SILVER NANOPARTICLES OF *CLINACANTHUS NUTANS* AQUEOUS EXTRACT: BIOSYNTHESIS, PHYTOCHEMICAL AND SELECTED BIOLOGICAL ACTIVITIES

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

AgNPs	Silver nanoparticles
AgNP-L	Silver nanoparticles using leaves of *C. nutans*
AgNP-S	Silver nanoparticles using stems of *C. nutans*
Ag	Silver
Au	Gold
Ag^{+}	Silver ion
$AgNO_3$	Silver nitrate
ATCC	American type culture collection
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
$AlCl_3$	Aluminium chloride
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
C-AgNPs	Commercial silver nanoparticles
Cu	Copper
CNL	Leaves extract of *C. nutans*
CNS	Stems extract of *C. nutans*
C. nutans	*Clinacanthus nutans*
CO_2	Carbon dioxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic acid

DMEM	Dulbecco's modified Eagle's medium
dH_2O	Distilled water
DLS	Dynamic Light Scattering
EAC	Ehrlich Ascites Carcinoma
FTIR	Fourier Transform Infrared
FBS	Fetal bovine serum
GC-MS	Gas chromatography–mass spectrometry
GC-Q-TOF-MS	Gas Chromatography- Quadrupole Time of Flight -Mass Spectrometer
hrs	Hours
HT-29	Colon cancer cell
HeLa	Cervical cancer cell
IC_{50}	Inhibitory concentration 50%
ICDD	International Centre for Diffraction Data
JCPDS	Joint Committee on Powder Diffraction Standard
KBr	Potassium bromide
LC-MS-MS	Liquid chromatography–mass spectrometry- mass spectrometry
MIC	Minimum inhibitory concentration
MCF-7	Breast cancer cell
MBC	Minimum bactericidal concentration
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)- 2H-tetrazolium
mm	Millimiter
mM	Milli mol
mol L^{-1}	Mole per liter
mg/mL	Milligram per millilitre

min	Minutes
nm	Nanometer
mV	Millivolts
PBS	Phosphate buffer saline
Pd	Palladium
PDI	polydispersity index
ROS	Reactive oxygen species
Rpm	Revolutions per minute
SEM-EDX	Scanning electron microscope - Energy dispersive X-ray
SPR	surface plasmon resonance
SD	Standard deviation
TEM	Transmission electron microscopy
TFC	Total flavonoid content
TPC	Total phenolic content
v/v	Volume/volume
XRD	X-ray diffractometer
YRV	yellow-head rhabdovirus
Zn	Zinc
°C	Degree celsius
5-Fu	5-fluoracil
%	Percentage
µL	Micro litre
µg/mL	Micro gram per millilitre
µmol	Micro mol

NANOPARTIKEL PERAK DARIPADA EKSTRAK AIR *CLINACANTHUS NUTANS*: BIOSINTESIS, FITOKIMIA DAN AKTIVITI BIOLOGIKAL YANG TERPILIH

