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SYNTHESIS OF SILVER NANOPARTICLES BY CLINACANTHUS NUTANS EXTRACT  
SUPPORTED WITH IDENTIFICATION OF FLAVONOIDS BY UPLC  
ANTIMICROBIAL ACTIVITY

-QTOFMS AND ITS

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## ABSTRACT

The present study reported a simple, environmental-benign and cost effective method in synthesizing silver nanoparticles (AgNPs) by using *Clinacanthus nutans* methanolic extract at 37°C. The reduction of silver ions could be visually observed as indicated by the developed of light reddish brown colour after incubation period of 1 h. The synthesized AgNPs was further monitored by UV-visible spectroscopy. A characteristic surface plasmon resonance (SPR) band was showed at around 480 nm in UV-vis spectrum and the intensity was increased with the increased of volume ratio of plant extract and incubation period. The characterization of the AgNPs in terms detailed size and morphology was performed by Field Emission Scanning Electron Microscopy (FESEM). The FESEM micrograph revealed that AgNPs were in the size range of 77.8-85.3 nm and spherical in shape. Next, Energy Dispersive X-ray spectroscopy (EDX) was conducted and further confirming presence of elemental silver as indicated by the signal peak at 3 keV. With respect to Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) analysis, reduction of silver ions was remarkably indicated by decreased in intensities of several significant functional groups including C-N stretch, C-O stretch, C=C stretch, C-H stretch and C=O stretch. Furthermore, antibacterial activity of biosynthesized AgNPs shows effective inhibition against common pathogen bacterial strains including *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Ultra Performance Liquid Chromatography (UPLC)- Quadrupole Time-Of-Flight (QTOF) was conducted to investigate active metabolites in *C. nutans* methanolic extract and the results revealed that polyphenolic compounds including flavonoids and phenolic groups present in *C.nutans* were mainly responsible for the reduction and stabilization of AgNPs.

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## ABSTRAK

Kajian ini telah membentangkan satu kaedah yang mudah, kos efektif dan mesra alam untuk mensintesis nanopartikel argentum (AgNPs) dengan menggunakan ekstrak methanol *Clinacanthus nutans* pada suhu 37°C. Reduksi ion argentum dapat diperhatikan secara visual dari kejadian warna perang kemerahan selepas pengeraman selama satu jam. AgNPs yang disintesis seterusnya dimonitor dengan spektroskopi UV-visible. Satu jalur serapan (SPR) telah didapati pada 480 nm dalam UV Spektrum dan intensiti tersebut meningkat apabila mengalami peningkatan nisbah jumlah ekstrak tumbuhan serta tempoh pengeraman. Karakterisasi AgNPs dari segi saiz secara terperinci dan morfologi telah dilakukan dengan Field Emission Scanning Electron Microscopy (FESEM). Mikrograf FESEM telah menunjukkan bahawa AgNPs berada dalam julat saiz 77.8- 85.3 nm dan berbentuk sfera. Seterusnya, Energy Dispersive X-ray spectroscopy (EDX) telah dijalankan mengesahkan kehadiran unsur argentum seperti yang ditunjukkan oleh puncak pada 3 keV. Dari aspek analisis spektroskopi Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR), reduksi ion argentum telah didapati menerusi penurunan beberapa kumpulan berfungsi seperti getaran regangan C-N, C-O, C=C, C-H dan C-O. Tambahan pula, aktiviti antibakteria AgNPs telah menunjukkan perencatan yang efektif terhadap patogen biasa seperti *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli* dan *Pseudomonas aeruginosa*. Ultra Performance Liquid Chromatography (UPLC)-Quadrupole Time-Of-Flight (QTOF) telah dijalankan untuk menilai metabolit aktif dalam ekstrak metanol *C. nutans* dan hasilnya telah menunjukkan sebatian polifenolik termasuk flavonoid dan kumpulan fenolik dalam *C. nutans* merupakan peranan utama dalam menjalankan reduksi dan penstabilan AgNPs.

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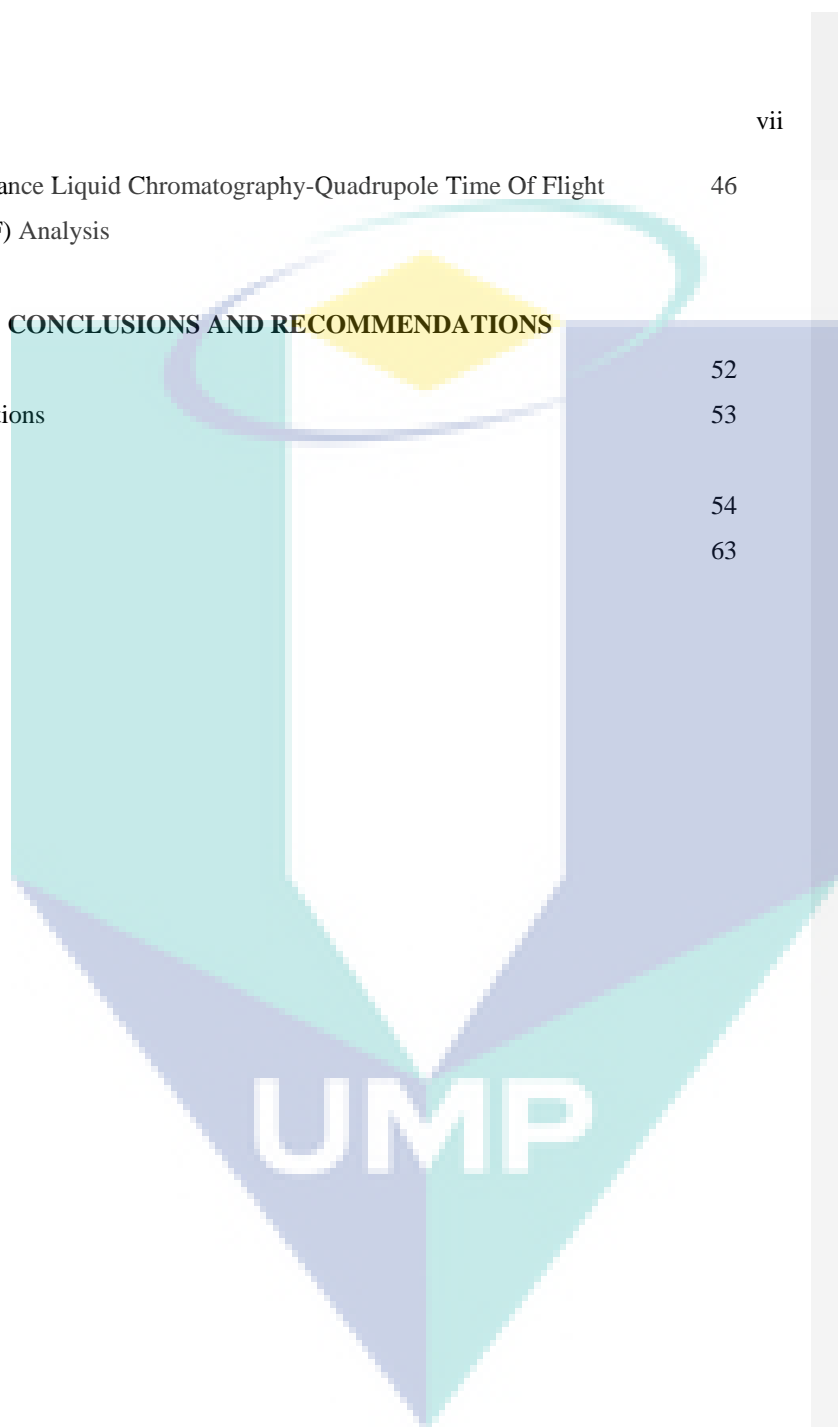
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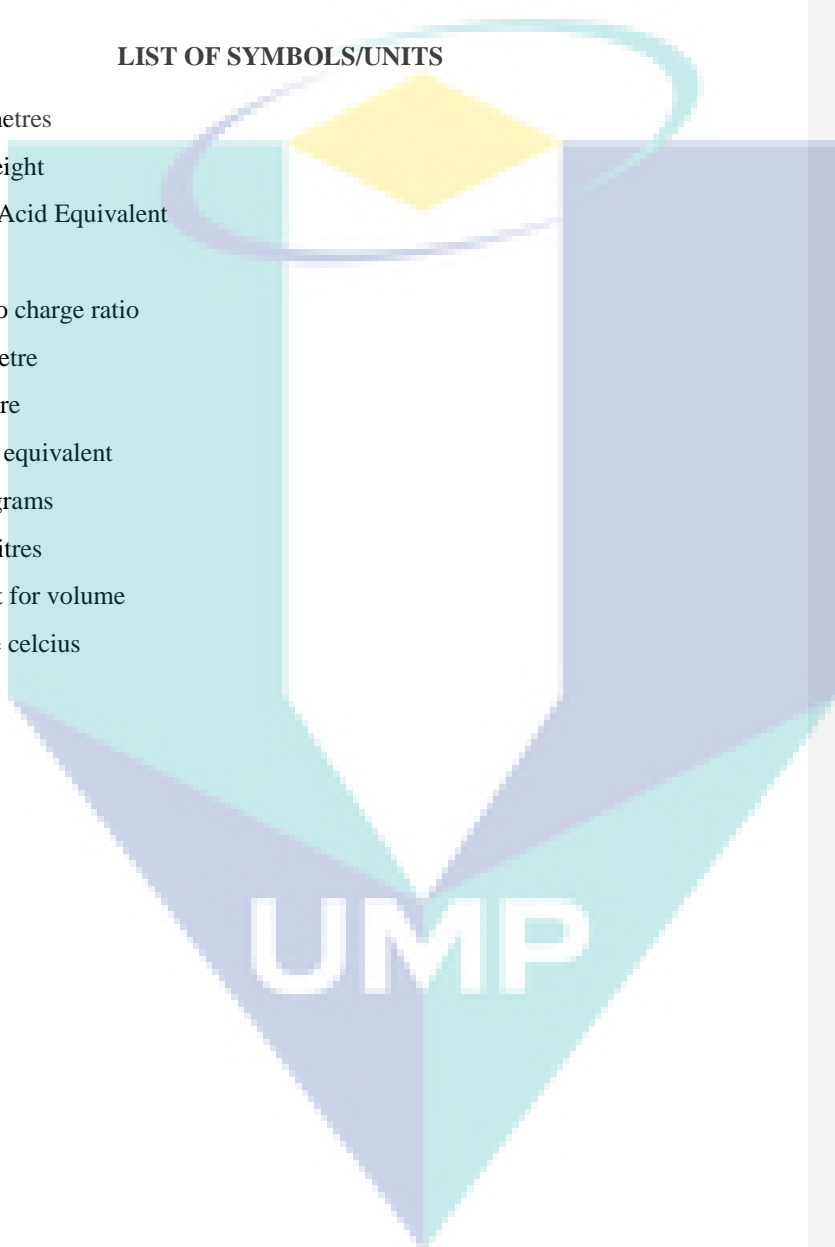
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**LIST OF SYMBOLS/UNITS**

cm	Centimetres
dw	Dry weight
GAE	Gallic Acid Equivalent
g	Gram
m/z	Mass to charge ratio
mm	Millimetre
mL	Millilitre
Teq	Trolox equivalent
µg	Micrograms
µL	Microlitres
w/v	Weight for volume
°C	Degree celcius

**LIST OF ABBREVIATIONS**

AgNO <sub>3</sub>	Silver nitrate
AgNPs	Silver nanoparticles
ATR	Attenuated Total Reflectance
<i>C. nutans</i>	<i>Clinacanthus nutans</i>
DPPH	2,2-diphenyl-1-picrylhydrazyl
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier Transform Infrared
HSV	Herpes Simplex Viruses
I.D.	Internal diameter
NA	Nutrient Agar
MS	Mass Spectroscopy
OGTT	Oral Glucose Tolerance Test
ROS	Reactive Oxygen Species
SPR	Surface Plasmon Resonance
TPC	Total Phenolic Content
TFC	Total Flavonoids Content
UV-vis	Ultraviolet Visible Spectroscopy



## CHAPTER 1

### INTRODUCTION

#### 1.1 BACKGROUND STUDY

Nanotechnology could be simply known as “technology at the nano-scale” in which it deals with the materials with the size ranged from 1 to 100 nm. It could be also defined in a more precise way as an emerging technology that are able to work at the molecular level to obtain novel materials and devices that display unique properties significantly as declared by the US National Nanotechnology Initiative (Ramsden., 2016). The advancement of nanotechnologies has been an emerging research area due to its wide applicability to almost every field of science and technology. In the nanotechnology industry, a number of promising products including silver, aurum, alumina, copper oxide are widely synthesized, for variety applications.

With respect to biomedical sciences, there are expanding of researches and analysis to investigate potential biological activities of metallic nanoparticles in recent decades. In light of this issue, silver has gained the highest interest since it had been discovered to exhibit an outstanding bactericidal and fungicidal activity in comparison with other metals (Sachindri and Kalaichelvan., 2011). Silver metals are established to have potent antimicrobial efficacy against a wide range of over 650 microorganisms from different classes including bacteria, fungi, viruses and eukaryotic microorganisms. The antimicrobial efficacy is getting better when the silver particles are developed in nano-scale regime, as it shows a larger surface area to volume ratio (Gong et al. 2007). Furthermore, Silver et al. (2007) had reported as silver exhibited low propensity in provoking microbial resistance.

Based on these distinctive properties, silver nanoparticles, have been projected as an alternative antimicrobial agents in the near future.

Previously, the metallic nanoparticles were synthesis by using chemical technique, where mostly toxic and hazardous chemicals are involved. Synthesis of nanoparticles by using plant extracts is preferred nowadays as it is a cost effective and ecological benign approach and the materials are easily available. The biomolecules and secondary metabolites of plants such as proteins, amino acids, alkaloids, tannins and many more are contributed in reduction and stabilization of silver ions throughout the biosynthetic process (Ahmed et al. 2016). Yet, the mechanism of the antimicrobial activities of silver nanoparticles was still investigated and well-debated. One of the widely recognized antimicrobial mechanism was the inhibitory effect of silver ions against the microorganisms was due to electrostatic attraction. Penetration followed by disruption of cell wall or cell membrane was taken place when positively charge nanoparticles attached to the negatively charged microorganism (Cao et al., 2001). However, more studies and investigations are required to verify the claimed.

In this study, *C. nutans* was introduced as the green syntheses as it is one of the popular locally grown medicinal plant. *C. nutans* is a plant species in family Acanthaceae which widely used for centuries as popular traditional medicines among Asia countries especially in Thailand. In Asia countries, this plant species has long been used as a traditional medicine for skin rashes, and antidotes for snake bites and animal sting. This plant species have been an active research topic of the scientific community in recent decades due to increasing evidence associates them with healthcare and the treatment and prevention of several diseases. Further studies on the medicinal potencies of this plant species had been reported in previous studies as treatment for glucose and lipid metabolism disorders including diabetes mellitus and hyperlipidemia and anti-cancer. Besides that, *C. nutans* extract have been demonstrated to have anti-inflammatory, antioxidant and antiviral such as HSV activities. Basically, these pharmacological effects of *C. nutans* are correlating to its bioactive compounds. Plants is one of the main source which rich of bioactive compounds with a wide array of potential applications. Most of the bioactive compounds are originated from their secondary metabolites which capable to possess pharmacological or toxicological affects either to human or animal. Hence, the bioactive

compounds in a medicinal plant is utmost important to be determined for efficacy of its usage in remedial purposes. Generally, there were a number bioactive constituents been found in plants including glycosides, flavonoids and proanthocyanidins, tannins, terpenoids, resins, lignans, alkaloids, furocoimarinines and proteins and peptides (Bernhoft., 2010). Production of secondary metabolites in plants was one of their defense responses in dealing with various of microorganisms including viruses, bacteria and fungi. Among all of these ingredients, some of these chemical constituents was existed in their biologically active forms in healthy plants, yet, some of the compounds such as cyanogenic glycosides and glucosinolates would be occurred as inactive precursor. These inactive precursors would only be activated in case of tissue damage or pathogen attack (Osbourn. 1996).

## 1.2 PROBLEM STATEMENT

In recent decades, several drawbacks of the current medicinal drugs had raised a great discussion among the society. The side effects of those modern medicines is gradually becoming a serious public health crisis to all level of society. One of the major crises facing nowadays was the problem of multidrug-resistant. It was an emergence of resistance among the pathogenic microorganism which resulted from continually usage of the antimicrobial drugs to suppress infections. These resistant pathogens were able to combat attack by current medicinal drug and led to ineffective treatment. Hence, in order to address this issue, development of novel antimicrobial agent had been becoming a focus among scientific communities in the present days in order to treat and control multidrug resistance bacterial infections. Silver nanoparticles have become an active research topic for its antimicrobial efficacy. Instead of using chemical synthesis, a synthesis protocol of nanoparticles could be introduced by working complementarily with medicinal plant extract. *C. nutans*, one of a popular vegetal species in Malaysia, was introduced to synthesis silver nanoparticles and antimicrobial activity of the product was further evaluated in this study.

