Studies on *Andrographis paniculata*

*(Burm. f.) Nees* *(HDM 15)*

A Medicinal Native Plant of Brunei Darussalam

Thesis submitted for the degree of

Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work described in this thesis is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Signed by: ________________

Kui Hong Chua

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Dedication

to

My Husband Huat Joo

&

Children Fong Ching, Beng Loon, and Fong Mei

“For their kind understanding, help, support and belief that I can complete this PhD project.”
“Modern science is founded on the belief that knowledge, as it progresses, accumulated new and improved concepts driving out the old and the fallible. It prides itself on being objective and rigorous; yet it fails to recognize that there can be other systems of thought. Phytotherapy, or herbal medicine, believes in the harmonious view that ‘the whole plant is greater than the sum of its parts’. Some of the wonder drugs of modern medicine have their roots in indigenous medicine.”

by Dr. U Ko Ko, 1991

Regional Direction
World Health Organization

“The principal ingredient of the plant or herb is a substance that provides the main therapeutic thrust, the other ingredients enhance or assist the therapeutic actions of the first.”

by Yuan & Lin, 2000
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Studies on *Andrographis paniculata*  
*(Burm. f.) Nees* (HDM 15)  
A Medicinal Native Plant of Brunei Darussalam

Thesis submitted for the degree of  
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March 2007
Declaration

I certify that except where due acknowledgement has been made, the work described in this thesis is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Signed by: ________________

Kui Hong Chua

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Dedication

to

My Husband Huat Joo

&

Children Fong Ching, Beng Loon, and Fong Mei

“For their kind understanding, help, support and belief that I can complete this PhD project.”
“Modern science is founded on the belief that knowledge, as it progresses, accumulated new and improved concepts driving out the old and the fallible. It prides itself on being objective and rigorous; yet it fails to recognize that there can be other systems of thought. Phytotherapy, or herbal medicine, believes in the harmonious view that ‘the whole plant is greater than the sum of its parts’. Some of the wonder drugs of modern medicine have their roots in indigenous medicine.”

by Dr. U Ko Ko, 1991

Regional Direction

World Health Organization

“The principal ingredient of the plant or herb is a substance that provides the main therapeutic thrust, the other ingredients enhance or assist the therapeutic actions of the first.”

by Yuan & Lin, 2000
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Publications & Communications

Conference proceedings


3. K.H. Chua, Edwin C.K. Pang, Charlie C.L. Xue, Chun Guang Li. Effects of herbal extracts of Andrographis paniculata (Burm. f.) Nees of Brunei Darussalam on compound 48/80-induced histamine release from rat peritoneal mast cells (RPMC). (This poster was recognised as a student poster award certification at the School of Health Sciences Conference, RMIT University, Bundoora West Campus on 31st May, 2005).

RAPD and RFLP Analyses. Paper presented at the Harmonisation of Traditional and Modern Medicine - An International symposium, 12-14th December, 2005, RMIT Storey Hall, 344 Swanston Street, Melbourne, Australia.


8. Participated in the Inaugural 1st Australia-China Biomedical Research Conference (ACBRC 2007) Walter & Eliza Hall Institute of Medical Research, 1G Royal Parade, Royal Melbourne Hospital, Melbourne, Australia (1st - 3rd February 2007).
Papers


2. Chua, K.H., Li, C.G., Xue, C.C.L. Comparison of the inhibitory effects of different sources of *Andrographis paniculata* (Burm. f.) *Nees* of Brunei Darussalam on iNOS mediated relaxation in LPS treated rat aorta. *Life Sciences. (submitted).*

Papers in preparation


2. Quantitative analysis of the contents of Andrographolide and Dehydroandrographolide in *Andrographis paniculata* (Burm.f.) *Nees* of Brunei Darussalam by HPTLC and HPLC Analysis. (in preparation).

## Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Andrographolide ($C_{20}H_{28}O_4$)</td>
</tr>
<tr>
<td>ADR</td>
<td>Adriamycin Drug Resistant Tumour cell lines</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP</td>
<td><em>Andrographis paniculata</em> (<em>Burm. f.</em>) <em>Nees</em></td>
</tr>
<tr>
<td>ASE</td>
<td>Accelerated Solvent Extraction, Dionex</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CRS</td>
<td>Chinese Reference Sample Herb (Herba Andrographitis)</td>
</tr>
<tr>
<td>CXL</td>
<td>Chuan Xin Lian</td>
</tr>
<tr>
<td>D</td>
<td>Dehydroandrographolide ($C_{20}H_{30}O_5$)</td>
</tr>
<tr>
<td>DPPH</td>
<td>1,1-Diphenyl-2-picrylhydrazyl</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Disodium ethylenediamine-tetraacetic acid</td>
</tr>
<tr>
<td>Ee</td>
<td>Ethanol extract</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>F</td>
<td>Fraction</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HDM</td>
<td>Herbarium Drug Museum of the Department of Agriculture, Ministry of Industry and Primary Resources, Brunei Darussalam</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HPTLC</td>
<td>High Performance Thin Layer Chromatography</td>
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iNOS  Inducible Nitric Oxide Synthase

K562  Human Tumour cells

LPS  Lipopolysaccharide Eschericia Coli Serotype 0127: B8

Me  Methanol extract

min  minutes

NO  Nitric Oxide

OPA  O-phthalaldehyde

P388  Mouse leukaemic lymphoblastic parental tumour cell line

P815  Mast cells tumour cell line

PCR  Polymerase chain reaction

PE  Phenylephrine

RAPD  Random amplified polymorphic DNA
\( R_f \)  
Retention factor (calculated as distance specimen travelled on a TLC plate divided by the distance the solvent travelled)

RFLP  
Restriction fragment length polymorphism

RMIT  
Royal Melbourne Institute of Technology Limited

RNA  
Ribonucleic acid

ROS  
Reactive Oxygen Species

RPMC  
Rat Peritoneal Mast Cell

RPMI-1640  
Roswell Park Memorial Institute Media 1640

s  
seconds

SDS  
Sodium Dodecyl Sulfate

SEM  
Standard Error of Mean

Std  
Standard

STIR  
Stir in solvent
TGA    Therapeutic Good Administration

UV    Ultraviolet

v/v    Volume for volume

We    Aqueous extract

WEHI-164    Human tumour cell line

WHO    World Health Organization

w/v    Weight for volume

w/w    Weight for weight

Yac-1    T-Lymphoblastic tumour cells

°C    degrees Celsius
Summary

Traditional and alternative medicines which are derived from wide range of plants, animals and minerals are used in the treatment of many disease conditions. Numerous studies have demonstrated that natural products are a rich source of novel compounds. There is a renewed interest in conducting studies to identify novel compounds with the potential to be developed into therapeutic agents.

This thesis provides an insight into the key issues in TM/CAM which remains a big challenge for Brunei Darussalam. As a worldwide scenario, the regulatory and legislative control of TM/CAM varies from country to country. In most developed countries, well established systems are in place, whereas in others they are regarded as food or nutrition and often their therapeutic claims are not permissible by the regulatory authorities of the countries concerned. However, the communities of most developing countries often have a wide ranging numbers of traditionally used herbal medicines and folk-knowledge about them from generations to generations. It is also highlighted that the three criteria required to legislating a pharmaceutical into the market through a legal framework of registration are quality, safety and efficacy. Recent development in TM/CAM harmonization and collaborative processes also spearhead into promoting these important criteria on TM/CAM towards achieving safe delivery of health for all.

There is an estimated 5,000 native species found in the tropical rain forests of Brunei Darussalam. Many medicinal plant species such as *Andrographis paniculata* (AP) have been long used by the local communities for the treatment of various disease conditions. However, so far little research has been done on the constituents or bioactivities of the native tropical
medicinal plants of Brunei Darussalam. It is likely that certain medicinal plants in Brunei Darussalam may have unique characteristics of secondary metabolites which are potential sources of therapeutic drugs. Therefore, prioritized research into these valuable medicinal plants in order to obtain valuable scientific information about the plants profile is necessary as they usually consist of many chemical constituents with highly complex pharmacological effects on the body.

Therefore, the main aims of this project are focused on two challenges. The first challenge was to develop the strategic framework on TM/CAM for Brunei Darussalam and the second challenge was that an interdisciplinary and systematic approach has been undertaken to investigate the genetic diversity, phytochemical profile, bioactivities and pharmacological actions of the tropical native medicinal plant *Andrographis paniculata* (Burm. f.) Nees (HDM 15) of Brunei Darussalam.

Firstly, the genetic diversity of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam was determined by Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) analyses. The genetic tree dendrogram was constructed based on the genetic variations generated by UPGMA followed by cluster analysis of two main clusters between the genotypes at maximum dissimilarity of 10%. Results indicated that the nine accessions from the three districts namely Brunei/Muara (Brunei AP), Kuala Belait (K.B.), Temburong (T) of Brunei Darussalam analysed in this study were found to be closely related to each other with only moderate variations. RAPD analysis demonstrated that it could be used effectively to survey the genetic variation of *Andrographis* species or varieties which will be beneficial in the characterisation and authentication of this tropical medicinal native plant of Brunei Darussalam.
Secondly the major phytochemical constituents Andrographolide (A) and Dehydroandrographolide (D) of *Andrographis paniculata* were determined by analytical High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC). The herbal samples collected from three districts namely Brunei/Muara (Brunei AP), Kuala Belait (KB), Temburong (T) of Brunei Darussalam compared with the Chinese Reference Sample herb (CRS) and Chuan Xin Lian (CXL) from China using the A and D reference standards to determine the levels of content of A and D respectively. Difference in the contents of A and D in these samples were observed. The content of A (%w/w) in Temburong and Brunei AP samples were higher than those from CXL, K.B. and Chinese Reference samples. On the contrary, the content of D (%w/w) in Temburong and Brunei AP samples were lower than that in CXL, K.B. and Chinese Reference samples. When combining A and D contents, the ranking order were Temburong, CXL, Brunei AP, Kuala Belait (KB) and Chinese Reference Sample.

Thirdly, the pharmacological effects of different extracts of *Andrographis paniculata* (*Burm. f.*) *Nees* of Brunei Darussalam were studied *in vitro*. These include studies on their anti-oxidant, anti-tumour, anti-hepatotoxicity activities, as well as their effects against compound 48/80-induced histamine release in rat peritoneal mast cells, and iNOS mediated smooth muscle relaxations in rat aorta tissues. Results showed that the aqueous (We), ethanol (Ee), methanol (Me) extracts of *Andrographis paniculata* (*Burm. f.*) *Nees* of Brunei Darussalam exhibited some significant scavenging activities towards 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical, hydrogen peroxide and nitric oxide in the cell-free systems with the exception of the aqueous extract which did not show any significant DPPH scavenging activity. In rat peritoneal mast cells, the extracts only significantly inhibited the compound 48/80-induced histamine release at a higher level of 1mg/ml which suggests that the mast cells were less responsive to
Andrographis paniculata. In functional studies of the rat aortic rings treated with LPS, Andrographolide and Dehydroandrographolide, the diterpenoid lactones of Andrographis paniculata, the ethanol extracts of Andrographis paniculata (Burm. f.) Nees of Brunei Darussalam exhibited a significant inhibition of the iNOS mediated relaxations. However, the aqueous extracts did not significantly inhibit iNOS responses. There is a correlation between the A and D contents and the inhibitory effects on iNOS responses in these extracts, indicating the important role of A and D in mediating the inhibitory actions of Andrographis paniculata on iNOS-mediated responses.

The tumouricidal activity was assessed against six tumour cell lines including mouse lymphoblastic parental PAR (P388), ADR, WEHI-164, K562 (Human Tumour cells), P815 (Mast cells), Yac-1 (T-Lymphoblastic) tumour cells. The general cytotoxicity against rat hepatocytes was assessed in freshly isolated rat hepatocytes.

Andrographolide (Std A) has the most potent anti-tumourigenic activity which inhibited the growth of all the six tumour cells by 50%, the IC\textsubscript{50} at a concentration range of 6-10µg/ml for P388, ADR, WEHI-164, K562, Yac-1 with the exception of the cell line P815 which showed less sensitivity to Std A, has a higher IC\textsubscript{50} value of 100µg/ml. The other extract fractions tested indicated that some Andrographis paniculata fractions inhibited the growth of tumour cell lines with varying potency.

It may suggest that the P815 cell line and the rat peritoneal mast cells are both derived from mast cells origin which was found to be less sensitive to Andrographis paniculata extract treatment. Possibly due to the fact that mast cells in general are less responsive to the treatment of Andrographis paniculata.
The general cytotoxicity of the extract fractions were evaluated by assessing the viability of the rat hepatocytes in the presence of two reference standards namely Andrographolide (A), Dehydroandrographolide (D) and seven Andrographis paniculata crude extract fractions. Results showed that significant toxicity was observed in all fractions but at varying degrees of cytotoxicity potency of the tested extract concentration at 100µg/ml. The most cytotoxic of all the fraction extracts was Std A which has IC₅₀ value of 60µg/ml at 50% inhibition of rat hepatocyte viability. At the concentration of 100µg/ml, the reference Std A, reducing rat hepatocyte viability by 68.78% ± 0.84%. At the concentrations tested indicated that all the fractions are moderately and relatively non cytotoxic.

This result suggests that Andrographis paniculata leaves may contain other active constituents other than Andrographolide with varying levels of %w/w content which may account for its varying potency of the anti-tumourigenic and cytotoxic activities. Preliminary results indicated that further cytotoxicity tests on Andrographis paniculata is warranted which is required to be elucidated in the future studies.

This finding offers support to the validity of the traditional medicine uses of this plant in which Andrographolide (A), the major active constituent of the extract of Andrographis paniculata (Burm. f.) Nees is a potential anti-cancer therapeutic agent.
In conclusion, the findings from the present project have demonstrated that *Andrographis paniculata* from Brunei Darussalam exhibited a distinctive genetic profile than the *Andrographis paniculata* from China. It has a wide range of anti-oxidant activities, pharmacological actions including anti-inflammatory and anti-tumour effects. Some of the pharmacological actions of *Andrographis paniculata* correlated with the active constituents Andrographolide (A) and Dehydroandrographolide (D). The findings have provided the first experimental evidence for this tropical medicinal native plant of Brunei Darussalam.
## Chapter 1  General Introduction

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1.1 Background

Traditional medicine (TM) has a very long history. These remedies mostly discovered from generations of practice and use, through trial and error, or simply by accident, had provided mankind with rich knowledge in plants, animals and minerals (Tyler, 1993) that has become part and parcel of life in many communities. It is also well documented that the world’s population relies upon natural products such as plants, animals and minerals based materials as the primary source of medicines for the treatment of disease conditions for many centuries. As officially endorsed by World Health Organization (WHO/EDM/TRM), ‘traditional medicine is the sum of total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses. The terms complementary/alternative/non-conventional medicine are used inter-changeably with traditional medicine in some countries.’

The World Health Organization (WHO) estimated in 2003 that the global market of traditional, complementary and alternative medicine (TM/CAM) usage was approximately US$60 billion and is on a rapid increasing trend (Robson, 2003). Herbs or medicinal plants or other natural products are now becoming one of the world’s most important commodities that are used with the intention to diagnose, treat, prevent or cure diseases, by all means are defined as drugs. Therefore, like any other drugs, they must be administered in proper doses for the appropriate duration of time and the intended purposes based on advice from the qualified health professionals. To ensure that TM/CAM practices are developed in the directions towards safeguarding public health, the development of strategic framework on TM/CAM remains an important challenge to many countries including Brunei Darussalam.
1.1.1 Definition of TM/CAM

In most developed countries, the term ‘complementary and alternative medicine’ (CAM) is usually used in the context where the dominant health care system is based on allopathic medicine, or where TM has not been incorporated into the national health care system. CAM has been recognised by many professionals and also non professionals in connection with health care needs. As a result of the paradigm shift, the term is used interchangeably as ‘traditional’, ‘complementary’, ‘alternative’, ‘non conventional’, ‘contemporary’, ‘herbal’, ‘natural’, ‘holistic’, ‘spiritual’ medicines with one common goal, that is to provide care to the patients, consumers and public at large. In the Bruneian context, the term TM/CAM encompass all the terms that are used on the basis of holistic approach for the enhancement of health and quality of life of the communities.

1.2 Use of traditional, complementary and alternative medicines (TM/CAM)

More than 80% of the world population use botanical preparations as medicines, and herbalism is experiencing an unprecedented acceptance (Atherton, 1994; Haper, 1994). This is supported by the increasing economic scales such as in the U.S. market amounts to about $1.5 billion per year (Marwick, 1995) and thrice as much as this was spent in the European market (Brevoort, 1996). In the developing world, similar trend was reported, such as in Malaysia, the annual market value of traditional remedies is about two billion ringgit which is twice as much as the amount spent on the pharmaceuticals (Balasubramaniam, 1998). The estimated national cost for both CAM preparations and practitioner visits were about one billion dollars when extrapolated to the Australian population and the increasing usage of CAM in the global market is overwhelming (Eisenberg et al., 1998; MacLennan et al., 1996; MacLennan et al., 2002). In the
recent national survey on the current usages of complementary medicines in Australia, 69% of the population used at least one of 17 forms of CAM in the last 12 months and for those used CAM, 64% of which consulted a CAM practitioner (Xue et al., 2005). The fact that China became a member of the World Trade Organization (WTO), had boosted the use of Chinese herbal medicines worldwide with a projected market value of $400 billion by 2010 (Zhen & Jun, 2002).

The use of TM/CAM in the primary health care of the world’s population is accounting for up to 80% of the low and middle income countries. Among the higher income countries, TM/CAM has gained its popularity with up to 65% of the population reporting that they have been exposed to the utilization of TM/CAM (Ernst, 2000; WHO, 2002b). Table 1.1 illustrated the % of population used CAM in the selected developed countries as reported by World Health Organization. The current fusion processes involved in the harmonious approach towards achieving common goal in the maintenance of health for all by parties concerned is very encouraging. Therefore, by embracing the concept of traditional medicine, perhaps one can meet the challenges of the health care needs of the public at present and the future. As far as Brunei Darussalam is concerned, this concept remains the mainstay of traditional medicine in the country, alike many developing countries as illustrated in table 1.2 (WHO, 2002a).
Table 1.1  Percentage of population used TM/CAM at least once in selected developed countries (Source: WHO Traditional medicine Strategy 2002-2005)

<table>
<thead>
<tr>
<th>Developed Countries</th>
<th>% of population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>31%</td>
</tr>
<tr>
<td>USA</td>
<td>42%</td>
</tr>
<tr>
<td>Australia</td>
<td>48%</td>
</tr>
<tr>
<td>France</td>
<td>49%</td>
</tr>
<tr>
<td>Canada</td>
<td>70%</td>
</tr>
</tbody>
</table>

Table 1.2  Percentage of population use of TM in Primary Health Care in selected developing countries (Source: TM-Growing Needs and Potential, WHO Policy Perspectives on Medicines, No. 2, May, 2002 Geneva)

<table>
<thead>
<tr>
<th>Developing Countries</th>
<th>% of population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uganda</td>
<td>60%</td>
</tr>
<tr>
<td>Tanzania</td>
<td>60%</td>
</tr>
<tr>
<td>Rwanda</td>
<td>70%</td>
</tr>
<tr>
<td>India</td>
<td>70%</td>
</tr>
<tr>
<td>Benin</td>
<td>70%</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>90%</td>
</tr>
</tbody>
</table>
The increasing global popularity of TM/CAM has drawn much attention from government bodies, academia and health care professionals with their keen interest to better understand the quality, efficacy and safety of TM/CAM. Despite the economic momentum and the wide use of TM/CAM, the better informed public of today are also interested in the safe use of TM/CAM. Many international, regional and national conferences, symposiums, meetings, discussions were conducted to share important updated information, research and development on the harmonisation of TM/CAM and modern medicines. As TM/CAM is being used in tandem with western medicine globally, a better understanding between the both worlds of practices would bring us closer to provide better health care to the communities.

1.3 Perception of TM/CAM

TM/CAM is commonly recommended to relieve minor ailments and illnesses. They are usually considered to be natural and generally safe (Koh & Woo, 2000) from the consumer’s perspective but many people do not perceive them in the same way as they take allopathic medicines. Like any other medications, the medical practitioner’s advice is for the public to treat TM/CAM with respect (Botting & Cook, 2000) and they are needed to be used with care because they may not be suitable for others as there is ‘no one size fits all’ and individualised therapy approach to optimise the medical condition of the patient is to be advocated. Obviously, they are not free of side effects and can also interact with western medicines in either a synergistic or antagonistic manner and may result in compromising the medical conditions of patients.

With the advancement in technology, the mass media promotion of TM/CAM has prompted the attention of health professionals on the benefit/risk ratio for patients. Young et al., (2001) examined the ethical and legal implications of the TM/CAM as requested to be used in the
mainstream intensive care practice by the better informed patients about their illness via accessing the internet which publicises and promotes the use of TM/CAM. As TM/CAM is distinguished by the lack of well conducted clinical trials in the determination of its efficacy and risks, as a result, decisions about its use cannot be based on benefit/risk analysis and genuine informed consent cannot be achieved which does not warrant its corporation in the intensive care practice (Young et al., June 2001).

1.4 Legislative and regulatory control of TM/CAM

In most countries, traditional medicine is regulated in an administrative framework to safeguard public health. In general, the regulatory control is less stringent than the legislative control on western medicines. The debate on whether traditional medicine should be subjected fully to conventional legal framework of legislative, regulatory and licensing procedures as imposed on the allopathic medicines in the assurance of quality, safety and above all, efficacy is still an on going agenda for discussions and research.

1.4.1 TM/CAM in developed countries

For most developed countries, the regulation and legislation on TM/CAM are changing rapidly for administering the appropriate course of direction. In the U.S., the Food and Drug Administration (FDA) classified the natural products as dietary supplements. Manufacturers must label these products on mandatory basis with a statement “this product has not been evaluated by the U.S. FDA and it is not intended to diagnose, treat, cure or prevent any disease,” whether products are foods or drugs is undecided (Rousseaux & Schachter, 2003). In Australia, the Therapeutic Goods Administration (TGA) regulates the quality, safety and efficacy of
traditional medicine (Briggs, 2005). The Australian Regulatory Guidelines for Complimentary Medicines (ARGCM) on the Evaluation of Complementary Medicine Substances constitutes a model scheme of listing and registration in the assessment of complementary medicines which are as rigorous as for pharmaceutical drugs (Therapeutic Goods Administration, 2005). The manufacturers (local or overseas) are required to comply with Good Manufacturing Practice (GMP) licences approved by the TGA to authenticate their starting materials, testing of the products and auditing the product performance for herbal products that are intended for sale in the market. This mechanism of control does not scrutinise the raw herbs dispensed by Chinese medicine practitioners in Australia (McEwen & Cumming, 2003).

1.4.1.1 Official guidelines, codes of practices and pharmacopoeial standards

for herbal medicinal products available online or publications

There are various official guidelines, codes of practices, pharmacopoeial and monographic standards which have been established and aim to safeguard the quality, safety and efficacy of medicinal herbs based on the searchable databases in the website. These sites provide information which ranges from comprehensive scientific references, botanical sources, names & composition, commercial, sources of drugs, chemistry, reported traditional & modern uses, dosage range (both traditional & clinical trials), safety profiles, product information (availability, combinations, packaging, storage), pharmacology, pharmacokinetics and regulatory status. These are subject to revision with features as listed in Table 1.3. These standards are available and are useful reference sources for countries with limited resources.
<table>
<thead>
<tr>
<th>No.</th>
<th>Countries/Institutions</th>
<th>Guidelines/Codes/Pharmacopoeial Standards</th>
<th>Modified and/or adopted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Australia</td>
<td>Australian Code of Good Manufacturing Practice (GMP) for medicinal plants</td>
<td>Guidelines on manufacturing of herbal medicinal products.</td>
</tr>
<tr>
<td>2</td>
<td>India</td>
<td>Ayurveda, Unani and Siddha Pharmacopoeia of India in two volumes</td>
<td>Provide information on standards for 158 single plant drugs. The 3rd volume on 100 more single plant drugs is in preparation.</td>
</tr>
<tr>
<td>3</td>
<td>United Kingdom</td>
<td>British Herbal Pharmacopoeial standards</td>
<td>Provides monographs of quality standards for 169 herbs as botanical drugs in U.K.</td>
</tr>
<tr>
<td>4</td>
<td>Central Institute of Medicinal &amp; Aromatic plants (CIMAP)</td>
<td>Central Institute of Medicinal &amp; Aromatic plants (CIMAP) Patents</td>
<td>Provides information on patents filed, production processes, phytochemicals, development of herbal preparations &amp; screening processes for biological activities of medicinal and aromatic plants.</td>
</tr>
<tr>
<td>5</td>
<td>Australia</td>
<td>Complementary Medicines-Therapeutic Goods Administration (TGA), Australia</td>
<td>Regulatory requirements of registration, evaluation, use of complementary medicines, GMP requirements, guidelines for labels, kinds of evidence to support indications, claims, alerts.</td>
</tr>
<tr>
<td>6</td>
<td>Europe</td>
<td>European Pharmacopoeia [Pharmacopoeial standards] in both English and French.</td>
<td>4th Edition provides 1,606 monographs with 268 new monographs of active substances of pharmaceuticals for human, veterinary use, herbal drugs &amp; homeopathic preparations.</td>
</tr>
<tr>
<td>9</td>
<td>WHO</td>
<td>Final Draft: Good Manufacturing Practices: Supplementary</td>
<td>Addressing the procedures &amp; techniques in manufacturing &amp; quality</td>
</tr>
<tr>
<td></td>
<td>Guidelines for the manufacturing of herbal medicinal products</td>
<td>control of herbal medicines exclusively.</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>India Homoeopathy Pharmacopoeia of India [Pharmacopoeial standards]</td>
<td>Provides information on standards for the preparation &amp; test of identity, quality &amp; purity of Homoeopathic drugs based on American, German &amp; British Homoeopathic pharmacopoeial principles &amp; standards.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>International Conference on Harmonization (ICH) ICH-Good Manufacturing Practices for Active Pharmaceutical Ingredients [Good Manufacturing Practices guidelines]</td>
<td>Provide guidelines on manufacturing of active pharmaceutical ingredients (APIs) from plant sources i.e. herbal extracts &amp; comminuted or dried herbs in addition to other pharmaceutical ingredients.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>India Indian Herbal Pharmacopoeia revised edition 2002 [Pharmacopoeial standards] -Two volumes of 40 monographs on 20 medicinal plants by IDMA in collaboration with Regional Research Laboratory (RRL), Jammu.</td>
<td>New edition covers 52 monographs on Indian medicinal plants. Information on authentic data based scientific studies &amp; investigations conducted by institutions &amp; research laboratories.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>India Indian Patents on Neem [Patent] -Working on the issue at the core of a worldwide debate on who controls genetic resources-traditional cultures or transnational corporations</td>
<td>Information on the patents filed on properties, active principles, their extraction &amp; stabilizing processes in US and Japan. Granted over 30 patents.</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>United Kingdom Licensing of Medicines: Policy on Herbal Medicines [Good</td>
<td>Provides information on regulatory requirements for licensing herbal</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Country</td>
<td>Description</td>
<td>Description</td>
</tr>
<tr>
<td>-----</td>
<td>------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>17</td>
<td>India</td>
<td>Patent Facilitating Center (PFC) [Patent]</td>
<td>Provides information about patents on processing &amp; manufacturing of herbal medicinal products &amp; their therapeutic uses.</td>
</tr>
<tr>
<td>19</td>
<td>United States of America</td>
<td>The American Herbal Pharmacopoeia &amp; Therapeutic Compendium-Analytical, Quality Control, &amp; Therapeutic Monographs [Monographic standard]</td>
<td>Provides information on safety &amp; efficacy of botanicals used as dietary supplements &amp; Chinese herbal medicines.</td>
</tr>
<tr>
<td>22</td>
<td>China</td>
<td>The People’s of China Pharmacopoeia [Pharmacopoeial standards]-Two volumes</td>
<td>The official &amp; authoritative compendium of drugs of 992 monographs of Traditional Chinese Medicines &amp; Western Drugs.</td>
</tr>
<tr>
<td>26</td>
<td>WHO</td>
<td>WHO Monographs on Selected Medicinal Plants [Monographic standards]</td>
<td>Comprehensive scientific reference for developing countries to develop national monographs on medicinal plants.</td>
</tr>
</tbody>
</table>
1.4.2 TM/CAM in developing countries

Therefore, with the existing framework of regulatory and legislative control on TM/CAM, developed and developing countries experience issues which may be similar or different in nature but of different magnitudes. Table 1.4 listed the countries with fully integrative approach to TM/CAM as compiled by World Health Organization (WHO, 2002b). These countries have long history of oriental practices and traditional use of natural remedies which has become fully integrated from generation to generation. On the other hand, for those countries which are listed in Table 1.5, there exists a broad set of health care practices which are already available to the public that are not readily integrated into the dominant health care model because they challenge dominant societal beliefs and practices such as cultural, economic, scientific, medical, and educational (Clark, 2000).
<table>
<thead>
<tr>
<th>Names of Countries</th>
<th>National policy on TM/CAM</th>
<th>TM/CAM Department in Ministry of Health</th>
<th>Regulation TM/CAM products/industries</th>
<th>TM/CAM National Research Institutes</th>
<th>Formal Institutional Education of TM/CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>1949 constitution contains policy on TM</td>
<td>State Administration of Traditional and Complementary Medicine (TCM)</td>
<td>-Regulation (Yes) -Chinese herbal Pharmacopoeia -Essential Drug List including herbals -Regulatory control on manufacturers, herbal farmers</td>
<td>170 National and state research institutes</td>
<td>Universities; Colleges; Medical technology schools of TCM</td>
</tr>
<tr>
<td>Republic of Korea</td>
<td>National policy on TM 1969</td>
<td>Oriental Medicine Bureau</td>
<td>-Regulation (Yes) -Herbal Pharmacopoeia</td>
<td>1 National research institute</td>
<td>11 oriental medicine universities</td>
</tr>
<tr>
<td>Vietnam</td>
<td>National policy on TM 1955</td>
<td>Department of TM</td>
<td>-Regulation (Yes) -Essential Drug List including herbals -State manufacturers</td>
<td>3 National research institutes</td>
<td>Colleges; Medical technology schools of TM</td>
</tr>
</tbody>
</table>
Table 1.5 Countries with an inclusive (recognized) approach to TM/CAM
(Ref. Sources: Compiled by WHO Traditional medicine Strategy 2002-2005)

<table>
<thead>
<tr>
<th>Names of Countries</th>
<th>National policy on TM/CAM</th>
<th>TM/CAM Department in Ministry of Health</th>
<th>Regulation of TM/CAM or herbal products or both</th>
<th>TM/CAM National Research Institutes</th>
<th>Formal Institutional Education of TM/CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>No</td>
<td>Yes (in some states)</td>
<td>Herbal products</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Canada</td>
<td>Yes</td>
<td>Yes</td>
<td>Both</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Germany</td>
<td>No</td>
<td>No</td>
<td>Both</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Ghana</td>
<td>Yes</td>
<td>Yes</td>
<td>Both</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>India</td>
<td>Yes</td>
<td>Yes</td>
<td>Both</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Indonesia</td>
<td>Yes</td>
<td>Yes</td>
<td>Both</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Japan</td>
<td>No</td>
<td>No</td>
<td>Both</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Nigeria</td>
<td>Yes</td>
<td>Yes</td>
<td>Both</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Norway</td>
<td>Yes</td>
<td>Staff in charge</td>
<td>Both</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Sri Lanka</td>
<td>Yes</td>
<td>Yes</td>
<td>Both</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>Yes</td>
<td>No</td>
<td>Both</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>USA</td>
<td>No</td>
<td>No</td>
<td>Both</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Brunei Darussalam</td>
<td>No</td>
<td>No</td>
<td>Guidelines on the import of Traditional medicine</td>
<td>National Herbarium Drug Museum, Department of Agriculture, Ministry of Industry &amp; Primary Resources, Brunei Darussalam</td>
<td>No</td>
</tr>
</tbody>
</table>
1.4.3 Recent development of plant natural products

The application of modern scientific technologies on selective analyses, biological assay and quality control assessments and evaluations of plant natural products are not usually straightforward due to the complexities of crude plant extracts which are not standardised.

Numerous studies have shown that natural plant products are a rich source of novel compounds. Although isolation, identification, characterization, quantification of phytochemicals and evaluation of their potential benefits were undertaken on selected and prospective plant species, only some plant species actually were developed as pharmacotherapeutic drugs.

For instance, the anti-cancer drugs such as the *vinblastine*, *vincristine* alkaloids isolated from *Catharanthus roseus*, *topotecan* which is a *camptothecin* analogue from *Camptotheca acuminata*, *etoposide* and *teniposide* (semisynthetic analogs of *epipodophyllotoxin*) isolated from *Podophyllum peltatum*. *Taxol*, a more recent significant drug isolated from the bark of *Taxus brevifolia* and currently newer plant derived pharmacophores are still undergoing different phases of clinical development. Flavopiridol, a flavone isolated from *Dysoxylum binectiferum* (Shapiro *et al.*, 2001), *genestein* from soyabeanse (Wang, 2000), indole 3-carbinol from cruciferous vegetables i.e. Brussels sprout and broccoli, *curcumin* from *curcuma root* (Bradlow *et al.*, 1999), *resvertrol* from red wine (Bhat & Pezzuto, 2002) and *epigallocatechin* from green tea (Fujiki *et al.*, 2002), *homoharringtonine* from the Chinese tree *Cephalotaxus harringtonia* (Kantarjian *et al.*, 2001) and many others that are continually undergoing anti-cancer research such as *Andrographolide*, isolated from *Andrographis paniculata* (*Burm. f.*) *Nees* (Rajagopal *et al.*, 2003).
Currently, a traditional Chinese Medicine labelled PHY906 was firstly described about 1,800 years ago is still under phase II clinical trial study in order to provide evidence based clinical claims for the treatment of hepatocellular carcinoma as conducted by PhytoCeutica Inc. in USA (Cheng, 2005).

The novel study on *Qinghaosu* (*Artemisia annua L.*) containing *Arteannuin* known as ‘sweet wormwood’ in english has been developed commercially into anti-malarial drug which is effective for anti-chloroquinines malaria (WHO, 1997). This further advances into the largest clinical trial on 1500 patients which was conducted in Bangladesh, Burma, India and Indonesia funded by the Wellcome Trust which compared the effectiveness of two plant-derived drugs; Quinine as currently the drug of choice for severe malaria in most malaria-affected regions, against artesunate, a drug derived from artemisinin. The results were compelling in which artesunate reduced mortality by 35% and was found to be safer and easier to administer (Dondorp et al., 2005). It is evidental that research and development on the plant natural products will provide avenues for the development of future therapeutically active compounds which are beneficial in the treatment of many disease conditions.

### 1.5 Classifications of TM/CAM

Hawks and Moyad (2003) have catogorised CAM into five types or classifications of therapies. These are alternative medical systems, mind body intervention, biologically based therapies, manipulative, body-based methods and energy therapies (Hawks & Moyad, June 2003). The major forms of traditional medicine on the therapeutic basis are the procedure-based therapies which include acupuncture, chiropractic, osteopathic, energy enhancement known as qigong,
taiji, yoga, dietary advice, massage & exercise techniques, physical, mental, spiritual, mind-body therapies and all the other therapies that are pertaining to improve the well-beings of the person.

In the application of these therapeutic procedures, the use of traditional medicine products derived from plant, animal and mineral based-materials as adjuncts are also prescribed for patients on complementary therapies. Table 1.6 listed the categories of TM/CAM disciplines in the United Kingdom (Mills, 2001) as compared to the major forms of TM/CAM illustrated in Table 1.7 (Zhang, 2001).
Table 1.6 Categories of Complementary (CAM) and alternative therapies of United Kingdom

(Ref. Source: (Mills, 2001).

<table>
<thead>
<tr>
<th>Categories of CAM in United Kingdom</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
</tr>
<tr>
<td>1.1 Professionally organised alternative therapies</td>
</tr>
<tr>
<td>Acupuncture</td>
</tr>
<tr>
<td>Chiropractic</td>
</tr>
<tr>
<td>Herbal medicine</td>
</tr>
<tr>
<td>Homeopathy</td>
</tr>
<tr>
<td>Osteopathy</td>
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<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Group 3 Alternative disciplines**

<table>
<thead>
<tr>
<th><strong>3.1 Traditional systems of healthcare</strong></th>
<th><strong>3.2 Other Alternative disciplines</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthroposophical medicine</td>
<td>Crystal therapy</td>
</tr>
<tr>
<td>Ayurvedic medicine</td>
<td>Dowsing</td>
</tr>
<tr>
<td>Chinese herbal medicine</td>
<td>Iridology</td>
</tr>
<tr>
<td>Eastern medicine</td>
<td>Kinesiology</td>
</tr>
<tr>
<td>Naturopathy</td>
<td>Radionics</td>
</tr>
<tr>
<td>Traditional Chinese Medicine (TCM)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.7 Categories of TM/Complementary (CAM) and alternative therapies of WHO

<table>
<thead>
<tr>
<th>Categories of TM/CAM of WHO (Zhang, 2001)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
</tr>
<tr>
<td>Major forms of TM/CAM – Therapeutic Basis</td>
</tr>
<tr>
<td>1.1 Medication</td>
</tr>
<tr>
<td>Medicinal plants</td>
</tr>
<tr>
<td>Mineral materials</td>
</tr>
<tr>
<td>Animal materials</td>
</tr>
<tr>
<td>Manual therapies</td>
</tr>
<tr>
<td>Qigong, Taiji, Yoga</td>
</tr>
<tr>
<td>Physical, mental, spiritual and mind-body therapies</td>
</tr>
</tbody>
</table>
1.6 Important key issues in TM/CAM

Traditional medicine in particularly herbal medicines which formed the basis of health care throughout the world since the beginning of mankind are still used by many communities and have considerable importance in the world trade. Medicinal plants are important for the pharmaceutical research and development, in the form of starting materials for the drug synthesis or as models for designing pharmacologically active compounds and in most instances as plant constituents taken directly as therapeutic agents for many disease conditions. Research and development on traditional medicine is usually taxing on time, financial and manpower resources.

The United Nations Convention on Biological Diversity stipulated that the conservation and sustainable use of biological diversity is of critical importance for ensuring that the needs of the growing world population are met on the basis of sharing both genetic resources and technologies worldwide (Jayasuriya, 1990).

The regulatory and legislative controls on TM/CAM vary widely from country to country. Most countries have prioritised and adopted various appropriate approaches to address the important key issues such as licensing, dispensing, manufacturing, trading systems to ensure the quality, safety and efficacy of TM/CAM in order to safeguard public health. With the paradigm shift in the evidence-based Western medicines, interest into research and development on TM/CAM encompasses education, practices, training and access to the world population to ensure the appropriate delivery of TM/CAM alongside mainstream healthcare is expanding. Development and research on the key strategies to improve research rationales, promote collaborative
relationships between researchers and generate key resources to fund priority research are among
some of the important challenges of TM/CAM (Bensoussan, 2005).

The quality assurance and control measures such as the guidelines on the specifications and
standards on good manufacturing practices (GMP), good clinical practices (GCP), good
laboratory practices (GLP), good agricultural and collection practices (GACP) of the starting
materials of herbal origin to the complex finished end products and various other guidelines
which are needed to ensure a steady, affordable and sustainable supply of medicinal plant
materials of quality, safety and efficacy (WHO, 2003). The progress on the quality assurance and
quality control on herbal medicines from its starting raw materials right through to its complex
finished end products is a mammoth task. As it is also known that the efficacy of herbal
medicines has a characteristic of a complex mixture of constituents present in a single herb and a
combination of herbs or medicinal plants are used together for the treatment of many ailments.

Such practices pose many difficulties in the quality assurance and control evaluations and
assessments of herbal medicines in order to ensure quality, safety and efficacy of the herb or
herbal preparations (Liang et al., 2004). Such a scenario calls for the collaborative efforts from a
multidisciplinary approach such as biochemistry, molecular biology, and cell biology in
establishing quantifiable and reproducible bioassays to provide assurance of quality, safety,
efficacy and consistency. Research and development across the board of interdisciplinary
approach among many researchers, hoping to discover the novel therapeutics which is comprised
of multiple chemical constituents is still a long journey.
1.7 Pharmacovigilance of TM/CAM

Worldwide efforts have been made recently to monitor possible adverse reactions and drug-herb interactions to various TM/CAM products. As a result of the increasing pressure to regulate these products to pharmaceutical industry standards of quality and safety, the challenge for the regulatory authorities and TM/CAM industry is to provide a level of postmarketing surveillance to the equivalent level of pharmacovigilance of pharmaceuticals. A total of 16,154 suspected herbal case reports were compiled in the WHO database and the most common conditions reported are illustrated in Table 1.8 (Mohamed, 2006). In line with the WHO guidelines on safe monitoring of herbal medicines, the Ministry of Health Brunei Darussalam, alike many countries have embarked on the monitoring of the adverse effects associated with TM/CAM based on the similar mechanism of pharmacovigilance activities of the WHO Collaborating Centre at Uppsala, Sweden.

The regulators worldwide face the biggest challenge in the necessary assessment and reviewing of traditional medicine to safeguard public health but as more stringent criteria and greater obstacles are imposed on pharmaceuticals and the greater is the temptation and tendency for the industries to exploit TM/CAM in the light of commercial interest of gain. As it is mandatory for western medicines to declare their side effects and toxicities, coupled with the pharmacovigilance activities which seems to place the risks over the benefits, this tends to push the consumers turning towards traditional medicine which in most cases usually claim to be safer and effective with lesser emphasis on risks.
For many years, researches have been trying to provide evidence-based medicines from the traditional medicine but most are unattainable or unsuccessful as it is not an easy and simple task at all. Therefore, just because traditional medicine contains natural ingredients in a product does not necessarily mean it is an automatic proof of that product’s safety and efficacy. At the same time, one should also remember that many important modern drugs are derived from plant, animal, or mineral origins. As a result, a better understanding of the traditional medicine can help to perform benefit/risk analysis for the patients.

The biggest challenging question among all key players is still “quality, safety and efficacy”. Such scenario calls for a concerted effort among key players in the field of traditional medicine with the objective to ensure the continuity of good complementary health care to the patients, consumers and public at large with confidence and minimal risks involved.

### Table 1.8 Suspected herbal adverse reactions case reports in WHO database.

<table>
<thead>
<tr>
<th>Commonly reported reactions</th>
<th>Most commonly reported critical terms for ADRs on herbal adverse reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pruritus</td>
<td>Hypertension</td>
</tr>
<tr>
<td></td>
<td>829</td>
</tr>
<tr>
<td>Rash</td>
<td>Hepatitis</td>
</tr>
<tr>
<td></td>
<td>751</td>
</tr>
<tr>
<td>Urticaria</td>
<td>Face oedema</td>
</tr>
<tr>
<td></td>
<td>751</td>
</tr>
<tr>
<td>Rash erythematous</td>
<td>Death</td>
</tr>
<tr>
<td></td>
<td>733</td>
</tr>
<tr>
<td>Nausea</td>
<td>Angioderma</td>
</tr>
<tr>
<td></td>
<td>682</td>
</tr>
<tr>
<td>Vomiting</td>
<td>Convulsions</td>
</tr>
<tr>
<td></td>
<td>561</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>Chest pain</td>
</tr>
<tr>
<td></td>
<td>546</td>
</tr>
<tr>
<td>Headache</td>
<td>Thrombocytopenia</td>
</tr>
<tr>
<td></td>
<td>501</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>Purpura</td>
</tr>
<tr>
<td></td>
<td>497</td>
</tr>
<tr>
<td>Fever</td>
<td>Dermatitis</td>
</tr>
<tr>
<td></td>
<td>460</td>
</tr>
</tbody>
</table>
1.7.1 Adverse reactions

An adverse reaction is defined by the Uppsala WHO Monitoring Centre as ‘a response to a medicine which is noxious and unintended, and which occurs at doses normally used in man’. Such description is of importance that it concerns the response of a patient, in which individual factors may play an important role, and that the phenomenon is noxious (an unexpected therapeutic response, for example, may be a side effect but not an adverse reaction).

An adverse event or experience is defined as ‘any untoward medical occurrence that may present during treatment with a medicine but which does not necessarily have a causal relationship with this treatment’. In this case, it is the coincidence in time without any suspicion of a causal relationship. With the rapid increase in the worldwide use of TM/CAM, unfortunately the number of reports on adverse drug reactions or events with negative health consequences on consumers which affect the quality of life and in certain cases resulting in death, is also on the increase (WHO, 2000).

It is a difficult task however, in ascertaining the causality of the adverse reactions in association with the TM/CAM products in many cases as experienced by most national collaborating centres worldwide. Assessment of adverse reaction case reports in accordance with the procedures of pharmaceutical assessments requires combined expertise in specialised fields such as clinical medicine, pharmacology, toxicology and epidemiology. And specialists in such fields are limited although great interest in the research of establishing the benefit of TM/CAM versus risks ratio is increasing. Polypharmacy is a common practice in many countries with self medicated community, the increased incidence of adverse reactions is further complicated by the widespread use of polyherbal combinations. Rational use of drugs and traditional medicine
require, among others, adequate assessment of the benefit versus risk. Well informed consumers demand continuous monitoring of drugs or traditional medicine for any adverse events throughout the drug’s or traditional medicine’s commercial life span as the educated public of today is very much involved in making their health care decisions.

