UNIVERSITI PUTRA MALAYSIA

EFFECTS OF COBALT-60 GAMMA IRRADIATION ON MICROBIAL CONTAMINANTS AND PHYTOCHEMICAL CONSTITUENTS OF DIFFERENT MEDICINAL PLANTS

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By

SYAFIQAH BINTI MHD JAMAL

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

To my beloved parents, Mhd Jamal Misni and Lena Kasmiran, as well as my sister, Syafiqah Nabilah Mhd Jamal, this valuable research work is dedicated to all of you. I could have never done this without your love, supports and encouragements.

Thank you.

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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Chair : Azmiza Syawani Binti Jasni, PhD
Faculty : Medicine and Health Sciences

Medicinal plants have been utilized worldwide for many centuries to treat diseases and enhance human's health since they are rich in phytochemical constituents. Nowadays, medicinal plants still play an important role in healthcare sectors across many countries include Europe and Southeast Asia. Recently, medicinal plants such as *Eurycoma longifolia*, *Ficus deltoidea* and *Centella asiatica* have been widely commercialized as herbal-based products in Malaysia. However, high occurrence of microorganisms in medicinal plants can cause hazards to the consumers and change the therapeutic effects. For this reason, the study is focused on the effectiveness of cobalt-60 gamma irradiation in reducing the microbial contaminants and preserving the phytochemical constituents in common medicinal plants; *Orthosiphon aristatus* (Blume) Miq., *Labisia pumila* and *Piper betle* L. This study provides preliminary data on the effectiveness of gamma irradiation as an efficient food sterilizer and provide specific dosages to sterilize herbs in Malaysia.

The medicinal plants were processed as powder, individually packaged and exposed to 0, 3, 6, 9 and 12 kGy cobalt-60 gamma irradiation at Malaysia Nuclear Agency. The microbial contaminants present in non-irradiated and irradiated medicinal plants were evaluated at 0, 3 and 6 months by conducting the microbial enumeration tests; Total Aerobic Microbial Counts (TAMC) and Total Yeast and Mold Counts (TYMC), bacterial identification using selective media and 16S rRNA PCR amplification. The microbial enumeration tests results showed that the bacterial, yeast and mold loads were significantly reduced after irradiation. There were significant changes ($P < 0.05$) observed in the microbial counts after irradiation at 3 and 6 kGy, whereas no significant changes ($P > 0.05$) observed after higher dosages. Interestingly, *P. betle* showed low microbial loads (< 10^2 CFU/g) and no significant changes ($P > 0.05$) were observed pre- and post- irradiation.

The identification results revealed the presence of bacteria from Gammaproteobacteria and Clostridia classes in non-irradiated *O. aristatus* and *L. pumila*, while bacteria from Bacilli class mostly isolated from irradiated medicinal plants. Dose of 6 kGy was able to eliminate pathogenic *Bacillus cereus* in *O. aristatus*, whereas 9 kGy was able to eliminate pathogenic *B. cereus* in *L. pumila*. Interestingly, no pathogenic bacteria detected in *P. betle* pre- and post-irradiation. The data clearly showed that gamma irradiation dose is plant-dependent where irradiation at 6 and 9 kGy were needed to eliminate pathogenic bacteria in *O. aristatus* and *L. pumila*, respectively. This highlights that specific dosages are needed in eliminating pathogenic bacteria in different medicinal plants. Meanwhile, *P. betle* is considered as a microbial low plant and gamma irradiation seems not necessary to be applied on the plant. Concurrently, there were no changes in the phytochemical contents of medicinal plants in which constituents including saponins, tannins, steroids and triterpenes were detected in both non-irradiated and irradiated medicinal plants. In conclusion, cobalt-60 gamma irradiation is effective in reducing the microbial contaminants in medicinal plants and maintaining the phytochemical constituents.

