UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF KAEMPFERIA SPECIES AND ACTIVITYGUIDED SELECTION OF *Kaempferia parviflora* WALL. EX BAKER FOR AGRONOMIC IMPROVEMENT

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By

CATHERINE DHARSHINI LABROOY

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

August 2018

DEDICATIONS

FOR MY FAMILY

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

CHARACTERIZATION OF KAEMPFERIA SPECIES AND ACTIVITY-GUIDED SELECTION OF *Kaempferia parviflora* WALL. EX BAKER FOR AGRONOMIC IMPROVEMENT

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Chairman : Associate Professor Thohirah Lee Abdullah, PhD
Faculty : Agriculture

Kaempferia is a genus of gingers has promising potential to be discovered for their ornamental and medicinal value. Nuclear ribosomal internal transcribed spacer (ITS) sequences and morphological characteristics were used to characterize six *Kaempferia* species including *Kaempferia parviflora* Wall. ex Baker, *Kaempferia galanga* Linn., *Kaempferia elegans* Wall. ex Baker, *Kaempferia pulchra* Ridl., *Kaempferia angustifolia* Roscoe, *Kaempferia marginata* Carey ex Roscoe. Sequence data showed nucleotide diversity (0.458), estimated values of transition/transversion bias (0.89) and divergence pattern in the *Kaempferia* genus was inferred using maximum parsimony analysis. The resulting phylogenetic tree from morphological and molecular data both showed congruence with three distinctive groupings within selected *Kaempferia* species. ITS 4 and 5 sequences are proposed as DNA barcodes for identification of *Kaempferia* species. In terms of morphological traits, petiole length, plant habit, rhizome colour and leaf variation can be used for preliminary identification of this genus.

Preliminary screening for phytochemical and bioactivity comparing *Kaempferia* extracts have provided baseline information on activities and potentials of each species. Plant extracts from each of the above-mentioned species were screened with cytotoxicity, anti-inflammatory and anti-histamine bioassays. All extracts were evaluated for total phenolic content, total flavonoid content and antioxidant activity. Most of the extracts were found to have phenolic compounds, flavonoids and anti-oxidant potential. Some extracts have shown positive activity in all three bioassays. Among the selected species, *K. parviflora* showed positive activity in three bioassays and higher phytochemical activity in comparison to other species. *K. parviflora* was selected for further studies. *K. parviflora* cultivars, KPM (*K. parviflora* from

Malaysia) and KPT (*K. parviflora* from Thailand) both gave the most selective cytotoxic activity against HCT116 cells (IC_{50} 9-13 µg mL^{-1}). KPM and KPT extracts also exhibited high β-hexosaminidase inhibitory effects (>50%) at concentrations 7.5 to 10 µg mL^{-1} without affecting cell viability in RBL-2H3 cells. Both extracts exhibited high inhibition against NO production at the highest concentration (50 µg mL^{-1}) but affected the viability of RAW 264.7 macrophage cells (<80%). Total phenolic content (TPC) and total flavonoid content (TFC) was higher in KPM 3.14 mg gallic acid equivalent (GAE)/g DW, 1.83 mg rutin equivalent / g DW. The results demonstrate that *K. parviflora* extracts have therapeutic properties, but its beneficial qualities may differ between cultivars.

To improve propagation of *K. parviflora*, multiple shoot regeneration was found most successful on Murashige and Skoog (MS) medium supplemented with 35.52 µM N^6-benzyladenine (BA) in terms of highest number of shoots (22.4 ± 1.84), leaves (29.27 ± 1.30), and roots (17.8 ±1.72) per explant. A batch of regenerated *in vitro* plantlets were then successfully induced to form micro rhizomes in MS medium free of plant growth regulators with an optimal concentration of 6% (*w/v*) sucrose. Increase in micro rhizome biomass (35.7 ± 2.59 g per flask), number of micro rhizomes (5.2 ± 0.78), shoots (8.5 ± 1.58) and roots (8.5 ± 1.58) were observed under this treatment.