### 1.3 SCOPE OF STUDY

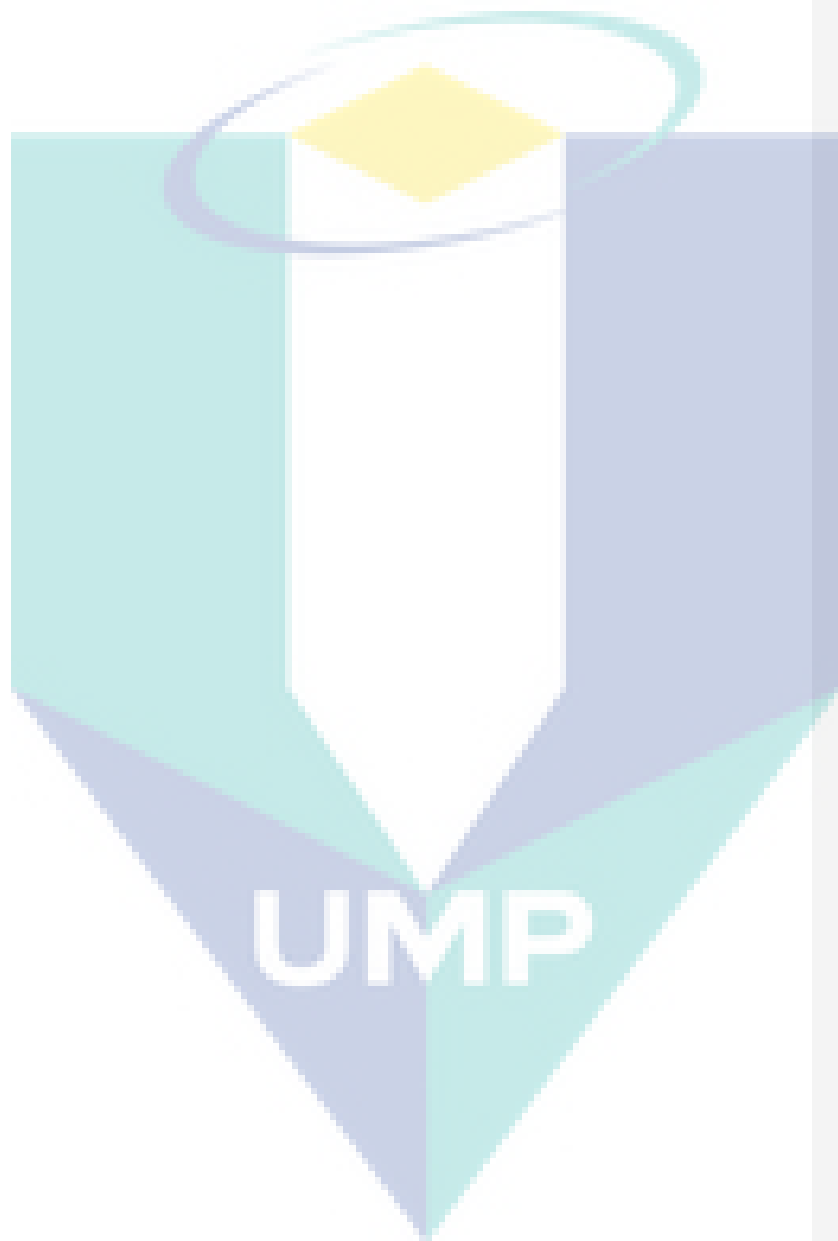
This study is basically started with preparation of methanolic extract of *C. nutans* and 1.0 mM of silver nitrate solution. Different volume ratio of plant extract were subsequently added to the silver nitrate solution followed by visual observation at different time intervals after incubation. Other than visual observation, the silver nanoparticles synthesized were further characterized through UV-vis spectroscopy, Fourier Transform Infrared analysis, Field Emission Scanning Electron Microscopy. In the same time, the *C. nutans* methanolic extract was subjected to Ultra-Performance Liquid Chromatography (UPLC) coupled to Quadrupole Time-of-Flight (QTOF) for screening and identification of bioactive constituents. Next, the antimicrobial activity of silver nanoparticles by using agar disk diffusion method was conducted. The silver nanoparticles were tested against two Gram positive microorganisms (*Bacillus subtilis* and *Enterococcus faecalis*.) and two Gram negative microorganisms (*Escherichia coli* and *Pseudomonas aeruginosa*).

### 1.4 SIGNIFICANCE OF STUDY

This study is an extensive study on biosynthesis of silver nanoparticles by using *Clinacanthus nutans* plant extract. Next, it is important in investigating the antimicrobial capability against the common pathogens, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and *Pseudomonas aeruginosa*.

### 1.5 OBJECTIVES OF THE STUDY

- I. To synthesis silver nanoparticles from silver salts by using *Clinacanthus nutans* extract.
- II. To characterize the synthesized silver nanoparticles.
- III. To study the antimicrobial activity of silver nanoparticles against a few common bacteria.







## CHAPTER 2

### LITERATURE REVIEW

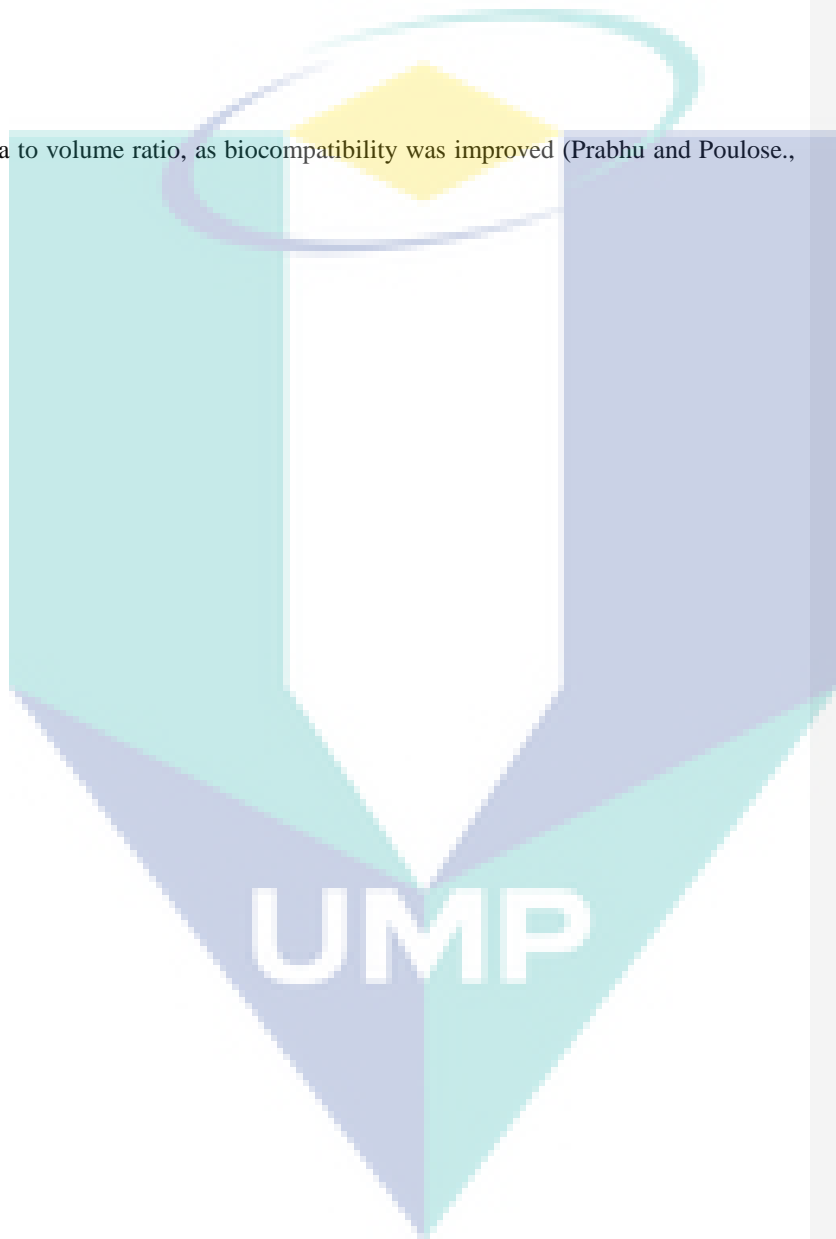
#### 2.1 SILVER NANOPARTICLES

Silver is commonly known as one of the transition metal, with white and lustrous appearance. In fact, silver could be present in a number of forms in the environment or in living organisms, in either metallic, salts, complexes or colloidal form (Panyala et al., 2008). The prevalence of using metallic silver was started as early as 4,000 B.C.E., after gold and copper (Alexander, 2009). It becomes a topic of interest when era of nanotechnology came into existence, as it was the most often incorporated in nano-functionalized consumer products including polymeric materials, soaps, foods, textiles and many more. In comparison with other metals, silver nanoparticles showed unique physic-chemical properties, including high thermal and electrical conductivity, chemical stability, catalytic activity and so forth (Tran et al., 2013). The general competitive edge of nanoparticles was their large surface to volume ratio, thus enhance their efficiency in various applications (Prabhu and Poulouse, 2012). Owing to a variety of potentials, silver nanoparticles had been widely applied in diverse fields ranging from medical fields to water treatments (Tran et al., 2013).

#### 2.2 MEDICAL USE OF SILVER NANOPARTICLES

In recent publications, silver ions or silver-based compounds was found to exhibit high inhibitory effects to microorganisms including 16 major species of bacteria. When silver was synthesized in nanoscale, the antimicrobial effects were further enhanced due to

high surface area to volume ratio, as biocompatibility was improved (Prabhu and Poulouse., 2012).



According to Alexander (2009), application of silver ions or silver-based compounds in medical fields had started over millennia ago, and they could be found in many forms, such as vessels for liquid, coins, foils, sutures, solutions and colloids. The medical applications of silver were for wound healing, as a counterirritant, purgative and for the treatment of burn injuries, and other infectious diseases. Also, it was used in preservation of water or any other beverages by preventing bacterial growth in the past. With respect to current medical fields, silver nanoparticles were widely used as a bactericidal and as a therapeutic agent. Moreover, it could be used in coating of medical devices, dental resin composites and wound dressing. According to Monteriro et al. (2009), medical devices with silver-based polymer were important in protecting the inner and outer surfaces of the devices by preventing attachment of microorganisms. The antimicrobial efficacy of silver also makes it to be used in air sanitizer spray (Prabhu and Poulouse., 2012). Besides that, owing to high thermal and electrical conductivity of silver nanoparticles, they have been employed as medical imaging and microelectronics too.

### **2.3 SYNTHESIS OF SILVER NANOPARTICLES**

Nanotechnology had become the main focus among scientific community nowadays and substantial researches were carried out owing to its distinctive properties which beneficial to various science and technology applications. There was a number of techniques were reported for the synthesis of metallic nanoparticles by using physical, chemical and biological ways. Fundamentally, the synthesis of metallic nanoparticles could be conducted in two ways, either is “top to bottom” approach or a “bottom to up” approach. The top-bottom approach involved in breaking down the bulk material into fine particles by size reduction. While in bottom-up approach, atom would be self-assembled to lead the formation of new nucleic which would grow further into metal cluster or aggregates in nanoscale, either through chemical or biological routes (Ahmed et al., 2016). Currently, numerous methods were proposed and their advantages and disadvantages were always been discussed including costs, scalability, particles sizes and size distribution.

### 2.3.1 Physical Synthesis of Metallic Nanoparticles

Generally, physical approach for synthesis nanoparticles involved the utilization of physical energies such as thermal, ac power and arc discharge to produce the particles with nearly narrow size distribution (Tran et al., 2013). One of the commonly used physical techniques was evaporation-condensation, which could be conducted by using a tube furnace at atmospheric pressure. Other than evaporation-condensation, laser ablation, thermal-decomposition method, local heating by using ceramic heater and several more physical synthesis were proposed and compared. Physical approach was preferred as compared to other approaches due to it permitted production large quantities of nanoparticles without complicated process. Furthermore, Iravani et al. (2014) had reported that physical synthesis was favored due to uniformity of nanoparticles distribution. However, there were some drawbacks found associated with this technique especially the primary costs for investment of equipment. Tube furnace used in evaporation-condensation technique occupied a large space and it was energy consuming while raising environmental temperature around the source materials. In the same time, time consuming was another issue of this technique for achieving thermal stability. Also, power consumption of more than several kilowatts was required for a typical tube furnace (Magnusson et al., 1999; Kruis et al., 2000).

### 2.3.2 Chemical Synthesis of Metallic Nanoparticles

Chemical synthesis was the most common method used in synthesizing metallic nanoparticles. Generally, there were three main components required in synthesizing nanoparticles including metal precursors, reducing agents and capping agents (Tran et al., 2013). In this context, the reducing agents used could be either organic or inorganic. The commonly used reductants were sodium borohydride ( $\text{NaBH}_4$ ), sodium citrate, ascorbate, elemental hydrogen and many more. Chemical reduction could be considered as the easiest and simplest method of synthesizing nanoparticles, and large amount of products could be obtained in short span of period. However, the application of toxic chemicals and released of non-ecofriendly byproducts were the major disadvantages of this approach (Ahmed et al.,

2016). In light of this issue, the focus of synthesis of metallic nanoparticles was gradually shifted to green synthesis instead of chemical reduction.

### 2.3.3 Biological Synthesis of Metallic Nanoparticles

Owing to certain limitations from various aspects, synthesis of nanoparticles through biological routes had attracting much attention in recent researches as it was claimed as it possessed a series of pros to either environment or industry. Generally, biological synthesis of nanoparticles was similar with the chemical approach, with the difference of employment of molecules living organisms as reducing agents and stabilizers such as bacteria, fungus or plant extract. Vijayakumar et al. (2013) had proposed that the three main steps involved in biological synthesis were including selection of solvent medium, selection of eco-friendly reducing agents and selection of toxic-free substances for the stability of metallic nanoparticles. Biological approach was the most favorable method as it free from chemical contamination and was relatively low-cost than physical and chemical synthesis. Moreover, this approach was easily scale up for large scale synthesis of metallic nanoparticles and energy saving as high temperature, pressure, and energy were need not for the process (Forough and Farhad, 2010). Table 2.1 shows some important examples of reducing agents used in biological synthesis of metallic nanoparticles.

**Table 2.1:** Some important examples of organisms used in biological synthesizing nanoparticles.

Bacteria	Fungi	Algae	Plants
<i>Aeromonas</i> sp. SH10	<i>Fusarium oxysporum</i>	<i>Spirulina platensis</i>	<i>Aloe vera</i> leaf extract
<i>Klebsiella pneumonia</i>	<i>Phaeneroechaete chrysosporium</i>	<i>Oscillatoria willer</i>	<i>Azadirachta indica</i>
<i>Lactobacillus</i> strains	<i>Verticillium</i> sp.	<i>Gelidiella acerosa</i>	<i>Cinnamomum camphora</i>

<i>Pseudomonas stutzeri</i> AG259	<i>Aspergillus flavus</i>	<i>Emblica Officinalis</i>
<i>Corynebacterium</i> sp. SH09	<i>Aspergillus fumigatus</i>	<i>Pelargonium graveolens</i> leaves (Geranium)
<i>Enterobacter cloacae</i>	<i>Fusarium oxysporium</i> <i>Fusarium semitectum</i>	<i>Pinus eldarica</i>

Source: Iravani et al. (2014)

## 2.4 Plant-mediated Synthesis

In compared to microbe-mediated synthesis, substantial of researches were emphasizing more on plant-mediated synthesis of nanoparticles. It is a well-known fact that bio-based synthetic route were cost effective and environmentally-benign in parallel, however, microbe-mediated synthesis was less preferred due to relatively inferior in industrial feasibility. It was relatively difficult in ensure industrial feasibility of microbe-mediated synthesis due to requirements of high aseptic conditions and their maintenance. Besides, biohazard and elaborate process of maintaining cell cultures of the microbe-mediated synthesis was another important issue. Therefore, plant extract-mediated synthesis was more favorable and recognized as the best platform for nanoparticles synthesis. Biosynthesis based on plant-extract was also advantageous as it reduced the cost used for isolation of microorganisms and their culture media (Ahmed et al., 2016). Fundamentally, mechanism of biosynthesis by the plant extract was closely relevant with the plants' phytochemicals constituents or secondary metabolites, for instance, proteins, amino acids, alkaloids, phenolics, flavonoids, vitamins and so on. These constituents act as a natural capping agent and responsible for reduction and stabilization of silver ions into elemental silver particles. The compounds of the plant extract had drawn attention in recent research due to their potential medicinal value, yet chemically complex structure (Kulkarni

and Muddapur., 2014). The development of green synthesis of nanoparticles were highlighted and a large number of medicinal plants were reported to facilitate synthesis of metallic nanoparticles. The protocols of green synthesis of silver nanoparticles were basically including mixing of silver nitrate solution with certain amount of plant extract, followed by incubation at specific temperature under agitation for hours (Kuppusamy et al., 2015; Jagtap and Bapat, 2013; Logeswari et al., 2015; Espenti et al., 2016).