Several reports have been published on the adverse effects of herbal and traditional remedies and the causality assessments of these reports are difficult to establish. With the establishment of the WHO Collaborating Centre for International Drug Monitoring, Uppsala Monitoring Centre in 1968 focusing on the safety monitoring of medicinal products which also encourage the reporting of adverse events experienced by practitioners and consumers who are using traditional remedies. Currently, there are more than 3.7 million suspected adverse reactions in the WHO database.

In July, 2006, a total of 81 countries had joined the WHO Drug Monitoring Programme with another 17 countries as associate members (WHO, 2006). This figure is on the increase which serves as an indicator that many countries are concern about safety issues and the way forward would be to conduct pharmacovigilance activities. Such reporting mechanism on safety issues is very important for the prevention of drug or traditional remedies induced human suffering which affect the quality of life and also to avoid financial risks associated with unexpected adverse effects.

Therefore, traditional remedies are of no exception as these remedies are consumed with the notion that it is for improving quality of life, but not necessarily risks free. We should make efforts to prevent tragedy of Thalidomide in 1960’s (deformities of limbs) or Mu Tong in 1990’s (kidney failure) or Slim 10 (herbal slimming product adulterated with thyroid gland extracts and
Fenfluramine) that resulted in liver failure and eventual death tragedy. According to the survey conducted by the government and restructured hospitals of the Ministry of Health Singapore in 1994, there were 154 cases of complications arising from traditional medicine treatment between August 1994 and January 1995 (Tan, 2005). Table 1.9 illustrated some of the adverse effects compiled by the authorities as a result of drug interactions or due to the scheduled poisons such as steroids which can result in some serious complications (Koh & Woo, 2000).

It was also highlighted that more recent concerns in the use of traditional medicine which poses potential interactions when use in conjunction with prescription or over-the-counter medicines (Smith, 2005). The need for scientific information on the toxicity and interaction potential of the herbal medicines and the dissemination of their safety profile to the practitioners/prescribers and patients for ensuring safety is of paramount importance (Majewski & Ahokas, 2005). Long term toxicity associated with the chronic use of traditional medicine remains a major issue in the absence of safety data of traditional medicine.
Table 1.9  Names of TM/CAM products with brands or manufacturer’s names where available, and the Scheduled Poisons detected (compiled by the Pharmacy Enforcement Unit, Ministry of Health Brunei Darussalam, 2002).

<table>
<thead>
<tr>
<th>No.</th>
<th>TM/CAM products (brand and/or manufacturer’s names)</th>
<th>Scheduled Poisons detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Air Akar-Akar Kayu Asli</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>2</td>
<td>Air Ikan Haruan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>3</td>
<td>Akarsom</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>4</td>
<td>Akarsom Jawa Asli</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>5</td>
<td>Ba Bao Feng Shi Huo Luo Dan</td>
<td>Diclofenac</td>
</tr>
<tr>
<td>6</td>
<td>Bai Feng Wan with Ginseng &amp; Pearls</td>
<td>Berberine</td>
</tr>
<tr>
<td>7</td>
<td>Bansetong Rheumatic Pill</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>8</td>
<td>Bantulin Rheumatic Pill</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>9</td>
<td>Benzi-Lin</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>10</td>
<td>Bestrim</td>
<td>Fenfluramine</td>
</tr>
<tr>
<td>11</td>
<td>Chinccough Caps</td>
<td>Dextromethorphan</td>
</tr>
<tr>
<td>12</td>
<td>Chinicough Caps</td>
<td>Dextromethorphan</td>
</tr>
<tr>
<td>13</td>
<td>Chin Toon Lin Rheumatic Caps</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>14</td>
<td>Chu Fong Kee Sak Wan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>15</td>
<td>Chu Fong Kee Sar Wan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>16</td>
<td>Chufeng Toukuwan</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>17</td>
<td>Chui Fong Toukuwan Caps</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>18</td>
<td>Dahuo Luodan</td>
<td>Berberine</td>
</tr>
<tr>
<td>19</td>
<td>Dr Yap Condensed Honey</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>20</td>
<td>Dura Tonic 20ml</td>
<td>Sildenafil</td>
</tr>
<tr>
<td>21</td>
<td>FB Slymer</td>
<td>Fenfluramine</td>
</tr>
<tr>
<td>22</td>
<td>Fuchingsong Shaiodu Chie Yang Caps (Moon Bells Brand)</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>23</td>
<td>Fukuwan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>24</td>
<td>Gan Man Wan</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>25</td>
<td>Gen Tong Ping</td>
<td>Tetrahydropalmatine</td>
</tr>
<tr>
<td>26</td>
<td>Ginkutan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>27</td>
<td>Ginseng G Pontocyn</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>28</td>
<td>Ginseng Jachiowan</td>
<td>Diazepam, Dexamethasone</td>
</tr>
<tr>
<td>29</td>
<td>Ginseng Lin-Zi Calyco Pil</td>
<td>Cyproheptadine, Dexamethasone</td>
</tr>
<tr>
<td>30</td>
<td>Ginseng Lopa Kupuwan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>31</td>
<td>Ginseng Zaizaowan</td>
<td>Berberine</td>
</tr>
<tr>
<td>32</td>
<td>Glaxi Rheumatic Caps</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>33</td>
<td>Gu Ben Wan</td>
<td>Indomethacin, Hydrochlorothiazide, Diazepam, Dexamethasone, Prednisolone, Caffeine</td>
</tr>
<tr>
<td>34</td>
<td>Guci Dih Mai Ji Wan</td>
<td>Promethazine</td>
</tr>
<tr>
<td>35</td>
<td>Herba Sari Resdung</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>36</td>
<td>Hoong Heng Tse Koo Choy</td>
<td>Santonin</td>
</tr>
<tr>
<td>37</td>
<td>Hukutan Rheumatic Caps</td>
<td>Indomethacin</td>
</tr>
<tr>
<td>38</td>
<td>Hu Ku Wan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>39</td>
<td>Huo Lu Wan Caps</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>40</td>
<td>Jamu Ajaib</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>41</td>
<td>Jamu Jarum Emas Cap Jarum Emas</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>42</td>
<td>Jamu Kapsul Tupai Jantan</td>
<td>Betamethasone</td>
</tr>
<tr>
<td>43</td>
<td>Jamu Madura</td>
<td>Betamethasone</td>
</tr>
<tr>
<td>44</td>
<td>Jamu Sumber Sehat Sesak Nafas</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>45</td>
<td>Jawa Tonik Akarsom</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>46</td>
<td>Jung Fei Pill</td>
<td>Cyproheptadine</td>
</tr>
<tr>
<td>47</td>
<td>Kapsul Farlin Jamu Cap Farlin</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>48</td>
<td>Kapsul Pahlawan Chap 100 plus Bagus</td>
<td>Prednisolone</td>
</tr>
<tr>
<td></td>
<td>Product Name</td>
<td>Main Ingredient(s)</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>49</td>
<td>Life Blood Medicine</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>50</td>
<td>Linzetan</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>51</td>
<td>Loong Tan Rheumatism Caps</td>
<td>Cyproheptadine, Dexamethasone</td>
</tr>
<tr>
<td>52</td>
<td>Maajun 500</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>53</td>
<td>Maajun Ajaib</td>
<td>Prednisolone</td>
</tr>
<tr>
<td>54</td>
<td>Maajun Ajaib Ikan Linang dan Korean Ginseng Cap Korean Ginseng</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>55</td>
<td>Maajun Kuat</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>56</td>
<td>Maajun Lebah</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>57</td>
<td>Maajun Traditional Cap Bulan</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>58</td>
<td>Makjun Kuat Tupai Jantan</td>
<td>Betamethasone</td>
</tr>
<tr>
<td>59</td>
<td>Makjun Lebah</td>
<td>Betamethasone</td>
</tr>
<tr>
<td>60</td>
<td>Makjun Tupai Jantan</td>
<td>Betamethasone</td>
</tr>
<tr>
<td>61</td>
<td>Man Che Too Chong Chooi Fong Wan</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>62</td>
<td>Nasalin High Strength</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>63</td>
<td>Nasalin Tab</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>64</td>
<td>Oleiviticus Negundo Caps</td>
<td>Theophylline</td>
</tr>
<tr>
<td>65</td>
<td>Pearl &amp; Antelope Throat Powder (spray)</td>
<td>Berberine</td>
</tr>
<tr>
<td>66</td>
<td>Pegalin Ekstrak</td>
<td>Betamethasone</td>
</tr>
<tr>
<td>67</td>
<td>Pien Tze Huang Sore Throat Powder</td>
<td>Berberine</td>
</tr>
<tr>
<td>68</td>
<td>Pemin Kan Wan (nasal clear)</td>
<td>Dex Chlorpheniramine</td>
</tr>
<tr>
<td>69</td>
<td>Pil Majun</td>
<td>Betamethasone</td>
</tr>
<tr>
<td>70</td>
<td>Primadona Pegal Linu</td>
<td>Mefenamic Acid, Phenylbutazone</td>
</tr>
<tr>
<td>71</td>
<td>Pytazone</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>72</td>
<td>Qiangli Kuben Su Caps</td>
<td>Methyltestosterone</td>
</tr>
<tr>
<td>73</td>
<td>Rheumatism Pill</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>74</td>
<td>S-Magon Morning and Night Tab</td>
<td>Theophylline, Promethazine</td>
</tr>
<tr>
<td>75</td>
<td>S-Magon Tab</td>
<td>Betamethasone</td>
</tr>
<tr>
<td>76</td>
<td>Sedolin-C Caps</td>
<td>Phenylbutazone, Prednisolone</td>
</tr>
<tr>
<td>77</td>
<td>Sepilin Rheumatism Caps</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>78</td>
<td>Sewtonlin</td>
<td>Codeine, Promethazine</td>
</tr>
<tr>
<td>79</td>
<td>Sha Hsien Chin Ointment</td>
<td>Clotrimazole</td>
</tr>
<tr>
<td>80</td>
<td>Shenchin Herb caps</td>
<td>Diazepam, Ibuprofen</td>
</tr>
<tr>
<td>81</td>
<td>Shen Po Caps</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>82</td>
<td>She Xiang Zhui Feng Tou Gu Wan</td>
<td>Diclofenac</td>
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<td>83</td>
<td>Shebanlin Caps</td>
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</tr>
<tr>
<td>84</td>
<td>Shetonlin Caps</td>
<td>Phenylbutazone, Prednisolone</td>
</tr>
<tr>
<td>85</td>
<td>Shewtonlin</td>
<td>Codeine, Promethazine</td>
</tr>
<tr>
<td>86</td>
<td>Siow Lim Rheumaryin Caps</td>
<td>Cyproheptadine, Dexamethasone, Prednisolone</td>
</tr>
<tr>
<td>87</td>
<td>Sitonpoh</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>88</td>
<td>Skimtan</td>
<td>Berberine</td>
</tr>
<tr>
<td>89</td>
<td>Slim 10</td>
<td>Fenfluramine</td>
</tr>
<tr>
<td>90</td>
<td>Speman Forte</td>
<td>Reserpine</td>
</tr>
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<td>91</td>
<td>Sukenlo</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>92</td>
<td>Sumber Rimba Majon Tongkat Ali</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>93</td>
<td>Super Rheumatic Caps</td>
<td>Prednisolone</td>
</tr>
<tr>
<td>94</td>
<td>Super Tunglin</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>95</td>
<td>Suxiao Gan Mao Pian</td>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>96</td>
<td>Tablet Cap Wau</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>97</td>
<td>Tackolin Caps</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>98</td>
<td>Te Xiao Zhi (Zhu Cheng Brand)</td>
<td>Berberine</td>
</tr>
<tr>
<td>99</td>
<td>Tean Mao Tou Chong Pill</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>100</td>
<td>Thien Ma Toh Chung Chen Kuo Won</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>101</td>
<td>Thien Ma To Jung Rheumatic</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>102</td>
<td>Tian Ma Tou Gu Wan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>103</td>
<td>Tiean Ma Shoo Chin Wan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>104</td>
<td>Toh Teong Par Kit Chap</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>105</td>
<td>Tongkat Ali Super Power</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>106</td>
<td>Tongkat Ali Tonik Mujarab Cap Bulan Kris</td>
<td>Dexamethasone</td>
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<td>Tonpilin Tab</td>
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<td>Tonik Jawa Akarsom</td>
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<td>Tonik Semangat</td>
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<td>112</td>
<td>Tonik Semangat Asli</td>
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</tr>
<tr>
<td>113</td>
<td>Tsaitsaowan (Ginseng Hui Sheng)</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>114</td>
<td>Tu Chon Fu Kuo Wan</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>115</td>
<td>Tuchong Tianmar Huko Wan</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>116</td>
<td>Tung Shueh Wan Caps</td>
<td>Diazepam, Indomethacin, Prednisolone</td>
</tr>
<tr>
<td>117</td>
<td>Tunglin Caps</td>
<td>Dexamethasone, Phenylbutazone</td>
</tr>
<tr>
<td>118</td>
<td>Ubat Demam Kanak-kanak</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>119</td>
<td>Ubat Sakit Pinggang jamu (Toko Air Pancur, Indonesia)</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>120</td>
<td>Ungentum Flucciononidi</td>
<td>Fluocinonide</td>
</tr>
<tr>
<td>121</td>
<td>101 Wei Yao Ling (Mei Hua Brand)</td>
<td>Diclofenac</td>
</tr>
<tr>
<td>122</td>
<td>Wei Loong Oral Solution 20ml</td>
<td>Sildenafil</td>
</tr>
<tr>
<td>123</td>
<td>Wuchaseng Wan Caps</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>124</td>
<td>XC Rheumatic Pill</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>125</td>
<td>Zhen Gu Wan</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>126</td>
<td>Zhen Zhu Tou Tong Ling (Fai Yien Pai Brand)</td>
<td>Diclofenac</td>
</tr>
<tr>
<td>127</td>
<td>Zhong Gan Ling (Yang Cheng Brand)</td>
<td>Dipyrrone</td>
</tr>
<tr>
<td>128</td>
<td>Zhung Gu Wan</td>
<td>Dexamethasone</td>
</tr>
</tbody>
</table>
There have been many adverse reactions reported since 1997 on the safety issues concerning the adverse reactions associated with the use of herbal medicines in Australia (Drew & Myers, 1997) caused by a number of reasons as illustrated by examples in Table 1.10.

Table 1.10 Reasons why adverse reactions are associated with TM/CAM

<table>
<thead>
<tr>
<th>No.</th>
<th>Reasons</th>
<th>Examples</th>
<th>References</th>
</tr>
</thead>
</table>
| 1   | Misidentification:  
-Herbal ingredients or products with confusion between species. | *Angelica polymorpha* or *Angelica archangelica* species due to Chinese herbal name is “dong quai or danggui or tang kuei”. | (But, 1993) |
| 2   | Lack of standardisation:-  
-Therapeutic/toxic components of plants may vary depending on:  
-plant used,  
-stage of ripeness,  
-geographical location of growth and storage conditions, etc | Content of *ginsenoside* varied from (1.9% to 9% w/w) in 44 products:  
-Six products with absence of active component ginsenoside;  
-One product contains a large amount of ephedrine. | (Cui *et al.*, 1994) |
| 3   | Contaminations of products:  
-by pesticide residues,  
-microorganisms,  
-aflatoxins,  
-radioactive substances and heavy metals such as lead, cadmium, mercury, arsenic and thalium | Toxic heavy metals detected in the products:  
-Lead,  
-Mercury,  
-Arsenic,  
-Copper. | (Bisset, 1994; Koh & Woo, 2000) |
<table>
<thead>
<tr>
<th></th>
<th>Substitution of herbs either intentional or mistakenly due to the herbs being look alike</th>
<th>Aristolochia fangchi containing the nephrotoxic component aristolochic acid in the place of Stephania tetrandra resulted in terminal renal failure.</th>
<th>(Vanhaelen et al., 1994; Vanherweghem J-L et al., 1993)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Adulteration with pharmaceuticals and chemicals intentionally by the unethical herbalists or the manufacturers/distributors for commercial gains in their compounded preparations for individual patients which resulted in severe adverse reactions.</td>
<td>Adulteration of pharmaceuticals Scheduled Poisons containing diazepam and mefenamic acid in “Tung Shueh” pills for arthritic complaints</td>
<td>(Diamond &amp; Pallone, 1994; Pharmacy Enforcement Unit, 2002).</td>
</tr>
<tr>
<td>5</td>
<td>Incorrect preparation/dosage due to failure of non-compliance of the patients/consumers</td>
<td>Failure to comply with herbalist’s specific instruction to boil aconite accordingly. Increased the dose &amp; shortened the boiling time, unable to reduce the toxicity of alkaloids timely</td>
<td>(De Smet PAGM &amp; Tognoni, 1992)</td>
</tr>
</tbody>
</table>
Chapter 2 Development of Strategic Framework on TM/CAM for Brunei Darussalam

2.1 Brunei Darussalam – The Abode of Peace

2.1.1 Geographical description

2.1.2 The National Heritage of Brunei Darussalam

2.1.2.1 Tropical Medicinal Native Plants of Brunei Darussalam

2.1.3 Challenges of TM/CAM in Brunei Darussalam – A Bruneian Viewpoint

2.1.3.1 The practice of TM/CAM in the Primary Health Care of Brunei Darussalam

2.1.3.2 TM/CAM care system in Brunei Darussalam

2.2 Policy on TM/CAM

2.2.1 Existing guidelines of the Ministry of Health Brunei Darussalam on TM/CAM

2.2.2 Legislative and regulatory control of drugs (pharmaceuticals) and TM/CAM of Ministry of Health Brunei Darussalam

2.2.3 Legislations and Regulatory control on medicinal products (drugs/pharmaceuticals)

2.3 Definition of products in the official guidelines

2.3.1 Drug or medicine (pharmaceutical)

2.3.2 Herbal remedies

2.3.3 Homeopathic medicines

2.4 Official guidelines on the importation and regulatory control of TM/CAM in Brunei Darussalam

2.5 Medicinal Native Plants of Brunei Darussalam

2.5.1 Community use of medicinal plants in Brunei Darussalam
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<th>Title</th>
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<td>2.9.3</td>
<td>Research and development strategies on TM/CAM</td>
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<td>2.9.4</td>
<td>Herbal pharmacovigilance activities</td>
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<td>2.9.5</td>
<td>Collaborations on the exchange and sharing of information on TM/CAM</td>
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<td>2.9.6</td>
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<tr>
<td>2.10</td>
<td>Guidelines on the Product Listing</td>
<td>78</td>
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</tbody>
</table>
2.10.1 Product listing of TM/CAM

2.10.2 Implementation of quality assurance on traditional medicine

2.10.3 Monitoring control/inspection system

2.10.4 Post Marketing Surveillance (P.M.S)

2.10.5 Clinical evaluation and assessments

2.10.6 Identification and development of a model list of medicinal plants used in Primary Health Care (P.H.C.)

2.10.7 Implementation of a national herbal formulary for more effective and proper use of TM/CAM

2.10.8 Research and development strategies

2.10.9 Collaborative and integrative roles of relevant ministries in the research and development of TM/CAM

2.10.10 Financial support & Funding mechanism

2.10.11 Public education on the proper use of TM/CAM
2.1 Brunei Darussalam – The Abode of Peace

2.1.1 Geographical description

Brunei Darussalam is situated on the north-west coast of the island of Borneo and lies in the heart of South East Asia. It is a peaceful and beautiful country close to the equator between east longitudes 114° 04’ and 11° 23’ and north latitudes 04° 00’ and 05° 05’. It has a total land area of 5,765 square kilometres and a coastline of about 161 kilometres and bounded on the north by the South China Sea, on the other sides by the Malaysian state of Sarawak. It comprises of four districts namely the Brunei/Muara, Tutong, Kuala Belait and Temburong (Figure 2.1).

Figure 2.1. Map of Brunei Darussalam

(Map by ©K. Becek, Geography Department, University of Brunei Darussalam, 2005 published in the Brunei yearbook 2006, Borneo Bulletin). This map is intended for use to illustrate the geographical location only).
2.1.2 The National Heritage of Brunei Darussalam

Brunei Darussalam has a tropical climate with uniform temperature, high humidity and annual rainfall ranges from around 2,790 millimetres in many parts of the interior with daily temperature ranging between 22ºC and 28ºC. Tropical rainforest covers 80% of the country’s total land area. Of this figure, 70% are virgin, primary forest, one of the most diverse and unique vegetative assemblages in the world, comprising of 5,000 species of plants, including 2,000 species of forest (Department of Information, 2003). The conservatorium of plants biodiversity under the Forestry Department, Ministry of Industry and Primary Resources, Brunei Darussalam was developed to protect the plants species from their extinction for the benefit of the Bruneians for the future generations ahead.

2.1.2.1 Tropical Medicinal Native Plants of Brunei Darussalam

Ethnobotanical surveys have revealed that Brunei Darussalam has a rich source of tropical medicinal plants (Department of Agriculture, 2000). As 80% of the country’s land is covered by tropical rainforest, Brunei Darussalam may have some medicinal plants with unique characteristics of secondary metabolites. Some plants such as Catharanthus roseus (L.) G. Don and Eurycoma longifolia Jack have long been used by the local communities to treat various disease conditions. However, no research has been done in terms of the constituents or pharmacological or biological activities of the Brunei Darussalam medicinal native plants.
2.1.3 Challenges of TM/CAM in Brunei Darussalam

- A Bruneian Viewpoint

The vast range of indigenous, tribal folklore and traditional systems of medicines, part of the heritage of Brunei Darussalam still needs to be adequately explored. Although herbal medicines are already extensively used in this country, the potential for their wider use in primary health care and in modern systems of medicines have been largely unrealised. The use of current scientific knowledge and analytical assay methods in the extraction, isolation, purification and analysis of such extracts in a systematic approach has always been the priority of the research and development in herbal remedies. This still remains as the biggest challenge in obtaining the scientific information on their quality, safety and efficacy of the medicinal native plants of Brunei Darussalam.

2.1.3.1 The practice of TM/CAM in the Primary Health Care of Brunei Darussalam

The Ministry of Health Report of Brunei Darussalam (MOH, 2000) stated that the population growth is at an approximate annual rate of 3%. The population census of 2002 was 340,800 and projected to be over 436,000 by the year 2011. There are many ethnic communities comprising of the majority Malays, followed by Chinese, Indians and other indigenous groups living in harmony.

Traditional medicine has always been practised in Brunei Darussalam from generation to generation due to its rich cultural and traditional background. The herbalists and traditional medicine practitioners include the Malay ‘bomoh’, the Chinese ‘sin seh’ and the Indian
‘ayurvedic healers/guru/yoga therapists’ and Indonesian religious and spiritual healers, all delivering contemporary primary health care in one form or another to members of the public. These natural or traditional healers are allowed to practice their long term trades of traditional/complementary/alternative medicines provided they do not contravene the existing legal and regulatory framework on the control and practices of allopathic medicine and the traditional medicine practices guidelines endorsed by the Ministry of Health Brunei Darussalam.

Currently, TM/CAM practices are not integrated in the public sector as it is still considered to be a separate discipline of alternative care therapy as compared to the conventional allopathic system of medicine. However, patients on treatment and consumers at large normally refer to TM/CAM therapies as supplementary alternative treatment. This is a very common practice especially in terminally ill patients who will try almost any therapies that will help to support the patients emotionally and spiritually. This is in agreement with the Risk Adaptation Model (RAM) by Ritvo, P et al. (1999) demonstrated that the motivations of cancer patients in seeking TM/CAM complementary therapies are, fundamentally, self-healing motivations which when engaged appropriately, can contribute to the patient’s psychological and physical well being (Ritvo et al., 1999).

From the consumer’s standpoint, the increased in the self-care approach continues to expand locally, national, regionally and internationally. The escalating medical cost and managed care restricts the access to conventional medical care, the choice towards using natural products and other non-prescription medications will also increased in the worldwide scenario. The simple reason which accounts for the popularity of traditional medicine is affordability and accessibility.
This is coupled by the offers to heal from many directions such as advertisements, infomercials, magazines, promotional meetings/seminars with the objective to provide contemporary health care to the public. Therefore, it has become an important issue in our health care delivery system and the need to ensure its rational use and practices remains a big challenge for many policy debates, regulatory questions, clinical and scientific arguments about natural products in the market and their evidence-based therapies will always be debated. Like any other developing and developed countries, the health care system in Brunei Darussalam is dominated by modern western medicine. Table 2.1 shows the socioeconomic and health indicators for year 2004 and the trend of the TM/CAM practices in the present health care domain is on the increase with 16 traditional Chinese Medicines (TCM) clinics in operation and others operating TM/CAM practices.

With the adoption of Primary Health Care delivery system to all, the alternative is TM/CAM which is widely practised in all communities. Currently, this practice plays an important role in the health systems but it is required to be defined clearly by conducting more research and development to evaluate the patterns of TM/CAM usage and the outcomes of these alternative therapies in the country. The Ministry of Health in the National Health Care Plan (2000-2010) continues to ensure that health for all by the year 2000 (Anon, 1993; MOH, 2000) and beyond is focused on the Primary Health Care approach accessible to all.

2.1.3.2 TM/CAM care system in Brunei Darussalam

As a global trend, in principle, the training and practices of TM/CAM traditionally involved an on-the-job apprenticeship with knowledge conveyed from patriarch to offspring, but this system is on the decline due to more practitioners are receiving formal institutional training, coupled
with the fact that more facilities are available for upgrading of standards, education, training and practices of TM/CAM. It has also been reported that fragmentation, disagreements between various groups of experts, and their concentration/focus on the differences rather than common aims and objectives were identified as one of the main problems with the existing professional bodies in the TM/CAM practices (Gauld, 1998). In general, TM/CAM often suffers from a poor research infrastructure and a lack of high quality research and the understanding of research ethics and methodology, the unwillingness to evaluate evidence, and an acute and chronic shortages of resources focusing on training on research into TM/CAM (Roach, 2000). In the project study on the practice and regulatory requirements of naturopathy and western herbal medicine conducted (Lin et al., 2005) and the study points to gaps in the key areas of professional conduct, educational standards and access.

Such a scenario calls for a formal regulatory and legislative framework in countries with increasing popularity in TM/CAM in order to protect and safeguard public health from any potential risk of TM/CAM therapies but at the same time maximising the beneficial potential of the TM/CAM. It is imperative to provide equal opportunities for both modern drugs and TM/CAM therapies to be used side by side for better health care needs of the communities.
Table 2.1 Data on Socioeconomic and Health Indicators for 2004

<table>
<thead>
<tr>
<th>Indicator (s) for Brunei Darussalam</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Area (in 1000 sq.km.)</td>
<td>5,765</td>
</tr>
<tr>
<td>2. Estimated population (‘000)</td>
<td>359.7</td>
</tr>
<tr>
<td>3. Annual population growth rate</td>
<td>2.9</td>
</tr>
<tr>
<td>4. Percentage of population:</td>
<td></td>
</tr>
<tr>
<td>0-4 years</td>
<td>13.2</td>
</tr>
<tr>
<td>5-19 years</td>
<td>27.2</td>
</tr>
<tr>
<td>20-54 years</td>
<td>54.0</td>
</tr>
<tr>
<td>55-64 years</td>
<td>3.3</td>
</tr>
<tr>
<td>65+ years &amp; Over</td>
<td>2.3</td>
</tr>
<tr>
<td>Median Age</td>
<td>23.5</td>
</tr>
<tr>
<td>5. Number of population (total no. by sex) (‘000)</td>
<td>189.4 Male</td>
</tr>
<tr>
<td></td>
<td>170.3 Female</td>
</tr>
<tr>
<td>6. Rate of natural increase of population (% per annum)</td>
<td>2.9</td>
</tr>
<tr>
<td>7. Crude birth rate (per 1000 population)</td>
<td>19.9</td>
</tr>
<tr>
<td>8. Crude death rate (per 1000 population)</td>
<td>2.8</td>
</tr>
<tr>
<td>9. Life expectancy at birth</td>
<td>74.6 Male</td>
</tr>
<tr>
<td></td>
<td>77.5 Female</td>
</tr>
<tr>
<td>10. Infant mortality rate (per 1000 live births)</td>
<td>8.8</td>
</tr>
<tr>
<td>11. Estimated population for the year 2011 (‘000)</td>
<td>436</td>
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</table>
### 12. Main causes of morbidity

<table>
<thead>
<tr>
<th>ICD-9 code</th>
<th>Description</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>460-465</td>
<td>acute upper respiratory infection</td>
<td>680</td>
</tr>
<tr>
<td>630-639</td>
<td>abortion</td>
<td>1,009</td>
</tr>
<tr>
<td>493</td>
<td>asthma</td>
<td>788</td>
</tr>
<tr>
<td>401-405</td>
<td>hypertensive diseases</td>
<td>695</td>
</tr>
<tr>
<td>009</td>
<td>gastroenteritis/diarrhoea</td>
<td>771</td>
</tr>
<tr>
<td>250</td>
<td>diabetes mellitus</td>
<td>606</td>
</tr>
<tr>
<td>466,480-487</td>
<td>acute lower respiratory infections</td>
<td>743</td>
</tr>
<tr>
<td>393-398, 410-429</td>
<td>heart diseases</td>
<td>680</td>
</tr>
</tbody>
</table>

### 13. Ten leading causes of death

<table>
<thead>
<tr>
<th>ICD-I0 code</th>
<th>Description</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>C00-C97</td>
<td>malignant neoplasms</td>
<td>218</td>
</tr>
<tr>
<td>100-109</td>
<td>heart diseases</td>
<td>185</td>
</tr>
<tr>
<td>E10-E14</td>
<td>diabetes mellitus</td>
<td>78</td>
</tr>
<tr>
<td>I60-I69</td>
<td>cerebrovascular diseases</td>
<td>72</td>
</tr>
<tr>
<td>J40-J46</td>
<td>bronchitis, chronic &amp; unspecified emphysema &amp; asthma</td>
<td>48</td>
</tr>
<tr>
<td>V01-110-115</td>
<td>hypertensive diseases</td>
<td>42</td>
</tr>
<tr>
<td>V01-V99</td>
<td>transport accidents</td>
<td>38</td>
</tr>
<tr>
<td>P00-P96</td>
<td>conditions originating in the perinatal period</td>
<td>38</td>
</tr>
<tr>
<td>Q00-Q99</td>
<td>congenital malformations, deformations &amp; chromosomal abnormalities</td>
<td>17</td>
</tr>
<tr>
<td>14. No. of hospitals by organizations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------------------------</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Government hospital</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Private hospital</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Private general practitioners</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Private dentists</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Specialist clinics</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Health clinics</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Health centers</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Maternity &amp; Child Health clinics</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Travelling Health clinics</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Services to remote areas (flying medical team)</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>15. No. of pharmaceutical premises</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Retail pharmacy (Guardian chain)</td>
<td>2</td>
</tr>
<tr>
<td>Pharmaceutical wholesalers</td>
<td>26</td>
</tr>
<tr>
<td>Agrochemical retailers</td>
<td>19</td>
</tr>
<tr>
<td>Agrochemical wholesalers</td>
<td>7</td>
</tr>
<tr>
<td>Chemical suppliers</td>
<td>33</td>
</tr>
<tr>
<td>Veterinary retailers</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>16. Traditional, Complementary, Alternative Medicine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TM/CAM practices</td>
<td></td>
</tr>
<tr>
<td>Medical halls</td>
<td>15</td>
</tr>
<tr>
<td>Homeopathic clinics</td>
<td>2</td>
</tr>
<tr>
<td>Service Type</td>
<td>Count</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Ayurvedic clinics</td>
<td>Nil</td>
</tr>
<tr>
<td>Chiropractic clinics</td>
<td>1</td>
</tr>
<tr>
<td>Acupuncture clinics</td>
<td>1</td>
</tr>
<tr>
<td>Herbalists/Herbal clinics</td>
<td>16</td>
</tr>
<tr>
<td>Traditional Chinese Medicine clinics</td>
<td>4</td>
</tr>
<tr>
<td>Massage therapy clinics</td>
<td>5</td>
</tr>
<tr>
<td>Nutriceutical companies/Health supplements</td>
<td>9</td>
</tr>
<tr>
<td>Supermarket chains (Sale of O.T.C., nutritional products vitamins, minerals, etc.)</td>
<td>11</td>
</tr>
</tbody>
</table>

### 17. No. of health manpower

#### 17.1 Public Sector: Doctors/specialists

<table>
<thead>
<tr>
<th>Profession</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doctors/specialists</td>
<td>400</td>
</tr>
<tr>
<td>Dentists</td>
<td>53</td>
</tr>
<tr>
<td>Pharmacists</td>
<td>26</td>
</tr>
<tr>
<td>Nurses</td>
<td>1,923</td>
</tr>
<tr>
<td>Dispensers</td>
<td>70</td>
</tr>
<tr>
<td>Pharmacy Enforcement Staff</td>
<td>6</td>
</tr>
</tbody>
</table>

#### 17.2 Private Sector: Doctors/specialists

<table>
<thead>
<tr>
<th>Profession</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doctors/specialists</td>
<td>63</td>
</tr>
<tr>
<td>Dentists</td>
<td>15</td>
</tr>
<tr>
<td>Pharmacists</td>
<td>15</td>
</tr>
<tr>
<td>Nurses</td>
<td>42</td>
</tr>
</tbody>
</table>

### 18. Ratio of health professionals to population

<table>
<thead>
<tr>
<th>Profession</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doctors/specialists</td>
<td>1:777</td>
</tr>
<tr>
<td>Dentists</td>
<td>1:5,290</td>
</tr>
<tr>
<td>Pharmacists</td>
<td>1:8,773</td>
</tr>
<tr>
<td>Nurses</td>
<td>Dispensers</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>1:183</td>
<td>1:5,138</td>
</tr>
</tbody>
</table>

19. Total Health budget (B$Millions) – Government based | 233.32 |

20. Health Budget as percentage of National budget | 6.63 |

21. Per capita health budget (B$) | 649 |

Reference Sources:


5. Budget & Tender Section, Ministry of Finance, Brunei Darussalam
   Ledger Section, Treasury Department, Ministry of Finance, Brunei Darussalam.
2.2 Policy on TM/CAM

2.2.1 Existing guidelines of the Ministry of Health Brunei Darussalam on TM/CAM

In Brunei Darussalam, currently there is no National Policy or Traditional medicine Act to regulate TM/CAM. Control on TM/CAM is only administered on guidelines stipulated by the Ministry of Health, Brunei Darussalam. According to the official guidelines, traditional medicine is defined as any product consisting of one or more substances derived from natural sources such as plants, animals and minerals-based materials or a combination of any one or more of these materials, but shall not include any medicinal product to be injected into the human body, any vaccine to be used by human beings, any product derived from human blood or any other item listed in the Poisons list under the Medicines and Poisons Act of Ministry of Health Brunei Darussalam.

Herbal medicine in the raw or processed forms consumed by the communities are normally imported mainly from China, India, Indonesia, Thailand, United Kingdom, Australia, the United States on a commercial licence or by custom permit or by personnel imports. This is a matter of personal choice among the various ethnic communities in Brunei Darussalam and screening of product imports are scrutinised by the Pharmacy Enforcement Unit on the import declarations from the importers.
2.2.2 Legislative and regulatory control of drugs (pharmaceuticals) and TM/CAM of Ministry of Health Brunei Darussalam

The following existing legislations and regulations of the Ministry of Health Brunei Darussalam are executed based on the following Acts:

- Poisons Act 1956 Cap 114 and Rules 1957 enforced by the Ministry of Health:
  This Act regulates the importation, possession, manufacture, compounding, storage, transport and sale of poisons.

- Misuse of Drugs Act Cap 27 (Revised 1984) (MDA) and Misuse of Drugs Regulations (MDR) enforced by the Narcotic Control Bureau (NCB) of the Prime Minister’s Department. This Act incorporates further additional provisions for the regulation of the import, export, manufacture, possession and consumption of controlled drugs as well as dangerous or otherwise harmful substances. Special provisions relating to the jurisdiction of courts in respect of offences are also stipulated.

- Registration of Pharmacist Order 2001 enforced by the Ministry of Health
  This Order incorporates provisions with respect to the registration of the pharmacy practice and pharmacy profession. The key requirements which are stipulated by most Pharmaceutical Societies of developed countries which cover the main aspects of drug practices and control are based on the official operations of the Pharmacy Board of the Royal Pharmaceutical Society of Great Britain (R.P.S.G.B), American Pharmaceutical
Association (A. Ph. A), the Registration of Pharmacists Regulations 1953 of Malaysia and Pharmaceutical Society of Singapore and other developed countries.

2.2.3 Legislations and regulatory control on medicinal products (drugs/pharmaceuticals)

The Poisons Act 1956 Cap 114 and Rules 1957 stipulated that the medicinal product is defined as any substance or article (not being an instrument, apparatus or appliance) which is manufactured, sold, supplied, imported or exported for use wholly or mainly in either or both of the following ways:

- Use by being administered to one or more human beings or animals for a medicinal purposes;
- Use as an ingredient in the preparation of a substance or article which is to be administered to one or more human beings or animals for a medicinal purpose.

2.3 Definition of products in the official guidelines

2.3.1 Drug or medicine (pharmaceutical)

A drug or medicine is defined by the Uppsala WHO Monitoring Centre as ‘a pharmaceutical product, used in or on the human body for the prevention, diagnosis or treatment of disease, or for the modification of physiological function’ (WHO, 2000).
2.3.2 Herbal remedies

Herbal remedy is defined as ‘any product consisting of substances produced by subjecting plant materials to drying, crushing or other process or a mixture whose ingredients are two or more substances so produced, or of a combination of such mixture with water or other inert substances as specified by the licensing authority’ (WHO, 2000).

2.3.3 Homeopathic medicines

Homeopathic medicine is defined ‘as any substances used in the system of therapeutics. Diseases are treated by the use of minute amounts of such substances which are capable of producing in healthy person’s symptoms similar to those of the disease being treated’ (WHO, 2000).

2.4 Official guidelines on the importation and regulatory control of TM/CAM in Brunei Darussalam

These official guidelines on TM/CAM products are in place and executed to ensure quality and safety in order to safeguard public health (MOH, 2004b):

i Traditional medicine in their raw unprocessed form is permitted to be imported into Brunei Darussalam and it does not contain any substance controlled under the Poisons Act.
ii Traditional medicine which have been processed into dosage forms must be properly packed, labelled with ingredients containing the proportions and indications. Any packaging without stating the ingredients listed is not permitted to be imported.

iii The ingredients and the mode of use must be labelled in Malay or English. Other additional languages can be used in addition to either Malay or English.

iv The ingredients of the Traditional medicine should not contain any modern medicines at all.

v Traditional medicine must not contain any substance controlled and listed in the Poisons Act.

vi Traditional medicine must not contain any injectables.

vii Traditional medicine with dubious or unreasonable claims on the labels are not allowed.

viii Traditional medicine for immoral or criminal use are not permitted to be imported.

ix Traditional medicine exported from the countries of origin which are subjected to the registration requirements must be registered with the authority in the exporting country.

x Any registration number from the country of origin or exporting country must be stated on the packaging labels if applicable.

xi The batch number, expiry date and the name and address of manufacturer/sponsor must be stated on the packaging.

xii Traditional/herbal medicines are not permitted to contain any heavy metals exceeding the permissible levels as follows:-

Arsenic - 5 PPM (weight)

Mercury - 0.5 PPM (weight)

Lead - 10 PPM (weight)
The importers are held responsible and are liable for the sale and distribution of traditional medicine as covered by the official guidelines stipulated by the Ministry of Health Brunei Darussalam. These restrictive guidelines are executed through the Pharmacy Enforcement Unit of the Pharmaceutical Services to ensure that the guidelines imposed are strictly complied by the importers in order to control traditional medicine containing scheduled poisons being imported into the country as listed in Table 1.9. These guidelines are limited in the aspect of safety and efficacy of the products as evidence-based TM/CAM is quite a recent development and the assessments/evaluations for the safety and efficacy of TM/CAM still remain as the biggest challenge for many regulatory authorities and key players worldwide.

2.5 Medicinal Native Plants of Brunei Darussalam

2.5.1 Community use of medicinal plants in Brunei Darussalam

In line with this worldwide trend, TM/CAM therapies in particularly from the plants origin is used widely by all communities in Brunei Darussalam, a country in the Western Pacific Region of the World Health Organization (WHO-WPRO). The herbal species are easily accessible in the open markets or in the backyard gardens for consumptions either as fresh vegetables or as herbal remedies for minor illnesses to meet the everyday needs. It is therefore of great interest to explore into these folklore traditional medicine which are being practised in the country especially when there is no written literature to provide the evidence to substantiate the authenticity of the intended medicinal benefits obtained from these natural remedies.

In Brunei Darussalam, the warm and humid equatorial climate, coupled with varying soil conditions, has contributed to extremely diverse natural plant species. It has been estimated that
there are up to three hundred native species of plants used in traditional medicine. Such wealth of plant resources should not only be conserved and treasured, but explored to provide more natural plant materials that are beneficial to mankind, at the same time without jeopardizing the environment or disrupt the perpetuity of nature.

Therefore, from the Brunei Darussalam research and development perspective, continued research on identifying novel compounds in the plant species with the potential to be developed into beneficial therapeutic agents has become the centre of interest and promoting the rationale use of medicinal plants alike many neighbouring countries in South East Asia.

2.5.2 Development of herbal pharmacopoeia or monograph

Currently, there is no formal herbal pharmacopoeia or monograph in documenting the herbal formulations or medicinal plants indicated for the beneficial treatment for a wide range of ailments. There is a need for the development of herbal pharmacopoeia or monograph documenting the medicinal plants of Brunei Darussalam which will be revised regularly to include the recent development and advancement in phytotherapy research. With the continued support from the government and proper scientific research and development, this will provide better understanding on the proper and rational use of traditional, complementary and alternative medicine (TM/CAM). Such a scenario calls for collaborative, concerted and integrated effort from all the professionals practising in the state of the art in traditional, complementary and alternative medicine (TM/CAM) for the actualization of the rational practice of TM/CAM.
2.5.3 Culture and tradition of TM/CAM

Brunei Darussalam has a national heritage of communities with long history of culture and traditions in which traditional medicine practices have become a way of life. Diversity in the ethnic communities influences each other in a harmonious manner in terms of the various types of traditional medicine which are easily accessible within reach. Locally grown medicinal plants are mainly used by the Malay communities with some ingredients imported from Malaysia and Indonesia. As for the Chinese community, they tend to compound the traditional medicine based on the ingredients imported from China or Hong Kong as some of the ingredients are not available in Brunei Darussalam. Similarly, the Indian communities import the materials from India. As for the folklore medicines, it is used by all communities across the board due to the harmonious integration and fusion process of culture and traditions (Lim, 2005) within the country.

An influx of TM/CAM are being imported from overseas such as China, Indonesia, India, Malaysia, Thailand (South East Asia), United States, United Kingdom and Australia as a result of the free trade exchange in the region and the industries marketing strategies due to demand and supply. In Brunei Darussalam, most of the traditional medicine are prepared by combining ten, twenty or even more different ingredients from plants, animals, minerals and compounded into a unit system of formulation. For the herbalists, single herbal species of any of the plant parts such as leaves, stem, twigs, barks, roots, flowers, seeds, etc are used as herbal remedies and the most commonly use medicinal native plants of Brunei Darussalam are listed in Table 2.2.
Table 2.2 Commonly used medicinal plants by the local communities in Brunei Darussalam

(Adapted from the Medicinal Plants of Brunei Darussalam, published by Department of Agriculture, Ministry of Industry and Primary Resources, Brunei Darussalam Revised Edition 2000, for the intended purpose of the studies on the native medicinal plants of Brunei Darussalam).