Keywords: Gamma irradiation, medicinal plants, microbial contaminants, phytochemical contents

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

KESAN PENYINARAN KOBALT-60 GAMMA TERHADAP KANDUNGAN MIKROBIAL DAN KONSTITUEN FITOKIMIA TUMBUHAN-TUMBUHAN UBATAN YANG BERBEZA

Oleh

SYAFIQAH BINTI MHD JAMAL

Julai 2020

Pengerusi : Azmiza Syawani Binti Jasni, PhD
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Tumbuhan-tumbuhan ubatan telah diaplikasikan selama berabad lamanya di seluruh dunia untuk merawat penyakit dan meningkatkan tahap kesihatan manusia kerana kaya dengan kandungan fitokimia. Pada masa kini, tumbuhan-tumbuhan ubatan masih memainkan peranan penting dalam sektor penjagaan kesihatan di kebanyakan negara seperti Eropah dan Asia Tenggara. Saat ini, tumbuhan-tumbuhan ubatan seperti Tongkat Ali, Mas cotek dan Pegaga telah dikomersialkan sebagai produk herba di Malaysia. Walau bagaimanapun, kandungan mikroorganisma yang tinggi boleh membahayakan pengguna dan juga mampu mengubah kesan terapeutik. Oleh hal yang demikian, kajian ini memfokuskan kepada keberkesanan sinaran kobalt-60 gamma dalam mengurangkan kandungan microbial dan memelihara kandungan fitokimia dalam Misai kucing, Kacip Fatimah dan Sireh. Kajian ini menyediakan data awal tentang keberkesanan sinaran kobalt-60 gamma sebagai pensteril makanan yang efektif dan menetapkan dos standard yang boleh digunakan untuk mensteril herba di Malaysia.

Tumbuhan-tumbuhan ubatan telah diproses menjadi serbuk, dibungkus secara berasingan dan didedahkan kepada sinaran gamma pada 0, 3, 6, 9 and 12 kGy di Agensi Nuklear Malaysia. Kandungan mikrobial yang ada dalam serbuk tumbuhan-tumbuhan ubatan sebelum dan selepas sinaran gamma telah dinilai selama 0, 3 and 6 bulan dengan menjalani ujian pengiraan mikrob; 'Total Aerobic Microbial Counts' (TAMC) dan 'Total Yeast and Mold Counts'

(TYMC) dan identifikasi bakteria menggunakan media selektif dan amplifikasi 16S rRNA PCR. Ujian pengiraan mikrob menunjukkan bahawa kandungan bakteria, yis dan kulat telah berkurang secara ketara selepas menggunakan sinaran. Perubahan yang ketara terhadap kandungan mikrobial ($P < 0.05$) telah diperhatikan selepas penyinaran gamma pada dos 3 dan 6 kGy, manakala tiada perubahan yang ketara ($P > 0.05$) diperhatikan pada dos yang lebih tinggi. Menariknya, Sireh menunjukkan kandungan mikrob yang sangat rendah (< 10^2 CFU/g) dan tiada perubahan diperhatikan ($P > 0.05$) sebelum dan selepas penyinaran gamma.

Keputusan identifikasi menunjukkan kehadiran bakteria daripada kelas Gammaproteobakteria dan Clostridia dalam Misai kucing dan Kacip Fatimah sebelum penyinaran, sementara bakteria daripada kelas Bacilli telah dikenalpasti selepas penyinaran. Dos 6 kGy telah berupaya menghapuskan patogenik *Bacillus cereus* dalam Misai kucing, manakala dos 9 kGy telah berupaya menghapuskan patogenik *B. cereus* dalam Kacip Fatimah. Menariknya, tiada bakteria patogenik dikenalpasti dalam Sireh. Berdasarkan data, penyinaran gamma menunjukkan bahawa dosnya bergantung terhadap jenis tumbuhan yang digunakan, seperti 6 dan 9 kGy telah berupaya untuk menghapuskan bakteria patogenik dalam Misai kucing dan Kacip Fatimah. Ini menjelaskan bahawa dos-dos spesifik yang berbeza diperlukan untuk menghapuskan bakteria patogenik yang terdapat dalam tumbuhan-tumbuhan ubatan yang berbeza. Sementara itu, Sireh pula dianggap sebagai tumbuhan ubatan rendah mikrobial dan penggunaan penyinaran gamma seperti tidak perlu diaplikasikan terhadap tumbuhan ini. Pada masa yang sama, tiada perubahan dalam kandungan fitokimia di mana konstituen seperti saponin, tannin, steroid dan triterpin dikesan dalam sampel tumbuhan ubatan sebelum dan selepas penyinaran gamma. Secara konklusinya, penyinaran kobalt-60 gamma telah dapat mengurangkan kandungan mikrobial yang terdapat dalam tumbuhan-tumbuhan ubatan dan mengekalkan kandungan fitokimia.

