

***IN VITRO* ANTIBACTERIAL ACTIVITY OF
Acanthus ilicifolius-SILVER NANOPARTICLES AGAINST
SELECTED BACTERIA**

By

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DECLARATION

I hereby declare that I am the author of this dissertation; entitled "*In Vitro* Antibacterial Activity of *Acanthus ilicifolius*-Silver Nanoparticles against Selected Bacteria". I hereby declare that this research has been sent to Universiti Sains Malaysia (USM) for degree of Master of Science. The dissertation has been sent for the respective degree and is not concurrently submitted in candidature of any other degree. I also declare no conflict of interest in this work.

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In the name of Allah, Most-Gracious, Most-Compassionate

*Beautified for people is the love of that which they desire – of women and sons, heaped-up sums of gold and **silver**, fine branded horses, and cattle and tilled land. That is the enjoyment of worldly life, but Allah has with Him the best return.*

Holy Al-Quran (3:14)

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LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
ATCC	American Type Culture Collection
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
AgNO ₃	Argentum / silver nitrate
Ag	Argentum / silver
UV-Vis	Ultraviolet-visible
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MIC	Minimal inhibitory concentration
MBC	Minimal bactericidal concentration
TEM	Transmission electron microscope
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
i.e.	In example
FTIR	Fourier-transform infrared spectroscopy
XRD	X-ray powder diffraction
LC-MS	Liquid chromatography-mass spectrometry
SEM	Scanning electron microscope

LIST OF SYMBOLS / UNITS

%	Percentage
g	gram
°C	degrees Celcius
psi	pounds per square inch
g/L	Grams per liter
mg	milligram
mL	milliliter
μg	microgram
μL	microliter
mg/mL	milligram per milliliter
mM	millimolar
Mm	Millimetre
nm	nanometer
% (v/v)	volume per volume percent
x g	relative centrifugal force (RCF)
L	liter
rpm	Revolution per minute
Mm	millimeter
M	meter
mV	millivolt
mS / cm	millisiemens per centimeter

AKTIVITI ANTI-BAKTERIA *IN VITRO* NANOPARTIKEL PERAK- *Acanthus ilicifolius* TERHADAP BAKTERIA TERPILIH

ABSTRAK

Nanopartikel perak (AgNPs) telah mendapat perhatian kerana kegunaan yang meluas dalam pelbagai bidang seperti perubatan, industri makanan dan sains bahan. Di antara kegunaan nanopartikel ini dalam perubatan adalah sebagai agen anti-bakteria. AgNPs yang dihasilkan melalui kaedah biosintesis hijau menggunakan ekstrak tumbuhan sebagai agen penurun adalah kurang berbahaya dan lebih ekonomi berbanding melalui kaedah fizikal dan kimia. Dalam kajian ini, tumbuhan yang digunakan untuk sintesis AgNPs telah diperolehi dan dikenalpasti sebagai *Acanthus ilicifolius Wallich, N.* dan telah digunakan untuk menghasilkan ekstrak akues yang kemudiannya digunakan untuk mensintesis AgNPs. Campuran 100 mg/mL ekstrak akues dan 5 mM perak nitrat pada nisbah 1:10 telah dieram pada 40 °C dengan 150 rpm di dalam inkubator. Pembentukan nanopartikel perak dipantau untuk perubahan warna dan ditentukan menggunakan spektrometri UV-Vis yang kemudiannya diikuti dengan pencirian dengan analisis potensi dan saiz zeta. Spektra UV-Vis AgNPs menunjukkan penyerapan maksimum pada 440 nm manakala analisis potensi dan saiz zeta pula menunjukkan saiz purata AgNPs yang dihasilkan adalah 94 nm dengan potensi zeta sebanyak -4.08 mV yang menunjukkan AgNPs tidak begitu stabil. Aktiviti *A. ilicifolius*-AgNPs terhadap *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa* melalui teknik serapan agar berlubang menunjukkan zon perencatan (dalam milimeter, mm) sebanyak 16, 15, 14 and 20. Kepekatan minimum perencatan (MIC) dan kepekatan minimum bakterisidal (MBC) melalui asai MTT bagi nanopartikel perak ini adalah lebih rendah daripada 0.078125 mM bagi kesemua bakteria yang diuji. Kesimpulannya, ekstrak berair *A. ilicifolius* berpotensi menghasilkan AgNPs melalui kaedah hijau dan boleh digunakan sebagai agen anti-bakteria terutamanya terhadap *S. aureus*, *B. cereus*, *E. coli* dan *P. aeruginosa*

