



UNIVERSITI PUTRA MALAYSIA

**GENETIC VARIATION AND ANTIVITY OF NDROGRAPHIS
PANICULATA GERMPLASM FROM MALAYSIA**

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FP 2005 18



**GENETIC VARIATION AND ANTICANCER ACTIVITY OF *NDROGRAPHIS
PANICULATA* GERMPLASM FROM MALAYSIA**

By

JEBRIL ALI ABDALLA

Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirement for the Degree of Master of
Agricultural Science

October 2005



DEDICATION

I would like to dedicate this dissertation to honor my parents for their extraordinary contribution in my life. Last but not least, a great appreciation goes to my brothers, my wife and my son for their love.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Agricultural Science

GENETIC VARIATION AND ANTICANCER ACTIVITY OF *ANDROGRAPHIS PANICULATA* GERMPLASM FROM MALAYSIA

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October 2005

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Andrographis paniculata (Acanthaceae) is a medicinal herb endowed with curative properties against a variety of ailments including cold, fever, liver diseases, diabetes and cancer. Pharmacological studies have demonstrated that the major compound of the plant, diterpene lactone andrographolide, have *in vitro* antitumour activity against human breast cancer cell lines MCF-7. Twenty-six *A. paniculata* accessions were collected from three states in Peninsula Malaysia (Selangor, Negeri Sembilan and Perak) to study the genetic variations, phytochemical variations and cytotoxicity activity against MCF-7 cells, in order to identify promising accessions.



Genetic variations of 26 *A. paniculata* were investigated using inter simple sequence repeat (ISSR) analysis. Of the 28 primers screened, 17 produced highly reproducible ISSR bands. Using these primers, 123 discernible DNA fragments were generated with 35 (29 %) being polymorphic, indicating low levels of polymorphism at the populations level. At the 0.84 Jaccard's similarity the *A. paniculata* accessions were divided into 7 distinct groups, the genetic clustering was in agreement with the geographical locations / distances.

Dry material of *A. paniculata* was extracted in petroleum ether dichloromethane, methanol, and mixture of dichloromethane/methanol (1:1) for bioactivity testing. The results indicate that the dichloromethane extracts retains the active compounds contributing for the anticancer activity. Dichloromethane extracts significantly inhibits the proliferation of MCF-7 breast cancer cell lines at low concentrations (11.0µg/ml).

Analysis of the contents of active components of *A. paniculata*, namely andrographolide (ANDRO), neoandrographolide (NAG) and 14-deoxy-11, 12-didehydroandrographolide (DDAG), was carried out using high performance liquid chromatography. Accession 11179S was most superior in terms of andrographolide content, whereby it had 1% in dry weight. Moreover, accessions 11269P, 11265P, 11261P and 11284P also showed higher content of ANDRO of 0.84, 0.78, 0.76 and 0.70% respectively in dry weight.

The lowest amounts for the 3 active compounds were found in accession 11169S, with value of 0.11 – 0.25%.

Dichloromethane (DCM) extracts of the 26 accessions were tested for cytotoxic effect on human breast cancer cell lines, MCF-7, using a microculture tetrazolium (MTT) assay. The accessions had similar cytotoxic activity, determined by the 50% inhibitory concentrations (IC_{50}). Accession 11276P had the lowest IC_{50} value of 2.2 $\mu\text{g/ml}$, and the highest IC_{50} value of 6.6 $\mu\text{g/ml}$ was shown by accession 11212NS.

In correlation study, the IC_{50} values were negatively correlated with ANDRO ($P \leq 0.05$) and NAG ($P \leq 0.01$), which indicates the choice of accessions with high contents of ANDRO and NAG, results in good activity against MCF-7 human breast cancer cell lines. Also heritability for phytochemicals ANDRO, NAG and DDAG in the present study confirms that these compounds are mainly under genetic control in *A. paniculata* germplasm. Accessions 11179S, 11269P, 11265P, 11261P and 11284P are considered to be the most valuable accessions with high potential for anticancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains Pertanian

**VARIASI GENETIK DAN KESAN ANTIKANSER GERMPLASMA
ANDROGRAPHIS PANICULATA DI MALAYSIA**

Oleh

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Andrographis paniculata Nees merupakan tumbuhan herba yang berupaya untuk merawat pelbagai jenis penyakit seperti selsema, demam, penyakit hepar, diabetes dan kanser. Kajian farmakologi menunjukkan sebatian utama yang terkandung dalam tumbuhan ini ialah lakton diterpena andrographolide yang mempunyai aktiviti antikanser terhadap sel kanser payudara manusia, iaitu MCF-7 secara *in-vitro*. Dua puluh enam aksersi germplasma *A. paniculata* telah dikumpul dari tiga negeri di Semenanjung Malaysia (Selangor, Negeri Sembilan, Perak) untuk kajian variasi genetik, variasi fitokimia dan aktiviti sitotoksiknya terhadap sel MCF-7 dan seterusnya menentukan aksersi yang bermanfaat.