ABSTRAK

Penggunaan tumbuhan untuk mensintesis nanopartikel perak (AgNPs) dianggap sebagai salah satu teknologi hijau. Dalam kajian ini, satu kaedah yang tidak toksik, kos efektif dan mesra alam telah digunakan untuk mensintesis AgNP dengan menggunakan ekstrak air daripada daun dan batang *Clinacanthus nutans (C. nutans)*. Nanopartikel perak yang telah disintesis diuji untuk aktiviti antimikrob, antioksidan dan sitotoksisitinya. Pembentukan AgNPs disahkan oleh spetroskopi UV-Vis pada jarak gelombang 420 nm (AgNP-L) dan 430 nm (AgNP-S). Saiznya telah diukur pada 114 nm (AgNP-L) dan 129 nm (AgNP-S) menggunakan zetasizer. Daripada analisis TEM, saiznya berjulat diantara 10-300 nm dan 10-180 nm bagi AgNP-L dan AgNP-S dengan bentuk sfera. Potensi zeta direkodkan pada -42.8 mV untuk AgNP-L dan -43.9 mV untuk AgNP-S. Analisis XRD dipadankan dengan struktur kubik berpusatkan muka (fcc) perak. Analisis FTIR menunjukkan kehadiran beberapa kumpulan fenol dan flavonoid yang berfungsi sebagai agen penurunan dalam sintesis AgNPs. Nanopartikel perak telah menunjukkan perencatan yang baik terhadap bakteria, tetapi tidak kepada kulat. Perencatan yang paling tinggi ialah ke atas *S. aureus* dengan 11.35 mm (AgNP-L) dan 11.52 mm (AgNP-S), manakala yang paling rendah ialah *E. coli* dengan 9.22 mm (AgNP-L) dan 9.25 mm (AgNP-S) dengan menggunakan kaedah penyebaran cakera. Pola yang sama juga ditunjukkan untuk kaedah penyebaran perigi. Bagi asai-asai antioksidan, AgNP-L menunjukkan IC_{50} pada 417.05 μg/mL dan AgNP-S pada 434.60 μg/mL (DPPH); manakala untuk asai ABTS adalah 304.31 μg/mL

(AgNP-L) dan 326.83 µg/mL (AgNP-S). Asai FRAP menunjukkan AgNP-L [872.389 µmol/L Fe(II)] dan AgNP-S S [612.770 µmol/L Fe(II)] secara signifikasinya mempunyai aktiviti antioksidan yang tinggi berbanding dengan ekstrak tumbuhan (CNL; 152.260 µmol/L Fe(II) dan CNS; 110.445 µmol/L Fe(II)). Nanopartikel perak juga terbukti mempunyai kesan kesitotoksikan pada sel selanjar kanser MCF-7, HT-29 dan HeLa yang bergantung kepada dos. AgNP-S dan AgNP-L menunjukkan kesan kesitotoksikan yang lebih tinggi ke atas MCF-7 (117.43 µg/mL) dan HT-29 (78.47 µg/mL). Sebagai kesimpulannya, biosintesis AgNPs daripada ekstrak air daun dan batang daripada *C. nutans* mempunyai bioaktiviti antioksidan, anti mikrob dan anti kanser yang baik.

SILVER NANOPARTICLES OF *CLINACANTHUS NUTANS* AQUEOUS EXTRACT: BIOSYNTHESIS, PHYTOCHEMICAL AND SELECTED BIOLOGICAL ACTIVITIES

ABSTRACT

The use of plant to synthesise silver nanoparticles (AgNPs) is considered as a green technology. In this study, a non-toxic, cost effective and eco-friendly method has been applied to synthesise AgNPs using aqueous extract of leaf and stem of *Clinacanthus nutans (C. nutans).* The synthesised AgNPs were tested on antimicrobial, antioxidant and cytotoxicity activities. The formation of AgNPs was confirmed by UV-Vis spectroscopy at the wavelength of 420 nm (AgNP-L) and 430 nm (AgNP-S). The size were measured at 114 nm (AgNP-L) and 129 nm (AgNP-S) using a zetasizer. From TEM analysis, the size ranged from 10-300 nm and 10-180 nm for AgNP-L and AgNP-S with spherical shape. Zeta potentials recorded at -42.8 mV for AgNP-L and -43.9 mV for AgNP-S. XRD analysis matched the face-centered cubic (fcc) structure of silver. FTIR analysis revealed the presence of few functional groups of phenolic and flavonoid compounds which acts as the reducing agent in AgNPs synthesis. The AgNPs showed good inhibition against bacteria, but not fungus. Inhibition showed that highest against *S. aureus* with 11.35 mm (AgNP-L) and 11.52 mm (AgNP-S), while the lowest inhibition against *E. coli* with 9.22 mm (AgNP-L) and 9.25 mm (AgNP-S) in disc diffusion method. Same trend of result was reported for well diffusion method. In antioxidant assays, AgNP-L showed IC_{50} at 417.05 µg/mL and AgNP-S at 434.60 µg/mL (DPPH); 304.31 µg/mL (AgNP-L) and 326.83 µg/mL (AgNP-S) for ABTS, respectively. FRAP assay showed AgNP-L [872.389 µmol/L Fe(II)] and AgNP-S [612.770 µmol/L Fe(II)], respectively were significantly