***IN VITRO* ANTIBACTERIAL ACTIVITY OF *Acanthus ilicifolius*-SILVER NANOPARTICLES AGAINST SELECTED BACTERIA**

ABSTRACT

Silver nanoparticles (AgNPs) has gained attention due to their wide application in various area such as medical, food industry and material sciences. Among medical uses of this nanoparticles is as antimicrobial agent against pathogenic bacteria. AgNPs produced by green biosynthesis approach using plant extracts as reducing agent is less harmful and economical in comparison with physical and chemical methods. In this study, plant material was collected and identified as *Acanthus ilicifolius Wallich, N* and was being used to prepare the plant aqueous extract which later being used to synthesis AgNPs. Ratio of 1: 10 mixtures of 100 mg/mL of aqueous extract and 5 mM of silver nitrate was incubated for 24 hours at 40 °C with 150 rpm in incubator shaker. The formation of silver nanoparticles was monitored by colour changes and was characterised by UV-Vis spectrometry followed by zeta (potential) sizer analyses. The UV-Vis spectrum of AgNPs showed maximal absorption at 440 while zeta (potential) sizer analyses revealed the average size of synthesized AgNPs was 94 nm with zeta potential value of -4.08 mV which the AgNPs quite unstable. Antibacterial activities of synthesised *A. ilicifolius*-AgNPs by well diffusion method on *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa* showed an inhibition zone (in millimeter, mm) of 16, 15, 14 and 20 respectively. Minimal inhibitory concentration (MIC) as well as minimal bactericidal concentration (MBC) of this silver nanoparticles accessed by MTT assay was lower than 0.078125 mM on all tested bacteria. Therefore, it is concluded that *A. ilicifolius* aqueous extract has potential in producing AgNPs by green synthesis approach and may be used as antibacterial agent against particularly on *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa*

CHAPTER 1

INTRODUCTION

Nanotechnology is considered an emerging technology of interdisciplinary research encompassing many areas of science ranging from chemistry, physics, biology including biotechnology (Tarafdar et al., 2013). Nanoparticles (NPs) refer to a group of nanoscale substances that are synthesised using a variety of metals or non-metal elements with tremendous potential in many applications (Hasan, 2014). Among them are silver nanoparticles (AgNPs). AgNPs has been reported to have good electrical conductivity and also being used for optical applications, biolabeling, as spectrally selective coatings, catalysts in chemical reactions and intercalation materials for electric batteries. Besides, it also has potential applications in drug delivery and as antimicrobial agents (Abou El-Nour et al., 2010).

Several reports AgNPs have been used and tested for prevention of bacterial colonization and removal of microorganisms on various medical devices, disinfection in sewage treatment plants, silicone rubber seals for the protection and transport of food and textiles, preservatives and additive in food industry, as drug carrier and as antimicrobial agents to combat bacterial infection in clinical treatment (Jaiswal and Mishra, 2018; Wang et al., 2018).

Bacterial infections are one of the leading causes of infectious disease, mortality and death. The preferred treatment for bacterial infections are antibiotics due to their cost-effectiveness and effective results (Huh and Kwon, 2011). However, the excessive and widespread of antibiotics usage has led to rising number of multi-drug resistance bacteria. AgNPs has broad spectrum antibacterial activity against wide range of pathogenic bacteria (Le Ouay and Stellacci, 2015). The inhibition of bacterial growth by AgNPs is the result of the release of Ag^+ ions, which interact with the sulphur-containing groups in bacterial proteins and affect replication of DNA (Marini et al., 2007).

Although AgNPs synthesis by physical and chemical methods are technically complex, expensive economically and more harmful to the environment, these method has been widely studied worldwide since the methods are clearly established (Gopinath et al., 2012) . Recently, biological methods have been proposed for the AgNPs synthesis by using enzymes, algae, microorganisms and plants as the possible alternatives to the physically and chemically synthesised AgNPs (Mohanpuria et al., 2008).

AgNPs production using plant extracts is more advantageous compared to other biological method as this method eliminate the need for extensive cultivation and maintenance of biological cells and up-scaling maybe possible for mass biosynthesis of nanoparticles (Saxena et al., 2012). Overall, plant mediated AgNPs biosynthesis is considered an environmentally friendly and inexpensive method that can be used in a single step biosynthetic process and that is safe for various therapeutic and food-based applications (Keat et al., 2015). Thus, the promising potential of green biological

synthesis of AgNPs is required to fulfil the increasing need of AgNPs to be synthesised and utilised as alternative antibacterial agent to combat antibiotic resistance bacteria.

