ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES, PHYTOCHEMICAL AND TOXICITY ANALYSES OF *MELINJO* (*Gnetum gnemon* L.) LEAF EXTRACT

By

TRISHA MANSURA RAHMAN

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

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DEDICATION

This thesis is dedicated to my beloved family, supervisors, and friends

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

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Global concern about foodborne pathogens is heightened due to their potential to cause widespread illness, economic loss, and worldwide undermining of food safety and security measures. Plant-derived antimicrobial compounds typically have a wider range of efficacy against foodborne pathogens than synthetic alternatives, in addition to being biodegradable and eco-friendly. Incorporating natural antimicrobial agents is crucial to mitigate the threat of antimicrobial resistance, promote sustainable practices, and lessen the possible adverse effects linked to synthetic substances. *Melinjo* (*Gnetum gnemon* L.) is one of the possible plant options. In Malaysia and Indonesia, several parts of the *Melinjo* plant, including leaves, seeds, and fruits, are utilized in culinary practices.

The antibacterial activities of ethanolic *Melinjo* leaf extract were determined using standard methods of CLSI, in terms of well diffusion assay (WDA),

minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill analysis. Total phenolic content (TPC) and antioxidant activities were performed using 2,2-diphenyl-1-picrylhydryzyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay. *Melinjo* showed antibacterial activities against tested foodborne bacteria. The inhibition zone in WDA ranged from 8.50 to 13.50 mm, MIC ranged from 6.25 to 50.00 mg/mL and MBC values were between 12.5 to >50 mg/mL. Results from time-kill analysis showed that at 4×MIC bacterial strains were completely killed within 1 to 4 h incubation time. Total phenolic content was demonstrated at 39.4 ± 2.69 mg GAE/g, and DPPH and ABTS assay showed scavenging activity with the IC_{50} value of 2.51 ± 0.26 mg/mL and 5.02 ± 0.30 mg/mL, respectively. Ferric-reducing antioxidant power activity was determined at 696.67 ± 13.56 µmol TEAC/g DW. The LC-MS analysis revealed the presence of various non-volatile compounds like anonaine, diosmetin 7-rutinoside, rhapontigenin, hamamelitannin, heteroflavanone C, tryptophan, [10]-gingerdiol. GC-MS analysis identified several volatile compounds such as phytol, vitamin E, ascorbic acid 2,6-dihexadecanoate, linoleic acid, gamma-sitosterol, and gamma-tocopherol. These identified compounds may have contributed to the antibacterial and antioxidant activities of *Melinjo* leaf extract. The LC_{50} of leaf extract was found to be 41.08 mg/mL which is more than 1 mg/mL; meaning that the extract is non-toxic and safe for human consumption.

In conclusion, ethanolic *Melinjo* leaf extract exhibits antibacterial activities against selected foodborne bacteria, and antioxidant potential due to the presence of bioactive compounds. It also is safe for consumption. Therefore, ethanolic *G. gnemon* L. extract leaf extract can be established as a natural anti-bacterial agent to be used in the development of natural food preservatives.

Keywords: Antibacterial, Antioxidant, *Gnetum Gnemon* L., Phytochemical, Toxicity.

SDG: GOAL 2: Zero Hunger, GOAL 3: Good Health and Well-Being

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

AKTIVITI ANTIBAKTERIA DAN ANTI-OKSIDAN, ANALISIS FITOKIMIA DAN TOKSISITI EKSTRAK DAUN *MELINJO* (*Gnetum gnemon* L.)

Oleh

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Kebimbangan global tentang patogen bawaan makanan meningkat kerana potensinya menyebabkan penyakit yang meluas, kehilangan ekonomi, dan menjejaskan keselamatan makanan dan langkah keselamatan di seluruh dunia. Sebatian antimikrob yang berasal dari tumbuhan biasanya mempunyai julat keberkesanan yang lebih luas terhadap patogen bawaan makanan daripada alternatif sintetik, selain boleh terbiodegradasi dan mesra alam. Menggabungkan agen antimikrob semulajadi adalah penting untuk mengurangkan ancaman rintangan antimikrob, menggalakkan amalan mampan, dan mengurangkan kemungkinan kesan buruk yang dikaitkan dengan bahan sintetik. *Melinjo* (*Gnetum gnemon* L.) adalah salah satu pilihan tumbuhan yang mungkin. Di Malaysia dan Indonesia, beberapa bahagian tumbuhan *Melinjo*, termasuk daun, biji, dan buah-buahan, digunakan dalam amalan masakan.