Kata kunci: Penyinaran gamma, tumbuhan-tumbuhan ubatan, kandungan mikrobial, kandungan fitokimia

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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:

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Declaration by graduate student

I hereby confirm that:

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This is to confirm that:

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AE	Elution buffer
AL	Lysis buffer
atm	Atmosphere
AW1	Wash buffer 1
AW2	Wash buffer 2
CFU/g	Colony-forming unit per gram
C-O	Carbon-Oxygen bond
dH2O	Distilled water
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EPP	Entry Point Project
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GAP	Good Administration Procedure
GIC	Gamma irradiation chamber
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
Gy	Gray
g	Gram
h	Hour
IAEA	International Atomic and Energy Agency
ICGFI	International Consultative Group on Food Irradiation
IFST	Institute of Food Science and Technology
kb	Kilobase
kg	Kilogram
kGy	Kilogray
Log CFU/g	Logarithm Colony-Forming Unit per gram
MANOVA	Multivariate Analysis of Variance
mins	Minutes
ml	Milliliter
NCBI	National Center for Biotechnology Information
NKEA	New Key Economic Area
PCR	Polymerase Chain Reaction
QS	Quorum sensing
rpm	Revolutions per minute
RM	Ringgit Malaysia
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSV	Rappaport Salmonella Vasiliadis broth
s	Second
spp	Species

SDA	Sabouraud Dextrose agar
SOP	Standard Operating Procedure
SPSS	Statistical Package for the Social Sciences
TAMC	Total Aerobic Microbial Counts
TBE	Tris-Borate-EDTA
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TYMC	Total Yeast and Mold Counts
UK	United Kingdom
US$	United States Dollar
UV	Ultraviolet
V	Volt
w/v	Weight per volume
WHO	World Health Organization
XLD	Xylose, Lysine and Deoxycholate agar
27F	27 Forward primer
1492R	1492 Reverse primer
16S	Ribosomal 16S
µl	Microliter
%	Percentage
ºC	Degree celsius

CHAPTER 1

INTRODUCTION

1.1 Study background

Medicinal plants or herbs have made great contributions to human healthcare and in the development of modern medicines. They become the useful natural resources for improving human's health and cure various type of diseases and inflammations across various human communities (Alviano & Alviano, 2009; Khan et al., 2013; Alsarhan et al., 2014; Mustafa et al., 2017). Thus far, the usage of medicinal plants is still relevant in healthcare sectors around the world including Europe, South America, Asia and Southeast Asia. In Malaysia, there are different medicinal plants that can be found such as *O. aristatus*, *L. pumila*, *P. betle*, *Clinacanthus nutans*, *Curcuma longa* and *Phyllanthus niruri* which have their own functions and efficacy. Different parts of the plants such as rhizomes, roots, stems, leaves, seeds and fruits are widely utilized by the locals for centuries and believed to have variety of phytochemical constituents which are beneficial for medicinal purposes.

