ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF BAY [Syzygium polyanthum (Wight) Walp.] LEAF EXTRACT AGAINST PLANKTONIC AND BIOFILM GROWTH OF Listeria monocytogenes

ILI SYUHADA BINTI MOHD DAUD

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By

ILI SYUHADA BINTI MOHD DAUD

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

Adhesion of microorganism or biofilm formation to food contact surface can become a source of microbial contamination and give major implication in food industries. Moreover, interest has recently grown in discovery of natural antimicrobial agents such as plant extract. The aim of this study was to investigate the antibacterial and antibiofilm activity of bay [*Syzygium polyanthum* (Wight) Walp.] leaf extract against planktonic and biofilm growth of *Listeria monocytogenes* strains. In this study, 9 strains of *L. monocytogenes* isolated from vegetables and one standard *L. monocytogenes* ATCC®19112™ were used. The standard strain (ATCC®19112™) and the local isolates were used to study the difference of antibacterial resistance towards bay [*S. polyanthum* (Wight) Walp.] leaf extract. Antibacterial activity of bay [*S. polyanthum* (Wight) Walp.] leaf extract against planktonic *L. monocytogenes* strains were investigated using the methods described by Clinical and Laboratory Standards Institute (CLSI) in term of disc diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time-kill curve analysis. Whereas *in vitro* biofilm formation ability of *L. monocytogenes* and antibiofilm activity of the extract against *L. monocytogenes* biofilms were evaluated in pre-sterilized 96-well microplate using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium-hydroxide (XTT) reduction assay. Moreover, the effect of bay [*S. polyanthum* (Wight) Walp.] leaf extract on *L. monocytogenes* ATCC®19112™ biofilms were visually observed using scanning electron microscopy (SEM). Finally, the effects of ethanolic extract of bay [*S. polyanthum* (Wight) Walp.] leaf were tested on bean sprout (*Vigna radiata*) that has been spiked with approximately 10^6-10^8 CFU/mL of *L. monocytogenes*. The results showed that all tested *L. monocytogenes* strains were susceptible to bay [*S. polyanthum* (Wight) Walp.] leaf extract with clear zone ranging from 6.50 mm to 8.00 mm for ethanolic extract and 6.50 mm to 7.50 mm for methanolic extract. The extract can inhibit the growth of *L. monocytogenes* with MICs ranging from 0.31 to 1.25 mg/mL. The extract also can kill completely the *L. monocytogenes* strains with MBCs ranging from 0.63 to 5.00 mg/mL. Based on time-kill curves established at 0× MIC, 1/2× MIC, 1× MIC, 2× MIC and 4× MIC of bay [*S.*

polyanthum (Wight) Walp.] leaf extract, the bactericidal endpoints for tested *L. monocytogenes* was fast acting from 0.63 to 5.00 mg/mL. Furthermore, four *L. monocytogenes* strains; 58a, 58b, 90g and ATCC®19112™ were classified as strong biofilm producer *in vitro*, with optical densities (ODs) ranging from 0.66 to 1.77, which were greater than optical density of negative control (ODc) ranging from 0.09 to 0.12. The sessile minimum inhibitory concentrations (SMICs) for both ethanolic and methanolic extract against the strong biofilm producer ranged from 0.63 to 2.50 mg/mL and 1.25 to 5.00 mg/mL, respectively. The minimal biofilm eradication concentrations (MBECs) of both ethanolic and methanolic leaf extract were varied from 5.00 mg/mL to >5.00 mg/mL. From the observation using SEM, most of the *L. monocytogenes* vegetative cells and biofilms were noticeably disrupted after treated with bay [*S. polyanthum* (Wight) Walp.] leaf extract at concentration of 5.00 mg/mL. In the simulation study, generally the significant reduction of natural microflora in bean sprout (*Vigna radiata*) spiked with *L. monocytogenes* after treated with bay [*S. polyanthum* (Wight) Walp.] leaf extract was at concentration of 0.05% for 5 min exposure time. Overall, the results showed that bay [*S. polyanthum* (Wight) Walp.] leaf extract possess strong antibacterial and antibiofilm activity against *L. monocytogenes*. In conclusion, bay [*S. polyanthum* (Wight) Walp.] leaf extract can be developed as a natural food sanitizer or preservative.