Aktiviti antibakteria ekstrak daun *Melinjo* etanolik ditentukan menggunakan kaedah standard CLSI, dari segi ujian resapan telaga (WDA), kepekatan perencatan minimum (MIC), kepekatan bakteria minimum (MBC), dan analisis bunuh masa. Jumlah kandungan fenolik (TPC) dan aktiviti antioksidan dilakukan menggunakan ujian 2,2-difenil-1-picrylhydryzyl (DPPH), ujian kuasa antioksidan pengurangan ferik (FRAP) dan ujian 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic asid (ABTS). *Melinjo* menunjukkan aktiviti antibakteria terhadap bakteria bawaan makanan yang diuji. Zon perencatan dalam WDA adalah antara 8.50 hingga 13.50 mm, MIC berkisar antara 6.25 hingga 50.00 mg/mL dan nilai MBC adalah antara 12.5 hingga >50 mg/mL. Keputusan daripada analisis bunuh masa menunjukkan bahawa pada 4×MIC strain bakteria terbunuh sepenuhnya dalam masa inkubasi 1 hingga 4 h. Jumlah kandungan fenolik ditunjukkan pada 39.4 ± 2.69 mg GAE/g, dan ujian DPPH dan ABTS menunjukkan aktiviti mengais dengan nilai IC_{50} masing-masing 2.51 ± 0.26 mg/mL dan 5.02 ± 0.30 mg/mL. Aktiviti kuasa antioksidan yang mengurangkan ferrik telah ditentukan pada 696.67 ± 13.56 mikron TEAC/g DW. Analisis LC-MS mendedahkan kehadiran pelbagai sebatian tidak meruap seperti anonaine, diosmetin 7-rutinoside, rhapontigenin, hamamelitannin, heteroflavanone C, tryptophan, [10]-gingerdiol. Analisis GC-MS mengenal pasti beberapa sebatian meruap seperti phytol, vitamin E, asid askorbik 2,6-dihexadecanoate, asid linoleik, gamma-sitosterol, dan gamma-tokoferol. Sebatian yang dikenal pasti ini mungkin menyumbang kepada aktiviti antibakteria dan antioksidan ekstrak daun *Melinjo*.

LC_{50} ekstrak daun didapati 41.08 mg/mL yang lebih daripada 1 mg/mL; bermakna ekstrak itu tidak toksik dan selamat untuk keg naan manusia. Sebagai kesimpulan, ekstrak daun *Melinjo* etanolik mempamerkan aktiviti antibakteria terhadap bakteria bawaan makanan terpilih, dan potensi antioksidan kerana kehadiran sebatian bioaktif. Ia juga selamat untuk dimakan. Oleh itu, ekstrak daun ekstrak *G. gnemon* L. etanol boleh ditubuhkan sebagai agen anti-bakteria semula jadi untuk digunakan dalam pembangunan pengawet makanan semula jadi.

Kata kunci: Antibakteria, Antioksidan, *Gnetum Gnemon* L., Fitokimia, Ketoksikan.

SDG: MATLAMAT 2: Sifar Kelaparan, MATLAMAT 3: Kesihatan dan Kesejahteraan Yang Baik

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LIST OF ABBREVIATIONS

ATTC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
EMLE	Ethanolic *Melinjo* Leaf Extract
FAO	Food and Agriculture Organization
GC-MS	Gas Chromatography-Mass Spectrometry
GRAS	Generally Recognised as Safe
HPLC	High-Performance Liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
LC_{50}	Median Lethality Concentration
LC-MS	Liquid Chromatography-Mass Spectrometry
m/z	mass/charge ratio
MBC	Minimum Bactericidal Concentration
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum Inhibitory Concentration
MPa	Mega Pascal
NIST	National Institute of Standards and Technology
PG	Peptidoglycan

CHAPTER 1

INTRODUCTION

1.1 Background

The variety of edible plants found in nature has sparked numerous breakthroughs in the research domain. The significant abundance of bioactive compounds within these plants plays a pivotal role in their application in the pharmaceutical and food industries as valuable resources. Edible plants are plants that are safe for human consumption and primarily used as food for their nutritional value and taste. They provide essential nutrients and can be part of a regular diet (Panda et al., 2019). Fruits, vegetables, grains, nuts, seeds, and certain herbs are examples of edible plants.