<table>
<thead>
<tr>
<th>No.</th>
<th>Plant Names</th>
<th>Local Names</th>
<th>Family Names</th>
<th>Plant parts &amp; Method of administration</th>
<th>Medicinal uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Aloe vera</em> L.</td>
<td>Lidah buaya</td>
<td><em>Aloeaceae</em></td>
<td>-Mucilage of leaves</td>
<td>-Poulticing wounds</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Skin and hair</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Conditioner</td>
</tr>
<tr>
<td>2.</td>
<td><em>Alpinia</em> galangal <em>(L.</em>) Sw.</td>
<td>Languas; Langkuas</td>
<td><em>Zingiberaceae</em></td>
<td>-Decoction of rhizomes &amp; base of pseudostem</td>
<td>-To cure stomachache, vomiting, diarrhoea &amp; indigestion.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Possibly a useful aphrodisiac.</td>
</tr>
<tr>
<td>3.</td>
<td><em>Andrographis paniculata</em> <em>(Burm. f.) Nees.</em></td>
<td>Daun pahit</td>
<td><em>Acanthaceae</em></td>
<td>-Decoction of leaves</td>
<td>-To cure diabetes;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Leaf poultice</td>
<td>-To reduce blood pressure</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-To relieve skin irritation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&amp; insect bites</td>
</tr>
<tr>
<td></td>
<td>Scientific Name</td>
<td>Common Name</td>
<td>Family</td>
<td>Uses</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>--------------------------</td>
<td>-------------</td>
<td>----------</td>
<td>----------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td><em>Areca catechu</em> L.</td>
<td>Pinang</td>
<td>Palmae</td>
<td>Seeds are chewed with betel leaf &amp; lime paste.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- For stomachache.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- For headache.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td><em>Catharanthus roseus</em> (L.) G. Don.</td>
<td>Bunga pasar; Tahi ayam</td>
<td>Apocynaceae</td>
<td>Decoction of roots</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- As contraceptive</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Control Hypertension</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td><em>Centella asiatica</em> (L.) Urb.</td>
<td>Penggaga; Pegaga</td>
<td>Umbelliferae</td>
<td>Raw or cooked young leaves are eaten as vegetables</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- To cure urinary tract infection and stones</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td><em>Cosmos caudatus</em> Kunth.</td>
<td>Rancah-rancah</td>
<td>Compositae</td>
<td>Young leaves &amp; shoots are eaten as raw vegetable</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- To treat gaseous stomach &amp; mild gastric pains.</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Scientific Name</td>
<td>Local Name(s)</td>
<td>Family</td>
<td>Uses</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>----------------</td>
<td>--------------</td>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Curcuma longa L.</td>
<td>Kunyit, Tamu Kunyit, Kunyit Biasa</td>
<td>Zingiberaceae</td>
<td>- Rhizomes are chewed and swallowed after each rubbing on the abdomen - To relieve uncontrollable and frequent urination due to the infection of the urinary system</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Curcuma zedoaria (Berg.) Rosc.</td>
<td>Tamu putih (derived from the white-coloured primary rhizome)</td>
<td>Zingiberaceae</td>
<td>- Rhizomes are pounded, soaked in hot water &amp; warm infusion are taken after child birth - To maintain good health</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Cymbopogon nardus Rendle</td>
<td>Serai wangi</td>
<td>Gramineae</td>
<td>- Infusion of leaves is used as herbal bath after childbirth; - Decoction of stems - To maintain good health - For stomachache, Indigestion</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Dillenia suffruticosa (Griff.) Mart.</td>
<td>Simpor</td>
<td>Dilleniaceae</td>
<td>- Exudates of the broken twigs, calyx, leaf stalks - Applied to wound to stop it from bleeding</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td><em>Eurycoma longifolia</em> Jack.</td>
<td>Tungkat Ali; Langir siam; Pasak bumi; Kayu raja; Petagar Ali; Serirana</td>
<td><em>Simaroubaceae</em></td>
<td>Decoction of roots</td>
<td>Infusion of roots</td>
</tr>
<tr>
<td>14.</td>
<td><em>Hedychium longicornutum</em> Baker.</td>
<td>Halia hantu; Kunyit hantu</td>
<td><em>Zingiberaceae</em></td>
<td>Decoction of roots &amp; rhizomes</td>
<td>Pounded roots for topical application</td>
</tr>
<tr>
<td>15.</td>
<td><em>Manilkara zapota</em> (L.) P.Royen.</td>
<td>Ciku</td>
<td><em>Sapotaceae</em></td>
<td>Pounded unripe fruits mixed with warm water to be taken orally</td>
<td>For treating diarrhoea</td>
</tr>
</tbody>
</table>
### 16. Morinda citrifolia L. (Mengkudu, Rubiaceae)
- Ripe or unripe fruits to be brushed onto the teeth of children
- To prevent tooth decay

### 17. Rhodomyrtus tomentosa (Ait.) Hassk. (Keramunting, Myrtaceae)
- Leaves extracts & fruits
- To alleviate anaemia
- For wound healing

### 18. Stenochlaena palustris Bedd. (Lamiding, Blechnaceae)
- Baked fronds
- Young fronds are consumed as vegetables
- Pregnant women at late stage of pregnancy for facilitating easy birth

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The indications of traditional medicine are normally based on empirical and folklore experiences. Unlike modern drugs such as pharmaceuticals, no data are required to substantiate the claims of these traditional remedies. Some natural products are of therapeutic potential, others are still in progress study and majority of the traditional medicine have not undergone any scientific study due to the fact that they are not prospective for the industries and also the lack of funding to carry out expensive research and development. This is particularly true for medicinal
plants used in traditional medicine for which no research on the clinical studies has been reported to date.

Worldwide experience indicated that extensive clinical trials have been conducted on those prospective natural remedies but the results usually require further elucidation and investigation to achieve evidence-based medicine. In most developed countries, clinical trials on traditional medicine are almost impossible due to the costly preclinical safety requirements of the drug regulatory agencies. Therefore, allowances are given to these traditional medicine that have been used from generations to generations and if the authority is to curtail these products which will result in the risk of loss of these valuable ethnomedical knowledge among the future generations.

It is to be noted that some medicinal plants that have been used for years by various ethnic groups are often not adequately documented in writing. The knowledge about the plants profile and usage is usually passed on verbally from person to person, i.e. grandfather to father to son, a generation game of knowledge handed over within the protected family circle. This knowledge is at risk of being forgotten, diminishing over the years and may be lost forever if studies on these medicinal plants are not to be taken off the ground. Ethnomedical knowledge in its indigenous context is utmost important especially in the chase for the search of novel therapeutic compounds.
2.5.4  Supply and demand of TM/CAM in Brunei Darussalam

2.5.4.1 General use of TM/CAM in Brunei Darussalam

The worldwide resurgence of interest in traditional medicine is evidenced by the numerous literature publications from the professionals such as policy makers, administrators, educators, clinicians, researchers, scientists, TM/CAM therapists and health personnel. The primary reason cited for its revival is a widespread fear of adverse effects associated with the western drugs and the belief that natural product is harmless. In addition to that, the high cost of treatment has turned many people to alternative remedies which are more affordable and widely available and accessible due to supply and demand.

Although there is no research as yet been carried out on the ethnobotanical survey on the usage of TM/CAM among the various communities in Brunei Darussalam, the Ministry of Industry and Primary Resources revealed that many native plant species are extensively being used as natural remedies for various symptomatic diseases or minor ailments from generation to generation. There are a rapid increased number of traditional medicine retail outlets throughout Brunei Darussalam selling and supplying the natural products due to its demand by the consumers. There also exists a few small scale industries processing the raw materials into various dosage forms either as single entity or compounded preparations.

The commercialisation of the traditional medicine and many other natural products has resulted in the influx of products in the capital which gives an indication of its popularity, used by many ethnic communities, not just solely by the oldfolks in rural areas. The growth of the natural
products market has turned into an industry of health care products of TM/CAM, may perhaps overtaking the orthodox medicine in the private sector in future.

2.5.4.2 TM/CAM formulations and preparations in Brunei Darussalam

There are many formulations of preparations on natural products which are available in the Brunei Darussalam market ranging from various origins of raw materials such as plants, animal parts, vitamins, minerals used as health food and nutritional/tonifying supplements. These are either processed or compounded locally or imported. These products are being used based on personal choice and handover practices. Generally, they are categorised as follows:

- Available as various pharmaceutical dosage forms in the form of tablets, capsules, pills, suspensions, lotions, creams, ointments, sachets and mixtures, etc. Some preparations resemble the pharmaceutical dosage forms.

- Strongly medicated wines which are highly alcoholic in contents usually soaked with animal organs and parts which are believed to be kept “the longer the better” for their medicinal effects. This is usually practised by the older generations as tonifying agents and believed to improve blood circulation. Similar practice can also be observed in Asia.

- Herbal remedies such as herbal tea leaves and other plant parts which requires further processes before consumption in the form of maceration techniques, infusion or decoction or mixtures, etc. Prescriptions of mixtures of all plant parts such as dried leaves, flowers, twigs, seeds, barks, stalks, stems and roots that are raw in nature and are
cooked in a specified manner, sometimes with meat for many hours as tonifying agent for health promotion and supplements. These preparations are believed to strengthen the body.

- Ayurvedic homeopathic remedies such as series of dilutions of the minute ingredient derived from the stock tinctures usually used in ayurvedic therapies and healing processes.

- Aromatherapeutic preparations such as the range of external preparations which usually have soothing effect, with odour and essence that relieve stress and relax the mind and body. Aromatherapy oils used in body massages and steam baths are also gaining popularity especially among the young generations.

- Miscellaneous preparations including mixtures that are derived from plants, animals, minerals in any other forms such as pastes, spice powders, dyes, food, emulsions, liquids, solutions, drinks and etc that are also available for commercial purposes.

Among these many formulations and preparations available in Brunei Darussalam, therefore the accepted classification of traditional medicine as natural remedies is a natural product that must contain one or more naturally occurring substance(s) of plants, animals, minerals in their raw and unextracted or crude extract forms. Such classification is intended to avoid confusion.
Novel research study on medicinal plants extracts demonstrated that *Catharanthus roseus (L.) G. Don* from the *Apocynaceae* family which is also one of Brunei Darussalam important collection of medicinal plants, Herbarium Drug Museum (HDM 36), local name ‘*bunga pasir; tahi ayam*’ contains the vinca alkaloids, vincristine, vinblastine and vindesine are commercially available as pharmaceutical products for the treatment of acute leukaemia, lymphomas, and some solid tumours such as breast and lung cancer. And vinorelbine, a semi-synthetic vinca alkaloid was further developed for treating advanced breast cancer and non-small cell lung cancer where anthracycline-containing regimens have failed (BNF, 2005). All vinca alkaloids are intended for intravenous administration only. They are contraindicated for intrathecal administration due to severe neurotoxicity which is usually fatal.

In the case of herbal remedies from the TM/CAM perspective, it is particularly important to illustrate their potential mechanisms of action at a molecular level by identifying the superior genotype with active components which are critical to understand their important role in the bioassays and as well as their potential risk to public health. At present, it is not surprising relatively little is proven about the efficacy and safety of TM/CAM due to the limited financial resources and laborious processes involved in the time consuming empirical study and keeping track of the performance of TM/CAM.
2.5.4.3 Nomenclature of the plants

The nomenclature of plants can vary tremendously from country to country. For instance, at least fifty three vernacular names of Andrographis paniculata were used by the communities throughout Asia recorded by World Health Organization as listed in Table 2.3. Many problems of incorrect or misnaming of the plant species which can cause confusion when the plant species is not identified and authenticated appropriately which will have massive implications in the quality, safety and efficacy analysis of the plant. For example, confusion may arise in the following ways:

- the same name (i.e. one particular name) may have been given to more than one plant.
- the same plant is being called/addressed differently by different people from the same or different districts, regions, countries.
- changes in the botanical name of that particular plant.
- botanical name was given wrongly to different species.
- same species was given different botanical names by different botanists.
- inaccurate identification of plant species resulting in the wrong botanical name being given to the species.
- wrong description of the plant species.
- lack of standardisation in the naming of the plant species locally, nationally, regionally and internationally.
- difficulties in establishing uniformity in the naming of plant species.
Table 2.3 Vernacular names of *Andrographis paniculata* (Burm. f.) Nees
(Adapted from the WHO monographs on selected medicinal plants

<table>
<thead>
<tr>
<th>Index</th>
<th>Vernacular names used for <em>Andrographis paniculata</em> (Burm. f.) Nees by communities in Asia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Akar cerita bidara, Alui, Andrographidis Kraut.</td>
</tr>
<tr>
<td>2</td>
<td>Bidara, bhoomiba, bhunimo, bhulimb, bhuninba.</td>
</tr>
<tr>
<td>3</td>
<td>Charayeta, charayetha, charita, cheranta, cherota, chiraita, chiretta, chuan-hsin-lien, chuanxinlian, cong cong.</td>
</tr>
<tr>
<td>4</td>
<td>Daun pahit</td>
</tr>
<tr>
<td>5</td>
<td>Faathalaichon, fathalaai, fathalaichon, fathalaijone.</td>
</tr>
<tr>
<td>6</td>
<td>Halviva, humpedu bumu, herba sambiloto, hinbinkohomba.</td>
</tr>
<tr>
<td>7</td>
<td>I-chien-his</td>
</tr>
<tr>
<td>8</td>
<td>Kalafath, kalmegh, kan-jang, kariyat, khee-pang-hee, king of bitters, kiriathu, kirta, kiryata, kiryato.</td>
</tr>
<tr>
<td>9</td>
<td>Lanhelian</td>
</tr>
<tr>
<td>10</td>
<td>Mahatikta, mahatita, mompoit</td>
</tr>
<tr>
<td>11</td>
<td>Naelavemu, naynahudandi, nelavemu</td>
</tr>
<tr>
<td>12</td>
<td>Quasab-uz-zarirah</td>
</tr>
<tr>
<td>13</td>
<td>Rice bitters</td>
</tr>
<tr>
<td>14</td>
<td>Sambilata, sambiloto, senshinren, sinta</td>
</tr>
<tr>
<td>15</td>
<td>Xuyen tam lien</td>
</tr>
<tr>
<td>16</td>
<td>Yaa kannguu yijianxi</td>
</tr>
</tbody>
</table>

It is a common phenomena that plants are called under more than one vernicular name which can cause a lot of confusion as shown in the Table 2.2, listing the commonly used medicinal plants by the local communities in Brunei Darussalam with their pictures depicted. Such a scenario is a worldwide problem.
2.6 Adoption of the WHO Policy and Strategy on TM/CAM

In line with the WHO Policy in the promulgation of TM/CAM globally, it is suggested that the development strategies need to incorporate the following objectives in line with WHO policy in the promotion of use of TM/CAM for the delivery of health care to all. This policy will encompass all the important issues identified and to rectify the current situations and to improve further the infrastructure of TM/CAM in Brunei Darussalam, particularly in the Western Pacific Region Organization (WPRO):

2.6.1 National Policy and Act on TM/CAM

This policy is to integrate TM/CAM with the national health care systems as appropriately as possible through the development and implementation of national TM/CAM policies and programmes in the areas of importance and priority for Brunei Darussalam. There is a need for Brunei Darussalam to formalise the legislation on TM/CAM which aims to ensure that quality, safe TM/CAM practices and products are available to the people in the country. Legislation would take the pragmatic approach of the needs of the communities and to maximise the benefits but minimise the risks involved in all aspects of TM/CAM therapy.

2.6.2 Collaboration with regional and international centres on ensuring quality, safety and efficacy

Brunei Darussalam needs to promote quality, safety and efficacy of TM/CAM by strengthening and expanding the knowledge-base on TM/CAM, and the implementation of quality assurance programmes on TM/CAM with the collaborating centres worldwide are to be encouraged.
2.6.3 Accessibility of TM/CAM

The increase in the demand of TM/CAM should be in line with their increase of supply. Such a balance should be maintained to ensure there is a continuous availability of TM/CAM in the market which is affordable, effective, of good quality and safe. Therefore, in order to increase the availability and affordability of TM/CAM as rationally to the population, there is a need to promote the rational therapeutic use of TM/CAM by the health providers and consumers for the intended use.

2.6.4 Pharmacovigilance on TM/CAM

Continued efforts on the collaborations and networking with the international, regional, national MADR Centres on Adverse Drug Reactions (ADRs) or adverse events associated with TM/CAM use are very important. The needs to conduct post marketing surveillance on TM/CAM in the market and to step up research and development on their pattern of use, benefit versus risks ratio of these products are of paramount importance.

2.6.5 Harmonisation process

The identification of key areas which are ready for some degree of integration to take place that is of priority to the health care needs of the people (Kairullah, 2005; Merican, 2005). Development and research into areas of common core for community health needs to be explored further. The key to the future of TM/CAM in Brunei Darussalam would be in the integrative and collaborative partnerships through bilateral and multilateral exchange and sharing of information on all aspects of TM/CAM processes and practices at national, regional, and
international levels. This would be for better use of resources and manpower in the most cost effectiveness manner and attainable.

2.7 Recommendations on the proposed strategic framework of TM/CAM for Brunei Darussalam

The use of native medicinal plans at the primary health care level has many advantages and should always be encouraged for the effective treatment of many ailments with good safety profile. In retrospect, during war experiences, there was a deprivation of modern medicines which has highlighted the importance of traditional medicine among many people in South East Asia. Communities would prefer to take herbal remedies which they grow in their own gardens, countries and will always be easily accessible and affordable when needed. For these old folks, they still remember this war experience and the need for essential medicine such as modern western drugs and for self-sufficiency medication; there is a clear need to encourage more widespread use of herbal medicine as natural reserves. These will, in fact, be an insurance in the communities’s perspective against such a situation from arising again and their endless endeavour to develop a reservoir of herbal armamentarium as alternative treatment for the next generations in need is clearly understood.

Integration of the traditional and allopathic system and a unified approach to health care, not only at the primary health care level but at all levels, remains as the future long term goals. In our endeavor to achieve the ultimate goals, doctors, clinicians, pharmacists and nurses and other health care professionals should be trained both in the allopathic system as well as the traditional system in order to complement each other in the delivery of health care to the community. In a better position to treat patients either using allopathic medicine or with medicines from the
traditional system, both systems of medicines can be practised side by side in an integrated manner for the benefit of mankind.

### 2.8 Development of national strategy on traditional and complementary medicines (TM/CAM) in Brunei Darussalam

The Ministry of Health Brunei Darussalam recognises the immense potential benefits of the national TM/CAM heritage for the people of Brunei Darussalam and the future generations to come. In order to ensure that safe use of TM/CAM is in tandem with the mainstream healthcare infrastructure, appropriate legislation and regulation stipulated in the National TM/CAM Health Policy should be in place. With the promulgation of the Traditional medicine Act which aims to ensure that quality and safe TM/CAM practices, standards and products are available to the people of Brunei Darussalam, actualization of healthcare for all can be achieved. Therefore, the health policy and TM/CAM Act in place should address important issues such as safe practices, standards, education, training, products and to prioritize research and development in TM/CAM.

Research and development strategies on the legislative and regulatory framework on TM/CAM with the objectives of improving quality health care to the communities in Brunei Darussalam. These strategies can be used in many settings to assess potential problems in TM/CAM use and to prioritize and focus subsequent efforts to ensure that TM/CAM is to be appropriately integrated into the Bruneian healthcare system in order to achieve a holistic approach in the improvement of quality of life of the Bruneians.

TM/CAM is readily available at an affordable level by all communities worldwide. Due to its increasing demand and supply, it is used by all communities in Brunei Darussalam either
obtained locally in their crude forms as raw materials or as processed forms mostly imported from China, Malaysia, Singapore, Thailand and Indonesia. The health and nutritional supplements in the form of pharmaceutical preparations are normally imported from U.S.A., U.K., and Australia.

Many factors such as easy availability of herbal remedies, accessibility to natural herbalists at all times and affordability coupled with the inherent faith, particularly in the ‘natural belief’, also complement the desire of large sectors of the population, to use medicinal herbs for therapeutic purposes. The influx of traditional medicine/herbals remedies into Brunei Darussalam is on the increase although there is no specific regulation or legislation on the importation, sale and use of TM/CAM. Currently, the Pharmacy Enforcement Unit of the Pharmaceutical Services of Ministry of Health exercise screening and control activities such as:

- to screen all the import declarations for drugs (pharmaceuticals) containing scheduled poisons, OTC drugs, traditional medicine/herbal remedies as well as various classification of medicines for personal use.

- to analyse on samples taken at random from sales premises and wholesalers’ warehouses to ensure that the importers comply to the official guidelines (as described fully in section 2.4 of Chapter 2) on the importation on traditional medicine as imposed by the Ministry of Health. The traditional medicine should contain the ingredients and strengths as stated on the label as well as to eliminate the inclusion of certain adulterated harmful substances.
• to detect traditional medicine/herbal remedies which are adulterated with Scheduled Poisons/Prescription Only Medicines (P.O.M.) such as steroids and heavy metals upon laboratory analysis at random (Table 1.9). Therefore, it is imperative for us to consider regulatory and legislative control on the use of these substances. Linked to this are issues of quality control, both of the raw materials and of the finished products, and of standardization of traditional medicine/herbal remedies.

As traditional medicine has become part of the cultural and traditional beliefs and practices of the communities, it is therefore important to bring the use of these traditional remedies into an existing framework of rational scientific use of medicines.

The national strategies are aimed at enhancing the regulatory status and rational scientific framework of the TM/CAM in Brunei Darussalam. The following approach is adapted from the existing framework of pharmaceuticals setting but with some modifications which are appropriately integrated into the existing health care system of Brunei Darussalam. The integration process is proposed to be incremental in magnitude and efforts are to be made into identifying key areas for harmonisation and integration on TM/CAM in Brunei Darussalam.
2.9 Objectives

The main objective is to propose to the Brunei Darussalam government a national strategy regarding the proper use, research & development and control of TM/CAM in the country. It is focused on the future collaborations in the key areas on the exchange and sharing of information on quality, safety and efficacy of TM/CAM. This framework is in line with the WHO promulgation of the use of TM/CAM worldwide on the basis of collaborative efforts of integration and harmonisation processes.

2.9.1 Quality assurance of TM/CAM

The following procedures are based on a pragmatic approach on TM/CAM for Brunei Darussalam to serve as an impetus in order to ensure that the quality assurance of TM/CAM is achieved:

- Implementation of product listing of TM/CAM.
- Laboratory analysis and assessment on samples submitted for application at random.
- Monitoring control and inspection system.
- Post Marketing Surveillance (P.M.S.).
2.9.2 Clinical evaluations and assessments

Development of clinical evaluations, assessments and trials on the clinical use of TM/CAM are required:

- Clinical evaluation and assessments on the TM/CAM.
- Well planned and properly conducted clinical trial methodology and to make certain the results are analysed properly.
- Conduct multicentre clinical trials on the treatment of uncomplicated disease conditions by using the medicinal native plants of Brunei Darussalam.

2.9.3 Research and development strategies on TM/CAM

This objective aims at obtaining up-to-date scientific information on quality, safety and efficacy of the tropical medicinal native plants of Brunei Darussalam in order to spearhead the planning of various research strategies on the community use of medicinal plants:

- Identification and development of a model list of medicinal plants used in Primary Health Care settings (P.H.C.) in line with the WHO model list illustrated in Table 2.4.
- Implementation of herbal formulary for more effective and proper use of traditional medicine/herbal remedies and improved herbal therapeutics.
- Development of medicinal plant pharmacopoeia/monographs on the tropical medicinal native plants of Brunei Darussalam.
• Official guidelines developed by WHO and other collaborating centres on TM/CAM are useful reference guides and can be cost effective for setting up development of TM/CAM in developing countries with limited financial and manpower resources.

2.9.4 Herbal pharmacovigilance activities

Pharmacovigilance monitoring activities to encompass the adverse reactions and events associated with traditional medicine or use in conjunction with polypharmacy are for collecting important data on safety issues associated with traditional medicine. Evaluation, assessment, interpretation and analysis of data collected can be useful for early detection of risk associated with TM/CAM therapies or products. Brunei Darussalam as a member to the WHO Collaborating Centre will benefit from this collaborative effort and support on pharmacovigilance:

• Reporting suspected adverse reactions associated with traditional medicine to the Uppsala Monitoring Centre, WHO Collaborating Centre for International Drug Monitoring, Stora Torget, S-75320 Uppsala, Sweden. Establishment of world databank on the adverse drug reactions and herbal reactions for sharing of critical management of these adverse events in the most effective and efficient manner.
2.9.5 Collaborations on the exchange and sharing of information on TM/CAM

Effective and efficient integration in the collaboration of sharing and exchange of information on many aspects TM/CAM in order to achieve quality TM/CAM care to the communities:

- Strengthening the mechanisms on the activities of sharing and exchange of information among technical working groups on TM/CAM areas of collaborations at national, regional and international levels.

- Create increased efficiencies through greater bilateral and multilateral collaborations, economies of scale and sharing resources with participating countries.

- Collaborating with WHO Collaborating Centers on traditional medicine which plays a very important role in global collaboration as these centers are officially mandated and designated to ensure that the WHO policy on the strategy on TM/CAM in the propagation of the safe, quality assurance and efficacious TM/CAM to the communities globally is achieved.

- Networking to the Global Information Hub regarding potential clinical benefits, safety and risks of TM/CAM products and practices made available to all participating member countries should be encouraged and well supported for the benefit of communities.
2.9.6 Public education

- Public education and information on the proper and rational use of TM/CAM and other natural products.

- Sharing, exchange of information and experiences on the use of traditional medicine of common interest among the public in public forums and group discussions to promote rational and quality use of traditional medicine.

The national research and development plan on TM/CAM are geared to address key issues in public health, resources sharing through collaborative efforts and provision of funds for conducting scientific research and development on TM/CAM. The integration process requires good coordination, coherency, consistency in all areas of collaborations should be in tandem with the need to position TM/CAM in the best interest of all parties towards achieving the common goal of holistic approach in enhancing the health and quality of life of the community at large.

2.10 Guidelines on the Product Listing

On the basis of the definition of ‘traditional use’ adopted by the Complementary Medicine Evaluation Committee of the TGA for the purpose of the guidelines for levels and kinds of evidence to support indications and claims for non-registrable medicines, including complementary medicines, and other listable medicines (ARGCM, 2005) and reviews on the developments of TM/CAM from other countries, it is proposed that the Ministry of Health
Brunei Darussalam adopt a similar system but with some modifications. Such modifications are taken into considerations of the wide range of products available in the country. It is therefore required to categorize products which are defined under the existing official guideline of the Ministry of Health as a traditional medicine containing one or more naturally occurring substance(s) of plants, animals or minerals in their unextracted or crude extract forms. These guidelines need to address the levels and kinds of evidence to support claims for therapeutic products.

2.10.1 Product listing of TM/CAM

Listing of products is proposed to be categorized according to the claims made for the following categories of products containing plants, animals and minerals origin which are pertaining to the needs of the population of Brunei Darussalam:

- Traditional medicine (Non scheduled poisons)
- Raw materials
- Crude or pure extracts
- Pharmacopoeial items
- Monographic herbs (format of the medicinal plant monograph as illustrated in Table 2.4 for documentation of each medicinal plant)
- Standardized materials
- Essential oils, aromatherapeutic agents
- Homeopathic (Ayurvedic) preparations
- Miscellaneous and other vague origin that may pose health risks to the public.
<table>
<thead>
<tr>
<th>Outline of the Medicinal native plant Monograph Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Scientific name:</td>
</tr>
<tr>
<td>2. Synonyms:</td>
</tr>
<tr>
<td>3. Common names of the plant (districts, country, region):</td>
</tr>
<tr>
<td>4. Definition of plant parts used:</td>
</tr>
<tr>
<td>5. Active and inactive chemical constituents (complete listing):</td>
</tr>
<tr>
<td>6. Qualitative and quantitative control:</td>
</tr>
<tr>
<td>• Certificate of authenticity (including scientific name, part of plant used, site of harvest, date of harvest and storage conditions of plant)</td>
</tr>
<tr>
<td>• Microscopic description of drug (pharmacognosy)</td>
</tr>
<tr>
<td>• Assays procedure, what is measured, %w/w contents of active constituents, references</td>
</tr>
<tr>
<td>7. Pharmacology - review of current available scientific data and information</td>
</tr>
<tr>
<td>8. Toxicology - review of available data and information on safety</td>
</tr>
<tr>
<td>9. Historical/traditional/folkloric claims for short and long term uses</td>
</tr>
<tr>
<td>10. Acceptable claims/indications for non-prescription use, denoting those claims which are supported by:</td>
</tr>
<tr>
<td>- human data;</td>
</tr>
<tr>
<td>- animal data;</td>
</tr>
<tr>
<td>- traditional/folkloric use.</td>
</tr>
<tr>
<td>11. Contraindications/cautions/warnings/interactions: groups at risk, include a standard statement in most cases that herb is not recommended for use in children under the age 12, pregnant or lactating women.</td>
</tr>
<tr>
<td>12. Dosage and directions for use:</td>
</tr>
<tr>
<td>• Accept doses found in the British Herbal Pharmacopoeia in most cases</td>
</tr>
<tr>
<td>• Pharmacopoeial and/or folk medicine doses</td>
</tr>
<tr>
<td>• Doses specified for different dosage forms (e.g., extracts and tinctures), as well as “equivalent doses of crude dried herb” which is obtained by calculation when the specification of the extract or tincture is known. Specifications of extracts and tinctures (1:1, 1:10 etc) clearly stated.</td>
</tr>
<tr>
<td>• Single dose, dosage frequency and total daily dose wherever possible.</td>
</tr>
<tr>
<td>13. Official pharmacopoeial preparations (Ref: USP, BP, NF, Indian, Chinese, EP and others) - past and present - Updated Scientific information</td>
</tr>
<tr>
<td>15. Closely related herbs e.g. other varieties or other species.</td>
</tr>
<tr>
<td>16. References: (Specific statements in the monograph should be numbered to relate to the appropriate reference, whenever possible. A general reference list could also be used). Other up-dated information on clinical benefits, safety and risks.</td>
</tr>
</tbody>
</table>
2.10.2 Implementation of quality assurance on traditional medicine

The main objective of the categorized product listing for products which are intended for commercial marketing purposes is to ensure that all TM/CAM available in Brunei Darussalam are safe, of acceptable quality and effective which is geared primarily for the benefits of the public so that such remedies will remain useful for alleviating or curing diseases without undue risks to the population. This objective can be achieved by carrying out the following activities:

- to receive and screen applications for product listing purposes before it is approved for use in the market.
- to list the number of TM/CAM to be marketed. Product listing number is issued for each product approved.
- to assess product quality prior to evaluation.

In applying for finished product listing, the manufacturer or importer must first request permission to produce or import sample products to be listed. Sufficient samples must be submitted for screening together with certificate of free sale. All information pertaining to the product must be completed with the following basic product profile for evaluations and assessments by the relevant evaluation committees:

- product formulation(s)
- indication(s)
- dosage form
- dosage regimen
• chemical and herbal data if applicable
• method of assay
• pharmacological data
• toxicological data
• clinical trials
• label claims
• package insert
• any other information which are important to assist in the overall evaluations and assessments of the products
• adverse reactions or events associated with the product which may be suspected, probably, certain, etc which are useful in the assessments of benefits versus risks.
• pamphlets, inserts of the product information profile including side effects, precautions, interactions and contraindications.
• Information on clinical benefits, safety and risks of TM/CAM products.

The following evaluation and assessment criteria are applicable for the herbal/plant species which are intended for commercial marketing purposes. Basic information is required as follows:

• Name of plant (genus, species, authority and family)
• Plant part used (leaves, stems, barks, roots, seeds, etc)
• Quality requirement (percentage of active principle, if known)
• Purity (foreign matter, adulterant, other contaminants)
• Physicochemical analysis:
- Qualitative (including micro-chemical tests)
- Quantitative (if major active ingredients are known, analytical procedures - e.g. thin layer chromatography (TLC), high pressure liquid chromatography (HPLC), other newer techniques which were developed to quantify the total and individual constituents. Even if active principles are known, one can still obtain characteristic special profiles for the plant material.

  - Packaging.
  - Storage conditions, shelf life and stability monitoring and re-testing.
  - Deterioration of the plant materials and re-assessments of the contents of active constituents.

The schematic flowcharts of Figures 2.2, 2.3, 2.4, 2.5 illustrated the processes involved in the listing of products by submission of applications to the Division of TM/CAM Authority of Ministry of Health Brunei Darussalam. These recommended procedures are needed to safeguard the interest of public health and at the same time to fully support the rational or quality use of TM/CAM in Brunei Darussalam.
STAGE 1

Application submits Letter of intent to TM/CAM evaluation Committee

Not Approved

(Application for product listing will not be accepted)

Preliminary screening

Approved

Application requests for permit to import/manufacture product concerned

Upon importation/manufacture of product concerned

STAGE 2

Application submits sample to TM/CAM evaluation committee for analysis

I. Ensure all necessary documents are submitted
II. Applicant is informed of analysis results from laboratory
III. Laboratory analysis fee is paid

Laboratory analysis

Fail Pass

(Application for product listing will not be accepted)

STAGE 3

 Applicant submits application for product listing

Processing of application

I. Only applications with complete documentation will be accepted.
II. Processing fee to be paid

Figure 2.2. Procedure for Processing Application for Product Listing of TM/CAM
Figure 2.3. Procedure for Laboratory Analysis of Samples for Product Listing of TM/CAM
Figure 2.4. Procedure for Processing Application of Product Listing of TM/CAM
1. New application
2. Renewal of licences
   Incomplete (returned)

1. Unsatisfactory weaknesses in documentation, etc.
2. Non-compliance to Good Manufacturing Practice or Good Storage Practice

Premise Inspection

Figure 2.5. Flowchart for Application of Manufacturer’s, Wholesalers’ and Importer’s Licences on TM/CAM
2.10.3 Monitoring control/inspection system

Monitoring control and implementation of inspectorate system are aimed at ensuring that traditional medicine comply with the criteria set by the Ministry of Health up to time that they are delivered to the consumers, by way of inspection, sampling test, analysis, guidance and punitive action which are in line with the existing framework of inspection with modifications. Regular inspection and suspected or petitioned inspection include:

- Inspection of importers’ premises, herbal outlets and medical halls in terms of standard regulations, operation, documentation and stocks.
- Confiscation of substandard quality, misleading labels or packages with false claims.
- Storage facilities of the premise i.e. storage conditions with appropriate temperature, moisture and humidity control.
- Regular stock turnover first in first out (FIFO).
- Continuous availability of affordable fresh raw herbal materials.
- Maintenance of product quality and safety in ensuring safe delivery at the wholesale and retail sectors throughout the country.

2.10.4 Post Marketing Surveillance (P.M.S)

Pre-listing evaluation and assessment of the product is to provide some form of control measures on its quality, safety and efficacy. Many aspects of the product’s performance can only be
established through cumulative experience of its use in general practice. The performance, therefore needs to be constantly appraised throughout the commercial and marketing lifespan of a product such that it will continue to meet the required standards of quality and safety. P.M.S. is carried out by:

- Adverse reactions/events monitoring programmes and activities.
- Voluntary reporting on suspected adverse reactions or events associated with products by health professionals and consumers.
- Encourage notification culture on suspected products concerning risks and safety issues.
- Reevaluation and re-assessment of the product.
- Product decomposition and deactivation of constituents which affect the quality, safety and efficacy of products.

2.10.5 Clinical evaluation and assessments

To achieve the research objectives, this component is prompted to set up protocols on the appropriate clinical evaluation and assessment of medicinal native plants in Brunei Darussalam. It is important that clinical investigators to carry out clinical trial methodology, especially pertaining to medicinal plants is properly planned, coordinated and well conducted and make certain that the results are analysed properly and that correct interpretations are drawn from the results. The traditional medicine model approach consists of the following important primary steps/protocols for the research and development of medicinal plants:

- Literature review on the background and understanding of the plant profile.
• Identification of the plant reportedly in use
• Collection of the plant.
• Transport of the plant to the research laboratory.
• Storage of the plant materials.
• Preparation of extracts for testing.
• Administration of the extracts to animal models, protocols and designs.
• Identification of the active principles and chemical structure.
• Synthesis of the active substances.
• Short and long term goals in establishing the clinical evaluation and assessment of the medicinal native plants of Brunei Darussalam.

In ensuring the safety profile of the medicinal plants species, toxicity testings are required for the confidence use of the communities and the following steps need to be undertaken:

• Toxicity testing of the plant species.
• A modified and shorter duration toxicity testing to avoid long term risk.
• Administration of the total extract or combination of plants, if used, in exactly the same way as it is prepared and used by the population for the actual intended purpose of the plant species.

Community based studies on the efficacy and safety of the medicinal plants in multicentre clinical trials setting on the actual use of the plants to reflect real life scenario need to be conducted:
• test for efficacy in human to be approved by the ethics committee.

• safety profile of the plants investigated and duration of the toxicity studies to be decreased for plants being used by human to avoid risk due to long term exposure.

• plant is administered to human subjects in exactly the same manner as is being used in traditional medicine approach.

• conduct multicentre clinical trials on large number of patients with uncomplicated conditions of tropical diseases using native tropical medicinal plants such as similar studies conducted on traditional medicine *Qinghaosu* for the treatment of malaria.

### 2.10.6 Identification and development of a model list of medicinal plants used in Primary Health Care (P.H.C.)

As an initial set up programme and for long term planning, it is necessary for Brunei Darussalam to adopt the model list for biodiversity on cultivar development which should be based on the following important criteria:

• Availability of the planting area so that it could be readily used.

• Allocation of lands for cultivation and also replantation of forest with the plant species that will be exhausted and facing treat of depletion if these species are not conserved.

• Regulation on the prevention of medicinal plants from being over-harvested.
  
  Implementation of forest reserve scheme and policy to protect these plants from
extinction. Zoning of the forest areas for cultivating and farming the medicinal plants to be gazetted as plantations for medicinal plants.

- Set up nursery for small and large scale holdings.
- Conservatorium and herbarium are to be further developed for the research and development of important plant species and their potentials for therapeutic benefits.
- Compilation of scientific information on quality, safety and efficacy of these native medicinal plants of Brunei Darussalam for treating various disease conditions.

There are many disease conditions need to be identified which are normally encountered at the primary health care level for which herbal remedies could be used by the communities in the treatment of these ailments. The important criteria for the selection of plants for each of these conditions are:

- Actual use of these medicinal plants in Brunei Darussalam.
- Scientific literatures indicating the quality, safety and efficacy of the plants in certain diseases and common conditions.
- Use of these medicinal plants for therapeutic purposes in countries outside Brunei Darussalam.
- Documentation of these medicinal plants profile is essential to achieve the research and development objectives on medicinal plants of Brunei Darussalam.
2.10.7 Implementation of a national herbal formulary for more effective and proper use of TM/CAM

The objective of herbal remedies as in line with the national drug formulary is to provide guidance for the traditional medicine practitioners and to health personnels of the modern system of medicine and allied para-professionals of this system. This will ensure that the knowledge about the native plants and their profiles are not lost in time for the benefit of the young generation and the future generation to come. Such herbal formulary is to be prepared only after we have identified and produced a list of plants that are used at the primary health care level. The herbal formulary (Chaudhury, 1992) comprises of the following basic structure about the medicinal plant with additional scientific data and information:

- Name of plant (genus, species, authority and family).
- Plant part(s) used for disease conditions.
- Quality requirement (percentage of active principle, if known).
- Purity (foreign matter, adulterant, other contaminant).
- Physicochemical analysis:
  - Qualitative (including micro-chemical tests).
  - Quantitative (if major active ingredients are known, analytical procedures e.g. thin layer chromatography, high pressure liquid chromatography, chemical fingerprinting techniques should be developed to authenticate the plant species or varieties, quantify individual and total constituents.
- Bioassay testings
- *In vitro* assay
- *In vivo* assay

- Pharmacological/biological category for which the herbal product is indicated.
- Posology (correct dose and directions for use).
- Toxicity/contraindications with appropriate warning information.
- Packaging and storage.
- Proper packaging, storage and shelf life.
- Scientific information on clinical benefits, safety and risks on the plant.

### 2.10.8 Research and development strategies

Fully integrated, coordinated and concerted efforts among all health care professionals should be encouraged and to ensure national coherency and consistency in the key areas of research and development on the medicinal native plants:

- developing a coordinated scientific framework to assess the medicinal native plants.
- promoting the acceptance and integration of phytomedicines in all levels of health care practices.
- supporting and initiating clinical and experimental research in phytotherapy,
- improving and extending international data banks for updated scientific and knowledge on quality, safety and efficacy of the medicinal native plants.
- information on clinical benefits, safety and risks on the medicinal native plants.
- furthering collaboration and cooperation among established
associations/institutions/centres on a worldwide scale.

- taking all possible initiatives to advance these research and development objectives on medicinal native plants worldwide.
- sharing and exchange findings through publications of any clinical benefits, safety, efficacy and risks associated with the plant.

2.10.9 Collaborative and integrative roles of relevant ministries in the research and development of TM/CAM

The relevant ministries which play important roles as joint venture partnership with assured consistency and coherency in the national strategies to embark on the research and development of the medicinal plants of Brunei Darussalam are:

- Ministry of Industry and Primary Resources
- Ministry of Health
- Ministry of Home Affairs
- Ministry of Education
- Other relevant ministries/institutions/organizations/sectors of common interest in the field of medicinal plants.

2.10.10 Financial support & Funding mechanism

Collaborative efforts from the various ministries and private organizations/institutions/sectors that are involved in the research and development projects:
• Public sector
• Private sector
  - TM/CAM Industries
  - Pharmaceutical Industries
  - Research Laboratories,
  - Manufacturers and distributors in TM/CAM products.

National strategies are aimed to achieve the following main objectives on a long term basis:

• to encourage broader knowledge based and recognition of the importance and value of the medicinal plants of Brunei Darussalam.
• to advance the science, technology and practice of medicinal plants by modern scientific techniques/approach.
• to promote high standards of quality and safety in herbal remedies.
• to foster research in phytotherapy, exploring the vast potential of medicinal plants and its medicinal benefits.
• to gather and document all scientific information on the three important criteria such as quality, safety and efficacy i.e. clinical benefits and risks of the medicinal plants of Brunei Darussalam.
• to develop important medicinal plants into potentially therapeutic agents for the treatment of the uncomplicated disease conditions, accessible to the communities.
Increased support from the public and private sectors are needed in order to achieve successful research on medicinal plants leading to new drug development in the following areas:

- Pooling and concentrating resources from the best brains and medicinal plants expertise to this important field of research and development of therapeutically active substances from the medicinal plants of Brunei Darussalam.
- Use of appropriate methods and new technological advancement for cost-effectiveness management.
- Development of viable infrastructure and step up research and development into medicinal plants of Brunei Darussalam.
- Effective and efficient use of limited resources such as funding, facilities and manpower.
- Training of personnel or key professionals in medicinal plants research and development.
- Renewing interest of pharmaceutical industries in medicinal plants drug development.
- Establishment of the databank of genetic information on the medicinal plants species and varieties of Brunei Darussalam and other countries.
- Ensuring the continuous availability of the important species and varieties of medicinal plants of Brunei Darussalam through biotechnological advancements in identifying the molecular markers of the superior genotype(s) for quality cultivar development.
Conservation of the important cultivar of the selected medicinal plant species which produces high yields of active constituents for future research on their therapeutic effects on many disease conditions.

2.10.11 Public education on the proper use of TM/CAM

Strengthening the promotion of public education and information on the rational use of TM/CAM and other natural products in order to ensure continuing health care for the communities is based on the following activities:

- to encourage public to discuss and communicate with the health professionals about the use of TM/CAM in daily practices.
- to take herbal remedies/TM/CAM according to the directions and instructions prescribed by the practitioners.
- to report any adverse reactions/events experience by the consumers to the National Monitoring Centre on TM/CAM.
- to provide platforms for the discussion and communication on the proper use and safety of TM/CAM among the public.

In conclusion, the ultimate goal of the strategic framework is to focus on the integration and harmonisation process of TM/CAM and to ensure safe delivery of TM/CAM in the enhancement of health and improving quality of life of the communities in Brunei Darussalam. There is also the need to explore further into the important native medicinal plant species of Brunei Darussalam which may contain important secondary metabolites of therapeutic significance and benefits to the community at large.
Chapter 3  Andrographis paniculata (Burm. f.) Nees of Brunei Darussalam

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3.1 Why *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam?

The search for effective phytotherapeutic agents for the treatment of various diseases or conditions, such as oxidative stress, inflammation, anti-allergies, tissue injuries, hepatotoxicity, cancer and other pathological complications remain the quest for medicines. In this context, great efforts have been made in studying natural plants/herbs, especially those from tropical or natural diversity areas or regions. Nevertheless, in certain countries or regions limited resources are available, thus evidence for chemistry and actions of most plants/herbs which have long been used in the community is still lacking.

Brunei Darussalam has a wealth of natural plant resources with significant potential for molecular analysis to conserve the genetic diversity and consequently provides avenues for studies on phytochemical content and bioactivities of the plant species. Among the 340 plant species recorded, only 160 species were positively identified. *Andrographis paniculata* (AP) (Burm. f.) Nees of Brunei Darussalam, is a widely used medicinal herb in Asia in particularly China, India, South East Asia and Brunei Darussalam for many disease conditions.

It was reported that there are 28 species in the world, 25 out of these are distributed in India and 23 occurred in peninsular region (*Index Kewensis, Royal Botanic Garden, Oxford University Press, Oxford, 1977-87*). *Andrographis* species such as *A. elongata, A. viscosula, A. echioides* were identified but among these, *Andrographis paniculata* species is widely distributed in South-East Asia and is one of most popular herbs used among the communities in Asia. However, literature reviews indicated that the genetic diversity and variation information of the *Andrographis paniculata* species and varieties is very limited. There is no documented genetic diversity analysis of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam. Therefore,
the genetic profile of genus *Andrographis* collected from different phytogeographical distributions need to be assessed in order to provide important linkages for further research into the plants medicinal benefits. The understanding of the correlation between the genetic profile and the phytochemical profile may provide vital information on the intraspecific variation of the biochemical profile. Such a profile may lead to the understanding of the mechanisms behind the various important aspects of the phytochemical profile and the pharmacological effects of this important medicinal plant.

3.2 Medicinal Uses of *Andrographis paniculata* (Burm. f.) Nees

*Andrographis paniculata* (Burm. f.) Nees is a member of the plant family *Acanthaceae*, with pharmaceutical name *Herba Andrographitis paniculatae*. It is known as *Chuan Xin Lian* (CXL) in China, mainly cultivated and is collected or harvested in early autumn when foliage branch growing luxuriantly, and dried in the sun. The leaves are extremely bitter in taste. It is documented in the Chinese Pharmacopoeia, 2000 to be used for a number of disease conditions including removing heat, counteract toxicity, and induce subsidence of swelling. In addition, it is also applied for influenza with fever, sore throat, ulcers in the mouth, cough, colitis, dysentery, urinary infection and snake bite (Martz, 1992; Poolsup *et al.*, 2004; Singha & Roy, 2003; Thamlikitkul *et al.*, 1991). Some of these indications are alike those of use in many other Asian countries, mostly in India, South East Asia including Brunei Darussalam. Fasihuddin B. Ahmad (2004) conducted research study on the medicinal plants used by various ethnic groups such as Dusun, Kadazan and Murut in Sabah, a neighbouring Malaysian state close by to Brunei Darussalam reported that *Andrographis paniculata* known as ‘Mompoit’ (vernacular name in Sabah) is widely used by the local communities to treat a range of diseases such as hypertension and diabetes (Ahmad, 2004). The *Andrographis paniculata* leaves are usually consumed steeped
in hot water for drinking. In addition to that, the ASEAN Regional Centre for Biodiversity Conservation (ARCBC) in 2004 data collection listed in the checklist of medicinal plants in South East Asia reported that the upper ground aerial part of *Andrographis paniculata* is used as tonic, diuretic, anti-pyretic, stomachache, typhus, diabetes, counters eczema, against itchiness, hypertension, tonsillitis, flu and chest pain (ARCBC, 2004). *Andrographis paniculata* is also well known as “King of Bitters” in India and is found in the Indian Pharmacopoeia in many ayurvedic formulae. In Scandinavian countries, it is used to prevent and treat common colds.