Keyword: Antibacterial, antibiofilm, bacterial biofilm, *Syzygium polyanthum* (Wight) Walp., *Listeria monocytogenes*

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

AKTIVITI ANTIBAKTERIAL DAN ANTIBIOFILEM EKSTRAK DAUN SALAM [*Syzygium polyanthum* (Wight) Walp.] TERHADAP PERTUMBUHAN PLANKTONIK DAN BIOFILEM *L. Monocytogenes*

Oleh

ILI SYUHADA BINTI MOHD DAUD

September 2019

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

Lekatan mikroorganisma ataupun pembentukan biofilem pada permukaan makanan boleh menjadi sumber kontaminasi mikrobiologi dan memberikan implikasi utama dalam industri makanan. Selain itu, baru-baru ini, minat tentang penemuan agen antimikrobiologi semulajadi seperti ekstrak tumbuhan semakin berkembang. Tujuan kajian ini dijalankan adalah untuk mengkaji aktiviti antibakteria dan antibiofilem ekstrak daun salam [*Syzygium polyanthum* (Wight) Walp.] terhadap planktonik dan biofilem bagi strain *L. monocytogenes.* Dalam kajian ini, 9 strain *L. monocytogenes* yang diasingkan daripada sayur-sayuran dan satu standard *L. monocytogenes* ATCC®19112™ telah digunakan. Strain standard (ATCC®19112™) dan pencilan tempatan telah digunakan bagi mengkaji perbezaan ketahanan antibakterial terhadap ekstrak daun salam [*S. polyanthum* (Wight) Walp.]. Aktiviti antibakterial ekstrak bagi daun salam [*S. polyanthum* (Wight) Walp.] terhadap strain *L. monocytogenes* telah dikaji menggunakan kaedah seperti yang dinyatakan oleh standard institut makmal dan latihan (CLSI) dari segi resapan cakera, kepekatan perencatan minimum (MIC), kepekatan bakterisidal minimum (MBC), dan analisis lengkung masa pembunuhan. Manakala, keupayaan *L. monocytogenes* untuk membentuk biofilem *in vitro* dan kegunaan ekstrak sebagai aktiviti antibiofilem telah dinilai di dalam telaga plat mikrotiter 96 pra-steril menggunakan asei pengurangan, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium-hydroxide (XTT). Tambahan pula, kesan ekstrak daun salam [*S. polyanthum* (Wight) Walp.] ke atas biofilem *L. monocytogenes* ATCC®19112™ boleh dilihat menerusi mikroskop imbasan electron (SEM). Kemudian, kesan ekstrak etanol [*S. polyanthum* (Wight) Walp.] telah diuji ke atas tauge (*Vigna radiata*) yang telah dicemari dengan 10^6-10^8 CFU/mL *L. monocytogenes*. Keputusan menunjukkan bahawa semua strain *L. monocytogenes* yang diuji mudah terkesan dengan ekstrak daun salam [*S. polyanthum* (Wight) Walp.] dengan julat zon perencatan daripada 6.50 mm hingga 8.00 mm untuk ekstrak etanol dan dari 6.5 mm hingga 7.50 mm untuk ekstrak metanol. Ekstrak tersebut mampu merencatkan pertumbuhan *L. monocytogenes* dengan bacaan kepekatan perencat minimal (MICs) dari 0.31 hingga 1.25 mg/mL. Ekstrak daun salam itu juga mampu membunuh sepenuhnya strain *L. monocytogenes* dengan bacaan kepekatan bakterisidal minimal (MBCs) dari 0.63 hingga 5.00 mg/mL. Berdasarkan analisis lengkung masa pembunuhan ekstrak daun salam [*S. polyanthum* (Wight) Walp.] yang diukur pada 0× MIC, 1/2× MIC, 1× MIC, 2× MIC dan 4× MIC, titik akhir bakterisidal bagi *L. monocytogenes* yang diuji ialah dari 0.63 hingga 5.00 mg/mL. Tambahan lagi, empat strain *L. monocytogenes*, *L. monocytogenes* asingan L58a, L58b, L90g dan *L. monocytogenes* ATCC®19112™ telah diklasifikasikan sebagai pembentuk biofilem yang kuat *in vitro* dengan julat kepadatan optik yang berbagai-bagai dari 0.66 hingga 1.77 yang mana mempunyai kepadatan optik yang lebih tinggi daripada kawalan negatif dari 0.09 hingga 0.12. Kepekatan perencatan minimum sesil (SMICs) untuk kedua-dua ekstrak etanol dan metanol terhadap pembentukan biofilem yang kuat adalah masing-masing antara 0.63 hingga 3.50 mg/mL dan 1.25 hingga 5.00 mg/mL. Sementara itu, kepekatan pembasmian minimum biofilem (MBECs) untuk kedua-dua ekstrak etanol dan methanol berbeza-beza daripada 5.00 mg/mL. hingga >5.00 mg/mL. Daripada pemerhatian menggunakan SEM, didapati kebanyakan sel-sel vegetatif *L. monocytogenes* dan biofilem ternyata terganggu selepas dirawat dengan ekstrak daun salam [*S. polyanthum* (Wight) Walp.] pada kepekatan 5.00 mg/mL. Dalam kajian simulasi, umumnya pengurangan mikroflora semulajadi dalam tauge (*Vigna radiata*) yang telah dicemar dengan *L. monocytogenes* selepas dirawat oleh ekstrak daun salam [*S. polyanthum* (Wight) Walp.] adalah pada kepekatan 0.05% untuk masa pendedahan 5 minit. Secara keseluruhannya, hasil menunjukkan bahawa ekstrak daun salam [*S. polyanthum* (Wight) Walp.] mempunyai aktiviti antibakteria dan antibiofilem terhadap *L. monocytogenes.* Sebagai kesimpulan, ekstrak daun salam [*S. polyanthum* (Wight) Walp.] boleh dikembangkan lagi menjadi sanitasi makanan atau pengawet makanan semula jadi.