Above all, medicinal plants such as *O. aristatus* (Misai kucing), *L. pumila* (Kacip Fatimah) and *P. betle* (Sireh) are commonly consumed by the Malaysian locals to promote well-being and treat various diseases such as diabetes mellitus, hypertension, kidney disease, bladder inflammation, gonorrhea, dysentery and maintain oral hygiene (Chua et al., 2012; Singh et al., 2015; Mohd Fuad et al., 2017; Ali et al., 2018). The healing properties in these plants are attributable to the presence of major phytochemical constituents including flavonoids, tannins, terpenoids, triterpenes, phenolics, alkaloids, saponins and steroids (Alavijeh et al., 2012; Khan et al., 2018). On top of that, *O. aristatus*, *L. pumila* and *P. betle* leaves extracts are reported to have high level of phytochemical constituents such as triterpenes, saponins, flavonoids, phenolics and alkaloids which show high antimicrobial activity against various type of microorganisms including bacteria and fungi (Pattiram et al., 2011; Ali et al., 2018). Nowadays, the commercialization of these medicinal plants is actively taking part in Malaysia and they are available in the current markets as health supplements, anti-ageing cream, herbal teas and feminine wash (Mohd Hafizudin et al., 2019). Recently, due to the various benefits of traditional medicines and herbs, the societies are starting to claim that herbal products are more safe, effective and affordable.

As the use of medicinal plants continues to grow and more herbal-based products are introduced in the markets, nonetheless concerns over safety and quality of medicinal plants are also rising. The presence of microorganisms in medicinal plants poses as risks since they can harm the consumers and lead to many health problems such as gastrointestinal diseases and bloody diarrhea (Bugno et al., 2006; Vitullo et al., 2011; Araujo & Bauab, 2012; Aiko & Mehta, 2016). Previously reported that dried powder of *L. pumila* is contaminated with high level of microbial contaminants (> 10^8 CFU/g) while more than 10^7 CFU/g of microbial counts, multidrug resistant *Salmonella* spp. and *Escherichia coli* are detected in *P. betle* leaves (Ahmad Ramli, 2010; Fakruddin et al., 2017; Kamal et al., 2018). Limited data though reported on *O. aristatus* in terms of its microbial levels. Furthermore, there are also foodborne disease outbreaks related to contaminated medicinal plants and herbal-based products with *Salmonella* spp., *Escherichia coli*, *Bacillus cereus*, *Shigella* spp., and *Staphylococcus aureus* have been revealed between the years 2014 to 2018 (Canadian Food Inspection Agency, 2019). Above all, *Salmonella* spp. and *E. coli* account for most food infections associated with medicinal plants (CDC, 2010; Zweifel & Stephan, 2012; Public Health Ontario, 2015). The high level of microbial contaminants and pathogens such as *B. cereus*, *E. coli* and *Salmonella* spp. result in food infections as these microorganisms can produce toxins, for instance enterotoxins which are detrimental to humans. Although the data on the prevalence of foodborne diseases associated with *O. aristatus*, *L. pumila* and *P. betle* are limited, yet previous studies have shown that *L. pumila* and *P. betle* consisted of high level of microbial contaminants, therefore the microbial quality of these plants need to be assessed (Ahmad Ramli, 2010; Kamal et al., 2018).

In attempt to have a safer and higher-quality herbal product, various preservation techniques such as oven drying, freeze drying, air drying and fumigation are used to reduce the microbial contaminants. These preservation methods, though are less effective because most of the methods are time-consuming and utilize high temperature (> 60ºC), whereas for fumigation, it has been banned from use since it contains carcinogenic and mutagenic agents. Therefore, a new preservation method known as cobalt-60 gamma irradiation is introduced to reduce the microbial contaminants in medicinal plants and ensure the safety of herbal-based products. Cobalt-60 gamma irradiation is a non-thermal preservation technique which employs carbon cobalt-60 to sterilize various type of foods and raw materials including herbs, spices, vegetables, fruits, fish and meats at very low temperatures (< 40ºC) (Bruhn, 2017). This technique is classified as an ionizing radiation that contains a short wavelength of radiation and carries energy to irradiate foods without causing any radioactive effects (Morehouse & Komolprasert, 2004). In order to reduce the microbial contaminants and enhance the shelf-life, irradiation dosages below 30 kGy are suggested by the Food and Drug Administration (FDA) to be used on medicinal plants, while dosages below

15 kGy are recommended in Malaysia (Malaysian Standard, 2005; FDA, 2016). Nonetheless, scientific reports about the specific dosages that need to be applied on different medicinal plants to eliminate microorganisms are still limited.

