UNIVERSITI PUTRA MALAYSIA

MICROPROPAGATION OF Zingiber montanum Koenig AND EVALUATION OF ITS ESSENTIAL OILS FOR ANTIOXIDATIVE AND ANTIMICROBIAL ACTIVITIES

DEANNE RUDIM

IB 2013 25

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MASTER OF SCIENCE
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2013

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By

DEANNE RUDIM

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

December 2013

DEDICATION

Dedicated to my beloved parents (Rudim Bowie and Marysia Malik) and siblings for their love and support.

Also dedicated to my relatives for their endless encouragements.

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

MICROPROPAGATION OF *Zingiber monatnum* Koenig AND EVALUATION OF ITS ESSENTIAL OILS FOR ANTIOXIDATIVE AND ANTIMICROBIAL ACTIVITIES

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Chair: Associate Professor Faridah Qamaruz Zaman, PhD
Faculty: Institute of Bioscience

Zingiber montanum or locally known as bonglai is a rhizomatous perennial plant belonging to Zingiberaceae. This species is highly valued for its medicinal properties as it has been used in folk medicine for centuries. Pharmacological studies indicated that *Z. montanum* extracts possess antioxidant and antimicrobial agent. But in Malaysia, there is little study confirming these activities for *Z. montanum*. Moreover, this plant has been considered as underutilized especially in Malaysia. Conventional propagation means was hampered by slow propagation rate and the risk of disease transmittance through division by sectioning of the rhizomes. The fact that more planting materials are needed for further pharmacological importance discovery and for commercial planting, alternative propagation method by means of tissue culture is required. This study was conducted to provide scientific evidence for its antioxidative and antimicrobial potential and technique for its mass propagation to overcome the conventional propagation constraints of *Z. montanum*. Shoot of *Z. montanum* was induced in Murashige and Skoog (MS) basal media enriched with 4.44µM BAP + 4.65µM KIN and produced 6.3 shoots. The shoots were multiplied in 4.44µM BAP + 4.65µM KIN + 2.69µM NAA supplemented MS media produced 5.8 shoots per explant with shoot length of 7.0 cm. Shoots in the MS medium incorporated with 0.56 µM NAA showed good rooting response producing 20.4 roots per explant with root length of 6.3 cm. While, the essential oil analysis of the *Z. montanum* mother plant showed that the highest percentage of oil based on their fresh weight was obtained in rhizome (1.75% v/w), followed by the leaves (0.05% v/w) and pseudo-stem (0.02% v/w). GC-MS analysis of the rhizome oil revealed that 28 compounds were detected where β-phellandrene (31.94%), terpinen-4-ol (24.27%), 1-4-bis(methoxy)triquinacene (24.03%) were identified as major compounds while in the leaf oil, 84 compounds were detected with the dominant compounds being 1,4,-bis(methoxy)triquinacene (17.00%), followed by β-phellendrene (13.79%) and β-sesquiphellandrene (12.86%). Total phenolic assay indicated that *Z. montanum* had high phenolic content as high as 343.42 mg Gallic acid equivalent GAE/100g. The antioxidant activity of the oils was higher than that of BHT as revealed by DPPH free radicals scavenging and β–carotene bleaching assay proving that *Z. montanum* is a good source of antioxidant agents. On the other hand, antimicrobial assay of the oils revealed different

susceptibility with *Bacillus subtilis* observed as the most susceptible microbe with Minimum Inhibitory Concentration (MIC) value of 0.025mg/µl. Fungi showed considerable resistance over the oils. The micropropagation protocol of *Z. montanum* in this present study could be potentially applied for its mass propagation to exploit its medicine properties. The present study revealed that even though *Z. montanum* oils exhibited weak to moderate antimicrobial activity but it exhibited relatively good antioxidant activity, confirming the potential of *Z. montanum* as a source of natural antioxidant to be used as natural preservatives in food, cosmetic and pharmacological products.