In this present work, the synthesis of AgNPs with the *Acanthus ilicifolius* was performed in a green way. *Acanthus ilicifolius* is a species of shrub or herb, from the plant family Acanthaceae (Juffe Bignoli, 2013). Studies on the biological activities of *A. ilicifolius* have shown that this plant having variety biological potential as antiparasitic leishmania, osteoblastic, hepatoprotective, anti-inflammatory, antiulcer, antioxidant, antimicrobial and antimicrobial (Bandaranayake, 2002). *Acanthus ilicifolius* is known to contain bioactive compounds including alkaloids, triterpenoids, lignan, phenolic compounds, steroids, flavonoids and terpenoids (Firdaus et al., 2013). The compounds have potential as reducing agent in biosynthesis of silver nanoparticles. However, up to now there is no study has yet been done to synthesis the AgNPs from this plant extract. Furthermore, this plant is abundantly found in swampy area and can provide economical method of green synthesis of AgNps. Thus, the potential application as an antibacterial compound for biological synthesis of AgNPs using *Acanthus ilicifolius* was evaluated. The goals of this study are:

- To synthesis and characterise *Acanthus ilicifolius*-AgNPs
- To study and evaluate *in vitro* antibacterial activities of *A. ilicifolius*-AgNPs against selected bacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 Nanotechnology

Nanotechnology is the extension of the existing sciences to nanoscale, that involve the manipulation of matter between 1 to 100 nanometers ($1\text{ nm} = 10^{-9}\text{ meters}$). The smaller the objects, the greater the ratio between the surface area and the volume and this has allowed the creation of interesting materials and new application (Hasan, 2014). Nanotechnology is considered an emerging technology with tremendous potential in many applications. Besides having various industrial applications, major innovations in biotechnology, metrology, electronics, medicine and medical technology are rising. In medical technology, the potential applications are predominantly in diagnostics, therapeutics, monitoring, the availability of longer lasting and better prostheses and new drug delivery systems for potentially drugs (Tarafdar et al., 2013).

2.1.1 Nanotechnology as Alternative Antimicrobial Approach

The resistance of bacterial strains to currently used antibiotics are becoming a serious public health problem. Therefore, there is a strong demand for the development of new approaches and materials that may resolve these issues (Huh and Kwon, 2011). Advancement of the nanotechnology has spawned many new antimicrobial agents' option. For this purpose, metal nanoparticles such as silver nanoparticles, gold nanoparticles, titanium nanoparticles and other metals may serve for this purpose. The types of organic, metal and additional nanoparticles have demonstrated good potential as bactericidal and fungicidal elements, showing their potential as an efficient antimicrobial agent in care of wound and associated medical problems.

Various nanomaterial activities exhibit antimicrobial activity against various types of viruses and pathogenic bacteria (Abou El-Nour et al., 2010; Le Ouay and Stellacci, 2015). The properties of these nanoparticles such as size, shape and concentration influence their effectiveness. In addition, the abundance of atoms on the surface of the particles plays a role in the properties of the substance. The smaller size of a particle, the atoms proportion at the surface increases in proportion to the number of atoms of the matter, thereby increasing the activity (Baker et al., 2005; Hasan, 2014). Nanomaterials are today a promising platform for alternative measures to combat bacterial infections. Among the metals nanoparticles, silver nanoparticles gained increasing attention for the diverse application in biomedicine particularly as antimicrobial agent.

2.2 Silver nanoparticles (AgNP)

Silver nanoparticles, which are generally smaller than 100 nm and contain 20 to 15,000 silver atoms, have different physical, chemical, and biological properties compared to their original precursors (Zhang et al., 2016). The optical, thermal and catalytic properties of silver nanoparticles are strongly influenced by their size and shape (Pal et al., 2007). Due to their broad-band antimicrobial capability, silver nanoparticles are also the most commonly used sterilizing nanomaterials in consumer and medical products such as textiles, food bags, refrigerator surfaces and personal care products (Guerra et al., 2012).

2.2.1 Synthesis and Characterisation of AgNP

AgNP can be synthesized by various methods including chemicals, physical as well as biological method. In general, conventional chemical and physical methods are very costly and dangerous to the health of workers as well as harmful to the environment (Abou El-Nour et al., 2010; Gurunathan et al., 2015). In contrast, the biological approach has been proven to be a cheaper, simple, reliable and environmentally friendly approach with high production AgNP using biological systems including plants extract, fungi, bacteria and small biomolecules such as vitamins, amino acids, sugars and secondary metabolites (Gurunathan et al., 2009; Khodashenas and Ghorbani, 2015). Thus, biological methods currently the method of choice in synthesising AgNPs compared to well established chemically and physically methods.

There were some studies reported on the synthesis of AgNPs using bacteria such as *Pseudomonas stutzeri*, strain of *Lactobacillus*, *Bacillus licheniformis*, *Escherichia coli* (*E. coli*), *Brevibacterium casei* and *Actinomyces* (Gurunathan et al., 2009; Kalimuthu et al., 2008; Klaus et al., 1999). In addition, a higher organism like fungus including *Fusarium oxysporum*, *Ganoderma neojaponicum* Imazeki as well as plant extracts such as *Allophylus cobbe*, *Artemisia princeps*, *Azadiracta indica* and *Typha angustifolia* has also been used to syntheses AgNPs (Ahmed, Saifullah, et al., 2016; Gurunathan et al., 2013, 2015; Shankar et al., 2003). Table 2.1 summarise different approach of silver nanoparticles biosyntheses and their characteristic with their intended application reported recently.

