Evaluation of Antifungal and Phytochemical Activity from Different Parts of *Cerbera odollam* Gaertn

by

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LIST OF ABBREVIATIONS

BST	Brine Shrimp Test
DFB	Diflubenzuron
ESI	Electron Spray Ionization
FCX	Flucycloxuro
LCMS	Liquid Chromatography Mass Spectrometry
MIC	Minimum Inhibition Concentration
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
TIC	Total Ion Current
TLC	Thin Layer Chromatograhy
NMR	Nuclear Magnetic Resonance

Penilaian Aktiviti Antikulat dan Fitokimia daripada Bahagian-bahagian Berbeza *Cerbera Odollam* Gaertn

ABSTRAK

Penggunaan racun kulat kommercial berlebihan dalam sektor pertanian telah mengakibatkan pencemaran alam sekitar yang akan membahayakan kesihatan manusia. Kajian ini bertujuan untuk meneroka kegunaan ekstrak tanaman (botani) sebagai pengganti kepada sintetik racun kulat. Ekstrak Etanol daripada bahagian tumbuhan *Cerbera odollam* seperti daun, bunga, buah, biji benih, kayu, dan kulit kayu telah diuji bagi menentukan sifat antikulat. Bioesei antikulat telah dijalankan dengan menggunakan kaedah pencairan dengan kepekatan yang berlainan (500 ke 3000 ppm) terhadap kulat berikut: *Aspergillus niger, Fusarium oxysporum* and *Penicilium citrinum* dan seterusnya dinilai dengan *Minimum Inhibitory Concentration* (MIC). Toksikologi tanaman dikaji dengan kaedah *Brine Shrimp Test* (BST) di mana kepekatan yang berbeza digunakan (5 ke 1280 ppm). Kajian fitokimia pula telah dijalankan melalui prosedur piawai untuk mengetahui sebatian fitokimia yang terlibat dalam aktiviti antikulat. Kaedah *Liquid Chromatography Mass Spectrometry* (LCMS) telah dijalankan untuk menentukan sebatian antikulat yang wujud dalam ekstrak tanaman. Keputusan kajian secara umumnya telah menunjukkan zon inhibition terhadap kulat di semua bahagian yang dikaji. Antara bahagian yang dikaji, daun menunjukkan kesan antikulat yang penting ($P \leq 0.05$) terhadap *Aspergillus niger* and *Fusarium oxysporum* dalam semua kepekatan (500 ke 3000 ppm) di mana 3000 ppm adalah terbaik antaranya. Walau bagaimanapun, tiada kesan ketara apabila ekstrak diuji ke atas *Penicilium citrinum*. Dalam kajian MIC, dos yang paling rendah dari daun dan kulit kayu adalah 250 ppm terhadap *Aspergillus niger* manakala biji benih, buah, bunga dan kayu menunjukkan 500 ppm; *Penicilium citrinum* and *Fusarium oxysporum* merekodkan 500 ppm untuk semua ekstrak. Bagi kajian toksikologi, nilai kritikal selamat ialah 20 ppm ke atas dan 2 ppm ke bawah adalah tidak selamat untuk manusia. Kayu (5619.97 ppm), buah (2116.66 ppm), kulit kayu (1745.04 ppm) dan bunga (64.47 ppm) selamat diggunakan manakala tahap selamat bagi daun (8.31 ppm) dan biji benih (3.62 ppm) adalah sedikit toksik dan harus digunakan dengan waspada. Kajian LCMS daripada ekstrak bunga dan buah *Cerbera odollam* adalah yang pertama. Sebatian antikulat yang telah dikesan ialah *neriifoli*n, asid hidroksibenzoik, asid cerberinik, asid salisilik dan asid terepthtalik. *Neriifolin* dan asid hidroksibenzoik merupakan sebatian yang pertama kali dikenal pasti dalam ektrak buah, bunga, dan kayu; asid cerberinik, asid salisilik dan asid terepthtalik dalam ekstrak kulit kayu. Kajian ini menunjukkan daun *Cerbera odollam* mempunyai potensi untuk dijadikan racun kulat bio.