## 2.5 Antibacterial Effect of Silver Nanoparticles through Plant-Mediated Synthesis

It is a well-known fact regards to excellence of antimicrobial activity of silver nanoparticles. There was growing evidence that ensure the antimicrobial efficacy of silver nanoparticles synthesized by using green approach as its inhibitory effect was accessed by using various plant extracts against different microorganisms were investigated as shown in Table 2.2.

**Table 2.2:** Antimicrobial activities of silver nanoparticles synthesized by using plant extracts.

Plant extracts	Tested microorganisms	References
Boerhaavia diffusa	<i>Aeromonas hydrophila</i> , <i>Pseudomonas fluorescens</i> , <i>Flavobacterium branchiophilum</i>	Kumar et al. (2014)
<i>Calliandra haematocephala</i>	<i>Escherichia coli</i>	Raja et al. (2015)
<i>Carica papaya</i>	<i>Staphylococcus aureus</i> , <i>Bacillus subtilis</i> , <i>Micrococcus lutues</i> , <i>E.coli</i> , <i>Klebsiella pneumonia</i> , <i>Pseudomonas putida</i>	Banala et al. (2015)
<i>Centella asiatica</i>	<i>S. aureus</i> , <i>Pseudomonas aeruginosa</i> , <i>E. coli</i> , <i>K. pneumonia</i>	Logeswari et al. (2015)

<i>Citrus sinensis</i>	<i>S. aureus, P. aeruginosa, E. coli, K. pneumonia</i>	Logeswari et al. (2015)
<i>Cucumis sativus</i>	<i>E. coli, K. pneumonia</i>	Sharma et al. (2016)
<i>Lagenaria siceraria</i>	<i>E. coli, K. pneumonia</i>	Sharma et al. (2016)
<i>Luffa cylindrical</i>	<i>E. coli, K. pneumonia</i>	Sharma et al. (2016)
<i>Ocimum tenuiflorum</i>	<i>S. aureus, P. aeruginosa, E. coli, K. pneumonia</i>	Logeswari et al. (2015)
<i>Solanum lycopersicum</i>	<i>E. coli, K. pneumonia</i>	Sharma et al. (2016)
<i>Solanum melongena</i>	<i>E. coli, K. pneumonia</i>	Sharma et al. (2016)
<i>Solanum tricobatum</i>	<i>S. aureus, P. aeruginosa, E. coli, K. pneumonia</i>	Logeswari et al. (2015)
<i>Syzygium cumini</i>	<i>S. aureus, P. aeruginosa, E. coli, K. pneumonia</i>	Logeswari et al. (2015)
<i>Terminalia chebula</i>	<i>B. subtilis, E. coli</i>	Espenti et al. (2016)
<i>Urtica dioica</i> Linn.	<i>Bacillus cereus, B. subtilis, S. aureus, Staphylococcus epidermis</i>	Jyoti et al. (2016)

However, the mechanism of its antimicrobial activity was still in investigations as some contradictions were occurred during study of this topic. According to Feng et al. (2000), antimicrobial activity of silver ion was due to interaction with thiol group in the protein, causing inactivation of bacterial proteins. Consequently, DNA of the microbes would lose the replication abilities and lead to cell death. There were several studies suggested that antimicrobial activity of silver nanoparticles was induced by electrostatic attraction between the positive charged nanoparticles with negative charged cell membrane of microorganism (Cao et al., 2001; Hamouda et al., 2001; Dibrov et al., 2002). It also been



proposed that the antimicrobial activity was induced by free radicals of silver nanoparticles based on electron spin resonance spectroscopy. These radicals were claimed to have the ability to penetrate the bacterial cell membrane and lead to cell death when they were in contact. (Danilczuk et al., 2006; Kim et al., 2007). Shrivastava et al. (2007) reported that the mechanism of antibacterial activity of silver nanoparticles was induced by alteration of phosphotyrosine profile of bacterial peptides. In this proposed mechanism, silver nanoparticles modulated cellular signaling of bacterial strains by dephosphorylating putative key peptide substrate on tyrosine residues. As a result, signal transduction was inhibited and bacterial growth was stopped. However, further studies have to be conducted to verify the claimed.

## **2.6 CLINACANTHUS NUTANS**

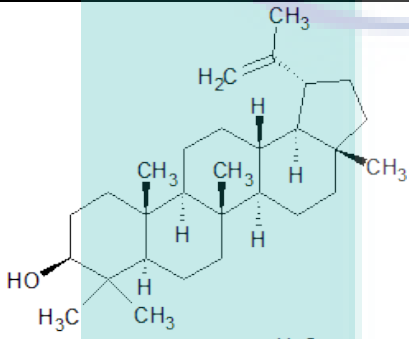
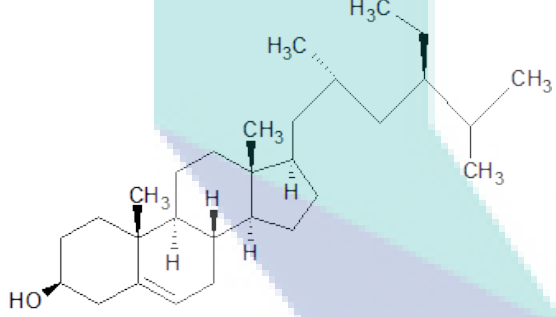
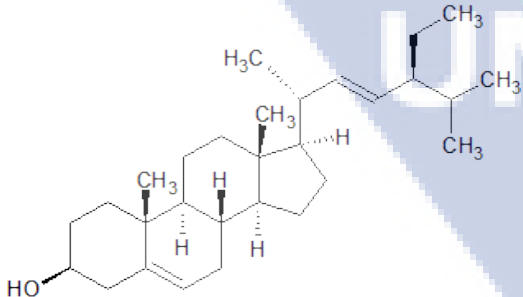
*Clinacanthus nutans* is a famous plant species in family Acanthaceae which is native in South East Asia, such as Thailand, Malaysia, Indonesia and South China. In Malaysia, this plant is locally known as “belalai gajah” or Sabah snake grass. It is a rambling shrub which consists of cylindrical, straight green stem and often growing up to 0.5 to 3.0 meter in height. The leaves are generally pale green in color and oppositely paired and elliptic-oblong in shape with acute apex size 2.5 to 13.0 cm long and 0.5 to 1.5 cm wide. The flowers of the *C. nutans* were dull red with green base and yellow streaks on lower lips. The flowers were found in dense cymes at the top of the branches. Each of the flower had glandular-pubescent calyx, which about 1 cm long and corolla glandular-pubescent, which about 3.5 cm (Kunsorn et al., 2013). Generally, *C. nutans* could be either drunk as raw vegetable or mixed with other juices and served as refreshing beverages (Shim et al. 2013). Other than that, this plant is also popularly with its medicinal potency and it was always used as traditional medicine for primary healthcare especially in Thailand.

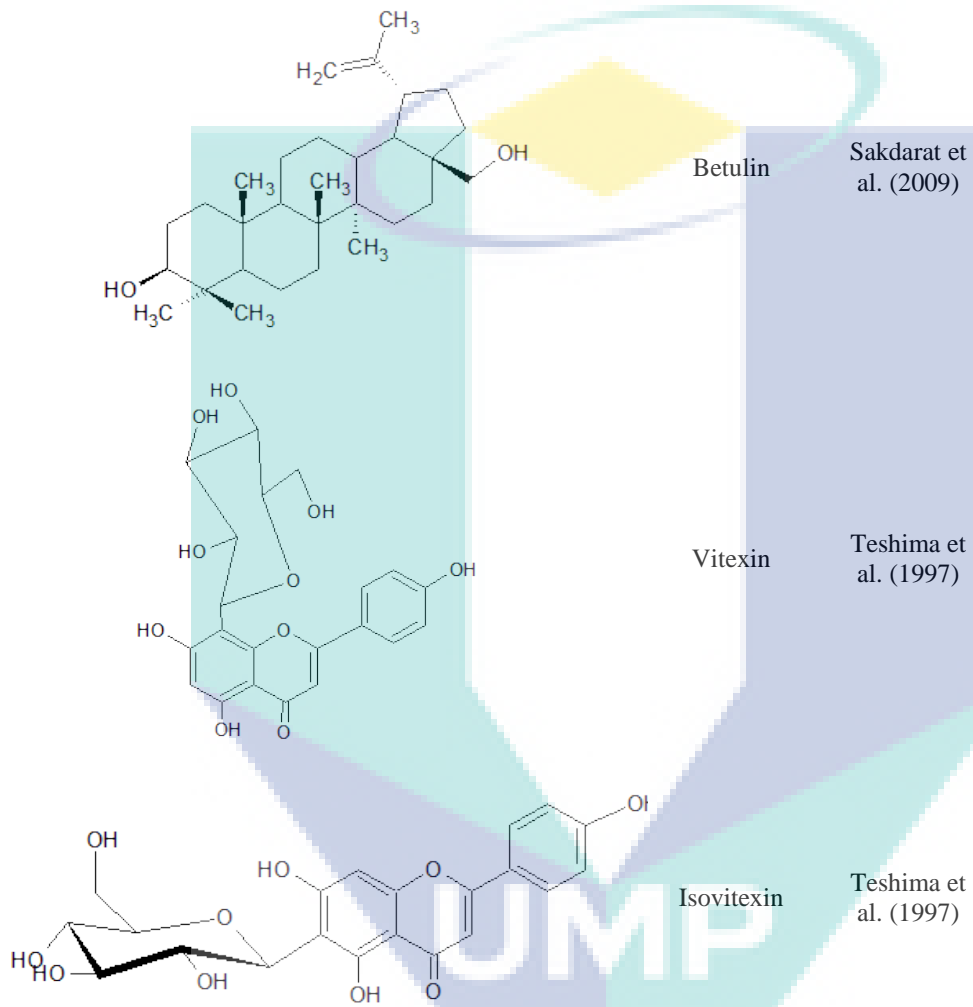
## **2.7 BIOACTIVE CONSTITUENTS**

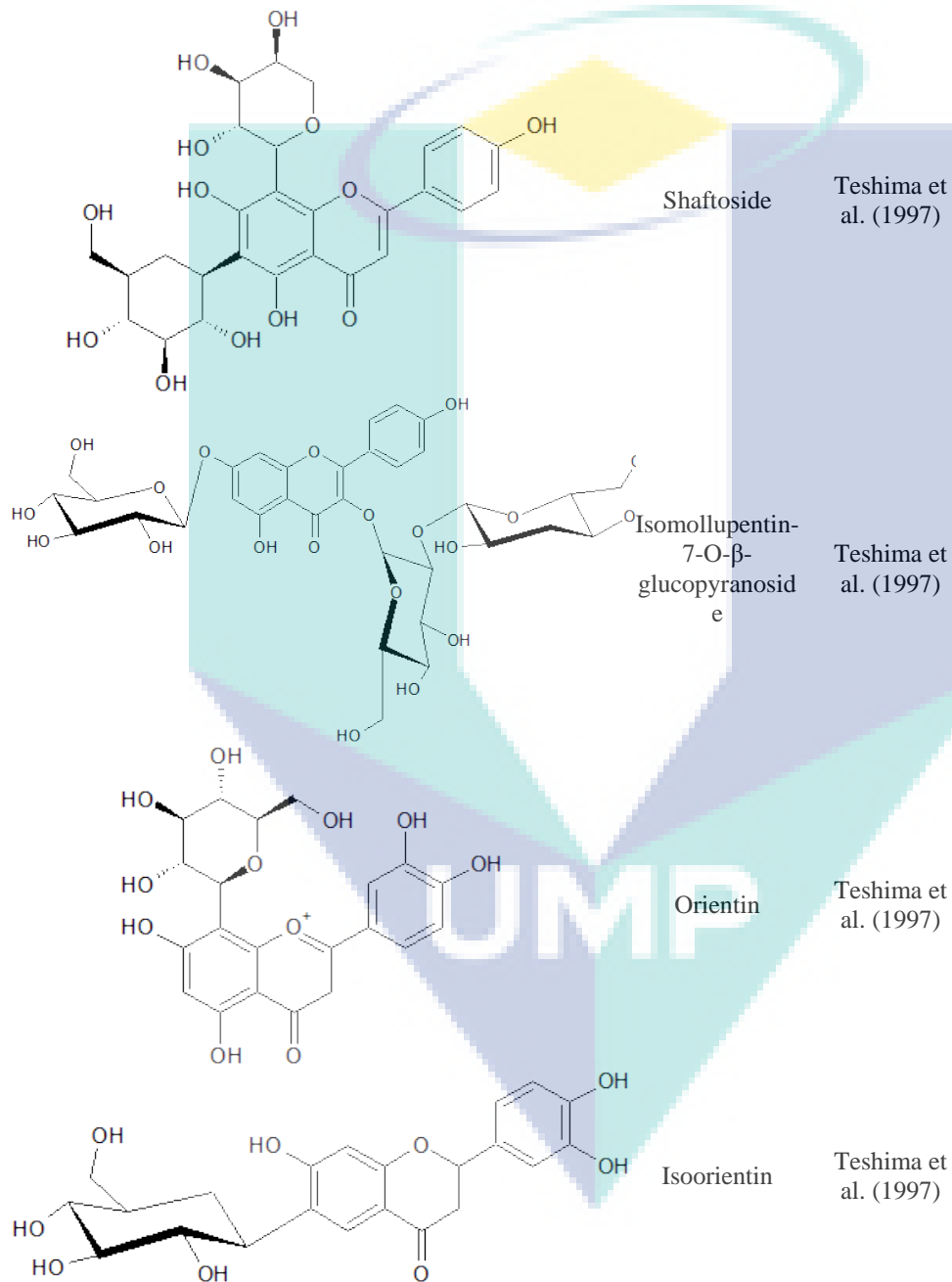
The therapeutic potential of medicinal plants was always with the presence of their bioactive compounds. These pharmacologically active plant derived compounds were

