GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *PIPER BETLE* LEAF EXTRACTS AND THEIR ANTIMICROBIAL PROPERTIES AGAINST ISOLATED PHYTO-PATHOGEN MODEL

by

NOORAMALINA BINTI AZHAR

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APPROVAL AND DECLARATION SHEET

This project report titled Green Synthesis of Silver Nanoparticles Using *Piper Betle* Leaf Extract and their Antimicrobial Properties against Isolated Phyto-Pathogen Model was prepared and submitted by Nooramalina binti Azhar (131141658) and has been found satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the Bachelor of Engineering (Bioprocess Engineering) in Universiti Malaysia Perlis (UniMAP).

Checked and Approved by

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(DR. KHAIRUL FARIHAN KASIM)
Project Supervisor

School of Bioprocess Engineering
Universiti Malaysia Perlis

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SINTESIS HIJAU SILVER NANOPARTIKEL DENGAN MENGGUNAKAN EKSTRAK DAUN *PIPER BETLE* DAN CIRI-CIRI ANTIMIKROB TERHADAP MODEL FITO-PATOGEN TERPENCIL

ABSTRAK

Nanopartikel perak telah dikenali sebagai salah satu agen antibakteria yang penting. Projek ini bertujuan untuk menilai nanopartikel perak yang disintesis daripada *Piper betle* dan sifat antimikrob mereka terhadap model fito-pathogen. Pembentukan nanopartikel perak dalam ekstrak akueus telah disahkan dengan perubahan warna dari kekuningan kepada coklat. Untuk mengesahkan lagi kehadiran nanopartikel perak, spektrum UV-Vis telah digunakan dan penyerapan nanopartikel perak dianalisis pada 452 nm. Aktiviti antimikrob oleh yang disintesis daripada P. betle telah dinilai dengan menggunakan plat telaga agar terhadap *Escherichia coli* (bakteria gram-negatif), *Pseudomonas aeruginasa* (bakteria gram-positif) dan *Aspergillus niger* (kulat). Keputusan menunjukkan yang disintesis nanopartikel perak pada 1 mM kepekatan pameran zon terpuji perencatan terhadap *E. coli, P. aeruginasa* dan *A. niger* (14.1 ± 0.13, 14.5 ± 0.17 dan 14.7 ± 0.40 mm, masing-masing). Semua nanopartikel perak yang disintesis menunjukkan aktiviti antimikrob yang signifikan berbanding ekstrak akueus *P. betle*. Kepekatan perencatan minimum (MIC) terhadap nanopartikel perak terbaik (1 mM) telah dinilai dan menunjukkan bahawa *P. aeruginasa, E. coli* dan *A. niger* boleh melihat di tiga kali ganda, dua kali ganda dan satu kali ganda masing-masing. Untuk mencirikan saiz dan bentuk nanopartikel perak, pelepasan bidang imbasan mikroskop elektron (FESEM) telah digunakan dan hasil yang nanopartikel perak itu dalam bentuk bulat yang pelbagai 15-19 nm dalam saiz. Kehadiran perak unsur telah diperolehi dengan menggunakan tenaga serakan spektroskopi (EDX), yang menunjukkan bahawa perak adalah tersedia sebagai elemen utama pada jarak penyerapan 2.8 keV.

ABSTRACT

Silver nanoparticles are well known as one of the promising agent especially for antibacterial activity. This project was aimed to evaluate the synthesized silver nanoparticles from *Piper betle* and their antimicrobial properties against phyto-pathogen model. The formation of silver nanoparticles in aqueous extract were confirmed by colour changes from yellowish to brown. To further confirm the presence of silver nanoparticles, UV-Vis spectrum was used and the absorbance of the silver nanoparticles were analysed at 452 nm. Antimicrobial activity of the synthesized silver nanoparticles of *P. bettle* was evaluated by using agar well plate on *Escherichia coli* (gram-negative bacteria), *Pseudomonas aeruginasa* (gram-positive bacteria) and the *Aspergillus niger* (fungi). The results show that synthesized silver nanoparticles on 1 mM concentration exhibits admirable zone of inhibition against *E. coli, P. aeruginasa* and *A. niger* (14.1±0.13, 14.5±0.17 and 14.7±0.40 mm, respectively). All the synthesized silver nanoparticles show significant antimicrobial activity compared to the aqueous extract of *P. bettle*. Minimum inhibition concentration (MIC) of the best synthesized silver nanoparticles of *P. bettle* (1 Mm) was further evaluated and shows that *P. aeruginasa*, *E. coli* and *A. niger* was perceived at three-fold, two-fold and one-fold dilution from the original samples respectively. In order to characterize the size and shape of silver nanoparticles, field emission scanning electron microscope (FESEM) was used and revealed that silver nanoparticles were in spherical shape that range from 15 to 19 nm in size. The presence of elemental silver was obtained by using energy-dispersive spectroscopy (EDX), which suggests that silver is presence as the primary element at absorption range 2.8 keV.