Evaluation of Antifungal and Phytochemical Activity from Different Parts of *Cerbera Odollam* Gaertn

ABSTRACT

Heavy usage of commercial fungicide in the agricultural sector has resulted in environmental pollution that has imposed a significant risk to human health. The current research intended to explore the use of plant extracts (botanical) as alternative to synthetic fungicide. Ethanolic extracts of *Cerbera odollam* from leaf, flower, fruit, seed, wood and bark were tested for antifungal properties. Antifungal bioassay was performed through dilution method at various concentrations (500 ppm to 3000 ppm) against fungi: *Aspergillus niger*, *Fusarium oxysporum* and *Penicilium citrinum* and assessed based on the Minimum Inhibitory Concentration (MIC). Plant toxicity was tested by Brine Shrimp Test (BST) at different concentrations (5 to 1280 ppm). Phytochemical tests were done using standard procedures to identify the phytochemical compounds involved in the antifungal activity and Liquid Chromatography Mass Spectrometry (LCMS) was performed to determine possible antifungal compounds existed in all extracts. The results of the research showed that the inhibition zone for the tested fungi generally exhibited antifungal activity from different parts. Among the treatments, leaf extracts had recorded significant antifungal effects ($P \leq 0.05$) against *Aspergillus niger* and *Fusarium oxysporum* at all concentrations (500 to 3000 ppm) with 3000 ppm showing the best inhibition zone. However, there was no significant difference when extracts were tested against *Penicilium citrinum*. For the MIC study, the lowest dosage recorded for leaf and bark was 250 ppm against *Aspergillus niger*, while seed, fruit flower and wood showed the effect at 500 ppm; *Penicilium citrinum* and *Fusarium oxysporum* recorded MIC value at 500 ppm from all treatments. In toxicology test, the safe value is an amount over 20 ppm while 2 ppm and below is considered unsafe or toxic for human being. Wood (5619.97 ppm), fruit (2116.66 ppm), bark (1745.04 ppm) and flower (64.47 ppm) extracts showed safe levels while leaf (8.31 ppm) and seed (3.62 ppm) were slightly toxic and should be used with caution. LCMS study of *Cerbera odollam*'s flower and fruit crude extracts were first reported in the research. Antifungal compounds detected were neriifolin, hydroxybenzoic acid, cerberinic acid, salicylic acid and terephtalic acid. Neriifolin and hydroxybenzoic acid were identified for the first time from fruit, flower and wood extracts and cerberinic acid, salicylic acid and terephtalic acid from bark extracts. The results of current study indicated that *Cerbera odollam* leaf extracts has a potential of being a biofungicide.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Post harvest management in agricultural production has been growing considerably over the past four decades due to the growing populations across the Asia-pacific region. In 2004, global production for fruits and vegetables in the Asia-pacific regions was approximated between 31% and 42%. However, due to diseases, poor handling technique and management, the losses in postharvest was reported between 24% to 40% (Asian Productivity Organization, 2006).

Post harvest diseases are mainly caused by fungi and bacteria. The fungi responsible for most post harvest disease are *Fusarium*, *Alternaria* and *Penicilium* (Coates & Johnson, 2013). In order to overcome the problem, commercial fungicide such as Imazalil, Benomyl, and Thiabenzole are extensively applied for postharvest disease control in fruits and vegetables (Singh *et al*., 2011). Some of the fungicide are reported to be hazardous to human health, include vinclozolin, flucycloxuron (FCX) and diflubenzuron (DFB) (Rouabhi, 2010). These fungicides are not only able to have an effect on natural non-target organisms, but also accumulate as residues in food for human consumption (Okwute, 2012). Negative effects of synthetic fungicide are therefore raising grave concern globally.

