



UNIVERSITI PUTRA MALAYSIA

***TEA LEAF [CAMELLIA SINENSIS (L.) KUNTZE]
EXTRACTS SYNTHESIZED
SILVER NANOPARTICLES AS ANTIMICROBIAL AGENT
AGAINST FOODBORNE PATHOGENS***

LOO YUET YING

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By

LOO YUET YING

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

July 2018

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment
of the requirement for the degree of Doctor of Philosophy

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SILVER NANOPARTICLES AS ANTIMICROBIAL AGENT AGAINST
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LOO YUET YING

July 2018

Chairman : Professor Son Radu, PhD
Faculty : Food Science and Technology

Foodborne illness has emerged as an important public health problem in many countries in the last decade. The main purpose of this research is to study the antibacterial activity of silver nanoparticles against foodborne pathogens. Silver nanoparticles (AgNP) are well-known as antimicrobial agents due to their strong biocidal effect against microbial species. In this study, the synthesis of AgNP using the Chinese tea extracts from *Camellia sinensis* was reported. The synthesized nanoparticles were characterized using UV-vis spectroscopy, X-ray diffraction (XRD), transmission electron microscopy (TEM), and fourier transform infrared (FTIR) spectroscopy. The XRD analysis shows that the synthesized AgNP are of face-centered cubic structure. Well-dispersed AgNP with an approximate size of 4 nm were observed in the TEM image. The antibacterial activity of silver nanoparticles was tested against 12 foodborne pathogens namely *Escherichia coli*, *Escherichia coli* O157:H7, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Enterococcus faecalis*, *Enterococcus durans*, *Listeria monocytogenes*, *Bacillus subtilissubsp. spizizenii*, and Methicillin-resistant *Staphylococcus aureus*. The antibacterial activity of AgNPs was determined by using disc diffusion method, resazurin microtitre-plate assay (minimum inhibitory concentration, MIC), minimum bactericidal concentration test (MBC), and time-kill curve assay. The disc diffusion test showed that all the foodborne pathogens were susceptible to AgNPs. The MIC and MBC value of AgNPs against these 12 foodborne pathogens were ranged from 3.9 to 15.6 µg/mL. The time kill activity of AgNPs against these foodborne pathogens was tested; the reduction in the number of CFU/ml was >3 log₁₀ units (99.9%) in 1-2 h. This study indicates that AgNPs has antibacterial activity. The toxicity study using the brine shrimp nauplii demonstrated that AgNPs have medium toxic to *A. salina* (LC₅₀ = 107.52 µg/mL). The applications of AgNPs were done by adding AgNPs into the washing-up liquid as an antibacterial additive in washing the artificially contaminated chopping board. Washing the artificially contaminated chopping board by using washing-up liquid with AgNPs reduced the bacteria count by 1.60 ± 0.11 log₁₀ CFU/g for *S. aureus*; while for *S. Typhimurium*, the bacteria count was reduced

by $2.90 \pm 0.02 \log_{10}$ CFU/g. The decontamination efficacy of washing using washing-up liquid with AgNPs was found to be significant ($P < 0.05$) and the most effective way in reducing the microbial contamination in chopping board. This is the first report to demonstrate that the application of AgNPs in washing-up liquid has significant antibacterial activity against foodborne pathogens. Overall, the findings show that AgNPs have strong antibacterial activity and can be an alternative antimicrobial agent.