There are three important factors in the green synthesis of nanoparticles; reducing agent; solvents and non-toxic substances (Gurunathan et al., 2009). The main advantages of this biological methods are the availability of biomolecules such as amino acids, proteins or secondary metabolites contain in the biological system that can eliminate the additional steps of preventing particle aggregation. In addition, the use of those biological molecules for the synthesis of AgNPs is environmentally friendly with minimal contaminants. Furthermore, this method appears to provide a controlled size and shape of a particle (Zhang et al., 2016). With bacterial cell lysate or plant extracts as reducing agents as well as the capping agent, we can control the shape and size of nanoparticles. In addition, the advantage of this method is also the extensive availability of biological resources (plant microorganism and biomolecules), reduced time requirements, stability, high density and solubility in water (Gurunathan et al., 2009; Thakkar et al., 2010).

Table 2.1: Summary of different approach of silver nanoparticles biosynthesis and their characteristic

Biological Material Used to Synthesize AgNPs	Aqueous Extract Preparation used to Synthesize AgNPs	Average size by Zeta Sizer Analysis (nm)	Zeta Potential Value (mV)	Intended Use	Reference
<i>Cornus officinal</i> (plant)	Sonication of chopped dried plant in water	12.8	Not done	Anticancer activity against human prostate cancer (PC-3) and human liver cancer (HepG2) cell lines	He et al., (2017)
<i>Azadirachta indica</i> (plant)	Boiling of chopped dried plant in water	34	Not done	Antimicrobial activity by disc diffusion method on <i>S. aureus</i> and <i>E. coli</i>	Ahmed et al., (2016)
<i>Vernonia cinereal</i> (plant)	Boiling of dried ground plant coarse powder in water	15.29 (by TEM analysis)	-29.50	Cytotoxicity on Kasumi-1 cells	Radhiyatul et al., (2016)
<i>Streptomyces xinghaiensis</i> OF1 strain (Bacteria)	Cell extract of the bacteria culture in water	64	-15.7	Antimicrobial activity on <i>B. subtilis</i>, <i>S. aureus</i>, <i>E. coli</i> and <i>K. pneumoniae</i>	Wypij et al., (2018)
<i>Nostoc sp.</i> strain HKAR-2 (Bacteria)	Cell extract of the bacteria culture in water	255.8	+1.80	- Antifungal activity <i>A. niger</i> and <i>T. harzianum</i> - Antibacterial activity on <i>R. solanacearum</i> and <i>X. campestris</i> - Cytotoxic effect on MCF-7 cell lines	Sonker et al., (2017)

<i>Pleurotus sajor-caju</i> (Mushroom)	Boiling of dried ground mushroom powder in water	89.76	-8.54	• Antifungal activity against <i>C. albicans</i>	Musa et al., (2017)
<i>Laminaria japonica</i> (algae)	Boiling of the dried algae powder in water	23	+29 - +47	• Study on seed germination (no effect) • Plant growth	Kim et al., (2018)
<i>Acanthus ilicifolius</i> (plant)	Boiling of dried ground plant coarse powder in water, followed by freeze drying of the extract and finally the freeze-dried extract ground to fine powder. The final fine powder used to prepare standardize extract	94	-4.08	• Antimicrobial activity on <i>B. cereus</i>, <i>S. aureus</i> <i>E. coli</i> and <i>P. aeruginosa</i>	This study (2018)

Various kinds of biomolecules such as proteins, sugars, amino acids, enzymes and other traces of metals are found in plant extracts. Those metabolites are suggested to be involved strongly in the biological reduction process silver ion. Reaction was proposed as Ag^+ ions reduction into metallic Ag nanoparticles with the presence of those metabolites and redox enzymes (Kuppusamy et al., 2016). Figure 2.1 summarise the silver nanoparticles biosynthesis mechanism using plant extracts.