Variasi genetik bagi 26 aksersi *A. paniculata* tersebut dikaji dengan menggunakan primer 'Inter Simple Sequences Repeat' (ISSR). Daripada 28 primer yang dikaji, 17 primer menghasilkan jalur ISSR yang dapat diulangi. Melalui primer ini, 123 pecahan DNA dihasilkan dengan 35 (29%) adalah polimorfik dan ini menunjukkan polimorfisme yang rendah. Persamaan Jaccard pada 0.84 menunjukkan aksersi *A. paniculata* digolongkan kepada 7 kumpulan dengan jelas, dimana pengelompokan genetik adalah sejajar dengan kedudukan geografi.

Bahan kering diekstrak daripada *A. paniculata* dengan menggunakan eter petroleum, diklorometana, metanol dan campuran diklorometana/metanol (1:1). Kajian menunjukkan ekstrak diklorometana mengekalkan sebatian aktif yang memaparkan aktiviti antikanser. Ekstrak diklorometana merencatkan proliferasi sel MCF-7 yang signifikan pada kepekatan rendah. Selain itu, aksersi 11269P, 11265P, 11261P dan 11284P juga mempunyai kandungan ANDRO yang tinggi, iaitu masing-masing sebanyak 0.84, 0.78, 0.76 dan 0.70% berasaskan berat kering. Kandungan ketiga-tiga sebatian aktif yang terendah dilaporkan pada aksersi 11169S dengan nilai 0.11-0.25%.

Ekstrak diklorometana (DCM) untuk 26 aksersi tersebut diuji untuk kesan sitotoksiti terhadap sel kanser payudara, MCF-7 dengan menggunakan ujian mikrokultur tetrazolium. Aktiviti sitotoksiti ditentukan berdasarkan nilai kepekatan perencatan 50% (IC_{50}). Aksersi 11276P mempunyai nilai IC_{50}

terendah iaitu 2.2 $\mu\text{g/ml}$, manakala aksersi 11212NS mempamerkan nilai IC_{50} yang tertinggi iaitu 6.6 $\mu\text{g/ml}$.

Analisa kolerasi menunjukkan nilai IC_{50} adalah berkorelasi secara songsang dengan kandungan ANDRO ($P \leq 0.05$) dan NAG ($P \leq 0.01$), dimana pilihan aksersi yang mempunyai kandungan ANDRO dan NAG yang tinggi akan memberi aktiviti terhadap sel kanser payudara manusia, MCF-7. Dalam kajian ini, variasi kandungan fitokimia ANDRO, NAG dan DDAG adalah dibawah kawalan genetik. Aksersi 11179S, 11269P, 11265P, 11261P dan 11284P merupakan aksersi yang paling bermanfaat dan berpotensi dalam perubatan antikanser.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisor, Assoc. Prof Dr. Mohd Said Saad and supervisor committee members; Dr. Johnson Stanslas and Assoc. Prof Dr. Mihdzar Abdul Kadir, for their guidance, encouragement, and patience throughout this study.

I would like to thank the members of the Cancer Research and Drug Discovery Group (CRDD), especially to Miss Audrey Young, who taught, guided and helped me with HPLC analysis. I would also like to thank Miss Ramisah Mohamed, Harouna Sow and the Cell Biology Laboratory students for their help and friendship. I further extend my gratitude to Mr. Mohd Shahril who helped me during my field work experiment.

I greatly appreciate the Libyan government for the financial support. Finally, I thank my family and my friends for their support, advice and encouragement.



TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvi
LIST OF FIGURES	xviii
LIST OF ABBREVIATIONS	xix
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	4
2.1 <i>A. paniculata</i>	4
2.1.1 Botany of <i>A. paniculata</i>	4
2.1.2 Origin and Distribution	6
2.1.3 Chemical Constituents of <i>A. paniculata</i>	7
2.1.4 Medicinal uses of <i>A. paniculata</i>	11
2.2 Molecular Markers in Plant Studies	13
2.2.1 Molecular Markers for Genetic Analysis in Plant	14
2.2.2 Molecular Genetic Studies in <i>A. paniculata</i>	20
2.3 Conservation and Utilization of the Genetic Variations in Medicinal Plants	21
2.4 Medicinal Plants in Treatment of Cancer	24
3 GENETIC VARIATIONS OF <i>A. PANICULATA</i> GERMPLASM IN MALAYSIA AS REVEALED BY INTER-SIMPLE SEQUENCE REPEATS MARKERS (ISSRs)	27
3.1 Introduction	27
3.2 Materials and Methods	29
3.2.1 Plant Material	29
3.2.2 Field Trial and Agronomic Practices	29
3.2.3 DNA Extraction	31
3.2.4 Determination of DNA Purity	32
3.2.5 Screening of ISSR Primers	34
3.2.6 ISSR-PCR Analysis	36
3.2.7 Detection of Amplification Product	36
3.2.8 Gel Scoring	38
3.2.9 Data Analysis	38