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L.	Linn
R^2	Correlation coefficient value
V_1	Volume of solution before dilution
C_1	Concentration of the microbial suspension before dilution
V_2	Volume of solution after dilution
C_2	Concentration of the microbial suspension after dilution

LIST OF ABBREVIATIONS

UV-Vis	Ultraviolet-visible
MIC	Minimum Inhibition Concentration
FESEM	Field Emission Scanning Electron Microscope
EDX	Energy-dispersive Spectroscopy

CHAPTER 1

INTRODUCTION

1.1 Background Study

Medicinal plants are proven by its value as the ability in therapeutics with the increase of resistant to pathogens that typically used in antibiotics and the emergence of new infectious diseases. *Piper betle* L. is one of the tropical plant and is native plant from eastern part and central of peninsular Malaysia. The unique part of this plant is having leaves in the love shaped and known as 'Daun Sirih'. *P. betle* L. is one of the invaluable medicinal plants, where it is focus more on its leaf that have been used for many medicinal purposes (Foo et al., 2015). This natural product are in great demand owing to their extensive biological properties and bioactive components which have been proved to be useful against large number of diseases (Chakraborty & Shah, 2011). *P. betle* leaf has a significant antimicrobial against broad spectrum of microorganisms.

In recent years, silver nanoparticles have been widely used in many consumer goods, such as medical devices, cleaning agents, and clothing, due to its unique microbial properties. Biological methods of synthesis have paved way for the "green synthesis" of nanoparticles and this is proven to be better methods (biological methods) use due to slower kinetics where they often better manipulation and control over crystal growth and their stabilization. The use of the environmentally materials like plants extract, bacteria, fungi and enzymes for the synthesis of silver nanoparticles after numerous benefits of eco-friendliness and suitable for pharmaceutical and other biomedical application as they do not involve the uses of toxic chemicals for the synthesis protocol (Praba et al., 2014).

Emeka et al. (2014) reported the silver nanoparticles are nontoxic to human but inhibit the growth of bacteria, virus and other eukaryotic microorganisms. Silver nanoparticles, also termed a new-generation antimicrobials, are used in consumer products such as cosmetics, textiles, dietary supplement and food packaging. They are in medicine against multidrug susceptible bacterial strains and for surgical coatings and medical implants (Carrillo-gonzález et al., 2016).

The advancement green synthesis of nanoparticles is a key branch of nanotechnology; where the use of biological entities like microorganisms and plant extract for the production of nanoparticles as an alternative to chemical and physical method in an eco-friendly manner. The use of plant extracts is preferable for this purpose because it is potentially advantageous over microorganisms due to the ease of improvement, the less biohazard and elaborate process of maintaining cell cultures. It is the best platform for syntheses of silver nanoparticles; being free from toxic chemicals. Moreover, by using the plant extract, it is also reduces the cost of microorganism isolation and their culture media which enhance synthesis of silver nanoparticles (Prabhu & Poulose, 2012).

Therefore, this present study will focus on the synthesis of silver nanoparticles using *P. betle* leaf extracts and to evaluate their antimicrobial properties against isolated phyto-pathogens model.

1.2 Problem Statement

Silver nanoparticles can be synthesized by using physical, chemical and biological methods. However, the problem with most of the chemical and physical methods of silver nanoparticles production is that they are extremely expensive and also involve the use of toxic, hazardous chemicals, which may pose potential environmental and biological risks. Thus, interest method (biological methods) has been moved to synthesis silver nanoparticle in a safer ways as green synthesis technique derived from plant and microbe. *P. betle* leaf is a promising antimicrobial agents against the plant pathogens. The incorporation between silver nanoparticle and this plant will clearly demonstrate the

newly synthesized of silver nanoparticles. Hence, the green synthesized of silver nanoparticles is one of the good source, which is easily produced and extensively useful in biomedical and agricultural applications. Therefore, this study will be carried out to synthesis silver nanoparticle using *P. betle* leaf and their antimicrobial properties.