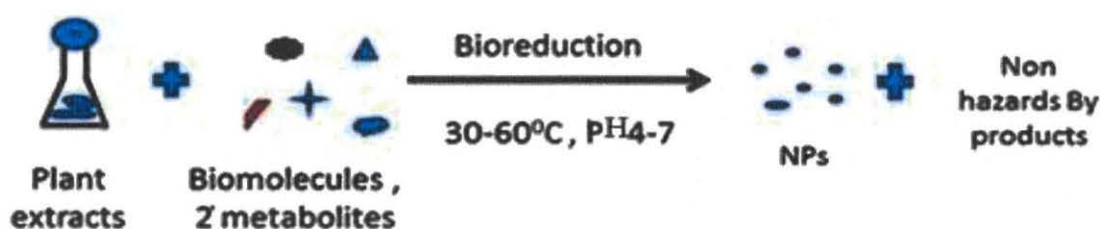


Figure 2.1: Proposed mechanism of silver nanoparticles biosynthesis (Adapted from Kuppusamy *et al.*, 2016)

The proper and accurate characterisation is required after the synthesis of AgNPs because the physico-chemicals of particle properties can influence the biological properties. The properties of nanomaterials such as shape, size, size distribution, surface area, solubility, aggregation, etc. should be assessed prior to assessment of biocompatibility, bioactivity or toxicity (Zhang et al., 2016).

There are many techniques used in the characterisation of synthesised nanomaterials, including ultraviolet (UV-vis) spectroscopy, dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), dynamic light scattering (DLS), Atomic Force Microscopy (AFM), X-ray photoelectron spectroscopy (XPS), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and so forth (Fissan et al., 2014; Inagaki et al., 2013; Manna et al., 2001; Murali Sastry et al., 1998; Waseda et al., 2011; Zhang et al., 2016).

UV-Vis spectroscopy and Zeta (potential) sizer analysis could be employed to characterise synthesised AgNPs as both of this method can be non-laborious and cheaper compared to other characterisation methods. UV-Vis spectroscopy may be employed as initial characterization method for monitoring the synthesis and AgNPs stability as well as initial assumption of AgNPs shape (Musa et al., 2017; Zhang et al., 2016). In the other hand, Zeta (potential) sizer analysis can provide useful information on the average size of the produced AgNPs as well as the Zeta potential value of the AgNPs.

2.2.2 Antibacterial Activity of AgNPs

Development of AgNPs as antibacterial agents is necessary as alternative and complementation to antibiotics to overcome bacterial resistance towards antimicrobial issues (Le Ouay and Stellacci, 2015). The AgNP properties, such as crystallographic surface structures and high surface-to-volume ratios, made it possible to be an antibacterial agent along the other promising nanomaterials such as gold, zinc, titanium and other metals (Abou El-Nour et al., 2010).

The smaller particles with a larger surface-to-volume ratio has a higher antibacterial activity compare to bigger particles (Baker et al., 2005). Beside the size, the antibacterial activity of AgNPs also influenced by shape. The synthesized AgNPs using different saccharide types and other types of biomolecules and biological materials increased antimicrobial activity against bacteria, including highly multidrug-resistant strains such as methicillin-resistant *Staphylococcus aureus*, MRSA and carbapenem-resistant Enterobacteriaceae (CRE) (Ali et al., 2018; Buszewski et al., 2018; Pal et al., 2007).

The AgNPs usually interact with the Gram-negative organism such as *E. coli*. The efficacy of the antimicrobial effects of AgNPs on *E. coli*, *S. aureus* and yeast was examined by Kim et al., (2007) and showed that at low AgNP concentrations, complete growth inhibition was observed in *E. coli* and yeast, whereas a mild effect was observed in *S. aureus*. The efficacy of various antibiotics such as amoxicillin, clindamycin erythromycin, penicillin G, tetracycline, kanamycin, neomycin, gentamycin and vancomycin against different type of bacteria was synergistically increased in the presence of Ag-NPs (Buszewski et al., 2018; Shahverdi et al., 2007).

2.3 *Acanthus ilicifolius*

Acanthus ilicifolius is a species of shrubs or herbs, of the plant family Acanthaceae (Juffe Bignoli, 2013). 'Acanthus' comes from the Greek word 'Acantha' which means thorns or thistle, referring to spiny leaves of several species. The word 'ilicifolius' means the 'ilex' leaf in Latin and refers to a leaf like holly. *Acanthus* is the only genus of the Acanthaceae family that occupy a mangrove habitat (Ramanathan et al., 2015). This species is widespread Southeast Asia including Indochina, Indonesia, the Philippines and northern Australia (Xie et al., 2005) and not forgetting Malaysia. In India, it is usually found on the east and west coast of Indian ocean as well as in Meghalaya and Andamans (Aeri and Singh, 2013). It is usually growing on the banks of the river or on the banks of the canals or low swamp areas in the mangroves and surrounding areas. It is vinyshrub or tall ingredient, up to 1.5 m high, hardly woody, dense, with very dense growth (Xie et al., 2005).