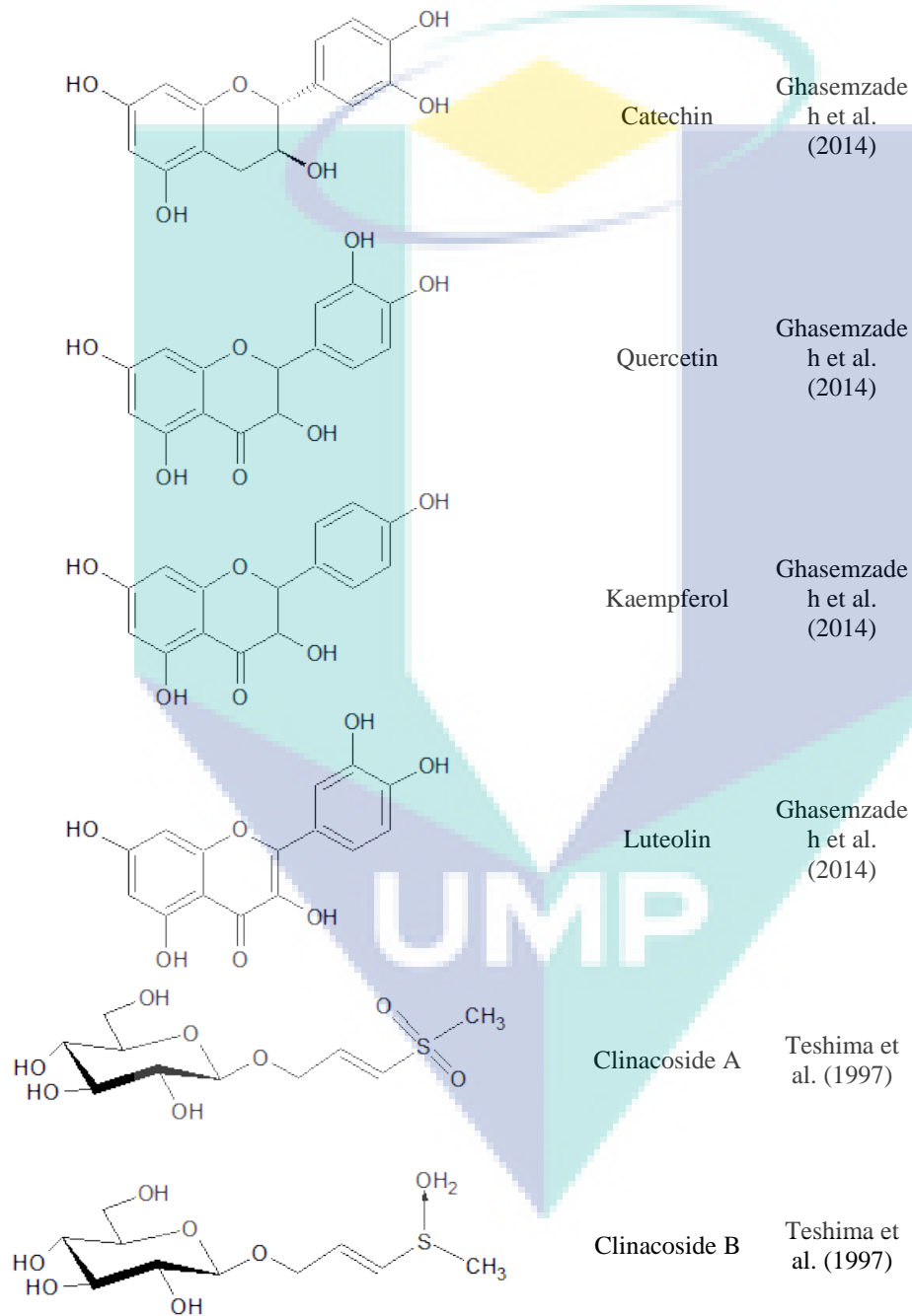
secreted for the defense purpose against various of pathogenic microorganisms. The bioactive constituents in *C. nutans* which previously reported were lupeol,  $\beta$ -sitosterol, stigmasterol, betulin, six known C-glycosyl flavones namely, vitexin, isovitexin, shaftoside, isomollupentin-7-O- $\beta$ -glucopyranoside, orientin, isoorientin (Dampawan et al., 1997; Sakdarat et al., 2009; Teshima et al., 1997). Other than C-glycosyl flavones, other flavonoids also been reported, namely catechin, quercetin, kaempferol and luteolin (Ghasemzadeh et al., 2014). Besides, five sulfur-containing glucosides (clinacoside A, -B, -C, cycloclinacoside A1 and A2), monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) also been reported by Teshima et al. (1997) and Kunsorn et al. (2013) respectively. Besides those, there were four new sulfur-containing compounds were reported by Tu et al. (2014), namely clinamides A, clinamides B, clinamides C and 2-cis-entamide A, which exhibited both sulfur atoms and acrylamide functionalities. Chlorophyll derivatives were also considered as one part of the bioactive ingredients within *C. nutans* according to the study of Sakdarat et al. (2009). Based on the findings, the chlorophyll derivatives including chlorophyll a and chlorophyll b namely, 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-S)-phaeophytin a and 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-R)-phaeophytin a and 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-R)-phaeophytin b. These chlorophyll derivatives could be utilized as anti-herpes simplex virus agent too other than MGDG and DGDG (Sakdarat et al. 2009). As reported in previous study, the phytochemicals within *C. nutans* also included 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-S)-chlorophyll-b, 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-S)-phaeophytin-b, 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-R)-phaeophytin-b, 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-S)-phaeophytin-a, 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-R)-phaeophytin-a, purpurin-18-phytylester and phaeophorbide-a when it was extracted with chloroform and hexane (Sakdarat et al. 2006). In addition, the phenolic groups found in the *C. nutans* were cinnamic acid, Proto-Catechuic acid, vanillic acid, gallic acid, caffeic acid, ferulic acid, chlorogenic acid and p-coumaric acid (Sarega et al., 2016). According to Khoo et al. (2015), several new compounds were discovered by using tandem mass spectrometry, including gendarucin A, a gendarucin A isomer, 3, 3-di-O-methylelagic acid, ascorbic acid and two isomeric oxoprolinates. All isolated phytochemicals are shown in Table 2.3.

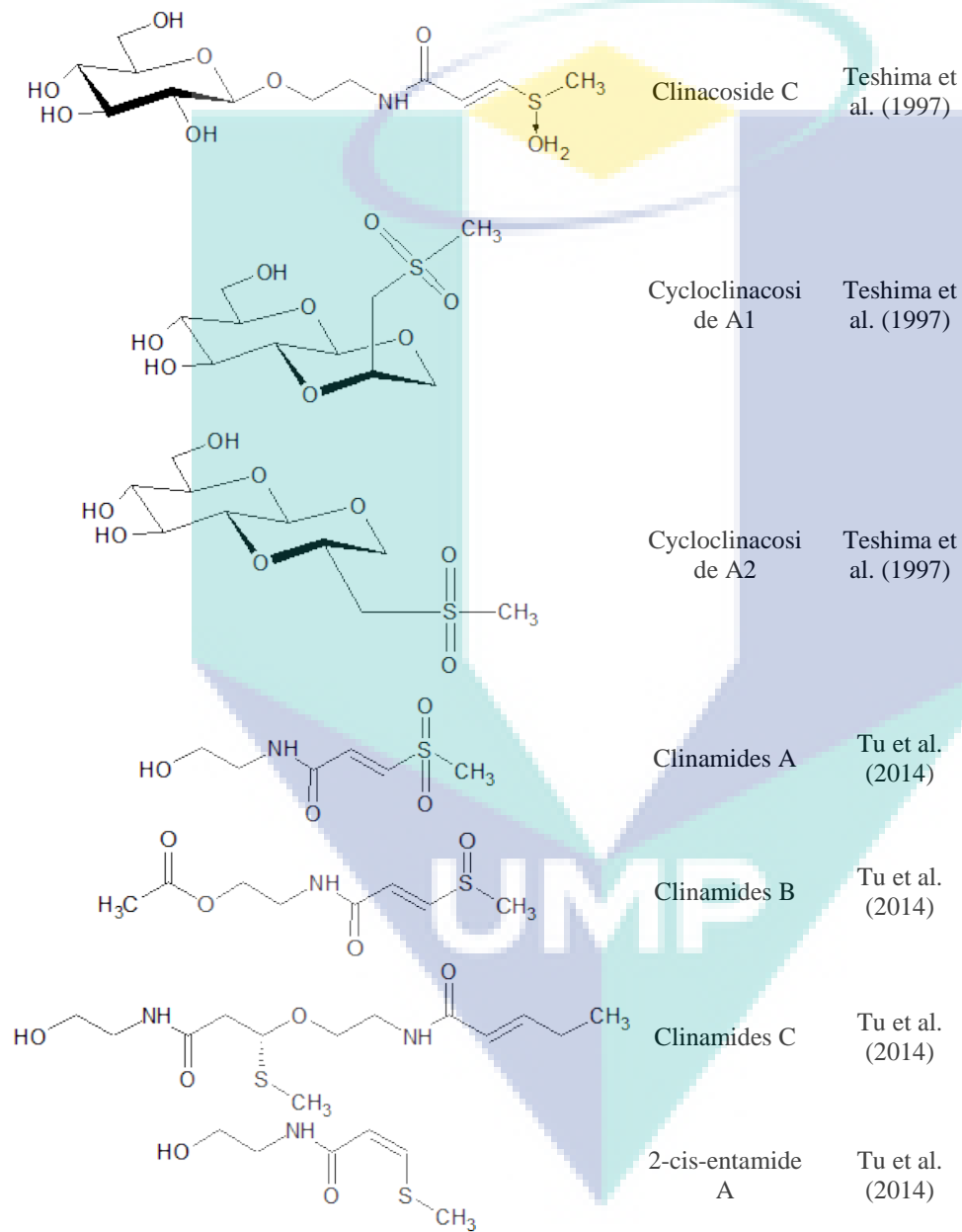
**Table 2.3:** Structure of various bioactive compounds in *C. nutans*

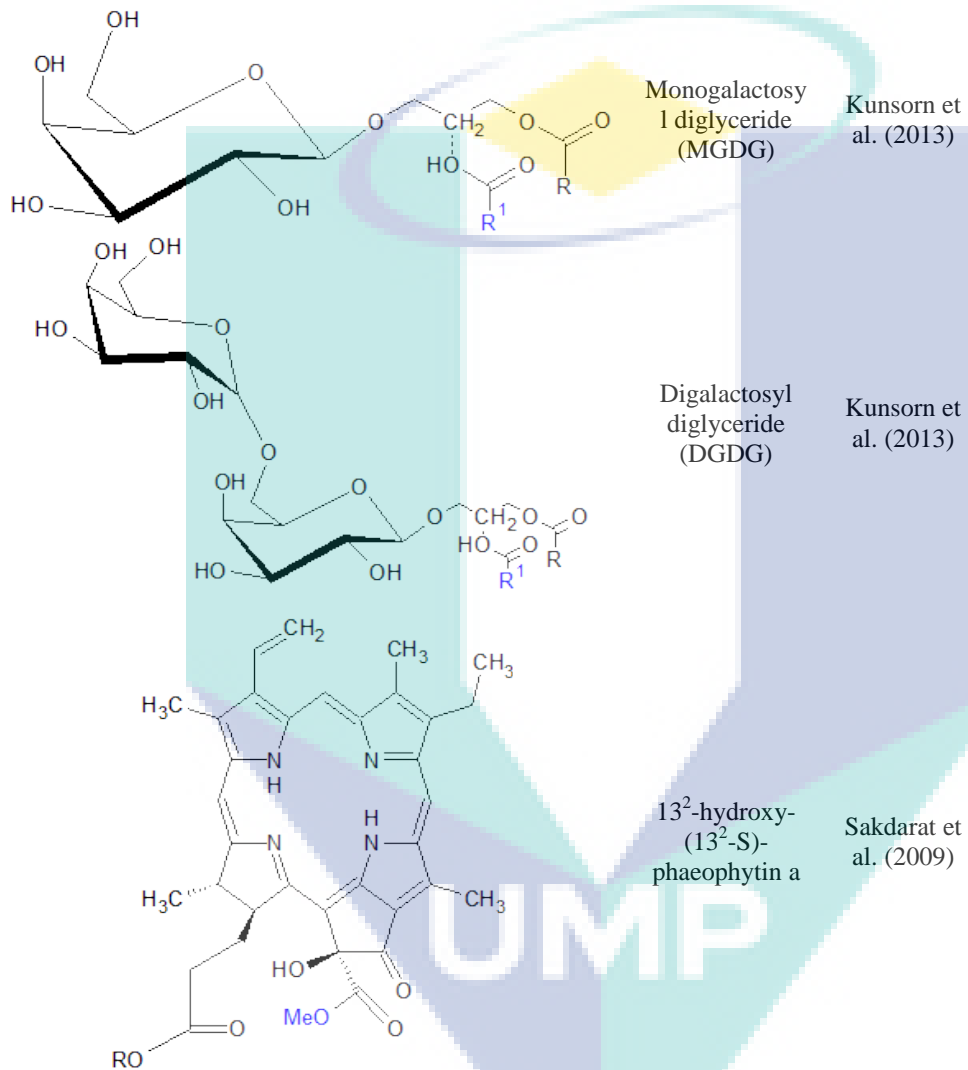
Structure of the compound	Name	Sources
	Lupeol	Dampawan et al. (1997)
	$\beta$ -sitosterol	Dampawan et al. (1997)
	Stigmasterol	Sakdarat et al. (2009)