1.3 Objectives

The goals of this research is to synthesis silver nanoparticle using *Piper betle* L. extracts and evaluate the antimicrobial properties against isolated phyto-pathogens model. The specific objectives are:

- To synthesize silver nanoparticle using *P. betle* leaf extract and analysis with Ultraviolet-visible spectra.
- To evaluate antimicrobial activity of synthesis silver nanoparticles against isolated phyto-pathogens model by using agar well plate and minimum inhibition concentration (MIC).
- To characterize the best synthesized silver nanoparticle by using Field Emission Scanning Electron Microscope (FESEM) and Energy-dispersive Spectroscopy (EDX).

CHAPTER 2

LITERATURE REVIEW

2.1 *Piper betle* L.

Piper betle L. (family Piperaceae) which cultivated as deep green heart shaped leaves is a woody, perennial and dioecious vine. Stems are swollen at the nodes. Leaves are alternate, simple, and yellowish green to bright green with 2 or 3 pairs of secondary veins. Betel leaves are a specialised type of ulam, best known as an essential component of betel quid, consisting of areca nut slices wrapped in fresh betel leaves with slaked lime, tobacco or spices added for flavouring. Betel leaves are supposed to ameliorate bad breath, improve vocalisation, harden the gum, and prevent indigestion, bronchitis, constipation, congestion, cough and asthma. The species has been reported to possess antimicrobial, insecticidal, antioxidant, antinociceptive, antidiabetic, gastroprotective and anticoagulant properties (Tan & Chan, 2014).

Below showing the taxonomic classification and nomenclature of *P. betle* L. (Rekha et al., 2014):

Kingdom : Plantae
Division : Magnoliphyta
Class : Magnolipsida
Order : Piperales
Family : Piperaraceae
Genus : Piper
Species : *betle*
Binomial name: *Piper betle* L.

P. betle is native to central and eastern Malaysia and was taken into cultivation more than 2500 years ago throughout Malaysia and tropical Asia. With known ethno-medicinal properties, this plant is widely use in India, Indonesia and other countries of the Indo-China region which in Malaysia, Vietnam, Laos, Kampuchea, Thailand, Myanmar and Singapore. *P. betle* thrives under humid forest conditions with high relative's humidity. It prefers deep, well-drained, friable loamy and clayey soils, rich in organic matter with a pH of about 7-7.5. It flourishes in areas with 2250-4750 mm annual rainfall and is cultivated at altitudes up to 900 m (Pradhan et al., 2013). The leaves of *P. betle* are as shown in Figure 2.1:

Figure 2.1: Picture of *P. betle* plant (Praba et al., 2014).

2.2 Antimicrobial property of *P. betle* leaf

P. betle leaf has a significant antimicrobial against broad spectrum of microorganisms. The *P. betle* shows the antimicrobial activity against *Streptococcus pyrogenes, Staphylococcus aures, Proteus vulgaris, Escherichia coli, Pseudomonas aeruginosa* etc. Besides, the leaf extract also possess the bactericidal activity against the urinary tract pathogenic bacteria such as *Enterococococcus faecalis, Citrobacter koseri, Citrobacter fruendi, Klebsiella pneumonia* etc. The bioactive molecule thought to be

responsible for anti-bacterial activity is sterol, which has been obtained in large quantities in *P. betle* extract. The mode of action may be due to surface interaction of sterol molecule present interaction of sterol molecule present in the extracts with the bacterial cell wall and membrane leading to alteration in the primary structure of cell wall, ultimately lead to pore for formation and degradation of the bacterial components. It is reported that sterol act through the disruption of the permeability barrier of microbial membrane structures (Pradhan et al., 2013).

Gram-positive bacteria are more susceptible to the inhibitory effects of the plant extract because of single layer and lack the natural sieve effect against large molecules, whereas gram negative bacteria are multi layered and complex cell wall structure. The leaf has also poses the antifungal activity against many fungal infections. One of this is *dermatophytosis*. *Dermatophytosis* is a disease of the keratinized parts of the body (skin, hair and nail) cause by a three genera (*Trichophyton, Microsporum and Epidermophyton*) of highly specialized fungi called the *Dermatophytes*. The chloroform extract of *P. betle* shows the better efficiency than methanol fraction against *dermatophytes* because of presence of non-polar components in the fraction (Pradhan et al., 2013).

2.3 Extraction in *P. betle* leaf

P. betle is a multifunctional medicinal plant that have antimicrobial properties. Previous study shows that *P. betle* had a broad spectrum of antibacterial activity against pathogens including *Rastonia, Xanthomonas* and *Erwinia*. Study of crude aqueous extract of *P. betle* showed activity against the bacteria, with the greatest zone of inhibition by the ethanol extract against Gram negative and Gram positive bacteria (Shah et al., 2016).

