UNIVERSITI PUTRA MALAYSIA

IN VITRO REGENERATION AND EVALUATION OF PHYTOCHEMICAL AND ANTIMICROBIAL ACTIVITY OF KUNYIT PUTIH [Curcuma zedoaria (Christm.) Roscoe]

KHALID ABUBAKER ALGHANNAY

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By

KHALID ABUBAKER ALGHANNAY

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

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DEDICATION

To those who have spent their lives to be always happy, **My Parents**

To those who stood with me morally and physically, **My Brothers and Sisters**

To which endure my circumstances and supported my suffering, **My Wife**

To those who I aspire and look forward to get benefit from my career and be better than me, **My Children**

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

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Chairman : Associate Professor Halimi Moh bin Saud, PhD
Faculty : Agriculture

Today's healthcare system is hampered with numerous health problems such as degenerative disorder, chronic diseases, resistant infection etc. Plants are source of precursors of many natural products and secondary metabolites with pharmacological and therapeutic potentials but some major set-back in plant natural product medicines are non-availability of medicinal plant material and in-ability to extract the bioactive compounds with the appropriate solvent. Therefore, there is highly need to develop an in vitro micro propagation technique for rapid multiplication to produce high quality planting materials of *C. zedoaria*, which is suffering from persistent endophytic and epiphytic microbial contamination and from low response to media, and to evaluate the phytochemicals screening method to explore their antioxidant compounds and antimicrobial properties. Some procedures do exist but generally do not address well the initial stage of culture establishment and phytochemical screening as well. Hence, the objectives of this study is to establish an in vitro regeneration protocol for *C.zedoaria* using tissue culture techniques. Secondly to optimize ideal solvent and concentrations suitable for screening the phytochemicals of *C. zedoaria* leaves and rhizome for Total Phenolic Content (TPC), Total Flavonoids Content (TFC) and Antioxidant activity (AO). The most suitable solvents (isopropanol for TPC and AOC/ methanol for TFC), from last experiment results, were applied as the third objective, as comparative study, to examine the extent of the difference between (NFGP) and (TCPP). Eventually, the evaluation of the antimicrobial activity of the different solvents and oils extracted from the leaves and rhizomes of *C. zedoaria* against pathogenic bacteria was carried out. Surface sterilization were assessed on explants (rhizome with apical buds), with 3 different sterilizing agents (NaOCl, HgCl2 and Nano Silver) at different concentrations and immersion times, in attempt to determining the most suitable method of reducing explant contamination. We detect the ability of 6-benzylaminopurine (BAP) alone for shoot induction, while (BAP), kinetin, and thidizuron (TDZ) were individually evaluated for shoot formation, however IBA and NAA was used separately to stimulate root formation. To screen for phytochemicals constituents, the leaf and rhizome of *C. zedoaria* were extracted with different polar solvents including ethanol, methanol, dimethyl sulfoxide, acetonitrile, acetone, isopropanol, and glycerol with different concentrations (0.0, 10, 30, 50, 70, 90, and 100%), We used Folin Ciocalteu's reagent, DPPH scavenging assay and colorimetric method using Alumunium Chloride, to determined TPC, AOX and TFC successively, while Disk Diffusion Test and also Minimum and Bactericidal Inhibitory Concentration (MIC and MBC) tests, was used to evaluate the antimicrobial activities. The results revealed that NaOCl was the most suitable sterilizing agent with 77.77% of sterilized success and survived explants compared to HgCl2 and NS after decontamination stage. The shoot induction and formation results showed that 3 mg/L of BAP strongly stimulated the shoot formation of C. *zedoaria* by producing 4.3 shoots per explants when compared to kinetin and TDZ. In evaluating the effects of different auxins on root induction of *C. zedoaria* revealed that 2.0 mg/L of NAA produced the highest root number by the mean of 6.3 roots per explant in *C. zedoaria* compared to other auxin hormonal treatments. The shoot induction and formation results showed that 3 mg/L of BAP strongly stimulated the shoot formation of *C. zedoaria* by producing 4.3 shoots per explants when compared to kinetin and TDZ. In evaluating the effects of different auxins on root induction of *C. zedoaria* revealed that 2.0 mg/L of NAA produced the highest root number by the mean of 6.3 roots per explant in *C. zedoaria* compared to other auxin hormonal treatments. The result of the phytochemical assay revealed that the highest flavonoid content in leaf tissue (3.309 mg) was achieved using dimethyl sulfoxide solvent (70%), methanol at 90% (3.01 mg), followed by 90% ethanol with a value of 2.7 mg, while in the rhizome, methanol displayed the highest concentration at 100 and 90 % by 4.8 and 4.5 mg respectively. Our finding showed that the Isopropyl alcohol was promising option for evaluating (AOC) and (TPC) due to its extractability potential on both Leaves & Rhizome tissues, while the Methanol is most suitable solvent for (TFC) in both cases too, despite competition and convergence of solvent impact. However, there was no significant difference in leaf and rhizome content for phytochemicals measured (TFC, TPC and AOC) in both NFGP and TCPP, hence there were no real differences using this propagation method (tissue culture) in certain phytochemical content. The antimicrobial inhibitory effect of *C. zedoaria* against certain pathogenic gram positive and gram negative revealed that these positive effects were present at all levels of all extracts (solvents, concentrations and type of tissues) and same is true for the effect of oil samples. This effect is incremental by increasing concentration each time. The effect of rhizome extract is relatively higher than that of leaf extract at all concentrations whereas, the solvent type did not have any obvious effect on inhibition, although minor effect was observed with isopropanol. The most MIC & MBC results is within the range of medium-impact rates, which has positive connotations and distinguished implications. In conclusion, *C. zedoaria* can be effective to treat diseases caused by bacterial infections.