On the other hand, medicinal plants are those that have properties or compounds with potential health benefits and are used for medicinal purposes. These plants may have specific therapeutic properties and are often utilized to treat or alleviate various health conditions. Medicinal plants can be consumed as tea, extracts, and capsules, or used topically as ointments or poultices (Kurhekar, 2021). *Gnetum gnemon* L. has been used as both an edible and medicinal plant for therapeutic purposes among local and tribal people native to Southeast Asia and the western Pacific Ocean islands, ranging from Assam to Indonesia, Malaysia, Philippines, and Fiji (Barua et al., 2015).

Gnetum gnemon L. is nutritionally valuable, boasting a wealth of minerals and proteins. It is a promising candidate for providing healthy sustenance in underprivileged regions with limited access to protein-rich foods (Bhat & Binti, 2014). Moreover, this plant is known to be abundant in various beneficial compounds, including antioxidants, tyrosinase inhibitors, antimicrobial agents, anti-aging substances, and other bioactive compounds (Barua et al., 2015; Agarwal et al., 2020). *G. gnemon* L. holds significant importance as a leafy vegetable, particularly among tribal communities within Arunachal Pradesh, India. Additionally, the young twigs of this plant are readily available in local markets (Bharali et al., 2018). Despite the potential of different plants to treat various pathological conditions, a significant portion remains understudied, with only 15% having been analyzed for their phytochemical composition (Thomford et al., 2018).

It is widely recognized that numerous antimicrobial compounds derived from plants exhibit high efficacy against both Gram-positive and Gram-negative bacteria (Hochma et al., 2021). Over the period from 1981 to 2019, a total of 162 new antimicrobial drugs received approval, and intriguingly, 94 of them were derived from plant sources (Newman & Cragg, 2020). Edible plants are abundant sources of bioactive compounds that exert various physiological effects, encompassing anticancer, antioxidant, anti-inflammatory, and antimicrobial activities (Aisa et al., 2019). These natural plant extracts find frequent application in extending the shelf life of both fresh and processed foods, ensuring their quality and safety are preserved.

Phytochemical investigations into these extracts and their diverse biological activities are of significant importance in the food and human nutrition industries (Rapoport & Drausal, 2013). Antibiotics are essential in combating bacterial infections, but their effectiveness is being compromised by antibiotic resistance. Overuse and misuse of antibiotics have led to increased residual antibiotics in food and environments, causing the rapid spread of antibiotic-resistant bacteria (Ben et al., 2019). This poses a significant threat to public health, as infections become harder to treat, leading to longer recovery times, increased healthcare costs, and higher mortality rates.

The emergence of antibiotic-resistant bacteria, particularly multidrug resistance, is a serious problem in medical care, animal husbandry, food industry, and public health (Monger et al., 2021). The development of new antibiotics is lagging behind the spread of multidrug-resistant bacteria, requiring significant investment in research and development. Addressing antibiotic resistance and promoting responsible antibiotic use is crucial for public health (Kim & Ahn, 2022).

1.2 Problem Statements

Food is an essential requirement for sustaining human life nutritionally. Undesirable microbes can contaminate food products, food processing facilities, or manufacturing environments, posing a risk of human diseases (Yousef & Abdelhamid, 2019; Abdelhamid & El-Dougdoug, 2020).

Atabila et al. (2018) stated that access to safe and nutritious food is crucial for preserving life and improving health. They stated that contaminated meals including microbial infections and chemicals increase global illness and death rates. Food pathogens are microorganisms such as bacteria, viruses, or parasites present in food, beverages, or water that can lead to infections, intoxication, or toxin-mediated illnesses. They are categorized according to the categories of foods they contaminate (Madilo et al., 2023). Pathogens such as *Campylobacter*, *Salmonella*, *Shigella*, *Escherichia coli*, *Clostridium botulinum*, *Clostridium perfringens*, *Vibrio cholera*, *Staphylococcus aureus*, *Enterobacter*, *Bacillus* species, and *Listeria monocytogenes* are associated with foodborne illnesses.