Light is one of the main limiting factors for phenolic compound biosynthesis in plants. KPM and KPT were investigated for growth and secondary metabolite content under four shade levels (0%, 30%, 50%, and 70%). Photosynthetic capability, plant biomass and total flavonoid content were highest in both accessions under 30% shade after nine months of growth cycle. 5,7-dimethoxyflavone (DMF) a chemical marker for *K. parviflora*, was greatest in KPM grown under 30% shade. KPT had higher biomass than KPM under 30% but lower DMF content. *K. parviflora* is a semi shade loving plant suitable to grow under 30% shading for high yield and secondary metabolite production.

This research gives an overall overview and comprehensive comparison of *Kaempferia* species for further research.

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

PENCIRIAN SPESIS KAEMPFERIA DAN PEMILIHAN *Kaempferia parviflora* WALL EX. BAKER BERPANDU AKTIVITI BAGI PENAMBAHBAIKAN AGRONOMI

Oleh

CATHERINE DHARSHINI LABROOY

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Kaempferia adalah genus ginger yang mempunyai potensi untuk dijadikan sebagai nilai hiasan dan perubatan. Nuclear ribosomal internal transcribed spacer (ITS) dan pencirian morfologi digunakan untuk mengkategorikan enam spesies *Kaempferia*, termasuk *Kaempferia parviflora* Wall. ex Baker, *Kaempferia galanga* Linn., *Kaempferia elegans* Wall. ex Baker, *Kaempferia pulchra* Ridl., *Kaempferia angustifolia* Roscoe, *Kaempferia marginata* Carey ex Roscoe. Urutan data menunjukkan kepelbagaian nukleotida (0.458), anggaran nilai peralihan / peralihan bias (0.89) dan corak perbezaan dalam genus *Kaempferia* telah disimpulkan dengan menggunakan analisis parsimon maksimum. Hasil kajian pokok phylogenetic daripada data morfologi dan molekul menunjukkan keseragaman dengan tiga kumpulan tersendiri dalam spesies *Kaempferia* yang terpilih. Urutan ITS 4 dan 5 dicadangkan sebagai kod bar DNA untuk mengenal pasti spesies *Kaempferia*. Dari segi ciri-ciri morfologi, kepanjangan tangkai, sifat tanaman, warna rhizome dan variasi daun boleh digunakan untuk peringkat awal mengenalpasti genus ini.

Pemeriksaan awal untuk fitokimia dan bioaktiviti membandingkan ekstrak *Kaempferia* telah menyediakan maklumat asas mengenai aktiviti dan potensi setiap spesies. Ekstrak tumbuhan dari setiap spesies yang disebutkan telah menjalani proses penyaringan total phenolic content, total flavonoid content dan activity anti-pengoxidaan. Semua ekstrak juga dinilai untuk jumlah kandungan fenolik, jumlah kandungan flavonoid dan aktiviti pengantioksidaan. Kebanyakan ekstrak didapati mempunyai sebatian fenolik, flavonoid dan potensi anti-oksidan. Beberapa ekstrak telah menunjukkan aktiviti positif dalam tiga bioassay. Antara spesies dikaji, *K. parviflora* menunjukkan aktiviti positif dalam tiga bioassay dan aktiviti phytochemical yang lebih tinggi berbanding spesies lain. *K. parviflora* dipilih untuk

dikaji lebih lanjut. Kedua-dua kultivar *K. parviflora*, KPM (*K. parviflora* dari Malaysia) dan KPT (*K. parviflora* dari Thailand) menunjukkan aktiviti sitotoksik paling selektif terhadap sel HCT116 (IC50 9-13 μg mL^{-1}). Ekstrak KPM dan KPT juga menunjukkan kesan perencatan β-hexosaminidase yang tinggi (> 50%) pada kepekatan 7.5 hingga 10 μg mL^{-1} tanpa menjejaskan kebolehidupan sel dalam sel RBL-2H3. Kedua-dua ekstrak mempamerkan dasar toleransi yang tinggi terhadap pengeluaran NO pada kepekatan tertinggi (50 μg mL^{-1}) tetapi mempengaruhi kebolehidupan RAW 264.7 sel makrofag (< 80%). Jumlah kandungan fenolik (TPC) dan jumlah kandungan flavonoid (TFC) lebih tinggi pada KPM 3.14 mg asid gallic equivalent (GAE) / g DW, 1.83 mg setara rutin / g DW. Hasil menunjukkan bahawa ekstrak *K. parviflora* mempunyai sifat terapeutik, tetapi kemanfaatan kualiti mungkin berbeza antara kultivar.