(p<0.05) higher in antioxidant activity compared to plant extracts (CNL; 152.260 µmol/L Fe(II) and CNS; 110.445 µmol/L Fe(II)). The AgNPs were also proven to possess cytotoxicity effect on cancer cell lines of MCF-7, HT-29 and HeLa in dose-dependent manner. AgNP-S and AgNP-L showed significantly (p<0.05) higher cytotoxicity against MCF-7 (117.43 µg/mL) and HT-29 (78.47 µg/mL), respectively. In conclusion, the biosynthesised AgNPs from aqueous extract leaves and stem of *C. nutans* provide promising bioactivities towards antioxidant, anti-microbial and cytotoxicity activities.

CHAPTER 1

INTRODUCTION

1.1 Research background

There have been remarkable developments in the field of nanotechnology and this has attracted the attention of researchers due to their wide range of applications. Generally, nanoparticles are denoted as particles within the range of 1 to 100 nm (Horikoshi and Serpone 2013). The physical and chemical properties of nanoparticles are different compared to bulk materials. The properties of nanoparticles is unique because of high surface area to volume ratio and small in size (Hosokawa *et al.*, 2012). The increase in surface area increases the biological activities and hence, increases the surface energy (Willems and Wildenberg 2005). Nanoparticles such as silver nanoparticles (AgNPs) are widely used in food industries, biomedical, dentistry, catalysis, diagnostic biological probes, and sensors (Sadowski 2009; Paula *et al.*, 2012; Priyadarshini *et al.*, 2013; Zarei *et al.*, 2014).

The synthesis of AgNPs is becoming popular and many research have been conducted using various methods either chemically, physically or biologically (Ghorbani *et al.*, 2011). Sodium borohydride, hydrazine and ethylene glycol are the most common chemicals used to synthesise AgNPs (Song *et al.*, 2009(a); Guzmán *et al.*, 2009; Kheybari *et al.*, 2010). However, this chemically synthesised of AgNPs is expensive and harmful to living organisms and hence not preferable. Therefore, biological synthesis has gained more interest among researchers which is cost effective, less energy consumption and non-toxic (Ghorbani *et al.*, 2011).

Indeed, many studies have been reported using natural resources like plants, bacteria, fungi and yeast to synthesise AgNPs (Thakkar *et al.,* 2010). Nevertheless, the use of plant extracts has become major interest in AgNPs synthesis. In the formation of AgNPs, the plant extracts act as reducing and capping agent due to the presence of various bioactive compounds (Song & Kim 2009; Tripathy *et al.,* 2010; Kaviya *et al.,* 2011(a); Loo *et al.,* 2012; Pasupuleti *et al.,* 2013; Shirmohammadi *et al.,* 2014; Maruti *et al.,* 2015). Synthesis of AgNPs using plant extracts is an advantage because it eliminates the process of maintaining cell culture and the process could be scaled up under a non-aseptic environment (Shankar *et al.,* 2004). Therefore, we are optimistic to venture this research by synthesising the AgNPs using *Clinacanthus nutans* (*C. nutans*) extracts. In this study, two types of AgNPs were biosynthesised by using the leaf and stem extracts of *C. nutans*.

Silver nanoparticles are also well known as an effective antimicrobial agent against bacteria and fungus (Kim *et al.,* 2007; Rai *et al.,* 2012). Hence, AgNPs have been studied in many bactericidal applications such as medical, food, cosmetics and textile industry (Chen and Schluesener 2008; Zhang *et al.,* 2009; Kokura *et al.,* 2010; Antony *et al.,* 2016). Various skin diseases triggered by pathogenic microorganisms are treated by antibiotic. Commonly prescribed antibiotics are erythromycin, clindamycin, tetracycline, penicillin and ciprofloxacin (Gupta *et al.,* 2013). Due to the development of antibiotic resistance, researchers are keen to look for alternatives in developing new antibacterial agents. The biosynthesis of AgNPs may overcome the resistance and reduce the cost compared to conventional antibiotics (Rai *et al.,* 2009).

