BIOACTIVITIES AND PHYTOCHEMICAL AND TOXICITY ANALYSES OF ETHANOLIC KAYU MANIS HUTAN [*Cinnamomum iners* (Reinw. ex Nees and T. Nees) Blume] LEAF EXTRACT AND ITS APPLICATION IN FOOD

By

KHAIRUL NAIM BIN MD PADZIL

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

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Chairman : Yaya Rukayadi, PhD
Faculty : Food Science and Technology

In the food industry, the foodborne bacteria and spoilage microorganisms have capabilities to cause foodborne diseases and spoilage. This has been prevented with utilization of synthetic antimicrobial agents in food products. However, its application has long term affected human health. This concern needs to be controlled which involves the utilization of safe natural plant such as Kayu Manis Hutan [*Cinnamomum iners* (Reinw. ex Nees and T. Nees) Blume] which reported to have antimicrobial and antioxidant activities. The study aims to determine the antimicrobial activity of ethanolic young and old *C. iners* leaves extracts against selected pathogenic microorganisms namely *Bacillus cereus*, *B. megaterium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Aspergillus flavus*, *A. niger*, *Rhizopus oligosporus* and *R. oryzae*. The result of antibacterial activity has exhibited inhibition zone at range

between 9.00 to 10.00 mm against all tested bacteria strains. The tested bacteria growths were inhibited at MICs ranged from 0.16 to 2.50 mg/mL, and were killed at MBCs ranged from 1.25 to 5.00 mg/mL. Time kills curve study showed that all tested bacteria can be killed at 4× MIC within 4 h. The antifungal activity of the extracts has exhibited zones of inhibition at range of 6.50 to 7.25 mm against all tested food spoilage microorganisms. The MICs of the extracts ranged from 0.63 to 5.00 mg/mL, and the MFCs at ranges of 1.25 to 5.00 mg/mL. In qualitative analysis, *A. niger* and *R. oryzae* showed no growth after being treated with young leaves extracts at 4× MIC while *R. oligosporus* and *A. flavus* at 2× MIC for 14 days. Meanwhile, no growth was shown on all tested fungi after being treated with old leaves extracts at 2× MIC for 14 days. In quantitative analysis, using 4× MIC for 24 h, the conidia germination was fully inhibited for all tested fungi. The antimicrobial activities of both extracts were shown not significantly different after being treated at different pHs and temperatures. The SEM analysis showed morphological features in selected treated microorganisms namely, *B. cereus*, *K. pneumoniae* and *A. flavus* were changed in the cell wall shape, ruptured, and the cytoplasm leaked. Both leaves extracts have exhibited high total phenolic content and antioxidant activities. The toxicity study demonstrated that both extracts was not toxic to *Artemia salina* with LC_{50} at 19.62 mg/mL and 13.20 mg/mL, respectively. GC-MS analysis has identified the presence of active volatile compounds in both extracts included phytol, hexadecanoic acid, dihydro(-)-neoclovene-(II), 14-β-pregnane and 2-hexadecenal. LC-MS have detected coniferyl alcohol, isorhamnetin-3-O-rutinoside in young leaf extract while α-terpineol, and 4-methylcatechol in old leaf extract. The decrease of 3

Log_{10} CFU/g of total place count, total viable mold count, *B. cereus*, *E. coli*, and *K. pneumoniae* count on raw chicken meat sample was observed when exposed to 5.00% (v/v) of *C. iners* extracts and maintain its microbiological quality with longer exposure within 21 days in different storage conditions. In conclusion, *C. iners* extracts and its compounds have shown potential of antimicrobial and antioxidant activities against foodborne pathogens and spoilage microorganism and thus can be developed as natural antimicrobial agent for food preservatives.

Keywords: *Cinnamomum iners* leaf extracts, antibacterial, antioxidant, toxicity, microbial population

SDG: Goal 3 - Good Health and Well Being

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

BIOAKTIVITI DAN ANALISIS FITOKIMIA DAN KETOKSIKAN EKSTRAK DAUN KAYU MANIS HUTAN [*Cinnamomum iners* (Reinw. Ex Nees dan T. Nees) Blume] DAN PENGGUNAANNYA DALAM MAKANAN