3.3	Results	40
3.3.1	DNA Extraction	40
3.3.2	Identification of Primers	43
3.3.3	ISSR Polymorphisms	43
3.3.4	Genetic Relationships among <i>A. paniculata</i> Germplasm	49
3.4	Discussion	52
4	PHYTOCHEMICAL CONTENTS OF <i>A. PANICULATA</i> GERMPLASM IN MALAYSIA	55
4.1	Introduction	55
4.2	Materials and Methods	57
4.2.1	Chemicals	57
4.2.2	Plant Materials	57
4.2.2.1	Field Layout and Experimental Design	57
4.2.2.2	Agronomic Practices and Field Management	58
4.2.3	Extractions of <i>A. paniculata</i>	58
4.2.3.1	Sample Preparation	58
4.2.3.2	Extraction Method	60
4.2.3.3	Preparation of Samples	61
4.2.4	Preparation of Standards	61
4.2.5	Phytochemical Analysis	61
4.2.6	Heritability	62
4.2.7	Data Analysis	64
4.3	Results	65
4.3.1	Yields and Cytotoxicity Activities of <i>A.</i> <i>paniculata</i> Extracts	65
4.3.2	Crude Extracts of <i>A. paniculata</i> Germplasm	65
4.3.3	Analysis of ANDRO, NAG and DDAG of <i>A.</i> <i>paniculata</i> Germplasm	69
4.3.4	Phytochemical Variability	72
4.3.5	Heritability	75
4.3.5	Grouping of Accessions	75
4.4	Discussion	81
5	GENETIC VARIATION OF CYTOTOXIC EFFECTS OF <i>A. PANICULATA</i> GERMPLASM IN MALAYSIA	84
5.1	Introduction	84
5.2	Materials and Method	86
5.2.1	Plant Materials and Extraction Method	86
5.2.2	Preparation of Extracts for <i>In Vitro</i> Test	87
5.2.3	Cell Culture Reagents	87
5.2.4	Cell Culture	88
5.2.5	Microculture Tetrazolium Cell Viability Assay	88
5.3	Results	91

5.3.1	Cytotoxicity of Various Accessions	91
5.3.2	Cellular Morphological Changes	94
5.4	Discussion	97
6	ASSOCIATION BETWEEN PHYTOCHEMICALS AND CYTOTOXICITY EFFECTS OF <i>A. PANICULATA</i> GERMPLASM IN MALYSIA	99
6.1	Introduction	99
6.2	Materials and Methods	100
6.2.1	Correlation	100
6.3	Results and Discussion	101
6.3.2	Association Between Phytochemicals and Cytotoxicity	101
7	GENERAL DISCUSSION	105
8	CONCLUSIONS	109
	REFERENCES	111
	APPENDICES	122
	BIODATA OF THE AUTHOR	131