The plant has a wide broad range of therapeutic applications. In the cardiovascular system it induces dilation of coronary blood vessels offering support for conditions such as hypertension and angina. It also stimulates cerebral and peripheral circulation enhancing conditions such as memory loss, chronic tiredness and numbness of extremities. In the immune system *Andrographis paniculata* enhances phagocytic activity of macrophages and migration of T-lymphocytes to peripheral organs.

### 3.3 Genetic diversity profile of *Andrographis paniculata* (Burm. f.) Nees

Currently, there is no documented genetic profiling analysis of *Andrographis paniculata* (Burm f) *Nees* of Brunei Darussalam. Literature search reported that the genus *Andrographis* also known as ‘King of Bitters; Kalmegh of Ayurveda’ as a whole is of potential significance to India as 25 out of 28 species (Padmesh *et al*., 1999) in the world are distributed mainly in India. However, with the development of biotechnology, new methods/techniques are available to study genetic profile of medicinal plants such as DNA fingerprinting, etc. Molecular techniques have aided in the assessment of genetic diversity in plants with profound results as compared to the traditional methods which relied on the use of morphological differences between individual
species or varieties. Further more, the number of easily scorable morphological differences are limited and are sensitive to environmental influences. Many diversity assessments are currently performed using RAPDs, RFLP and actual gene sequences.

The analysis by agarose gel electrophoresis is probably the most reliable approach, especially for the crude DNA preparations in order to estimate the concentration of genomic DNA extracted from *Andrographis paniculata* and to evaluate the quality of the DNA. In this study, the Polymerase Chain Reaction (PCR) is performed on *Andrographis paniculata* DNA extracted from its air dried powdered samples in order to generate its molecular marker profiles.

Andrographolide (C_{20}H_{30}O_{5}; MW 350.44), the plant-specific bicyclic diterpenoid lactone is intensively bitter and have demonstrated a wide spectrum of pharmaco-biological activities as described. Andrographolide content on dry weight basis was found to be highest at 1.47% in the AP36 genotype population from Tamil Nadu and showed an overall mean value of 0.95% for all the populations (Sabu, 2002). For the biodiversity rich countries in the tropics, chemical and genetic prospecting of the plant is a high priority and important not only for identifying superior genotypes/DNA molecules of potential economical importance but also for developing therapeutically active medicines.

The study conducted by Sabu (2002) and Padmesh et al., (1999) have shed some light in the intraspecific variations. The genetic distances were moderate among different populations which substantiated previous report on the RAPD analysis of *Andrographis paniculata* by Padmesh et al., 1999. *Andrographis paniculata* is unlike many other plant species that is facing threat from extinction due to its reduced genetic variability and increased susceptibility to agents of stress.
It is cultivated and distributed widely in many countries in Asia such as India, China and Thailand which at present is facing threat of genetic depletion as a result of over-exploitation due to its wide popularity of medicinal uses in the primary health care system. Measures are therefore initiated for the conservation of *Andrographis* species with its varied diversity, otherwise the potential variants with highest productivity to be developed as superior cultivars may disappear once for all and resulting in extinction of such an invaluable species. Sabu (2002) concluded in his study that by selection of an improved natural variant for the development as cultivar and the isolation of a high yielding cultivar from the selected superior genotype through chemical mutagenesis would be the most fruitful methods of achieving the dual objectives of conservation of the existing genetic diversity and sustainable utilization of this important medicinal resources which is affordable by the needy population of the developing world.

3.4 Phytochemical profile of *Andrographis paniculata* (Burm. f.) Nees

3.4.1 Morphology profile

Sabu (2002) reported that *Andrographis paniculata* (Burm f) *Nees*, an important medicinal plant of India, presumably having its center of origin and diversity in southern India and Sri Lanka, grows abundantly in southeastern Asia (Sabu, 2002). It is also cultivated extensively in China, Thailand (Sandberg, 1994), east and west Indies, Mauritius (Gupta *et al.*, 1990). *Andrographis paniculata* is such a hardy plant that grows in all types of soil, in pine, evergreen and deciduous forests, roadsides, and villages. It can also thrive on soil types such as serpentine soil which is relatively high in aluminium, copper and zinc where almost no other plant can be cultivated. Such hardiness accounts for its world wide-distribution and easy accessibility.
Andrographis paniculata appears to grow best in the tropical and subtropical areas of India, China and South East Asia including Thailand, Malaysia, Brunei Darussalam and Indonesia. The highest concentration of the active components is found just before the plant blooms, and harvested timely before the leaves fall.

It is an annual-branched, erect up to around one meter in height. Light green coloured stems are squarish, with green leaves of dimensions 5 cm x 1.5 cm, simple, elliptic with acuminate tips. The axillary and stem terminal in panicles with upright flowers; pods to 1.7 cm long, 2-celled, containing 12 seeds as described (Chin, 2000).

3.4.2 Phytochemical profiles of Andrographis paniculata

Table 3.1 illustrates the known chemical contents of Andrographis paniculata. Gorter was the first to isolate the bitter water soluble diterpene lactone, Andrographolide (C$_{20}$H$_{30}$O$_{5}$) from the leaves of Andrographis paniculata. Two other diterpenes, viz. Deoxandrographolide and Neoandrographolide with medicinal properties and clinical applications were further isolated by (Bright et al., 2001). It is reported to contain three active components diterpene lactones Andrographolide as the major compound, Deoxandrographolide and Neoandrographolide. Recent studies by Pramanick et al. (2005) and Shen et al. (2005) has isolated new ent-labdane type diterpenoids from the leaves of Andrographis paniculata (Pramanick et al., 2005; Shen et al., 2005) which are still undergoing various bioactivities testings.

There are various laboratory techniques available in studing the content of Andrographolide in Andrographis paniculata products including leaves powders, liquid extracts or tinctures, such as thin-layer chromatography, ultraviolet spectrophotometry, liquid chromatography, conventional
methods of volumetric and colorimetric systems. It was reported that the leaves contain the highest amount of Andrographolide (2.39%), being the most medicinally active phytochemical in the plant whereas the seeds contain the lowest (www.altcancer.com/andcan.htm). The levels of other medicinal phytochemicals extracted from the leaves are low but may be active in nature (Sharma et al., 1992).

Table 3.1 Phytochemical content of *Andrographis paniculata* leaves

<table>
<thead>
<tr>
<th>Phytochemical content of <em>Andrographis paniculata</em> leaves</th>
<th>References: (Chem &amp; Liang, 1982; Sharma et al., 1992; Shen et al., 2005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Andrographolide</td>
<td></td>
</tr>
<tr>
<td>2 Deoxyandrographolide</td>
<td></td>
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<tr>
<td>3 -19ß-D-glucoside</td>
<td></td>
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<tr>
<td>4 Neo-andrographolide</td>
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<tr>
<td>5 14-deoxy-11,12-didehydroandrographolide (andrographolide D)</td>
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<td>6 Homoandrographolide</td>
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<td>7 Andrographan</td>
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<td>8 Andrographon</td>
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<td>9 Andrographosterin</td>
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<td>10 Stigmasterol</td>
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<td>11 3-O-ß-D-glucopyranosyl-14, 19-dideoxyandrographolide (ent-labdane diterpenoids)</td>
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<td>12 14-deoxy-17-hydroxyandrographolide (ent-labdane diterpenoids)</td>
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<td>13 19-O-[ß-D-apiofuranosyl(1→2)-ß-D-glucopyranosyl]-3, 14-dideoxyandrographolide (ent-labdane diterpenoids)</td>
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<td>14 3-O-ß-D-glucopyranosylandrographolide (ent-labdane diterpenoids)</td>
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<td>15 12S-hydroxyandrographolide (ent-labdane diterpenoids)</td>
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<tr>
<td>16 Andrographatoside (ent-labdane diterpenoids)</td>
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</tr>
</tbody>
</table>
3.5 Pharmacological actions

Some of the important milestone discoveries as reported in the literature review and monographs (WHO, 2002c) based on the previous studies conducted on *Andrographis paniculata* (Burm. f.) *Nees* leaves which have led to our current understanding of the plant’s pharmacological actions and its medicinal value to the Asian communities. There is a renewed interest into conducting research on the mechanism of actions, cultivar development of *Andrographis paniculata* (Burm. f.) *Nees* and its potential as the therapeutic anti-cancer drug (Rajagopal et al., 2003).

3.5.1 Anti-bacterial and anti-microbial activities

George and Pandalai (1949) showed that the ethanol extract of *Andrographis paniculata* leaves inhibited the growth of Escherichia coli and Staphylococcus aureus *in vitro* (George & Pandalai, 1949). 50% methanol extract of *Andrographis paniculata* leaves were found to inhibit growth of Proteus vulgaris *in vitro* (Nakanishi et al., 1965). However, there was no *in vitro* activity observed by Leelarasamee et al., (1990) when the dried powder of the leaves of *Andrographis paniculata* was tested against E.coli, Staphylococcus aureus, Salmonella typhi or Shigella species based on three assays conducted (Leelarasamee et al., 1990). Firstly, direct assay of *Andrographis paniculata* crude powder in water at a concentration of 25g/Litre. Second assay was performed on testing the serum bacterial activity after oral ingestion of *Andrographis paniculata* stem and leaves by ten healthy volunteers at 1, 2, 3 & 6 g. Third study was carried out on culture media testing on the lung parenchyma and liver tissue from rats which were fed on high doses of *Andrographis paniculata* from 0.12-24g/kg body weight for six months before the rats were sacrificed. Andrographolide and arabinogalactan proteins from *Andrographis*
*paniculata* showed significant anti-microbial activity may be due to their combined effects in the aqueous extract (Prajjal *et al.*, 2003).

### 3.5.2 Anti-pyretic activity

Vedavathy and Rao (1991) investigated the intragastric administration of the ethanol extract of *Andrographis paniculata* leaves at 500mg/kg to rats on yeast-induced pyrexia. This extract was as effective as 200mg/kg body weight of aspirin (at equipotency dosage) with no toxicity observed at doses up to 600mg/kg body weight (Vedavathy & Rao, 1991). Intragastric administration of the major active component Andrographolide at 100mg/kg body weight to mice decreased brewer’s yeast-induced pyrexia (Madav *et al.*, 1995). Similar study was conducted on deoxyandrographolide, andrographolide, or 11, 12-didehydro-14-deoxyandrographolide at 100mg/kg body weight to mice, rats or rabbits were found to reduce pyrexia induced by 2, 4-dinitrophenol or endotoxins (Chang & But, 1986; Deng W *et al.*, 1982). The leaves have been widely used for relieving fever and lowering body temperatures by the Asian communities for many years.

### 3.5.3 Anti-Familial Mediterranean Fever

ImmunoGuard, a standardized fixed combination of *Andrographis paniculata* (Burm. *f.*) *Nees*, Eleutherococcus senticosus Maxim, Schizandra chinensis Bail and Glycyrrhiza glabra L. extracts showed significant improvement of the clinical symptoms such as abdominal, chest pains, temperature, arthritis, myalgia, erysipelas like erythema in patients with Familial Mediterranean Fever (Amaryan *et al.*, 2003).
3.5.4 Urinary tract infection

Study on fifty patients post Extracorporeal Shock Wave Lithotripsy (ESWL) pyuria and hematuria found that *Andrographis paniculata* tablets at an oral dose of 250mg reduced symptoms in ESWL urinary tract infection (Muangman *et al.*, 1995).

3.5.5 Anti-diarrhoeal activity

Gupta *et al.* (1990) found that ethanol, chloroform or 1-butanol extracts of *Andrographis paniculata* leaves at 300mg/ml inhibited the E. coli enterotoxin-induced secretory response-diarrhoeal syndrome in rabbit but the aqueous extract was ineffective. Andrographolide and Neoandrographolide exhibited potent antisecretory activity *in vivo* against E. coli enterotoxin-induced diarrhoea at 1mg of equivalent potency as loperamide when tested against heat labile E. coli enterotoxin-induced diarrhoea by acting through the stimulation of adenylate cyclase. However, at the same concentration, Andrographolide was more effective than loperamide when compared to Neoandrographolide against heat stable enterotoxin-induced diarrhoea by acting through the activation of guanylate cyclase (Gupta *et al.*, 1990).

3.5.6 Anti-inflammatory activity

Intragastric administration of deoxyandrographolide, andrographolide, neoandrographolide or 11, 12-didehydrodeoxyandrographolide to mice inhibited the increase in cutaneous or peritoneal capillary permeability induced by xylene or acetic acid. Among the tested components, 11, 12-didehydrodeoxyandrographolide exhibited the most potent anti-inflammatory activity *in vivo* (Chang & But, 1986).
3.5.7 Anti-malarial activity

Ethanol extract of the *Andrographis paniculata* leaves was found to inhibit the growth of plasmodium berghei both *in vitro* at 100mg/ml and also by intragastric administration in mice (1g/kg body weight). Andrographolide (5mg/kg body weight) and Neoandrographolide (2.5mg/kg body weight) were found to be effective when administered by gastric lavage in the treatment as anti-malarial agents.

In *vitro* and *in vivo* studies of *Andrographis paniculata* (Najib *et al.*, 1999) demonstrated the anti-malarial properties which can be further researched into the development of future potent anti-malarial drugs. The schizonticidal activity of *Andrographis paniculata* was found to be as potent as G. scortechinii in the inhibition of the sensitive strain, D10 of plasmodium falciparum but less effective inhibition towards the resistant strain, Gombak A (Najila *et al.*, 2002).

3.5.8 Anti-filarial activity

Dutta and Sukul found that the aqueous extract of *Andrographis paniculata* leaves caused mortality against Dipetalonema reconditum microfilariae within 40 min *in vitro* (Dutta & Sukul, 1982). The anti-filarial activity of *Andrographis paniculata* was measured by the relative movability value of the adult worms (Zaridah *et al.*, 2001).
3.5.9 Anti-platelet effect

Study on 63 patients of cardiac and cerebral vascular diseases taking *Andrographis paniculata* extract showed that it could inhibit the releasing of dense and alpha agranules for platelet and dilatation of canalicular system which suggested that *Andrographis paniculata* exerts its anti-platelet effect by raising the platelet cAMP level (Zhang et al., 1994). The possible effect of Andrographolide on the biosynthesis of eicosanoids and the platelet-activating factor (PAF) indicated that its mechanism of action is most likely to be associated with the cardiovascular and antithrombotic activity of *Andrographis paniculata* (Amroyan et al., 1999).

3.5.10 Choleretic effect

Andrographolide demonstrated a dependent choleretic effect at a dose range of 1.5-12 mg/kg as indicated by the increase in bile flow, bile salt, and bile acids in conscious rats and anaesthetized guinea pigs (Shukla & Visen, 1992).

3.5.11 Enhancement of intestinal digestion

Both *Andrographis paniculata* leaves extract and Andrographolide increased intestinal digestion and absorption of carbohydrate in a dose related and time dependent characteristic activation of brush border membrane-bound hydrolases (Choudhury & Poddar, 1985).
3.5.12 Anti-fertility

Male albino rats fed at a dose of 20mg/day of dried leaf powder of *Andrographis paniculata* for 60 days resulted in the cessation of spermatogenesis, degenerative changes in the seminiferous tubules, regression of Leydig cells in the epididymis seminal vesicle, ventral prostate and coagulating gland demonstrated the antispermatic and antiandrogenic effect of the plant (Akbarsha *et al*., 1990). Female mice on *Andrographis paniculata* at 2gm/kg daily for a period of 6 weeks were not pregnant compared to the majority of the control female mice (95.2%) without *Andrographis paniculata* were pregnant indicated its potent antifertility effect (Zoha *et al*., 1989), indicating a precaution against use during pregnancy. Extra contraception is necessary as *Andrographis paniculata* has not been proven as an absolute contraceptive.

3.5.13 Anti-venom activity

*Andrographis paniculata* is indicated for snake bites as traditional medicine therapy. Intraperitoneal injection of an ethanol extract of the leaves (25g/kg body weight) to mice poisoned with cobra venom markedly delayed the occurrence of respiratory failure and death. This extract also induced contractions in guinea-pig ileum at concentrations of 2mg/ml, and these contractions were enhanced by physostigmine and blocked by atropine, but were unchanged by antihistamines. Studies indicated that the extracts do not modify the activity of the nicotinic receptors but possibly mediated by producing significant muscarinic activity, accounting for its anti-venom effects (Chang & But, 1986; Nazimudeen *et al*., 1978).
3.5.14 Anti-hyperglycaemic effect

Diabetes is a condition strongly related to oxidative stress. Complications of the diabetes such as neuropathy, retinopathy, nephropathy and multi-organ atherosclerosis are believed to be implicated by oxygen free radicals namely superoxide (O$_2^-$), hydrogen peroxides (H$_2$O$_2$) and hydroxyl radicals (OH$^-$) (Sabu & Kuttan, 2002). Experimental study on the anti-hyperglycaemic and anti-oxidant properties of ethanolic extract of *Andrographis paniculata* in streptozotocin-diabetic rats at doses of 0.1-0.4g/body weight significantly reduced the fasting serum glucose level in a dose dependent effect similar to the metformin treatment group compared to the vehicle (Zhang & Tan, 2000a). The liver & kidney thiobarbituric acid-reactive substances (TBARS) were significantly decreased, whereas liver GSH concentrations were significantly higher (P<0.05) in extract and metformin treated diabetic rats. Hepatic superoxide dismutase (SOD), catalase (CAT) & glutathione peroxidase (GSH-PX) activities were significantly lowered in vehicle treated diabetic rats. However, *Andrographis paniculata* and metformin significantly increased the SOD and CAT activities but had no significant effect on GSH-PX activity in the diabetic rats. The results indicated that *Andrographis paniculata* possess anti-hyperglycaemic property and may also reduce oxidative stress in diabetic rats (Zhang & Tan, 2000b).

In contrast, *Andrographis paniculata* (Burm. f.) Nees does not lower fasting blood sugar level in non-diabetic rabbits after chronic administration of aqueous extract at 10 mg/kg body weight over a duration of 6 weeks, probably due to its prevention on the glucose absorption from the gut (Borhanuddin et al., 1994).
3.5.15 Cardiovascular activity

Zhang & Tan (1997) studied the mechanism of hypotensive activity of *Andrographis paniculata* in the anaesthetized Sprague-Dawley (SD) rats and found that it was mediated through blockade of α-adrenoceptors, autonomic ganglion and histaminergic receptors but not by the β-adrenoceptor, muscarinic cholinergic receptor and angiotensin-converting enzyme (Zhang & Tan, 1997). Study on the cardiovascular activity of 14-Deoxy-11,12-Didehydroandrographolide in the anaesthetised rat and isolated right atria (Zhang et al., 1998) demonstrated its bradycardia-inducing and β-adrenoceptor antagonistic properties. A study has shown that *Andrographis paniculata* (Burm. f.) Nees and fish oil can alleviate atherosclerosis artery stenosis induced by both de-endothelialization and high cholesterol diet (HCD) as well as lower restenosis rate after experimental angioplasty in rabbits which demonstrated that *Andrographis paniculata* (Burm. f.) Nees is superior (Wang & Zhao, 1994).

The cardiovascular actions of *Andrographis paniculata* may also be related to its activity on calcium ion channels as demonstrated by Burgos et al. (2000) that *Andrographis paniculata* (Burm. f.) Nees selectively blockades the voltage-operated calcium channels (VOCs) by inhibiting the Ca$^{2+}$ influx (Burgos et al., 2000). *Andrographis paniculata* (Burm. f.) Nees may improve the activity of sarcolema ATPase in alleviating the Ca$^{2+}$ and Na$^{+}$ overloading by decreasing the harmful effect of oxygen free radical in dog model (Guo et al., 1995).

Eight dogs pretreated with the root extracts of *Andrographis paniculata* (Burm. f.) Nees showed that there was no elevation of the ST segment, plasma 6-k-PGF 1 alpha and platelet cAMP were increased, the production of TXB2 and aggregation of platelets were inhibited, and no thrombus
or myocardial infarction was induced. The data obtained (Zhao & Fang, 1991) suggested that *Andrographis paniculata (Burm. f.) Nees* might promote the synthesis of PGI2, inhibit the production of TXA2, stimulate the synthesis of cAMP in platelets, impede aggregation of platelets, and prevention of thrombus formation and development of myocardial infarction. Chiou et al. (1998) demonstrated that Andrographolide inhibited the expression of an inducible isoform of nitric oxide synthase linked to endotoxin-induced circulatory shock (Chiou et al., 1998). The protective effects of *Andrographis paniculata (Burm. f.) Nees* on post infarction myocardium in experimental dogs (Zhao & Fang, 1990) suggested that it may limit the expansion of ischemic area focus, exert marked protective effect on reversibly ischemic myocardium and demonstrated a weak fibrinolytic action.

### 3.5.16 Immunostimulatory activity

Intragastric administration of an ethanol extract of the *Andrographis paniculata* leaves at 25mg/kg body weight or the purified andrographolides at 1mg/kg body weight to mice stimulated antibody production. The crude extract was found to be more effective than either Andrographolide or Neoandrographolide alone indicating that the effect may be due to the other active constituents involved in the immuno-stimulant response (Puri et al., 1993).

### 3.5.17 Anti-human immunodeficiency virus (HIV) activity

Study on the aqueous and methanol extracts of *Andrographis paniculata* leaves inhibited HIV-1 infection and replication in the lymphoid cell line MOLT-4. Dehydroandrographolide inhibited HIV-1 and HIV-1 (UCD123) infection of H9 cells at 1.6µg/ml and 50µg/ml respectively (Otake et al., 1995).
3.5.18 Anti-tumour activity

Recent studies have found that *Andrographis paniculata* (*Burm. f.*) *Nees* is a potential anti-cancer agent. *Andrographis paniculata* plant extract contains diterpenes, flavonoids and stigmasterols with Andrographolide as the major active diterpenoid which have shown to possess potent cytotoxic activity against KB (human epidermoid carcinoma) and P388 (Mouse leukaemic lymphoblastic parental tumour cell line) (Siripong *et al.*, 1992). Among the three diterpene compounds isolated, the major active component Andrographolide showed anti-cancer activity on diverse cancer cells representing different types of human cancers and all enhanced proliferation and interleukin-2 (IL-2) induction in HPBLs (Rajagopal *et al.*, 2003). Study using different cancer cell lines on the modulatory influence of *Andrographis paniculata* (*Burm. f.*) *Nees* on mouse hepatic and extrahepatic carcinogen metabolizing and anti-oxidant status (Singh *et al.*, 2001) also indicated that it has chemoprotective potential against chemotoxicity including carcinogenicity.

The research conducted by Rajagopalan *et al.* (2003) demonstrated that Andrographolide at a concentration range of 5-15µM inhibits growth of human cancer cell lines such as Leukemia (CCRF-CEM, K562), Renal (ACHN, A498), Prostate (DU145, PC3), Ovarian (ES2, SKOV3, OVCAR8, PA-1), Melanoma (UACC62, A431, M14), Lung (A549, NCI-H23, HOP62, MES-SA, MES-SA-DX5, H522), Colon (SW620, HT29, HCT116, KM12, COL0205), CNS (U251, SF268, SNB 19), Breast (MDA-MB-453, MCF7, T47D, MCF7/ADR) with similar potency, except the COLO205 cell line which showed more sensitivity to Andrographolide treatment.

The findings suggested that Andrographolide exerts direct anti-cancer activity on cancer cells by cell-cycle arrest at G0/G1 phase through the induction of cell-cycle inhibitory protein p27 and
decreased expression of cyclindependent kinase 4 (CDK4). The indirect anti-cancer activity is by enhancing the tumor necrosis factor-a production and CD marker expression, causing an increased cytotoxic activity of lymphocytes against cancer cells. Its immunostimulatory activity is shown by the increased proliferation of lymphocytes and production of interleukin-2. This indicated that Andrographis paniculata (Burm. f.) Nees is a beneficial pharmacophore with anti-cancer and immunomodulatory activities which warrant for its continued research & development in anti-cancer therapy.

Results from the study on the increased tumor necrosis factor alpha (TNF-alpha) and natural killer cell (NK) function in late stage cancers using an integrative approach of a combination of natural products such as Transfer Factor Plus; IMUPlus; Ascorbic acid; Agaricus Blazeii Murill teas; Immune Modulator Mix (a combination of vitamin, mineral, antioxidants and immune-enhancing natural products) indicated high levels of genistein and dadzein and Andrographis paniculata at 500 mg twice daily was quite promising as an anti-cancer agent (See & Mason, 2002).

### 3.5.19 Anti-hepatotoxicity activity

The Andrographis paniculata leaves extract protected carbontetrachloride-induced hepatic toxicity better than Andrographolide (Choudhury & Poddar, 1984). Studies have demonstrated the in vivo hepatoprotective effect of Andrographolide against carbontetrachloride (Handa & Sharma, 1990b; Rana & Avadhoot, 1991), galactosamine or paracetamol-induced hepatotoxicity in rats (Handa & Sharma, 1990b). The significant hepatoprotective activity of aqueous extract of Andrographis paniculata was reported on hexachlorocyclohexane (BHC) induced severe liver damage in Swiss male mice by estimating serum ALT & AST and parameters such as alkaline
phosphatase, \( \gamma \)-Glutamyl transpeptidase, glutathione and lipid peroxidase (Trivedi & Rawal, 2000).

A comparative study by Kapil et al. (1993) with the known hepatoprotective agent silymarin demonstrated that Andrographolide exhibited a lower protective potential than Andrographiside and Neoandrographolide, which were as effective as silymarin in the formation of the degradation products of lipid peroxidation and release of glutamic-pyruvate transaminase (GPT) and alkaline phosphatase. The greater hepatoprotective activity of the latter two diterpenes could be due to their glucoside groups which may act as strong antioxidants, possibly due to the antioxidant activities of superoxide dismutase (SOD), catalase, glutathione peroxidase and reductase as well as the level of glutathione (Trivedi & Rawal, 2001).

Intraperitoneal administration of methanol extract of *Andrographis paniculata* leaves and also Andrographolide to mice inhibited the hepatotoxicity induced by carbon tetrachloride (CCl\(_4\)), and reversing the CCl\(_4\)-induced histopathological changes in the liver by suppressing the increased activity of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase and bilirubin–induced by CCl\(_4\). Andrographolide being the major antihepatotoxic component of the plant, exhibited hepatoprotective effect in rats against hepatotoxicity induced by CCl\(_4\), D-galactosamine, paracetamol and ethanol, more effective than silymarin, the standard hepatoprotective agent (Kapil *et al.*, 1993; Visen *et al.*, 1993).
3.5.20 Toxicological studies

*Andrographis paniculata* did not produce reproductive toxicity in male rats after 60 days of intragastric administration of 20-1000mg/kg body weight daily and is not mutagenic *in vitro* study. It possesses anti-mutagenic activity (Burgos *et al.*, 1997). It is contraindicated in pregnancy due to the suggestion that it has abortifacient activity, however, no interruption of pregnancy, fetal resorption or decrease in the number of live offspring was observed in pregnant rats fed with 2g/kg body weight during the first 9 days of gestation (Zoha *et al.*, 1989). As a general rule of thumb, any medicinal or herbal product is contraindicated unless proven safe for use in pregnancy.

Due to the potential antagonism between *Andrographis* and endogenous progesterone, as a caution, it is contraindicated in pregnancy (Panossian *et al.*, 1999) or lactation or in cases with known allergies to the *Acanthaceae* family. It is not recommended to be injected in various routes of administration to avoid adverse effects such as potential anaphylactic reactions. It has been reported to interact with Isoniazid in a synergistic manner (Chang & But, 1986). Ingestion of large doses of *Andrographis paniculata* orally have been reported to cause gastric discomfort, vomiting and loss of appetite as associated adverse reactions or side effects of the extremely bitter taste of Andrographolide. Cases of urticaria have also been reported (Melchior *et al.*, 1997). The evaluation of the safety of subchronic and testicular toxicity of *Andrographis paniculata* was conducted and concluded that the dried extract did not produce such toxicity effects in male rats (Burgos *et al.*, 1997).
3.5.21 Clinical studies on *Andrographis paniculata*

3.5.21.1 Common cold and related symptoms

In a placebo-controlled, double-blind clinical trial on 61 adult patients were conducted to assess the efficacy of a standardized *Andrographis paniculata* leaves extract of 1200mg extract daily containing 4% Andrographolides for the treatment of common cold. Result showed a significant reduction of $p<0.0001$ in the clinical symptoms of the patients such as sore throat, tiredness, muscular ache and malaise observed on day four, compared to the placebo controlled group. Both groups did not exhibit any adverse drug reactions (Hancke *et al.*, 1995b).

In a randomized, placebo-controlled, double-blind study was conducted to evaluate the efficacy of a standardized *Andrographis paniculata* leaves extract of 200mg extract daily for 3 months containing 4% Andrographolides for the prophylaxis of common cold on 107 school children during the winter season. On evaluation weekly by the physician, no difference was observed on the occurrence of common cold between the two groups at the first two months of treatment. There was a significant difference $p<0.05$ in the third month between the treated group (30%) as compared to the placebo group (62%) (Caceres *et al.*, 1997).

A group of 150 adult patients in a randomized double blind placebo trial received *Andrographis paniculata* dried extract (1,200 mg/day), using visual analogue scale measurements (VAS) have shown its effectiveness in the relief of common cold symptoms (Caceres *et al.*, 1999). Two randomized double-blind, placebo-controlled parallel group clinical trial studies (Melchior *et al.*, 2000) showed that throat symptoms and signs were found to be significantly improved. In a randomized, placebo-controlled, double-blind pilot trial on 50 adult patients were conducted to
evaluate the efficacy of a standardized *Andrographis paniculata* leaves extract of 1020mg extract daily for five days containing 4% Andrographolides for the treatment of initial symptoms of common cold and uncomplicated sinusitis.

Results demonstrated that the patients on the treatment group (0.21 days) were off sick less than the placebo group (0.96 days). In addition to that, 68% of the treated patients were totally recovered compared to the 36% of the placebo group. And 55% of the treated patients felt that the course of illness was much easier than normal compared to only 19% of the placebo group (Melchior et al., 1997). In a randomized, placebo-controlled, double-blind comparison study of 152 adult patients with pharyngotonsilitis evaluate the efficacy of powdered *Andrographis paniculata* leaves 6g daily and paracetamol of 1 capsule of 325mg for improving symptomatology. No significant difference between the two groups at baseline comparison. However, the crude drug was as effective as paracetamol in the reduction of sore throat and fever after three days of treatment (Thamlikitkul et al., 1991).

In a study, treatment of patients on *Andrographis paniculata* leaves extract containing 4% Andrographolides reduced the incidence of fever associated with common cold. The body temperature of patients was lowered in less than 48 hours post treatment (SICMM, 1975). This finding was confirmed in another study (Hancke et al., 1995a).
3.5.21.2 Urinary infections

A clinical trial comparative study on the efficacy between *Andrographis paniculata*, co-trimoxazole (sulfamethoxazole + trimethoprim) and norfloxacin in the prevention of urinary tract infections after extracorporeal shock wave lithotripsy. The dosage of *Andrographis paniculata* was four tablets of 250mg, three times daily, co-trimoxazole two tablets of 25mg, twice daily, norfloxacin was one tablet of 200mg, twice daily. The urinalysis results of 100 patients post 1 month treatment demonstrated that pyuria, haematuria and proteinuria were reduced in all treatment groups with no significant difference between the three treatments (Muangman et al., 1995).

3.5.21.3 Dysentery

It has been reported in some clinical studies that the combination of Andrographolide and Neoandrographolide were found to be more effective than either furazolidine or chloramphenicol in the treatment of bacillary dysentery (Chang & But, 1986). In a randomized, placebo-controlled, double-blind comparison study of 200 adult patients on treatment with *Andrographis paniculata* and tetracycline at a dosage of 500mg, four times daily for 3 days for treating acute diarrhoea and bacillary dysentery (Chaichantipyuth & Thanagkul, 1986; Thanagkul & Chaichantipayut, 1985). *Andrographis paniculata* was found to decrease the diarrhoea in terms of frequency and amount of discharge. It was also more effective in treating diarrhoea of shigellosis rather than from cholera.
3.6 Objectives of the thesis and research aims

Little is known about the genetic diversity, phytochemical profile, pharmacological actions of the content of the active components of Andrographolide and Dehydroandrographolide present in *Andrographis paniculata (Burm. f.) Nees* species grown in Brunei Darussalam. Despite the long traditional use of *Andrographis paniculata* by the communities in Brunei Darussalam. Therefore, such study will serve as the pioneering project to assess this medicinal native plant of Brunei Darussalam and future research on other medicinal native plants of Brunei Darussalam based on the fact that plants from different sources may have different phytochemical profiles and pharmacological actions. This study used various interdisciplinary approach to assess the genetic diversity, phytochemical contents, and pharmacological actions to establish the profile of *Andrographis paniculata (Burm. f.) Nees* species grown in Brunei Darussalam.

The tropical forest of Brunei Darussalam in the Asia region remains a national heritage of bioprospecting avenues for the future developments of medicinal native plants. Medicinal plants of Brunei Darussalam available in abundance still need to be explored and subjected to rigorous research in order to ascertain the associated claims of medicinal uses and clinical applications. Traditional medicines (‘ubat kampong’) need to be researched further for scientific proof of their efficacy and safety to ensure the rational and proper use. It is one of the nineteen species of *Andrographis* belonging to the family *Acanthaceae* (Saxena S. et al., 1997). One of the most widely used plants in Ayurvedic preparations in which twenty-six out of forty Indian polyplant formulations contain *Paniculata* species (Handa & Sharma, 1990a).

The objective of this research, therefore, is to evaluate the bioactivities and investigate the correlation of the genetic fingerprinting of *Andrographis paniculata* species/varieties and its
bioactivities. The findings of this study may provide critical information on the potential therapeutic use of *Andrographis paniculata* (*Burm. f.*) *Nees* of Brunei Darussalam as promising therapeutic drugs.

### 3.6.1 Aims of the thesis

There are several research aims in the present project based on extensive literature reviews and an interdisciplinary systematic approach of scientific analyses, which are:

- to develop the strategic framework on TM/CAM for Brunei Darussalam

- to study the genetic diversity profile of *Andrographis paniculata* (*Burm. f.*) *Nees* of Brunei Darussalam using DNA fingerprinting techniques of RAPD and RFLP analyses.

- to study the phytochemical profile of *Andrographis paniculata* of Brunei Darussalam by HPTLC and HPLC analyses.

- to determine the %w/w content of the active components Andrographolide (A) and Dehydroandrographolide (D) in *Andrographis paniculata* of Brunei Darussalam.

- to evaluate the pharmacological actions of *Andrographis paniculata* (*Burm. f.*) *Nees* extracts on oxidative radicals, iNOS-mediated response, mast cell derived histamine release, tumour cell growth as well as its anti-tumour and general hepatocyte toxicity.
## 4. Chapter 4 Genetic Diversity of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam

### 4.1 Introduction

4.1.1 RAPD-PCR and PCR-RFLP

### 4.2 Materials and methods

4.2.1 Plant materials

4.2.2 DNA extraction and PCR primers

4.2.3 RAPD analysis

4.2.4 PCR-RFLP analysis

4.2.5 Data collection and analysis

### 4.3 Results

### 4.4 Discussion
4.1 Introduction

Brunei Darussalam has a wealth of natural plant resources with great potential for discovering new molecules with bioactive activities. An estimated 5,000 native species have been found in the tropical rain forests which constitutes 80% of the total land area. Ethnobotanical surveys recorded 340 species with 160 species were positively identified (Department of Agriculture, 2000). Out of these species, *Andrographis paniculata* is a widely used medicinal plant in Asia in particularly India, China and South East Asia including Brunei Darussalam for many disease conditions. It is a popular plant from the ‘Acanthaceae’ family used by the communities to lower high blood pressure, managing diabetes and to relieve abdominal pains. However, at present there is a lack of study on the genetic diversity of of *Andrographis paniculata* of Brunei Darussalam. The aim of this study was to use RAPD and PCR-RFLP at 5S-rRNA region to determine the intraspecific genetic variability of nine genotypes of *Andrographis paniculata* collected from three districts of Brunei Darussalam.

Studies on the genetic diversity information of this species or varieties are important not only for the authentication of the plant species but also for future studies of correlations between genetic markers and their metabolic compounds or chemotypes. Literature search reported that the genus *Andrographis* also known as ‘King of Bitters; Kalmegh of Ayurveda’ is of potential significance to India as 25 out of 28 species in the world are distributed mainly in India with 23 of them occurring in the peninsular region. These different species and varieties of *Andrographis paniculata* are used interchangeably as traditional medicine worldwide as described in Table 2.3 of Chapter 2. *Andrographis paniculata* has also been listed as herbal medicines in the Chinese
With the advent of the development of molecular cloning and polymerase chain reaction (PCR) techniques, DNA based markers are advocated as one of the important techniques to identify and authenticate plant species or varieties of plant medicinal materials (Joshi et al., 2004). The application of conventional chemical analysis is limited as identification and authentication tools since the amount and profiles of the plant species ginsenosides are affected significantly due to variable factors such as the growth, storage conditions, samples freshness and post-harvest processing (Ngan et al., 1999). The DNA-based markers are less affected by age, physiological conditions of samples and environmental factors and is popularly used for the identification and authentication of natural products such as plant and animal species due to its robustness (Shaw et al., 2002).

DNA profiling can specifically identify herbal species/varieties or cultivar, to ensure the quality if there is any linkage of its genetic identity with either their chemical constituents or bioactivities and to ascertain the genetic uniformity of herbal materials. Therefore, continued research on the establishment of the correlations between the genetic diversity profile and the chemical constituents and compositions of medicinal plants used by the community at large need to be supported.
4.1.1 RAPD-PCR and PCR-RFLP

Random Amplified Polymorphic DNA (RAPD) is a useful tool in genetic analysis, in classification of species and in taxonomic studies. The key to RAPD is that oligonucleotides of arbitrary sequence are used as PCR primers and if these primers are short, then complementary sequences will occur frequently in the target genome (Malyshev & Kartel, 1997). There is a finite chance that not only the pairs of sequences are complementary to the primer and at the same time they will be arranged with 3’ ends pointing towards each other. Under appropriately optimised thermal cycling conditions, annealing of the primer to the target genome will result in the production of an amplified fragment (Malyshev & Kartel, 1997). RAPD analysis enables good differentiation between closely related organisms in the form of the different banding patterns in PCR products generated due to the difference in DNA of different strains of species (Foster et al., 1993; Tommerup et al., 1995). RAPD is a commonly used molecular technology which has been extensively applied to study the genetic diversities and authenticate different species or varieties of plant materials (Shaw et al., 2002). PCR-RFLP has also been used to study plant profiling. It reveals variation in DNA sequences between organisms that can be recognised through the use of restriction endonucleases which identify particular sequences and consistently make double stranded cuts in DNA.

RAPD and PCR-RFLP analyses have become a popular means for the identification and authentication of natural products such as plant and animal species. For instance, they have been used to characterise species such as Epimedium species (Nakai et al., 1996) and to examine the genetic relationships within genera of herbal species (Kim et al., 2004a; Roser et al., 2001).
Interspecies variation studies on various genera of *Glycyrrhiza* (Yamazaki *et al*., 1994), *Echinaceae* (Kapteyn *et al*., 2002), *Curcuma* (Chen *et al*., 1999) were also conducted. Wang *et al*., (2004) revised the phenotypic relationship between 20 species from the subgenus *Yulania* based on RAPD technique (Wang *et al*., 2004). The significant genetic distance between the species derived from different geographical locations of Asia and America were investigated among 17 species of subgenus *Yulania* using RAPD analysis which generated high levels of polymorphisms. In general, RFLP markers are much less polymorphic, more expensive and labour intensive compared to RAPD.

### 4.2 Materials and methods

#### 4.2.1 Plant materials

The *Andrographis paniculata* plant materials were collected from the various districts (Figure 4.1). *Andrographis paniculata* leaves samples were authenticated by the Department of Agriculture, Ministry of Industry and Primary Resources, Brunei Darussalam. The reference control *Chuan Xin Lian* (CXL) leaves were authenticated by the Chinese Medicine Clinic of RMIT University, Bundoora West Campus, Melbourne, Victoria.
Figure 4.1. Collection of herbal accessions

The fresh leaves from nine accessions of *Andrographis paniculata* were obtained from the following districts of Brunei Darussalam. Bandar Seri Begawan from the Brunei/Muara district (designated as AP2, AP4, AP6, AP8, AP11, AP14, AP15); Kuala Belait (district designated as K.B.); Temburong district (designated as T). The materials were air dried and transported to RMIT University, Australia. Dried herbal materials *Chuan Xin Lian* (CXL) were also obtained from China by the Chinese Medicine Clinic, Chinese Medicine Division of Royal Melbourne Institute of Technology, Bundoora West Campus, Australia.
4.2.2 DNA extraction and PCR primers

Total DNA from air-dried fresh leaves were extracted using the DNeasy Plant Mini Kit (Qiagen). Thirty four random oligonucleotide primers-10mer (Operon Technologies Inc.) and 5S-rRNA sequences using primers-20mer 5SP1 (forward) (5’-GTG CTT GGG CGA GAG TAG TA-3’) and 5SP2 (reverse) (5’-TTA GTG CTG GTA TGA TCG CA-3’) purchased from Geneworks were designed for Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (PCR-RFLP) analyses respectively.

4.2.3 RAPD analysis

The RAPD analysis was modified from that described in published literature (Cui et al., 2003; Shaw & But, 1995). The 25μl PCR mixture comprised of 40ng of template DNA, 2.5μl of 10xPCR buffer (100mM Tris-HCl, 500mM KCl, 0.01% gelatin, Invitrogen, Australia), 0.75μl of 50mM MgCl₂, 6μl of 1mM dNTP, 1μl of 10μM primer and 1 unit Taq Polymerase (Invitrogen, Australia). PCR reaction consisted of 3 min at 94°C, followed by 15 seconds at 94°C (denaturing), 1 min at 40°C (annealing), 1 min at 72°C (extension) for 35 cycles, and terminating with 5 minutes at 72°C for RAPD analysis by Px2 Thermal Cycler (Thermal Electron Corporation, UK). Each PCR product was electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining. The Discovery Series Quantity One 1-D Analysis Software (BioRad, Australia) was used for imaging the electrophoresis gels.
4.2.4 PCR-RFLP analysis

As for the PCR-RFLP analysis, a pair of PCR-primers, 5SP1 (forward) 5’-GTG CTT GGG CGA GAG TAG TA-3’ and 5SP2 (reverse) 5’-TTA GTG CTG GTA TGA TCG CA-3’ were used. They are designed to amplify the 5S ribosomal RNA (5S-rRNA) spacer (Wolters & Erdmann, 1988). The 25μl PCR mixture containing 40ng leaf DNA, 2.5μl of 10×PCR buffer, 0.75μl of 50mM MgCl₂, 6μl of 1mM dNTP, 1μl of 10μM forward primer, 1μl of 10μM reverse primer and 1 unit Taq polymerase (Invitrogen, Australia). PCR reaction was performed using 38 cycles consisting of 5 min at 94°C, 1 min at 94°C, 1min at 60°C, 1 min at 72°C, terminating with 10 min at 72°C and hold at 4°C in a Px2 Thermal Cycler (Thermal Electron Corporation, UK). Restriction enzyme 10×Buffer was recommended for use by the manufacturer’s product information.

Briefly, 4μl of the PCR products from the 5S-rRNA gene were then digested with the 13 restriction enzymes in purified BSA 100x (New England Biolabs, USA) were incubated at 37°C for 2hr (Lin et al., 2001b) and out of these, *HinfI*, *MnlI*, *MspI*, *HaeIII*, *NlaIV*, *Tsp 509I* were found to be suitable (Table 4.3). Each PCR products was fractionated in 2.0% agarose gel and visualized by Ethidium Bromide staining. The Discovery Series Quantity One 1-D Analysis Software (BioRad, Australia) was used for imaging the electrophoresis gels. Only clear, coherent and consistent banding patterns revealed by RAPD analysis were scored. RAPD bands were scored as present (1) or absent (0) for each genotype.
4.2.5 Data collection and analysis

On the basis of Nei’s coefficient, a matrix of genetic distances estimation between the accessions of *Andrographis paniculata* from different districts of Brunei Darussalam based on dissimilarity (\(D=1-S_{XY}\)) indices was obtained by POPGENE version 1.31, a Microsoft Window-based free software for population genetic analysis (Yeh *et al.*, 1999). The Molecular Evolutionary Genetics Analysis 2 (MEGA 2) was used for reconstruction and comparing the genetic distances between all individuals by an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Kumar & Tamura, 2000; Kumar *et al.*, 2001). The linearised tree was expressed as percentage of the dissimilarity indices.