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

MIKROPROPAGASI *Zingiber monatnum* Koenig DAN PENILAIAN MINYAK PATINYA UNTUK AKTIVITI ANTIOKSIDAN DAN ANTIMIKROB

Oleh

DEANNE RUDIM

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Zingiber montanum atau lebih dikenali sebagai bonglai merupakan tumbuhan saka yang mempunyai rizom dan tergolong dalam keluarga Zingiberaceae. Spesies ini mempunyai nilai perubatan yang tinggi dan telah lama digunakan dalam perubatan tradisional. Kajian farmakologi menunjukkan ekstrak *Z. montanum* mempunyai agen antioksida dan antimikrob. Walau bagaimanapun di Malaysia, kajian yang mengesahkan kehadiran bioaktiviti ini adalah kurang. Selain itu, tumbuhan ini dianggap sebagai tumbuhan yang belum digunakan sepenuhnya terutama sekali di Malaysia. Pembiakan konvensionalnya pula terencat disebabkan kadar pembiakan yang perlahan dan risiko pemindahan penyakit daripada rizom yang digunakan sebagai benih. Lebih banyak anak pokok diperlukan bagi tujuan kajian famakologi dan untuk penanaman komersial, oleh itu kaedah pembiakan alternatif melalui kultur tisu adalah perlu. Kajian ini telah dijalankan untuk memberi bukti saintifik tentang potensi *Z. montanum* sebagai antioksidan dan antimikrob agen dan teknik penanaman secara *in vitro* untuk menghasilkan anak pokok yang banyak bagi mengatasi masalah penanaman konvensionalnya. Pucuk *Z. montanum* dihasilkan dalam Murashige dan Skoog media yang mengandungi hormon 4.44µM BAP + 4.65µM KIN dengan bilangan 6.3 pucuk. Pucuk digandakan dalam MS media yang mengandungi 4.44µM BAP + 4.65µM KIN + 2.69µM NAA dengan bilangan pucuk sebanyak 5.8 dan kepanjangan pucuk iaitu 7.0 cm. Manakala, MS+0.56 µM NAA menunjukkan tindak balas yang baik untuk penghasilan akar *Z. montanum* dengan menghasilkan sebanyak 20.4 akar dengan kepanjangan akar iaitu 6.3 cm. Sementara itu, analisis minyak pati induk *Z. montanum* menunjukkan peratusan hasil minyak tertinggi berdasarkan berat segar diperolehi daripada rizom (1.75% v/w), diikuti oleh daun (0.05%v/w) dan batang pseudo (0.02%v/w). Dengan analisis GC-MS, 28 sebatian telah dikesan dalam minyak rizom di mana sebatian utamanya adalah β-phellandrene (31.94%), terpinen-4-ol (24.27%) dan 1-4-bis (methoxy) triquencene (24.03%) manakala dalam minyak daun, sebanyak 84 sebatian telah dikesan yang didominasi oleh 1,4 bis (methoxy) triquinacene (17.00%), β-phellandrene (13.79%) dan β-sesquiphellandrene (12.86%). Analisis kandungan fenolik menunjukan *Z. montanum* mempunyai kandungan fenolik yang tinggi (343.42 mg GAE/100g). Aktiviti antioksidan minyak pati *Z. montanum* adalah lebih tinggi daripada BHT yang dibuktikan melalui memerangkapan radikal bebas DPPH dan pelunturan β-karotena assay, seterusnya membuktikan *Z. montanum* merupakan agen antioksidan yang baik. Analisis antimikrob pula menunjukkan sensitiviti mikrob yang berbeza dimana *Bacillus subtilis* merupakan mikrob yang paling mudah direncatkan

pertumbuhannya dengan nilai MIC yang kecil iaitu 0.025µl. Sementara itu, minyak pati tidak mempunyai kesan terhadap pertumbuhan kulat. Hasil kajian mikropropagasi *Z. montanum* ini boleh digunakan untuk pembiakannya secara besar-besaran bagi tujuan komersial. Berdasarkan hasil kajian ini, walaupun minyak pati *Z. montanum* menunjukkan aktiviti antimikrob yang lemah dan sederhana tetapi ia mempunyai ia aktiviti antioksida yang baik, seterusnya mengesahkan potensi *Z. montanum* sebagai sumber antioksida semulajadi dan boleh digunakan sebagai bahan pengawet semulajadi dalam produk makanan, kosmetik dan farmakologi.