Kata kunci: Antibakterial, Antibiofilem, *Syzygium polyanthum* (Wight) Walp., *Listeria monocytogenes*

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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

Nor Khaizura Mahmud @ Ab Rashid, PhD
Senior lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

____

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

Date:

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Name and Matric No.: Ili Syuhada binti Mohd Daud (GS 45965)

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This is to confirm that:

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Signature : ________________________
Name of Chairman of
Supervisory Committee : Assoc. Prof. Dr. Yaya Rukayadi

Signature : ________________________
Name of Member of
Supervisory Committee : Dr. Nor Khaizura Binti Mahmud @ Ab Rashid

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BHI	Brain heart infusion
CFU	Colony forming unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethylsulfoxide
h	Hour
IBS	Institute of Bioscience
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
min	Minute
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
PBS	Phosphate buffered saline
PCA	Plate Count Agar
rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
spp.	Species
UV	Ultraviolet
UPM	Universiti Putra Malaysia
EPS	Extracellular polymeric substances
TW	Tap water
TPC	Total plate count

CHAPTER 1

INTRODUCTION

1.1 Background

Foodborne pathogens are major causes of foodborne diseases that triggered worst outbreaks related with different kind of foods, which is a pandemic with huge impact on human health. Moreover, foodborne pathogens have developed less susceptibility or even resistance due to rampant use of antibiotics (Newell et al., 2010). Foodborne illnesses emerge because of the entrance of bacteria, viruses or parasites in the food which proliferate speedily in the intestine and cause sickness or consumptions of non-communicable disease agents like chemicals and toxin (Linscott, 2011). Diarrhea is an important symptom of foodborne diseases which sometimes came together with nausea, vomiting, fever and abdominal cramp (FDA, 2018).

Malaysia is one of the countries that have top occurrence of foodborne illnesses related to the optimum temperature and fast adapting microorganisms to the condition suitable for growth. In 2014, Ministry of Health (MOH) Malaysia reported that the outbreak rate of reported food poisoning statistic in 2013 is 47.79 cases for each 100,000 populations, with mortality rate of 0.04. However, there are numerous under reported foodborne poisoning diseases in the country. There are many elements that are responsible to the incidence of foodborne diseases, such as regularly contaminated raw food products, mistakes in food preparation, food handling at home and purpose intake of undercooked animal sources (Kaferstein, 2003).

Blackman and Frank (1996) reported that *L. monocytogenes* are able to attach to a variety of surfaces, including stainless steel, polystyrene, and glass and are able to form biofilms. The direct contamination of foods or food processing equipment with *L. monocytogenes* can result in outbreaks of food borne disease called listeriosis (Allenberger et al., 2010). It poses a serious threat to public health, and the majority of cases of human listeriosis are associated with the consumption of contaminated food. This pathogen is commonly found on contaminated dairy products prepared meat products, including turkey, deli meats, hot dogs and seafood, and raw vegetables that have been contaminated from the soil (Rodrigues et al., 2005).

Previously, antibiotics were used through treatment of livestock to control or reduce the spread of foodborne pathogens. However, problems within animals and humans keep occuring because of the antibiotic resistant of foodborne pathogens (Kurekci et al., 2013). Recently, there is much interest in medicinal and herbal plant as they consist many chemical constituents expressing antimicrobial activity, antioxidant activity and natural preservative components that can be replaced antibiotic purposes (Mabona et al., 2013). Apparently, plants are reviewed *in vitro* to be abundant in a variety of phytochemicals including flavonoids, alkaloids, tannins and terpenoids that are significant to exhibit antimicrobial activities (Talib and Mahasneh, 2010).