Oleh

KHAIRUL NAIM BIN MD PADZIL

Januari 2024

Pengerusi : Yaya Rukayadi, PhD
Fakulti : Sains dan Teknologi Makanan

Dalam industri makanan, bakteria bawaan makanan dan mikroorganisma perosak mempunyai keupayaan untuk menyebabkan penyakit bawaan makanan. Ini telah dicegah dengan penggunaan agen antimikrob sintetik dalam produk makanan. Walau bagaimanapun, penggunaannya telah menjejaskan kesihatan manusia jangka panjang. Kebimbangan ini perlu dikawal dengan menggunakan produk tumbuhan semulajadi yang selamat seperti Kayu Manis Hutan [*Cinnamomum iners* (Reinw. Ex Nees and T. Nees) Blume] yang dilaporkan mempunyai aktiviti antimikrob dan antioksidan. Matlamat kajian ini adalah untuk menentukan aktiviti antimikrob ekstrak daun *C. iners* muda dan tua dalam etanol terhadap bakteria bawaan makanan terpilih iaitu *Bacillus cereus*, *B. megaterium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Aspergillus flavus*, *A. niger*, *Rhizopus*

oligosporus dan *R. oryzae*. Hasil daripada aktiviti antibakteria telah menunjukkan zon perencatan pada julat antara 9.00 hingga 10.00 mm terhadap semua bakteria yang diuji. Pertumbuhan bakteria yang diuji telah terhalang pada MIC berjulat 0.16 hingga 2.50 mg/mL, dan dibunuh dengan MBC pada nilai antara 1.25 hingga 5.00 mg/mL. Kajian pembunuhan masa menunjukkan bahawa semua bakteria yang diuji boleh dibunuh oleh ekstrak pada 4× MIC dalam tempoh 4 jam. Aktiviti antikulat oleh ekstrak menunjukkan penghalang kawasan perencatan yang berkisar antara 6.50 hingga 7.25 mm terhadap semua spesies kulat berfilamen yang diuji serta MIC ekstrak berjulat antara 0.63 hingga 5.00 mg/mL, dan MFC pada julat 1.25 hingga 5.00 mg/mL. Dalam analisis kualitatif, *A. niger* dan *R. oryzae* tidak menunjukkan pertumbuhan selepas dirawat dengan ekstrak daun muda pada 4× MIC manakala *R. oligosporus* dan *A. flavus* pada 2× MIC selama 14 hari. Sementara itu, tiada pertumbuhan ditunjukkan pada semua kulat yang diuji selepas dirawat dengan ekstrak daun tua pada 2× MIC selama 14 hari. Dalam analisis kuantitatif, dengan menggunakan 4× MIC selama 24 jam, percambahan konidia telah dihalang sepenuhnya untuk semua kulat yang diuji. Aktiviti antimikrob bagi kedua-dua ekstrak tidak menunjukkan perbezaan yang signifikan selepas dirawat pada pH dan suhu yang berbeza. Analisis SEM menunjukkan bahawa ciri morfologi dalam mikroorganisma B. cereus, *K. pneumoniae* dan *A. flavus* mengalami perubahan dalam bentuk dinding sel, kerosakan, dan kebocoran sitoplasma. Kedua-dua ekstrak telah mempamerkan jumlah kandungan fenolik yang tinggi dan aktiviti antioksida. Kajian ketoksikan menunjukkan kedua-dua ekstrak tidak beracun kepada *Artemia salina* dengan nilai LC_{50} pada 19.62 mg/mL dan 13.20 mg/mL. Analisis

GC-MS telah mengenal pasti kewujudan sebatian meruap yang aktif dalam kedua-dua ekstrak termasuk fitol, asid heksadekanoik, dihydro(-)-neoklovin-(II), 14-β-pregnan dan 2-heksadesenal. LC-MS telah mengesan koniferil alkohol, isorhamnetin-3-O-rutinosid dalam ekstrak daun muda manakala α-terpineol, 4-methylkatekol dalam ekstrak daun tua. Penurunan 3 Log_{10} CFU/g dalam jumlah kiraan piring, jumlah kiraan kulat hidup, jumlah *B. cereus*, *E. coli* dan *K. pneumoniae* pada daging ayam mentah diperhatikan apabila terdedah pada 5.00% (v/v) ekstrak *C. iners* dan kualiti mikrobiologi dapat dikekalkan dengan pendedahan yang lebih lama selama 21 hari dalam keadaan penyimpanan yang berbeza. Kesimpulannya, ekstrak *C. iners* dan sebatian-sebatian dalamnya telah menunjukkan potensi aktiviti antimikrob dan antioksidan terhadap bakteria bawaan makanan dan mikroorganisma perosak, dan oleh itu ia boleh dibangunkan sebagai agen antimikrob semulajadi untuk pengawet makanan.