Over the past 150 years, botanical pesticides have been traditionally used in the agricultural community. Anti-microbial activity of botanical has been reported by many researchers in their work (Ashikur *et al*., 2011; Cowan, 1999; Duke *et al*., 2010; Ghassan *et al*., 2012). However, due to the regulatory procedures associated with product development, there are only a small number of commercial botanical pesticide available in the market today, such as *pyrethrins*, *rotenone*, *neem*, *ryania, nicotin, sabadilla, garlic oil* and *Capsicum* oleoresin (Okwute, 2012). Extract from natural plant products are preferred for replacing synthetic fungicide because of safe usage, as they are biodegradable.

Cerbera odollam Gaertn belonging to Apocynaceae family is commonly known as "Pong-Pong tree". It is also known as "Buta-buta" in Malay. In peninsular Malaysia, locations of *Cerbera odollam* are distributed mostly at coastal mangrove swamps, sometimes inland as a roadside tree at Perlis, Kedah, Perak, Selangor, Melaka, Pahang and Johor. This species is able to produce flowers and fruits throughout the year (Kiew *et al*., 2011).

In the past decades, *Cerbera odollam* has been widely studied on its effects as antiproliferative, anticancer, antiestrogenic, antimicrobial, antinociceptive and sedative effect at different dosage (Ahmed *et al*., 2008; Ahmed *et al*., 2006; Shen *et al*., 2007). The bio-compounds reported in *Cerbera* includes cardiac glycoside, lignans, terpenoids, flavonoids, and some other compounds (Shen *et al*., 2007; Yamauchi *et al*.,1987). Secondary metabolites which have the potential to defend against pathogens are classified into three main chemical constituent family, i.e. alkaloids, terpenoids and phenolic (Brusotti *et al*., 2014). These secondary metabolite compounds have indicated their anti-microbial effects (Cowan, 1999; Cunha *et al*., 2012; Soković *et al*., 2009).

Thus, potential of *Cerbera odollam* as a replacement to conventional fungicide in post harvest management led the author to pursue in the anti-fungal effect in postharvest management.

In this research, the focus was on the evaluation of antifungal properties of different parts from *Cerbera odollam*, i.e. leaf, flower, fruit, seed, wood and bark. Ethanolic extracts of the plant parts were investigated for their antifungal activities and the bio-compounds through Liquid Chromatography Mass Spectrometer (LCMS).

1.2 Problem Statement

Cerbera odollam has been widely studied on its antimicrobial or anticancer in medical application. However, little investigation has been conducted in inhibiting agricultural diseases. Roles of this species in agro industry are still in the infancy stage. Therefore, further study could be done in this area. Besides, up to date, there are no reports available regarding the bio-compounds obtained from *Cerbera odollam* flowers and fruits. Hence, further analysis of these plant parts could be done by using LCMS.

1.3 Objective

1.3.1 Overall Goal

The aim of this research is to evaluate *Cerbera odollam* extracts as an alternative to commercial fungicide in post harvest management.

1.3.2 Specific Objectives

The objectives of this research are:

i. To evaluate the antifungal effect from different parts of *Cerbera odollam*, such as leaf, flower, fruit, seed, wood and bark on the growth of *Aspergillus niger*, *Penicilium citrum* and *Fusarium oxysporum*.

ii. To evaluate the toxicity (LC_{50}) of the plant extracts using Brine Shrimp Test (BST).

iii. To determine the correlation between phytochemical compounds and antifungal activity from different parts of plant extracts.

1.4 Scope of Study

Aspergillus niger, Fusarium oxysporum and *Penicilium citrum* are common post-harvest pathogens. The scope of the study is to investigate the anti-fungal activity from different parts of crude extracts of *Cerbera odollam* e.g leaf, flower, fruit, seeds, wood and bark against these pathogens. Group of bio-compounds were determined by standard phytochemical test and the antifungal bioassay were carried out on laboratory scale.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The residual effects of synthetic fungicide due to long usage in post-harvest management are causing health and environmental issues. Commercialized botanical or plant-based fungicides are still low in global market. Industrial and researchers are in search of novel compounds which will be able to denote a marketable technical advantage over their own or competitors' products. Furthermore, increase of fungal infection in post-harvest also aspire researcher to discover the new antifungal agents providing new mechanisms of action with a wide spectrum of antifungal activity. In our context, *Cerbera odollam* is studied in this research, in term of its antifungal activity, phytochemical and toxicity properties, as well as their bio-compounds. This chapter reviewed the bio-compounds of *Cerbera odollam*, the application and few methodology techniques employed in this study.

