerated Dose (MTD) of AP extract	120
5.4.2	Validation of HPLC method	121
5.4.3	Pharmacokinetic Studies Of AGP, NAG And DDAG In NU/Nu Mice Administered With 250 mg/kg Standardised AP Extract	123
5.4.4	Pharmacokinetic Studies Of AGP, NAG And DDAG In BALB/C Mice Administered With 250 mg/kg p.o. Standardised AP Extract	125
5.4.5	Pharmacokinetic Studies of AGP, NAG and DDAG in Sprague Dawley Rats administered with 250 mg/kg p.o. Standardised AP extract	127
5.5	Discussion	128
6	PRECLINICAL THERAPEUTIC STUDIES OF <i>A. PANICULATA</i> EXTRACTS IN ANIMAL WITH TUMOUR XENOGRAFTS	134
6.1	Introduction	134
6.2	Materials	137
6.2.1	Test Sample - AP Dichloromethane Extract	137
6.2.2	Cell Lines	137
6.2.3	Animals	138
6.2.4	Chemicals and Reagents	138
6.2.5	Laboratories Wares and Consumables	138
6.3	Method	139

6.3.1	Animals and Environmental Control	140
6.3.2	Development of Subcutaneously Implanted DU 145 Human Prostate and MDA-231 Breast Tumour Xenografts	149
6.3.2.1	AP Extract <i>in vivo</i> Antitumoural Activity Evaluation Against DU 145 and MDA-MB-231 Tumour Xenografts	141
6.3.3	Data Analysis	142
6.3.4	Statistical Analysis	143
6.4	Results	144
6.4.1	Effect of AP extract on the Growth of DU 145 Xenografts in Nude Mice	144
6.4.2	Effect of AP extract on the Growth of MDA-MB-231 Tumour Xenografts in Nude Mice	148
6.5	Discussion	152
7	GENERAL DISCUSSION AND CONCLUSION	154
7.1	Discussion	154
7.2	Conclusion	163
7.3	Recommendation For Future Research	164
	REFERENCES	165
	APPENDICES	221
	BIODATA OF STUDENT	239