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

PENJANAAN *IN VITRO* DAN PENILAIAN FITOKIMIA DAN AKTIVITI ANTIMIKROBIAL BAGI KUNYIT PUTIH [*Curcuma zedoaria* (Christm.) Roscoe]

Oleh

KHALID ABUBAKER ALGHANNAY

Mac 2019

Pengerusi : Profesor Madya Halimi Moh bin Saud, PhD
Fakulti : Pertanian

Sistem penjagaan kesihatan hari ini terjejas dengan banyak masalah kesihatan seperti gangguan degeneratif, penyakit kronik, jangkitan tahan dan lain-lain. Tumbuh- tumbuhan adalah sumber utama produk-produk semulajadi dan metabolit sekunder dengan potensi farmakologi dan terapeutik namun halangan utama dalam ubat berasaskan produk tumbuhan semula jadi adalah ketidakmampuan untuk mengeluarkan sebatian bioaktif dengan pelarut yang sepatutnya. Justeru, ianya sangat diperlukan untuk membangunkan teknik penanaman mikro in vitro untuk penggandaan yang cepat untuk menghasilkan bahan penanaman *C. zedoaria* yang berkualiti tinggi, yang mana mengalami pencemaran mikrob endophytic dan epiphytic yang berterusan dan mengalami tindak balas yang rendah terhadap media, dan juga untuk menilai kaedah pemeriksaan fitokimia untuk meneroka sifat sebatian antioksidan dan antimikrob. Terdapat beberapa prosedur yang ada tetapi pada amnya tidak sesuai untuk tahap awal pembentukan kultur dan juga pemeriksaan fitokimia. Oleh itu, objektif kajian ini adalah untuk mewujudkan protokol regenerasi in vitro untuk *C. zedoaria* menggunakan teknik kultur tisu. Kedua adalah untuk mengoptimumkan pelarut dan kepekatan yang sesuai untuk pemeriksaan fitokimia daun *C. zedoaria* dan rizom untuk Total Kandungan Fenolik (TPC), Total Kandungan Flavonoid (TFC) dan aktiviti Antioksidan (AO). Pelarut yang paling sesuai (isopropanol untuk TPC dan AOC / methanol untuk TFC), dari hasil eksperimen lepas, digunakan sebagai objektif ketiga, sebagai kajian perbandingan, untuk melihat sejauh mana perbezaan antara (NFGP) dan (TCPP). Akhirnya, penilaian aktiviti antimikrob bagi pelarut dan minyak yang berbeza yang diekstrak dari daun dan rizom *C. zedoaria* terhadap bakteria patogen telah dijalankan. Pensterilan awal dinilai pada eksplan (rizom dengan tunas apikal), dengan 3 agen steril yang berbeza (NaOCl, HgCl2 dan Nano Silver) pada kepekatan dan masa penyerapan yang berlainan, dalam usaha untuk menentukan kaedah yang paling sesuai untuk mengurangkan pencemaran pada eksplan. Kami mengesan keupayaan 6-benzylaminopurine (BAP) sendirian untuk induksi pucuk, sementara (BAP), kinetin, dan thidizuron (TDZ) secara individu dinilai untuk pembentukan pucuk, namun IBA dan NAA digunakan secara berasingan untuk merangsang pembentukan akar. Untuk memaparkan unsur-unsur fitokimia, daun dan rizom *C. zedoaria* diekstraks dengan pelarut polariti yang berbeza termasuk etanol, metanol, dimetil sulfoksida, asetonitril, aseton, isopropanol, dan gliserol dengan kepekatan yang berbeza (0.0, 10, 30, 50, 70, 90, dan 100%), kami menggunakan reagen Folin Ciocalteu, kaedah DPPH pengarkian dan kaedah kolorimetrik menggunakan Alumunium Chloride, untuk menentukan TPC, AOX dan TFC secara berturut-turut, manakala ujian Penyebaran Cakera dan ujian