2.2 *Cerbera odollam* Gaertn

2.2.1 Botanical Description and Localization

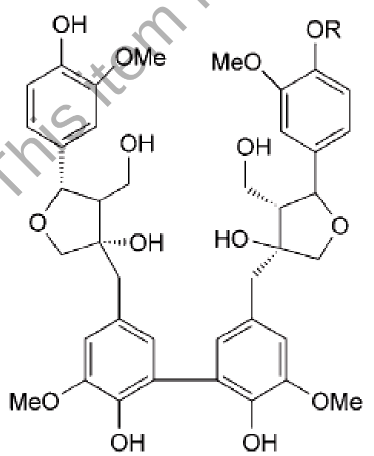
Genus *Cerbera* belongs to the poisonous Apocynaceae family. This genus comprises of 10 to 15 species, e.g *Alstonia spatulata*, *Alstonia pneumatophora*, *Cerbera manghas* L., *Cerbera odollam* Gaertn, *Rauvolfia verticillata*, *Dyera costulata*, *Ochrosia* *oppositifolia*, *Anodendron nervosum* Kerr, *Carissa spinarum*, and they are widely distributed in coastal area of South East Asia and the Indian Ocean (Kiew *et al*., 2011; Shen *et al*., 2007). In Peninsular Malaysia, there are only 2 species recorded, i.e. *Cerbera odollam* and *Cerbera manghas* (Kiew *et al.*, 2011). This two species are often described as synonym in some literature. However, both plants are actually divided into different species by taxonomists (Abe & Yamauchi, 1977; Shen *et al*., 2007). They can be differentiated through the yellow-eye white corolla of *Cerbera odollam* and red-eye white corolla of *Cerbera manghas* (Cheenpracha *et al*., 2004).

Figure 2. 1: (A) flowers (B) fruits (C) tree of *Cerbera odollam*.

Cerbera odollam, commonly referred as “Pong-Pong tree” (English name) and “Buta-buta” (Malay name) is a tree that grow about 6 to 15m height (Fig. 2.1) (Kiew *et al*., 2011). The leaves are dark green in color. The flowers’ are white and have scent reminiscent of jasmine and fruits are spherical in shape (Ahmed *et al*., 2008; Kiew *et al*., 2011). Locations of *Cerbera odollam* in Malaysia are mostly distributed at coastal mangrove swamps, sometimes inland as a roadside tree at Perlis, Kedah, Perak, Selangor, Melaka, Pahang and Johor and are able to flower and fruit throughout the year (Kiew *et al*., 2011).

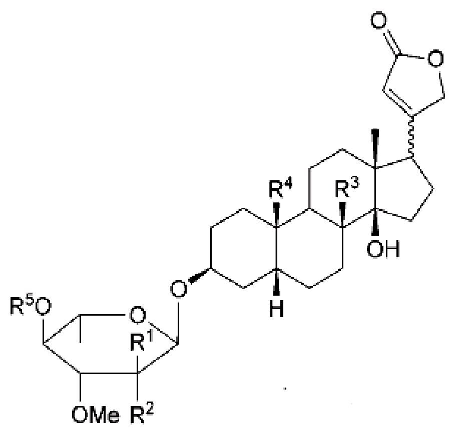
2.2.2 Bio-compounds in *Cerbera odollam*

The review of bio-compounds focused on earlier and current researches. *Cerbera odollam* are well known to be rich in variety of compounds such as lignans, cardiac glycoside and terpenoids (Abe, Yamauchi, & Wan, 1989; Shen *et al*., 2007; Yamauchi, Abe, & Wan, 1987a). Compounds structures are as shown in Fig. 2.2.