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

**NANOPARTIKEL PERAK YANG DISINTESIS OLEH DAUN TEH
[*Camellia sinensis* (L.) Kuntze] EKSTRAK SEBAGAI AGEN
ANTIMIKROBIAL TERHADAP PATOGEN BAWAAN MAKANAN**

Oleh

LOO YUET YING

Julai 2018

Pengerusi : Professor Son Radu, PhD
Fakulti : Sains dan Teknologi Makanan

Penyakit bawaan makanan telah muncul sebagai satu masalah kesihatan awam di kebanyakan Negara sejak dekad yang lalu. Tujuan utama kajian ini adalah untuk mengkaji antibakteria aktiviti nanopartikel perak terhadap penyakit bawaan makanan. Nanopartikel perak telah dikenali sebagai agen antimikrobial adalah disebabkan oleh kesan biocidalnya yang kuat terhadap spesies microbial. Dalam kajian ini, nanopartikel perak telah disintesis menggunakan ekstrak daun teh cina dari *Camellia sinensis* dilaporkan. Nanopartikel perak yang disintesis telah dicirikan dengan spektroskopi UV-vis, pembelauan sinar-X (XRD), spektroskopi jelmaan Fourier inframerah (FTIR), dan mikroskop electron penghantaran (TEM). Analisis XRD menunjukkan nanopartikel perak mempunyai struktur berpusatkan muka padu. Permerhatian imej TEM menunjukkan nanopartikel perak yang terdispersi dengan baik mempunyai purata saiz 4 nm. Antibakteria aktiviti nanopartikel perak telah diuji terhadap 12 patogen bawaan makanan iaitu *Escherichia coli*, *Escherichia coli* O157:H7, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Enterococcus faecalis*, *Enterococcus durans*, *Listeria monocytogenes*, *Bacillus subtilis* subsp. *spizizenii*, dan Methicillin-resistant *Staphylococcus aureus*. Antibakteria aktiviti nanopartikel perak telah ditentukan dengan menggunakan kaedah penyebaran cakera, ujian penentuan nilai kepekatan perencatan minimum (MIC), ujian penentuan nilai kepekatan minimum bakterisidal (MBC), dan asai waktu-membunuh. Ujian penyebaran cakera menunjukkan semua patogen bawaan makanan rentan terhadap nanopartikel perak. Nilai-nilai MIC dan MIC nanopartikel perak untuk 12 patogen bawaan makanan adalah dalam lingkungan antara 3.9 – 15.6 µg/mL. Aktiviti waktu membunuh nanopartikel perak terhadap pathogen bawaan makanan telah diujikan; pengurangan nombor CFU/mL adalah >3 log₁₀ unit (99%) dalam 1-2 jam. Kajian ini menunjukkan nanopartikel perak mempunyai antibakteria aktiviti. Kajian sitotoksik telah diuji menggunakan nauplii udang air garam menunjukkan nanopartikel perak mempunyai sederhana toksik terhadap *A. salina* (LC₅₀ = 107.52 µg/mL). Aplikasi nanopartikel perak telah dilakukan dengan menambahkan nanopartikel perak ke dalam cecair pembersih sebagai aditif antibakteria dalam pembasuhan papan pencincang yang

dicemari secara buatan. Pembasuhan papan pencincang yang dicemari secara buatan dengan menggunakan cairan pembersih yang mengandung nanopartikel perak telah mengurangi bilangan bakteri sebanyak $1.60 \pm 0.11 \log_{10}$ CFU/g untuk *S. aureus*; manakala untuk *S. Typhimurium*, bilangan bakteri telah dikurangkan sebanyak $2.90 \pm 0.02 \log_{10}$ CFU/g. Keberkesanan dekontaminasi basuh menggunakan cairan pembersih yang mengandung nanopartikel perak telah dijumpai signifikan ($P < 0.05$) dan cara yang paling berkesan dalam mengurangkan pencemaran mikroorganisma dalam papan pencincang. Ini merupakan laporan yang pertama untuk menunjukkan aplikasi nanopartikel perak dalam cairan pembersih mempunyai antibakteria aktiviti yang signifikan terhadap patogen-patogen bawaan makanan. Secara keseluruhan, penemuan-penemuan ini menunjukkan nanopartikel perak mempunyai antibakteria aktiviti yang kuat dan boleh dijadikan sebagai satu alternatif agen antimikrobial.