Recently, there are several studies reported that nanoparticles have been effectively improved the antioxidant activity. Synthetic antioxidant such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) had a side effects. According to Labrador *et al.,* (2007), the low doses BHA tested appears to be toxic to Vero cell with loss of mitochondrial function based on MTT assay. The AgNPs inhibited free radical to prevent any tissue or cell damage (Sivakumar *et al.,* 2015(a); Phull *et al.,* 2016). Elemike *et al.,* (2017) reported that synthesised AgNPs using aqueous leaf extract of *Costus afer* showed the antioxidant activity of the nanoparticles were higher compared to the leaf extract only. Thus, The AgNPs can be one of new antioxidant agent, which may avoid the side effects caused by synthetic antioxidant agents.

Apart from antibacterial and antioxidant, plant AgNPs are widely studied on the cytotoxicity of different cancer cell lines (Sunita *et al.,* 2015). As far as we concern, cancer is one of the major health concerns worldwide. Cancer cells are formed from the transformation of normal cells in several stages based on the interaction between the genetic with external influences such as physical, chemical and biological carcinogen (Lokina *et al.,* 2014). Drug resistance may occur even after chemotherapy (Liu, 2009). Thus, alternative biocompatible and cost-effective chemotherapeutic drugs are needed. Recently, many reports on plant derived AgNPs have the potential to control tumour cell growth (Nakkala *et al*., 2015; Ovais *et al.,* 2016). Cytotoxicity of AgNPs was reported to induce apoptosis of the cancer cells via mitochondria damage, oxidative stress which led to cell death (Hackenberg *et al.,* 2011). Therefore, this project aims to investigate the antimicrobial, antioxidant and cytotoxicity effects of the synthesised plant AgNPs.

1.2 Objectives

This project embarks the following objectives:

i. To determine the classes of phytochemicals in aqueous extract of leaf and stem of *C. nutans.*
ii. To biosynthesise and characterise the AgNPs.
iii. To evaluate the antimicrobial activities of the AgNPs on selected microorganisms.
iv. To study the antioxidant activities of the AgNPs.
v. To investigate the cytotoxicity effects of the AgNPs.

CHAPTER 2

LITERATURE REVIEW

2.1 Metal Nanoparticles

Nanotechnology has been one of the most active research areas in modern science. Nanoparticles synthesis has gained interest among researchers, due to their unique physicochemical characteristics compared to bulk materials, especially their high surface area to volume ratio (Hosokawa *et al.,* 2012). Two different methods have been developed to synthesise metal nanoparticles; *top-down* and *bottom-up* approach. The reduction in size of a bulk material into smaller pieces by mechanical or physical methods to get the desired product is known as the *top-down* approach. Meanwhile, the *bottom-up* approach is the synthesis of an atom or molecule to form the desired product by chemical reactions (Kheybari *et al.,* 2010; Mavani and Shah 2013) as well as biological methods (Marchiol, 2012).

Various research have been reported using metal nanoparticles with several applications (Carbone *et al*., 2016). Nanoparticles have been widely explored in the medical, environment and industrial for various functions such as implants, drug delivery, imaging and sensor (Furno *et al.,* 2004; Schröfel *et al.,* 2014). Several metal nanoparticles that are commonly used include silver (Ag) (Gardea-Torresday *et al.,* 2002), Zinc (Zn) (Salari *et al.* 2017), palladium (Pd) (Nadagouda & Varma 2008; Petla *et al.,* 2012), copper (Cu) (Hashemipour *et al.,* 2011) and gold (Au) (Song *et al.,* 2009(b)).