Acanthus ilicifolius has been used traditionally for several human medications. It is used as an aphrodisiac, blood purifier, diuretics, asthma, diabetes, dyspepsia, hepatitis, leprosy, neuralgia, paralysis, ringworm, rheumatic, skin diseases, snakebite, abdominal pain, leucorrhoea and leukaemia (Bandaranayake et al., 2017; Xie et al., 2005). In Ayurvedic practice, this plant current being used to ease rheumatic problem and may also being use for as antioxidant (Paul and Ramasubbu, 2017; Singh and Aeri, 2013)

2.3.1 Bioactivity of *Acanthus ilicifolius*: Antimicrobial Activity

It is known that *Acanthus ilicifolius* contain bioactive compounds including alkaloids, triterpenoids, lignan, phenolic compounds, steroids, flavonoid, and terpenoids (Firdaus et al., 2013; Wöstmann and Liebezeit, 2008). Studies on the biological activities of *A. ilicifolius* revealed that this plant has antiparasitic (*Leishmania spp*), osteoblastic, hepatoprotective, anti-inflammatory, antiulcer, antioxidant, anticancer, and antimicrobial activities (Ramanathan et al., 2015). As proposed by Kuppusamy et al., (2016), biomolecules contained in *A. ilicifolius* such as sugars, protein, alkaloids, triterpenoids, lignan, phenolic compounds, steroids, flavonoid, and terpenoids may contributed to the reduction and antioxidant processes in AgNps biosynthesis.

A. ilicifolius has a strong bioactive compound such as 6-hydroxybenzoxazolinone, (Z) - 4-coumaric acid-4-O-D-glucopyranoside and 3,5-dimethoxy-4-hydroxymethylbenzoate that have been identified as an antibacterial agent (Ravikumar et al., 2012). Alcoholic and chloroform extracts of leaves showed potent inhibitory activity against *B. subtilis*, *C. albicans*, *S. aureus*, *A. niger*, and *A. fumigatus* with moderate inhibitory activity against *P. vulgaris* and *P. aeruginosa* (Khan et al., 2018; Rai et al., 2017).

Extracts of aqueous root and ethanol-methanol mixture on leaves showed significant activity against *Aspergillus flavus*, *Bacillus megaterium*, *Candida albicans*, *Escherichia coli*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Salmonella paratyphi* B, *Shigella dysenteriae*, *Streptococcus mutans*, *Staphylococcus albus* and *Klebsiella pneumoniae* (Ganesh and Vennila, 2009). The white growth of *V. harveyi* in vitro was inhibited by the n-butanol fraction of *A. ilicifolius* leaves. In addition, *A. ilicifolius* was potentially active against *E. coli*, *K. pneumonia* and *Vibrio cholera* (Ramanathan T, 2011).

Chloroform extract from *A. ilicifolius* leaves showed systemic antifungal activity against mice infected with *Aspergillus fumigatus* (Kalaskar et al., 2012). Benzoate and phenylethanoid derivatives isolated from chloroform leaf extract produced inhibitory activity against infectious pathogens of the urinary tract (Ravikumar et al., 2012). Chloroform extract demonstrated maximal activity (MBC value: 0.5-3 mg/mL, MIC value: 2-4 mg/mL) against skin pathogens (Govindasamy and Arulpriya, 2013). 2-Benzoxazolinone showed activity against some plant pathogens (Singh and Aeri, 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 *Acanthus ilicifolius Wallich*, N Plant Materials

Plant material was collected at Kampung Sungai Pial, Merbok, Kedah (5.7171891 °N, 100.3566836 °E). The whole plant that consists of stems, barks, leaves and root was collected.

3.1.2 Bacteria Culture

Glycerol stock of bacteria cultures used for the experiments which were *Staphylococcus aureus* ATCC 33591, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 was kindly provided by Associate Professor Dr Khamsah Suryati Mohd, Dean of Fakulti Biosumber dan Industri Makanan, Universiti Sultan Zainal Abidin, Kampus Besut

3.1.3 Chemicals and Reagents

The chemicals and reagent used throughout the study were analytical or higher grades and obtained from various suppliers as listed in Table 3.1

Table 3.1: List of chemicals and reagents

Chemical / Reagent	Manufacturer
Hydrochloride acid	Merck (Germany)
Sodium hydroxide	R&M (Malaysia)
Silver nitrate	R&M (Malaysia)
Glycerol	Merck (Germany)
Dimethylsulphoxide	Merck (Germany)
Technical grade ethanol	R&M (Malaysia)
[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], MTT	Promega (USA)
McFarland standard	Oxoid (UK)
30 µg vancomycin antibiotic disc	Oxoid (UK)
120 µg gentamycin antibiotic disc	Oxoid (UK)
Penicillin 10000 units/mL-Streptomycin 10000 µg/mL solution	Nacalai Tesque (Japan)
Mueller Hinton Agar	Merck (Germany)
Mueller Hinton Broth	Merck (Germany)

3.1.4 Equipment

To carry out the project, different types of equipment as well as apparatus were used as listed in Table 3.2