LIST OF TABLES

Table		Page
2.1	A comparison of DNA - markers, Amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD) and intersimple sequence repeats (ISSR) used to determine genetic diversity in plant populations	16
3.1	List of 26 accessions of <i>A. paniculata</i> collected from three states (Selangor, Negeri Sembilan and Perak) in Peninsular Malaysia that used in the study	30
3.2	List of 28 random UBC ISSR primers that were screened for the present study	35
3.3	Condition for ISSR-PCR reaction	37
3.4	Ratio of UV Spectrophotometer reading for 26 <i>A. paniculata</i> accessions	42
3.5	List of the ISSR Primers tested and selected on the Screening of <i>A. paniculata</i> germplasm	44
3.6	ISSR Analysis using 17 primers on 26 accessions of <i>A. paniculata</i> germplasm from Malaysia	45
3.7	Jaccard similarity index among the 26 accessions of <i>A. paniculata</i> from Malaysia based on ISSR analysis using 17 primers-	50
4.1	Analysis of variance (ANOVA) key out, for accessions effects of each trait in the study	63
4.2	Yield and IC ₅₀ values of <i>A. paniculata</i> extracts obtained using various solvents	66
4.3	Yield of the crude extracts of 26 <i>A. paniculata</i> accessions obtained from 100 g dry material of the aerial parts using (DCM)	68
4.4	Mean squares of contents of ANDRO, NAG and DDAG of the 26 accessions of <i>A. paniculata</i> in Malaysia	73
4.5	Mean values of ANDRO, NAG and DDAG contents (% of dry weight) in the <i>A. paniculata</i> accessions in Malaysia	74
4.6	Genotypic and phenotypic variance and heritability values for phytochemicals ANDRO, NAG and DDAG in dry weight of the 26 <i>A. paniculata</i> germplasm in Malaysia	76
4.7	Distribution of <i>A. paniculata</i> accessions, revealed by cluster analysis, in the state of Selangor, N. Sembilan and Perak of Malaysia with mean values of ANDRO, NAG and DDAG in each group	78
4.8	Overall mean, range and coefficient variation for three active phytochemicals in 26 <i>A. paniculata</i> accessions collected from Selangor, N. Sembilan and Perak, Malaysia	80



5.1	Cytotoxic activity of <i>A. paniculata</i> accessions on MCF-7 breast cancer cells	92
6.1	Analysis of variance (ANOVA) key out, for accessions effects of each trait in the study	102



LIST OF FIGURES

Figure		Page
2.1	The plant of <i>A. paniculata</i>	5
2.2	Distribution map of <i>A. paniculata</i> . Boxed region in the inset (Upper right) is enlarged	6
2.3	Chemical constituents of <i>A. paniculata</i>	8
3.1	Schematic representation of DNA extraction protocol of <i>A. paniculata</i>	33
3.2	Image of ethidium bromide stained gel electrophoresis of <i>A. paniculata</i> genomic DNA isolation indicating good quality of DNA which is suitable for PCR analysis	41
3.3	Clear polymorphic bands generated by primers 807, 809, 811, nissr2, 835, and nissr1 which produced 53% fragments for the 26 <i>A. paniculata</i> accessions	46
3.4	Dendrogram revealed by UPGMA cluster analysis on the 26 <i>A. paniculata</i> germplasm based on Jaccard's similarity generated from ISSR analysis using 17primers	51
4.1	Field planting of 26 <i>A. paniculata</i> germplasm at field 2, UPM	59
4.2	A representative of a 96-hours dose-response cytotoxic effect of DCM (11.0 µg/ml), MeOH (13.5 µg/ml), MeOH+DCM (12.0 µg/ml) and PE (> 100 µg/ml) crude extraction on MCF-7 cells	67
4.3	Standard calibration curves of ANDRO, NAG and DDAG--	70
4.4	HPLC chromatogram of the extract of accession 11179S showing clear separation of the three main active compounds of ANDRO, NAG and DDAG	71
4.5	Dendrogram from cluster analysis on the 26 <i>A. paniculata</i> accessions collected from Selangor, N. Sembilan and Perak, Malaysia with respect to phytochemicals contents	77
5.1	Conversion of soluble yellow MTT into insoluble purple formazan by the living cells after 4-hours incubation	89
5.2	Representative of a 4-day dose-response cytotoxic effect of DCM extracts of accessions 11276P and 11212NS on MCF-7cells. From this figure the IC ₅₀ of 2.2 µg/ml and 6.6 µg/ml were obtained for accessions 11276P and 11212NS, respectively	93
5.3	Morphological change of DCM crude extract of accession 11179S induced by 10 µg/ml and 100 µg/ml on MCF-7 cells at 48 and 96 hour time points	95
5.4	Morphological changes induced by (a) 10 µg/ml (apoptosis mode of cell death) and (b) 100 µg/ml (necrosis mode of cell death) of <i>A. paniculata</i> DCM extract in MCF-7 cells at 48 hour time points	96
6.1	Simple linear regression of ANDRO (a) and NAG (b) with IC ₅₀ values. Both show negative correlation	103



LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ANDRO	Andrographolide
CDK 4	Cyclin-dependent kinase 4
CRDD	Cancer Research and Drug Discovery
cpDNA	Chloroplast deoxyribonucleic acid
CV	Coefficient of variations
DCM	Dichloromethane
DDAG	14-deoxy-11, 12-didehydroandrographolide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dntp	Deoxynucleoside triphosphate
DTH	Delayed type hypersensitivity
EDTA	Ethylene-diamine-tetra acetic acid
EtBr	Ethidium bromide
FCS	Foetal calf serum
H ²	Heritability
HHT	Homoharringtonin
HIFCS	Heat inactivated foetal calf serum
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC ₅₀	50% inhibitory concentration
ISSR	Inter-simple sequence repeat
KB	Human epidermoid carcinoma
MCF-7wt	Breast cancer cell lines (hormone-dependant)
MDA-MB-231	Breast cancer cell lines (hormone-independent)
MeOH	Methanol
MSE	Mean square for error
MSG	Mean square for genotypes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide



