SHOOT MULTIPLICATION, ESSENTIAL OILS, ANTIOXIDANT CONTENT AND ANTIMICROBIAL ACTIVITIES OF Alpinia conchigera Griff.

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By

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

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DEDICATION

I dedicate this thesis to my mother Mrs. Hairunnishah binti Mat Jaffari for your patience, love and encouragement.

Also to my beloved Mr. Halim bin Ahmad for your support and love.

Thank you very much for everything

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

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Supervisor: Assoc. Prof. Faridah Qamaruz Zaman, PhD
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The research on medicinal value of *Alpinia conchigera* (Zingiberaceae) in Malaysia have been growing extensively for its antifungal properties. In order to meet the demand for commercial cultivation, the research on the *in vitro* propagation and the potential of rhizome and leaf oil of this species had been conducted. The whole plant of *Alpinia conchigera* Griff. (lengkuas padi) were collected from the Conservatory Park, Institute of Bioscience, Universiti Putra Malaysia. This research was divided into two experiment. Experiment 1 involved the establishment of the aseptic cultures of *A. conchigera* through *in vitro* propagation for shoot multiplication. The rhizome buds were cultured and directly regenerated in the Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP), Kinetin (Kin) at different concentrations (0, 1, 3, 5, 7 mg/L) and naphthalene acetic acid (NAA) at the concentrations of (0.5, 1.0, 1.5 mg/L). The optimum concentration for shoot initiation of *A. conchigera* was produced in MS medium supplemented with 0.5 mg/L NAA with 3.2 ± 0.9 shoots. Whereas, MS medium supplemented with 1.5 mg/L NAA was optimum for root initiation which gave 16.6 ± 1.4 roots. The effect of adenine on shoot multiplication was also investigated. The highest shoot number was produced in the medium containing 5 mg/L BAP and 0.5 mg/L NAA with the addition of 80 mg/L of adenine with 3.4 ± 0.5 shoot number. However, the medium supplemented with 80 mg/L adenine alone was observed to give good results for both shoots and roots, therefore was chosen for the rooting stage. Among all the sucrose concentrations, the sucrose supply of 60 g into the medium containing 80 mg/L adenine showed better response of plantlets. Healthy regenerated plantlets were selected for the hardening in a sterile mixture of husk and peat moss with the ratio 1:3. Experiment 2 was conducted to screen the constituents, antioxidant and antimicrobial properties of *A. conchigera* essential oils. The rhizomes and leaves were subjected to hydrodistillation using Clevenger-type apparatus. Then, the essential oils obtained from each part was analyzed for its volatile constituents by GC-MS analysis. Rhizome oil indicated 38 compounds with major compound detected was eucalyptol (60.58%) whereas the leaf oil produced 51 compounds with the most abundant compound was β-bisabolene (46.70%). The essential oils were then subjected to antioxidant tests namely

2,2-diphenyl-1-1-picrylhydrazyl (DPPH) free radical scavenging activity and β-carotene-linoleic acid assay followed by the total phenolic content (TPC) test. The rhizome oil indicated the highest antioxidant activities and the most abundant phenolic and polyphenolic compounds (IC_{50}=151.7 mg/µl, antioxidant activity=106.01% and TPC=203.3 GAE/100g) followed by leaf oil (IC_{50}=309.8 mg/µl, antioxidant activity=84.96% and TPC=94.1 GAE/100g) when comparing with the synthetic antioxidant, butylated hydroxytoluene (BHT). The antimicrobial assay namely disc diffusion assay and minimum inhibitory concentration (MIC) was conducted against selected microbes. The rhizome oil also showed inhibitory activity against all six bacteria and fungi. In contrast, the leaf oil only showed inhibition against two Gram positive bacteria. Overall, this study had provided the useful evidence on the shoot multiplication of tissue culture plantlets and the essential oils potential of *A. conchigera* for the its cultivation and commercialization.

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

PENGGANDAAN PUCUK, MINYAK PATI, KANDUNGAN ANTIOKSIDA DAN AKTIVITI ANTIMIKROB *Alpinia conchigera* Griff.

