UNIVERSITI PUTRA MALAYSIA

SCREENING OF ANTIFUNGAL COMPOUND ISOLATED FROM CATHARANTHUS ROSEUS L. (PINK) FOR BIOLOGICAL CONTROL OF SELECTED PLANT DISEASES

ROZIHAWATI BINTI ZAHARI

FPAS 2015 3

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

July 2015

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in fulfillment of the requirements for the degree of Doctor of Philosophy

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July 2015

Chairman: **Normala Binti Halimoon, PhD**
Faculty: **Environmental Studies**

Diseases of rubber (*Hevea brasiliensis*) caused by pathogens, *Rigidoporus microporus, Ganoderma philippii* and *Phellinus noxius. Fusarium oxysporum, F. solani* and *Colletotrichum gloeosporioides* on chilli (*Capsicum annuum*) are currently being kept under control with chemical fungicides. However, these fungicides have been shown to have hazardous effects to humans and the ecosystems. To address these problems, the search for an effective and environmentally safe compound to control these harmful pathogens is highly warranted. Thus, in this study, antifungal compounds from selected plants, *Aglaia argentea, A. leucophylla, A. grandis, A. odoratissima, A. variisquama, Cassia alata, Catharanthus roseus, Derris elliptica* and *Tinospora baenzigeri* were screened for their effectiveness in controlling the growth of selected fungal pathogens on seedlings. The result showed that *C. roseus* extract was the most effective in inhibiting the growth of pathogens as the extract contains various antifungal compounds such as phenolics, alkaloids, essential oils and flavonoids. Although the extract of *C. roseus* contains abundant bioactive compounds, detailed studies on their biological activities on those fungal pathogens have yet to be reported. The *C. roseus* extracted with dichloromethane (DCM) showed the greatest inhibition zone diameter in controlling *R. microporus* and *F. oxysporum* at the values of 11.29 mm and 8.10 mm, respectively, compared to other selected medicinal plant extracts. The *C. roseus* extract assessed based on conidia and hyphae growth of *F. oxysporum* and *R. microporus,* respectively showed minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC). MIC and MFC results showed that the extract at 270 µg/mL with inhibition value of 0.0 µg/mL was the most effective in controlling the growth of *F. oxysporum* and *R. microporus* compared to 200, 140, 70 and 10 µg/mL. The antifungal compound isolated from *C. roseus* extract was determined through thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) analysis. Each *C. roseus* of DCM extracts

was marked as CRD5a, CRD5b, CRD5c, CRD5d, CRD5e, CRD5f and CRD5g. The TLC results showed that all of the *C. roseus* extracts peak with red in colour at *Rf*= 0.61 at 366 nm wavelength, except for CRD5g. The CRD5d extract was the most effective against *G. philippii* and *R. microporus* with inhibition zones of 3.5 and 1.9 mm, respectively, compared to other extracts. However, CRD5g extract was the most effective against *F. oxysporum* with a value of 3.0 mm compared to other extracts. HPLC results also showed the major peak is at 210 nm. The CRD5d extract isolated contained single compound such as ursolic acid after being detected by NMR analysis. The compound was effective to control *R. microporus* and *G. philippii* with inhibition zone values of 4.0 and 3.0 mm, respectively. In addition, the efficacy of *C. roseus* extracts against *F. oxysporum* and *R. microporus* was assessed based on healthy effects percentage of the chilli and rubber seedlings, respectively, by assessing the symptoms on leaves and roots. The assessment was based on disease incident (DI%) and disease suppression (DS%). The DI% results showed that an extract at 2,000 µg/mL was the most effective in controlling *F. oxysporum* on chilli seedlings with value of 0.0%, compared to 1000 and 1500 µg/mL with values of 60 and 80%, respectively. The DS% results also showed that the extract was most effective at 2000 µg/mL in controlling the growth of *R. microporus* on rubber seedlings with a value of 100% compared to 1500 and 1000 µg/mL with a value of 0.0%. In conclusion, *C. roseus* extracted with DCM contains an effective toxin that is detrimental on the plant pathogenic fungi. The *C. roseus* of DCM extract isolated contains ursolic acid and the compound effective against *R. microporus* and *G. philippii*. Hence, *C. roseus* extract should also be developed as a biofungicide for controlling *R. microporus* and *F. oxysporum* on rubber and chilli, respectively.