NAG	Neoandrographolide
NCI	National Cancer Institute
NTSYS-PC	Numerical Taxonomy and Multivariate Analysis System
OD	Optical density
PCR	Polymerase chain reaction
PCR Buffer	[100mM Tris-HCl (pH 9.0) at 25°C; 500mM KCl; 1% Triton X 100]
PE	Petroleum ether
R	Number of replications
RAMP	Random amplified microsatellite polymorphisms
RAPD	Random amplified polymorphic DNA
RCBD	Randomized Complete Block Design
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI 1640	Roswell Park Memorial Institute – 1640
SAHN	Sequential, Agglomerative, Hierarchical and Nested clustering
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SIMQUAL	Similarity for Qualitative Data
SRBC	Sheep red blood cells
SSR	Simple Sequences Repeat
TBE	[89 mM Tris-base, (pH 8.3); 89 mM Boric acid; 2 mM EDTA]
tBHP	Tert-butyl hydroperoxide
TE	Tris-EDTA buffer
t_R	Retention time
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
URI	Upper respiratory tract infection
USA	United states of America
UV	Ultraviolet
σ^2_e	Environmental variance



$\sigma^2 g$ Genotypic variance
 $\sigma^2 p$ Phenotypic variance



CHAPTER 1

INTRODUCTION

Throughout the ages, humans have been relying on plants for medicine. Such plants or parts of the plant are known as herbs (Ralph 1996). Among the many medicinal plants, *Andrographis paniculata* (Nees) from the family of Acanthaceae, known commonly as King of Bitters, has been identified to possess a great deal of medicinal values and traditionally has been used in treating various illnesses and disorders (Ong and Nordiana 1999).

Andrographis paniculata is believed to be originated from India and was introduced into Malaysia by the migration of Indian labours. It is known locally in most parts of Malaysia as 'Hempedu Bumi' although several other names are being used by the local people from different areas of the country. It grows as a wild plant in the open fields but at times planted specially for medicinal purposes (Zhang and Tan 1997).

Chemical investigations of *A. paniculata* began as early as 1911 when Gorter (1911) isolated a bitter constituent from its leaves. The compound was trivially named andrographolide. Cava *et al.* (1965) elucidated the complete structure of andrographolide and found that it was a diterpene lactone.



The major ones are andrographolide (ANDRO), neoandrographolide (NAG) and 14-deoxy-11, 12-didehydroandrographolide (DDAG). The main component andrographolide, an unsaturated γ -lactone has been reported to have anticancer activity (Siripong *et al.* 1992).

Despite its enormous medicinal and economic importance, attempts to cultivate *A. paniculata* have seldom been undertaken in Malaysia, in particular, and in most parts of the world. It should be noted that even basic biological data are lacking for *A. paniculata* as also the case with many other medicinal plants. It is needless to say that in the wilderness of nature, the populations of a sexually reproduced species like *A. paniculata* are presumably so varied, that selection of high biomass and product yielding genotypes is distinctly possible and holds much promise for up grading into cultivars.

The involvement of *A. paniculata* in research work and breeding programs for genetic improvement has not been fully realized until recently and also studies on the germplasm of the species are very much lacking. Information on germplasm variations is very important for genetic improvement of the plant. Recent work in our laboratory has shown moderate variation of the quantitative morphological characters within the Malaysian *A. paniculata* germplasm (Melaku 2003).

Several studies has been conducted to evaluate and test the medicinal properties of *A. paniculata*. However genetic variations of the species particularly for the effects on certain illness have been a subject of little investigation (Sabu *et al.* 2001).

Thus, the present study was undertaken to analyse the genetic variations at molecular level and anticancer activity of *A. paniculata* germplasm in Malaysia. The specific objectives were;

- 1- To study genetic variations of *A. paniculata* germplasm in Malaysia as revealed by Inter–Simple Sequence Repeats (ISSR) markers.
- 2- To determine Phytochemical variation of the active components of *A. paniculata* namely andrographolide (ANDRO), neoandrographolide (NAG) and 14-deoxy-11, 12-didehydroandrographolide (DDAG).
- 3- To determine the variation in cytotoxic effect of *A. paniculata* germplasm in Malaysia.