2.2 Silver nanoparticles

Among the metal nanoparticles, silver (Ag) has gained the most attention among researchers. Silver is a naturally occurring element known since ancient history. During the Greek and Roman Era, people used silver vessel to store their water (Barillo and Marx 2014). In the 1960s, silver compounds have been used for burn wound applications. As time passed, in the 21st century, silver has been incorporated into catheters (Barillo and Marx 2014). In the household, silver nanoparticles (AgNPs) are embedded in chitosan to remove pesticides in water (Saifuddin *et al.,* 2011). The AgNPs are also used in the textile industry (Zhang *et al.,* 2009) and personal care products such as cosmetics and as an enhancement in shampoo for anti-dandruff purpose (Kokura *et al.,* 2010; Pant *et al.,* 2013).

There are several methods to synthesise AgNPs such as chemical, physical and biological methods. The physical methods include laser ablation, photochemical reduction and ultrasonic irradiation (Tsuji *et al.,* 2002; Lu *et al.,* 2003; He *et al.,* 2014). The chemical method to synthesise AgNPs uses borohydride, citrate and hydrazine (Guzmán *et al.,* 2009; Song *et al.,* 2009(b); Sileikaite *et al.,* 2009). Nevertheless, chemically synthesised nanoparticles requires toxic solvents and produce hazardous by-products. Likewise, physical methods may require high amounts of energy during synthesis (Thakkar *et al.,* 2010; Ghorbani *et al.,* 2011).

Reliable and environmental friendly way to synthesise AgNPs is important for development as well as applications in the nanotechnology. Therefore, biological methods have gained interest due to the advantages over other conventional methods. The biological synthesis of AgNPs derived from plants, fungus, bacteria, yeast and

viruses (Mohanpuria *et al.,* 2008; Thakkar *et al.,* 2010). This method is simple and used as an alternative to more complex synthesis procedure.

2.2.1 Synthesis of silver nanoparticles using plants

The biological synthesis of AgNPs show more compatibility to be used for biomedical application compared to physical and chemical synthesis (Vijayaraghavan and Nalini 2010; Choudhary *et al.*, 2015). The biological synthesis of AgNPs can be from plants, fungus, bacteria, yeast and viruses (Mohanpuria *et al.,* 2008; Thakkar *et al.,* 2010). The biosynthesis of AgNPs using plant extract is preferable compared to other biological methods. Unlike chemical method, it requires a stabiliser to be added during the synthesis process to avoid agglomeration. On the contrary, biological method using plant extract does not require any stabiliser because the plant extract itself acts as stabiliser in the synthesis process (Ghaffari-moghaddam *et al.,* 2014). Different parts of plant have been used to synthesise AgNPs like leaves, stems, roots, flowers, seeds and barks (Ghaffari-moghaddam *et al.,* 2014). Table 2.1 shows the synthesis of AgNPs using various parts of plant extracts.

The use of plant extract in the formation of AgNPs through reducing agents present in the extracts, which can be the metabolites of phenolic compounds, flavones, alkaloids and sterols derived from various parts of the plant (Iravani *et al*., 2014; Makarov *et al*., 2014; Singh *et al*., 2016) The usage of plant is inexpensive, easily available and easy to scale up for industrial use without purifications or maintaining cultures compared to microorganism (Mittal *et al.,* 2013). The used of microbe requires aseptic condition and high maintenance. Therefore, the use of plant extract is better over microorganism due to less cost and easy to handle (Ahmed *et al.,* 2016). Also, the time required for the AgNPs to form is faster using plant extract compared to microorganisms. The rate

of reaction to synthesise AgNPs using microorganism can take up to 120 hrs compared to plant extract ranging from few hours to maximum of 48 hours (Sabri *et al.*, 2016). Thus, the plant-based method to synthesis AgNPs showed an optimistic choice as biological method compared to other biological methods.

Table 2.1: Various plants used to synthesis silver nanoparticles.