1.2 Problem statements

High occurrence of microorganisms in medicinal plants have raised concerns as they can cause hazards to the consumers. The toxigenic microorganisms such as *B. cereus*, *Salmonella* spp., *E. coli* and *S. aureus* can alter the structure of bioactive components and change the therapeutic effects of medicinal plants which can cause nausea, vomiting, infectious diarrhea and gastroenteritis (Ratajczak et al., 2014). The usage of *O. aristatus*, *L. pumila* and *P. betle* either as raw materials or herbal products is increasing, as well as their commercialization as cosmetics and health products, however the safety and quality of these medicinal plants are still in doubts. Due to the safety issues regarding high occurrence of microbial contaminants and pathogens in medicinal plants, and also the inefficient previous preservation methods, cobalt-60 gamma irradiation with dosages less than 15 kGy are suggested to be used in Malaysia to decontaminate medicinal plants. Therefore, it is critical to find a specific dosage that can be applied on different medicinal plants to reduce and eliminate the microbial contaminants without disturb its phytochemical constituents, as well as maintain the microbial counts at an appropriate level during longer storage.

1.3 Objectives

In order to have a safer and higher-quality herbal product, this study therefore evaluated the microbial quality of three medicinal plants including *Orthosiphon aristatus* (Blume) Miq. (Misai kucing), *Labisia pumila* (Kacip Fatimah) and *Piper betle* L. (Sireh).

1.3.1 General objective

To evaluate the effects of gamma irradiation on microbial contaminants and phytochemical constituents present in *O. aristatus* (Blume) Miq. (Misai kucing), *L. pumila* (Kacip Fatimah) and *P. betle* L. (Sireh).

1.3.2 Specific objectives

1. To examine the Total Aerobic Microbial Counts (TAMC) of non-irradiated and irradiated different medicinal plants at 0, 3 and 6 months of storage duration.
2. To examine the Total Yeast and Mold Counts (TYMC) of non-irradiated and irradiated different medicinal plants at 0, 3 and 6 months of storage duration.
3. To identify the pathogenic bacteria that present in non-irradiated and irradiated different medicinal plants using 16S rRNA PCR.
4. To detect the presence of phytochemical constituents in non-irradiated and irradiated medicinal plants by performing phytochemical contents screening.
5. To determine the standard dosage of gamma irradiation for the different medicinal plants.

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BIODATA OF STUDENT

Syafiqah Mhd Jamal was born in Hospital Kluang, Johor on July 9th, 1995. She attended her primary school at Sekolah Kebangsaan Parit Bingan from 2002 to 2007 and continued her secondary school at Sekolah Menengah Kebangsaan Tun Ismail (SMKTI) from 2008 to 2012. After she finished her Foundation in Biological Science at Universiti Malaysia Sarawak, she then furthered her Bachelor of Science (Hons) Resource Biotechnology at the Faculty of Resource Sciences and Technology (FRST), Universiti Malaysia Sarawak from 2014 to 2017. She has been actively involved as one of the committee members under the Majlis Perwakilan Pelajar UNIMAS and Allamanda Residential College for 3 years. Apart from that, she was also a part of Sukarelawan Mahasiswa UNIMAS (SMU) as one of the committee members and she actively took part in volunteering since 2015 until 2017. She then graduated with CGPA of 3.25 in 2017. In 2018, she pursued her Master's degree in Medical Microbiology under the supervision of Dr. Azmiza Syawani Jasni from the Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, UPM.

PUBLICATIONS

Article

Mhd Jamal, S., Bahari, H., Arapoc, D. J., Adnan, M. A., Mad Diah, I. J., & Jasni, A. S. (2020). Evaluation of the microbial contaminants and phytochemical properties of Sireh (Piper betle L.). *International Journal of Research in Pharmaceutical Sciences*, *11*, 2745-2750.

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