Kepekatan Minimum dan Bakterisidal (MIC dan MBC) digunakan untuk menilai aktiviti antimikrob. Keputusan menunjukkan bahawa NaOCl adalah agen penyerap yang paling sesuai dengan 77.77% kejayaan steriliti dan eksplan yang terselamat berbanding dengan HgCl2 dan NS selepas peringkat dekontaminasi. Keputusan induksi dan perbentukan pucuk menunjukkan bahawa 3 mg/L BAP sangat merangsang pembentukan tembakau *C. zedoaria* dengan menghasilkan 4.3 pucuk per eksplan apabila dibandingkan dengan kinetin dan TDZ. Dalam menilai kesan-kesan auksin yang berbeza pada induksi akar *C. zedoaria* mendedahkan bahawa 2.0 mg / L NAA menghasilkan bilangan akar tertinggi dengan purata 6.3 akar per eksplan *C. zedoaria* berbanding dengan rawatan hormon auxin yang lain. Hasil ujian fitokimia menunjukkan bahawa kandungan flavonoid tertinggi dalam tisu daun (3.309 mg) dicapai menggunakan pelarut dimetil sulfoksida (70%), metanol pada 90% (3.01 mg), diikuti oleh 90% etanol dengan nilai 2.7 mg, manakala dalam rizom, metanol menunjukkan kepekatan tertinggi pada 100 dan 90% masing-masing sebanyak 4.8 dan 4.5 mg. Penemuan kami menunjukkan bahawa alkohol Isopropil adalah pilihan yang bagus untuk menilai (AOC) dan (TPC) kerana potensi kebolehan ekstrasi pada kedua-dua Daun & Rhizome tisu, sementara Metanol adalah pelarut paling sesuai untuk (TFC) dalam kedua-dua kes, walaupun terdapat persaingan dan pertindihan kesan pelarut. Walau bagaimanapun, tidak terdapat perbezaan yang signifikan dalam kandungan daun dan rizom untuk ujian fitokimia yang direkod (TFC, TPC dan AOC) pada kedua-dua NFGP dan TCPP, oleh itu tidak terdapat perbezaan yang ketara menggunakan kaedah penanaman (kultur tisu) dalam kandungan fitokimia tertentu. Kesan penghalang antimikrob daripada *C. zedoaria* terhadap sebahagian pathogen gram positif dan gram negatif mendedahkan bahawa kesan positif ini berlaku pada semua peringkat ekstrak (pelarut, kepekatan dan jenis tisu) dan sama juga berlaku untuk sampel minyak. Kesan ini semakin meningkat dengan setiap kali peningkatan dalam kepekatan. Kesan ekstrak rizom adalah lebih tinggi daripada ekstrak daun pada semua kepekatan sedangkan jenis pelarut tidak mempunyai sebarang kesan jelas pada perencatan, walaupun kesan kecil diperhatikan dengan isopropanol. Hasil MIC & MBC yang paling banyak adalah dalam lingkungn kadar kesan sederhana, yang mempunyai impak positif dan implikasi yang berbeza. Kesimpulannya, *C. zedoaria* berkesan untuk merawat penyakit yang disebabkan oleh jangkitan bakteria.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Halimi bin Mohd Saud, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Uma Rani a/p Sinniah, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Intan Safinar binti Ismail, PhD
Associate Professor
Faculty of Sience
Universiti Putra Malaysia
(Member)

____

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: ______________________________

Name of Member of Supervisory Committee: Professor Dr. Uma Rani a/p Sinniah

Signature: ______________________________

Name of Member of Supervisory Committee: Associate Professor Dr. Intan Safinar binti Ismail