Table 3.2: List of equipment and apparatus

Equipment	Manufacturer
Incubator	MMM Incucell 111 (Germany)
Biosafety Cabinet Level 2	Esco (Singapore)
Incubator Shaker	New Brunswick Innova 42 (USA)
Chiller	Berjaya (Malaysia)
-80°C deep freezer	Thermo Forma 900 Series (USA)
Refrigerated Centrifuge	Eppendorf 5810 R (Germany)
Convection Oven	Memmert UN110 (Germany)
Industrial Oven	Kimah Industrial Supply (Malaysia)
Freeze Dryer	Christ Alpha 1-4 LSC (Germany)
-80°C deep freezer	ARDO CV382 (Italy)
Glass Vacuum Desiccator	Nalgene (USA)
Microplate Reader	BioTek Powerwave XS (USA)
Dryer	Protech FSD 380 (Malaysia)
Hotplate Stirrer	Fisher Scientific (USA)
Micropipette	Eppendorf (Germany) Gilson (USA)
Multichannel Pipette	Eppendorf (Germany)
Weighing Balance	Sartorius CP3245 (Germany)
Autoclave	Tuttnauer 3870 ELVC (The Netherlands)

UV-Vis Spectrometry	Agilent Technology Cary 60 (USA)
Zeta (Potential) Sizer	Malvern (UK)

Apparatus	Manufacturer
Syringe Filter 0.2 μm	Sartorius (Germany)
96-well plate	SPL (South Korea)
Centrifuge Tubes	SPL (South Korea)

3.1.5 Glassware and Apparatus

All glassware and apparatus used for the experiment were either obtained / purchased sterile or sterilised by autoclaves at 121 °C, 15 psi for 15 minutes.

3.2 Methods

3.2.1 Preparation of Microbiological Media

Mueller-Hinton agar was prepared by suspending 34 g/L of the media powder in distilled water while Mueller-Hinton broth was prepared by mixing 21 g/L of the powder in distilled water. Broth medium was dispensed into 20-mL universal bottles 10 mL each. All media were autoclaved at 121 °C, 15 psi for 15 minutes. The molten agar was cooled down to 45 °C before being poured into sterile Petri dish. All media were kept in 4 °C prior to usage.

3.2.2 Maintenance of Bacteria Culture

Bacteria cultures were inoculated on Mueller-Hinton agar (MHA) using standard microbiological method with aseptic techniques. Cultured media was then incubated 37 °C incubator for 24 hours. The plate containing bacteria colonies can be stored at 4 °C up to 14 days for the usage in the experiment or next sub-culture. For long term storage, a single colony of the bacteria culture was suspended in Mueller-Hinton broth (MHB) containing 25 % (v/v) sterile glycerol and stored in -80 °C. To recover the cells, frozen tube was thawed rapidly before being inoculated on agar by using wire loop and incubated for 24 hours at 37 °C.

3.2.3 Plant Botanical Identification and Herbarium Preparation

Four samples of intact whole plant including stems, barks, leaves and root were sent to Herbarium room, Biodiversity Reference Complex (D32), School of Biology, Universiti Sains Malaysia for herbarium preparation and botanical identification of plant material.

3.2.4 Preparation of Plant Aqueous Extract

The whole plant including stems, barks, leaves and root were cut to smaller pieces by using universal snip. The whole plant material was thoroughly washed using tap water to remove dirt and unwanted particles. The sample then soaked in distilled water for 5 minutes before being dried in an oven at 45 °C for 48 hours. Dried samples were then grounded into coarse powder using blender and was stored in the zip lock plastic bag at -20 °C until being used in future.

The aqueous extract was prepared by mixing 50 g of fine powder with 950 mL of distilled water in a 2-liter bottle. The mixture was boiled for 30 minutes until the colour of the aqueous solution turn from greenish to brownish dark green. The extracts were cooled down at ambient temperature and filtered through double-layered clean cloth tea strainer to remove larger particles. The filtrates were then centrifuged at 12000 x g for 10 minutes at 4 °C to pallet down any smaller particles. The supernatant was collected and filtered using Whatmann filter paper No 4.

The final filtrates of the extracts were then frozen at -20 °C in freeze dryer compatible round bottle. The frozen extracts were subjected to freeze drying process for 36 hours until the brownish crystalized-like material formed at the bottom of the round bottle. The crystallised-like material was then collected and macerated using mortar and pestle in addition of the liquid nitrogen. The powder formed after maceration is then collected in a pre-weigh aluminium foil wrapped conical tube. The conical tube containing the fine powder was then dried in a glass vacuum desiccator containing blue silica gel to remove remaining moisture. The extract powder was kept in 4 °C prior to further use.