Plant species	Characteristics of AgNPs		Activity	References
	Size(nm)	Shape		
Bergenia ciliata (whole plant)	35	spherical	Antioxioxidant, (DPPH and total antioxidant assay) cytotoxicity on brine shrimp eggs and antimicrobial (*M. luteus, S. aureus, B. bronchiseptica, E. aerogens, A. fumigatus, F. solani, A. niger* and *A.flavus*)	(Phull *et al.,* 2016)
Solanum muricatum (leaf)	20-80	Irregular and rounded	Cytotoxicity against HeLa cell line	(Gorbe *et al.,* 2016)
Momordica charantia (leaf)	16	spherical	Antimicrobial against *E. coli, Pseudomonas* spp., *Bacillus* spp., *A. niger, A. flavus* and *Penicillium* spp.	(Ajitha *et al.,* 2015)
Vigna radiata (seed)	15-20	Oval and spherical	Antimicrobial against *E. coli and S. aureus*	(Choudhary *et al.,* 2015)
Datura inoxia (leaf)	13-60	spherical	Cytotoxicity against breast cancer cell lines (MCF-7)	(Gajendran *et al.,* 2014)
Tea leaf	20-90	nearly spherical	Antimicrobial against *E. coli*	(Sun *et al.,* 2014)

Table 2.1: Continued

Acacia leucophloea (bark)	17-29	spherical	Antimicrobial againt *S. aureus, B. cereus, L. monocytogenes* and *S. flexneri*	(Murugan *et al.,* 2014)
Podophyllum hexandrum (leaf)	12-40	spherical	Cytotoxicity on HeLa cell lines	(Jeyaraj *et al.,* 2013)
Artemisia nilagirica (leaf)	10-60	Spherical and nanotriangles	Antimicrobial againsts *S. aureus, E. coli, B. subtilis* and *P. mirabilis*	(Vijayakumar *et al.,* 2013)
Dioscorea bulbifera (tuber)	13.54	triangular	Antimicrobial against *A. baumannii, E.cloacae, E. coli, H. influenza, K. pneumoniae, N. mucosa* and *P. mirabilis*	(Ghosh *et al.,* 2012)
Ficus benghalensis (leaf)	16	spherical	Antimicrobial against *E. coli*	(Saxena *et al.,* 2012)
Melia azedarach (leaf)	78	cubical and spherical	Cytotoxicity against HeLa cell lines	(Sukirtha *et al.,* 2012)
Tribulus terrestris (fruit)	20-70	spherical	Antimicrobial againsts *S. pyogens, E. coli, P.aeruginosa, B. subtilis* and *S. aureus*	(Gopinath *et al.,* 2012)

2.2.2 Mechanism of AgNPs synthesis

Various studies have been conducted on screening and identification on plants for biosynthesis of AgNPs. Several hypotheses have been proposed for the AgNPs synthesis. The formation of AgNPs occurs mainly from the reduction process of plant biomolecules such as polysaccharides, protein and organic compounds. The biomolecules with functional groups of C=C, C=N, O-H, N-H, C-H and COO are involved in the formation of AgNPs (Kasthuri *et al*., 2009; Prod *et al*., 2014; Ajitha *et al*., 2015; Kuppusamy *et al*., 2016).

The mechanism of AgNPs formation could be described using phenolic compounds as an example. Phenolic compounds are antioxidants with high reducing capacity. This compound reduces the silver ion from $AgNO_3$ to AgNPs due to electron donor of the phenolic compound (Figure 2.1) (Prod *et al.,* 2014). According to Martinez-Castanon *et al.* (2008), gallic acid was used to synthesise AgNPs with three different sizes (7, 29 and 89 nm) using three different procedures. Other phenolic compounds that have the potential as reducing agent such as phyllanthin from the *Phyllanthus amarus* plant, the $–OCH_3$ group from phyllanthin extract may play a role in the formation of AgNPs (Kasthuri *et al.,* 2009). Besides, *apiin* compound from henna leaf contain secondary hydroxyl and carbonyl group which have been used to synthesise AgNPs. Other studies also suggested phenolic compound as the reducing agent in the process of AgNPs including rosmarinic acid from basil extract (Ahmad *et al.,* 2010).