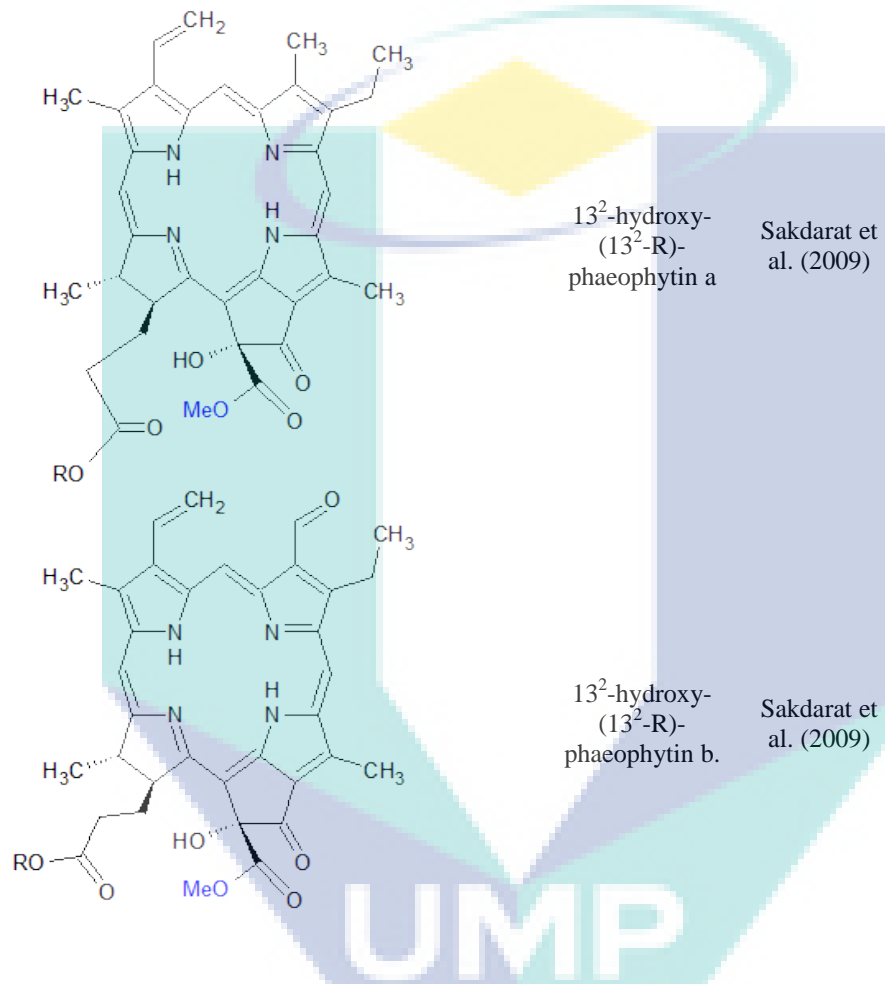


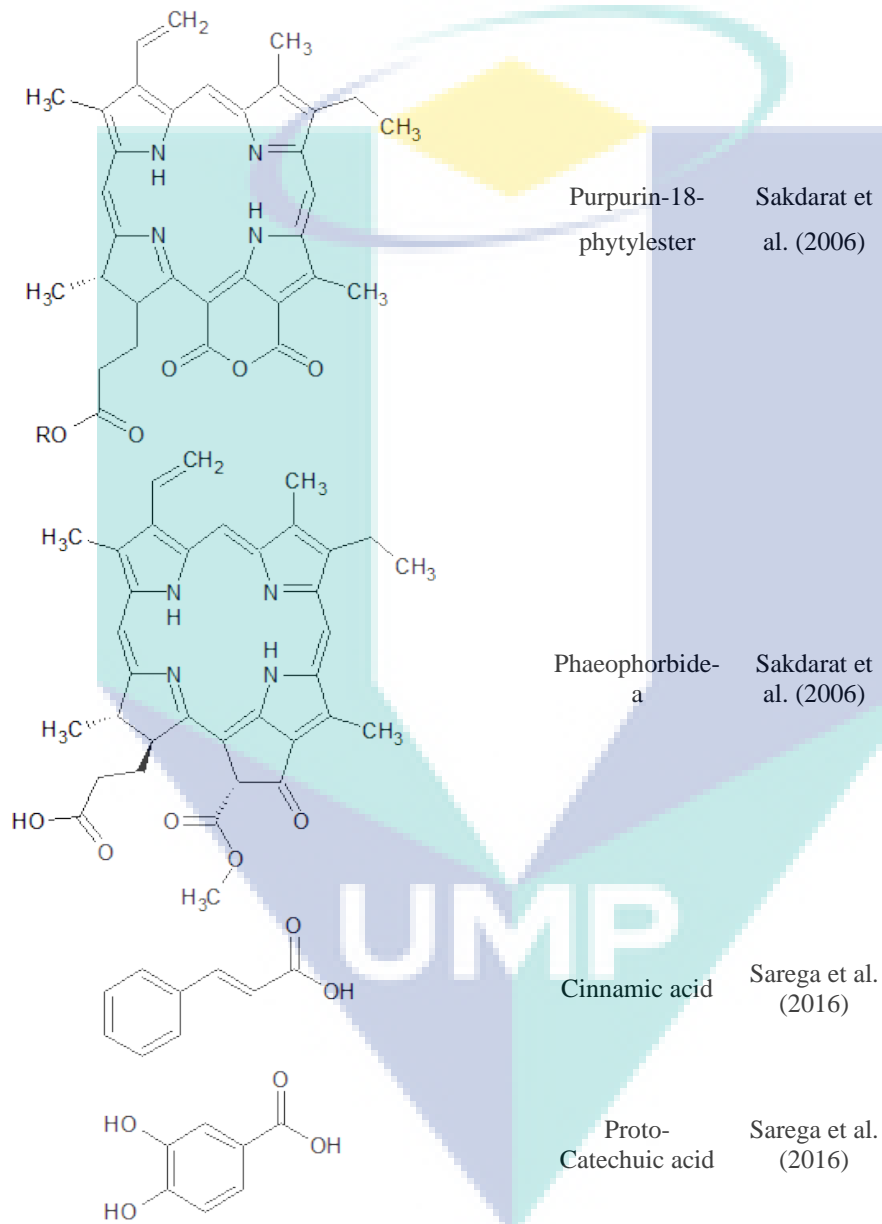


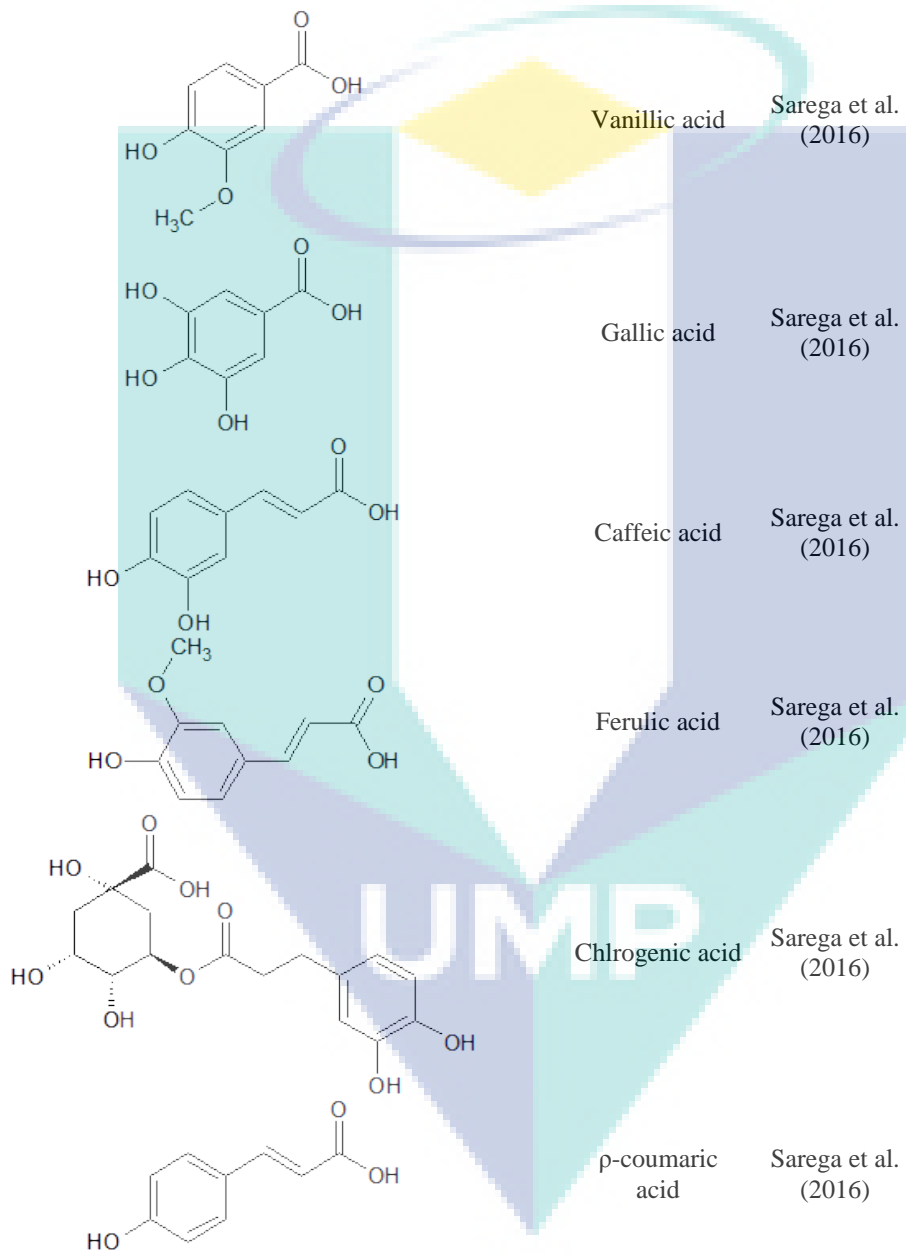












## 2.8 TRADITIONAL MEDICINAL USES

In past decades, it was reported that the *C. nutans* had been traditionally used for relieve of skin rashes, dysentery and fever (Cheeptham and Towers., 2002). Besides, it was reported that *C. nutans* could be used as antidotes for snake bites (Daduang et al., 2005). In previous study, it was reported the *C. nutans* could help in neutralizing other components in venom snake by exhibiting the ability to neutralize the inhibitory effects of neurotoxins of *Naja-naja siamensis* venom on neuromuscular transmission (Cherdchu et al., 1977). Other than snake bites, it could be used to cure the scorpion stings too (Uawonggul et al. 2006).

## 2.9 BIOLOGICAL ACTIVITY

### 2.9.1 Anti-cancer

Cancer is one of the major health threats among population all around the world. Recently, extensively study of medicinal value of *C. nutans* on cancer treatment had been reported (Yong et al. 2013). Based on the findings, capability of *C. nutans* on inhibiting the growth of cultured cancer cell lines was revealed. The anti-proliferation activity was significantly shown by the chloroform extract of *C. nutans* extract in chloroform on the several tested cancer cell lines, namely HepG2, NCL-H23, SNU-1, HeLa, LS-174T, K562 and Raji cells. Besides, the suppression of proliferation on the cancer cell line, IMR32 also been found by using *C. nutans* extracts in methanol and water. Thus, this herb was recommended as an alternate adjudicative remedy for cancer prevention or treatment. This anti-cancer potency of *C. nutans* also in agreement with Nasir and Bohari (2015) and this plant species was investigated to exhibit lesser cytotoxic effect. Furthermore, anti-proliferative effect on tumour cell in vivo was also been reported. In the study, *C. nutans* had displayed significant inhibitory effect on the volume and weight of tumor. Also, hepatoma cell also underwent apoptosis with *C. nutans* treatment, through Hematoxylin and eosin (H& E) staining and TUNEL assay (Huang et al. 2015).

### 2.9.2 Anti-diabetes mellitus

*C. nutans* is also well-known for treatment of diabetes mellitus in Asia countries in the past decades. Based on recent investigation on antidiabetic activity of *C. nutans*, it was evidenced that the blood glucose serum level could be significantly lowered by this plant extract (Nurullita et al., 2008). It was found that the antidiabetic capabilities was possessed by chlorogenic acid, which is one of the significant active ingredients within *C. nutans* extract. In this context, chlorogenic acid acts as an antidiabetic agent by stimulating glucose uptake in both insulin-sensitive and insulin-resistant adipocytes (Meng et al. 2013). The antidiabetic characteristic of chlorogenic acid was also in agreement with Zhang et al. (2007) on the inhibitory effects on  $\alpha$ -glucosidase and lowered the postprandial blood glucose level. Next, recent report from Sarage et al. (2016) had documented the medicinal potency of *C. nutans* in managing hyperlipidemia. It is one of the common disorder in recent days which resulted by unhealthy food practices. These food practices were including high fat and high cholesterol (HFHC) diet which would subsequently induced insulin resistance. The worsening of insulin resistance was induced through transcriptional modulation of insulin signaling genes. Based on the OGTT (Oral Glucose Tolerance Test) data, it was shown that the *C. nutans* was able to normalize the plasma glucose level and improving insulin resistance via prevention of some of the transcriptional changes on insulin signaling genes resulted by HFHC. The prevention included meditation by multiple bioactive ingredients of the *C. nutans* such as protocatechuic acid and chlorogenic acid. Besides, other bioactive compounds found in *C. nutans* namely cinnamic acid and caffeic acid were found to exhibit anti-diabetic characteristics by regulating glucose utilization. These compounds were demonstrated to maintain glucose homeostasis via modulating gluconeogenesis and glycogenesis in insulin-resistant mouse hepatocyte model (Huang and Shen, 2012).

### 2.9.3 Anti-inflammatory

*C. nutans* was widely known as an anti-inflammatory agent. There was a study was done on anti-inflammatory activities by practicing on models of EPP-induced ear oedema

and carrageenan-induced paw oedema in the rat. As a result, this plant species had showed the in-vitro inhibitory effects on neutrophil functional responsiveness without revealing cytotoxic effect. (Wanikiat et al. 2007). Besides, this medicinal potency of *C. nutans* as anti-inflammatory agent was in agreement with that Tu et al. (2014) who reported that 80% ethanol extract of *C. nutans* showed anti-inflammatory activities with in bioscreening. 10µg/mL of the *C. nutans* had the strongest elastase which imposed the inhibitory effect of 68.33%.

#### **2.9.4 Anti-oxidant activity**

In addition, *C. nutans* also well-known for its antioxidant properties. It was believed that a natural antioxidant possessed a potential therapeutic intervention for defense body from oxidative stress or against free radical damage. Based on the findings from Pannangpetch et al. (2007), it was found that the *C. nutans* did shown moderate free radical scavenging activity with the maximum effect of  $67.65 \pm 6.59\%$ . Also, the protective effect against free-radical induced hemolysis had been reported. On the other hand, the strong antioxidant capabilities also been agreed with that the petroleum ether extracts of this plant had possessed the highest radical scavenging activity as  $82.00 \pm 0.02\%$  as compared to ascorbic acids and  $\alpha$ -tocopherol. In this study, the reason of higher radical scavenging activity as compared to previous study by Pannangpetch et al. (2007) might due to geographical are difference and the presence of certain phytochemical constituents such as alkaloids, flavonoids and flavones (Arullappan et al. 2014). The strong antioxidant properties also been reported by Sulaiman et al. (2015), as high phenolic compounds within ethanol and ethyl extracts of *C. nutans* could carry out neutralization of harmful free radicals (ROS). According to Yong et al. (2013), it was found that chloroform extracts of *C. nutans* performed well the antioxidant activity against DPPH and galvinoxyl radicals too, with the antioxidant capacity value of  $7852.63 \pm 449.90 \mu\text{g Teq/g extract}$  and  $12248.82 \pm 173.50 \mu\text{g Teq/g extract}$  respectively.

#### **2.9.5 Anti-viral against HSV**

Herpes simplex viruses (HSV-1 AND -2) is generally known as one kind of common human pathogens which can lead to a series of human threats. In light of this issue, efficacy of *C. nutans* in treatment against Herpes Simplex Virus was reported had discovered the antiviral activities of extracted and purified monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) from *C. nutans* against HSV (Pongmuangmul et al., 2016). In addition, other bioactive constituents of the extract from the genus *Clinacanthus* such as polyphenolic, glycoside, terpenes were discovered to be suitable as anti-HSV agents (Kunsorn et al., 2013). In fact, natural products from medicinal plant including *C. nutans* always treated as a potent medicinal herb in treating HSV infections instead of Acyclovir (ACV) and other nucleoside derivatives. It was due to the medicinal potency of natural products is associated with several benefits such as lesser side effects would be possessed, less resistance and the lower toxicity would be exerted as compared to current anti-HSV drugs (Hassan et al., 2015).

## **2.10 TOXICITY OF SILVER NANOPARTICLES TO HUMAN**

Without a doubt, various distinctive properties of silver nanoparticles had made it an excellent candidate for wide applications in sciences and technology especially in medical field. Nevertheless, adverse effects of silver nanoparticles to human was currently under intense discussion since some studies which concerned with toxicity of silver nanoparticles were reported. According to Panyala et al. (2008), it was reported that chronic exposure to silver would lead to the health crisis such as permanent bluish-grey discoloration of the skin (argyria) and eyes (argyrosis). Also, exposure to soluble silver compounds would harm to liver and kidney and caused the irritation of various human organs including eyes, skin, respiratory and intestinal tract. The literature also proved that silver nanoparticles led to cellular morphological modifications, lactase dehydrogenase (LDH) leakage and impaired mitochondria function in an in-vitro toxicity assay of silver nanoparticles in rat liver cell (Hussain et al., 2005). Besides that, high concentration of silver nanoparticles also been reported to exhibit a significant cytotoxic effect on peripheral blood mononuclear cells (Shin et al., 2007). According to McAuliffe and Perry (2009), male reproductive organ, including testes and male germline cells would be also affected

followed by adverse reproductive outcomes due to toxicity of silver nanoparticles. Kim et al. (2010) had investigated the subchronic oral toxicity of silver nanoparticles on treated animals. It was found that slight liver damage was occurred as a consequences of exposure of more than 125 mg/kg of silver nanoparticles. High incidence of bile-duct hyperplasia, with or without necrosis, fibrosis, and pigmentation was found too through histopathologic examination.



UMP





## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 GENERAL

The general procedures in this experiment was including collection of *C. nutans* leaves, preparation of five reaction mixtures of 1.0 mM silver nitrate solution with different volume ratio of *C. nutans* methanolic extract and various characterization techniques to investigate silver nanoparticles followed by antimicrobial susceptibility testing. Furthermore, profiling of active ingredients in *C. nutans* methanolic extract was done by using Waters ACQUITY UPLC I-Class systems (Waters, Milford, USA) equipped with a binary pump, an autosampler, a degasser, and a diode-array detector (DAD). The system was controlled with Waters UNIFI Vion software. The chromatographic column UPLC HSS T3 C18 (2.1 mm×100 mm, 1.8 μm) was used and eluted with a linear gradient of A (0.1% formic acid in deionized water) and B (ACN) at a flow rate of 0.5 mL/min for 16 minutes. The injection volume was 3 μL.

For extraction and purification procedures, the organic solvents used were industrial grade of hexane and methanol were used. In the same time, the apparatus used for solvent extraction were a separating funnel, a retort stand and a 500 mL Erlenmeyer flask. Prior to extraction and purification, an oven and mechanical grinder was required for drying and grinding respectively. Also, an A251 Branson electric sonicator was used for extraction and a Buchi Rotavapor R-II system associated with an Eyela A-1000 S vacuum pump and Buchi R-II heating bath as unit components for rotary evaporation.

Various characterization techniques were utilized to investigate silver nanoparticles including UV-Visible Spectroscopy (UV-vis), Field Emission Scanning Electron Microscopy (FESEM), Energy Dispersive X-ray (EDX) analysis and Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR). For the antimicrobial testing, four testing microorganisms were used including two Gram-positive strains; *B. subtilis*, and *E. faecalis* and two Gram-negative strains; *E. coli* and *P. aeruginosa*. All the microbial strains were provided by Microorganism Laboratory, Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang. Other materials required were including Nutrient agar powder (NA) for agar making, gentamicin antibiotics. The apparatus needed in this assay were cotton swabs, inoculating loop, Petri dishes, and glass tubes.

### **3.2 COLLECTION OF PLANT MATERIALS**

The whole plants/ plant leaves were collected in Jelebu, Negeri Sembilan. The plant was authenticated by Dr. Shamsul Kamis, Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen number (SK 2874/75) and deposited at the Herbarium Unit of Universiti Putra Malaysia, Malaysia. The collected leaves were washed thoroughly with running tap water for two to three times. Next, the *C. nutans* leaves were dried in the oven under temperature of 40°C, and subsequently powdered to a coarse consistency by mechanical grinder. The powdered dried leaves were then stored at -80°C prior to further analyses.

### **3.3 PREPARATION OF *C. NUTANS* METHANOLIC EXTRACT**

300 g of the powdered dried leaves were first extracted via maceration with industrial grade hexane for defatting purpose. The maceration process was conducted by using 1000 mL of hexane in a covered beaker and sonicated at 60°C for 30 minutes and repeated for three times. The plant extract was then filtered using Whatman filtered paper No. 1 and the solvent subsequently been evaporated and concentrated to one-fourth by using a rotary evaporator under reduced pressure at 40°C. The plant residue obtained from

the previous filtration process was next macerated with methanol by sonication for 30 minutes for three cycles. Subsequently, the plant extract was filtered as previous step and concentrated to one-fourth by using rotary evaporator. The weight of the methanolic crude extract was measured. In the end of the extraction, *C. nutans* methanolic fraction was obtained and kept in vial and stored at  $-80^{\circ}\text{C}$  for further in vitro investigation.

#### **3.4 SYNTHESIS OF SILVER NANOPARTICLES (AgNPs)**

In order to synthesis the silver nanoparticles, 10,000 ppm *C. nutans* methanolic plant extract were prepared and added to 1.0 mM of  $\text{AgNO}_3$  solution in different volume ratios as followed: 1: 50, 2: 50, 3: 50, 4: 50 and 5: 50 mL. Five prepared mixture were then incubated for 48 hours in an ambient shaker incubator (Protech, Model SI-100D) with 200 rpm at  $37^{\circ}\text{C}$ . At the time intervals of 24-h, 15 mL of each mixtures are centrifuged in refrigerated centrifuge (Kubota, Model 5922) at 6000 rpm for 15 minutes to eliminate the unwanted biomolecules. Subsequently, the pellet was re-dispersed with deionized water after discarding the supernatant and dried in oven at  $50^{\circ}\text{C}$  for 24 hours. The centrifugation and drying procedures were repeated again after the mixtures undergone 48 h incubation.