4.3 Results

For RAPD analysis, 34 random primers were screened and of these, 13 primers (Table 4.1) were found to be suitable for the identification of DNA from the leaf samples investigated but 21 primers were not suitable (Table 4.2). *Andrographis paniculata* populations are genetically and moderately different in Brunei Darussalam on the basis of RAPD analysis. The genomic DNA fingerprinting by RAPD among fresh accessions from *Andrographis* species showed distinctive DNA fragments.

There was a considerable level of polymorphism among the genotypes investigated. Of the total 67 loci scored, 61% were polymorphic with an average of 3 bands per primer (Table 4.1). The
number of bands produced per primer ranged from 1 (OPA-11) to 9 (OPW-04) with an average of 5 bands per primer and the products ranged in size from 280bp to 3000bp.

Table 4.1  Polymorphic bands generated by 13 RAPD primers which were suitable

<table>
<thead>
<tr>
<th>RAPD Primers (Operon Technologies)</th>
<th>Sequence 5’ - 3’</th>
<th>Total No. of bands</th>
<th>No. of Polymorphic bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA-11</td>
<td>CAATCGCCGT</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>OPB-08</td>
<td>GTCCACACGG</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>OPC-19</td>
<td>GTTGCCAGCC</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>OPG-13</td>
<td>CTCTCCGCCA</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>OPG-14</td>
<td>GGATGAGACC</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>OPM-04</td>
<td>GGCCTTGCCTAC</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>OPN-04</td>
<td>GGACTTGAGT</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>OPP-10</td>
<td>TCCCGCTAC</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>OPT-13</td>
<td>AGGACTGCCA</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>OPW-04</td>
<td>CAGAAGCGGA</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>OPW-09</td>
<td>GTGACGGAGT</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>OPX-17</td>
<td>GACACGGACC</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>OPZ-10</td>
<td>CCGACAAACC</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

Total No. of bands: 67
Mean per primer: 5.15 ≈ (5)

Number of Polymorphic bands: 41
Mean per primer: 3.15 ≈ (3)
Table 4.2  RAPD primers screened but not suitable

<table>
<thead>
<tr>
<th>RAPD primers</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPZ-02</td>
<td>CCTACGGGGGA</td>
</tr>
<tr>
<td>OPZ-04</td>
<td>AGGCTGTGCT</td>
</tr>
<tr>
<td>OPZ-12</td>
<td>TCAAGGGGAC</td>
</tr>
<tr>
<td>OPZ-16</td>
<td>TCCCCCATCAC</td>
</tr>
<tr>
<td>OPAW-03</td>
<td>CCATGCGGAG</td>
</tr>
<tr>
<td>OPAW-05</td>
<td>CTGCTTCGAG</td>
</tr>
<tr>
<td>OPV-06</td>
<td>ACGCCCAGGT</td>
</tr>
<tr>
<td>OPU-03</td>
<td>CTATGCGGAC</td>
</tr>
<tr>
<td>OPT-17</td>
<td>CCAACGTGCT</td>
</tr>
<tr>
<td>OPT-03</td>
<td>TCCACTCCTG</td>
</tr>
<tr>
<td>OPA-07</td>
<td>GAAACCGGTTG</td>
</tr>
<tr>
<td>OPB-18</td>
<td>CCACACGAGT</td>
</tr>
<tr>
<td>OPB-17</td>
<td>AGGGAACGAG</td>
</tr>
<tr>
<td>OPB-12</td>
<td>CCTTGACGCA</td>
</tr>
<tr>
<td>OPB-07</td>
<td>GGTGACGCAG</td>
</tr>
<tr>
<td>OPB-06</td>
<td>TGCTTCGCCCT</td>
</tr>
<tr>
<td>OPB-04</td>
<td>GGACTGGAGT</td>
</tr>
<tr>
<td>OPB-03</td>
<td>CATCCCCCTG</td>
</tr>
<tr>
<td>OPA-14</td>
<td>TCTGTGCTGG</td>
</tr>
<tr>
<td>OPZ-06</td>
<td>GTGCGGTTCA</td>
</tr>
<tr>
<td>OPZ-08</td>
<td>GGTTGGGTAA</td>
</tr>
</tbody>
</table>

Nine genotypes were grouped into two main clusters based on the maximum dissimilarity of 10% between genotypes (Figure 4.2). The maximum genetic distance was 0.3545 between accessions AP6 and CXL. The minimum genetic distance was 0.0303 between accessions AP15 and AP6 as shown in Figure 4.3. *Chuan Xin Lian* (CXL) from China (Family: *Acanthaceae*, Species: *Andrographis paniculata*, Genus: *Andrographis*) as reference control, was found to be the most genetically distant from all other accessions where the greatest dissimilarity was between CXL and AP6 (15%). Cluster I consisted of AP8, KB, AP4 and Cluster II consists of AP11, T, AP2, AP14, AP15 and AP6 (Figure 4.4). *Chuan Xin Lian* (CXL) is about 4% dissimilar from all the Brunei Darussalam accessions by combining Clusters I and II together as one major group. On an individual basis, CXL is 6% dissimilar to Cluster I and 9% to Cluster II.
PCR amplification using the 5S-rRNA primers produced a 400bp amplicon. Restriction enzyme digestion using six endonucleases revealed that all the restriction fragments were identical among all genotypes (Table 4.3).

<table>
<thead>
<tr>
<th>Restriction Enzymes</th>
<th>Number/Colour of Buffer</th>
<th>Sequence (Promega Technologies)</th>
<th>Length of Amplifications of the 5S-rRNA region</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hinf</em>I</td>
<td>No. 2/Blue Buffer</td>
<td>G-ANT C C TNA-G</td>
<td>150bp 250bp</td>
</tr>
<tr>
<td><em>Mnl</em>I</td>
<td>No. 2/Blue Buffer</td>
<td>CCTC(N)\textsubscript{7} GGAG(N)\textsubscript{6}</td>
<td>70bp 100bp 220bp</td>
</tr>
<tr>
<td><em>Msp</em>I</td>
<td>No.2/Blue Buffer</td>
<td>C-CG G G GC-C</td>
<td>100bp 125bp 150bp</td>
</tr>
<tr>
<td><em>NlaIV</em></td>
<td>No.4/Green Buffer</td>
<td>GGN-NCC CCN-NGG</td>
<td>200bp 200bp</td>
</tr>
<tr>
<td><em>HaeIII</em></td>
<td>No.2/Blue Buffer</td>
<td>GG-CC CC-GG</td>
<td>70bp 70bp 100bp 150bp</td>
</tr>
<tr>
<td><em>Tsp 509I</em></td>
<td>No.1/Yellow Buffer</td>
<td>-AATT TTAA-</td>
<td>180bp 220bp</td>
</tr>
<tr>
<td><em>HhaI</em></td>
<td>No.4/Green Buffer</td>
<td>G CG-C C-GC G</td>
<td>Restricted amplicon into two equal fragment</td>
</tr>
<tr>
<td><em>AluI</em></td>
<td>No.2/Blue Buffer</td>
<td>AG-CT TC-GA</td>
<td>No restriction</td>
</tr>
<tr>
<td><em>BsaI</em></td>
<td>No.2/Blue Buffer</td>
<td>C-CNNNG G G GNNGC-C</td>
<td>No restriction</td>
</tr>
<tr>
<td><em>BstXI</em></td>
<td>No.3/Red Buffer</td>
<td>CCAN NNN-NTGG GGTN-NNN NACC</td>
<td>No restriction</td>
</tr>
<tr>
<td><em>Ddel</em></td>
<td>No.3/Red Buffer</td>
<td>C-TNA G G ANT-C</td>
<td>No restriction</td>
</tr>
<tr>
<td><em>ApoI</em></td>
<td>No.3/Red Buffer</td>
<td>A-AATT T T TTAA-A</td>
<td>No restriction</td>
</tr>
<tr>
<td><em>MseI</em></td>
<td>No.2/Blue Buffer</td>
<td>T-TAA AAT-T</td>
<td>No restriction</td>
</tr>
</tbody>
</table>
Dissimilarity matrix

Figure 4.2. A dendrogram showing genetic relationship of the nine accessions of *Andrographis paniculata* from Brunei Darussalam compared to the reference control *Chuan Xin Lian* (CXL) (matrix scale is expressed as percentage x100).

<table>
<thead>
<tr>
<th></th>
<th>CXL</th>
<th>T</th>
<th>KB</th>
<th>AP2</th>
<th>AP4</th>
<th>AP6</th>
<th>AP8</th>
<th>AP11</th>
<th>AP14</th>
<th>AP15</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXL</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.2729</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KB</td>
<td>0.2729</td>
<td>0.1616</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP2</td>
<td>0.3129</td>
<td>0.0938</td>
<td>0.1974</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP4</td>
<td>0.1793</td>
<td>0.2157</td>
<td>0.1793</td>
<td>0.2534</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP6</td>
<td>0.3545*</td>
<td>0.0938</td>
<td>0.2344</td>
<td>0.0616</td>
<td>0.2927</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP8</td>
<td>0.3129</td>
<td>0.0938</td>
<td>0.1272</td>
<td>0.1442</td>
<td>0.1974</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP11</td>
<td>0.2157</td>
<td>0.0776</td>
<td>0.2157</td>
<td>0.1103</td>
<td>0.1616</td>
<td>0.1103</td>
<td>0.1793</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP14</td>
<td>0.3335</td>
<td>0.1103</td>
<td>0.1793</td>
<td>0.0458</td>
<td>0.2729</td>
<td>0.0458</td>
<td>0.1793</td>
<td>0.1272</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>AP15</td>
<td>0.3129</td>
<td>0.0938</td>
<td>0.2344</td>
<td>0.0616</td>
<td>0.2927</td>
<td>0.0303*</td>
<td>0.1974</td>
<td>0.1103</td>
<td>0.0458</td>
<td>1.000</td>
</tr>
</tbody>
</table>
In a related HPLC study, Chua et al., (2006, unpublished) attempted to obtain the correlation between the polymorphisms of three genotypes CXL, T & K.B. and the levels of %w/w Andrographolides (A) and %w/w Dehydroandrographolides (D). Their genetic differences and the medicinally active principles were analysed quantitatively using HPLC to study their phytochemical diversity from different geographical locations. In addition to the cluster analysis, principal component analysis of the similarity coefficient was performed and the diagram of the components extracted was constructed as illustrated in Figure 4.5. The HPLC analysis of three accessions CXL, T and K.B. as mentioned in section 5.3.3.2 of Chapter 5 accumulating significant concentrations of %w/w Andrographolide were 1.14%, 3.43% and 0.67% and %w/w Dehydroandrographolide were 0.66%, 0.12% and 0.24% respectively, may be potentially useful for breeding and cultivar development. The results of the dendrogram and principal component analysis were in agreement with the overall representation of relationships among the genotypes studied.
Figure 4.5. **Principal component diagram based on RAPD analysis.**

The two main principal components extracted from the PCA were plotted and analysed. The first component accounted for 59.55% and the second component was 26.60% of the total variance. The combined cumulative was 86.14% among the genotypes studied. Three clusters of genotype-specific RAPD markers were identified from the PCA plot as shown in Figure 4.5. These markers may be employed in future studies to identify these genotypes, but additionally may be useful as predictors of Andrographolide and Dehydroandrographolide levels in accessions of *Andrographis paniculata*. However, further studies are required to confirm the linkages of these RAPD polymorphisms with the gene loci responsible for Andrographolide production.
4.4 Discussion

The data obtained in this study reflected the utility of RAPD and PCR-RFLP in the analysis of genetic variability distribution within *Andrographis paniculata*. The finding of the close genetic distances between all the nine accessions from Brunei Darussalam and *Chuan Xin Lian* (CXL) are in agreement with the other studies on lotus (Campose *et al.*, 1994), sweet potato (Cannoly *et al.*, 1994), and *Andrographis paniculata* of India (Padmesh *et al.*, 1999) which suggests that RAPD is more appropriate for the analysis of genetic diversity in closely related genotypes. This study provides a better understanding of genetic variation at the intraspecific level would help in identifying superior genotype(s) for cultivar development. To the best of our knowledge, this is the first study conducted on *Andrographis paniculata* from Brunei Darussalam. The polymorphism obtained can be used as a marker for screening the genotypes. The genomic DNA fingerprinting by RAPD among fresh accessions from *Andrographis* species showed distinctive DNA fragments which may be used for the authentication, identification and quality assessment of the plant species or varieties.

This finding is in agreement with the RAPD analysis in the study by Padmesh *et al.*, (1999) and Sabu (2002) on the intraspecific genetic diversity of *Andrographis paniculata* where the genetic dissimilarity is at 0.51 between AP-29 from Assam and AP48 from Thailand (Padmesh *et al.*, 1999; Sabu, 2002). The nine accessions of *Andrographis paniculata* in the current study were collected from Brunei Darussalam, whereas the accessions in previous studies were obtained from different parts of India, Thailand, Malaysia and Indonesia. It is possible that the genotypes from different distantly geographical regions can be genetically similar to genotypes with
immediate spatial relationships association which may be attributed to the unique, broad genetic base combinations, seed movement and gene flow since *Andrographis paniculata* (Burm. f.) Nees is an introduced species into Thailand and Brunei Darussalam. This species is also reported from Hongkong, Borneo, Sulawesi, Jamaica, Barbados, Bahamas and Christmas Island in Indian Ocean but there is no precise data with regards to the introduction and naturalisation of *Andrographis paniculata* in these countries. As this study focused on the intraspecific genetic diversity of the species, the future study of interspecific variability of the different species of *Andrographis paniculata* from different near and distant geographical locations may be useful and lead to better understanding of these existing findings.

In contrast, the RAPD analysis potentially explores genetic polymorphism across the entire genome therefore may potentially reveal greater genetic diversity with higher degree of polymorphism (Lynch & Milligan, 1994). Such characteristic features of the RAPD are useful for the identification of medicinal plants despite the weakness of RAPD method with low reproducibility and high sensitivity of contaminants during the hash processing of DNA (Penner *et al.*, 1993). The PCR-RFLP of multi copy genes, such as 5S-rRNA, may perhaps be more appropriate for the differentiation and authentication of medicinal plants as in it’s application in differentiating four medicinal *Codonopsis* species from their related adulterants, *Campanumoea javania* and *Platycodon grandiflorus* (Joshi *et al.*, 2004).

The findings from 5S-rRNA digestions indicated that the nine accessions of *Andrographis paniculata* from Brunei Darussalam were closely related. There are different varieties of *Andrographis* in India, the native homeland of the plant which has always been the interest of
researchers to analyse them genetically. At present, no information is available whether the different varieties have arisen due to more than one introduction to Brunei Darussalam or as a result of genetic mutation. PCR-RFLP digestion can be a useful tool for resolving subspecies taxonomic status, but in this analysis the 5S-rRNA region is not variable enough for taxonomic comparison. However, future study using a larger number of restriction enzymes may possibly uncover differences in the taxonomic pattern. Although in this section PCR-RFLP was not a useful diagnostic tool, PCR-RFLP’s were reported to be useful for deciphering phylogenetic relationships between two varieties of *Imperata cylindrica* L. Beauv. (*Congograss*) (Chou & Tsai, 1999).

The study on DNA fingerprinting profiles of *Andrographis paniculata* from Brunei Darussalam was established using RAPD technique. The RAPD profile revealed the genetic relationship of the medicinal materials and demonstrated the genetic dissimilarities through RAPD markers. PCR-RFLP analysis was also performed in which the 5S-rRNA region was used for the establishment of DNA fingerprints. The findings shows similar patterns indicating that they are closely related which is in agreement with the fact reflecting that they are within the same species. RAPD is the preferred method for identification in this study. It would be most encouraging to expand into future study on the application of RAPD and PCR-RFLP analyses in the different species of *Andrographis*. The RAPD analysis in the genetic diversity revealed moderate variation within the species which provide sensitive and rapid technique to identify and evaluate *Andrographis paniculata* which may contribute to the quality assessment of this important medicinal plant.
The findings of this study using RAPD and PCR-RFLP of 5S-rRNA region of the phylogenetic diversity of nine accessions samples collected from the three districts namely Bandar Seri Begawan from the Brunei/Muara district (AP2, AP4, AP6, AP8, AP11, AP14, AP15), Kuala Belait district (K.B.), Temburong district (T) and Chuan Xin Lian (CXL) from China constituted as the first report on the genetic diversity study on *Andrographis paniculata* from Brunei Darussalam. *Andrographis paniculata* populations are genetically and moderately different in Brunei Darussalam on the basis of RAPD analysis.

This study also provides a better understanding of the correlation of the level of contents of A and D to the genetic variation at the intraspecific level as discussed earlier in this chapter. Geographical location and season may also explain the differences in the contents of the active components in the herb (Hu & Wu, 1995). Such finding would help us in identifying superior genotype(s) for cultivar development of *Andrographis paniculata* (Burm. f.) Nees. Further studies are needed to elucidate this correlation in particularly the genotype of the *Andrographis paniculata* from the Temburong sample.
Chapter 5  Phytochemical analysis on the profile of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam

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5.1.2.4 Electrophoretic methods

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5.2.2.2 Ethanol extraction

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5.2.4 Discussion
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5.3.1 High Performance Liquid Chromatography (HPLC) Analysis

5.3.2 Materials and Methods

5.3.2.1 Plant materials

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5.3.2.4 Data collection and statistical analysis

5.3.3 Results

5.3.3.1 Standard curves

5.3.3.2 Contents of A and D in the *Andrographis paniculata* herbal samples

5.3.3.3 Contents of A and D in the herbal extract fractions used for anti-tumour and general cytotoxicity assays

5.3.4 Discussion
5.1 Introduction

Herbal medicines are complex mixtures of active and inert ingredients/constituents which contain usually hundreds of chemically different constituents to which only a few, if not one, compounds are responsible for the beneficial therapeutic as well as the hazardous effects (Pavel & Jitka, 2004). Therefore, efficient and effective methods of identification, extraction, standardisations, qualitative, quantitative, bioassays and pharmacological testings are essential and must be highly selective in order to achieve the optimal outcomes.

Advances in analytical techniques in particular chromatographic, spectroscopic, plant cell, tissue culture and genetic manipulation techniques have paved the way for the better understanding of not only the profile of medicinal plants but also providing means for economic production of important plants with markers of high cultivar. The important secondary metabolites are often used as important markers for quality control in the chemotaxonomy, chemical ecology, agriculture and food industry.

Based on a simplistic approach, the analysis of the two components A and D by conventional thin layer chromatography (TLC) method was not well described. For instance, a conventional TLC identification method based on compositions of CHCl₃-Ethyl acetate-MeOH mobile phase used by the Pharmacopoeia of People Republic of China (PPRC, 2000) was further modified in our study on the basis of an automated system (CAMAG) by adjusting and optimizing the separation conditions in order to separate these two constituents effectively. In this study, we described an improved HPTLC method for determining the contents of these two components in dried raw leaves samples of Andrographis paniculata. Table 5.1 illustrated the various
chromatographic techniques which have been reported to be useful for the phytochemical analyses of _Andrographis paniculata_.

### 5.1.1 Common chemical techniques used in herbal analysis

Generally, the methods for quality control of herbal medicines involve sensory inspection (macroscopic and microscopic examinations) and analytical inspection using instrumental techniques such as TLC, HPLC, GC-MS, LC-MS, near infrared (NIR), and spectrophotometer, etc (Choi _et al._, 2002). HPLC is a popular method for the analysis of herbal medicines because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. It can also be used to analyse almost all the compounds in the herbal medicines and has received the most extensive applications (Cawthray, 2003; Desai _et al._, 1993; Imanari _et al._, 1996; Leonard _et al._, 2003; Li _et al._, 1999; Lin & Chen, 2003; Lin _et al._, 2001a; Loganathan _et al._, 1990; Sanyal _et al._, 2003; Thanawiroon & Linhardt, 2003; Tsai _et al._, 2002) For instance, reversed-phase (RP) columns may be the most popular columns used in the analytical separation of herbal medicines. It is important to note that the good experimental design for the optimal separation condition for the HPLC involves many factors, such as the different compositions of the mobile phases, the pH adjustment, pump pressures, etc.
5.1.2 The Chromatography and chemical fingerprinting techniques of herbal medicines

Some of the important chromatographic analytical methods which are useful as reported in the literature reviews from previous studies employed for analysing herbal medicines (Liang et al., 2004).

5.1.2.1 Thin Layer Chromatography (TLC)

TLC was the most common conventional method of choice for herbal analysis before the newer and more sophisticated instrumental chromatographic methods was developed. It is a convenient method of determining the quality and possible adulteration of herbal products which is employed in multiple samples analysis. The advantages of TLC techniques are simplicity, versatility, high velocity, specificity and simple sample preparation and ease of use.

5.1.2.2 Gas Chromatography and volatile components in herbal medicines

Many pharmacologically active components present in herbal medicines contain volatile chemical compounds. The advantages of Gas Chromatography (GC) analysis of compounds containing volatile oil provide good resolution on the fingerprinting which serve as an identity of the plant. Furthermore, it can detect the components both quantitatively and qualitatively. The useful features of GC include the ease of identification, standardisation, and monitoring of the relative quantities and characterisation of the components and variations of compositions of the volatile oil. With the help of FID detection and GC-MS are highly sensitive in the detection of all
volatile oil composition and also its highly selective capillary column technique which has good resolution in the separation of volatile compounds within short duration.

5.1.2.3 High Performance Liquid Chromatography (HPLC)

By far, HPLC has been the most popular method used to analyse almost all compounds in herbal medicines and still remain as the most extensive application which has optimal separation of the compounds with reversed-phase (RP) columns, suitable mobile phases, pH adjustment, pump pressures and optimised analytical conditions. Other newer techniques which will provide new opportunities for good separation of specific extracts of herbal medicines such as micellar electrokinetic capillary chromatography (MECC) (Sanyal et al., 2003), high speed counter-current chromatography (HSCCC), low-pressure size-exclusion chromatography (SEC) (Pervin et al., 1995), reversed-phase ion-pairing HPLC (RP-IPC-HPLC) (Karamanos et al., 1997) and strong anion-exchange HPLC (SAX-HPLC) (Rice & Lindardt, 1989). Over the recent years, there has been an increased use of HPLC coupled with evaporative light scattering detection (ELSD) which is ideal in the detection of non-chromophoric compound (Christie, 1992; Nebinger et al., 1983).

5.1.2.4 Electrophoretic methods

Capillary electrophoresis is a versatile and powerful separation tool with high separation efficiency and selectivity in the analysis of mixtures of low molecular mass charged components ranging from simple inorganic ions to DNA.
Table 5.1. Chromatographic techniques used for the phytochemical analyses of *Andrographis paniculata*

<table>
<thead>
<tr>
<th>No.</th>
<th>Project areas to determine active constituents inclusive of (A), (D) and other diterpenoids in <em>Andrographis paniculata</em> leaves</th>
<th>Methods of determination/Techniques/Optimal conditions</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Microemulsion Electrokinetic Chromatographic (MEEKC) for simultaneous determination of A and D in TCM and Chinese medicinal preparations</td>
<td>Heptane 0.81%w/w, SDS 3.31%w/w, Butan-1-ol 6.61%w/w, 10mM sodium tetraborate buffer pH 9.2</td>
<td>Achieved good reproducible Separation</td>
<td>(Yanfang <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>2</td>
<td>HPLC for simultaneous determination of Andrographolide (AP1), 14-deoxy-11,12-didehydroandrographolide (AP3) and Neoandrographolide (AP4) in the AP samples from Thai markets</td>
<td>Methanol &amp; Water mobile phase, wavelength at 220nm</td>
<td>Demonstrated that AP1 was more stable than the others, Content of AP3 increased and AP4 fluctuated during storage time. Different levels obtained during storage resulting in significant effect on clinical efficacy of the</td>
<td>(Pholphana <em>et al.</em>, 2004)</td>
</tr>
</tbody>
</table>
3. HPLC for simultaneous determination of Andrographolide & Dehydroandrographolide in common powdered or ultra-fine powdered AP

Dissolution rates of the size of powders

Ultra fine powder technique promotes the dissolution rates of A & D (Qui et al., 2004)

4.

4.1 HPLC for simultaneous determination of the chemical fingerprinting of Andrographolide & Dehydroandrographolide in AP.

HPLC reagents: Hexane, Chloroform, Methanol and water extracts

The analyses showed that Andrographolide & Neoandrographolide were absent in the hexane extracts but in substantial concentration in the methanol extract. Chromatograms are beneficial in the chemical fingerprinting for quality control and formulation preparations of AP (Srivastava et al., 2004)
<p>| 5   | 2D NMR spectroscopy method in the determination of structures of flavonoids Andrographolide diterpenoids and others in the whole plant of AP | Phytochemical analysis using 2D NMR spectroscopy | The structures of flavonoids and Andrographolides from AP were established based on 2D NMR spectroscopic (Koteswara et al., 2004) |
| 6   | Extraction technology of active ingredients using orthogonal experiment with supercritical carbon dioxide | Ethanol solvent, extractor pressure 25 MPa, extractor temperature 46°C, separator I pressure 6 MPa, separator I temperature 65°C, separator II pressure 6 MPa, separator II temperature 45°C, CO₂ rate of flow 40kg/h. | Higher purity and more stable quality than the conventional extract technology (Ge et al., 2002) |</p>
<table>
<thead>
<tr>
<th>Page</th>
<th>Method/Technique</th>
<th>Solvent System</th>
<th>Conditions</th>
<th>Bioactive Diterpenes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Separation of bioactive diterpenes using high-speed counter-current chromatography</td>
<td>Water-methanol-ethyl acetate-n-hexane (2.5:2.5:4:1) solvent system</td>
<td>AT 280 min separation, electrospray MS, one dimensional NMR, circular dichroism, optical rotation dispersion &amp; specific optical rotation [alpha] D</td>
<td>Andrographolide &amp; Neoandrographolide were successfully separated by high speed counter current chromatography</td>
<td>Qizhen et al., 2003</td>
</tr>
<tr>
<td>8</td>
<td>Determination of active diterpenes Andrographolide, deoxyandrographolide &amp; Neoandrographolide by micellar electrokinetic capillary chromatographic (MEKC) method</td>
<td>Water:ethanol extract, 15mM sodium dodecyl sulphate in 30mM borate buffer (pH 9.5) with UV detection wavelength at 214nm and constant voltage of 16 kV.</td>
<td>The MEKC separation method demonstrated efficiency, sensitivity, linearity and repeatability, easier and less expensive to use and does not suffer from column contamination.</td>
<td>(Zhao et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Determination of active diterpenes Andrographolide, deoxyandrographolide &amp; Neoandrographolide by micellar electrokinetic capillary chromatographic (MEKC) method</td>
<td>Water:ethanol extract, 20mM sodium dodecyl sulphate in 20mM borate buffer, 10mM sodium cholate (pH 8.3), with fused-silica capillary tube UV detection wavelength at 214nm and constant voltage of 25 kV.</td>
<td>The MEKC separation method demonstrated efficiency, sensitivity, linearity and repeatability, easier and less expensive to use and does not suffer from column contamination</td>
<td>(Cheung et al., 2001)</td>
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<tr>
<td>10</td>
<td>Validated analytical methods (HPLC, CE and GC-MS) were used for the determination of active diterpenes in Pharmacokinetic study</td>
<td>Detector UV diode-array detection method were used</td>
<td>Maximum plasma levels of Andrographolide at 393 ng/ml (approximate 1.12µM) at 1.5-2 hours. Half life &amp; mean residence times were 6.6 &amp; 10.0 hours (one compartmental model). In two compartmental model, the max. plasma level was 660 ng/ml</td>
<td>(Panossian et al., 2000)</td>
<td></td>
</tr>
</tbody>
</table>
It has been reported in literatures that Andrographolide is the major bitter active components responsible for the pharmacological properties of *Andrographis paniculata*, and its related component Dehydroandrographolide is lesser in activity. Our main objective in this Chapter 5 is to investigate the %w/w of Andrographolide (A) and Dehydroandrographolide (D) of the *Andrographis paniculata* leaves samples collected from Brunei Darussalam compared with the samples of CRS (China Reference Standard Herbs) and *Chuan Xin Lian* (CXL) from Chinese Medicines Clinic, RMIT University by HPTLC and HPLC analyses.

As this is the first study conducted on *Andrographis paniculata* (Burm. f.) Nees from Brunei Darussalam based on the HPTLC and HPLC analyses of the contents of active components Andrographolide and Dehydroandrographolide which are often used as marker compounds for the quality control of *Andrographis paniculata* related herbal products. The structures of these two compounds are quite similar as shown in Figures 5.1 and 5.2, and the separation of these two compounds in some analysis process may be relatively difficult. HPTLC and HPLC analytical methods have been reported to be useful for quantitative determination of the contents of *Andrographis paniculata* (Handa & Sharma, 1990a; Saxena et al., 1999).

**Figure 5.1. Structural formula of Andrographolide (A)**
5.2 Determination of Andrographolide and Dehydroandrographolide in *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam by High Performance Thin Layer Chromatography (HPTLC).

5.2.1 High Performance Thin Layer Chromatography (HPTLC)

The analysis of these two components A and D by conventional thin layer chromatography (TLC) method was not well described. For instance, a conventional TLC identification method based on compositions of CHCl$_3$-Ethyl acetate-MeOH mobile phase used by the Pharmacopoeia of People Republic of China (PPRC, 2000) was further modified in our study on the basis of an automated system (CAMAG) by adjusting and optimizing the separation conditions several
times in order to separate these two constituents effectively and efficiently. In this study, we described an improved HPTLC method for determining the contents of these two components A and D in the dried leaves samples of *Andrographis paniculata*.

5.2.2 Materials and Methods

5.2.2.1 Plant materials

As previously described in Chapter 4, the identity of the plant was confirmed by the Herbarium Museum of the Department of Agriculture and the reference herbarium voucher specimen (HDM 15) of the Ministry of Industry and Primary Resources, Brunei Darussalam. In this HPTLC experiment, the herbal extract fractions investigated were CRS and CXL from China and Brunei AP, Kuala Belait, Temburong from Brunei Darussalam.

5.2.2.2 Ethanol extraction

Cold extraction of the pulverised *Andrographis paniculata* leaves in absolute ethanol solvent at room temperature was sonicated and left overnight. The *Andrographis paniculata* leaves samples CRS, CXL, Brunei AP, Kuala Belait, Temburong were prepared in 1.67%w/v compared to the reference standards of Andrographolide (A) and Dehydroandrographolide (D) purchased from National Institute for the Control of Pharmaceutical and Biomedical Products (NICPBP) at 0.1%w/v. The stock solutions were stored at -80°C and diluted (with the same solvents) to the desired concentrations on the day of experiment.
5.2.2.3 HPTLC Methods

5.2.2.3.1 Composition of the mobile phase

After testing the solvents in various composition ratio of the mobile phase, the HPTLC conditions were then optimised to result in effective separation of the A and D in the herbal samples investigated. Proper selection of the sample concentrations and the mobile phase CHCl₃-Ethyl acetate-MeOH compositions were optimised at 4:3:0 (PPRC, 2000) to reduce the tailing effect of the spots. Exact volume of the samples was injected precisely by automation into each track using Linomat 5 CAMAG automated system.

5.2.2.3.2 HPTLC development plates (CAMAG)

The HPTLC development was conducted in a horizontal developing chamber (CAMAG), with HPTLC silica gel 60 F₂₅₄ precoated plate (20 x 10 cm) from Merck spotted with extracts and reference standard using the CAMAG Nanomat and Capillary Dispenser autojet syringe for spotting precision. After plate development in mobile phase, dried on a hot plates and visualised under ultra violet light of wavelength at 254nm and the migration of the reference standards and the extract fractions were quantified by calculation of the retention factor ($R_f$) values and the distance migrated by each band divided by the distance travelled by the solvent front.

5.2.2.3.3 VideoScan and VideoStore 2 software (CAMAG)

The CAMAG winCATS-Planar Chromatography Manager serves as the automated Multiple Development, VideoScan evaluates the images captured by the CAMAG VideoStore 2 software
which is used for both qualitative and quantitative HPTLC evaluation to calculate the amount of unknowns in addition to the co-chromatographed standards on the same plate. Both evaluation modes will transfer the image pixel data into densitogram curves and the $R_f$, peak height, height %, peak area and area% and the calibration curve for the quantification of the unknown samples are generated for each detected and integrated substance.

5.2.2.4 Data collection and statistical analysis

The results were expressed as a percentage w/w of *Andrographis paniculata* leaves powder samples. Values were expressed as mean ± Standards Error of Mean (S.E.M.), n denotes the number of experiments.

5.2.3 Results

By Video scanning the HPTLC plate, chromatography peaks (densitogram) for Andrographolide and Dehydroandrographolide standards and herb samples were obtained as shown in Figure 5.3. The content of Andrographolide and Dehydroandrographolide in the leaves sample was calculated from a formula derived from the standard calibration curves using CAMAG VideoStore 2 and VideoScan equipment. The CAMAG video scan facilities demonstrated good chromatography peaks of Andrographolide and Dehydroandrographolide standards illustrated in Figure 5.4 and Figure 5.5.

The densitograms of the *Andrographis paniculata* leaves extract samples investigated were shown in the Figures 5.6 to 5.10. HPTLC analysis of the relative components of the extract fractions of *Andrographis paniculata* leaves by comparison to the reference standards
Andrographolide (A) and Dehydroandrographolide (D) were conducted. Among the tested sample fraction, %w/w Andrographolide content in CRS were (0.52 ± 0.04%), CXL (1.36 ± 0.12%), Brunei AP (1.84 ± 0.1%), Kuala Belait (0.86 ± 0.15%) and Temburong (2.50 ± 0.26%) as illustrated in Figure 5.11. The %w/w Dehydroandrographolide content in CRS were (0.54 ± 0.05%), CXL (1.41 ± 0.07%), Brunei AP (0.84 ± 0.07%), Kuala Belait (0.56 ± 0.07%) and Temburong (0.40 ± 0.05%) as illustrated in Figure 5.12.

The HPTLC results indicated that the %w/w content of the major active constituent of Andrographolide in the *Andrographis paniculata* leaves samples were higher level in the Brunei samples compared to the CXL and CRS from China. Conversely, the %w/w of Dehydroandrographolide level is higher in the CXL and CRS than in the Brunei Darussalam samples. The official method of the Chinese Pharmacopoeia (as benchmarking) which stipulated that the China Reference Standards of the *Andrographis paniculata* leaves should contain not less than 0.8%w/w of the total amount of A (C$_{20}$H$_{28}$O$_{4}$) and D (C$_{20}$H$_{30}$O$_{5}$). Figure 5.13 indicated that the %w/w A and D of the herbal samples combined were CRS (1.06 ± 0.04%), CXL (2.77 ± 0.03%), Brunei AP (2.68 ± 0.05%), Kuala Belait (1.42 ± 0.19%) and Temburong (2.90 ± 0.22%) which is in agreement with the assay limits stipulated in the Chinese Pharmacopoeia of People Republic of China (PPRC, 2000). As a result indicating that the samples fractions investigated actually comply with the assay limits of the Chinese Pharmacopoeia. Results are mean ± SEM for n=6, expressed as a percentage of weight for weight of the dried *Andrographis paniculata* leaves samples.
Figure 5.3. The CAMAG video scan facilities

Demonstrated good chromatography peaks of the Andrographolide & Dehydroandrographolide reference standards and the extract of dried Andrographis paniculata herbal leaves samples.

Track 1-8: 0.1% w/v Andrographolide; Dehydroandrographolide Standards: 1μl, 3μl, 9μl, 18μl; 0.5μl, 2.0μl, 6.0μl, 12μl respectively.

Track 9-18: 1.67%w/v Samples CRS, CXL, Brunei AP, KB, T: 30μl each.

Video scan the developed HPTLC plate above, chromatographic peaks (densitograms) for the reference standards and the samples were obtained.
5.2.3.1 Standard curves

The % w/w content of Andrographolide and Dehydroandrographolide were determined from the formula derived from the calibration standard curves of A and D using CAMAG VideoStore 2 and VideoScan Equipment samples can be obtained as shown in Figures 5.4 and 5.5. The linear regression calibration method generated the y-axis which represents the height/peak area of the substances A and D. The x-axis represents the amount detected for A (µg) and D (ng).

Calibration results

Area calibration for substance: Andrographolide 0.1%

![Calibration curves for the determination of A in the herbal samples](image)

Figure 5.4. Calibration curves for the determination of A in the herbal samples
Calibration results

Area calibration for substance: Dehydroandrographolide 0.1%

Figure 5.5. Calibration curves for the determination of D in the herbal samples

The y-axis of the densitograms illustrated represents the peak areas of the substances (peak 1 denotes A) and (peak 2 denotes D) and the x-axis represents the $R_f$ values.

Figure 5.6. Densitogram of CRS Ee (*Andrographis paniculata* herbal sample) containing A and D

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Figure 5.7. Densitogram of CXL Ee (*Andrographis paniculata* herbal sample) containing A and D

Figure 5.8. Densitogram of Brunei AP Ee (*Andrographis paniculata* herbal sample) containing A and D
Figure 5.9. Densitogram of Kuala Belait Ee (*Andrographis paniculata* herbal sample) containing A and D

Figure 5.10. Densitogram of Temburong Ee (*Andrographis paniculata* herbal sample) containing A and D
Figure 5.11. Andrographolide (A) content (%w/w) in *Andrographis paniculata* leaves powder samples analysed by HPTLC (CAMAG). Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.

Figure 5.12. Dehydroandrographolide (D) content (%w/w) in *Andrographis paniculata* leaves powder samples analysed by HPTLC (CAMAG). Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.
Figure 5.13. The Andrographolide (A) + Dehydroandrographolide (D) content (%w/w) in *Andrographis paniculata* leaves powder samples analysed by HPTLC (CAMAG). Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.

5.2.4 Discussion

TLC was the common method of choice for herbal analysis before instrumental chromatography methods like GC and HPLC were established. TLC is still frequently being used for the analysis of herbal medicines as various pharmacopoeias such as American Herbal Pharmacopoeia (AHP), Chinese drug monographs and analysis, Pharmacopoeia of the People’s Republic of China (PPRC, 2000) and many others are still using TLC to provide first characteristic fingerprints of herbs (Liang *et al.*, 2004). HPTLC has the advantages of many-fold possibilities of detection in the analysis of herbal medicines. HPTLC is rather simple to use and can be employed for multiple sample analysis for instance, more than a series of 30 spots of samples can be studied simultaneously in one time.
As a result of its usefulness to get qualitative and quantitative information from the developed HPTLC plate, it is still a popular convenient method of choice in the analysis of herbal medicines. The CAMAG video store system (CAMAG, Switzerland) and TLCQA-UV methods, and the image analysis and digitalized technique developed in computer science, the evaluation of similarity between different samples is also possible (Chau et al., 1998). The advantages of HPTLC to construct the fingerprints of herbal medicines are its simplicity, versatility, high velocity, specific and simple sample preparation. For improvement in the agriculture, drug analysis development, more sensitive and accurate analytical method is required for the quantitation of important of diterpenoids which are present in the plant (Hu & Zhou, 1982; Jain et al., 2000; Qizhen et al., 2003; Reddy et al., 2003; Zhao et al., 2002).

Among the tested samples fractions, %w/w of Andrographolide and Dehydroandrographolide (A and D) contents were much similar in trends to those obtained using the HPLC analytical technique which validated the HPTLC procedures. Such procedures based on the computerised densitometer applied to the two dimensional spectrographic image analysis of the HPTLC plates employed indicating that HPTLC is a useful diagnostic tool in the analysis of *Andrographis paniculata*.

The findings demonstrated that the content of Andrographolide and Dehydroandrographolide in *Andrographis paniculata* extracts varied significantly, ranging from 0.52% - 2.50%. The highest Andrographolide content was found in the Temburong sample (2.50 ± 0.26%), followed by Brunei AP (1.84 ± 0.1%), CXL (1.36 ± 0.12%), Kuala Belait (0.86 ± 0.27%) and CRS (0.52 ± 0.04%). In contrast, the highest Dehydroandrographolide content was found in CXL (1.36 ± 0.12%) followed by Brunei AP (0.84 ± 0.07%), Kuala Belait (0.56 ± 0.07%), CRS (0.54 ± 0.05%) and Temburong (0.39 ± 0.07%). This ranking order of the content of A and D levels is
also in close agreement of the HPLC analysis on the Andrographis paniculata extracts found in section 5.3.3.2 of Chapter 5.

The HPTLC determination of Andrographolide and Dehydroandrographolide contents in the Andrographis species is relatively simple and easy to apply. The stable nature of the A and D molecules (Huang et al., 2002) which is not subject to decomposition during the extraction process produced good separated chromatographic peaks (densitograms). The content of Andrographolide and Dehydroandrographolide combined is not less than 0.8% in the raw herb which is in accordance with the recommended conventional TLC methods of the Pharmacopoeia of People Republic of China (PPRC, 2000). The Pharmacopoeia has only a descriptive identification method for the raw herb on the content of Andrographolide and Dehydroandrographolide using TLC analytical method but the HPTLC quantitative analytical method was not mentioned.

In this study, a sensitive and useful HPTLC method for quantitative determination of Andrographolide and Dehydroandrographolide in raw herb samples of Andrographis paniculata has been developed. Compared to the conventional analysis method of HPLC, LC-MS or TLC, this method provides more direct and audio-visual results of many spots of samples on a single HPTLC plate, and is both cost and time effective. It may be useful for routine and quick analysis of Andrographis paniculata contents in samples containing Andrographis species.
5.3 Determination of Andrographolide and Dehydroandrographolide in *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam by High Performance Liquid Chromatography (HPLC) Analysis

### 5.3.1 High Performance Liquid Chromatography (HPLC) Analysis

Generally, the methods for quality control of herbal medicines involve sensory inspection (macroscopic and microscopic examinations) as well as analytical inspection using instrumental techniques such as TLC, HPLC, GC-MS, LC-MS, near infrared (NIR), and spectrophotometer, etc. HPLC is a popular method for the analysis of herbal medicines because it is easy to learn, adapt and use and is not limited by the volatility or stability of the sample compound. It can also be used to analyse almost all the compounds in the herbal medicines and has received the most extensive applications in a timely and cost effective manner. For instance, reversed-phase (RP) columns may be the most popular columns used in the analytical separation of herbal medicines. It is important to note that the good experimental design for the optimal separation condition for the HPLC involves many factors, such as the different compositions of the mobile phases, the pH adjustment, pump pressures and all conditions need to be optimised prior to the analytical process.

### 5.3.2 Materials and Methods

#### 5.3.2.1 Plant materials

Plant materials were described previously in section 5.2.2.1 of Chapter 5.
5.3.2.2 Stability of diterpenes

It has been reported that Andrographolide (A) is quite stable for months. Andrographolide is by far the most stable active constituent compared to the other diterpenes. Dehydroandrographolide (D) is not a metabolite of Andrographolide. On prolong heating treatment during the production of formulations of *Andrographis paniculata* into tablets and other dosage forms, it is quite possible that Dehydroandrographolide content may increase (Huang *et al.*, 2002). By chemical treatment such as esterification of Andrographolide can produce esters which can be active compounds such as 14-deoxy-11,12-didehydroandrographolide and others. The HPLC fractions of *Andrographis paniculata* samples were prepared in absolute ethanol by cold extraction and stirred for one hour according to the Chinese Pharmacopoeia Volume I for HPLC method of analysis. There was actually no heat or chemical treatment at all which do not affect the stability of the A and D in the HPLC samples analyzed. Huang *et al* (2002) demonstrated that by avoiding or reducing heating treatment, time is the main factor to protect and preserve the effective active ingredients in the herb (Huang *et al.*, 2002). The stability of the crystalline Andrographolide was highly stable at 70 °C (75% relative humidity) over a period of three months whereas the amorphous phase degraded promptly.

5.3.2.3 Extraction methods

5.3.2.3.1 Cold extraction in ethanol

The pulverised *Andrographis paniculata* leaves were stirred with magnetic stirrer in ethanol at room temperature overnight. The extracts were centrifuged at 5000 rpm for 10 min and the supernatant filtered with Millipore vacuum filtering system (XX10 090 20-Billerica, MA, USA).
A rotary evaporator (Buchi Rotavapor, Brinkman company, Westbury, NY, USA) was used to remove the solvent. Extract fractions were then redissolved in absolute ethanol. The stock solutions were stored at -80ºC and diluted with the same solvents to the desired final concentration of 200µg/ml on the day for HPLC analysis. Sample fractions CRS, CXL, Brunei AP, Kuala Belait and Temburong were analyzed.

5.3.2.3.2 Cold extraction for fractions tested in anti-tumour and general cytotoxicity assays

Cold extraction fractions of CRS Ee STIR, CXL Ee STIR and Brunei AP Ee STIR were prepared in 200µg/ml final concentration for HPLC analysis prior to their investigation in the anti-tumour and general cytotoxicity assays as described in Chapter 9 and 10 respectively.