These pathogens can be found in various raw or undercooked foods, meat, poultry, eggs, under-pasteurized milk, improperly canned foods, unpasteurized milk, as well as soft cheese (Havelaar et al., 2015). At present, food safety concerns have escalated globally because of the rise of foodborne illness due to microbial contamination (World Health Organization, 2021). Worldwide, almost 600 million individuals suffer foodborne diseases from consuming contaminated food, resulting in roughly 420,000 deaths daily (Szakály et al., 2020). To combat this situation, natural antimicrobial agents derived from plants could be the safest alternative to chemical antimicrobial agents and antibiotics.

Parhusip and Sitanggang (2011) reported the antimicrobial activity of *Melinjo* seed and peel extract against some pathogenic bacteria including *Bacillus* *cereus* ATCC10876, *Staphylococcus aureus* ATCC25953, and *Enterobacter aerogenes* ATCC13048. Another study conducted by Dayoh et al. (2021) stated that ethanolic *Melinjo* leaf extract has antibacterial activity against *S. aureus* ATCC25923. One of the members of the *Gnetum* family is *G. africanum* Welw. which is a native plant of Africa. Ilodibia et al. (2015) reported antibacterial and anti-fungal activity of aquas and ethanolic *G. africanum* leaf extract against selected pathogens such as *S. aureus* (NR 201), *Escherichia coli* (NR 202), *Salmonella* Typhi (NR 204) and fungal strain *Aspergillus niger* (NR 241) and *Candida albican* (NR 242).

The antibacterial activity of *G*. *gnemon* L. leaf extract against various foodborne bacteria has not been previously reported. The current study aimed to address this gap in knowledge by investigating the potential of *G*. *gnemon* L. leaf extract as a natural antimicrobial agent. Due to the increasing concerns over the development of antibiotic resistance and the limitations associated with synthetic preservatives, there is a growing interest in exploring natural alternatives. The findings of this study can contribute to this area of research by suggesting that *G*. *gnemon* L. leaf extract may possess antibacterial properties against a range of foodborne pathogens.

Kato et al. (2011) and Santoso et al. (2010) documented the presence of many bioactive substances, such as saponins, tannins, and flavonoids, inside *G. gnemon* L. The *Melinjo* seed is known for its high content of resveratrol, a specific type of stilbenoid that is predominantly found in the form of dimers, namely gnetin C. This unique dimer form of resveratrol has been the subject of research for over a decade, with particular focus and contributions from Japanese scientists (Barua et al., 2015; Wazir et al., 2011) who analyzed antioxidant activities of different parts of *G. gnemon* L. to determine the total phenolic and antioxidants activity of the plant. The quantification of total phenolic content in the plant extracts was conducted using the Folin-Ciocalteu method. Additionally, the assessment of antioxidant activities in the plant extracts was performed using three different assays: 2,2-diphenyl-1-picrylhydryzyl (DPPH), ferric reducing antioxidant power (FRAP), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays.

To the best of our knowledge, no previous study was done to determine the ABTS scavenging activity of *Melinjo* leaf extract and DPPH, FRAP, and total phenolic content (TPC) of ethanolic *Melinjo* leaf extract at the concentration used in this current study compared to the previous studies. Our findings not only shed light on the antioxidant potential of *Melinjo* leaf extract but also pave the way for future investigations into its application in various food products. Utilizing the antioxidant properties of plant extracts offers the possibility of reducing reliance on synthetic antioxidants, thereby meeting consumer preferences for clean-label and natural food products.

Phytochemical compounds are chemical substances found in plants that exhibit pharmacological and biological activities. These compounds are naturally produced by plants as part of their metabolic processes and can have various effects on living organisms. Phytochemicals are divided into two categories, namely primary and secondary metabolites, based on their roles in plant metabolism. Primary metabolites, essential for plant survival, encompass carbohydrates, amino acids, proteins, lipids, purines, and pyrimidines found in nucleic acids.