#### **3.5 CHARACTERIZATION OF SILVER NANOPARTICLES**

The biosynthesis of the AgNPs in various mixture was monitored by measuring the UV-visible spectra and were recorded with Genesys 10S UV-VIS Spectrophotometer, (Thermo Scientific) from 380 nm to 700 nm at room temperature. The dried AgNPs was diluted with chloroform and subjected to Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) by using Perkin-Elmer. In the same time, the dried AgNPs were determined for its detail size, morphology and agglomeration by using Field Emission Scanning Electron Microscope (FESEM).

#### **3.6 CULTURE AND MAINTENANCE OF MICROORGANISMS**

The test microorganisms used in this study including two Gram-positive microbes and two Gram negative microbes. The Gram-positive strains were *B. subtilis* and *E. faecalis*, while the Gram-negative strains were *E. coli* and *P. aeruginosa*. Pure culture of these test microorganisms were obtained from FIST Laboratory. All the bacterial strains were maintained in the Nutrient agar medium and incubated at around 37°C.

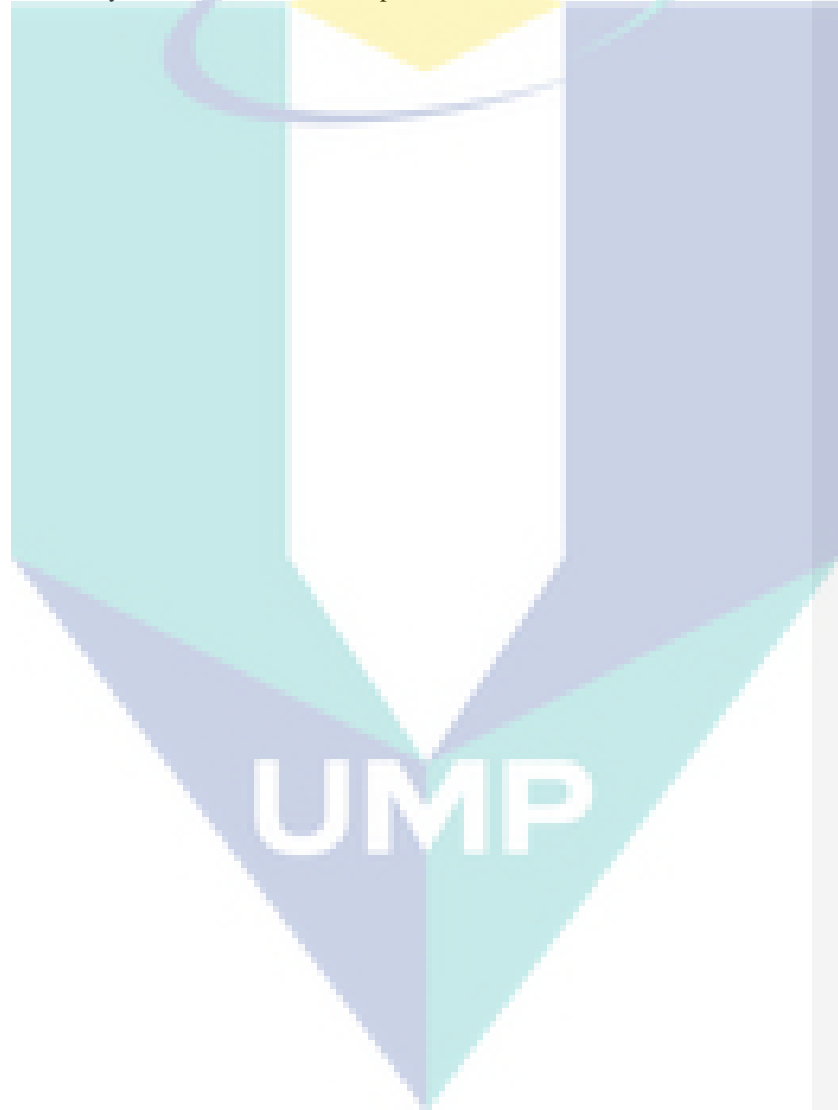
### 3.7 AGAR DISK DIFFUSION METHOD

28 g of agar powder was suspended in 1 L distilled water and the mixture was subjected to heating while stirring. The dissolved mixture was subsequently autoclaved at 121°C for 15 minutes. The nutrient agar was then left to cool until 50°C once the agar has been autoclaved. Next, the molten agar was poured into three 90 mm sterile Petri dishes to create a mean depth of 4.0 mm±0.5 mm and allowed to dry on the purpose of moisture removal before used. Similar agar preparation procedures were repeated for the preparation of potato dextrose agar plate. A sterile cotton wool swab was used to dip into broth culture of the organism and gently pressed against the wall of the tube followed by rotating the swab to remove excessive fluid. Next, the swab was used to spread the inoculum evenly over the entire surface of the agar plate for a lawn of growth. The streaking step was recommended done in one direction, then right angles to the first streaking and finally streaking all around the edge of the agar. The procedure was repeated for the remaining plates. The plates were left for 15 minutes inoculation before applying discs. After inoculation, the 6 mm filter paper discs were impregnated with AgNPs and placed in the plates by using sterile forceps, with about 2 cm apart from each other. Ensure all the plates were labelled with the chemical and microorganism used. The plates were inverted followed by incubation within 15 minutes of disc application. All of the plates were incubated for 24 hours. The gentamicin served as positive control for bacteria whereas the blank disc impregnated with 10000 ppm plant extract was used as a negative control.

### 3.8 DETERMINATION OF ZONE OF INHIBITION

The diameters of zones of inhibition for all tested microorganisms were measured by using a ruler in millimeter (mm). The measurement should be taken from edge to edge

across the zone of inhibition over the center of the disk. The zone of inhibition of all experimental bacterial strains was compared with the positive and negative control. The microorganism would only be considered as susceptible when there was a clear zone of inhibition.



## CHAPTER 4

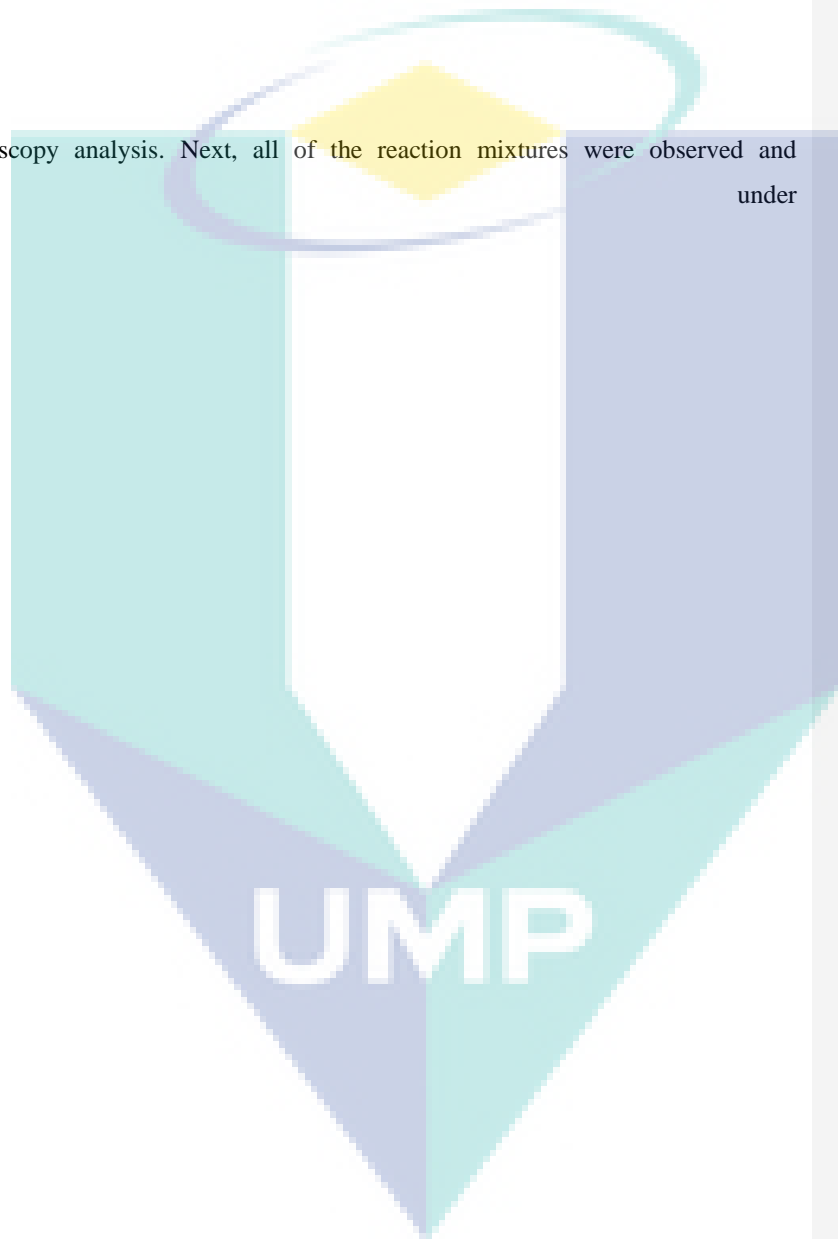
### RESULTS AND DISCUSSION

#### 4.1 VISUAL OBSERVATION AND UV-VISIBLE SPECTROSCOPY

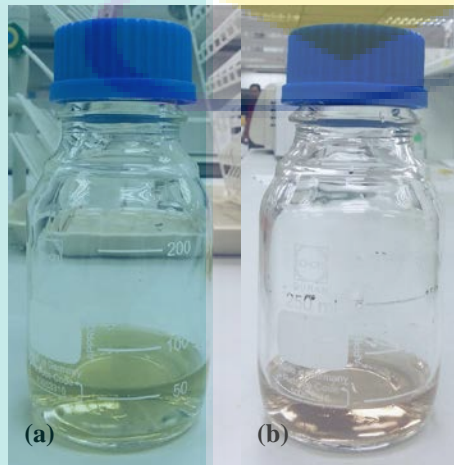
The detailed study on biosynthesis of silver nanoparticles by natural plant extract, *C. nutans* was carried out by visual observation and UV-vis spectrophotometer analysis. The reaction started within 1 h and the colour changes were observed visually. The colour of the solutions was further changed into deep reddish brown and dark brown after 24 h and 48 h of the reaction, which indicated the formation of silver nanoparticles (Figure 4.1). The changes of colour could be ascribed to generation of silver nanoparticles by active components in *C. nutans* methanolic extract, indicating bioreductive mechanism was carried out, reduced the silver ions into elemental silver.

Lu, Bravo-Suárez, Takahashi, Haruta, and Oyama (2005) have reported that the electronic transitions involving the  $\text{Ag}^+$  ion give rise to absorption bands located between 200 and 230 nm, whereas the electronic transitions of metallic  $\text{Ag}^0$  appear in the 250-330 nm spectral range. In this analysis, there was a characteristic absorption peak was observed as shown as Figure 4.1. The absorption peak was known as SPR band as it was owing to excitation of surface plasmon vibration. Based on the Figure, SPR band of silver nanoparticles was determined at around 480 nm. The obtained SPR was different as previously reported due to the size different as the larger the size of silver nanoparticles, the larger the wavelength of SPR peak. According to Cytodiagnostics (2016), presence of SPR peak at around 480 nm was contributed to the silver nanoparticles of the size of around 80 nm and this statement was further verified and discussed at Field Emission Scanning

Electron Microscopy analysis. Next, all of the reaction mixtures were observed and analysed under



two manipulating variables including effect of *C. nutans* methanolic extract and incubation period.

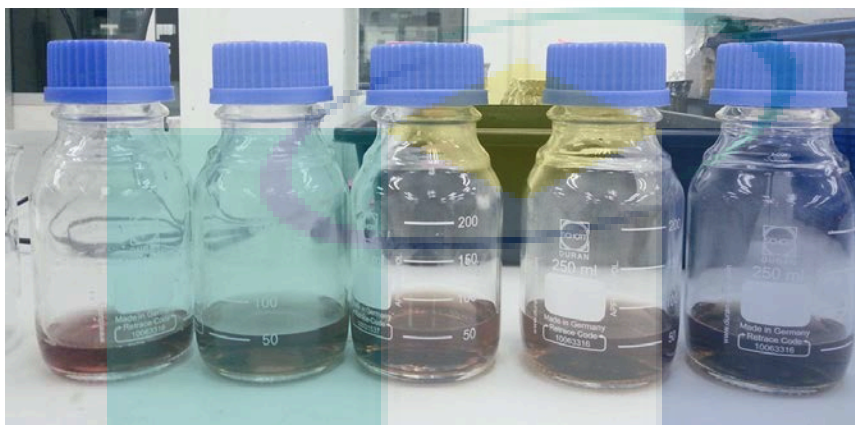


**Figure 4.1:** Reaction mixtures (a) before incubation and (b) after incubation for 1 h

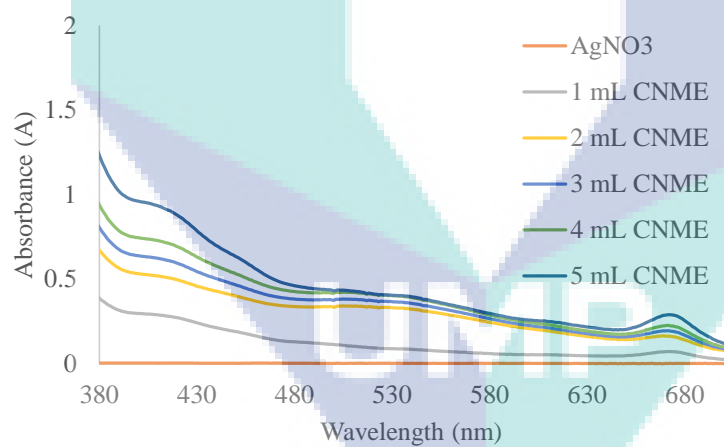
#### 4.1.1 Effect of Different Ratio of *C. nutans* Methanolic Extract

Initially, each of the reaction mixtures developed different colour intensity when different volume ratios of plant extracts were added into respective  $\text{AgNO}_3$  solutions. After a short period of time, the variations of colour intensity were become more apparent as showed in Figure 4.2. A light reddish brown colour were observed on the reaction mixtures which containing 1.0 and 2.0 mL of *C. nutans* methanolic extracts, while those containing 3.0 up to 5.0 mL of plant extract showed darker reddish brown colour. Furthermore, the SPR peaks were proportionally higher and the maximum peak intensity was observed at *C. nutans* methanolic extract content of 5.0 mL as shown in Figure 4.3. It is because of more active components of plant extract was involved in bio-reduction of silver ions into elemental silver.





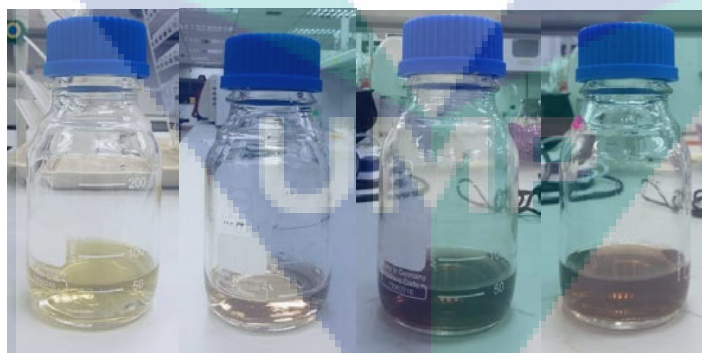
**Figure 4.2:** Reaction mixtures with different ratio of *C. nutans* methanolic extract: 1 mL, 2 mL, 3 mL, 4 mL and 5 mL (from left to right)



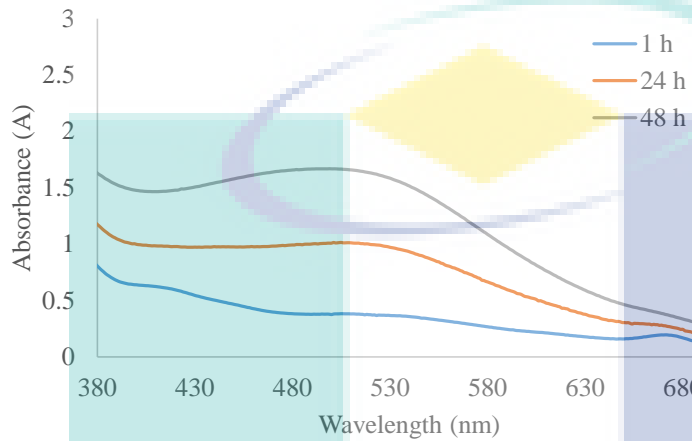
**Figure 4.3:** UV-visible absorption spectra of synthesized AgNPs at variable volume ratio of *C. nutans* methanolic extract

#### 4.1.2 Effect of Incubation Period

In the present study, the changes of the reaction mixture in terms of colour intensity and absorbance value were determined at different time intervals of incubation, 1 h, 24 h and 48 h. The colour of  $\text{AgNO}_3$  was initially colourless and developed into light yellow after *C. nutans* methanolic extract added. The reaction mixtures were gradually turning into light reddish brown after 1 h of incubation period. The colour intensity of this reaction mixture were subsequently becoming deeper reddish brown and dark brown at the following 24 h and 48 h of incubation period respectively (Figure 4.4). Also, the absorbance of the reaction mixtures were determined and showed an increasing trend with time, proportionally with colour intensity (Figure 4.5). The maximum absorbances of the reaction mixture were generally obtained after 48 h of incubation. The increase in absorbance with colour intensity could be ascribed to increase of the amount of silver nanoparticles as the time prolonged. This rapid generation of AgNPs in reaction mixture was owing to the great reducing potential of the active components in *C. nutans* methanolic extract.