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Son Radu, PhD

Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Yaya Rukayadi, PhD

Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Nor Khaizura Mahmud @ Ab Rashid, PhD

Senior Lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____

Name of

Chairman of

Supervisory Committee: Professor. Dr. Son Radu

Signature: _____

Name of

Member of

Supervisory

Committee: Assoc. Prof. Dr. YayaRukayadi

Signature: _____

Name of

Member of

Supervisory

Committee: Dr. NorKhaizura Mahmud @ Ab Rashid

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LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
AgNO ₃	Silver nitrate
ATCC	American Type Culture Collection
CDCIS	Communicable Diseases Control Information System
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CPRC	Crisis Preparedness and Response Centre
CPS	Capsule polysaccharide
DNA	Deoxyribonucleic acid
DAEC	Diffuse-adhering <i>Escherichia coli</i>
EAEC	Enterohaemorrhagic <i>Escherichia coli</i>
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EIEC	Enteroinvasive <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FTIR	Fourier–Transform Infrared
h	hour
HUS	Hemolytic uraemic syndrome
kV	kilovolts
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MHB	Mueller Hinton broth
min	minute
MOH	Ministry of Health
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAG	<i>N</i> -acetyl glucosamine
NAM	<i>N</i> -acetyl muramic acid
nm	nanometer
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	reactive oxygen species

rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TSA	Trypticase soy agar
TSB	Tryptic soy broth
UPM	Universiti Putra Malaysia
WHO	World Health Organization
<i>x g</i>	Unit gravity



CHAPTER 1

INTRODUCTION

1.1 Background

Foodborne illness is caused by consuming food contaminated with bacteria, viruses or chemicals. However, the most important source of foodborne illness is mainly related to the consumption of bacterial contaminated foods (Su *et al.*, 2005). Food especially minimal-processed food can be contaminated during pre-harvesting, post-harvesting, processing, transport, handling or preparation. The most common foodborne pathogens found in food are *Salmonella* spp. (Lee *et al.*, 2015; D'Ostuni *et al.*, 2016), *Listeria* spp. (Ferreira *et al.*, 2014; Välimaa *et al.*, 2015), *Escherichia coli* O157 (Heiman *et al.*, 2015), *Campylobacter* spp. (Kaakoush *et al.*, 2015) and *Clostridia* spp. (Chukwu *et al.*, 2016).

Recently, nanotechnology has emerged as a dynamically developing area of scientific interest in the world. Nanoparticles such as silver nanoparticles (AgNPs) are well-known to exhibit a strong antimicrobial activity against various microorganisms such as bacteria, viruses and fungi due to its smaller in size and large surface area (Franci *et al.*, 2015). Synthesis of AgNPs employing either biological microorganisms or plant extracts has emerged as a simple and alternative to chemical synthesis. Biological or plant extracts-mediated synthesis method provides advancements over chemical methods as it is environmental friendly and cost effective. Plant extracts-mediated synthesis of AgNPs can be advantageous compared with other biological processes as it does not require the process of maintaining the cell cultures and aseptic environments (Loo *et al.*, 2012). Several studies on the green synthesis of AgNPs using plant extracts have been reported (Medda *et al.*, 2015; Ahmed *et al.*, 2016; Dhand *et al.*, 2016; Selvam *et al.*, 2017).

Tea is a well-known beverage which produced from the plant *Camellia sinensis*. There are 3 types of tea: green tea (non-fermented), Oolong tea (semi-fermented) and black tea (fully fermented) (Liu *et al.*, 2015). According to Sentkowska *et al.* (2017), approximately 80% of all teas consumed is black tea. The leaves of *Camellia sinensis* contain polyphenolic compounds which known as tea catechins. The phenolic compounds in teas contain high antioxidants properties which are good in reducing metal ions. Thus, the phenolic compounds could act as the reducing and capping agents in the process of synthesis silver nanoparticles (Senthilkumar *et al.*, 2014).