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A. anitratus	Acinetobacter anitratus
AD	Air Drying
ATCC	American Type Culture Collection
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
CFU	Colony forming unit
DMSO	Dimethylsulfoxide
E. coli	*Escherichia coli*
FAO	Food and Agriculture Organization
IBS	Institute of Bioscience
K. pneumonia	*Klebsiella pneumonie*
L. monocytogenes	*Listeria monocytogenes*
MBC	Minimal bactericidal concentration
MHA	Mueller Hinton agar
MIC	Minimal inhibitory concentration
NFGP	Naturally Field-Grown Plant
P. acane	Propionibacterium acne
S. aureus	*Staphylococcus aureus*
S. mutans	*Streptococcus mutans*
TCPP	*Tissue Cultured Produced Plants*
UPM	Universiti Putra Malaysia
WHO	World Health Organization
DMSO	Dimethyl Sulfoxide
DPPH	DPPH radical scavenging assay
ISOPRO	IsoPropanol

OD	Oven Drying
GAE	Gallic Acid Equivalent
MeOH	Methanol
EtOH	Ethanol
Aq	Water
mL	MilliL
ºC	Degree in Celsius
ppm	Part Per Million
TAC	Total Antioxidant Contents
TFC	Total Flavonoid Content
TPC	Total Phenolic Contents

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Major challenges facing today's healthcare system are issues such as chronic diseases, resistant infections, autoimmune disorder, and degenerative disease of aging and insecure medicines, despite the advancement in medical sciences (Tholkappiyavathi et al., 2013). The rhizomes of Curcuma zedoaria have been extensively used in the treatment of ulcers, wounds, tumors, atherosclerosis, and inflammation as well as various traditional preparations used for the treatment of Ohyul syndrome, a major caused of blood stagnation (Seo et al., 2005; Tariq et al., 2016). Bharalee et al, (2005), confirms that their rhizomes, are traditionally used as appetizer and antipyretic and its useful in bronchitis, asthma, tuberculosis and also against enlargement of spleen. Rhizomes are also used as tonic to the brain and heart, expectorant and in treatment of pains, inflammation, toothache, bruises and sprains.

Curcuma zedoaria is a perennial herb and member of the genus Curcuma, family Zingiberaceae (Ullah et al., 2014; Tipthara et al, 2007). It is a potential source of therapeutic molecules for the treatment of a range of ailments as the genus is credited with anti-inflammatory, antibiotic, antiviral, anticancerous, hypocholestraemic, choleretic, antidiabetic, antihepatotoxic, antivenomous, and antirheumatic properties (Parthasarathy et al., 2006; Safitri et al., 2017; Xiong et al., 2017). The Curcuma has attracted attention since last 3 decades.

The *C. zedoaria* is associated with phenolic and flavonoid compounds possessing strong antioxidant activities (Avanço et al., 2017; Tipthara et al, 2007). However, there are conflicting reports on the phenolic content and contribution of phenolic compound to the total antioxidant activities of *C. zedoaria* (Kim et al., 2011; Ghasemzadeh et al., 2011; Hossain et al., 2011). It is also showed that the extraction yield of phenolic and flavonoid content is greatly depending on the solvent polarity (Senathilake et al., 2016). The antioxidant activity of polyphenols is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations (Złotek et al., 2016). Presently, the focus has been shifted to naturally occurring antioxidant. The use of natural antioxidant is considered to be safe rather than synthetic as latter has the potential for carcinogenic. The antioxidants present in diet can reduce attacks and risks of diseases by reducing the free radicals.

Tissue culture technique has been successfully used to grow wild plants that are difficult to propagate through the conventional ways or plants with certain defects (Lima et al., 2012). Especially, when the populations have decreased as a result of over exploitation by destructive harvesting, and when there is a growing demand for clonally uniform elite plants and, also, when species have been over collected by hobbyists for medicine, food or fragrance, then, in vitro propagation can provide an alternate source of plants and alleviate the pressures on wild populations (Kapai et al., 2010). Plant tissue culture is an enabling technology from which many novel tools have been developed to assist plant breeders to increase the speed or efficiency of the breeding process and to create a new variation for crop improvement (Pikulthong et al., 2016). However, the establishment of a good axenic culture is another inherent problem with Curcuma rhizome (Das et al., 2010a).