Untuk meningkatkan penyebaran *K. parviflora*, regenerasi anak pokok didapati paling efisien pada Murashige dan Skoog (MS) yang ditambah dengan 35.52 μM N6-benzyladenine (BA) dengan catatan tertinggi pucuk (22.4 ± 1.84), daun (29.27 ± 1.30), dan akar (17.8 ± 1.72) setiap peledakan. Sekelompok tumbuhan in vitro yang dihasilkan semula kemudian berjaya diinduksi untuk membentuk mikrorhizomes dalam medium MS tanpa pengawalselia pertumbuhan tumbuhan dengan kepekatan optimum 6% (w / v) sukrosa. Peningkatkan biomassa mikro (35.7 ± 2.59 g setiap lob), bilangan mikrorhizomes (5.2 ± 0.78), pucuk (8.5 ± 1.58) dan akar (8.5 ± 1.58) dilihat dalam rawatan ini.

Cahaya adalah salah satu faktor pembatas utama bagi biosintesis kompaun fenolik dalam tumbuhan. *K. parviflora* kultivar KPM (*K. parviflora* Malysia) dan KPT (*K. parviflora* Thai) dikaji untuk pertumbuhan dan kandungan metabolit sekunder di bawah empat tahap teduhan (0%, 30%, 50%, dan 70%). Keupayaan fotosintesis, biomas tumbuhan dan jumlah kandungan flavonoid tertinggi dalam kedua-dua kultivar dijumpai bawah teduhan 30% selepas jangkamasa sembilan bulan kitaran pertumbuhan. Penanda kimia 5,7-dimethoxyflavone (DMF) untuk *K. parviflora*, dicatatkan tertinggi di KPM yang membesar di bawah 30% teduhan. KPT mempunyai biomas yang lebih tinggi daripada KPM di bawah 30% tetapi kandungan DMF yang lebih rendah. *K. parviflora* adalah tumbuhan yang sesuai untuk tumbuh di bawah teduhan 30% untuk hasil pengeluaran optimum dan pengeluaran metabolit sekunder.

Kajian ini memberikan gambaran umum dan perbandingan komprehensif spesies *Kaempferia* untuk penyelidikan selanjutnya. Kajian agronomik pada *K. parviflora* meningkatkan potensi sepenuhnya untuk dijadikan herba yang penting dari segi ekonomi.

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

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%	Percentage
°C	Degree celsius
ANOVA	Analysis of variance
$ABTS^{\cdot+}$	2, 2' – azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation
BA	N^6-benzyladenine
Chl	Chlorophyll
cm	Centimeter
CRD	Completely Randomize Design
cv.	Cultivar
d	Day
DCM	Dichloromethane
DCPIP	2,6-dichlorophenolindophenol
DMEM	Dulbecco minimum essential medium
DMSO	Dimethylsulfoxide
DMRT	Duncan's multiple range test
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
e.g.	For example
et al.	And friends
etc.	et cetera
FBS	Foetal Bovine Serum
FRAP	Ferric reducing/antioxidant power
g	gram
GAE	Gallic acid equivalent
GI_{50}	Concentration that causes 50% growth inhibition of cells
h	Hour
HCT116	Human colorectal carcinoma cell line
HPLC	High performance liquid chromatography