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

PENGASINGAN BAGI KANDUNGAN ANTI-KULAT DARIPADA *CATHARANTHUS ROSEUS* L. (MERAH JAMBU) YANG TELAH DIPISAHKAN UNTUK KAWALAN BIOLOGI PADA PENYAKIT POKOK YANG TERPILIH

Oleh

ROZIHAWATI BINTI ZAHARI

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Pengerusi : Normala Binti Halimoon, PhD
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Ketika ini, penyakit getah (*Hevea brasiliensis*) yang diserang oleh perosak-perosak seperti *Rigidoporus microporus, Ganoderma philippii* dan *Phellinus noxius,* manakala *Fusarium oxysporum, F. solani* dan *Colletotrichum gloeosporioides* terhadap cili (*Capsicum annuum*) adalah dikawal oleh pelbagai racun kulat kimia. Bagaimanapun, racun-racun kulat ini mempunyai kesan-kesan yang sangat merbahaya terhadap manusia dan ekosistem alam. Penyelesaian masalah-masalah tersebut dengan menumpukan kandungan kesan alam sekitar yang selamat untuk mengawal perosak-perosak adalah sangat diperlukan. Justeru itu, dalam kajian ini, kandungan anti-kulat daripada tumbuhan yang terpilih iaitu *Aglaia argentea, A. leucophylla, A. grandis, A. odoratissima, A. variisquama, Cassia alata, Catharanthus roseus, Derris elliptica* dan *Tinospora baenzigeri* adalah telah disaring untuk keberkesanan tumbuhan tersebut dalam mengawal kulat-kulat perosak tersebut terhadap anak pokok. Keputusan ini telah menunjukkan bahawa ekstrak *C. roseus* adalah yang paling berkesan dalam merencatkan pertumbuhan kulat-kulat perosak, yang mana ekstrak ini terkandung pelbagai kandungan anti-kulat seperti phenolics, alkaloids, essential oils dan flavonoids. Sungguhpun ekstrak *C. roseus* ini mengandungi banyak kandungan bioaktif, kajian terperinci terhadap aktiviti biologi pada kulat-kulat perosak tersebut masih belum dilaporkan. *C. roseus* yang telah diekstrak dengan dichloromethane (DCM) menunjukkan perencatan zon diameter yang terbesar untuk mengawal *R. microporus* dan *F. oxysporum* dengan nilai 11.29 mm dan 8.10 mm masing-masing, berbanding dengan ekstrak-ekstrak tumbuhan ubatan yang lain. Ekstrak *C. roseus* ini telah dinilai berdasarkan pada pertumbuhan spora bagi *F. oxysporum* and hyphae bagi *R. microporus,* telah menunjukkan minimum inhibition concentration (MIC) dan minimum fungicidal concentration (MFC). Keputusan-keputusan MIC dan MFC telah menunjukkan ekstrak ini pada 270 µg/mL dengan nilai perencatannya 0.0 µg/mL adalah yang paling efektif untuk

mengawal pertumbuhan *F. oxysporum* dan *R. microporus* berbanding dengan 200, 140, 70 and 10 µg/mL. Kandungan anti-kulat yang telah dipisahkan daripada ekstrak *C. roseus* adalah ditentukan melalui thin layer chromatography (TLC), high performance liquid chromatography (HPLC) dan nuclear magnetic resonance (NMR) analysis. Setiap ekstrak *C. roseus* bagi DCM ini telah dilabelkan seperti CRD5a, CRD5b, CRD5c, CRD5d, CRD5e, CRD5f dan CRD5g. Keputusan TLC telah menunjukkan kesemua peak yang berwarna merah berada di *Rf* = 0.61 pada 366 nm wavelength, kecuali CRD5g. Ekstrak CRD5d adalah yang efektif terhadap *G. philippii* dan *R. microporus* dengan zon perencatannya 3.5 dan 1.9 mm masing-masing, berbanding dengan ekstrak-ekstrak yang lain. Bagaimanapun, ekstrak CRD5g adalah yang paling efektif terhadap *F. oxysporum* dengan 3.0 mm berbanding dengan ekstrak-ekstrak yang lain. Keputusan HPLC juga telah menunjukkan peak yang besar pada 210 nm. Ekstrak CRD5d mengandungi kandungan tunggal iaitu ursolic acid setelah dikesan oleh analisis NMR. Kandungan ini adalah berkesan mengawal *R. microporus* dan *G. philippii* dengan nilai perencatannya 4.0 dan 3.0 mm, masing-masing. Tambahan pula, keberkesanan ekstrak *C. roseus* ini terhadap *F. oxysporum* dan *R. microporus* adalah telah dinilai berdasarkan peratus kesan kesihatan cili dan getah masing-masing, dengan menilai simptom-simptom pada daun dan akar. Pernilaian ini adalah berdasarkan disease incident (DI%) dan disease suppression (DS%). Keputusan DI% telah menunjukkan ekstrak 2,000 µg/mL adalah yang paling efektif untuk mengawal *F. oxysporum* pada anak pokok cili dengan nilainya 0.0%, berbanding dengan 1000 dan 1500 µg/mL dengan nilainya 60 dan 80%, masing-masing. Keputusan DS% juga telah menunjukkan ekstrak ini yang paling efektif pada 2000 µg/mL untuk mengawal pertumbuhan *R. microporus* terhadap anak pokok getah dengan nilainya 100% berbanding dengan 1500 dan 1000 µg/mL adalah nilai 0.0%. Kesimpulanya, ekstrak *C. roseus* daripada DCM mengandungi kesan racun yang efektif terhadap kulat perosak tumbuhan. Ekstrak ini juga yang telah diasingkan mengandungi ursolic acid dan ia efektif terhadap *R. microporus* dan *G. philippii*. Hakta itu, ekstrak ini seharusnya diusahakan untuk dijadikan sebagai bio-antikulat untuk mengawal *R. microporus* and *F. oxysporum* terhadap anak pokok getah dan cili, masing-masing.