Kata Kunci: Ekstrak daun *Cinnamomum iners*, antibakteria, antioksidan, ketoksikan, populasi mikrob

SDG: Matlamat 3 - Kesihatan Baik dan Kesejahteraan

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Yaya Rukayadi, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Faridah binti Abas, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Maimunah binti Sanny, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Noor Azira binti Abdul Mutalib, PhD
Senior Lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

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

Date: 11 July 2024

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
CFU	Colony Forming Unit
CHX	Clorhexidine
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picryl-hydrazyl
EO	Essential oil
FRAP	Ferric reducing antioxidant potential
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography – Mass Spectrometry
IC_{50}	Inhibition Concentration at 50 percent
LC_{50}	Lethal Concentration at 50 percent
LC-MS	Liquid Chromatography – Mass Spectrometry
m/z	mass/charge ratio
MFC	Minimum Fungicidal Concentration
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MHz	Megahertz
NIST	National Institute of Standards and Technology
PBS	Phosphate Buffer Saline
SEM	Scanning Electron Microscopy
TE	Trolox equivalent

CHAPTER 1

INTRODUCTION

1.1 Background

Foodborne diseases represent a global health threat encountered by the food industry. It is caused by ingesting food that are deteriorated and contaminated by microorganisms, such as bacteria and fungi, as well as natural toxins (mycotoxins), chemicals, and physical agents (Sharif et al., 2018). Pathogenic bacteria such *Salmonella* Typhi*, Escherichia coli, Staphylococcus aureus, Vibrio cholera* and *Listeria monocytogenes* are the most frequent cause of outbreaks (Gourama, 2020). These pathogens and their toxins can enter the body through different modes and cause foodborne illness that may include abdominal cramps, nausea, vomiting, dehydration, diarrhea, and fever (Switaj et al., 2015). Foodborne bacteria also can be transmitted during food preparation, for example at the farm; the animals have already been infected by pathogenic microorganisms such as *E. coli*, *Bacillus cereus* and *Proteus mirabilis* (Abdul-Mutalib et al., 2015). Asa results, without prevention and preservation, food could transmit foodborne and spoilage contamination in food industry.

In order to avoid the growth of harmful bacteria in food, the use of synthetic or chemical antimicrobial compounds is involved in a number of techniques for inhibiting the growth of microorganisms (Uka et al., 2020). However, the threat posed by the quick emergence and spread of bacterial resistance caused by

synthetic antimicrobial treatments becoming more concerned. The synthetic antimicrobial has serious impacts on human health as their long run use causes liver damage, asthma, many allergic reactions, and cancer. Due to negative consumer perception of synthetic antimicrobial agents, the demand for safe natural antimicrobial has increased for food products (Ibrahim et al., 2021).

For many years, natural products have been recognized as an essential source of active substances (Mlala et al., 2019) with more than 200,000 chemical compounds are synthesized to possess medicinal importance (Yang et al., 2016). Plant derived antimicrobial often used to improve health in communities, providing health benefits, prevention diseases and numerous nutritional value (Santini et al., 2017). Worldwide, about 70% plant-based preparations are used as traditional medicines and also facilitate the base for 50% of prescription and/or over the counter drugs used in the Western-type practice of medicine (Pandey, 2019). Additionally, traditional medicines practices and herbal remedies has preference for using young leaves than old leaves of herb due to several factor, including the concentration of active compounds, taste, and safety considerations (Alamgir, 2017). One of the potential plants is *Cinnamomum iners* (Kayu Manis Hutan). It is commonly distributed in the tropical and subtropical regions, Asia and South and Central America, and Australia (Wuu-Kuang, 2011). It originated from Sri Lanka and widely distributed in Myanmar, Sumatra, India, Indonesia (Java), Malaysia, Thailand, Vietnam and Sulawesi (Udayaprakash et al., 2015). According to botanical classification, *C. iners* is one of the 250 species from the genus

Cinnamomum commonly known as wild cinnamon, kayu manis hutan, *pokok teja, medang kemangi* (Malaysia), *teja lawang* (Indonesia) and *deya gui* (China) (Ghalib et al., 2012; Wahab and Hussain, 2021).