ACKNOWLEDGEMENTS

First and foremost, praise to the Almighty Lord for His blessings bestowed upon me, grant me wisdom, knowledge and most importantly the strength and determination to complete this study.

I would like to express my sincere gratitude and appreciation to my supervisor, Assoc. Prof. Dr. Faridah Qamaruz Zaman for her supervision, optimism, patience, endless advice, guidance and support throughout this study. Special thanks to my supervisory committee members Assoc. Prof. Dr. Mihdzar Abdul Kadir and Assoc. Prof. Dr. Abdul Karim Sabo Mohamed for their constant advice and guidance, for their time and patience in making this study possible and also for their kindness.

Not forgetting for everyone who assisted me as the study progressed, the staff of Unit of Biodiversity and UNiCC especially Dr. Adil Hassan Ahmed Abdelmageed, Mr. Shamsul Khamis, Mrs. Satna Zuriah, Mr. Tajuddin Abd Manap, Mr. Ahmad Ikhmal, Miss Nor Hafizah and Mrs. Nooraishah for their help, advice, encouragement and friendship. Special thanks to Dean of Faculty of Food, Science and Technology for giving me the permission to conduct the experiment in the faculty. A million thanks to the staff in the faculty for their patience, guidance, kindness, assistance, generosity and technical support.

I would like to extend my gratitude to my friend, Raihana Ridzuan for her help, contribution, patience, support and encouragement. Last but not least, to my family to whom I dedicate this thesis for their endless love, support and great understanding. Finally, thanks to UPM for the financial support for this study which enabled me to complete my project works.

I certify that a Thesis Examination Committee has met on 9 December 2013 to conduct the final examination of Deanne Rudim on her thesis entitled "Micropropagation of *Zingiber montanum* Koenig and Evaluation of its Essential Oils for Antioxidative and Antimicrobial Activities" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Master of Science.

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LIST OF SYMBOLS AND ABBREVIATIONS

DMPBD	((E)-1(3,4-dimethylphenyl) butadiene
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl
2ip	2-isopentenyladenine
BAP	6-benzylaminopurine
ASTA	American Spice Trade Association
ANOVA	Analysis of Variance
AA	Antioxidant Activity
ASIUMAP	Asian Symposium on Industrial Utilization of Medicinal and Aromatic Plants
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CFU	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
DMRT	Duncan's Multiple Range Test
FIC	Ferrous Ion Chelating
FAO	Food and Agriculture Organization
GAE	Gallic Acid Equivalent
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
IBA	Indole butyric acid
IAA	Indole-3-acetic acid
KIN	Kinetin
MIC	Minimum Inhibitory Concentration
MS	Murashige and Skoog
NAA	Napthaleneacetic acid
NCCLS	National Committee for Clinical Laboratory Standards
NIST-MS	National Institute of Standards and Technology Mass Spectral
NIST08. LIB	NIST/EPA/NIH Mass Spectral Library
PGRs	Plant Growth Regulators
RCBD	Randomized Complete Block Design
RAP	Regional Office for Asia and the Pacific
RI	Retention index
Sp.	Species
SD	Standard division
SE	Standard error
TDZ	Thidiazuron
TPC	Total Phenolic Content
UV	Ultraviolet
USA	United States of America
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Introduction

Plant plays an important role in various aspects of human life. The use of plant dates back since the ancient time as a primary healthcare. Plants have been used as natural pesticides, flavorings, fragrances, medicinal compounds, fibers, beverages and food metabolites (Kaufman *et al*., 1999). The plant contains unique chemical profiles that offered pain relief, pleasant aromas and enhanced food flavors (Crake, 2007). These plants are known as medicinal and aromatic plants and their extracts became the main source for medicines, seasonings, colorings and preservatives used in societies (Crake, 2007).