Oleh

RAIHANA RIDZUAN

Februari 2014

Penyelia: Prof. Madya Faridah Qamaruz Zaman, PhD
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Kajian mengenai nilai perubatan daripada pokok *Alpinia conchigera* (Zingiberaceae) di Malaysia telah berkembang dengan meluas oleh kerana ciri-ciri antikulatnya. Dalam usaha untuk memenuhi permintaan untuk penanaman komersial, penyelidikan ke atas pembiakan *in vitro* dan potensi minyak rizom dan daun spesis ini telah dijalankan. Keseloruhan pokok *Alpinia conchigera* Griff. (lengkuas padi) telah diambil dari Taman Konservatori, Institut Biosains, Universiti Putra Malaysia. Kajian ini dibahagikan kepada dua eksperimen. Eksperimen 1 melibatkan pembiakan tunas *A. conchigera* melalui kultur *in vitro* untuk pertumbuhan pucuk kultur. Tunas rizom dikulturkan secara langsung di dalam Murashige dan Skoog (MS) media yang ditambah dengan 6-benzylaminopurine (BAP), Kinetin (Kin) pada kepekatan (0, 1 , 3, 5, 7 mg /L) dan naftalena asetik asid (NAA) pada kepekatan (0.5 , 1.0 , 1.5 mg /L). Kepekatan optimum untuk pertumbuhan pucuk *A. conchigera* dihasilkan dalam media MS yang dibekalkan dengan 0.5 mg / L NAA dengan penghasilan sebanyak 3.2 ± 0.9 pucuk. Manakala, media MS yang dibekalkan dengan 1.5 mg / L NAA adalah optimum untuk pertumbuhan akar dengan penghasilan sebanyak 16.6 ± 1.4 akar. Penambahan hormon adenina dalam media dan kesannya pada penggandaan pucuk dikaji. Bilangan pucuk tertinggi dihasilkan dalam media yang mengandungi 5 mg/ L BAP dan 0.5 mg/L NAA dengan tambahan 80 mg/L adenina 3.4 ± 0.5 bilangan pucuk. Walau bagaimanapun, media yang hanya ditambah dengan 80 mg/ L adenina telah menunjukkan hasil yang baik untuk penggandaan pucuk dan akar, oleh itu kepekatan ini telah dipilih untuk dibekalkan di dalam media peringkat perakaran. Antara semua kepekatan sukrosa, bekalan sukrosa sebanyak 60 g di dalam media yang dibekalkan dengan 80 mg/L adenina menunjukkan penggandaan akar yang lebih baik. Anak pokok kultur yang sihat telah dipilih untuk peringkat pengerasan di dalam campuran media tanah sekam dan tanah gambut dengan nisbah 1:3. Eksperimen 2 telah dijalankan untuk menyaring sebatian kimia, anti-oksida dan anti-mikrob minyak pati pokok *A. conchigera*. Rizom dan daun pokok lengkuas padi telah menjalani proses penyulingan menggunakan alat Clevenger. Kemudian, minyak pati yang diperolehi daripada setiap bahagian telah dianalisa untuk menentukan sebatian kimia melalui analisis GC-MS. Minyak rizom

menunjukkan 38 sebatian dengan sebatian utama dikesan adalah eucalyptol (60.58 %) manakala minyak daun menghasilkan 51 sebatian dengan sebatian yang paling banyak adalah β-bisabolene (46.70%). Ujian antioksida iaitu 2,2-difenil-1-1-picrylhydrazyl (DPPH) aktiviti mengaut radikal bebas dan β-karotena-linoleik asid diikuti dengan ujian jumlah kandungan fenolik (TPC) telah dijalankan. Minyak rizom menunjukkan aktiviti antioksida tertinggi, fenolik dan sebatian polifenolik yang paling banyak (IC_{50}=151.7 mg/µl, aktiviti antioksida=106.01% dan TPC=203.3 GAE/100g) diikuti oleh minyak daun (IC_{50} = 309.8 mg/µl , aktiviti antioksida=84.96 % dan TPC=94.1 GAE/100g) apabila dibandingkan dengan antioksida sintetik, hydroxytoluene butylated (BHT). Ujian antimikrob kaedah cakera penyebaran dan kepekatan minimum perencatan (MIC) telah dijalankan terhadap mikrob terpilih. Minyak rizom juga menunjukkan aktiviti perencatan terhadap kesemua bakteria dan kulat. Sebaliknya, minyak daun hanya menunjukkan perencatan terhadap dua bakteria Gram positif . Secara keseluruhannya, kajian ini telah memberikan maklumat yang berguna mengenai pembiakan *A. conchigera* melalui kultur tisu dan potensi minyak patinya untuk penanaman dan pengkomersialan.

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

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LIST OF SYMBOLS AND ABBREVIATIONS

DPPH	2,2-diphenyl-1-1-picrylhydrazyl
BAP	6-Benzylaminopurine
And	Adenine
AD	After Death (of Christ)
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
BC	Before Christ
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
Cm	Centimeter
°C	Degree Celsius
DMRT	Duncan's New Multiple Range Test
et al.	*et alia*
FSTM	Faculty of Food Science and Technology
F-C	Folin-Ciocalteau
GAE	Gallic Acid Equivalence
G	Gram
g/L	Gram per liter
HPLC	High Performance Liquid Chromatography
H	Hour
IBA	Indole butylated acid
IBS	Institute of Bioscience
Kin	Kinetin

L	Liter
$HgCl_2$	Mercury chloride
µM	Micro molar
Mg	Milligram
mg/L	Milligram per liter
Mm	Millimeter
MIC	Minimum Inhibitory Concentration
Min	Minute
M	Molar
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MS	Murashige and Skoog
MSO	Murashige and Skoog medium without supplemented hormone
NAA	Naphthalene acetic acid
NA	Nutrient Agar
NB	Nutrient Broth
%	Percentage
AA%	Percentage of antioxidant activity
OPC	Oropharyngeal candidosis
PGR	Plant Growth Regulator
±	Plus minus
pH	Potentiometric hydrogen ion concentration
ROS	Reactive Oxygen Species
NaOCl	Sodium hypochlorite

SE	Standard error
SPSS	Superior Performing Statistical Package
TPC	Total Phenolic Content
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

1.1 Introduction

Malaysia is one of the mega-biodiversity countries of the world which is not only rich with its flora and fauna but also as a living heritage of various herbal species. The jungles which are believed to be 130 million years old consist of around 14,500 species of flowering plants and trees (Richmond, 2010).