IC_{50}	Concentration that reduces the effet by 50%
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
ITS	Internal transcribed spacer
l	Litre
LC_{50}	Concentration that causes 50% cell death
m	Meter
min	Minutes
ml	Millilitre
mol	Mole
MCF-7	Breast cancer cell line
MTT	Microculturetetrazolium
MS	Murashige and Skoog
µg	Microgram
µmol	Micromole
ns	Non-significant
p	Probability
pH	Measurement of Acidity/Alkalinity
PBS	Phosphate buffer saline
PC3	Prostate cancer cell line
PCR	Polymerase chain reaction
PGR	Plant Growth Regulator
RCBD	Randomized Complete Block Design
RM	Ringgit Malaysia
RAW 264.7	Mouse macrophage cell line
RBL-2H3	Rat basophilic leukaemia cell line
RPMI	Roswell Park Memorial Institute medium
s	Second
TBE	Tris/Borate/EDTA
TPC	Total phenolic conteNT

CHAPTER 1

INTRODUCTION

Mankind have depended on herbal medicine as the basis of healthcare throughout the world since ancient times. Herbal medicine is still widely used and have considerable importance in international trade. The commercial value of herbal medicines on the international market is high and increasing greatly. The global herbal industry is estimated by World Health Organization to a value of US $ 14 billion with a growing demand rate of 15–25% annually and approximately US$17 billion was spent in the US on traditional herbal medicines in 2003 (Booker, Johnston, & Heinrich, 2012; Willis, 2017)

Malaysia is a melting pot of cultures and the herbal tradition of these cultures continues to flourish. The Malaysian government has taken a great interest in the herbal industries and its role in the national healthcare system. The herbal industry in Malaysia drives three main sectors, agriculture, tourism and manufacturing. Herbal industry sales in Malaysia is estimated at RM 2 billion Ahmad et al., 2015. There are more than 1250 plant species with medicinal value currently identified in Malaysia. Herbs have also been made into the first Entry Point Project (EPP1) for the nation's Agriculture NKEA (National Key Economic Areas) due to its potential in propelling the sustainability of the nation's Bio-economy sector (Ahmad, Farizah,Zaidi, Mohd Azlan, Sulaiman, Noorasiah & Majid, 2015).

The herbal medicine industry faces several major challenges in its development as well as government implementation of regulation. These challenges are related to regulation pertaining to herbal products, assessment of safety and efficacy of herbal products, quality control, safety monitoring and lack of knowledge about herbal medication (WHO, 2005). In the agriculture sector, it is important to address the standardization for quality control of both raw materials and final herbal products. This will not only solve poor quality problem but also promote value of local-natural resources (Putiyanan, Chansakaow, & Phrutivorapongkul, 2008).

Zingiberaceae species are a rich source of active phytochemicals. They have been widely used as spices, medicines, dyes, flavouring agents, ornamental plants and more in Malaysia (Ibrahim, Khalid, & Hussin, 2007). *Kaempferia* is a lesser known genus of Zingibereceae plants. They are commonly called peacock gingers with 60 species distributed from India to Southeast Asia (Sirirugsa, 1989). Some *Kaempferia* cultivated in Malaysia include *Kaempferia galanga* Linn., *Kaempferia pulchra* Ridl., *Kaempferia elegans* Wall. ex Baker, *Kaempferia parviflora* Wall. ex Baker, *Kaempferia angustifolia* Roscoe and *Kaempferia marginata* Roscoe.

Kaempferia species are not well investigated in Malaysia. They are difficult to identify and characterise due to morphological similarities in vegetative parts of Zingiberaceae (Techaprasan, Klinbunga, Ngamriabsakul, & Jenjittikul, 2010). Furthermore, there are no comparative studies on bioactivity and phytochemical contents between species. There is a need to establish baseline information of *Kaempferia* in Malaysia to fill knowledge gaps and improve its potential utilization in the herbal industry.

Agriculture plays a major role in producing raw materials and finished herbal products at highest quality. Agricultural improvements in terms of rapid propagation and improved secondary metabolite contents can increase economical value of herbal products. *Kaempferia* species are conventionally propagated through rhizome splitting which affords slow growth rates and low productivity. Many species also undergo dormancy periods during dry seasons limiting large-scale propagation (Techaprasan et al., 2010). It would be beneficial to use plant tissue culture technology to propagate *Kaempferia* species to overcome shortcomings of conventional propagation.