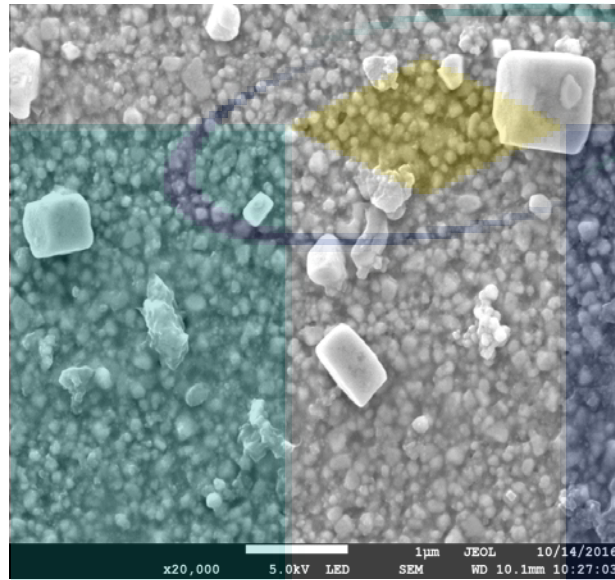
**Figure 4.4:** Reaction mixtures (a) before incubation; (b) 1 h of incubation period; (c) 24 h of incubation period and (d) 48 h of incubation period



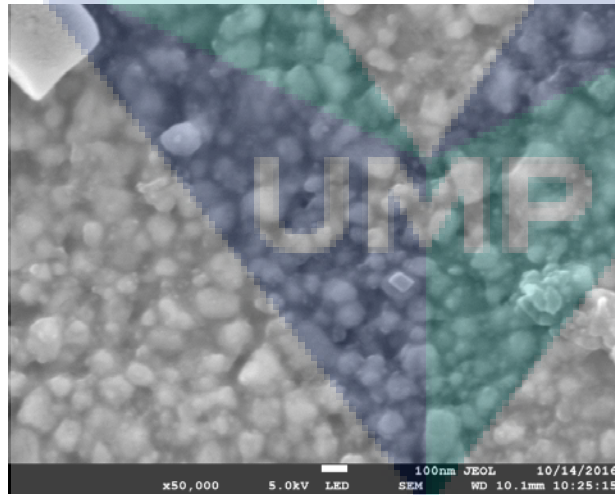
**Figure 4.5:** UV-vis absorption spectrum of 3:50 mL of Plant extract- $\text{AgNO}_3$  at different time intervals

#### 4.2 FIELD EMISSION SCANNING ELECTRON MICROSCOPY ANALYSIS

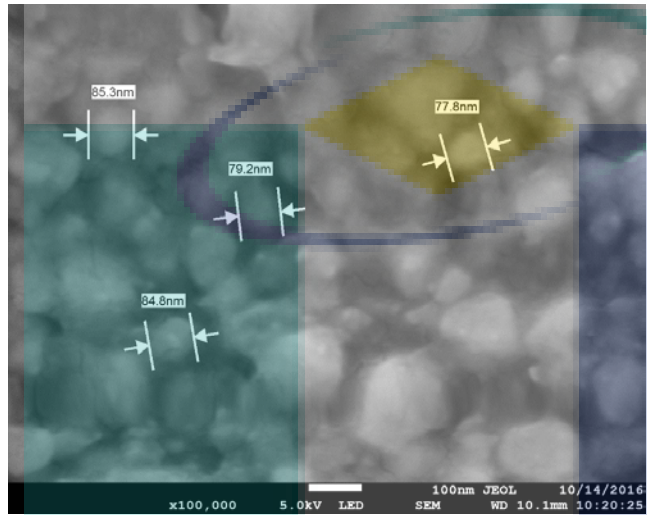
In this study, FESEM was employed to characterize the synthesized silver nanoparticles for its detailed size and morphology. The topographical image of synthesized AgNPs was showed below at various magnification, 20 kx (Figure 4.6), 50 kx (Figure 4.7), and 100 kx (Figure 4.8). Based on Figure 4.6 and 4.7, high density of silver nanoparticles synthesized by *C. nutans* could be observed and they were uniformly dispersed. Under 100 kx of magnification (Figure 4.8), the development of the silver nanostructures was further confirmed as spherical shape and clearly distinguishable in 77.8 to 85.3 nm in size.



**Figure 4.6:** FESEM micrograph of AgNPs of 20 kx of magnification



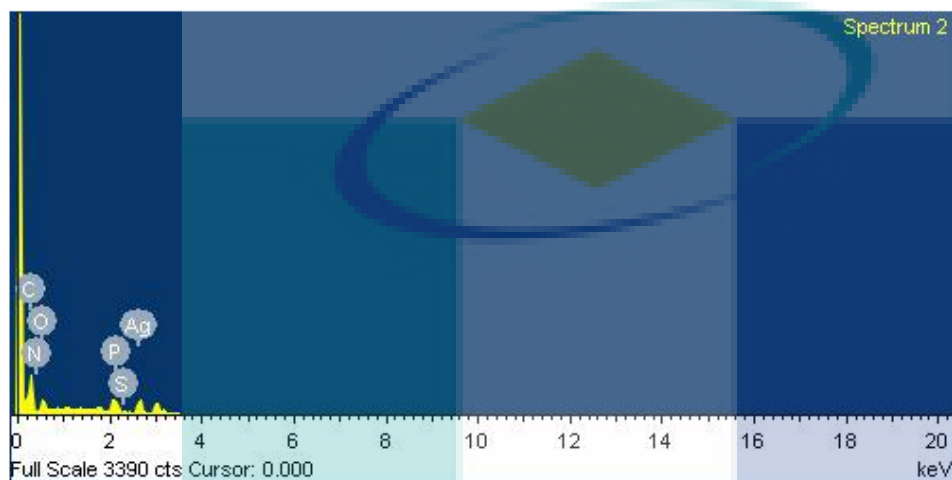
**Figure 4.7:** FESEM micrograph of AgNPs of 50 kx of magnification



**Figure 4.8:** FESEM micrograph of AgNPs of 100 kx of magnification

### 4.3 ENERGY DISPERSIVE X-RAY (EDX) ANALYSIS

Throughout the EDX analysis, a complete elemental distribution of the sample was revealed as shown in the Figure 4.9 and Table 4.1 Based on the results, a significant peak of silver was indicated at around 2.7 keV. Formation of AgNPs was confirmed as typical optical absorption peak of metallic silver nanoparticles generally take places approximately at 3 keV due to surface plasmon resonance. Besides, silver was appeared as the major constituent, which was 81.58 % of total weight of the sample. Other than the silver, presence of other elements such as carbon (8.55%), nitrogen (2.10%), oxygen (5.05%) phosphorus (1.47%) and sulphur (1.26%) were also assigned on respective signal peaks. Peak of C, N, O, P and S were corresponded to the phytoconstituent capping over the AgNPs. These elements were the active molecules of *C. nutans* which responsible for bio-reduction of silver ions to elemental silver.



**Figure 4.9:** EDX characterization spectrum of synthesized silver nanoparticles

**Table 4.1:** EDX results of percentage of elements in reaction product. Note: CK = carbon; NK = nitrogen; OK = oxygen; PK = phosphorus; SK = sulfur; AgL = silver

Element	Weight %	Atomic %
C K	8.55	35.23
N K	2.10	7.41
O K	5.05	15.63
P K	1.47	2.35
S K	1.26	1.94
Ag L	81.58	37.44
Totals	100	

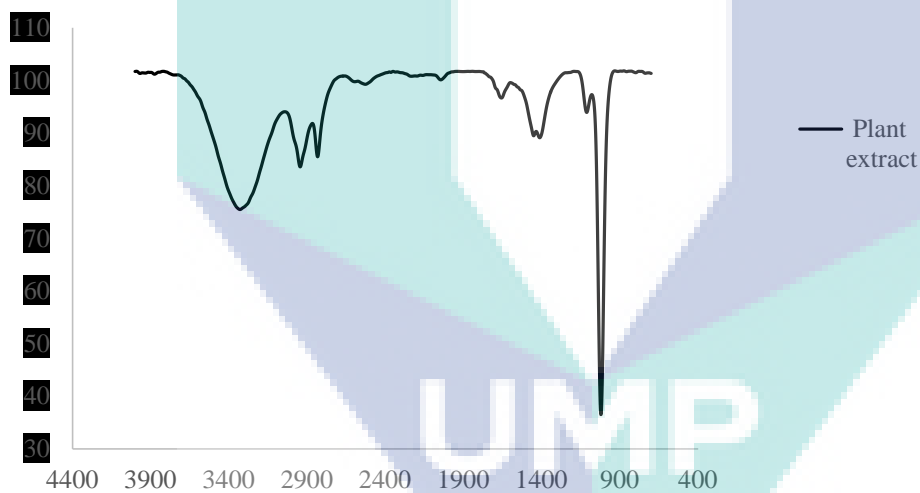
#### 4.4 ATTENUATED TOTAL REFLECTANCE FOURIER TRANSFORM INFRARED SPECTROSCOPY (ATR-FTIR) ANALYSIS

The FTIR analysis were carried out for identification of possible biomolecules in *C. nutans* extract used in biosynthesis of silver nanoparticles. The FTIR spectra of aqueous *C.*

*nutans* methanolic solution and synthesised AgNPs are shown in Figure 4.10. Based on the literature, the bioactive compounds are generally phenolic, flavonoids, sulphur-containing compounds. In the methanolic *C. nutans* extract, absorption peaks are observed at 1021.11, 1112.34, 1657.08, 2944.09 and 3328.76  $\text{cm}^{-1}$ . These peaks were assigned with their representative functional groups as showed in the Table 4.2. In comparison with the spectra of the supernatant, some of the FTIR peaks in *C. nutans* were found decreased significantly after the reaction and confirmed the process of bio-reduction. In this study, C-N stretch had showed a decreased in intensity, indicated the involvement of amine group in biosynthesis of AgNPs. These amine groups could be present in glycoprotein on the cell wall, enzymes and other proteinaceous substance within *C. nutans*. Furthermore, other absorption bands of interests are including C-O stretch, C=C stretch and C-H stretch as C-O stretch had showed a significant decrease in intensity while C=C and C-H stretch were disappeared after reaction. These bonding could probably come from the phenolic and flavonoid groups in the plant extract. In this study, hydroxyl group and carbonyl group did showed some contradictions with previous studies. Remarkably, these two peaks showed increment in absorbance intensity, which suggested as not involved in bio-reduction of silver ions. As for the absorbance peak of carbonyl group, this was due to the presence of silver nanoparticles in the supernatant. This was further confirmed as C=O stretch vibration had showed a slight shift from 1657.08  $\text{cm}^{-1}$  to 1638.78  $\text{cm}^{-1}$ , and a shift to lower wavenumber range was due to binding of the functional groups with the silver nanoparticles surface. Thus, carbonyl group was considered as one of the active components in involving bio-reduction of silver ions and it could be attributed to carboxylic acids, ketones or aldehyde groups of polysaccharides in plant cell wall. Besides, presence of carbonyl group could be found abundantly from phenolic and flavonoid compounds within *C. nutans*. For the increased intensity of hydroxyl groups, it was probably happened due to hydrolysis of polysaccharides in the plant (Subramaniyam et al., 2015). This condition was also probably due to deionized water used in the silver nitrate solution.

**Table 4.2:** FTIR peaks and their assigned functional groups

FTIR peaks, wavenumber (cm-1)	Assigned functional groups	References
1021.11 ; 1015.94	C-O stretch	Tiew et al., 2014
1112.34	C-N stretch	Subramaniyam et al., 2015
1412.86 ; 1451.26	C=C stretch	Tiew et al., 2014
1657.08 ; 1638.73	C=O stretch	Tiew et al., 2014
2944.09	C-H stretch	Tiew et al., 2014
3328.76 ; 3330.30	O-H stretch	Tiew et al., 2014

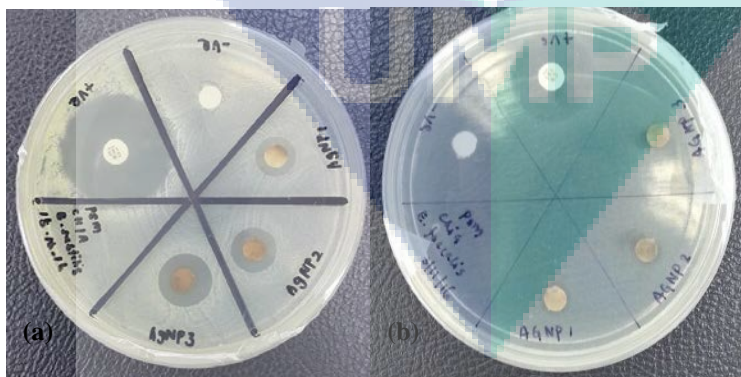


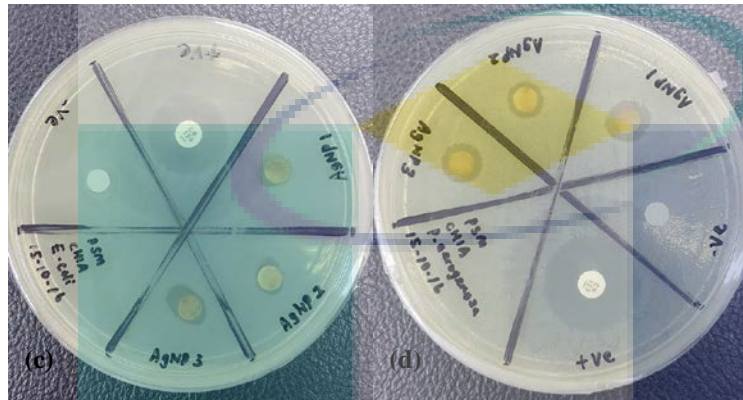
**Figure 4.10:** FTIR spectra of plant extract and synthesized AgNPs



#### 4.5 ANTIMICROBIAL ACTIVITY DETERMINATION

The antimicrobial activities of biosynthesized AgNPs were investigated against bacterial strains by using disk diffusion method. In this study, gentamicin was served as the positive control, while the *C. nutans* methanolic extract was used as negative control. The bacteria used could be classified into Gram-Positive and Gram-Negative bacterial strains. The Gram positive bacteria used in this antimicrobial susceptibility testing were *B. subtilis* and *E. faecalis* while the Gram negative were *E. coli* and *P. aeruginosa*. In this study, AgNPs had showed the remarkable inhibitory effect on these bacteria, where the average diameter of inhibition zone on *B. subtilis*, *E. faecalis*, *E. coli* and *P. aeruginosa* were  $11.50 \pm 1.22$ ,  $8.33 \pm 0.47$ ,  $8.50 \pm 0.50$  and  $9.00 \pm 0.94$  mm respectively (Table 4.3). In comparison, AgNPs revealed a better antimicrobial efficacy on Gram positive bacteria as larger diameter of zone of inhibition was possessed on *B. subtilis* as compared to the Gram negative bacteria (Figure 4.11). Gram positive bacteria exhibited larger susceptibility due to presence of peptidoglycan layers which allows the penetration of foreign substances without any barrier. The membrane structure of Gram negative was different with the former bacteria as it contains of lipopolysaccharides which served as a protective barrier for the cell from complement-mediated lysis from various antibiotics.