5.3.2.3.3 Solvent Extraction with Accelerated Solvent Extraction (ASE) Dionex System

The *Andrographis paniculata* leave powder was accurately weighed and extracted separately using the ASE extraction (Dionex system) in ethanol and MilliQ water as solvents into various herbal fractions CXL Ee ASE, Brunei AP Ee ASE, CXL We ASE and Brunei AP We ASE were prepared in 200µg/ml final concentration. The pure standards of Andrographolide (A) and Dehydroandrographolide (D) purchased from National Institute for the Control of Pharmaceutical and Biomedical Products, Ministry of Health Beijing, China (NICPBP) were prepared in 1.0µg/ml, 2.5µg/ml, 5µg/ml, 10µg/ml, 25µg/ml in ethanol solvent. After HPLC analysis, all these fractions were used in the anti-tumour and general cytotoxicity assays as described in Chapter 9 and 10 respectively.
5.3.2.3.4 HPLC analytical conditions

After testing different solvents in various composition ratio of the mobile phase, the HPLC conditions were then optimised to result in effective separation of the A and D in the herbal fractions investigated. Proper selection of the sample concentrations and the mobile phase Methanol: Water (52:48) compositions were optimised to reduce the tailing effect of the peaks eluted. Exact volume 20µl of the fractions were injected precisely using Shimazu (Japan), LC-10AT liquid chromatography instrument, equipped with and controlled by a model SCL-10AVP System, a model SIL-10ADVP Auto Injector, a 20ul sample loop and a multidimensional UV-VIS detector (SPD-M 10AVP), DGU-14A Degasser, CTO-10AVP Column Oven and FCV-10AL VP. The data is to be collected with a Pentium Computer (Datamini) and HP-deskjet printer. Solvents were filtered using a millipore system and the analysis is performed on a Waters make µ Bondapak C18 Column (300 x 3.9mm, I.D. 10 µm). A constant flow rate of 1ml/min, column temperature of 27ºC, detector wavelength at 254nm, the absorption maxima is close to all the compounds.

5.3.2.4 Data collection and statistical analysis

The results were expressed as a percentage of w/w of the Andrographis paniculata herbal leave powder. Values were expressed as mean ± Standards Error of Mean (S.E.M.), n denotes the number of experiments.
5.3.3 Results

HPLC analysis of the active components of *Andrographis paniculata* leaves detected the presence of Andrographolide (A) and Dehydroandrographolide (D) in all the extract fractions.

5.3.3.1 Standard curves

The HPLC system demonstrated good resolutions of the chromatography peaks of Andrographolide and Dehydroandrographolide reference standards and the extract fractions of *Andrographis paniculata* leaves samples as shown in Figure 5.14. In this study, an automated 10μl injection volume of Andrographolide (A) and Dehydroandrographolide (D) reference standards of concentrations at 1μg/ml, 2.5μg/ml, 5μg/ml, 10μg/ml, 25μg/ml were used. And an automated 20μl injection volume of all the extract fractions were analyzed by HPLC and the chromatographic peaks were illustrated in Figures 5.15 to 5.19.

The %w/w content of Andrographolide and Dehydroandrographolide were determined from the formula derived from the calibration standard curves of A and D. Linearity was determined by using five concentrations in a working range of 1-25μg/ml of both components A and D combined. Linear regression equations and good correlation coefficients for the A and D were obtained. Calibration plots of peak areas versus concentrations are linear, with *r* values between 0.9999 and 1.0000. These values indicated good linearity in the examined concentration range. The retention time (*Rt*) of A is 8.57 ± 0.05 mins, % relative standard deviation (RSD) is 1.55% and the retention time of D is 20.67 ± 0.17 mins, %RSD is 2.05%.
Figure 5.14. Calibration curves for the determination of A (a) and D (b) in the herbal fractions.
Figure 5.15. The HPLC Chromatogram Peaks for the sample fractions. Y axis represents the peak area and x-axis represents the retention time (mins).

(a) Standard Mix A + D at 10µg/ml

(b) CRS Ee STIR at 200µg/ml
(c) CXL (clinic) at 200µg/ml

(d) Brunei AP at 200µg/ml
(e) Kuala Belait at 200\(\mu\)g/ml

(f) Temburong at 200\(\mu\)g/ml
(g) CXL Ee ASE at 200µg/ml

(h) Brunei AP Ee ASE (Bru Ee ASE) at 200µg/ml
(i) CXL Ee STIR at 200µg/ml

(j) Brunei AP Ee STIR (Bru Ee STIR) at 200µg/ml
(k) CXL We STIR at 200µg/ml

(l) Brunei AP We STIR (Bru We STIR) at 200µg/ml
5.3.3.2 Contents of A and D in the *Andrographis paniculata* extract samples

The results of this HPLC study indicated that the %w/w of the important major active component Andrographolide which is responsible for the medicinal benefits of the plant is higher in the Temburong (3.43 ± 0.02%), Brunei AP (1.86 ± 0.02%), compared to the samples CXL (1.14 ± 0.01%), Kuala Belait (0.67 ± 0.02%), CRS (0.51 ± 0.01%) as shown in Figure 5.16. On the contrary, the %w/w Dehydroandrographolide, being the lesser potent component of the plant seems to be lower in the descending order of CXL (0.66 ± 0.01%), Brunei AP (0.24 ± 0.01%), Kuala Belait (0.24 ± 0.05%), CRS (0.14 ± 0.01%) and Temburong (0.12 ± 0.01%) illustrated in Figure 5.17.

The HPLC results indicated that the %w/w content of the major active constituent of Andrographolide in the *Andrographis paniculata* leaves samples were higher level in the Brunei samples compared to the CXL and CRS from China. Conversely, the %w/w of Dehydroandrographolide level is higher in the CXL and CRS than in the Brunei Darussalam samples. The official method of the Chinese Pharmacopoeia (as benchmarking) which stipulated that the China Reference Standards of the *Andrographis paniculata* herbal leaves should contain not less than 0.8%w/w of the total amount of A (C<sub>20</sub>H<sub>28</sub>O<sub>4</sub>) and D (C<sub>20</sub>H<sub>30</sub>O<sub>5</sub>).

Figure 5.18 indicated that the %w/w A and D of the herbal samples combined were CRS (0.65 ± 0.02%), CXL (1.80 ± 0.02%), Brunei AP (2.10 ± 0.03%), Kuala Belait (0.91 ± 0.06%) and Temburong (3.55 ± 0.03%) which is in agreement with the assay limits stipulated in the Chinese Pharmacopoeia of People Republic of China (PPRC, 2000). As a result indicating that the samples fractions investigated actually comply with the assay limits of the Chinese
Pharmacopoeia. Results are mean ± SEM for n=6, expressed as a percentage of weight for weight of the dried *Andrographis paniculata* leaves samples.
Figure 5.16. Andrographolide (A) content (%w/w) in *Andrographis paniculata* leaves powder samples analysed by HPLC. Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.

Figure 5.17. Dehydroandrographolide (D) content (%w/w) in *Andrographis paniculata* leaves powder samples analysed by HPLC. Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.
Figure 5.18. Andrographolide (A) + Dehydroandrographolide (D) content (%w/w) in the Andrographis paniculata extracts by HPLC. Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.

5.3.3.3 Contents of A and D in the herbal extract fractions used for anti-tumour and general cytotoxicity assays

On the basis of the extraction techniques of cold extraction by stirring (STIR) versus ASE (Dionex) system, among the tested standardised herbal fractions 200ug/ml, the %w/w Andrographolide (A) content in CRS Ee STIR (0.51 ± 0.01%), CXL Ee ASE (3.4 ± 0.02%), Bru Ee ASE (9.55 ± 0.03%), CXL Ee STIR (1.63 ± 0.02%), Bru Ee STIR (2.3 ± 0.03%), CXL We ASE (3.08 ± 0.04%) and Bru We ASE (3.40 ± 0.02%) as shown in Figure 5.19.

As for the Dehydroandrographolide (D) content in %w/w CRS Ee STIR (0.14 ± 0.13%), CXL Ee ASE (4.23 ± 0.19%), Bru Ee ASE (2.63 ± 0.04%), CXL Ee STIR (10.65 ± 0.04%), Bru Ee STIR (11.59 ± 0.12%), CXL We ASE (1.78 ± 0.01%) and Bru We ASE (0.31 ± 0.01%) as illustrated in Figure 5.20.
When combining both Andrographolide and Dehydroandrographolide contents, Bru Ee STIR (13.89 ± 0.15%), CXL Ee STIR (12.28 ± 0.06%) and Bru Ee ASE (12.18 ± 0.05%) were found to contain the higher levels among the seven extract fractions as shown in Figure 5.21.

![Figure 5.19. Andrographolide (A) content (%w/w) in *Andrographis paniculata* leaves powder samples analysed by HPLC. Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.](image)

![Figure 5.20. Dehydrographolide (D) content (%w/w) in *Andrographis paniculata* leaves powder samples analysed by HPLC. Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.](image)
Figure 5.21. Andrographolide (A) + Dehydroandrographolide (D) content (%w/w) in the *Andrographis paniculata* extract fraction samples for anti-tumour and general cytotoxicity assays by HPLC. Results are mean ± SEM for n=6, expressed as a percentage of weight of the dried herb.
5.3.4 Discussion

The findings of the HPLC analysis of the *Andrographis paniculata* fraction samples for the anti-tumour and general cytotoxicity assays as described in Chapter 9 and 10, demonstrated that the %w/w content of Andrographolide in the Brunei ethanol extract fraction (Bru Ee ASE) and CXL Ee ASE based on the Accelerated System Extraction (ASE) Dionex system was 9.55 ± 0.03% and 3.40 ± 0.02% respectively.

However, the %w/w content of Dehydroandrographolide of Bru Ee ASE (2.63 ± 0.02%) which was lower when compared to CXL Ee ASE at 4.23 ± 0.19%. This is again in close agreement with the HPTLC and HPLC analysis described earlier that the level of content of Andrographolide is higher and conversely, the Dehydroandrographolide is lower in the Brunei Darussalam fractions compared to the *Chuan Xin Lian* (CXL) from China.

As a result of the content of Andrographolide difference is higher between these two samples, our interest to look into the correlation of the contents of Andrographolide and Dehydroandrographolide of these two sample fractions were then further investigated for their anti-tumour and general cytotoxicity effects in Chapter 9 and 10.

This study demonstrated that Andrographolide and Dehydroandrographolide have obtained good linearity in the range of 1 mg/L to 25 mg/L with the correlation coefficients of 0.9999 and 1.0000 compared to the correlation coefficients of 0.9976 and 0.9986 respectively (Xu *et al.*, 2002) which is in agreement with the previous study. The active constituents of *Andrographis paniculata* were investigated by Jain *et al.*, 2000 using the mobile phase composition of varying the percentage of acetonitrile in water/methanol, in water giving rise to a good resolution of the
active components (Jain et al., 2000). The structures of three major Andrographolides namely Andrographolide, Neoandrographolide, 14-Deoxy-11,12-didehydroandrographolide were resolved in the optimised operating conditions such as acetonitrile-water (70:30, v/v), flow rate of 1ml/min, column temperature 26°C, detector wavelength 230nm, with the absorption maxima closed to all the compounds.

In the other previous study, the content of Andrographolide content in the leaf sample obtained from the experimental farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India were found to be 1.71% by HPTLC analytical method (Saxena et al., 1999) and 1.78% by HPLC analytical method (Jain et al., 2000). In our study, based on the similar chromatographic conditions, the Brunei AP samples were found to be 1.84 ± 0.1% (HPTLC) and 1.86 ± 0.01% (HPLC) which seems to be in close agreement with the previous studies conducted. This may suggest that the Andrographis paniculata sources from India (King of Bitters) and Brunei Darussalam (Daun pahit) are both originated from the tropics whereas Chuan Xin Lian (CXL) and CRS from China with temperate climate.

Based on the literature review, Andrographis paniculata leaves are rich and dark green in colour and thrives well in the tropics would provide a rich source of chlorophyll contents that may potentiate the level of active constituents in the leaves. This may suggest that the Andrographis paniculata source from the tropics may have higher level of active constituents which may be more potent that those derive from the temperate conditions. This requires further elucidation on the study of Andrographis paniculata source from countries with different climatic conditions and other factors that may affect the level of contents of active constituents in the leaves (Ngan et al., 1999). It has also been reported that geographical locations and seasons may explain the differences in the contents of the active components in the plants (Hu & Wu, 1995). However,
further studies are required to elucidate the different levels of contents of constituents in *Andrographis paniculata* (*Burm. f.*) *Nees* of Brunei Darussalam compared to *Andrographis paniculata* from other geographical locations in order to have a better understanding on this possible explanation.

The results obtained in this study reflected the utility of HPTLC and HPLC in the analysis of the contents of A and D variability distribution within *Andrographis paniculata*. The finding of the contents between extract samples investigated from Brunei Darussalam and *Chuan Xin Lian* (CXL) are in close agreement with the previous studies (Jain et al., 2000; Saxena et al., 1999). Both HPTLC and HPLC results demonstrated that the contents of A combined with D comply with the assay limits of not less than 0.8% which is in accordance with the Chinese Pharmacopoeia (PPRC, 2000) suggest the appropriateness of these two analytical methods for *Andrographis paniculata* (*Burm. f.*) *Nees*.

This study also provides a better understanding of the correlation of the level of contents of A and D to the genetic variation at the intraspecific level as discussed earlier in Chapter 4. Such finding would help us in identifying superior genotype(s) for cultivar development of *Andrographis paniculata* (*Burm. f.*) *Nees*. Further studies are needed to elucidate this correlation in particularly the genotype of the Temburong *Andrographis paniculata* sample. In conclusion, both the HPTLC and HPLC chromatographic procedures employed may also serve as simple and useful chemical fingerprint analysis of *Andrographis paniculata* for the quality control purposes and in the preparation of formulations based on the herb (Srivastava et al., 2004).
Chapter 6  Anti-oxidant properties of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam

6.1 Introduction

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6.1 Introduction

It is documented that extracts of many plants and natural products have anti-oxidant and free radical scavenging properties. Free radical oxidative stress, usually resulting from deficient natural anti-oxidant defenses (Halliwell & Gutteridge, 1984), has been implicated in the pathogenesis of a wide variety of clinical disorders, such as degenerative diseases (Cross, 1987). It may contribute as a factor in aging (Beckman et al., 1998) and progressive impairment of the immune function (Pike & Chandra, 1995).

*Andrographis paniculata* has been studied for its anti-oxidants activities in relation to the anti-hepatotoxicity effect of the diterpenoid components of the herb (Kapil et al., 1993; Singh et al., 2001; Trivedi & Rawal, 2001; Zhang & Tan, 2000b). However, only few reports on the anti-oxidant activities of *Andrographis paniculata* are available.

Therefore, it is beneficial to conduct this study on *Andrographis paniculata* (Burm. f.) Nees grown in Brunei Darussalam. In this study, the aqueous, ethanol and methanol extracts of *Andrographis paniculata* leaves were analyzed for their potential anti-oxidant activities. It is believed by many in the anti-oxidant therapy that the administration of compounds with anti-oxidant properties perhaps acts as an effective drug therapy in the preventative and curative therapeutic agents of many diseases conditions. This study provides a better understanding of the anti-oxidant profile of *Andrographis paniculata* (Burm. f.) Nees grown in Brunei Darussalam on their scavenging activities of 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical, hydrogen peroxide scavenging and the inhibition of nitric oxide formation in the cell-free systems.
6.1.1 Importance of anti-oxidants of natural products

It has been well established that oxidative stress caused by the imbalance between oxidants and anti-oxidant is involved in many pathological conditions such as mutagenesis, cancer, atherosclerosis, cardiovascular diseases, degenerative disorders and aging process (Darlington & Stone, 2001; MacNee, 2001; Wei & Lee, 2002). Thus anti-oxidants have an important role in the prevention or treatment of these diseases conditions. The natural food products industry in their continued effort to use alternative natural anti-oxidants to synthetic antioxidants in food manufacturing due to the beneficial safety profile of natural plant products. It has long been known that many plants possess potent anti-oxidant activities. Many research and development have been focused on the characterization of these anti-oxidants constituents which are responsible for the pharmacological and biological activities (Brown & Goodman, 1998).

6.1.2 Reactive oxygen species (ROS)

The formation of the reactive oxygen species such as the superoxide anion (O$_2^-$), hydroxyl radical (OH’), peroxyl radical (ROO’) and hydrogen peroxide (H$_2$O$_2$) in most cellular processes during phagocytosis of invading pathogens has been implicated in the pathogenesis of a series of clinical disorders resulting in the degenerative diseases. The state of oxidative stress occurs as a result of the imbalance of the levels of ROS thus causes reactions with cellular lipids i.e. lipid peroxidation, proteins and nucleic acids of cells. Such a state may also be a causative factor in ageing process, resulting in the deterioration and impairment of the immune function. Complications such as inflammatory, cardiovascular, cancer diseases become the end stage if the imbalance is not restored to normal state. Compounds with potent anti-oxidant properties are protective and help to reduce damages caused by ROS. It is known that many extracts of plants
have anti-oxidant and free radical scavenging which are beneficial and employed in the therapeutic management of most free radical oxidative stress (Chiang et al., 1994; Cos et al., 1998; Cross, 1987; Halliwell & Gutteridge, 1984).

Reactive oxygen species (ROS) are categorised into free radicals (superoxide radical anion $O_2^{-}$, Nitric oxide NO•, hydroxyl radical •OH) and non-radical compounds (hydrogen peroxide H$_2$O$_2$, hypohalous acids and their salts HOCl, singlet oxygen $^1$O$_2$, peroxynitrite ONOOH). These potent oxidants are often generated as by products of biological reactions or from exogenous factors. Their involvement in the pathogenesis of diseases including rheumatoid arthritis, artherosclerosis, diabetes mellitus, reperfusion injury, asthma, carcinogenesis and other medical complications have been well documented. Therefore, powerful scavengers of these ROS will protect and prevent tissue injuries and are of therapeutic importance. The most likely mechanism of the anti-oxidant is by interfering with the damages caused by •OH not by direct •OH scavenging but by scavenging or blocking formation of its precursors ($O_2^{-}$, H$_2$O$_2$, HOCl, ONOO•) and/or by binding with transition metal ions needed for •OH formation from $O_2^{-}$ and H$_2$O$_2$ (Halliwell et al., 1995). The use of synthetic anti-oxidants in the food industry as inhibitors of the reactive oxygen species such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), as due to its instability and has become the suspected carcinogenesis promoters. The use of natural plant products with potential anti-oxidant activities are now the centre of importance in the food industry (Candan F & Sokmen, 2004; Zaprorozhets et al., 2004).
6.1.3 Pathological roles of the ROS in the anti-inflammatory mechanism of *Andrographis paniculata*

Reactive oxygen species (ROS) play an important role in the pathogenesis and many symptoms of inflammatory processes of the respiratory tract triggered by potentially toxic and infective airborne molecules and particles as environmental impacts (Grabmann & Hippeli, 2000). Studies have shown that anti-inflammatory mechanism of actions of *Andrographis paniculata*, are associated with its inhibition of platelet-activating factor (PAF)-mediated inflammatory response and inhibition of expression of nitric oxide synthase in macrophages (Chiou *et al.*, 1998), but not via the inhibition of the biosynthesis of eicosanoids, different from NSAID pathways (Amroyan *et al.*, 1999).

ROS are generated by neutrophils or alveolar macrophages as defence mechanism against the invading microorganisms. Neutrophils, upon stimulation release superoxide which dismutated into \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \). Neutrophils also produce nitric oxide which reacts with superoxide to form peroxynitrite and myeloperoxidase (MPO), thus oxidize halides to hypohalous acids. Subsequently, reacts with \( \text{H}_2\text{O}_2 \) as oxidant to form singlet oxygen. ROS also include hydroxyl radical produced by iron-catalyzed decomposition of \( \text{H}_2\text{O}_2 \) which is an extremely powerful oxidant. Andrographolide was studied in the suppression of rat neutrophil reactive oxygen species production and adhesion suggested that its potential use as an effective anti-inflammatory agent (Shen *et al.*, 2002).
6.1.4 ROS and Neuronal pathways

The potential and beneficial use of a combination of a NOS inhibitor, (N^G nitro-L-arginine (LNA) and the anti-oxidant /superoxide scavenger, di-tert-butyl-hydroxybenzoic acid (DtBHB) are synergistic toward the reduction of neuronal damage during ischaemia-reperfusion and supported that NO, superoxide anion, and peroxynitrite, both independently and cooperatively play important roles in neuronal injury (Spinnewyn et al., 1999). Nitric oxide reacts with superoxide radical (Huie & Padmaja, 1993) to form peroxynitrite, an endogenous mediator of many forms of tissue injury ranging from neurodegenerative diseases, chronic inflammation and immunological disorders, atherosclerosis and other pathological diseases (Beckman et al., 1990; Darley-Usmar et al., 1995; Kaur & Halliwell, 1994; Swain et al., 1994). It induces lipid peroxidation (Radi & Beckman, 1991), oxidize protein and non protein thiol groups (Radi et al., 1991), damage DNA (Inoue & Kawanishi, 1995), react with tyrosine residues to 3-nitrotyrosine (Beckman, 1996; Ischiropoulos et al., 1992).

6.1.5 Types and sources of natural antioxidants

6.1.5.1 Medicinal herbs

Danchunhwan (Radix Salviae miltorrhiza and Rhizoma Cnidii) protects human neuroblastoma SH-SY5Y cells from apoptotic death by free radicals including peroxynitrite and NO via generation of anti-oxidation, GSH (Kim et al., 2001). The inhibitory effects of water extracts from fruiting bodies of cultured Cordyceps sinensis on raised serum lipid peroxide levels and aortic cholesterol deposition in atherosclerotic mice which may have beneficial effects on the process of artherogenesis and aging with few side effects (Yamaguchi et al., 2000).
6.1.5.2 Animal ingredients

The anti-inflammatory actions of *Uncaria tomentosa* known as cat’s claw or ‘una de gato’ (UG), a Peruvian traditional medicine, protects cells against oxidative stress by inhibiting lipopolysaccharide (LPS)-induced iNOS gene expression, nitrite formation, peroxynitrite-induced apoptosis and negated the activation of NF-κB (Sandoval-Chacon et al., 1998).

Quantification of total oxidant scavenging capacity of anti-oxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals was studied (Regoli & Winston, 1999). The quantification of the absorbance capacity of anti-oxidants on three potent oxidants such as hydroxyl radicals, peroxyl radicals, and peroxynitrite which were generated by the iron plus ascorbate-driven Fenton reaction, thermal homolysis of 2, 2’-azobis (2-methylpropionamidine) dihydrochloride (ABAP), 3-morpholinosydnonimine N-ethylcarbamide (SIN-1). Reactions between these three oxidants and α-keto-γ-methiolbutyric acid (KMBA), oxidized to ethylene. Quantification of their total oxidant scavenging capacity is measured on the ability to inhibit ethylene formation relative to the control reaction.

6.1.5.3 Green tea

The investigation by Dyke et al., (2000) on the anti-oxidant properties of the polyphenols in the green tea extract which markedly inhibit luminal-dependent chemiluminescence activated by peroxynitrite (a potent oxidant about 1000 times more active than equidose hydrogen peroxide) produced by the hydrolysis of linsidomine (SIN-1) (Dyke et al., 2000). The screening for the efficacy of ten kinds of green tea for their tyrosinase inhibitory activity of active components i.e.
(-)-epicatechin 3-O-gallate (ECG), (-)-gallocatechin 3-O-gallate (GCG), and (-)-epigallocatechin 3-O-gallate (EGCG) (No et al., 1999). The study suggested that the kinetic analysis for such inhibition of tyrosinase revealed a competitive nature of GCG with this enzyme for the L-tyrosine binding at the active site of tyrosinase.

The mechanism of how the increases in the levels of free radicals could be due to the increased production and/or decreased destruction. Anti-oxidant enzymes such as catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismuthase (SOD) and non-enzymatic scavenger components glutathione (GSH) and vitamin E offer good protection to cells and tissues against oxidative injury (Simmons, 1984).

6.2 Materials and Methods

6.2.1 Chemical materials

1,1-Diphenyl-2-picrylhazyl, ethanol, ascorbic acid, deoxyribose, potassium dihydrogen orthophosphate, potassium hydroxide, iron (III) chloride, EDTA, H₂O₂, TBA, TCA, NaOH, horseradish peroxidase, phenol red, sodium nitroprusside, griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) were commercially available from BDH Chemicals Australia (Kilsyth, Victoria, Australia), Sigma Chemical Co. (St. Louis, MO, USA).
6.2.2 Plant materials

The *Andrographis paniculata* leaves samples were described earlier in Chapter 4. The Brunei AP samples were used in the anti-oxidant assays.

6.2.3 Extraction with ASE (Accelerated Solvent Extraction) Dionex System

The herbal leave powder of *Andrographis paniculata* of Brunei Darussalam, air dried at room temperature was extracted in absolute ethanol, methanol and Milli-Q pure grade water as solvents respectively using the ASE 100 Dionex System (Dionex Pty Ltd, Lanecove, 1595, NSW). Operating procedure was based on the in built set up method 1 by Dionex with the following specifications and extraction conditions: Dionex Part No. 056780 Filter grade: D28, Size: 30mm; Temperature: 100°C; Pressure: 1,500psi (10MPa); Heating time: 5 min; Static time: 5 min; Flush time: 60%; Purge time: 100s; Static cycle: 3; Total extraction: 30 min per sample. The extracts were then filtered through a Whatman filter paper No. 1.

The ethanol and methanol were evaporated using a rotary evaporator under vacuum (Biichi Rotavapor, Brinkman Company, Westbury, NY, USA). The concentrated aqueous extracts were transported in liquid nitrogen for freeze drying using Freeze Drier Dynavac FD 12 (Airvac Engineering Pty Ltd, Melbourne, Australia) to remove water. The stock extracts were stored at -80°C and dissolved in the respective solvents to the appropriate concentrations on the day of use. The extracts were resuspended in absolute ethanol resulting in ethanol, methanol and aqueous extract used for the anti-oxidant assays.
6.2.4 Data collection and statistical analysis

Statistical Analysis was performed using Graph Pad Prism Version 4.0 for Windows (San Diego, CA, USA). Data was analyzed by One Way Analysis of Variance (ANOVA) followed by Bonferroni multiple comparison test. Where applicable values are given as mean ± Standard Error of Mean (SEM) of n observations. *P-value <0.05 was considered to be statistically significant.

6.3 Anti-oxidant screening assays

6.3.1 DPPH Scavenging Assay

The hydrogen-donating ability of each extract in the presence of a DPPH stable radical was examined according to the method developed by Blois and Cao et al., 1997 (Blois, 1958; Cao et al., 1997). All the Andrographis paniculata leaves extracts were diluted with vehicle control to final extract concentrations of 0.1, 0.2, and 0.3mg/ml of aqueous, ethanol and methanol extracts.

10µl, 20µl and 30µl of each extract with stock concentration of 10mg/ml were added to a 100µM solution of DPPH (2, 2-diphenyl-1-1-picrylhydrazyl. Absorbance of the ethanolic DPPH tincture from DPPH• (violet colour) to DPPH2 (clear) was measured with a spectrophotometer at 517 nm (Bondet V. et al., 1997), Beckman Coulter, CA, PUSA. Trolox which is a water-soluble analogue of Vitamin E was used as positive control compound over the same concentration range.
6.3.2 Hydroxyl radical scavenging assay

The hydroxyl radicals were generated by the Fenton reaction of an iron-EDTA complex (100µM FeCl₃; 104µM EDTA) with 1mM H₂O₂ in the presence of 100µM ascorbic acid. The degradation of the deoxyribose (2.8mM) by the hydroxyl radicals into products which then react with Thiobarbituric acid (1% w/v in 0.05M NaOH), form a pink chromogen on heating. Extracts with anti-oxidant properties scavenge hydroxyl radicals to reduce the amount of deoxyribose degradation and chromogen formation. The aqueous, ethanol and methanol extracts (1mg/ml) were added to the reaction mixture and incubated at 37°C for 15 minutes. Trichloroacetic acid (TCA, 2.8% w/v) was added to stop the reaction before the addition of Thiobarbituric acid (TBA) on 15 minutes incubation in boiling water. Absorbance reading on the production of the chromogen was then measured spectrophotometrically at 532nm using Trolox as reference positive control compound.

6.3.3 Phenol red peroxidase assay

Hydrogen peroxide (H₂O₂) scavenging assay was assessed based on the H₂O₂-dependent horseradish peroxidase (HRP)-mediated oxidation of phenol red, the reaction between H₂O₂ and phenol red which results in the appearance of a complex which absorbs at 610 nm. All aqueous, ethanol and methanol extracts (0.1mg/ml) were preincubated with 100µM H₂O₂ in potassium phosphate buffered saline (0.15M, pH 7.0) for 30 minutes, before the addition of 50µl of phenol red (2mg/ml) and 10µl of HRP (3.6mg/ml). The absorbance was measured spectrophotometrically after 5 minute against a sample blank at 610 nm (Tan et al., 2000) using Catalase 0.025mg/ml as reference positive control compound.
6.3.4 Nitric oxide scavenging assay

The nitric oxide generated from sodium nitroprusside, was measured as described by (Marcocci et al., 1994) using the griess reagent. 0.5ml of the stock concentrations of aqueous, ethanol and methanol extracts (2, 4 and 6mg/ml) were each incubated with 0.5ml of 25 mM sodium nitroprusside at 25°C for 30min. After incubation, 1ml of the extracts was added to 1ml of griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) giving a final concentration of 0.5, 1 and 1.5mg/ml were incubated at room temperature for 10 min. The absorbance of the chromophore formed was measured with a spectrophotometer (DU530, Beckman Coulter, CA, USA) at 540 nm.

6.4 Results

6.4.1 DPPH free radical scavenging

Both the ethanol and methanol extracts were found to significantly scavenge DPPH free radical at concentrations of 0.1, 0.2 and 0.3mg/ml with the exception of the aqueous extract which did not show any significant DPPH scavenging activity. The ethanol extract at concentrations of 0.1, 0.2 and 0.3mg/ml showed significant scavenging activity of DPPH free radicals at 25.07 ± 2.28%, 32.39 ± 3.58% and 33.44 ± 2.75% (Figure 6.1) and methanol extract at the same concentrations tested displayed scavenging activity at 27.37 ± 3.72%, 37.52 ± 1.64% and 30.88 ± 0.35% respectively. The positive control trolox over the same concentration range, showed %DPPH scavenging activity of 96.98 ± 3.34%, 95.32 ± 0.67 and 96.35 ± 1.89% respectively, significantly decrease in the absorbance compared to the control (extract blank), as a result validated the procedure of DPPH assay.
Figure 6.1. Effect of *Andrographis paniculata* extracts (0.1, 0.2, 0.3mg/ml) on DPPH scavenging activity.

Results are mean ± SEM n=4, *P<0.05 expressed as percentage scavenging of DPPH free radicals when compared to an extract blank (control).

### 6.4.2 Hydroxyl radical scavenging assay

All the aqueous, ethanol and methanol extracts were found to significantly scavenge hydroxyl radical at a concentration of 0.1mg/ml, exhibited significant inhibition of deoxyribose degradation. The aqueous, ethanol and methanol extracts displayed significant scavenging of hydroxyl radicals at 9.64 ± 3.63%, 19.60 ± 4.68% and 16.24 ± 4.64% respectively. Trolox, a relatively water soluble vitamin E analogue and known free radical scavenger, used as positive control was able to scavenge 87.93 ± 1.0% of the available hydroxyl radicals at 0.1mg/ml (Figure 6.2).
Figure 6.2. Effect of *Andrographis paniculata* extracts at concentration (0.1mg/ml) and the known hydroxyl radical scavenger Trolox on hydroxyl radical scavenging activity.

Results are mean ± SEM (n=4), *P<0.05 expressed as percentage of hydroxyl radical scavenging activity when compared to an extract blank (control).
6.4.3 Phenol red-peroxidase assay

All the aqueous, ethanol and methanol extracts were found to significantly scavenge hydrogen peroxide at a concentration of 0.1mg/ml. The aqueous, ethanol and methanol extracts displayed significant scavenging of hydrogen peroxide at 16.99 ± 2.07%, 17.25 ± 3.68% and 23.19 ± 6.6% respectively when compared to the control blank (Figure 6.3). Catalase was able to scavenge hydrogen peroxide at a significant inhibition of 69.96 ± 1.67% at 0.025mg/ml.

![Graph showing the effect of Andrographis paniculata extracts concentration (0.1mg/ml) and catalase on hydrogen peroxide scavenging activity by phenol red-peroxidase assay.](image)

**Figure 6.3.** Effect of *Andrographis paniculata* extracts concentration (0.1mg/ml) and catalase on hydrogen peroxide scavenging activity by phenol red-peroxidase assay.

Results are mean ± SEM (n=4), *P<0.05 expressed as percentage of hydrogen peroxide scavenging activity when compared to an extract blank (control).
6.4.4 Nitric oxide scavenging assay

All the aqueous, ethanol and methanol extracts at the tested concentrations of 0.5, 1.0, 1.5mg/ml, displayed significant inhibition of the nitric oxide between a range of 63 to 90%, 49 to 69% and 35 to 43% respectively (Figure 6.4) when compared to the absorbance control blank.

![Figure 6.4](image)

Figure 6.4. Effect of *Andrographis paniculata* extracts concentrations (0.5, 1.0, 1.5mg/ml) on the inhibition of Nitric oxide.

Results are mean ± SEM (n=4), *P<0.05 expressed as percentage of Nitric oxide reduction activity compared to an extract blank (control).
6.5 Discussion

Several *in vitro* methods have been developed to measure and assess the potential anti-oxidant activities of natural products from plants, animals and minerals origin. Free radicals and other reactive oxygen species (ROS) are oxidizing agents which can be a good anti-oxidant or good reducing agent (Halliwell, 2000). The anti-oxidant potential of a bioactive compound can vary from one assay to the other, thus a combination of several methods should be explored and used in the assessment and evaluation of the anti-oxidant potential of the test compound (Aruoma *et al.*, 1989).

In this study screening for anti-oxidant potential of *Andrographis paniculata* showed scavenging of all the assays performed with varying degree of potency indicating that the *Andrographis paniculata (Burm. f.) Nees* of Brunei Darussalam has anti-oxidant properties as previously reported by (Kapil *et al.*, 1993; Singh *et al.*, 2001; Trivedi & Rawal, 2001; Zhang & Tan, 2000b). Changes in the enzymatic and non-enzymatic anti-oxidant defense systems may reduce resistance to free radical-mediated damage (Jones *et al.*, 1988). Therefore, naturally occurring compounds with anti-oxidant potential have beneficial effects on the overall disease processes (Stanely & Menon, 1999).

The activities of *Andrographis paniculata* leaves extracts on scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, hydroxyl radical, hydrogen peroxide and nitric oxide were assessed in cell-free systems.

The DPPH free radical compound is relatively stable with an electron when paired off with an anti-oxidant will change the colour from deep violet to a bright yellow colour and such changes
is recorded spectrophotometrically as described in section 6.3.1 of Chapter 6. It was found that both the ethanol and methanol extracts at the concentrations tested displayed significant DPPH free radicals scavenging activities in the range of 25 to 38% with the exception of the aqueous extracts which did not show DPPH scavenging activity. However DPPH scavenging assays is not capable of detecting all anti-oxidants and therefore, there is a need to use a number of assays to demonstrate anti-oxidant activities.

Results showed that the aqueous (We), ethanol (Ee) and methanol (Me) extracts of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam exhibited some significant scavenging activities towards 1,1-diphenyl-2-picryhydrazyl (DPPH), hydroxyl radical, hydrogen peroxide and nitric oxide in the cell-free systems with the exception of the aqueous extract which did not show any significant DPPH scavenging activity.

The hydroxyl radical (OH·), being the most reactive and damaging radical species react readily with and damage DNA and other important cellular components as well as initiating lipid peroxidation reactions. All the aqueous, ethanol and methanol extracts at 0.1mg/ml displayed significant hydroxyl radical scavenging activities at a range of 10-20%.

The hydrogen peroxide scavenging activities were assessed based on the H₂O₂ molecule which is not a radical but a reactive oxygen species. Superoxide radicals (O₂⁻) which are formed *in vivo*, are mostly converted to H₂O₂ by superoxide dismutase (SOD) and non-enzymatic dismutation (Halliwell & Gutteridge, 1990). Some enzymes also produce hydrogen peroxide as a by-product. Despite that hydrogen peroxide has low reactivity but once converted into hydroxyl radical (OH·) through catalysis by transition metal ions are damaging to DNA and other important cellular components. Therefore, hydrogen peroxide remains an important target for scavenging by anti-
oxidants in the prevention of hydroxyl radical (OH·) formation. All the aqueous, ethanol and methanol extracts showed significant scavenging of hydrogen peroxide scavenging activities for the phenol red-peroxidase assays at a range of 17 – 23%.

In addition to ROS, nitric oxide (NO) is also implicated in inflammatory diseases, cancer, and many other pathological conditions (Sreejayan & Rao, 1997). High levels of NO induce host cell death and inflammatory tissue damage (Kim et al., 2000; Lee et al., 2000). Therefore, a number of natural plant materials were investigated as inhibitors of NO production, and the development of effective inhibitors for the NO production in inflammatory cells are of great interest and beneficial for the therapeutic treatment of disease conditions mediated by NO (Batkhuu et al., 2002; Chiou et al., 2000; Seo et al., 2001; Wang et al., 2000). All the aqueous, ethanol and methanol extracts at the tested concentrations of 0.5, 1.0, 1.5mg/ml, displayed significant inhibition of the nitric oxide (NO) between a range of 63 to 90%, 49 to 69% and 35 to 43% respectively.

In a previous study by Sheeja et al., (2006) demonstrated that the methanolic extract of *Andrographis paniculata* was found to inhibit the formation of nitric oxide at 42.8% (Sheeja et al., 2006) compared to the finding of this study which was 43.51% in vitro. Several studies on the anti-oxidant activities of *Andrographis paniculata* in various disease conditions demonstrated the inhibition of nitric oxide (NO), lipid peroxide (LPO), superoxide dismutase (SOD) (Sheeja et al., 2006; Singh et al., 2001; Wang et al., 1997; Zhang & Tan, 2000b).
These series of anti-oxidant assays demonstrated that the aqueous (We), ethanol (Ee), methanol (Me) extracts of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam displayed significant scavenging activities towards 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical, hydrogen peroxide and nitric oxide in cell-free systems, with the exception of the aqueous extract which did not show any DPPH scavenging activity. Other assays need to be attempted to demonstrate anti-oxidant activities of compounds as one single assay may not be able to detect all anti-oxidant activities.

The finding that extracts exhibited the scavenging activity of the free radicals may imply that *Andrographis paniculata* possesses anti-oxidant activities which are beneficial in the treatment of many disease conditions such as inflammatory, immunostimulatory, cardiovascular, cancer, HIV diseases and other pathological diseases related with oxidative stress. It has also been well-documented that there is an increased production of free radicals resulting in accelerated lipid peroxidation in disease conditions such as diabetes mellitus and hypercholesterolaemia or hyperlipidaemia (Stefano *et al.*, 1997). The elevated lipid peroxidation levels may be responsible for some of the pathological effects of the diseases mentioned (Slatter *et al.*, 2000). However the concentration of the inhibitory activity on these radicals seems relatively high, thus it is not clear if any of these anti-oxidant activities are involved at the therapeutic doses.

Further study is clearly needed to elucidate if these anti-oxidant activities may contribute to the pharmacological actions and bioactivities of *Andrographis paniculata* which exhibited significant effects in anti-allergy, anti-inflammatory, anti-tumouricidal and anti-hepatotoxicity effects as demonstrated in Chapters 7, 8, 9 and 10.
Chapter 7 Effects of Andrographis paniculata (Burm. f.) Nees of Brunei Darussalam (HDM 15) plant extracts on compound 48/80-induced histamine release from rat peritoneal mast cells

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7.1 Introduction

*Andrographis paniculata* has been demonstrated for its anti-allergic and anti-inflammatory actions *in vivo* and *in vitro* (Anand et al., 2001; Dahanukar et al., 2000; Gupta et al., 1998). Gupta et al., (1998) found two active diterpenes namely Andrographolide and Neoandrographolide of *Andrographis paniculata*, administered at an oral dose of 10mg/kg to rats daily for four days exhibited mast cell stabilizing activities against compound 48/80 (0.5µg/ml) and egg albumin induced mast cell degranulation. The activities were found to be comparable to disodium cromoglycate with Neoandrographolide exhibited higher potency than Andrographolide in the inhibition of compound 48/80 induced histamine release. Andrographolide also attenuated inflammation by inhibition of NFκB activation through covalent modification of reduced cysteine 62 of p50 (Xia et al., 2004). The inhibition on mast cell derived histamine release by *Andrographis paniculata* is considered as the main mechanism of its anti allergic action (Li & Zhang, 2002). However, little is known about the difference in the anti-histamine effects of *Andrographis paniculata* from different sources as well as that in solvent extracts such as aqueous, ethanol and methanol extracts.

Therefore, the aim of this study was to investigate the effect of aqueous, ethanol and methanol extracts of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam on compound 48/80-induced histamine release measured by HPLC in rat peritoneal mast cells (RPMC). Changes in growth conditions may also affect the pharmacological actions of the *Andrographis paniculata* from different sources such as Brunei Darussalam and China. To our understanding, this is the first study in comparing the actions of different extracts of *Andrographis paniculata* (Burm. f.) *Nees* of Brunei Darussalam on mast cell-derived histamine release.
7.2 Materials and Method

7.2.1 Plant Materials

In this experiment, the source of Andrographis paniculata leaves extracts samples was described previously in Chapter 4. An accurate weight of 5.0019g of herbal powder of Andrographis paniculata was extracted in 60ml of absolute ethanol, methanol and Milli-Q pure grade water respectively using the ASE-100 Dionex System (Dionex Pty Ltd, Lane Cove, 1595, NSW). Operating procedure was optimised by Dionex system with the following specifications and extraction conditions: Dionex Part No. 056780 Filter grade: D28, Size: 30mm; Temperature: 100°C; Pressure: 1,500psi (10MPa); Heating time: 5 min; Static time: 5 min; Flush time: 60%; Purge time: 100s; Static cycle: 3; Total extraction: 30 min per sample. The herbal powder was extracted three times on three static cycles. The extracts were then filtered through a Whatman filter paper No. 1.

The ethanol and methanol were then evaporated at 80°C using rotary evaporator under vacuum (Biichi Rotavapor, Brinkman Company, Westbury, NY, USA and the concentrated aqueous extract was transported in liquid nitrogen for freeze drying using Freeze Drier Dynavac FD 12 (Airvac Engineering Pty Ltd, Melbourne, Australia) to remove all the aqueous solvent. Accurate weights of these completely dried extracts were then resuspended in absolute ethanol/methanol or Milli-Q pure grade water. The solvent extracts were then filtered with 0.45µm syringe filter and stored at -80°C for further analysis.
7.2.2 Reagents and chemicals

All reagents and chemicals used were of commercial grades. The compound 48/80, spermidine trihydrochloride, o-phthalaldehyde (OPA), histamine, toluidine blue, N-2-hydroxyethylpiperazine-N’-ethanesulfonic acid (HEPES), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl₂), L-(+)-tartaric acid, sodium tartrate dihydrate, 1-octanesulfonic acid sodium salt, boric acid, sodium hydroxide (NaOH), and disodium ethylenediamine-tetra-acetic acid (EDTA) were purchased from Sigma-Aldrich (Australia). Calcium chloride (CaCl₂), perchloric acid (HClO₄), di-sodium hydrogen orthophosphate (NaH₂PO₄) and D-glucose anhydrous were purchased from Ajax Chemicals (Australia). Heparin sodium injection was obtained from BDL (Australia). HPLC grade methanol and ethanol were purchased from Merck (Australia).

7.2.3 Preparation and Isolation of Rat Peritoneal Mast Cells (RPMC)

Male Sprague Dawley rats at 8-12 weeks of age (250 – 350g) (n=22), AEC No: 0411 were purchased from Monash Animal Services, Victoria, Australia. All procedures employed in experimental animals were approved by the Animal Experimentation Ethics Committee of RMIT University and conformed to the Australian National Health and Medical Research Council guidelines. Animals were housed in the RMIT Bundoora animal facility. Animals were fed with standard chow with adlib water. Rats were sacrificed by asphyxiation with 95% CO₂ and then decapitated. The RPMC were prepared as previously described (Ikarashi et al., 2001). Briefly, 20ml of Tyrode’s buffer (NaCl, 137.0mM; KCl, 1.0mM; MgCl₂, 1.0mM; CaCl₂, 1.6mM; NaH₂PO₄·2H₂O, 0.41mM; HEPES, 10mM and L-(-)-Glucose, 1%; pH 7.4) containing 0.3% BSA and 5.0 units/ml heparin sodium was injected intraperitoneally. The abdomen was gently massaged for 2 min, followed by the collection of peritoneal solutions containing the mast cells.
The solution was then centrifuged at 1200rpm for 5min at 4°C. The pellet containing RPMC was washed three times with 15ml of Tyrode’s solution and resuspended in 10ml Tyrode’s buffer, pH 7.4, containing 0.1% BSA. Finally, the number of RPMC in the suspension was adjusted to 5×10^5 cells/ml for further analysis.