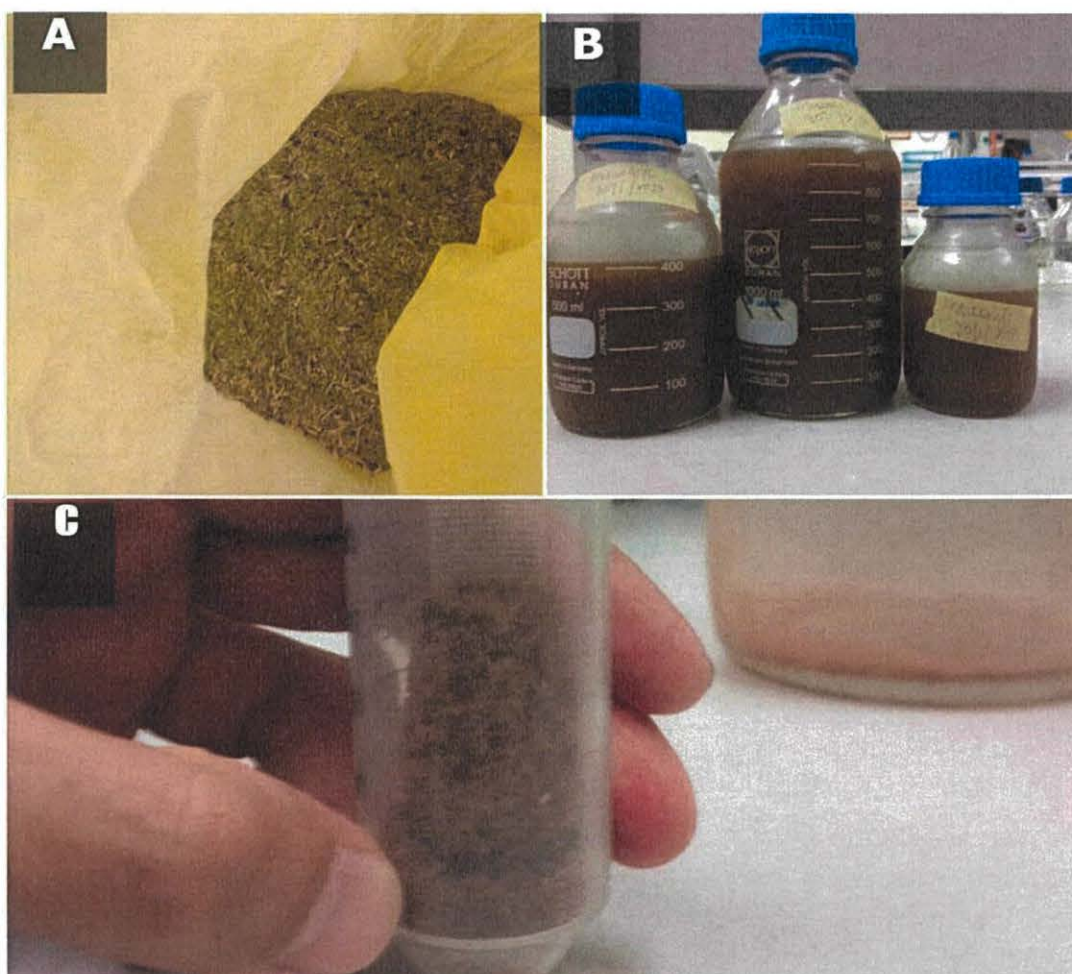


Figure 3.1: Coarse powder (A) and aqueous extract of whole plant of *A. ilicifolius* (B). Final fine powder of freeze-dried aqueous extract after being macerated with addition of liquid nitrogen (C).

3.2.5 Synthesis of *Acanthus ilicifolius*-AgNPs

Acanthus ilicifolius extract powder reconstituted with distilled water to form 100 mg/mL solution. The AgNPs were prepared by mixing 100 mg/mL *A. ilicifolius* solution with 5mM silver nitrate (AgNO_3) solution. A ratio of 1:10 (one part of 100 mg/mL plant extract with another 9 parts of 5 mM silver nitrate) was used in the mixture. In this study, several incubation periods were selected at 0, 1, 6, and 24 hours. The mixture then incubated in an incubator shaker at 40 °C with agitation of 150 rpm for 24 hours with minimal light exposed into the incubator shaker. Reduction of AgNO_3 to Ag^0 was confirmed by the change of colour from clear (before mixing) to colloidal brown. Then, the colloidal mixture was properly sealed and kept for future use.

3.2.6 Characterisation of *Acanthus ilicifolius*-AgNPs

The *Acanthus ilicifolius*-AgNPs synthesised at different incubation times were characterised by Ultraviolet-Visible (UV-Vis) Spectroscopy. Samples those are showing maximum absorption peak around 425-475 nm were chosen to be further characterised by Zeta (Potential) Sizer Analyses. (Banerjee et al., 2014)