1.2 Problem Statement and Justification

The screening of phytochemicals has been utilized for exploring antioxidant compounds in plants (Tiwari et al., 2011; Wadood et al., 2013; Ribeiro et al., 2016). However, the yield of extraction and antioxidant activity does not only depend on the extraction method, and also on the solvent used for extraction. The current increase in demand for *C. zedoaria* is the motivating factor to investigate its biochemical properties and the actual content in various tissue, due to lack of studied screening methods for solvent to be used. The solubility of antioxidant compounds in *C. zedoaria* with different polarities and chemical characteristics is influenced by the solvent of extraction. (Do et al., 2014). The increase in antibiotic resistance and failure of chemotherapeutics exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Du Toit and Rautenbach, 2000; Wilson et al., 2005; Wayne et al., 2012; Medini et al., 2014). However, the test systems ought to be simple, rapid, reproducible, and inexpensive and maximize high sample throughput in order to cope with a varied number of extracts and fractions (Dias et al., 2012).

The methods of propagation are slow, inconsistent, and it suffers frequent crop loss. Hence, it's imperative to provide more attractive, flexible and innovative way to accelerate the acquisition of healthy plants in reasonable quantities and these drawbacks is also coupled with high rate of contamination of tissue culture of *C. zedoaria* plant. Where the stages of in vitro propagation of *C. zedoaria* comprises of selection explants, aseptic culture establishment, multiplication of propagules, rooting, and acclimatization (Anisuzzaman et al., 2008). Sterilization is the most challenging step of explants aseptic culture establishment (Bhattacharya et al., 2014). Among the various explants previously tested, rhizome buds have been found to be very suitable material for in vitro propagation (tissue culture) of Curcuma (Islam et al., 2004; Roy and Raychaudhuri, 2004; Tipthara et al, 2007). Among the common problems of explants from wild plants include; diseased specimens, or plant parts located close to or below the soil that make it difficult or impossible to disinfect due to both endophytic and epiphytic microbes (Reed et al., 1995).

Thus, a tissue culture technique will play an important role in the study of *C. zedoaria* plants (Loc et al., 2005). It is deemed important to develop a Micropropagation technique to make commercially available of pathogen-free germplasm. The tissue culture offers an alternative tool for rapid multiplication and conservation of disease- free propagules, which will further enable uninterrupted supply of raw materials. In this project, *C. zedoaria* leaves and rhizome from Malaysian traditional plants which has been used in folkloric practices for the management of diseases was selected for this study.

Thus, the hypotheses of this study states that, the selected cytokines and auxins have distinctive potential to stimulate shoot growth and root induction respectively by micro propagation techniques and extract from in vitro culture of *C. zedoaria* with high antioxidative and flavonoid content will have antibacterial effect against pathogenic bacterial. Therefore, the main objectives of this study are:

1. To establish in vitro regeneration protocol for Kunyit Putih (*C. zedoaria*) using tissue culture techniques.
2. To optimize ideal solvents and its concentrations require for the selection and screening of various phytochemical extracts (secondary metabolite) of *C. zedoaria* in leaf and rhizome for total phenolic content (TPC) and total flavonoids content (TFC), and antioxidant activity (AO).
3. To compare extractability of phytochemicals contents using methanol and isopropanol on total flavonoids and phenolic/antioxidant contents of Kunyit Putih leafs and rhizomes before and after tissue culture for in vitro and in-vivo study.
4. To evaluate the antimicrobial activity of the different solvent extracts of leaves, rhizomes, and oils of *C. zedoaria* against gram-positive and negative pathogenic bacteria.

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BIODATA OF STUDENT

Khalid Abubaker Alghannay, was born on August 15, 1969 in Sebha city located in the southern province of Libya. He had obtained a high school certificate in basic sciences, Biology Division in 1988, from Brak Secondary School, and then obtained a Bachelor of Agricultural Sciences, Department of Plant Production, Sebha University in the 1993. He completed his specialization in the field of horticulture by being able to obtain a master's in agricultural sciences, by studying the nutrition of evergreen Orchids (fruit trees), specifically in 2001, from University of Tripoli, where his primary interests focused on foliar fertilization and its impact on the productivity of grape vines and their natural characteristics. He derived his experiences through his work as a faculty staff member at a university of Sabha, and his skills expanded by supervising some graduation research at that time. Currently, he is about to complete his PhD from the esteemed UBM University, Faculty of Agriculture, Department of Agriculture Technology, which focused on devising a sterilization protocol for one of the commonly used ginger varieties in Malaysia and developing a method for its propagation through tissue culture technology. His study also included surveying the methods of extracting some phytochemicals and exploring the extent of their effect on inhibiting the growth of some pathogenic bacteria.

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