Demetrio (2015) published a paper in which they described that using 80% methanol and 70% ethanol of leaf extracts have an effective performed against strains of *S. aureus, E. coli and P. aeruginosa*, with the methanolic extract exhibiting wider zones of inhibition in the agar well diffusion method.

Recent studies of Chakraborty and Shah (2011) show the antibacterial activity of four different extract of *P. betle* leaves (aqueous, methanolic, ethyl acetate and petroleum ether). Five concentrations of extracts were taken (5, 10, 25, 50 and 100 mg mL^{-1}) and tested against four different bacteria, namely *S. pyogens, S. aureus, P. vulgaris* and *E. coli*. Significant increase in the zone of inhibition is observed with the increasing of the concentration extracts. In case of aqueous extract, maximum inhibition is obtained against *E. coli* at the concentration of 100 mg/ml. Whereas, in case of methanolic extract maximum inhibition is obtained against *S. aureus*. Ethyl acetate give clear zone of inhibition against *E. coli* and ether extract against *P. vulgaris* respectively.

Study by Foo et al. (2015) found the yield of aqueous extract and ethanol (70%) extract. By using 10 grams of dried *P. betle* leaves, it can produce 1.57 grams of extract in aqueous extraction and 1.23 grams of extract in ethanol (70%) extraction. The yield of aqueous extraction better than ethanol (70%) extraction because of polarity water is greater than ethanol and the polar compounds are easier to be extract compared with non-polar compounds. Water and ethanol contain hydroxyl group and can form hydrogen bonding with the bioactive compounds. Aqueous extraction is more effective than ethanol extraction in antimicrobial activity because water has higher polarity and shorter chain than ethanol. Hence, it is consider that the aqueous extraction of dried *P. betle* leaves is a promising tool for antibacterial. Aqueous extraction is less cost and green process if compare to using ethanol as solvent because water is non-toxic and eco-friendly.

2.4 Synthesis Silver Nanoparticle by Using *P. betle* Leaf

Synthesis of nanoparticles is the field of nanoparticle synthesis and assembly by utilization of biological systems such as yeast, fungi, bacteria and plant extract. Silver is a well-known antimicrobial agent against a wide range of over 650 microorganisms from different classes such as gram-negative and gram-positive bacteria, fungi or viruses. Out of all the metals with antimicrobial properties, it was found that silver has the most effective antibacterial action and is least toxic to animal cells (Ahmed et al., 2016). The green synthesis of silver nanoparticles involves three main steps, which must evaluated based on chemistry perspectives, including (i) selection of solvent medium, (ii) selection

of environmentally benign reducing agent, and (iii) selection of nontoxic substances for the silver nanoparticles stability (Sharma et al., 2009).

The major advantage of using plant extracts for silver nanoparticle synthesis is that they are easily available, safe, and nontoxic in most cases, have a broad variety of metabolites that can aid in the reduction of silver ions, and are quicker than microbes in the synthesis.

Praba et al. (2014) proposed *P. betle* extract to produce silver nanoparticle where Ag^+ ions were reduced to Ag nanoparticles when plant extract is mixed with $AgNO_3$ solution and followed by on immediate change in yellowish to brown colour in aqueous solution of the plant extract due to the excitation of surface plasmon vibration in silver nanoparticle.

These natural products are in great demand due to its extensive biological properties and providing source for the discovery of effective bioactive compounds. *P. betle* has been recognized for their many pharmacological activities such as its antioxidant and antibacterial properties. It was found that the extract of *P. betle* contains fatty acids (stearic acid and palmatic acid) and hydroxyl fatty acid esters (hydroxyl esters of stearic, palmitic and myristic acids) and hydroxychavicol, with the latter as main component. The hydrochavicol is said to exhibit antibacterial activity. Fatty acids can act as anionic surfactants and have antibacterial and antifungal properties at low pH, in addition to being selective against gram-positive organisms by targeting the structure and function of bacterial cell walls and membranes (Nalina & Rahim, 2007).

As a conclusion, the use of plant extract to synthesize silver nanoparticles has drawn attention, because of its rapid, eco-friendly, non-pathogenic, economical protocol and providing a single step technique for the biosynthesis processes. The reduction and stabilization of silver ions by combination of biomolecules are already established in the plant extract that having medicinal values and are environmental friendly.

2.5 Phyto-pathogen model

Phyto-pathogen model is refer to the model use to study the antimicrobial properties. In this study, mango (*Mangifera indica*) is selected as a model. Mango is choose because the numerous production in Perlis and it is one of the big production in Perlis. Mango suffers from several diseases at all stages of its life. Growing and marketing of fresh produce are complicated by post-harvest losses in quantity and quality between harvest and consumption. Postharvest losses of same horticultural commodities in state farm and peasant sectors are estimated to be 25-35% caused by a combination of several factors. This high loss will contributed to the lack of packaging, storage facilities, poor means of transportation and handing. Several mango postharvest techniques have been developed for controlling disease and insets and for protection against injury during packaging and storage (Woldeselassie et al., 2015).