7.2.4 Effects of herbal extracts on compound 48/80-induced histamine release from RPMC

Briefly, 10µl of various concentrations of aqueous, ethanol and methanol extracts (final extracts concentrations, 0.001, 0.01, 0.1, and 1.0 mg/ml), an equal volume of methanol, absolute ethanol or Tyrode’s buffer (aqueous) (as vehicles control), a Ca^{2+} chelating agent EDTA (100 µM) (positive control) was added to the cell suspension of 5×10^5 cells/ml, pre-incubated in the water bath at 37°C for 10 min to a final volume of 500µl and incubated in the water bath at 37°C for 10 min. Histamine release from RPMC was then induced by the addition of 25µl of compound 48/80 (at final concentration 0.5µg/ml) into the cell supernatant. The Tyrode’s buffer (10mM) or 30% HClO_4, was used to determine the basal level of histamine release from mast cells or total histamine level of the mast cells (Ikarashi et al., 2001). After further incubation at 37°C for 10 min, the supernatant was chilled in ice for 10min to stop the reaction of histamine release from RPMC. Residual histamine in the cells was released by disrupting the cells with 30% HClO_4. Fifty microliter of aqueous Spermidine trihydrochloride (1.0mg/ml) was added into the mixture as an internal standard.
7.2.5 HPLC determination of histamine release from RPMC

The level of histamine released from RPMC was determined by HPLC using a Shimadzu HPLC instrument SCL-10A vp (Shimadzu, Japan) with the following conditions: a fluorescent detector (RF-10Axl, Shimadzu, Japan), a STR reverse-phase column (ODS-II; L 150 × 4.6 I.D. mm; 5μM; Shimadzu, Japan.), an attached post-column for derivatization with the OPA, which is a reaction coil (5.0×0.5mm stainless steel tubing). Mobile phase to the main column consisted of a mixture of 100mM sodium tartaric acid buffer (pH 4.4), containing 25mM L-(-)-tartaric acid, 75mM sodium tartrate dihydrate and 10mM 1-octanesulfonic acid sodium salt, and HPLC grade methanol (2:1), and maintained at the flow-rate of 1.0ml/min. The post column solution for derivatization contained a mixture of 400mM sodium borate buffer (pH 9.2), containing 400mM boric acid and 200mM NaOH, and 10mM OPA in HPLC grade methanol (2:1). The post column flow-rate was 0.5ml/min. The wavelengths for excitation and emission were 360nm and 440nm, respectively. The column temperature was set at 50°C and the injection volume was 10μl. Under these conditions, the retention time of histamine was 3.1min, and the internal standard, spermidine was 4.8min (Figures 7.1, 7.3 and 7.5).
Figure 7.1. **Representative chromatograms of histamine detection by HPLC**

Under the experimental conditions, the retention time of Histamine was at 3.1min and the internal standard Spermidine was detected at 4.8min.
Figure 7.2. The Effect of *Andrographis paniculata* ethanol extract (Ee) on inhibition of compound 48/80-induced histamine release from rat peritoneal mast cells.

The column bars represent the mean ± SEM for n=6, *P<0.05, Two Way ANOVA, followed by Bonferroni’s test compared to compound 48/80.
Figure 7.3. Representative chromatograms of histamine detection by HPLC.

Under the experimental conditions, the retention time of Histamine was at 3.1 min and the internal standard Spermidine was detected at 4.8 min.
Figure 7.4. The Effect of *Andrographis paniculata* aqueous extract (We) on inhibition of compound 48/80-induced histamine release from rat peritoneal mast cells.

The column bars represent the mean ± SEM for n=6, *P<0.05, Two Way ANOVA, followed by Bonferroni’s test compared to compound 48/80.
Figure 7.5. Representative chromatograms of histamine detection by HPLC

Under the experimental conditions, the retention time of Histamine was at 3.1min and the internal standard Spermidine was detected at 4.8min.
Figure 7.6. The Effect of *Andrographis paniculata* methanol extract (Me) on inhibition of compound 48/80-induced histamine release from rat peritoneal mast cells.

The column bars represent the mean ± SEM for n=6, *P<0.05, Two Way ANOVA, followed by Bonferroni’s test compared to compound 48/80.
7.2.6 Data collection and statistical analysis

The percentage (%) of inhibition by the different solvent extracts on compound 48/80-induced histamine release from RPMC was calculated using the following formula which is [the concentration of histamine release induced by compound 48/80 after treated by variable extract concentrations minus the concentration of histamine release induced by compound 48/80 after treated by an equal volume of absolute ethanol)/the concentration of histamine released induced by compound 48/80] expressed as %.

Statistical data were presented as Mean ± Standard Error of Mean (S.E.M.). Statistics of comparisons between groups were performed by One Way or Two Way Analysis of Variance (ANOVA) followed by Bonferroni’s test using the Prism 4.0 (GraphicPad Software, Inc., U.S.A.). Values of *P<0.05 indicate significant difference.

7.3 Results

7.3.1 Determination of the optimum concentration of the compound 48/80 on histamine release from RPMC

A basal level of histamine at 19.6 ± 3.5ng/ml (n=6) was produced from the unstimulated RPMCs in Tyrode’s buffer. Variable concentrations of compound 48/80 from 0.0001-0.5µg/ml caused a concentration-dependent increase of histamine release from RPMC, with the maximum optimised response at 0.5µg/ml of Compound 48/80 (422.9 ± 64.1 ng/ml, n=4). As a result, this optimal concentration of 0.5µg/ml of compound 48/80 was then used for the subsequent experiments. The vehicle control (compound 48/80 induced histamine release treated with an
equal volume of absolute ethanol) slightly inhibited the histamine release (9.5 ± 1.2%, n=6, P>0.05), compared with compound 48/80 alone induced histamine release. The increase in histamine release by compound 48/80 was significantly inhibited by 78.9 ± 5.0 % by 100 µM EDTA, compared with compound 48/80 alone induced histamine release (n=7, P<0.05).

7.3.2 Effects of different solvent extracts on compound 48/80 induced histamine release from RPMC

All three extracts when tested at 0.001, 0.01, 0.1mg/ml indicated a minor difference (intra) within the extract but moderate difference (inter) between the three solvent extracts. At lower concentrations of 0.001, 0.01, 0.1 mg/ml of ethanol (Figure 7.2), aqueous (Figure 7.4), and methanol (Figure 7.6) extracts showed a moderate inhibitory effect, but at a higher concentration of 1mg/ml, % inhibition of histamine release was significantly increased (Figures 7.1, 7.3 and 7.5). Among the tested concentrations, at 1 mg/ml of methanol, aqueous and ethanol extracts significantly inhibited histamine release by 73.33 ± 4.55%, 57.59 ± 6.57% and 32.34 ± 3.49% (*P<0.05, n=6) respectively compared to 8.38 ± 0.01% in the compound 48/80 only treated cells as shown in Figure 7.7.
Figure 7.7. The Effect of *Andrographis paniculata* aqueous, ethanol and methanol extracts (1mg/ml) on the inhibition of compound 48/80-induced histamine release from rat peritoneal mast cells.

The column bars represent the mean ± SEM for n=6, P<0.05.

One Way ANOVA followed by Bonferroni Multiple Comparison test compared to compound 48/80.
7.4 Discussion

This study is the first report to compare the differences in their pharmacological anti-allergic actions on mast cell derived histamine release from RPMC of aqueous, ethanol and methanol extracts of *Andrographis paniculata* (Burm. f.) Nees from Brunei Darussalam.

By determining the histamine levels using HPLC, this study demonstrated that all the extracts significantly inhibited the compound 48/80 induced histamine release from RPMC at higher concentration of 1mg/ml but not at lower concentrations of 0.001, 0.01, 0.1 mg/ml. Further more, this study demonstrated a similar trend among three different solvent extracts of the *Andrographis paniculata* (Burm. f.) Nees species, histamine release at a higher concentration of 1mg/ml but not lower.

Previous studies by Gupta et al., 1998 on the two active diterpenes Andrographolide and Neoandrographolide isolated from *Andrographis paniculata* which exhibit mast cell stabilizing activities against compound 48/80 at 0.5µg/ml and in sensitized mast cells, against egg albumin induced degranulation. The activities were found to be comparable to disodium cromoglycate and suggested its potential anti-allergic properties with Neoandrographolide exhibited higher potency than Andrographolide in the significant inhibition of compound 48/80 induced histamine release (Anand et al., 2001; Dahanukar et al., 2000; Gupta et al., 1998). Gupta et al., 1998 investigated the antiallergic activity of Andrographolides in *Andrographis paniculata* (Burm. f.) Wall (the former name of Nees) and found that the mast cells collected from the rats fed on Andrographolide 10mg/kg orally for 4 days, incubated with compound 48/80 at 0.5µg/ml produced a significant inhibition of 60% compared to 72% inhibition produced by Disodium
cromoglycate (DSCG) at the same experimental conditions. The antiallergic activity appear to be due to immunomodulating activity which requires further elucidation (Gupta et al., 1998).

Disodium cromoglycate (cromolyn) and sodium nedocromil are used in the treatment of allergic rhinitis via the nasal route of administration topically (Bush, 2004). Cromolyn sodium is a class of therapeutic drugs in which its mode of action is not completely elucidated (Holgate et al., 2001). The mode of action is suggested to be mast cell stabilisation activities (Mygind et al., 1996; Naclerio, 1991) potentially through blocking Ca\(^{2+}\) channels and oxidative phosphorylation or inhibiting phosphodiesterase (van Cauwenberge et al., 2000). Recent studies suggested the mechanism of action to include inhibitory effects on eosinophil, platelets and macrophages (Holgate et al., 2001).

However, it is not clear about the exact nature of the active component(s) involved and the actual mechanism of action in this anti-histamine release action given the complex nature of the active components in the herb as studies have indicated that *Andrographis paniculata* contains the active components diterpene lactones Andrographolide as the major compound, Dehydroandrographolide, Deoxyandrographolide and Neo-andrographolide. Recent studies by Pramanick et al (2005) and Shen et al (2005) isolated new *ent*-labdane type diterpenoids from the leaves of *Andrographis paniculata*. The contents of active ingredients in the various solvent extracts may vary due to the solubility of the active components in the extraction solvents. *Andrographis paniculata* (Burm. f.) Nees possesses Ca\(^{2+}\) antagonist activities which selectively block voltage-operated calcium channels in rat vas deferens and induce relaxation of uterus by blocking voltage operated calcium channels and inhibits Ca\(^{2+}\) influx (Burgos et al., 2001; Burgos et al., 2000).
As a result, the compound 48/80 may target on the peritoneal mast cells by increasing intracellular calcium concentrations hence inducing the release of histamine (Chakravarty & Yu, 1986; Marshall et al., 1994). *Andrographis paniculata* (Burm. f.) Nees may possibly contribute to the regulation of degranulation of mast cells in the studies on other herbs (Brown et al., 2001; Ma & Han, 1995; Mitsuo, 2001). Further study is necessary to establish a correlation between the effect on the inhibition of histamine release and the % composition and the active components present in the different solvent extracts.

Observation from this study indicated that the three samples, the inhibitory action for aqueous, ethanol and methanol extracts had only a marginal difference between lower concentrations of 0.001mg/ml and 0.1mg/ml. However at a higher concentration of 1mg/ml, all the three extracts from this species actually produced a significant inhibitory effect than that obtained from 0.001mg/ml to 0.1mg/ml. The findings indicated that the active component(s) which are of potential effect on the inhibition of histamine release only result in a significant effect at 1mg/ml of the solvent extracts. Such findings showed that *Andrographis paniculata* may not be beneficial to be used as anti-allergies or anti-histaminic therapeutic agents as the study demonstrated that it requires a much higher dose to exert the therapeutic effect when compared to other agents. The finding in this study demonstrated that the rat peritoneal mast cells is less responsive towards *Andrographis paniculata* extracts which is in close agreement with the previous study by (Gupta et al., 1998).

A better understanding is needed on the exact composition of *Andrographis paniculata* which would contribute to the optimization of the effective dose of *Andrographis paniculata* (Burm. f.) Nees species for its clinical application as an anti-allergic agent. The observation from this first screening on the effect of *Andrographis paniculata* (Burm. f.) Nees species of Brunei
Darussalam showed a similar trend in the inhibition on histamine release in the various solvent extracts. This indication may provide a useful profile of the pharmacological actions of the same species grown at different geographical region or locations such as India or China as geographical location and seasons may also explain the differences in the contents of the active components in the herb (Hu & Wu, 1995). A close observation and monitoring of the profiling of medicinal plants in terms of the medicinal effects of its active constituents need to be screened in terms of qualitative and quantitative analyses, which is usually time consuming and expensive. So far, there is limited study specifically on the inhibition of the compound 48/80-induced histamine release from rat peritoneal mast cells by *Andrographis paniculata* (Burm. f.) Nees extracts for assessing its anti-allergic properties.

Therefore, the present study indicated that there are varying percentage composition of the active components and the types of components present in the aqueous, ethanol and methanol extracts that may account for the differences in compound 48/80 induced anti-histamine from RPMC. The difference in extract concentrations may be important as it is unlikely to achieve the therapeutic anti-allergic and anti-histaminic effects at doses less than 1mg/ml. As a result, it is not appropriate to be recommended as an anti-allergic or anti-histaminic therapeutic agents.

In conclusion, all the three solvent extracts have similar trends of inhibition with moderate degree of potency in the inhibition of compound 48/80 induced histamine release in RPMC at 1mg/ml. The ethanol extract is significantly different from the aqueous and methanol extracts (P<0.05, n=6) but the aqueous extract is not significantly different from the methanol extract (P>0.05, n=6), One Way ANOVA followed by Bonferroni’s test, compared all groups (Figure 7.7). Such difference may have clinical implications if they are used interchangeable. Further investigation is necessary to investigate the active component(s) which are responsible in
the inhibition of compound 48/80 induced histamine release in RPMC to ascertain the optimized
dose of therapy for allergies.
Chapter 8  Inhibition of iNOS mediated relaxations by Andrographolide, Dehydroandrographolide and *Andrographis paniculata* of Brunei Darussalam in the rat aorta

8.1 Introduction

8.2 Materials and Methods

8.2.1 Plant materials

8.2.2 Data collection and statistical analysis

8.2.3 Pharmacological Studies

8.2.3.1 Animals

8.2.3.2 Functional studies in the inhibition of iNOS mediated relaxation in LPS treated rat aorta

8.2.3.2.1 Physiological Salt Solution (PSS)

8.2.3.2.2 Rat tissue preparation

8.2.4 Endothelium dependant relaxation in rat aorta rings

8.2.5 iNOS mediated relaxation in LPS treated aortic preparation

8.3 Results

8.3.1 Controls

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8.4 Discussion
8.1 Introduction

It has been demonstrated that inducible NO plays an important role in systemic inflammatory disorders, diabetes, arteriosclerosis and other diseases complications (Connor et al., 1995; Stefanovic-Racic et al., 1993; Wu & Thiemermann, 1996). Therefore, natural products which possess the inhibitory properties on the formation of NO are potential therapeutic agents for treating patients with septic shock or inflammatory diseases of both local and systemic disorders as well as other disease conditions. Among a range of pharmacological actions of *Andrographis paniculata*, the anti-inflammatory effects of *Andrographis paniculata* is important in its clinical use. In this regard to understand the actions of *Andrographis paniculata* on inflammatory iNOS mediators will enhance our understanding of the therapeutics of *Andrographis paniculata* and the possible mechanisms involved in the iNOS pathways.

The pharmacological and functional effects of *Andrographis paniculata* (Burm. f.) Nees grown in Brunei Darussalam has not yet been studied. In this study, we investigated the functional effects of *Andrographis paniculata* leaves samples from Brunei Darussalam in three different concentrations of ethanol (Ee) and aqueous (We) extracts at 1, 10, 100µg/ml in the inhibition of iNOS mediated relaxation in LPS treated rat aorta compared to the Chinese Reference Sample herb CRS and *Chuan Xin Lian* (CXL) from China. Large scale screening on Andrographolide (3-[2-[Decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-naph-thalenyl] ethyldene] dihydro-4-hydroxy-2(3H)-fura-none), a bicyclic diterpenoid lactone present in *Andrographis paniculata* leaves (Lu et al., 1981) demonstrated inhibition of NO synthesis. Study by Chiou et al (1998) showed that Andrographolide (10 & 30 µM) suppressed the expression of inducible nitric oxide synthase and restores the vasoconstriction in rat aorta treated with
lipopolysaccharide LPS with *P<0.05 represents significant differences when compared to control rings (Chiou et al., 1998).

Given that the growing condition of herbal species may affect the contents of chemical components and their pharmacological actions (Hu & Wu, 1995), we investigated the targeted pharmacological actions of *Andrographis paniculata* from different geographical locations of Brunei Darussalam. In this study we focused on the effects of *Andrographis paniculata* on inducible nitric oxide (iNOS)-mediated responses. It has been well established that nitric oxide (NO) is an important inflammatory mediator. NO generated through inducible nitric oxide synthase (iNOS) plays important roles in various pathophysiological conditions, such as vascular dysfunctions in septic shock (Julou-Schaeffer et al., 1990; Kilbourn et al., 1990; Rees et al., 1990; Thiemermann & Vane, 1990; Wu & Thiemermann, 1996) and inflammatory reactions in arthritis and diabetes (Connor et al., 1995; Stefanovic-Racic et al., 1993; Wu & Thiemermann, 1996).

Therefore, natural products which possess the inhibitory properties on the formation of inducible NO are potentially useful for treating certain inflammatory diseases or conditions. However it is not known if the actions of raw herbal extracts directly relates to Andrographolide content or other related active component(s). Thus, in this study, we compared the effects of Andrographolide, Dehydroandrographolide and *Andrographis paniculata* extracts from different locations in Brunei Darussalam as well as that from China on iNOS-mediated responses in the rat aorta *in vitro*
8.2 Materials and Methods

8.2.1 Plant materials

In this experiment, the *Andrographis paniculata* ethanol and aqueous extracts investigated were CRS and CXL from China and *Andrographis paniculata* from Brunei Darussalam. Aerial parts of *Andrographis paniculata* (Burm. f.) Nees of Brunei Darussalam samples were collected from Brunei/Muara district (Brunei AP Ee), Kuala Belait district (Kuala Belait Ee) and Temburong district (Temburong Ee) and air-dried. Commercial dried *Andrographis paniculata* leaves samples from China *Chuan Xin Lian*, (CXL Ee) were also collected from the Chinese Medicine Clinic, Chinese Medicine Division of Royal Melbourne Institute of Technology.

The plant materials were authenticated by the Department of Agriculture, Ministry of Industry and Primary Resources, Brunei Darussalam. The Chinese samples were authenticated by the Chinese Medicine Clinic of RMIT University, Victoria, Australia. Samples were crushed into powder with a mortar and pestle. 5 g of each powder was then extracted in 60ml of absolute ethanol by ASE-100 Dionex System (Dionex 100, USA) under the following specifications and extraction conditions: Dionex Part No. 056780 Filter grade: D28, Size: 30mm; Temperature: 100°C; Pressure: 1,500psi (10MPa); Heating time: 5 min; Static time: 5 min; Flush time: 60%; Purge time: 100s; Static cycle: 3; Total extraction: 30 min per sample. Each herbal powder was extracted three times on three static cycles. The extracts were then filtered through a Whatman filter paper and the ethanol was evaporated at 80°C using rotary evaporator under vacuum (Biichi Rotavapor, Brinkman, USA). Accurate weights of the extracts were then resuspended in absolute ethanol, then filtered with 0.45µm syringe filter and stored at -80°C for further analysis.
8.2.2 Data collection and statistical analysis

Results were expressed as Mean ± S.E.M of n observations, where n represent the number of animals or aortic vessels studied. Statistical analysis was performed with Student’s t-tests, or One Way Analysis of Variance (ANOVA) or Two Way Analysis of Variance (ANOVA), Bonferroni’s test. A *P-value less than 0.05 was considered to be statistically significant.

8.2.3 Pharmacological Studies

8.2.3.1 Animals

Male Sprague-Dawley of 200-350g (n=112), age 8-12 weeks old were used for the functional studies (AEC No: 0327 and 0411). These rats were purchased from Monash University, Clayton, Victoria, Australia. Groups of up to a maximum of four rats of the same species (SD) were caged in standard animal boxes, housed in the RMIT Bundoora Animal Facility with proper care and fed with standard chow with adlib water for a few days, sometimes up to a week before experimentation. All experimental procedures involving animals were approved by RMIT Animal Ethics Committee and conformed to National Health and Medical Research Council Guidelines.
8.2.3.2 Functional studies in the inhibition of iNOS mediated relaxation in LPS treated rat aorta

8.2.3.2.1 Physiological Salt Solution (PSS)

The composition of the physiological salt solution (PSS) was (mM): NaCl, 118; KCl, 4.7; NaHCO$_3$, 25; MgSO$_4$, 0.45; KH$_2$PO$_4$, 1.03; and, CaCl$_2$, 2.5. All reagents and chemicals used were of commercial grades.

8.2.3.2.2 Rat tissue preparation

The rats were killed by asphyxiation with 95% CO$_2$ and decapitated. Aortae were isolated and dissected into four rings of 5mm each in accordance with set up described by (Lenon et al., 2003). Endothelium was removed by gently rubbing the lumen with forceps. Each ring was mounted in a 6ml or 8ml or 10ml organ bath containing physiological salt solution (PSS) bubbled with 95% O$_2$ and 5% CO$_2$ maintained at 37ºC under a resting tension of 2g, then equilibrated at 60 min prior to the commencement of the experimental procedures. The tension was measured isometrically using Grass FT03 force-displacement transducers (Grass Instruments, Quincy, MA, USA), traces recorded by MacLab data acquisition system.
8.2.4 Endothelium dependant relaxation in rat aorta rings

Cumulative concentration response curves to acetylcholine from 0.1-10µM were constructed in Phenylephrine PE-precontracted (1µM) aorta tissue preparations before and after 15 minutes incubation with ethanol extracts such as CRS Ee, CXL Ee, Brunei AP Ee or aqueous extracts CRS We, CXL We and Brunei AP We of final concentrations 1, 10 and 100µg/ml or vehicle control (absolute ethanol or Milli-Q water) or L-NAME as positive control. Tests were carried out in endothelium-denuded preparations using the method described previously (Ellis et al., 2000; Griffiths et al., 1993). Briefly, the endothelium was denuded by gently rubbing the inner lumen with a pair of forceps and the failure of relaxation to acetylcholine 10µM indicated the successful endothelium denudation.

8.2.5 iNOS mediated relaxation in LPS treated aortic preparation

After the confirmation of the denudation of endothelium, the preparations were incubated in LPS (50µg/ml) and ethanol (Ee) or aqueous (We) extracts of CRS, CXL, Brunei AP at 1, 10 and 100µg/ml simultaneously for 6 hours before the contraction to PE. Cumulative concentration-dependant relaxations to L-arginine (1, 3, 10, 30 & 100µM) were constructed in PE precontraction aorta. L-NAME (100µM) as positive control was incubated 15 minutes prior to the experiment (Figure 8.1).
8.3 Results

8.3.1 Controls

Phenylephrine PE (1µM) induced contraction of around 1.71 ± 0.1 gw (n=22) and the cumulative concentration response curve to acetylcholine produced a concentration-dependent relaxation with maximal response with ethanol and aqueous as vehicle control at 61.49 ± 9.58% or aqueous vehicle control of at 67.46 ± 9.59% or L-NAME was (5.16 ± 1.02%), n=4 (Figure 8.2).

8.3.2 Effect of Ethanol extracts

Among the tested extract concentrations, Andrographolide Ee and Dehydroandrographolide Ee in ethanol at 10µM and all the ethanol extracts of CXL Ee, Brunei AP Ee, Kuala Belait Ee and Temburong Ee at 100µg/ml, significantly inhibited the iNOS mediated relaxation in LPS treated rat aorta (*P<0.05, n=4); Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control. The maximal relaxations to L-arginine 100µM (Log M=-4.0) were significantly inhibited by Andrographolide Ee (10µM) at 15.45 ± 1.97% (Figure 8.3) and Dehydroandrographolide Ee (10µM) at 21.07 ± 2.57% (Figure 8.4). The maximal relaxations were also significantly reduced by CXL Ee at 31.52 ± 4.02% (Figure 8.6), Brunei AP Ee at 26.12 ± 4.67% (Figure 8.7), Kuala Belait Ee at 30.40 ± 3.96% (Figure 8.8) and Temburong Ee at 22.98 ± 2.91% (Figure 8.9) when compared to the vehicle control at the high concentration of 100µg/ml. However, among all the ethanol extracts tested, CRS Ee (100µg/ml) did not show any significant inhibition at the maximal response of L-arginine 100µM (Log M=-4.0) in the inhibition of iNOS mediated relaxation in LPS treated rat aortae (Figure 8.5). All the ethanol extracts did not exhibit any significant difference between all groups of extracts at 100µg/ml.
(P>0.05, n=4), One Way ANOVA followed by Bonferroni Multiple Comparison tests, compared with all groups of ethanol extracts (Figure 8.10).

### 8.3.3 Effect of Aqueous Extracts

All the aqueous extracts such as Dehydroandrographolide We (10µM) (Figure 8.12), CRS We (Figure 8.13), CXL We (Figure 8.14) and Brunei AP We extracts (Figure 8.15) at 1-100µg/ml did not show any significant inhibition. However, the maximal relaxations to L-arginine 100µM (Log M=-4.0) was significantly reduced by Andrographolide We (10µM) to 32.29 ± 4.97% (Figure 8.11) in the iNOS mediated relaxation in LPS treated rat aortae, compared to the vehicle control. All the aqueous extracts did not exhibit any significant difference between all groups of extracts at 100µg/ml (P>0.05, n=4), One Way ANOVA followed by Bonferroni Multiple Comparison tests, compared with all groups of aqueous extracts (Figure 8.16).

### 8.3.4 Effects of Andrographolide and Dehydroandrographolide on iNOS-mediated relaxations

Under the experimental conditions (endothelium-denuded and LPS-pre-treated preparations), phenylephrine (PE, 1µM) induced a contraction at 1.7 ± 0.1 gw (n=22). L-arginine (1-100 µM) produced a concentration-dependent relaxation which was abolished by the NOS inhibitor L-NAME (100µM).

Both Andrographolide and Dehydroandrographolide caused a concentration-dependent inhibition of iNOS-mediated relaxations. The maximal relaxations to L-arginine (100µM) were significantly inhibited by Andrographolide (10 µM) from 61.49 ± 9.58% to 15.45 ± 1.97% and
Dehydroandrographolide Ee (10 µM) from 61.49 ± 9.58% to 21.07 ± 2.57% as shown in Figures 8.3 and 8.4 respectively.

8.3.5 Effects of *Andrographis paniculata* extracts on iNOS-mediated relaxations

The ethanol extracts of CXL Ee, Brunei AP Ee, Kuala Belait Ee and Temburong Ee concentration-dependently significantly inhibited the iNOS mediated relaxations, compared to the vehicles control (*P<0.05, n=4, Two-Way ANOVA followed by Bonferroni’s tests). At 100 µg/ml, the maximal relaxations to L-arginine (100µM) were significantly inhibited by CXL Ee (to 31.52 ± 4.02%), Brunei AP Ee (to 26.12 ± 4.67%), Kuala Belait Ee (to 30.40 ± 3.96%) and Temburong Ee (to 22.98 ± 2.91%) as illustrated in Figures 8.6, 8.7, 8.8 and 8.9 respectively, compared to the vehicle control.

8.3.6 Correlation of Andrographolide and Dehydroandrographolide contents to inhibitions of iNOS relaxations by *Andrographis paniculata* extracts

The content of Andrographolide and Dehydroandrographolide in *Andrographis paniculata* extracts varied significantly in *Andrographis paniculata* extracts, ranging from 0.51 – 3.43%. The highest Andrographolide content was found in Temburong Ee (3.43 ± 0.02%), followed by Brunei AP Ee (1.86 ± 0.02%), CXL Ee (1.14 ± 0.01%), Kuala Belait Ee (0.67 ± 0.02%) and CRS Ee (0.51 ± 0.01%). In contrast, the highest Dehydroandrographolide content was found in of CXL (0.66 ± 0.01%) followed by Brunei AP Ee (0.24 ± 0.01%), Kuala Belait Ee (0.24 ± 0.05%), CRS Ee (0.14 ± 0.01%) and Temburong Ee (0.12 ± 0.01%).
The extent of inhibition of iNOS-mediated responses by *Andrographis paniculata* extracts had a poor correlation with the content of Andrographolide and Dehydroandrographolide levels alone. However, it has a significant correlation with the combined content of Andrographolide and Dehydroandrographolide together (with $R^2 = 0.832$) as shown in Figure 8.17.

**Figure 8.1. MacLab Trace of the positive control L-NAME**

The relaxation to L-arginine (100µM) were significantly reduced by L-NAME (100µM) as positive control to around (5.16 ± 1.02%), n=4.
Figure 8.2. Effect of L-NAME as positive control (100µM) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta. n=4; *P<0.05, One Way ANOVA followed by Bonferroni’s tests compared to the vehicle control.

Figure 8.3. Effect of Andrographolide Ee (1, 3, 10µM) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta. n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.
Figure 8.4. Effect of Dehydroandrographolide Ee (1, 3, 10µM) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.

n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.

Figure 8.5. Effect of CRS Ee (1, 10, 100µg/ml) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.

n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.
Figure 8.6. Effect of CXL Ee (1, 10, 100µg/ml) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.

n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.

Figure 8.7. Effect of Brunei AP Ee (1, 10, 100µg/ml) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.

n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests,
compared to the vehicles control.

Figure 8.8. Effect of Kuala Belait Ee (1, 10, 100µg/ml) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta. n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.

Figure 8.9. Effect of Temburong Ee (1, 10, 100µg/ml) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta. n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.
Figure 8.10. Effects of *Andrographis paniculata* ethanol extracts (Ee) 100µg/ml on the maximal relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.
n=4; *P<0.05; One Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.

Figure 8.11. Effect of Andrographolide We (1, 3, 10µM) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.

n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.

Figure 8.12. Effect of Dehydroandrographolide We (1, 3, 10µM) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.
n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.

Figure 8.13. Effect of CRS We (1, 10, 100µg/ml) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.

n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.

Figure 8.14. Effect of CXL We (1, 10, 100µg/ml) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.
n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.

Figure 8.15. Effect of Brunei AP We (1, 10, 100µg/ml) on the relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.

n=4; *P<0.05; Two Way ANOVA followed by Bonferroni’s tests, compared to the vehicles control.
Figure 8.16. Effects of *Andrographis paniculata* aqueous extract (We) 100µg/ml on the maximal relaxations elicited by L-arginine in LPS-incubated endothelium-denuded aorta.

n=4; *P<0.05; One Way ANOVA followed by Bonferroni’s Multiple Comparison Tests, compared to the vehicles control.
Figure 8.17. Effect of % Inhibition of iNOS by %w/w of Andrographolide and Dehydroandrographolide (A plus D) in Andrographis paniculata
8.4 Discussion

To the best of our knowledge, this is the first study on *Andrographis paniculata* from Brunei Darussalam. By using a well established protocol we have demonstrated that the *Andrographis paniculata* extracts significantly inhibited iNOS-mediated responses, an effect have been previously shown to certain *Andrographis paniculata* active components and *Andrographis paniculata* extracts from other regions from Asia (Chiou et al., 2000; Chiou et al., 1998). In addition, we found that the *Andrographis paniculata* from Brunei were more potent in inhibiting iNOS mediated responses than those from China, indicating that the growing and climatic conditions of *Andrographis paniculata* may affect the potency of its action or the contents of chemical components which are responsible for mediating anti-iNOS actions of *Andrographis paniculata* as geographical location and seasons may also explain the differences in the contents of the active components in the herb (Hu & Wu, 1995). Nevertheless, the anti-iNOS effect of *Andrographis paniculata* seems highly reserved in *Andrographis paniculata* from different geographical locations in Asia.

The main active ingredients characterized in *Andrographis paniculata* are diterpenoid components, including Andrographolide, Dehydroandrographolide, Neoandrographolide, Deoxyandrographolide, etc (Pamanick et al., 2006; Zhao et al., 2006). Among these, Andrographolide has been most studied. The finding that Andrographolide inhibited iNOS mediated responses in the rat aorta in the present study is consistent with previous report on its protection of LPS-induced reduction of contractile responses to phenylephrine *in vitro* (Chiou et
Such effect of Andrographolide is most likely to be mediated by its inhibition of expression of iNOS through prevention of protein synthesis and decrease of the protein stability rather than affecting iNOS mRNA (Chiou et al., 2000). Similar effect was also reported for Neoandrographolide, which inhibited LPS-induced NO production in mouse peritoneal macrophages in vitro although it is weaker than Andrographolide (Batkhuu et al., 2002), possible through inhibition of p38 MAPKs action (Liu et al., 2006). Importantly, the present finding that Dehydroandrographolide, a related diterpenoid compound, significantly inhibited iNOS-mediated relaxations, indicates that Dehydroandrographolide also contributes to the anti-iNOS actions of Andrographis paniculata. Further work is necessary to find if similar biochemical mechanisms are involved in the action of Dehydroandrographolide.

Interestingly, Andrographis paniculata extracts from different districts of Brunei Darussalam produced a similar but varied inhibition on iNOS-mediated response, indicating that it is likely to be mediated by similar active components, although such active components may vary significantly among different samples. It is most likely a number of diterpenoid components are involved. The key question is whether the anti-iNOS effect of Andrographis paniculata directly correlated to the contents of Andrographolide related components.

Our finding suggested that the anti-iNOS effect of Andrographis paniculata extracts had a poor correlation with the content of Andrographolide and Dehydroandrographolide alone but it had a good with the total content of Andrographolide plus Dehydroandrographolide indicates the importance of multiple Andrographolide components working collectively in mediating anti-iNOS response by Andrographis paniculata. In this regards, other component(s) may also
contribute to the inhibitory effect by *Andrographis paniculata* extracts as significant portion of the observed effect seems not related to Andrographolide and Dehydroandrographolide alone. It would be interesting to further study the genetic profile and correlations between genetic markers and their metabolic compounds of *Andrographis paniculata* from Brunei Darussalam. The level of Andrographolide and Dehydroandrographolide in *Andrographis paniculata* extracts from China in the present study is similar to those reported in the literature (Chiou et al., 2000).

Given the importance of iNOS in various pathophysiological conditions, such as vascular dysfunctions in septic shock (Julou-Schaeffer et al., 1990; Kilbourn et al., 1990; Rees et al., 1990; Thiemermann & Vane, 1990; Wu & Thiemermann, 1996) and inflammatory reactions in arthritis and diabetes (Connor et al., 1995; Stefanovic-Racic et al., 1993; Wu & Thiemermann, 1996), the inhibitory properties of *Andrographis paniculata* are potentially useful for treating certain inflammatory diseases or conditions. In this regards, *Andrographis paniculata* extracts from Brunei Darussalam may serve as a better herbal source for treating iNOS related inflammatory conditions.

In conclusion, the ethanol extracts of *Andrographis paniculata* from different locations of Brunei Darussalam exhibits significant anti-iNOS mediated relaxations in the rat aorta, an effect partly correlated to the total content of Andrographolide and Dehydroandrographolide of the herbal extracts. Such anti iNOS mediated response may contribute to the beneficial effects of *Andrographis paniculata* in related disease conditions.
## Chapter 9  Anti-tumour activity of *Andrographis paniculata* (Burm. f.) Nees,

### HDM 15 - Medicinal Plant of Brunei Darussalam

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9.1 Introduction

It is known over the years that the world’s population relies upon plant species as the primary source of medicine for the treatment of diseases. The continuous search for natural heals for the treatment of minor to more severe complications such as potential anti-cancer agents are focused on the natural products as effective and safe remedies. It was reported that more than 10 million people world-wide are diagnosed with cancer every year, and more than 6 million die from cancer, according to World Health Organization (WHO). It is projected that in the next 20 years these numbers will be increased by 50%. It is reported that cancer is a major cause of mortality and morbidity in Australia. More than 34,000 Australians died of cancer, and a further 80,000 were diagnosed with some form of cancer. An estimated of 270,000 non-melanocytic skin cancers are diagnosed each year (AACR, 2001). In 2002, malignant neoplasms were the leading cause of all deaths (28%) in Australia (ABS, 2003).

In line with the worldwide trend, cancer is a major cause of mortality and morbidity in Brunei Darussalam. Currently, cancer is the number one killer in Brunei Darussalam which has increased to about 22% in year 2004, almost double compared to 12.1% in 1996 which was only ranking the third leading cause of death according to the statistics of death registry of Brunei Darussalam. The top four cancer cases diagnosed were lungs (47), cervix (47), breast (45) and colo-rectal (35) (MOH, 1996, 2004a). The rate is on the rapid increase as reflected in most developing nations and the continued search for an effective therapeutic anti-tumour agent is paramount.

Cytotoxic drugs are used in the chemotherapies of cancer which interfere with cell proliferation during the process of cell division. Unfortunately, such drug action also affects the normal
dividing cells, giving rise to untoward effects and complications such as immunosuppression and excessive hair loss (Tannock & Hill, 1998). Therefore, anti-tumour agent with specific toxicity targeting on tumour cells and at the same time without affecting the normal cells will become the ideal drug of choice in cancer therapies, with the aim to maximise the benefits and at the same time minimising the risks.

Many compounds from natural sources are constantly being screened for cytotoxic activity against tumour cell lines and normal cells, with the interest to differentiate between the specificity and non specific tumour activities. The general cytotoxicity of the same extract fractions on rat hepatocytes is discussed in the Chapter 10 on the rat hepatocyte toxicity screening assay.

Therefore, this study on Andrographis paniculata is of paramount importance for the future research and development of the medicinal native plants of Brunei Darussalam which are of therapeutic benefits to health.

9.2 Materials and Methods

9.2.1 Plant materials

The extract fractions of the Andrographis paniculata were described previously in section 5.3.2.3.2 and 5.3.2.3.3 of Chapter 5.
9.2.2 Biological materials

All biological materials were provided by the Key Centre for Toxicity, RMIT. Mouse lymphoblastic parental PAR (P388), ADR, WEHI-164, K562 (Human Tumour cells), P815 (Mouse mastocytoma cells), Yac-1 (T-Lymphoblastic) tumour cells (all six cell lines were provided by Associate Professor Paul Wright, the Key Centre for Toxicity, RMIT). RPMI-1640 was obtained from Sigma Chemical Company (St Louis, MO., USA). Freshly isolated rat hepatocytes (hepatocytes cells were provided by Dr. Simone Yendle, the Key Centre for Toxicity, RMIT).

9.2.3 Anti-tumour screening assay

In-vitro anti-tumour activity of extracts fractions were determined using mouse leukaemic lymphoblastic Parental PAR (P388), ADR, and other cancer cell lines WEHI-164, K562 (Human Tumour cells), P815 (Mouse mastocytoma cells), Yac-1 (T-Lymphoblastic) tumour cells (cell lines were provided by Associate Professor Paul Wright, the Key Centre for Toxicity, RMIT). Extract fractions were dissolved in 100% absolute ethanol to ensure sterility for cell culture testing and then serially diluted into final concentrations of 1, 3.3, 10, 33.3, 100µg/ml in RPMI-1640 media containing 2mM glutamine, 10% heat inactivated foetal calf serum (CSL, Parkville, Australia), 10mM 2-mercaptoethanol and 100µg/ml gentamicin sulfate) in a 96-well flat bottom cell culture sterile microtitre plate. Extract blank, ethanol solvent control and cell free wells were plated. The tumour cells of 1 x 10^5 cells/ml in RPMI-1640 were added to the extract fractions and incubated for 48 hours at 37°C, 5% CO₂. Cell viability was assessed by the addition of blue dye Resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide) C_{12}H_{6}NNaO_{4} (Sigma, USA) sodium salt solution 0.01mg/ml in each well, which is converted to pink in metabolically active cells and fluoresce. 6%w/v SDS (Sodium Dodecyl Sulfate) were added in final concentrations of 40µg/ml,
60µg/ml, 80µg/ml, 100µg/ml in four wells of extract free as a positive control and acts as the inhibitor of cancer cell lines. The fluorescence of the reaction product was measured at emission wavelength of 560nm and excitation wavelength of 590nm after 48 hours incubation (37ºC, 5% CO₂) using the 96 wells fluorescence flat, pico green black plates.

9.2.4 Data collection and statistical analysis

The results were expressed as a percentage of w/w of the herbal extract. Values were expressed as mean ± Standards Error of Mean (S.E.M.) and were analyzed using either student t-test, One Way Anova, Bonferroni Multiple Comparison Test (GB-Stat by Dynamic Microsystems Inc., MD, USA). Results are mean ± Standard Error of Mean (SEM), (*P<0.05, n=4).

9.3 Results

The anti-tumouricidal activity of *Andrographis paniculata* was investigated using six tumour cell lines as described earlier. The detection of cytotoxic compounds Andrographolide and Dehydroandrographolide was determined by comparison to the ethanol vehicle control. Andrographolide (Std A) has the most potent antitumourigenic activity which inhibited the growth of all the six tumour cells by 50%, the IC₅₀ at a concentration range of 6-10µg/ml for P388 (Figure 9.1), ADR (Figure 9.2), WEHI-164 (Figure 9.3), K562 (Figure 9.4), Yac-1 (Figure 9.5). However, the cell line P815 (Figure 9.6) which has a higher IC₅₀ value of 100µg/ml, showed lesser sensitivity to Std A when compared to the other cell lines investigated. The other extract fractions tested indicated that some *Andrographis paniculata* fractions inhibited the growth of these tumour cell lines with varying degree of potency which correlated with the content of Andrographolide.
The Brunei AP Ee ASE (Bru Ee ASE) fraction has the anti-tumourigenic activity which inhibited the growth of all the five tumour cells by 50%, the IC_{50} at a concentration range of 20-50µg/ml. However, the IC_{50} of CXL Ee ASE fraction has a higher concentration range of 40-95µg/ml. Both have no inhibition effect on P815 tumour cell line.

This anti-tumouricidal cytotoxicity finding offers support to the validity of the traditional medicinal uses of this plant as well as the earlier study on Andrographolide (A), the major active constituent of the extract of *Andrographis paniculata* (Burm. f.) Nees is a potential anti-cancer therapeutic agent.
Figure 9.1. Effect of *Andrographis paniculata* extracts at varying concentrations (1, 3.3, 10, 33.3, 100 µg/ml) on P388 (PAR) tumour cell viability after 48 hours.

Ethanol (1%v/v final concentration) used as a vehicle control (expressed as 100% viability). Data expressed as mean percent viability (relative to vehicle control) ± SEM (n=4) when compared to vehicle control. *Statistically significant P<0.05 when compared to vehicle control.
Figure 9.2. Effect of *Andrographis paniculata* extracts at varying concentrations (1, 3.3, 10, 33.3, 100 µg/ml) on ADR tumour cell viability after 48 hours.

Ethanol (1%v/v final concentration) used as a vehicle control (expressed as 100% viability). Data expressed as mean percent viability (relative to vehicle control) ± SEM (n=4) when compared to vehicle control. *Statistically significant P<0.05 when compared to vehicle control.*
Figure 9.3. Effect of *Andrographis paniculata* extracts at varying concentrations (1, 3.3, 10, 33.3, 100 µg/ml) on WEHI-164 tumour cell viability after 48 hours.

Ethanol (1%v/v final concentration) used as a vehicle control (expressed as 100% viability). Data expressed as mean percent viability (relative to vehicle control) ± SEM (n=4) when compared to vehicle control. *Statistically significant P<0.05 when compared to vehicle control.
Figure 9.4. Effect of *Andrographis paniculata* extracts at varying concentrations (1, 3.3, 10, 33.3, 100 µg/ml) on K562 tumour cell viability after 48 hours.

Ethanol (1% v/v final concentration) used as a vehicle control (expressed as 100% viability). Data expressed as mean percent viability (relative to vehicle control) ± SEM (n=4) when compared to vehicle control. *Statistically significant P<0.05 when compared to vehicle control.
Figure 9.5. Effect of *Andrographis paniculata* extracts at varying concentrations (1, 3.3, 10, 33.3, 100 µg/ml) on Yac-1 tumour cell viability after 48 hours.

Ethanol (1%v/v final concentration) used as a vehicle control (expressed as 100% viability). Data expressed as mean percent viability (relative to vehicle control) ± SEM (n=4) when compared to vehicle control. *Statistically significant P<0.05 when compared to vehicle control.
Figure 9.6. Effect of *Andrographis paniculata* extracts at varying concentrations (1, 3.3, 10, 33.3, 100 µg/ml) on P815 tumour cell viability after 48 hours.

Ethanol (1%v/v final concentration) used as a vehicle control (expressed as 100% viability). Data expressed as mean percent viability (relative to vehicle control) ± SEM (n=4) when compared to vehicle control. *Statistically significant P<0.05 when compared to vehicle control.
9.4 Discussion

The presence of tumouricidal activity was investigated using six types of cancer cell lines. The detection of cytotoxic compounds was made by comparison to the ethanol vehicle control blank. The Andrographolide (Std A) has the most potent antitumourigenic activity inhibited the growth of all the six types of cancer cells by 50% (IC$_{50}$) at a concentration range of 6-10µg/ml for P388 (PAR), ADR, WEHI-164, K562, Yac-1 except for the cell line P815 which showed lesser sensitivity to Std A has a higher IC$_{50}$ value of 100µg/ml. The other extract fractions tested indicated that some fractions inhibited the growth of tumour cells from the six different cancer types with varying degree of potency as shown from Figures 9.1 to 9.6.

The cell line P815 which showed lesser sensitivity to Std A, has a higher IC$_{50}$ value of 100µg/ml. This observation may suggest that P815 being the mastocytoma cell lines of the mouse mast cells is in agreement with the less responsiveness of the rat peritoneal mast cells (RPMC) in the weak anti-allergic effect of Andrographolide against mast cell-derived histamine release in RPMC as demonstrated in Chapter 7 in which both are mouse mast cells. However, further studies are required to elucidate the mechanism of actions of the Andrographolide on the mast cells in order to have a better understanding on this possible explanation.