The plant *C. iners* is used as a traditional medicine to relieve headaches, breathing and appetite problems, illnesses with bacterial symptoms such as fevers, digestive ailments, coughs, diarrhea and dysentery (Mustaffa et al., 2011). An ethnobotanical report reported that *C. iners* has medicinal properties of antiplasmodial, cytotoxicity, amylase inhibitor, anti-inflammatory, antimicrobial, antioxidant, anti-diarrheal, analgesic and carminative activity (Vigila et al., 2018). Accoding to Mustaffa et al. (2011), xanthorrhizol is known as the active compound isolated from *C. iners* methanolic leaves extract, and was shown to have antimicrobial activity against methicillin resistance *S. aureus* (MRSA). The extracts of *C. iners* contain numerous bioactive compounds such as cinnamic aldehyde (aldehyde), (*E*)-phytol acetate (acetate), linoleic acid (fatty acid) and linalool; *β*-pinene; *β*-selinene; *α*-humulene; *α*-pinene; *α*-selinene (terpinenes) (Kumar et al., 2019). Different leaf maturity exhibited different polyphenolic compound and bioactivity (Dziadek et al., 2019). The dried mature leaves of *C. iners* used as spices includes in flavoring stews, pilafs, curries, cornmeal porridge and jerk marinades. This study investigates the antimicrobial and antioxidant activities, phytochemical and toxicity analysis of ethanolic kayu manis hutan (*Cinnamomum iners*) leaves extract against foodborne pathogens and application in food.

1.2 Problem statements

The current existing methods of controlling food-borne illness are the misuse and overuse of antibiotics in food production which contribute to the development of antibiotic-resistant bacteria. Due to the increasing prevalence of this life-threatening microorganism infection and the ability of these pathogens to develop resistance to antibiotic, there is a great need to find new compounds to combat those (Stan et al., 2021). Herbs demonstrate a varied range of biological activities for managing diseases, including foodborne pathogenic microbes with the help of essential oils, phenolic compounds, flavonoids, alkaloids, and other bioactive molecules (Gupta and Birdi, 2017). By incorporating plant-derived antimicrobial agents into food products, these antimicrobial agents can inhibit the growth of spoilage-causing microorganisms, thereby extending the expiration date of the food products (Nogueira et al., 2020). This has inspired the present study to determine the antimicrobial activity of plants which have a wide range of medicinal properties.

The extracts of *C. iners* leaf show significant antibacterial () and antifungal activity against foodborne bacteria (Vigila et al., 2018; Anis et al., 2012). The antimicrobial activity was achieved through various combinations of phenols, triterpenoids, lignins, alkaloids, saponins, tannins and flavonoids (Buru et al., 2014). Unfortunately, the antibacterial and antifungal activities of ethanolic young and old *C. iners* crude leaves extracts against foodborne pathogens and spoilage microorganisms have not been assessed. The stability of *C. iners* extracts on different parameters such as pHs and temperatures against

selected foodborne pathogens and spoilage microorganisms were lacking in finding. To the best of the researcher's knowledge, the phytochemicals compounds in the ethanolic young and old *C. iners* crude leaves extracts that are responsible for the antibacterial and the antifungal activities against foodborne pathogens and spoilage microorganisms have never been examined. The extracts of *C. iners* have been reported to possess medicinal properties such as antioxidant activity (Mustaffa et al., 2016). Unfortunately, the total phenolic content and antioxidant assay of ethanolic young and old *C. iners* crude leaves extracts are not yet reported. The determination of toxicity of ethanolic young and old *C. iners* crude leaves extracts using brine shrimp are also not yet reported. During the storage period, poultry meat undergoes many undesirable changes due to microbial growth that leads to spoilage (Saleh et al., 2020). However, reduction of microbial population in poultry meat by ethanolic young and old *C. iners* crude leaf extracts is not yet reported. Thus, the general objective of the present study is to assess the antimicrobial and antioxidant activities, phytochemical and toxicity analyses of ethanolic kayu manis hutan (*Cinnamomum iners*] leaf extract and application in food.

1.3 Objectives

General of objective of this study is to discover the potential of ethanolic *C. iners* leaf extracts on antimicrobial activity against several type of pathogenic and spoilage microorganism, its phytochemical constituents, the toxicity activity, the antioxidant activity and to determine the ability of this plant extract as natural food preservatives. The specific objectives were:

1. To determine the antibacterial activity of young and old *C. iners* crude leaves extracts on *B. cereus*, *B. megaterium*, *E. coli*, *K. pneumoniae* and *P. mirabilis*, followed with extract stability of different pHs and temperatures and its mode of action by SEM.
2. To determine the antifungal activity of young and old *C. iners* crude leaves extracts on *A. flavus*, *A. niger*, *R. oligosporus* and *R. oryzae*, followed with extract stability of different pHs and temperatures and its mode of action by SEM.
3. To identify the bioactive compounds in young and old *C. iners* crude leaves extracts through GC-MS and LC-MS analyses.
4. To evaluate the toxicity of young and old *C. iners* crude leaves extracts by using brine shrimp (*Artemia salina*) lethality assay, the total phenolic content and the antioxidant activities in term of DPPH, FRAP and ABTS assay.
5. To evaluate the effect of young and old *C. iners* crude leaves extracts on microorganisms in raw meat chicken samples.

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