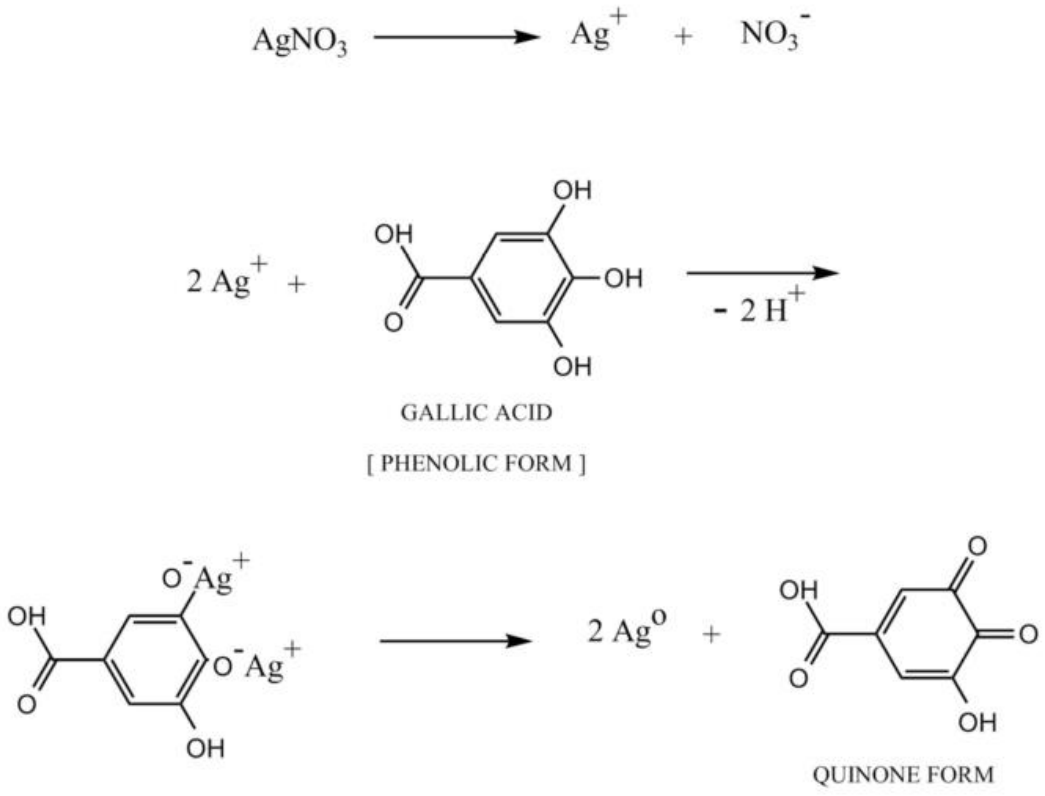

Figure 2.1: The possible mechanism for the formation of AgNPs from gallic acid (phenolic compound) (Prod *et al.*, 2014).

Flavonoids also involved in the formation of AgNPs. Flavonoids are secondary metabolites with basic structure C_6-C_3-C_6 (labelled as A,B and C) (Figure 2.2) and consist of six subgroups including flavones, flavonols, flavanones, flavan-3-ols, isoflavones and anthocyanidins (Ghasemzadeh and Ghasemzadeh 2011). These compounds are the key of bioreducing in aqueous plant extract as they can be electron or hydrogen donor (Pietta 2000). The ketoform on the backbone of a flavonoid reduces Ag^+ to Ag^0 (Ajitha *et al.*, 2015). Based on research conducted by Radhakrishnan *et al.* (2017), apigenin, a plant derived flavonoid isolated from the leaf extract of *Smilax perfoliate* showed formation of AgNPs after 5 minutes of incubation time. Besides apigenin, other flavonoid compounds used to synthesise AgNPs include hesperidin, naringin and diosmin (Sahu *et al.*, 2016). Thus, it can be concluded that flavonoids play an important role in the process of AgNPs synthesis.