ACKNOWLEDGEMENTS

Bismillahirrahmannirrahim,

Firstly I give thanks to merciful Allah for giving me the strength to complete this study. I would like to express my sincere appreciation and thanks to my chairman, Dr. Normala Binti Halimoon, my supervisor, Prof. Dr. Ahmad Said Bin Sajap and Dr. Mohd Farid Bin Ahmad as my committee member for their suggestions, advice, support and guidance throughout my study.

My appreciation and gratitude go to my mother, Hajah Habibah Binti Othman and my father, Haji Zahari Bin Abd. Rahman for their constant support, love and for being so patient throughout my graduate study. Thanks to my husband Mohd Fuzi Bin Bidin, my son Muhammad Firdaus Ikhwan Bin Mohd Fuzi (10 years) and my daughter Nur Farisha Adelia Binti Mohd Fuzi (1 year) for their support, wonderful patience and care. Thank you very much.

Sincere thanks and gratitude are also extended to all staff of Pathology Laboratory and Phytochemical Laboratory, FRIM, Kepong, especially Mrs. Anida Binti Zakaria, Dr. Ling Ki Siong for all their help. Thanks also to Dr. Mastura Binti Mansor and Mrs. Hanan from Anti-microbial Laboratory for their help towards the success of this project.

I certify that a Thesis Examination Committee has met on 27 July 2015 to conduct the final examination of Rozihawati binti Zahari on her degree of Doctor of Philosophy on her thesis entitled "Screening of antifungal compound isolated from *Catharanthus roseus* L. (pink) for biological control of selected plant diseases" 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 Doctor of Philosophy.

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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 Supervisor Committee were as follows:

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C	carbon
ºC	degree Celsius
cm	centre meter
CRD	Complete block design
DCM	dichloromethane
DMSO	dimetil sulfuric acid
g	gram
HPLC	high performance liquid chromatography
nm	nanometer
NMR	Neuron Magnetic Resonance
MIC	minimum inhibitory concentration
ME	malt extract
MFC	minimum fungicidal concentration
mg	milligram
mL	milliliter
mg/mL	milligram per milliliter
mm	millimeter
µL	micro liter
PDA	Potato dextrose agar
PDB	Potato dextrose borth
%	percentage
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION

1.1 Introduction

Rubber (*Hevea brasiliensis*) and chilli (*Capsicum annuum*) are two plants that receive high demand throughout the world and thus, contribute to a country's economy such as in Malaysia (Van Beilen and Poirier, 2007; Tey *et al*., 2008). For many years, rubber and chilli have been attacked by various fungal species. Reported by Baraka *et al.* (2011) and Mohd Farid (2010), *Phellinus noxius, Rigidoporus microporus* and *Ganoderma philippii* are among the most common fungi that cause root rot diseases in rubber plantations. As for chilli, the plant is commonly attacked by *F. oxysporum* and *F. solani* (Maja, *et al.,* 2012; Abu Taleb, *et al.,* 2011). In addition, *Colletotrichum gloeosporioides* has also been identified to cause serious leaf spot and fruit diseases in chilli (Ajay *et al*., 2012; Sittisack *et al*., 2010).