There are both scientific merit and commercial justifications to establish comparative baseline information on *Kaempferia* species in Malaysia. Morphological and molecular markers will improve identification and assist in improving scientific knowledge and quality control of *Kaempferia* herbal products. The bioactivities and phytochemical content of *Kaempferia* extracts can be used as a guideline for future pharmacology and phytochemical investigations into this genus. Selecting the most active *Kaempferia* species from the first two studies, agriculture technology will be applied to improve impediments in production of high-quality raw materials for the herbal industry.

Hence, the objectives of this present study were:

1. To identify and characterize *Kaempferia* species
2. To determine the activity profile of *Kaempferia* species
3. To determine the dose response activity in *K. parviflora*
4. To improve propagation of *K. parviflora*
5. To increase secondary metabolite content in *K. parviflora*

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BIODATA OF STUDENT

Catherine Dharshini Labrooy born on 22nd June 1987 in Batu Pahat Johor is the second child of the late Mr Louis Clinton Danzil Labrooy and Mrs Vinodha Nanoo. She received her primary education in SK Taman Inderawasih followed by her secondary education in SMK Taman Inderawasih and pre-university or STPM education in SMK Permatang Rawa. She was an active student in school activities being a school prefect and awarded school sports women of the year twice in 2005 and 2007. She completed her Bachelor in Horticulture Science with 1st Class Honours in the year 2011. During her course of study, she actively participated in various university activities. She was a committee member of Golden Key Association. She was also a committee member as well as a participant of the International Agriculture Students Symposium (IASS). After completing her degree, she was offered to continue working in the Tropical Spice Garden Penang and invited to continue her studies under Assoc Prof Dr. Thohirah Lee Abdullah. As adviced by her family she registered for a master's degree in Horticulture and was offered the fast track PhD programme by UPM. During her graduate studies she has participated in various workshops, seminars, conferences and symposiums. She has also published several articles in proceedings, journals and book chapters. She has also contributed to securing three research grants and an active participant of Canser Research and Drug Discovery (CRDD) group lead by Prof. Johnson Stanslas.

LIST OF PUBLICATIONS

Book chapters and Journal articles

Abdullah, T.L., Abdullah, N.A.P., Labrooy, C.D., (2011). Genetic Relationships of *Curcuma* Species Revealed by RAPDs Marker. In WAN Noordin Wan Daud; Adam Puteh; Muhammad Nazmin Yaapar. *Recent Advances in Crop Science*, (pp.81-96) Universiti Putra Malaysia Press, Malaysia: Selangor.

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Labrooy, C.D., Abdullah, T.L., Stanslas, J. (2012) Kaempferia parviflora: An understory ginger plant with promising ornamental and medicinal value; Urban Forestry Conference.

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Labrooy, C.D., Abdullah, T.L., Stanslas, J. (2013) Morphological description of Kaempferia parviflora and breaking bud dormancy with BAP and Ethephon treatment; 24th Maaysian Society of Plant Physiology Conference

Labrooy, C.D., Abdullah, T.L., Stanslas, J. (2014). Shade Loving Connoisseur Plants for Urban Landscapes; Urban horticulture conference

Grants (Co-authored)

Research University Grant Scheme (RUGS) - Improved propagation and enhancement of secondary metabolite production of Kunyit Hitam (*Kaempferia parviflora* Wall. Ex Baker) for evaluation of anti-allergy activities

Fundemental Research Grant Scheme (FRGS) - DNA Barcoding and screening for cancer cell cytotoxicity, anti-allergy and anti-inflammatory bioactivity of *Kaempferia* species in Malaysia

Niche Research Grant Scheme (NRGS) - Improved propagation, maximizing yield and enhancement of secondary metabolite production of Lempoyang (*Zingiber zerumbet* (L.) Smith) through optimal fertilization and planting density under suitable light intensity

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