Normally, the fruit part of mango are attacked by a number of pathogens including fungi, bacteria and algae. They cause several kinds of rot, die back, anthracnose, scab, necrosis, blotch, spots, etc. Infection on young fruits (less than 4-5 cm) appears as dark, irregular, sunken lesions and causes the fruit to abscise from the panicles. Infection of larger fruit usually remains latent (dormant) until the fruit ripens. Lesions are black, expand rapidly in size, and produce pinkish-orange spore masses under wet conditions (Woldeselassie et al., 2015).

Alternaria rot (black spot) is one of the major problem of mango pathogens during storage, which is also known as black spot, causes postharvest fruit rot. The pathogens affects leaves and panicles. They usually start near the stem end, but can expand and merge to cover much of fruit surface. Affected areas does not soften or penetrate more than 1-2 mm into the flesh until late in symptom development. Lesion centres are depressed and develop olive-brown conidia of the pathogens under moist conditions. Figure 2.2 and 2.3 shows the symptoms of *Alternaria* rot and anthracnose of mango fruit (Woldeselassie et al., 2015).

Anthracnose, *Colletotrichum gloeosporioides*, is also the most important disease of mango wherever it is grown. It is the limiting factor for mango production especially when the areas are wet. Small fruit can develop minute brown spots and abort if infected

early in their development. After fruit exceed 4-5 cm in diameter, abortion is less common and infections tend to stop development once on appressorium is formed. The fungus is ubiquitous and responsible for fruit disease of the fruits. The pathogens that cause the major disease of mango are summarized in Table 2.1.

Figure 2.2: Symtoms of a) *Alternaria rot* (Diedhiou et al., 2007).

Figure 2.3: Symptoms of anthracnose on the surface of mango (Abd-Alla & Haggag, 2010).

Table 2.1: Major diseases of mango (Wayne, 1971).

Fruit Disease	**Pathogens**
Anthracnose	*Colletotrichum gloeosporioides*
Stem-end decay	*Lasiodiplodia theobromae*
	Phomopsis mangiferae
	Dothiorella dominicana
Bacterial black spot	*Xanthomonas campestris*
Rhizopus soft rot	*Rhizopus stolonifer* *R. arrhizus*
Soft brown rot	*Hendersonia creberrima*

CHAPTER 3

METHODOLOGY

3.1 Collection and Raw Material Preparation of *P. betle* (L.)

Fresh *P. betle* leaves were bought from fresh market at Arau, Perlis. The leaves were washed with tap water to remove earthly matters. After washing, the fresh leaves were cut into small pieces and flatly spread in trays and dried in an oven-drier (Binder, Malaysia) at 50 ºC. After overnight of drying, the leaves were ground by using kitchen blender (Panasonic MX1800, Malaysia) for 3 minutes. The powdered *P. betle* leaves was keep in sterile bags for further analysis (Praba et al., 2014). Figure 3.1 shows the sequences for preparation of raw material.

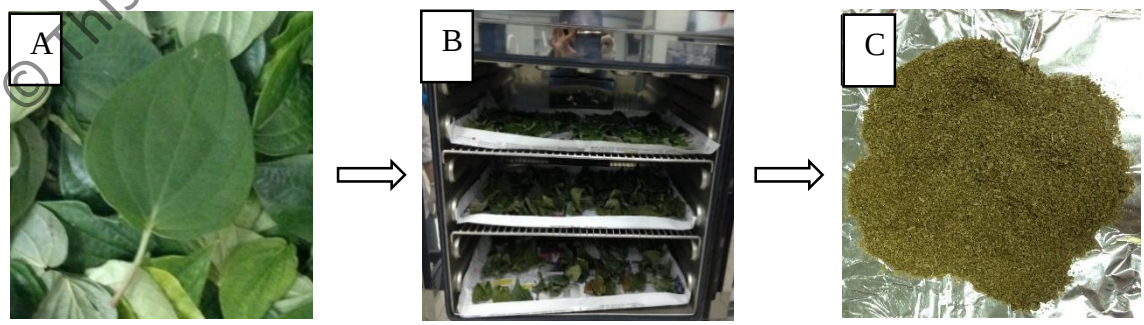

Figure 3.1: Sequences of raw material preparation A) *P. betle* leaf, B) Drying process, C) *P. betle* powder.