A report from Kurek et al. (2010) also proves that plants exhibit various kind of antimicrobial properties such like essential oils, phenols, and terpenes. Mariita et al. (2011) suggested that the presence of alkaloids with additional phytochemicals in plant species *Scadoxus multiflorus* and *Acacia nilotica* possibly contribute to the antibacterial activity. Moreover, botanical ingredients such like lemon and cherry have been reported as potential antimicrobials agent against *Clostridium perfringens* in ready to eat food (Jackson et al., 2011). Oleanolic acid extracted from variety of plants is also proven to possess important antilisterial activity (Yoon and Choi, 2010). These findings prove that the chemical structures and functional groups of the plants are important parts to determine their antimicrobial activity.

Former studies reported that the main components of bay [*S. polyanthum* (Wight) Walp.] leaf essential oil are eugenol, methyl chavicol and citric acid (Sumono and Wulan, 2008). Bay [*S. polyanthum* (Wight) Walp.] leaf have been widely used in Indonesian culinary as a food additive (Dalimartha, 2001). They have also been used for antiulcer, antidiabetes, anti-inflammatory and antidiarrhea treatments. These leaf demonstrated to have high antioxidative activity (Lelono et al., 2009) and clear from cytotoxicity consequence against lineage cell (Perumal et al., 2012). Matured fruits were reviewed by Kusuma et al. (2011) as high antimicrobial, antioxidant and high cytotoxicity activities. There are other previous studies which report that bay [*S. polyanthum* (Wight) Walp.] leaf also possesses biological activities such as antioxidant (Wong et al., 2006), antibacterial (Sumono and Wulan, 2008), and antifungal activities (Noveriza and Miftakhurohmah, 2010).

1.2 Problem Statements

Over the last few decades, health benefits are under threat as a lot of frequently used antibiotics have become less effective against particular diseases. This is because of the increase of resident antibiotic resistance bacteria and the toxic produced by them (Bhalodia and Shukla, 2011). However, there is promising alternative to substitute the drug-resistant bacteria. Nowadays, the search for chemical constituent of plant sources that competent in mitigating biofilm production is widely in search.

Plant extract and other naturally active constituents isolated from parts of plant such as roots, stems and leaf have acquired more attention in the antibiofilm effect (Essawi and Srour, 2000). Furthermore, there is also a report from Friedman et al. (2002), that studied on the effectiveness of essential oil of bay leaf (*Syzigium aromaticum* L.) against oral bacteria related to other oral diseases including periodontal disease and dental caries. It is also proven to be active against other bacteria for instance *Escherichia coli, Listeria monocytogenes* and *Salmonella enterica*.

Bae et al. (2012) in their report, have claimed that foodborne pathogens which grow as biofilm mode exist immensely in the food processing premises rather than in planktonic form. In addition, the existence of biofilm in dairy, fish processing, poultry, meat, and ready-to-eat (RTE) food industries are becoming problematic to public health with increased resistant to antimicrobial and antibiofilm agents (Costerton and Stewart, 2001). The arising of resistant bacteria to typical antimicrobials compound convey that advanced biofilm standard approach is compulsory.

L. monocytogenes is a pathogenic bacterium that can cause a rare but dangerous infection called listeriosis. It is commonly found and isolated from processed, ready-to-eat (RTE) and cold-stored meat and dairy products. However, an increasing number of recent reports show contamination and prevalence of *L. monocytogenes* in fresh produce. Thus, this study was conducted to offer a practical and novel solutions to give alternatives especially for food products consumers in reducing or eliminating completely the bacterial populations in food products using natural antibacterial and antibiofilm agents originate from plant sources. From the reports collected, there are nearly no review on the antibiofilm effect of crude extract of bay [*S. polyanthum* (Wight) Walp.] leaf on foodborne pathogens biofilm. Therefore, the antibiofilm activity was investigated in this study to examine whether it can perform as a substitute to the antimicrobial agent for food commodities.

1.3 Objectives

The objectives of this study are:

1. To determine the antibacterial activity of bay [*S. polyanthum* (Wight) Walp.] leaf extract against *Listeria monocytogenes* strains in term of disc diffusion, MIC, MBC, and killing curve.
2. To assess the ability of biofilm formation of *L. monocytogenes in vitro* using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium-hydroxide (XTT) reduction assay.
3. To evaluate the antibiofilm activity of bay [*S. polyanthum* (Wight) Walp.] leaf extract in term of sessile minimum inhibitory concentration (SMIC) and minimum biofilm eradication (MBE) against *L. monocytogenes* biofilms.
4. To examine the effect of bay [*S. polyanthum* (Wight) Walp.] leaf extract on natural microflora in food samples at different concentrations of extract.

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