(A)

Where,
R=H



(B)

Where,
R^1=17β OAC; R^2= H; R^3=H;R^4=Me; R^5=H

Figure 2. 2: Compounds structure of: A, Cerberalignan (lignan) ;
B, Cerberin (cardiac glycoside) (Shen *et al*., 2007)

The seeds are the earliest part to be investigated. Ghanekar & Ayyar (1927) had successfully detected glycorides of palmitic, staric, myristic, lignoceric, oleic and linolic acids in seed extract, followed by De Veij who had report the presence of cerberin in the seeds of *Cerbera odollam* (Bisset, 1961). The seeds are the earliest part to be investigated. The work was then further confirmed by Steldt & Chen (1943). Study of seeds was persistence until Abe & Yamauchi (1977) commenced to investigate different parts of *Cerbera odollam*, i.e. bark, leaf, stem and lignin (Abe, Yamauchi, & Wan, 1988; Abe *et al*., 1989; Abe & Yamauchi, 1977; Yamauchi *et al.*, 1987a; Yamauchi, Abe, & Wan, 1987b). The summary of the bio-compounds identified from *Cerbera odollam* is shown in Table 2.1.

Up to date, little reports are available on the bio-compounds obtained from flower, fruits and roots. Some researchers had engaged crude extracts from flowers and fruits to test on their termite decay resistance properties on particleboard (Hashim *et al*., 2009). Meanwhile, crude extract from roots were tested for their possibility of antinociceptive and antibacterial properties in mice (Ashikur Rahman *et al*., 2011) and the result reported were positive. Fruits crude extract, on the other hand, failed to exhibit significant antimicrobial effects on skin bacteria (Shankar *et al*., 2009). The current research is investigating bio-compounds from leaf, seed, stem, bark and latex. The findings on flower, fruit and roots are still limited.

Table 2.1: Summary of bio-compounds from different part of *Cerbera odollam* (leaf, seed, stem, bark and latex).

Parts	Compounds	Reference
Leaf	Neriiforlin deacetyltanghinin 17α- deacetyltanghinin Cerleaside A cerdollaside 17α- cerdollaside solanoside 17α- solanoside Oleagenin α-L-thevetoside (cerleaside A) Oleagenin-β-glucosyl-(1→4)-α-L-thevetoside (cerleaside B) 17β- and 17α-cerdollaside 17β- and 17α-solanoside 17β- and 17α- neriifolin 17β- and 17α-deacetyltanghinin Glucose-3-ulosyl-thevetoside 17β- and 17α- digitoxigenin Cerberidol Cyclocerberidol Cerberidol-3-*O*-β-D-allopyranoside Cyclocerberidol-3-*O*-β-D-allopyranoside Cardiac glycoside	(Abe *et al*., 1989; Pongpijid, Bunyapraphatsara, & Panvisavas, 2011; Shen *et al*., 2007; Yamauchi *et al*., 1987a, 1987b)
Seed	cerberin 2′-*O*-acetyl cerleaside A Cerleaside A 17β- and 17α- neriifolin Thevetin B	(Laphookhieo, 2002; Laphookhieo *et al*., 2004; Shen *et al*., 2007b)
Stem	Gentiobiosyl-thevetoside Tanghinigenin Thevetin B tanghinigenin Glocosyl-thevetosides of digitoxigenin 17α- digitoxigenin Cerleaside B (-)-olivil (+)-cycloolivil (-)-cycloolivil Cerberalignan A Cerberalignan B Cerberalignan C Cerberalignan D Cerberalignan E Cerberalignan F Cerberalignan G Cerberalignan H Cerberalignan I	(Abe *et al*., 1988; Shen *et al*., 2007; Yamauchi *et al*., 1987b)