Currently, these diseases are kept under control with chemical fungicides (Jahanshir and Dzhalilov, 2010; Anonymous, 2015) which result in maintaining better stands, in creating more vigorous plants and in increasing yields. However, these fungicides posed various problems to the environment and ecosystems (Kaewchai and Soytong, 2010). To overcome these problems, it is important to develop an environmentally safer method to control these diseases. According to Alam (2009), some plant extracts contain environmentally safe compound and have the potential to be used as the much needed biopesticides to control plant diseases.

Catharanthus roseus is one of the plants identified as containing environmentally safe compounds for controlling fungal pathogens. For example, the plant contains 2,3-dihydroxybenzoic acid, 3,10 dinitrodiftalone and desmethylomifensine which was shown to be effective against various fungi such as *Phytium aphaniderma*, *Aspergillus fumigatus, Candida albicans, P. chrysogenum* and *A. niger* (Moreno *et al*., 1994a; Balaabirami and Patharajan, 2012). The *C. roseus* also contains various chemical compounds such as alkaloid indole terpenoids, phenolics, alkaloids, essential oils and flavonoids (Arvind *et al*., 2008; Natali and Robert, 2007; Shashi *et al*., 2006; Tikhomiroff and Jolicoeur, 2002). Although *C. roseus* has bioactive compounds, not much attention has been given to this plant, and there is little research done on its effectiveness in controlling *P. noxius, R. microporus G. philippii*, *F. oxysporum, F. solani* and *C. gloeosporioides.* Thus, there is a need to carry out *in-vitro* and *in-vivo* tests to determine antifungal activities of the selected medicinal plant extracts as bio-control agent against fungal pathogens. Future studies could also investigate the effectiveness of the extracts of other medicinal plant species in determining their potential to be developed as bio-antifungal agents.

1.2 Objectives of the Study

The general objective or scope of the study is to screen selected medicinal plant extracts against common fungi; *P. noxius, R. microporus, G. philippii*, *C. gloeosporioides*, *F. oxysporum* and *F. solani*. Among the selected medicinal plant extracts, only the extract of *C. roseus* was found to be most effective in controlling fungal pathogens and in determining antifungal bioactive compounds. The extract of *C. roseus* was also tested to determine its effective against *F. oxysporum* on chilli (*C. annuum*) seedlings and *R. microporus* on rubber (*H. brasiliensis*) seedlings. The specific objectives are:

i. to screen and compare selected medicinal plant extracts for antifungal activities;

ii. to isolate and determine *C. roseus* extract compound based on the effectiveness of antifungal activities;

iii. to assess the efficacy of *C. roseus* extracts against *F. oxysporum* on chilli seedlings and *R. microporus* on rubber seedlings.

1.3 Significance of the study

Chemical crop protection has a vital role in securing food supplies for the growing global population. However, a challenge in crops protection management is to protect the agricultural commodities from harmful agrochemicals (Janna, 2008; Wayne, 2013). Some fungi have evolved to resist toxins in such agrochemicals; therefore, higher doses of chemical fungicides have to be used especially on rubber and chili crops which indirectly, increases environmental pollution and creates ecological disturbances (Chan *et al*., 1991; Jahanshir and Dzhalilov, 2010). In addition, a study on the effect of bio-fungicides at the seedling stage, before the plants are attacked by fungal pathogens is important to ensure adequate production of planting stock. Thus, production of new fungicidal products for crop protection should be effective in reducing pest populations and should have low toxicity to human and other mammals (Janna, 2008).

In order to achieve this, it is important to search for an effective and environmentally safe compound in controlling fungal pathogens. Maria *et al.* (2007) stated that organic fungicides from some medicinal plants contain alkaloids, terpenoids, phenolics, essential oils and flavonoids which are effective against fungal pathogens. These compounds provide a pool of rich biologically active compounds in agrochemical research. Thus, focus on selected medicinal plants especially *Aglaia argentea, A. leucophylla, A. grandis, A. odoratissima, A. variisquama, Cassia alata, Tinospora baenzigeri, Derris elliptica* and *Catharanthus roseus* that contain antifungal active compounds is needed to replace the existing chemical fungicides.

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