**Figure 4.11:** Antibacterial activity of biosynthesized AgNPs against tested microorganisms  
(a) *B. subtilis*; (b) *E. faecalis*; (c) *E. coli*; and (d) *P. aeruginosa*

**Table 4.3:** Zone of inhibition produced by silver nanoparticles, *C. nutans* extract and reference antibiotic gentamicin

Pathogenic Bacterial Strain	Zone of Inhibition (mm)		
	Positive control (Gentamicin)	Negative control (Plant extract)	AgNPs
<i>B. subtilis</i>	30.00	6.00	11.50±1.22
<i>E. faecalis</i>	20.00	6.00	8.33±0.47
<i>E. coli</i>	19.00	6.00	8.50±0.50
<i>P. aeruginosa</i>	21.00	6.00	9.00±0.94

#### 4.6 ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-QUADRUPOLE TIME OF FLIGHT (UPLC-QTOF) ANALYSIS

In this study, it is also important in developing a robust method for the metabolic profiling of the *C. nutans* methanolic extract. UPLC-QTOF analysis was carried out to identify the active ingredients in the plant extract which participating in biosynthesis of silver nanoparticles. According to the BPI plot showed in Figure,4.12 there were more than 70 signal peaks been separated.. Generally, 39 compounds were identified under 20 minutes gradient as presented in Table 4.4. Notably, the most abundant MS-signal was assigned to corymboside ( $m/z= 565.156$ ) with the retention time of 7.60 minute. Under the present MS conditions, there were five more significant peaks were determined at a retention time of 4.36, 6.63, 8.69, 11.27 minutes. These compounds were tentatively assigned as 5,7-dihydroxychromone-7- $\beta$ -D-glucoside ( $m/z= 363.072$ ), glabrol ( $m/z= 393.21$ ), viscumneoside II ( $m/z= 535.145$ ) kushenol U ( $m/z= 445.203$ ) and smiglanin ( $m/z= 363.072$ ) respectively by comparing their mass spectrums with the MS library (Figure 4.13). The structure of confirmed components are shown in Figure 4.14. On the basis of this information, the identified components could be categorized into two major groups, flavonoids and phenolics. The subgroups of flavonoids included C-glycosides, prenylflavonoids, flavanone, flavones, trihydroxyflavones, glycosyl flavones, prenylated chalcone, flavanol and flavanol glycosides. While, the groups under phenolics compounds were chromene, chromene glucoside and chromene glycoside. The presence of these components again confirmed the flavonoid and phenolic compounds were the found to play the principal role in bio-reduction of silver ions as discussed in previous sections. Based on the FTIR spectroscopy data, various functional groups namely carbonyl group and hydroxyl group could be found in flavonoids. It had been suggested that mechanism of bio-reduction by polyphenolic compounds was initiated with tautomerization. The release of a reactive hydrogen atom during tautomeric transformation from enol-form to the keto-form was possibly involved in reduction of silver ions into silver nanoparticles (Makarov et al.,2014). Also, redox mechanism might be the key role of the where ketone groups in the identified compounds, reduced the silver ions into elemental silver, by conversion into carboxylic groups. According to Symonowicz and Klanek (2012), interactions of some flavonoids with

metal ions could lead to chelate formation by using their carbonyl groups or  $\pi$  electrons and hydroxyl groups as coordination sites. For example, corymboside could chelate between 4-carbonyl and 5-hydroxyl groups and also between two hydroxyl groups at other sites. Such mechanisms probably explained on the ability of flavonoids and phenolic compounds on acting as capping agents and subsequently induced the formation of silver nanoparticles.

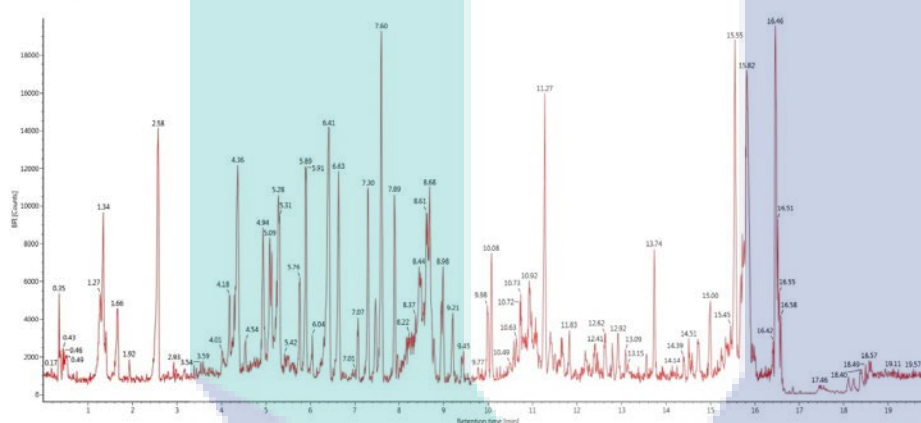


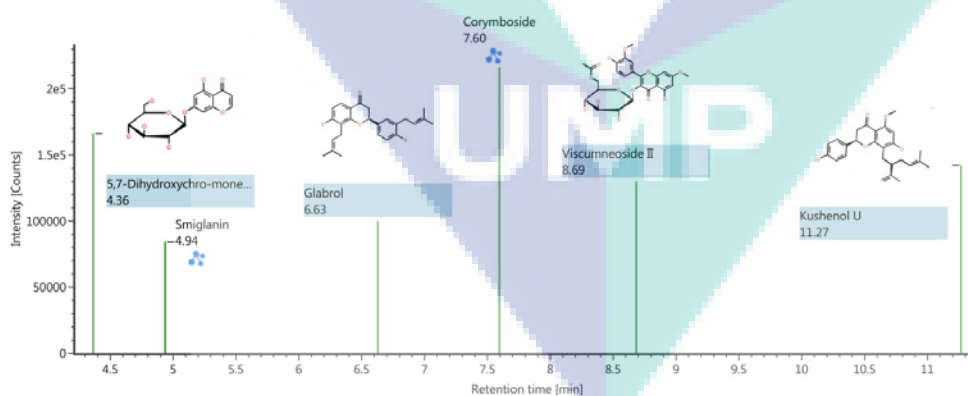
Figure 4.12: BPI chromatogram of *C. nutans* methanolic extract

Table 4.4: Summary of compounds identified in *C. nutans* methanolic extract through UPLC-QTOF analysis

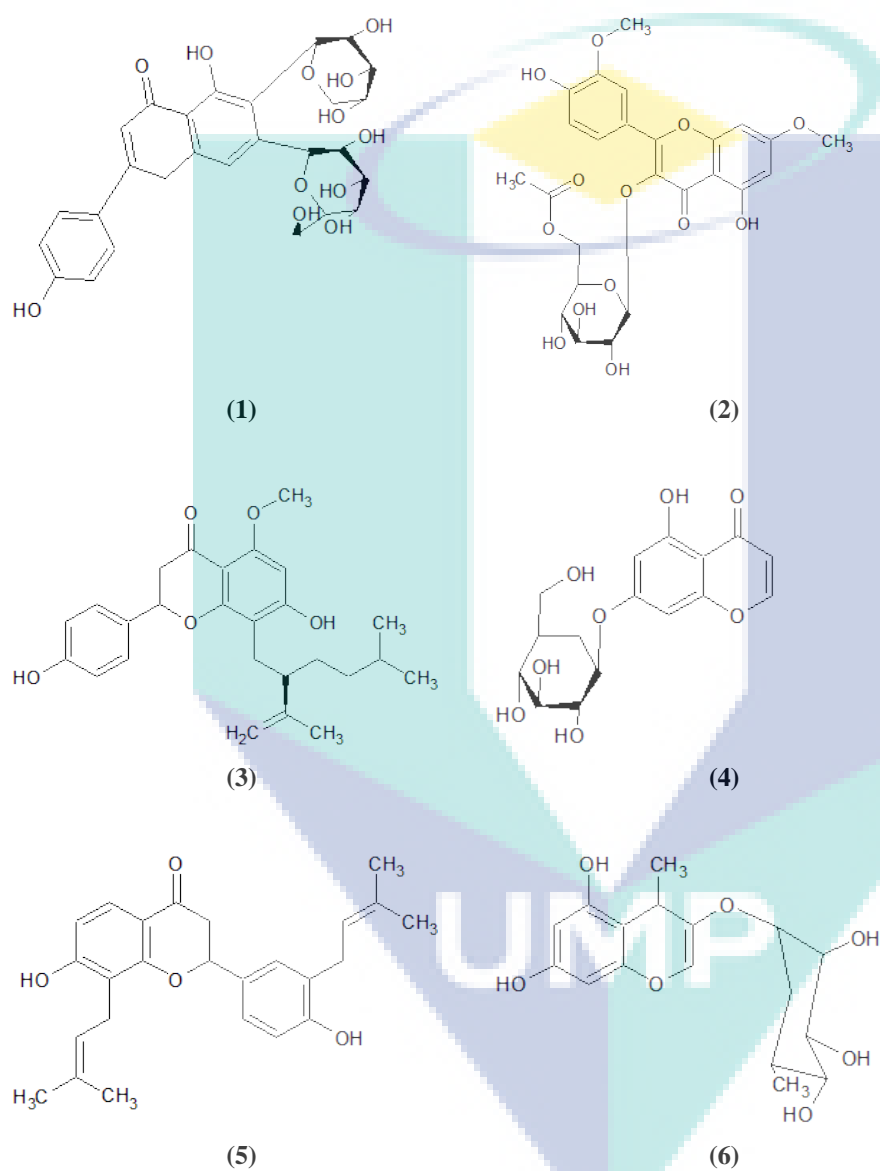
Retention time (min)	Mass to charge ratio (m/z)	Compounds	Phytochemical groups
0.45	581.152	Luteolin-7-O-[ $\beta$ -D-apiofuranosyl(1 $\rightarrow$ 6)] $\beta$ -D-glucopyranoside	Glycosyl flavones
0.45	581.152	Luteolin-7-O-[ $\beta$ -D-apiofuranosyl(1 $\rightarrow$ 6)] $\beta$ -D-glucopyranoside	Glycosyl flavones
1.28	305.067	Dihydromorin	Flavanonol
2.44	333.062	Patuletin	Flavonol
3.41	317.067	Pedalitin	Flavone
3.68	433.089	Aleoresin	Chromones

3.95	433.089	Aleoresin	Chromones
4.14	473.142	Epiafzelechin-3-O- $\beta$ -D-allopyranoside	Flavanol glycoside
4.17	363.072	5,7-Dihydroxychromone-7- $\beta$ -D-glucoside	Chromone glycoside
4.28	347.124	Bavachin (Corylifolin)	Prenylflavonoids
4.36	363.072	5,7-Dihydroxychromone-7- $\beta$ -D-glucoside	Chromone glycoside
4.61	291.087	Catechin	Flavanols
4.94	363.072	Smiglanin	Chromone glycoside
5.34		Cnidimol D	Chromones
5.77	465.138	Homoeriodictyol-7-O- $\beta$ -D-glucopyranoside	Flavanone glycosides
6.43	469.169	Prim-O-Glucosylcimifugin	Chromones
6.63	393.210	Glabrol	Prenylated flavonoid
6.83	361.138	Licochalcone A	Prenylated chalcone
6.89	527.112	Neocomplanoside	Flavonoids
6.99	581.152	Kaempferol-3-O- $\beta$ -D-glucoside-7-O- $\alpha$ -L-arabinofucranoside	Flavonol glycoside
7.15	439.159	Sec-O-glucosylhamaudol	Chromones
7.26	595.168	Apigenin-6-C-glucosylglucoside	Flavonol glycoside
7.59	445.115	Apigenin-7-O- $\beta$ -D-glucuronopyranoside	Glycosyloxyflavone
7.60	499.125	5-Hydroxy-6,4'-dimethoxy-flavone-7-O- $\beta$ -D-glucopyranoside	Trihydroxyflavone
7.60	511.125	Acacetin-7-O-(6"-O-acetyl)- $\beta$ -D-glucopyranoside	Flavone glycoside
7.60	529.135	Nevadensin-5-O- $\beta$ -D-glucoside	Flavone glucosides
7.82	565.157	Corymboside	C- glycosides
7.91	565.156	Corymboside	C- glycosides
8.24	499.124	5-Hydroxy-6,4'-dimethoxy-flavone-7-O- $\beta$ -D-glucopyranoside	Trihydroxyflavone
8.24	535.145	Viscumneoside II	Flavonoids
8.44	535.146	Viscumneoside II	Flavonoids
8.60	501.174	Baohuoside II	Flavones
8.69	469.114	Wogonoside (Wogonin-7-O- $\beta$ -D-glucuronide)	Glycosyloxyflavone
8.69	499.124	5-Hydroxy-6,4'-dimethoxy-flavone-7-O- $\beta$ -D-glucopyranoside	Trihydroxyflavone
8.69	535.145	Viscumneoside II	Flavonoids
9.20	493.262	Kushenol B	Flavanones

9.44	501.173	Baohuoside II	Flavones
9.87	271.060	Resokaempferol	Flavanols
9.88	433.114	Kaempferol-3-O-rhamnoside	Flavones
10.22	233.078	Aleosone	Chromones
11.27	445.203	Kushenol U	Flavanones
11.27	477.141	5-Hydroxy-6,4'-dimethoxy-flavone-7-O- $\beta$ -D-glucopyranoside	Trihydroxyflavone
11.3	515.189	Baohuoside I	Flavones
11.78	287.125	2'-Hydroxy-4',6'-dimethoxydihydrochalcone	Chalcone
12.86	579.184	8-C- $\beta$ -D-[2-O-(E)-p-Coumaroyl] glucopyranosyl-2-[2-hydroxy]propyl-7-methoxy-5-methyl-chromone	Chromones
13.47	579.189	8-C- $\beta$ -D-[2-O-(E)-p-Coumaroyl] glucopyranosyl-2-[2-hydroxy]propyl-7-methoxy-5-methyl-chromone	Chromones
13.74	461.149	7-Hydroxy-5,8-dimethoxyflavone-7-O- $\beta$ -D-glucoside	Trihydroxyflavone
13.75	365.100	4',5,7,8-Tetramethoxy-flavone	Flavones
13.76	271.062	Baicalein	Flavones
18.61	301.075	Koparin	Flavanones



**Figure 4.13:** UPLC-QTOF of *C. nutans* methanolic extract



**Figure 4.15:** Major components in *C. nutans* methanolic extract: (1)corymboside; (2)Viscumneoside II; (3)Kushenol U; (4)5,7-Dihydroxychromone-7-β-D-glucoside (5)Gabrol and (6)Smiglanin



## CHAPTER 5

### CONCLUSIONS

#### 5.1 CONCLUSIONS

In conclusion, quick formation of silver nanoparticles was accomplished by using *C. nutans* as reducing agent. The biosynthesized silver nanoparticles using *C. nutans* methanolic extract were further characterized by several techniques. The UV-vis spectra had confirmed the reduction of silver ions taken place at around 480 nm, and the formation of AgNPs was increased with increasing amount of plant extract and longer incubation period. Based on the result obtained from FESEM, AgNPs were evenly distributed, spherical in shape with average diameter of 77.8 to 85.3 nm. Presence of silver nanoparticles could be confirmed through the EDX analysis, where a high intensity peak signal was showed in the silver region. Furthermore, ATR-FTIR had showed some significant functional groups which participating in biosynthesis of AgNPs including C-N stretch, C-O stretch, C=C stretch, C-H stretch and C=O stretch. Synthesized silver nanoparticles revealed good antibacterial activity against tested microorganisms especially to *B. subtilis* bacterial strains. UPLC-QTOF had revealed the major compounds found in *C. nutans* were flavonoids and other phenolic compounds. The compounds which probably involved in biosynthesis of AgNPs were identified as cymboside ( $m/z=565.156$ ), 5,7-dihydroxychromone-7- $\beta$ -D-glucoside ( $m/z= 363.072$ ), Glabrol ( $m/z= 393.21$ ), Viscumneoside II ( $m/z= 535.145$ ) Kushenol U ( $m/z= 445.203$ ) and Smiglanin ( $m/z= 363.072$ ).



## 5.2 RECOMMENDATIONS

*C. nutans* is one of the popular medicinal plants which could be easily obtained in Southeast Asia. Thus, it is worthwhile to explore the medicinal value of this plant by extraction with different solvents such as ethyl acetate and chloroform to assure of absorption of different bioactive ingredients from the plant extracts. In order to improve the study on plant extract-mediated-synthesis, major compounds which probably involved in biosynthesis of nanoparticles could be isolated from the plant and subjected to further analysis and aided in high yield of AgNPs. In order to synthesize the silver nanoparticles in controlled size and morphological properties, variables such as concentration of plant extracts, incubation period, incubation temperature and other manipulating variables could be modified properly to match the purposes. Furthermore, the synthesized AgNPs could be tested against more microorganisms, in order to determine the extent of antimicrobial activity of silver nanoparticles. This is important in investigating biological activities of biosynthesized AgNPs on microbial human threats around the globe. Also, it would be helped in determining the toxicology of silver nanoparticles to intracellular organelles if the clinical trials could be conducted.

The logo of UMPA (Universiti Malaysia Perlis) is a large, stylized 'V' shape composed of four triangles meeting at the center. The top-left triangle is light blue, the top-right is light green, the bottom-left is light purple, and the bottom-right is light teal. The letters 'UMPA' are written in white, bold, sans-serif font across the center of the 'V' shape.

UMPA

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Comment [HAH2]: Remove the gantt chart

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