The finding also demonstrated that the antitumourigenic effect of both the Bru Ee ASE and CXL Ee ASE fractions correlated with the Andrographolide content of these sample fractions as described in section 5.3.4 of Chapter 5. This is in close agreement that Andrographolide displayed significant anti-tumourigenic effect on the tumour cell lines as demonstrated in previous studies.
The cytotoxic activities of Andrographolide and its analogues were investigated and found that
the changes in the structure of the analogues synthesized affect the cytotoxic activities of
Andrographolide (Nanduri et al., 2004). The cytotoxic activities of Andrographolide, Neoandrographolide, Andrographiside, Deoxyandrographiside, and 14-deoxy-12-methoxyandrographolide evaluated by (Tan et al., 2005) appeared to be non-cytotoxic against
the cell lines such as Caov-3 (human ovarian carcinoma cell line), T-47D (human breast
carcinoma cell line), Hs-578T (human breast carcinoma cell line), HepG2 (human
hepatocarcinoma cell line), and NCI-H23 (human lung adenocarcinoma cell line). Of these cell
lines tested using MTS assay after 72 hours of incubation, only 14-deoxyandrographolide and
14-deoxy-11, 12-didehydroandrographolide exhibited cytotoxic activities on T-47D (human
breast carcinoma cell line at concentration of IC\textsubscript{50} values of 2.8µg/ml and 1.5µg/ml respectively.
Among the active constituents examined, it was found that 14-deoxy-11,12-
didehydroandrographolide was the most potent. Our study demonstrated that the
Andrographolide (Std A) has the most potent anti-tumourigenic activity inhibited the growth of
all the five types of cancer cells investigated by 50% (IC\textsubscript{50}) at a concentration range of 6-
10µg/ml.

There are many active components apart from Andrographolide, Andrographiside,
Deoxyandrographolide and Neoandrographolide isolated by the researchers and newer ent-
labdane diterpenoids were isolated by Pramanick et al. and Shen et al., (2005) from the leaves of
Andrographis paniculata (Pramanick et al., 2005; Shen et al., 2005). Rajagopalan et al., (2003)
investigated the effect of Andrographolide in human cancer cell lines based on SRB assay where
the amount of dye bound to the cells after staining gives a measure of cell growth.
Andrographolide treatment resulted in a dose-dependent decrease in the viability of the human
cancer cell lines MDA-MB-453, MCF7, T47D, SW620, COLO205, NCI-H23, MES-SA, MES-
SA-DX5, A431, ES2, PA-1, PC3, A498, CCRF-CEM and NCI-ADR-RES, U251, SF268,
SNB19, HT-29, HCT116, KM12, H522, H226, A549, NCI-H23, HOP62, UACC62, M14,
SKOV3, OVCAR8, DU145 and ACHN (Rajagopal et al., 2003). With more new active labdane
diterpenoids isolated from the *Andrographis paniculata* leaves, it will be most interesting to
research further into the therapeutic potential effect of these newly isolated constituents and their
effects on human cancer and in *vitro* proliferation of different tumour cell lines.

Andrographolide inhibited the growth of many types of human cancer cells by 50% (IC$_{50}$) at a
concentration range of 5-15µM with the exception of COLO205 that showed more sensitivity to
Andrographolide treatment. Results illustrated that Andrographolide inhibits the tumour cells
growth of different cancer cell types with similar potency.

However, Andrographolide’s role as an anti-cancer or anti-tumour and immuno-modulatory
agents in human and its molecular mechanism of action has not been fully elucidated. It was
reported that the possible mechanism of action was by exerting direct anti-cancer activity on
cancer cells by cell-cycle arrest at G0/G1 phase through the induction of cell-cycle inhibitory
protein p27 and decreased expression of cyclin-dependent kinase 4 (CDK4). Immunostimulatory
activity of Andrographolide is evidenced by increased proliferation of lymphocytes and
production of interleukin-2. Andrographolide also enhanced the tumour necrosis factor-α
production and CD marker expression, resulting in increased cytotoxic activity of lymphocytes
against cancer cell lines, which may possibly contribute to its indirect anti-cancer activity. Study
by Rajagopal et al., 2003 demonstrated *in vivo* anti-cancer activity of the compound is further
substantiated against B16F0 melanoma syngenic and HT-29 xenograft models. These results
suggest that Andrographolide is an interesting pharmacophore with anti-cancer and
immunomodulatory activities and therefore has potential for being developed as a cancer
therapeutic agent. Among the three main diterpenoids, Andrographolide exhibited the highest degree of cytotoxicity followed by deoxyandrographolide while neoandrographolide was the least effective. It was suggested that *Andrographis paniculata* leaves and Andrographolide induce cell cycle arrest and affect an intrinsic mitochondria-dependent pathway of apoptosis by regulating the expression of some pro-apoptotic markers in HL-60 cells (Rajagopal *et al*., 2003).

Andrographolide showed selective cytotoxicity to prostate cancer PC-3 cell death. Using immunocytochemistry staining and cellular caspase-3 activity assay, Andrographolide-treated cells exhibited considerable caspase-3 activation and caspase-8 in PC-3 cells at 4 and 2 hours after treatment respectively demonstrating Andrographolide-induced cell death was achieved through the apoptotic pathway, via the activation of an intrinsic caspase cascade (Kim *et al*., 2005).

The in *vitro* and in *vivo* study on the effect of the Andrographolide and its novel semi-synthetic analogue DRF 3188 using FACS and western blot analysis of cell cycle proteins showed that both compounds block the cell cycle at the G0-G1 phase through the induction of the cell cycle inhibitor, p27 and the concomitant decrease in the levels of Cdk4. Results indicated that both the compounds exhibited anti-tumour activities by a similar mechanism of action (Satvanaravana *et al*., 2004). Kumar *et al*., (2004) showed that the anti-tumour and immunomodulatory activity of the dichloromethane (DCM) fraction of the methanolic extract of *Andrographis paniculata* exhibited the highest potency, contributing for both their anti-cancer and immunostimulatory activities. Dichloromethane fraction significantly inhibits the proliferation of HT-29 (colon cancer) cells and augments the proliferation human peripheral blood lymphocytes (HPBLs) at low concentrations. Further fractionation of this DCM resulting in three diterpenes such as Andrographolide, 14-deoxyandrographolide and 14-deoxy-11, 12-didehydroandrographolide
were found to exhibit anti-cancer activity on diverse cancer cells of different human cancers. All showed enhanced proliferation and interleukin-2 (IL-2) induction in HPBLs (Kumar et al., 2004).

The effects of LianBiZhi (LBZ) injection containing Andrographolide on macrophage phagocytotic function and natural killer cells cytotoxicity were examined by Peng et al., (2002). The results demonstrated that LBZ injection could promote IFN-alpha, IFN-gamma, TNF-alpha inductions of PBMCs, but had no effect on IL-8. LBZ injection could not only enhanced the phagocytosis activity of peritoneal macrophage from guinea pig to phagocytosis cock erythrocyte, but also augment the cytotoxicity mediated by natural killer cells from PBMCs to damage the K562 cell lines suggesting that Andrographolide, being an immunostimulant agent can modulate both antigen specific and nonspecific immune function by means of its natural killer cells and macrophage and cytokines induction (Peng et al., 2002).

Singh et al., (2001) investigated the effects of 80% hydroalcohol extract of Andrographis paniculata and butylated hydroxyanisole (BHA) at 50 and 100 mg/kg body weight/day content, lactate dehydrogenase (LDH) and lipid peroxidation in the liver of 6-8 weeks old Swiss albino mice as well as in the other organs such as lung, kidney and stomach. The finding indicated that Andrographis paniculata has the chemopreventive potential against chemotoxicity including carcinogenicity (Singh et al., 2001).

In conclusion, the anti-tumour activity of the crude extracts of Andrographis paniculata was determined by use of the six cancer cell types P388, ADR, K562, WEHI-164, Yac-1 and P815 tumour cells. All compounds displayed a concentration dependent effect on the tumour cell viability demonstrated the tumouricidal effect of the diterpenes with the most effective and
cytotoxic fraction at a range of 6-10µg/ml being the Std A. Studies by (Cheung et al., 2005; Kumar et al., 2004; Rajagopal et al., 2003) demonstrated that Andrographolide, the major active diterpenoid of *Andrographis paniculata* leaves exhibited specific tumouricidal activities on many cancer cell types.

In this study, only five tumour cells viability were inhibited by the extract fractions with the exception of P815 which is less sensitive to Std A (the most potent cytotoxic fraction). This may suggest that Andrographolide (Std A) did not exhibit any tumouricidal activity on the growth of P815 tumour cells possibly due to the different pathway of mechanism of action of the other five tumour cells.

Our findings offer support to the validity and in agreement with the traditional medicinal uses of this plant as well as the earlier studies on Andrographolide (A), the major constituent of the extract of *Andrographis paniculata* (Burm. f.) Nees is a potential cancer therapeutic agent (Cheung et al., 2005; Kumar et al., 2004; Rajagopal et al., 2003).

Further studies are clearly needed to elucidate the anti-tumouricidal effects of Andrographolide (Std A), Dehydroandrographolide (Std D) as well as the crude plant extracts of *Andrographis paniculata* leaves which was reported earlier to contain three active components diterpene lactones Andrographolide as the major compound, Deoxandrographolide and Neoandrographolide. However, the most recent studies by Pramanick et al. (2005) and Shen et al. (2005) has isolated newer *ent*-labdane type diterpenoids from the leaves of *Andrographis paniculata* (Pramanick et al., 2005; Shen et al., 2005) which are now being investigated for their various pharmacological actions and bioactivities of these newly found constituents which may be present in small amounts but active and potent in nature.
Chapter 10  Hepatocyte toxicity of *Andrographis paniculata* (*Burm. f.*) Nees

-A Medicinal Plant of Brunei Darussalam

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10.1 Introduction

10.1.1 General cytotoxicity on rat hepatocytes

Cytotoxic drugs are used in the chemotherapies of cancer which interfere with cell proliferation during the process of cell division. Unfortunately, such drug action also affects the normal dividing cells, giving rise to untoward effects and complications such as immunosuppression and excessive hair loss (Tannock & Hill, 1998). Therefore, anti-tumour agent with specific toxicity targeting on tumour cells and at the same time without affecting the normal cells will become the ideal drug of choice in cancer therapies.

Many compounds from natural sources are constantly being screened for cytotoxic activity against tumour cell lines and normal cells, with the interest to differentiate between the specificity and non specific tumour activites. The general cytotoxicity of the extract fractions on rat hepatocytes is discussed in this chapter on rat hepatocyte toxicity screening assay.

Freshly isolated hepatocytes have high metabolic activity that make the intact liver a common target of toxicity. In vitro study on hepatocytes are usually used to determined whether anti-tumour activity is specific or whether an extract is just generally toxic to most cell types.
10.2 Materials and Methods

10.2.1 Plant materials

The extract fractions of the *Andrographis paniculata* were previously described in Section 5.3.2.3.2 and 5.3.2.3.3 of the Chapter 5.

10.2.2 Biological materials

All biological materials were provided by the Key Centre for Toxicity, RMIT.

RPMI-1640 was obtained from Sigma Chemical Company (St Louis, MO., USA).

Freshly isolated rat hepatocytes (hepatocytes cells were provided by Dr. Simone Yendle, the Key Centre for Toxicity, RMIT).

10.2.3 General cytotoxicity assay

10.2.3.1 Rat Hepatocyte Toxicity

The evaluation of the general cytotoxicity of extracts against isolated rat hepatocytes HepG2 was carried out by the addition of blue dye of resazurin (7-Hydroxy-3H-phenoazin-3-one-10-oxide) sodium salt solution 0.01mg/ml in each well for the detection of cell viability. Extract fractions dissolved in 100% ethanol were serially diluted into final concentrations of 1, 3.3, 10, 33.3, 100µg/ml in RPMI-1640 media containing 2mM glutamine, 10% heat inactivated foetal calf
serum (CSL, Parkville, Australia), 10mM 2-mercaptoethanol and 100µg/ml gentamicin sulfate) in a 96-well flat bottom cell culture sterile microtitre plate. Extract blank, ethanol solvent control and cell free wells were plated. Freshly isolated rat hepatocytes HepG2 (1 x 10^5 cells/ml, provided by Associate Professor Paul Wright and Simone Yendle PhD, the Key Centre for Toxicity, RMIT) were added and the plate incubated for 48 hours at 37°C, 5% CO₂. Cell viability was assessed by the addition of blue dye of the resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide) C₁₂H₈NNaO₄ (Sigma, USA) sodium salt solution 0.01mg/mL in each well, which is converted to pink in metabolically active cells and fluoresce. 6% w/v SDS (Sodium Dodecyl Sulfate) were added in final concentrations of 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml in four wells of extract free as a positive control and acts as the inhibitor of cancer cell lines. The fluorescence of the reaction product was measured using resazurin assay protocol at emission wavelength of 560nm and excitation wavelength of 590nm after 48 hours incubation (37°C, 5% CO₂) using the 96 wells fluorescence flat, pico green black plates.

10.2.4 Data collection and statistical analysis

Statistical Analysis was performed using Graph Pad Prism Version 4.0 for Windows (San Diego, CA, USA). Data was analysed by One Way Analysis of Variance (ANOVA) followed by Bonferroni Multiple Comparison Test for rat hepatocyte toxicity. Results are mean ± Standard Error of Mean (SEM), (*P<0.05, n=4).

10.3 Results

The general cytotoxicity of the extract fractions were evaluated by assessing the viability of the rat hepatocytes in the presence of two reference standards namely Andrographolide (Std A),
Dehydroandrographolide (Std D) and seven other *Andrographis paniculata* crude extract fractions. Results showed that significant toxicity was observed in all fractions but at varying degrees of cytotoxicity potency of the tested extract concentration at 100µg/ml (Figure 10.1). All the extract fractions displayed a concentration dependent effect on cell viability with the most cytotoxic of all the fraction extracts was Andrographolide (Std A) which has half effective dose IC$_{50}$ value of 60µg/ml at 50% inhibition of rat hepatocyte viability (Table 10.2). At the concentration of 100µg/ml, the reference Std A, reducing rat hepatocyte viability by 68.78% ± 0.84%. At the concentrations tested indicated that all the fractions are moderately and relatively non cytotoxic.

Our results also demonstrated that the Brunei AP Ee ASE extract fraction at 100µg/ml reducing rat hepatocyte viability by 46.62% ± 4.95%. CXL Ee ASE extract fraction at 100µg/ml reducing rat hepatocyte viability by 11% ± 2.41% as illustrated in Table 10.1. Some extract fractions such as Std D (F2), Brunei AP Ee STIR (F4), CXL Ee ASE (F5), Brunei AP We ASE (F7), CXL We ASE (F8), and CRS Ee STIR (F9) exhibited inhibition on the percentage growth of the rat hepatocytes with varying degree of potency. Such finding demonstrated that for those fractions containing much lesser content of Andrographolide present in the extract fractions were relatively non-cytotoxic.

This finding suggests that the *Andrographis paniculata* (Burm. f.) Nees from Brunei Darussalam may contain other active constituents apart from Andrographolide that may account for its moderate cytotoxicity. This requires further elucidation on the study of *Andrographis paniculata* source from countries with different climatic conditions and other factors that may affect the level of contents of constituents in the herb (Ngan *et al.*, 1999). It has also been reported that geographical locations and seasons may explain the differences in the contents of the active
components in the plants (Hu & Wu, 1995). However, further studies are required to elucidate the different levels of contents and composition of constituents in *Andrographis paniculata* (Burm. f.) Nees obtained from other geographical locations in order to have a better understanding on this possible explanation.

Table 10.1  % reducing of rat hepatocyte viability at extract concentration of 100µg/ml

<table>
<thead>
<tr>
<th>Crude Extract Fractions of <em>Andrographis paniculata</em> at 100µg/ml</th>
<th>MEAN</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 Std A</td>
<td>68.78</td>
<td>0.84%</td>
</tr>
<tr>
<td>F2 Std D</td>
<td>14</td>
<td>3.38%</td>
</tr>
<tr>
<td>F3 Brunei AP Ee ASE</td>
<td>46.62</td>
<td>4.95%</td>
</tr>
<tr>
<td>F4 Brunei AP Ee STIR</td>
<td>17</td>
<td>3.54%</td>
</tr>
<tr>
<td>F5 CXL Ee ASE</td>
<td>11</td>
<td>2.41%</td>
</tr>
<tr>
<td>F6 CXL Ee STIR</td>
<td>16</td>
<td>2.58%</td>
</tr>
<tr>
<td>F7 Brunei AP We ASE</td>
<td>2.40</td>
<td>0.40%</td>
</tr>
<tr>
<td>F8 CXL We ASE</td>
<td>1.54</td>
<td>0.31%</td>
</tr>
<tr>
<td>F9 CRS Ee STIR</td>
<td>1.69</td>
<td>0.40%</td>
</tr>
</tbody>
</table>
Figure 10.1. Effect of *Andrographis paniculata* extracts at varying concentrations (1, 3.3, 10, 33.3, 100 µg/ml) on rat hepatocyte viability after 48 hours.

Ethanol (1%v/v final concentration) used as a vehicle control (expressed as 100% viability). Data expressed as mean percent viability (relative to vehicle control) ± SEM (n=4). Data was analysed by One Way Analysis of Variance (ANOVA) followed by Bonferroni Multiple Comparison Test for rat hepatocyte toxicity.
*Statistically significant P<0.05 when compared to vehicle control.

**Table 10.2**  Effect of nine *Andrographis paniculata* fractions (1, 10, 100µg/ml) on rat hepatocytes viability.

<table>
<thead>
<tr>
<th>Extract Fraction</th>
<th>IC50 Value (µg/ml)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 Std A</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>F2 Std D</td>
<td>#No effect</td>
<td></td>
</tr>
<tr>
<td>F3 Brunei AP Ee ASE</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>F4 Brunei AP Ee STIR</td>
<td>#No effect</td>
<td></td>
</tr>
<tr>
<td>F5 CXL Ee ASE</td>
<td>#No effect</td>
<td></td>
</tr>
<tr>
<td>F6 CXL Ee STIR</td>
<td>#No effect</td>
<td></td>
</tr>
<tr>
<td>F7 Brunei We ASE</td>
<td>#No effect</td>
<td></td>
</tr>
<tr>
<td>F8 CXL We ASE</td>
<td>#No effect</td>
<td></td>
</tr>
<tr>
<td>F9 CRS Ee STIR</td>
<td>#No effect</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SEM (n=4) expressed as a percentage of control viability.
# denotes that the other extract fractions showed no cytotoxicity towards the rat hepatocytes at the concentrations tested.

### 10.4 Discussion

General cytotoxicity of extracts was evaluated by assessing the viability of rat hepatocytes in the presence of Andrographolide, Dehydroandrographolide and the *Andrographis paniculata* extracts. The main findings of the present study showed that significant toxicity was only observed for the reference Std A at 100µg/ml. The most cytotoxic of all the tested fractions was reference Std A, reducing rat hepatocyte viability by 68.78% ± 0.84%. The other extract fractions were all relative non-cytotoxic. Solubility issues may also have affected the results of this assay, as the *Andrographis paniculata* extracts may contain substituents that were difficult to solubilise completely in the vehicle control.

Study by Kapil *et al.*, (1993) investigated the anti-hepatotoxic effects of major diterpenoids of *Andrographis paniculata* in hepatotoxicity induced in mice by carbon tetrachloride or tert-butylhydroperoxide (tBHP) intoxication. The mice were pretreated with Andrographolide, Andrographiside and Neoandrographolide at 100 mg/kg, i.p. for 3 consecutive days with significant reduction in malondialdehyde formation, reduced glutathione (GSH) depletion and enzymatic leakage of glutamic-pyruvate transaminase (GPT) and alkaline phosphatase in either pretreated groups using the known hepatoprotective agent Silymarin as the positive control. Results showed that Andrographolide exhibited a lower protective potential than Andrographiside and Neoandrographolide, which the latter two were as effective as the control with respect to their effects on the formation of the degradation products of lipid peroxidation and release of GPT and alkaline phosphatase in the serum. Neoandrographolide restored the GSH status to normal. Study suggested that the greater protective antihepatotoxic effects of
Andrographiside and Neoandrographolide could be due to their glucoside groups which may act as strong and potent anti-oxidants (Kapil et al., 1993).

The hepatoprotective effect of Andrographolide was studied by Handa and Sharma (1990) on acute hepatitis induced in rats by single dose of galactosamine (800 mg/kg, i.p.) or paracetamol (3g/kg, po). The hepatoprotective activity was monitored by estimating the serum transaminases (GOT and GPT), alkaline phosphatase and bilirubin in serum, hepatic triglycerides, and by histopathological changes in the livers of experimental rats. The results confirmed that the in vivo hepatoprotective effect of Andrographolide against galactosamine or paracetamol-induced hepatotoxicity were different in their primary mechanism of inducing hepatotoxicity which suggested that the protective mechanisms of Andrographolide which were non-specific in both intoxications may be responsible for the hepatoprotective activity of this major diterpenoid of Andrographis paniculata (Handa & Sharma, 1990a). In conjunction with this study, Andrographolide was found to normalize completely the Carbontetrachloride (CCl₄)-induced increase in the pentobarbitone induced sleeping time of mice. In the previous earlier study, Choudhury and Poddar (1984) demonstrated that the kalmegh, the Andrographis paniculata leaf extracts had more protective action on Carbontetrachloride (CCl₄)-induced hepatic toxicity than its bitter principle Andrographolide (Choudhury & Poddar, 1984).

Freshly isolated hepatocytes have high metabolic activity that make the intact liver a common target of toxicity. In vitro study on hepatocytes are usually used to determined whether anti-tumour activity is specific or whether an extract is just generally toxic to most cell types.

The most cytotoxic of all the Andrographis paniculata extract fractions was Std A which has IC₅₀ value of 60µg/ml at 50% inhibition of rat hepatocyte viability. At the concentration of Std A at 100µg/ml which reduced the rat hepatocyte viability by 68.78% ± 0.84%. In this study, all the
other fraction extracts and Std A did not exhibit any significant cytotoxicity at extract concentration less than 100µg/ml indicating that they are only moderate or relative non cytotoxic to hepatocytes. This is in agreement with the previous studies by Kumar et al., (2004) in the in vivo results from hollow fiber assay conducted in immunocompetent Swiss albino mice, demonstrated that Andrographolide significantly inhibited the cancer cell proliferation without showing any signs of cytotoxicity in healthy normal mice treated with high doses of Andrographolide (Kumar et al., 2004). The findings demonstrated that Andrographolide displayed the most potent tumouricidal activity, yet was less cytotoxic in the rat hepatocyte assay, which may indicate that this extract potentially has specific anti-tumour activity.

This finding offers support to the validity of the traditional medicinal uses of this plant as well as the earlier study on Andrographolide (A), the major active constituent of the extract of Andrographis paniculata (Burm. f.) Nees is non-cytotoxic and hepatoprotective (Handa & Sharma, 1990a).
Chapter 11  General discussion and future directions .................................286

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11.1 Introduction

The main objective of this thesis was to develop the strategic framework on TM/CAM for Brunei Darussalam in tandem with the global developments on TM/CAM. In conjunction with this objective, an interdisciplinary approach, as a case study, was also undertaken to investigate the genetic diversity, chemical profile, bioactivities and pharmacological actions of *Andrographis paniculata* (Burm. f.) Nees (HDM 15) from Brunei Darussalam involving different techniques in molecular biology, biochemistry and pharmacology for the systematic study of the tropical medicinal native plants of Brunei Darussalam. To the best of our knowledge, this is the first study on the *Andrographis paniculata* (Burm. f.) Nees (HDM 15) of Brunei Darussalam conducted.

There have been several challenges for the study. Firstly, although there is a strong need from Brunei Darussalam government in promoting the safe use of traditional medicine, there has been little research done in terms of strategy and policy development in this area in Brunei Darussalam. Given the significant use of traditional medicine in the general population in this country, it is important to develop a framework on traditional medicine. After an extensive review of the developments of traditional medicine in other countries, regions or organizations, a framework has been proposed. The main feature of the framework is to encourage the formalisation of TM/CAM with the aim of achieving integrated health care systems and promoting the quality, safety and efficacy of TM/CAM interventions through the interdisciplinary approach in the research and development. In line with the concerted efforts worldwide are pivotal in supporting the WHO’s global strategy for TM/CAM. With such a strategy framework in place will benefit Brunei Darussalam in the actualisation of TM/CAM.
practices as well as recognising the importance of the natural reserves of medicinal native plants available in the country, widely used by the local communities.

The second challenge is on the study of tropical medicinal native plants of Brunei Darussalam with the need for new drug development in the treatment of certain disease conditions which have also been highlighted. Natural products have always been a potential source of new therapeutic agents in which the desire for the development of novel drugs warrant continued investigation. Given the complex nature of the research required for studying the medicinal native plants of Brunei Darussalam, a multi-disciplinary approach was taken by using various techniques to document the blueprint for *Andrographis paniculata* (Burm. f.) Nees (HDM 15), a native medicinal plant grown in Brunei Darussalam. The techniques in this study have been shown to be useful and suitable for the phytochemical and biological profile of the activities of the crude plant extracts and their constituents. The laboratory investigations revealed that *Andrographis paniculata* from Brunei Darussalam exhibited a distinct genetic profile from the *Andrographis paniculata* of China. It has a wide range of anti-oxidant properties, pharmacological actions including anti-inflammatory and anti-tumour effects with relatively non-cytotoxicity on rat hepatocytes. Some of the pharmacological actions of *Andrographis paniculata* correlated with the active constituents Andrographolide (A) and Dehydroandrographolide (D). The findings have provided the first experimental evidence for tropical medicinal native plants of Brunei Darussalam, and also set up examples for studying other medicinal native plants in Brunei Darussalam. Given the rich native medicinal plant resources in Brunei Darussalam and the potential of understanding the pharmacological actions of native plants are of great significance in terms of health care and economic development of the region. Further work is clearly needed to the elucidation of potential targets of the active
components of the plants which can affect the ultimate outcomes on the quality, safety and efficacy of the plant.

11.2 The studies on Andrographis paniculata (Burm. f.) Nees (HDM 15)
– A Medicinal Native Plant of Brunei Darussalam

This study was initiated using RAPD and PCR-RFLP of 5S-rRNA region of the phylogenetic diversity of nine accessions samples collected from the three districts namely Bandar Seri Begawan from the Brunei/Muara district (AP2, AP4, AP6, AP8, AP11, AP14, AP15), Kuala Belait district (K.B.), Temburong district (T) and Chuan Xin Lian (CXL) from China. The genetic comparison as demonstrated for Andrographis paniculata from Brunei Darussalam constituted as the first report on genetic diversity of related medicinal plants in Brunei Darussalam on the basis of RAPD analysis. The data obtained in this study reflected the utility of RAPD and PCR-RFLP in the analysis of genetic variability distribution within Andrographis paniculata. The finding of the close genetic distances between all the nine accessions from Brunei Darussalam and Chuan Xin Lian (CXL) are in agreement with the other studies on lotus (Campose et al., 1994), sweet potato (Cannoly et al., 1994), and Andrographis paniculata of India (Padmesh et al., 1999) which suggests that RAPD is more appropriate for the analysis of genetic diversity in closely related genotypes. This study provides a better understanding of genetic variation at the intraspecific level would help in identifying superior genotype(s) for cultivar development. The polymorphism obtained can be used as a marker for screening the genotypes. The genomic DNA fingerprinting by RAPD among fresh accessions from Andrographis species showed distinctive DNA fragments which may be used for the authentication, identification and quality assessment of the plant species or varieties.
The chromatographic analysis of *Andrographis paniculata* extract fractions using HPTLC and HPLC procedures (Jain et al., 2000) was intended to elucidate the levels of Andrographolide and Dehydroandrographolide contents in the *Andrographis paniculata* samples obtained from different districts of Brunei Darussalam and compared to the China samples. Difference in the contents of A and D in these samples were observed. The content of A (%w/w) in Temburong and Brunei AP samples were higher than those from CXL, K.B. and Chinese Reference samples. On the contrary, the content of D (%w/w) in Temburong and Brunei AP samples were lower than that in CXL, K.B. and Chinese Reference samples. When combining A and D contents, the ranking order were Temburong, CXL, Brunei AP, K.B. and Chinese Reference.

Given that the different climatic, growing, storage conditions, freshness of the of herbal samples and the different post-harvesting processing may affect the contents of chemical components in the herbs (Hu & Wu, 1995; Ngan et al., 1999; Zhou, 1987), this may suggest why the content of A and D levels in *Andrographis paniculata* extracts are different from different region. Interestingly, the *Andrographis paniculata* extracts from different region for instance the Brunei samples were found to be higher in the Andrographolide content and lower in the Dehydroandrographolide may suggest that since the plant thrives best in the tropics and subtropical areas indicating that the climatic conditions may influence the levels of contents of this evergreen plant (Padmesh et al., 1999; Sabu, 2002).

From the pharmacological and biological perspectives, the *Andrographis paniculata* which has been assessed and evaluated for its anti-oxidant, anti-tumour and anti-hepatotoxicity properties, as well as its pharmacological actions such as the effects against compound 48/80-induced histamine release in rat peritoneal mast cells, and iNOS mediated smooth muscle relaxations in rat aorta tissues and their bioactivities look promising. Study showed that the aqueous (We),
ethanol (Ee) and methanol (Me) extracts of *Andrographis paniculata* (*Burm. f.*) Nees of Brunei Darussalam exhibited some significant scavenging activities towards 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical, hydrogen peroxide and nitric oxide in the cell-free systems with the exception of the aqueous extract which did not show any significant DPPH scavenging activity. Although it is not clear if *Andrographis paniculata* acts effectively *in vivo* as anti-allergies or anti-histaminic therapeutic agents since as it requires a relative higher dose (1mg/ml) for such effects *in vitro* as demonstrated in this study. The finding that the rat peritoneal mast cells is less responsive towards *Andrographis paniculata* extracts in this study is in close agreement with the previous study by (Gupta et al., 1998).

The most significant finding is the demonstration that *Andrographis paniculata* possesses beneficial effects with Andrographolide possesses the most potent anti-tumourigenic activity and relatively non-cytotoxic to rat hepatocytes which is in agreement with the previous studies (Handa & Sharma, 1990a; Kapil et al., 1993; Rajagopal et al., 2003). Extract fractions studies have shown that this is due to the presence of Andrographolide which has varying potential pharmacological effects (Chiou et al., 2000).

*Andrographis paniculata* leaves have been reported to contain three active components diterpene lactones Andrographolide as the major compound, Deoxandrographolide and Neoandrographolide. Recent studies by Pramanick et al. (2005) and Shen et al. (2005) has also isolated new *ent*-labdane type diterpenoids from the leaves of *Andrographis paniculata* (Pramanick *et al.*, 2005; Shen *et al.*, 2005) which are still undergoing various bioactivities testings. These active constituents may also be responsible for the pharmacological actions and bioactivities of *Andrographis paniculata* leaves.
The finding also suggests that *Andrographis paniculata* leaves may contain other active constituents other than Andrographolide with varying levels of %w/w content which may account for its varying potency of the anti-tumourigenic and cytotoxic activities. Preliminary results indicated that further anti-tumour and cytotoxicity tests on *Andrographis paniculata* is warranted which is required to be elucidated in the future studies to ensure the absolute safety of the leaves for long term consumption by the population.

This finding offers support to the validity of the traditional medicinal uses of this plant as well as the earlier studies on Andrographolide (A), the major active constituent of the extract of *Andrographis paniculata (Burm. f.) Nees* is a potential anti-cancer therapeutic agent (Rajagopal *et al.*, 2003).

This study also provides a better understanding of genetic variation at the intraspecific level would help us in identifying the superior genotype(s) for cultivar development such as the higher level of active Andrographolide and lower level of the less active Dehydroandrographolide contents found in the *Andrographis paniculata* from Brunei Darussalam in particularly, the Temburong sample. It would be interesting to carry out future anti-tumour assay on this particular extract fraction. The isolation and chemical fingerprinting of this plant has not yet been achieved in our study, but it is hope that this will be pursued further in the near future.
11.3 Limitations of this thesis

The limitations of this study which needs to be considered is that there are other chemical ingredients such as diterpene lactones active constituents other than Andrographolide such as Deoxyandrographolide and Neo-andrographolide and the recent studies by Pramanick et al., (2005) and Shen et al., (2005) isolated newer ent-labdane type diterpenoids from the leaves of *Andrographis paniculata* (Pramanick et al., 2005; Shen et al., 2005) which are also key players in the implication of the clinical benefits of *Andrographis paniculata* in total. It is also envisioned that *Andrographis paniculata* (Burm. f.) Nees will be the future potential anti-cancer pharmaceutical drug and ongoing studies are conducted to research into the other potent constituents and their pharmacological actions and bioactivities of this important medicinal plant.

It has been demonstrated that Dehydroandrographolide is much lesser in potency than the rest of the active components when compared to Andrographolide as shown by previous studies. It is to be bear in mind that in most instances, *Andrographis paniculata* leaves are usually taken as a whole rather than as active constituents alone. The oral administration of the raw herbal leaves or powder by the communities at large in the dosage amount which may be much greater than the tested concentrations within this study may also play an important role in the clinical effects. It would be interesting to conduct further studies on the different doses of *Andrographis paniculata* taken by the community. The results of this future project may contribute to further understanding the mechanism of herbal remedies in totality.

Although this project investigated only the individual dried plants of Andrographis species, some of the folklore herbal medicine may consist of brews which are mixtures of fresh plants directly source from the fields or gardens in their own countries or imported. *Andrographis paniculata* is
widely used by the local communities to treat a range of diseases such as hypertension and diabetes (Ahmad, 2004). The *Andrographis paniculata* leaves are usually consumed steeped in hot water for drinking. These mixtures may contain some chemical reactions by heat on boiling or brewing and the effect can be actually quite different from a single compound isolated from a dried plant or from its crude extract. There is also some evidence that customized TM/CAM might be more effective than standardized preparations (Bensoussan *et al.*, 1998). The fact that what the public actually consume might not be the exact components that are investigated in the laboratory. Therefore, from the consumers’ perspective, totality plays a major part of the TM/CAM system of therapies. Further studies are needed to elucidate the actual mechanisms of actions of *Andrographis paniculata* on the selective analyses of the laboratory interventions conducted in this project.

### 11.4 Future directions

*Andrographis paniculata* (*Burm. f.*) *Nees* grown in Brunei Darussalam is the first plant species being research at the University of the Royal Melbourne Institute of Technology. There is clearly a potential for using this approach to study other medicinal plants or natural products. There is also a clear need for the utilization of the research techniques employed and applies on other medicinal native plant species of Brunei Darussalam for the screening and hopefully resultant in the development of potential source of novel therapeutics.

Future studies to be undertaken include understanding the species distribution, habitat, phenology of flowering and fruiting, cytology, breeding system and seed dormancy/germination of the species. To conduct further research on the quantification of the amount of intra and inter-specificity genetic variability of *Andrographis paniculata* and its patterns of population
differentiation throughout the districts of Brunei Darussalam will be useful to gather more valuable scientific information on the profile of *Andrographis paniculata*. The significance of inbreeding and gene flow to the level and pattern of diversity in *Andrographis paniculata* need to be assessed as well as to test the hypothesis that genetic distance is considerably correlated with geographical distance and most importantly to identify superior genotype(s) with increased biomass and active component products i.e. Andrographolide synthesis.

There is also a need to study whether the external factors such as seasons and climatic changes (temperate versus tropical), geographical location, soil types, agricultural conditions, and various other factors that may affect the composition of the secondary metabolites of *Andrographis paniculata* and their relative pharmacological effects and bioactivities.

It would also be beneficial to conduct further studies on the *Andrographis paniculata* from other tropical and temperate regions and to assess their similarities and differences which may shed lights on the contents of its active ingredients and their clinical importance.

Further studies on pharmacological actions of *Andrographis paniculata* in particular its anti-tumour activities would be interesting, including the study on active ingredients of specific samples from Temburong region. Comparison studies on *Andrographis paniculata* and other medicinal plants with potential anti-cancer activity based on the similar anti-tumour assay should be encouraged.

The current and additional research data will certainly expand our existing knowledge into the Brunei Darussalam medicinal native plants and promote an update of monographs on the local medicinal native plants of Brunei Darussalam as compiled and published by the Ministry of
Industry and Primary Resources, Brunei Darussalam to serve as the niche for future research and development of these important medicinal native plants. Fortunately in Brunei Darussalam, under the National Forestry Policy, this tropical rain forest is classified as virgin and primary forest which are designated as protected forest reserves and conservation forest for natural habitats, wildlife sanctuaries, and also for specific area of scientific research and development of potential therapeutic drugs. The availability of natural ingredients for herbal medicines is therefore abundant in the country.

Finally, further research on the the potential tropical medicinal native plants in Brunei Darussalam will not only promote the development of the region but also may lead to the discovery of new therapeutic agents to benefit the general population in the world. In this regard, provisions on the future financial support for related projects are important to promote the evidence-based practice of traditional medicine in Brunei Darussalam.
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WHO. (2003). *WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants.*


## Appendices

### Appendix I  Milestones of *Andrographis paniculata* (*Burm. f.*) *Nees* research areas

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Appendix II  Chemicals and chemical suppliers

APS Ajax Finechem, 9 Short Street, Auburn, NSW, Australia

Calcium Chloride Dihydrate Ca Cl₂. 2H₂O, MW 147.02 (Analytical reagent)
Magnesium Sulphate Hydrated Mg SO₄.7H₂O, MW 246.67 (Analytical reagent)
Potassium Chloride KCl, MW 74.55 (Analytical reagent)
Sodium Hydrogen Carbonate NaHCO₃, MW 84.01, Assay 99.7, 100.3%
NaH₂PO₄ 0.41, MW 156.01

BDH AnalAr Merck Pty Limited Chemicals (Australia), Kilsyth, VIC 313, Australia

Ascorbic acid

1, 1-Diphenyl-2-picrylhydrazyl (DPPH)

Disodium ethylenediamine-tetra acetic acid [CH₂N(CH₂ COOH). CH₂COONa]₂ H₂O, EDTA,
Deoxyribose

Ethanol, C₂H₅OH, absolute, BDHA, 99.7-100% v/v

ETHYLENEDIAMINETETRAACETIC ACID EDTA

Horseradish peroxidase

Hydrogen peroxide

Iron (III) chloride

Methanol CH₃OH, absolute, 100% v/v, Density 0.79g/ml

Methanol CH₃OH, Hipersolv for HPLC

Potassium dihydrogen orthophosphate KH₂PO₄, MW 136.09

Potassium hydroxide KOH

Sodium hydroxide NaOH

Thiobarbituric acid TBA

Thiochloroacetic acid TCA
BDL

Heparin sodium injection

Fluka Chemie GmbH, ch-9471 Buchs, Switzerland

Di-sodium hydrogen phosphate dihydrate puriss.p.a.for HPLC, HNa$_2$P.2H$_2$O, MW 177.99, Assay 99% (T)

1-octanesulfonic acid sodium salt monohydrate, C$_8$H$_{17}$NaO$_3$.H$_2$O, MW 234.29

ICN Biomedicals Inc 1263 South Chillicothe Road, Aurora, Ohio.

Sodium dodecyl sulphate Ultra pure, MW 288.38

Sigma-Aldrich Chemical Co., St. Louis, MO, USA

Acetylcholine

Boric acid, H$_3$BO$_3$, MW 61.83

Bovine serum albumin (BSA)

Compound 48/80

D-(+)-Glucose (Dextrose, Corn Sugar Anhydrous) C$_6$H$_{12}$O$_6$, MW 180.2, > 99.5% (HPLC)

Glucose 1%, MW 180.16

Histamine injection

L-Arginine Hydrochloride C$_6$H$_{14}$N$_4$O$_2$.HCl, Sigma Ultra >99% (TLC)

Lipopolysaccharide Escherichia Coli Serotype 0127: B8, L-3129,

L-NAME

L-(+)-Tartaric acid, C$_4$H$_6$O$_6$, MW 150.09

Lyophilized powder prepared by phenol extraction

Magnesium Chloride Dihydrate, MW 95.21

N-2-hydroxyethylpiperazine-N’-ethanesulfonic acid (HEPES 10), MW 238.31

O-Phthalaldehyde (OPA)

Phenylephrine
Sodium Chloride NaCl, MW 58.44, 99.5%
Sodium Hydroxide NaOH, MW 40.00, 98%
Spermidine trihydrochloride,
Sodium tartrate dibasic dihydrate minimum 99.0% acid C₄H₄Na₂O₆. 2H₂O, MW 230.08
Tartaric acid, MW 150.1

**Invitrogen Corporation, Gibco, Auckland, NZ**
Trypan Blue Stain 0.4%

**Crown Science**
Whatman Filter paper No. 1, 150mm, Qualitative Circles, 150mm Dia Cat No 1001 150.

**Dionex Pty Ltd, Lanecove, 1595, NSW**
Dionex ASE Filter paper Part No. 056780 Grade D28, Size 30mm

**Chemsal Pty Limited, Lab Chem Class 6.1, Australia**
Celite (A Diatomite Product)

**E. Merck, Darmstadt, Germany**
HPTLC Aluminium sheets, silica gel, F₂₅₄, precoated
Kieselgel 60 (silica gel 60), particle size 0.040-0.063, 230-400 mesh ASTM

**Monash Animal Centre, Monash University, Clayton, VIC, Australia**
Spargue-Dawley rats, male, out bred

**Qiagen Pty Ltd, Clifton Hill, Victoria, Australia**
DNeasy Plant Mini Kit (50)
DNeasy Plant Maxi Kit (250)

**Reagents for PCR amplification**
PCR buffer (Tris-HCl, KCl, gelatin)

**Invitrogen, Pty Ltd, Australia**
Taq DNA Polymerase Recombinant
Taq Polymerase Recombinant (500 units)
Taq Polymerase Recombinant (250 units)
MgCl$_2$
100 mM dNTP Set (4x25µmol)
100 mM dTTP
100 Mm dGTP
100 Mm dATP
100 Mm dCTP
deoxynucleotide triphosphate mixture (dNTPs),
10-mer primers (Operon, Technologies, USA),
1.5% Agarose gel for electrophoresis
Loading Dye
Marker/Ladder
Polaroid 667 film
Appendix III Instrumentation and equipment

Alltech Associate, Australia Brookhollow Ave Baulkham Hills, NSW 2153

Alpha-bond C₁₈ Column (300x3.9mm, I.D. 10 µm) Part No. 7701

Beckman Coulter Inc., Fullerton, CA, USA

Beckman J2-21 M/E High Speed Centrifuge

Biofuge Heraeus Centrifuge in cold room.

Buchi Laboratortechnik AG, Flawil, Switzerland

Buchi RE 011 Rotavapor

Buchi Rotavapor, Brinkman company, Westbury, NY, USA

Brinkman Instruments Inc., Westbury, NY, USA

CAMAG Sonnenmattstr.11.CH-4132 Muttenz (Switzerland)

CAMAG Linomat 5

CAMAG Nanomat and Capillary Dispenser

CAMAG Horizontal Developing Chamber 2

Dionex Pty Ltd, Lanecove, 1595, NSW

Dionex ASE (Accelerated Solvent Extraction) 100 Extraction System

Dionex Corporation Filter sizes 25mm, 30mm, P/N 056781, Rev.1, GF/B Filter

Dupont Supa Refrigerants, Asheville North Carolina USA

Legaci Refrigeration System, Revco Freezer -80°C, Quantum Scientific

Dynavac FD 12 (Airvac Engineering Pty Ltd, Melbourne, Australia)

Eppendorf 5415 C centrifuge

HD Scientific (Hettich Zentrifugen)

CFC Free Centrifuge

Hitachi Ltd., Tokyo, Japan

F-2000 Fluorescence Spectrophotometer
U-3200 UV-Vis Spectrophotometer

IEC, Australia Industrial Equipment & Control Pty Ltd.

pH meter Magnetic Stirrer Model 208-1

Pierce Reacti-Therm Heating Module

Reacti-Vap and Evaporating unit, Rockford, Illinois, USA

Roche Diagnostic Australia Pty Ltd 31, Vic, Avenue

Thermo Electron Corporation PX2 Thermal cycle

Hot Plate Heater and Magnetic Stirrer

Radiometer Analytical SA, Lyon, France

PHM61 Laboratory pH Meter

Shimazu Corporation, Kyoto, Japan

Shimazu High Performance Liquid Chromatography system:

Shimazu SPD-M10Avp system Photodiode Array Ultraviolet/Visible Detector

SIL-10AD VP Shimazu Auto Injector

LC-10AD AT VP Liquid Chromatograph

FCV-10 AL. VP Leak

DGU-14A Degasser

RF-10A XL Fluorescence Detector

CTO-10A VP Column oven

SCL-10A VP System controller

LC-10AT VP Liquid Chromatograph

FRC-10A Fraction Collector

STR ODS-II reverse-phase column (4.6mm I.D.x 150 mm length, Shimadzu) with Fluorescent Detector

Microsoft Windows XP Pentium 4
Shimazu UV-160, UV-Visible recording Spectrophotometer

**Quincy, Mass, USA**

MacLab/4, Analog Digital Instruments Digitimer Ltd Multistim-system D330

MacLab/4e

Myograph Lab, Apple Computer Grass medical instrument, Model S88D

**Quantum Scientific**

Capsulefuge Tomy PMC-060