Among all the monocots, Zingiberaceae (ginger family) has the highest number of plants used as herbs and spices. Zingiberaceae is the monocotyledonous plant family constitutes a vital group of rhizomatous medicinal and aromatic plants. This family is characterized by the presence of its volatile oils and oleoresins. Taxonomically, this family consist of 52 important genera which are distributed mostly in the tropical and subtropical countries such as India, China, Malaysia, Thailand, and Philippines (Habsah *et al*., 2005).

Holttum (1950) listed two subfamilies under Zingiberaceae which are Zingiberoideae (aromatic) and Costoideae (non-aromatic). Among 52 genera and 1400 of Zingiberaceae species distributed in the region of Asia (Kasarkar and Kulkarni, 2010), at least 20 genera and 300 species of this family are found in Malaysia (Habsah *et al*., 2005). Some of the important and common genus found in Malaysia are *Alpinia* Roxb.*, Curcuma* L.*, Zingiber* Mill.*, Geostachys* (Baker) Ridl, *Etlingera* Giseke, *Globba* L., and *Kaempferia* L.

Most of Zingiberaceae plants such as *Alpinia* Roxb. are obtained mainly from natural growing areas and the demand for these plants is very high as for its medicinal value. With these high demands, the plants are being overexploited and are threatening the survival of many rare species (Nalawade and Tsay, 2004).

As aromatic herbs*, A. conchigera* is rich in volatile oils or essential oils. Essential oils are highly concentrated and not the same as perfume or fragrance oils because it is derived directly from the true plants whereas in contrast, perfume oils are artificially created fragrances and may contain artificial substances (Nigam & Ahmed, 1991). The extract from this plant have been shown to contains natural phenolics, polyphenolics, terpenes and other phytochemical compounds which responsible for antimicrobial, anti inflammatory, anti tumor and antioxidant activities (Habsah *et al*., 2000; Lee *et al*., 2006; Awang *et al*., 2010; Sulaiman *et al*., 2010).

Over the last few years in Malaysia, the research on the medicinal values of *A. conchigera* have been growing extensively and the demand on this plant also became higher. Same as other *Alpinia* species, *A. conchigera* can be propagated through underground rhizomes, however this part is susceptible to the various diseases of fungal, bacterial, viral, and mycoplasmal origins such as rhizome rot, *Fusarium* yellow disease, leaf spot and bacteria wilt (Samsudeen *et al*., 2000). Whereas the major pests of Zingiberaceae spp. namely shoot borer *Conogethes punciferalis* and root-knot nematode, *Meliodogyne incognita* cause the considerable crop losses (Kavyashree, 2009).

The propagation of this plant through underground rhizomes is considered very slow (Devasahayam and Koya, 2007). This plant produces seeds, however it resulted in genetic variations which affect the consistency of their character. In order to overcome this production constraint, the biotechnological approaches was attempted for the production of disease-free plants. Thus, the micropropagation method provides an alternative way to produce healthy and disease-free plants in a short period of time continuously. To this date, no research had been conducted on the establishment of the micropropagation protocols of the *A. conchigera* in Malaysia.

In addition, most of the research papers only reported on the medicinal properties of the underground rhizome and lack of investigation specifically on the other plant parts such as leaf. The selection of the rhizome part is mainly due to its underground occurence which aid in the storage of plant nutrient. However, the report on the potential of the leaf needs to be explored since the mass production of this plants may contribute to the waste agriculture biomass. Although previous research have been conducted and the volatile components of the *A. conchigera* have been identified, however the relationship between the phenolic compounds and their bioactivity are not completely known. Moreover, the study on the essential oils, antioxidant and antimicrobial potential, and cultivation of *A. conchigera* are not as widely available as those of common Alpinia, *A. galanga.*

1.2 Research objectives

The aim of this study is to mass propagate the species for multiplication and commercialization and to determine the medicinal properties of essential oils from leaf and rhizome parts of *A. conchigera*.

The specific objectives of this study are:

1. To establish the micropropagation protocol of *A. conchigera.*
2. To identify the essential oils constituents extracted from the rhizomes and leaves of *A. conchigera*.
3. To determine the total phenolic content of the rhizome and leaf oil of *A. conchigera.*
4. To determine the antioxidant and antimicrobial properties of the essential oils of *A. conchigera*.

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