On the other hand, secondary metabolites comprise the remaining plant chemicals that are produced through metabolic pathways derived from the primary ones (Bone and Mills, 2013; Hussein and El-Anssary, 2019). These secondary metabolites play crucial roles in protecting plants from pathogens as they possess antiviral, antifungal, and antibiotic properties. Additionally, they serve as essential UV-absorbing agents, safeguarding leaves from severe damage caused by light exposure (Bourgaud et al., 2001). Throughout history, these biologically active plant secondary metabolites have been utilized in traditional medicine, and the medicinal effects of plants can be attributed to these molecules (Jamwal et al., 2018). Multiple experiments were undertaken to investigate the presence of phytochemical substances in *G. gnemon* L. Previous research has identified various phytochemical compounds in multiple parts of *G. gnemon* L. plants, including stilbenoids, resveratrol, saponins, flavonoids, tannins, 3,4-dimethoxychlorogenic acid, gnemonols K and L, vitamin C, beta-carotene, lutein, polyphenols, and vitamin E (Anisong et al., 2022).

The present study represented the first attempt to analyze the ethanolic *Melinjo* leaf extract using LC-MS and GC-MS techniques. Despite the extensive research on phytochemical compounds in *Melinjo*, no prior investigations have been conducted using these analytical methods.

Over a significant period, individuals have incorporated *Melinjo* into their diets as both a leafy vegetable and for its medicinal attributes. *Melinjo* leaf has been included in culinary practices and utilized as remedies for health-related issues (Barua et al., 2015). This longstanding customary application suggests a reasonable level of safety. Nevertheless, there exists an absence of empirical evidence concerning the potential toxicity of *Melinjo* leaf, specifically when evaluated using scientific techniques such as the brine shrimp lethality assay. Therefore, it is crucial to undertake a systematic analysis of toxicity to substantiate the safety of *Melinjo* leaf, bridging the traditional usage with scientific validation.

1.3 Objectives

The general goals of this study were to explore the antibacterial and antioxidant potential of *Melinjo* (*Gnetum gnemon* L.) leaf extract. The specific objectives are:

1. To determine the antibacterial activity of *G. gnemon* L. leaf extract on foodborne pathogens in terms of well diffusion assay (WDA), minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC), and killing time curve analysis.
2. To determine the total phenolic content (TPC) and antioxidant activity of *G. gnemon* L. leaf extract.
3. To identify the bioactive compounds in *G. gnemon* L. leaf extract which are responsible for antimicrobial and antioxidant activity by Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS).
4. To evaluate the toxicity of *G. gnemon* L. leaf extract using brine shrimp (*Artemia salina*) lethality assay.

1.4 Research Questions

This study explores four research questions to investigate its core objectives. The research questions are:

1. Does the leaf extract of *Gnetum gnemon* L. exhibit antibacterial activity against foodborne pathogens when assessed through well diffusion assay (WDA), minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC), and killing time curve analysis?

2. Does *Gnetum gnemon* L. leaf contain phenolic compounds and possess antioxidant activity? If so, what are their concentrations?

3. What are the bioactive compounds present in *Gnetum gnemon* L. leaf extract that contribute to its antimicrobial and antioxidant activity, as identified by Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS)?

4. What is the toxicity level of *Gnetum gnemon* L. leaf extract as determined by the brine shrimp (*Artemia salina*) lethality assay?

1.5 Hypothesis

This study investigates its research questions through four hypotheses. The hypotheses are:

1. The leaf extract of *Gnetum gnemon* L. has significant antibacterial activity against foodborne pathogens, evidenced by the inhibition zones observed in the well diffusion assay, low MIC and MBC values, and a notable decrease in bacterial population over time in the killing time curve analysis.

2. *Gnetum gnemon* L. leaf contains significant levels of phenolic contents and has antioxidant activity.

3. There are bioactive compounds present in *Gnetum gnemon* L. leaf extract which may contribute to its antimicrobial and antioxidant activity, as detected by Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS).

4. *Gnetum gnemon* L. leaf extract exhibits no toxicity in the brine shrimp (*Artemia salina*) lethality assay.

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