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) have been used to evaluate the *in vitro* activity of new antimicrobial agents. MIC value of an antimicrobial agent is determined by broth dilution in 96-well microtitration plate while MBC value is determined by using an antibiotic free culture plate. Well- and disc-diffusion methods have been reported as qualitative indicators for testing the antimicrobial activity (Yemoa *et al.*, 2011). Time-kill assay

is a method in determining the bactericidal or fungicidal effect. The testing methods are standardised and described by the Clinical and Laboratory Standards Institute (CLSI)M100-S22 for antibiotic testing (CLSI, 2012).

Nanotoxicology research is important as the widely used of nanoparticles have increased the exposure of living organisms to nanoparticles direct- or indirectly. Brine shrimp lethality assay has been served as the alternative biological assay in evaluating the toxicity effect of AgNPs. Brine shrimp (*Artemia salina*) is the most widely used species among all the *Artemia* species. *Artemia* species have been applied as an experimental test organism in over 90% of the toxicological test (Hamidi *et al.*, 2014). Several studies reported that brine shrimps as the most suitable test organisms for toxicological test due to its rapid screening technique, simple, convenience, no aseptic technique required and cost-effective (Arulvasu *et al.*, 2014; Rajabi *et al.*, 2015; Phull *et al.*, 2016; Rajakumar *et al.*, 2017).

Several studies reported that cross contamination from raw product through different surfaces such as hands (Jensen *et al.*, 2017), dishcloth (Mazengia *et al.*, 2015), stainless steel utensils (Erickson *et al.*, 2015; Kuda *et al.*, 2016), and cutting board (Goh *et al.*, 2014; Faour-Klingbeil *et al.*, 2016) increased the risk of getting foodborne illnesses. Cutting board and utensils used in domestic kitchen are reported as the potential reservoirs for foodborne pathogens (Cliver, 2006; Kuan *et al.*, 2017). Washing is the important process during food preparation in reducing the risk of foodborne infections. Washing-up liquid with antibacterial agent is required as it helps in eliminating the food residues and microbial load on the kitchen utensil such as chopping board.

1.2 Problem Statement

Foodborne illness has increased in recent years. Foodborne illness is mostly caused by the consumption of bacteria contaminated foods. Cross-contamination during processing or food preparation is one of the causes of food contamination and lead to foodborne illness. Kitchen utensils such as cutting board in domestic kitchen should be washed properly by using antibacterial washing-up liquid as they could be a vehicle for the transmission of foodborne pathogens. However, bacteria may exhibit multiple drug resistance where the bacteria species are able to multiply after exposure to several types of antibiotics. Widely misuse and abuse of antibiotics are the leading cause of antibiotic resistance in the bacteria (O'Bryan *et al.*, 2018). Multidrug resistant bacteria infection may lead to several impacts including increase of mortality and morbidity rates, prolong of hospitalization period, and economic loss (Patel *et al.*, 2008). Thus, the development of a new and natural antimicrobial agent is needed as there is a growing concern in multidrug resistant foodborne pathogens. Synthesis of silver nanoparticles by tea extracts are reported as a simple, inexpensive and environmental friendly process. Silver nanoparticles are able to eliminate the bacteria as the nanoparticles are less likely to develop resistance in bacteria than antibiotics. Using tea extract-synthesized silver nanoparticles as antibacterial additive in washing-up liquid for cutting board, the bacteria can be reduced.

1.3 Objectives

The objectives of this study are:

1. to synthesis the silver nanoparticles by black tea leaves extracts
2. to determine the antibacterial activity of green synthesized silver nanoparticles against a diverse range of Gram-negative and Gram-positive foodborne pathogens
3. to evaluate the toxicity effect of tea leaves extracts synthesized AgNPs
4. to evaluate the effectiveness of AgNPs as an antibacterial additive in washing-up liquid



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