Approximately, 70-80% of the populations residing in the vast rural areas of the developing and under developed countries still rely mainly on traditional herbal medicine (Siahsar *et al*., 2011). Medicinal plants are the only affordable and accessible source of primary health care for them, especially in the absence of access to modern medical facilities. This traditional use of plant provides ethnomedical information which can be used to identify plants with potential therapeutic interest. In fact, many of the herbal remedies which have been used ages ago are proven as effective treatments for various illness and diseases and has been used in today's medicine and developed into pharmaceutical drugs through scientific and innovative research.

However, while using traditional practices as reference, one must consider the fact that the plants may have evolved over a period of time resulting in changes in their phytochemical composition and hence their medicinal properties. Therefore, scientific validations to prove their traditional health claims are required. Biological screening of the traditional medicine will not only provide a scientific basis for their traditional healing properties but it also needed to establish the therapeutic efficacy of the medicinal plants.

Zingiber montanum has been used for centuries as traditional medicine for treatments of various conditions however there is little scientific evidence regarding to its medicinal activities. Moreover, the fact that there are very limited report of its micropropagation in Malaysia and no large scale cultivation of *Z. montanum* as more planting materials are needed, have motivated this study to be conducted. Therefore the intervention of biotechnology through plant tissue culture is needed as a tool for mass propagation of *Z. montanum*.

In this study, *in vitro* propagation of *Z. montanum* will be attempted using cytokinins and auxins to boost the growth and development of *Z. montanum* which can be used further for its large scale cultivation. The essential oils of *Z. montanum* mother plant will be isolated from different plant parts and the biological activities of the essential oil from the rhizome will be evaluated in respect to its phenolic content, antioxidative and antimicrobial activities to explore its therapeutic potential.

1.2 Problem Statement

The traditional healings have received wide acceptance among indigenous people in rural areas but urban society considered traditional medicine as alternative medicine apart from the modern medicine available around them. Therefore, they need the scientific validation for the traditional medicine. *Zingiber montanum* have been used in traditional medicine and have high medicinal value as it has been reported very effective as anti-inflammatory and antioxidant. It is known that the same species of plant can produce a similar essential oil, however with different chemical composition, resulting in different therapeutic activities due to various factors such as agronomic, geographical and climatic factors. The challenge is to prove whether *Z. montanum* which is indigenous to Malaysia has biological activities specifically antioxidative and antimicrobial potential.

The interest for the cultivation of *Z. montanum* comes from the necessity to guarantee a constant supply of plant materials to exploit its antioxidative and antimicrobial potential. Therefore, more planting materials are needed. Moreover, since there is no record of commercial planting for *Z. montanum* in Malaysia and most of the time, it only can be found in backyard garden, mass production of this plant species is necessary. However, this plant has slow multiplication rate and high risks of disease transmittance from the rhizomes used as planting materials make the conventional propagation of *Z. montanum* unfeasible and cause shortage of plant material. Alternative propagation through biotechnological approaches specifically plant tissue culture is seen as effective tools to produce large quantity of disease-free planting materials of *Z. montanum*.

1.3 Research Objectives

i) to establish a micropropagation system for *Z. montanum*
ii) to determine the presence of essential oils, the identity and the quantity of the chemical constituents in different parts of *Z. montanum* mother plant
iii) to determine the presence of phenolic content and analyse the biological activities of the essential oil extracted from *Z. montanum* mother plant using antioxidant and antimicrobial assays

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