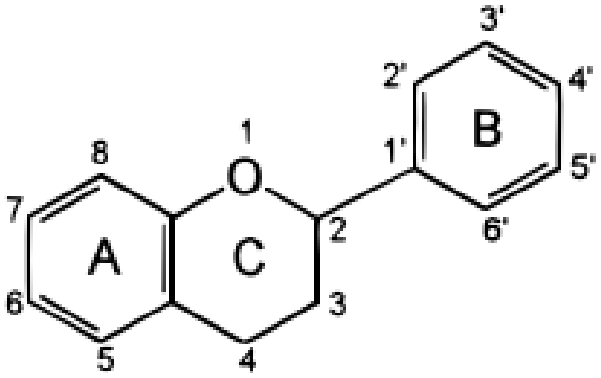

Figure 2.2: Basic structure of flavonoids

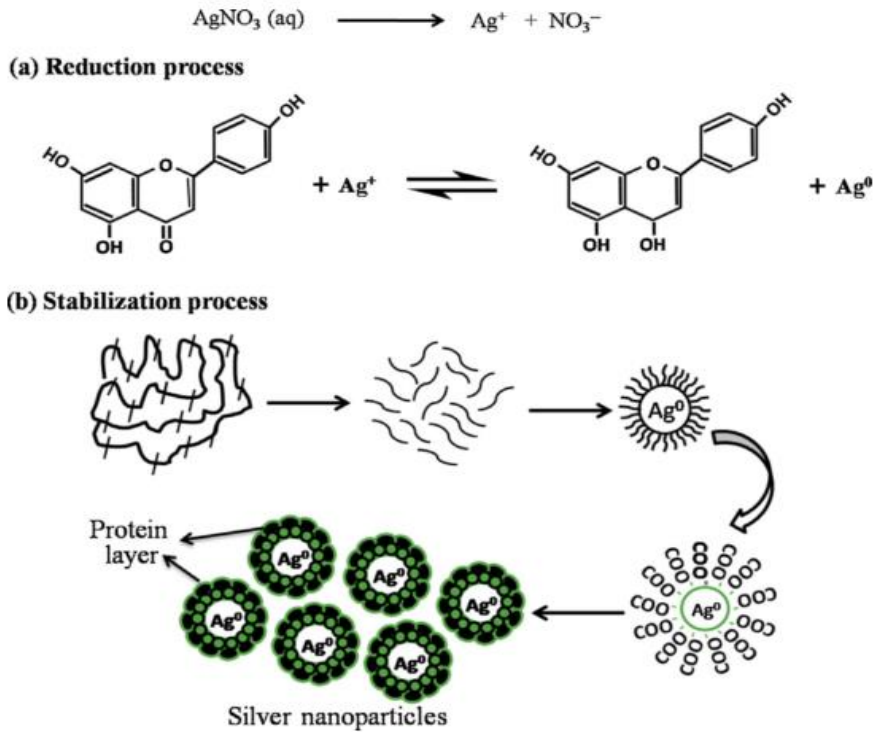

Figure 2.3: The mechanism of AgNPs formation from (a): flavonoids and (b): protein from plant extract (Ajitha *et al.,* 2015).

Unlike using chemically synthesis, it requires an added stabiliser to avoid agglomeration (Ghaffari-moghaddam *et al.,* 2014). Compared to the plant extract, it can act as stabiliser in the process of AgNPs synthesis. When the peptide bonds in a protein molecule break, carboxylate acts as a surfactant to form a layer on the nanoparticles, thus stabilise the AgNPs (Figure 2.3) (Ajitha *et al.,* 2015). AgNPs have

been directly synthesised using amino acid such as aniline (Khan *et al.,* 2017), tyrosine and tryptophan (Shankar and Rhim 2015). Amino acid contains $–NH_2$, -COOH and –R group that act as reducing agents in the process of AgNPs synthesis (Matos and Courrol 2017). Matos and Courrol (2017) used cystine, histidine, methionine, tyrosine and tryptophan while Babu *et al.* (2017) used tyrosine on *in vitro* cytotoxicity against mouse fibroblast (3T3) cells. Tyrosine with –OH group donates the electron for the reduction of Ag^+ to Ag^0. Due to complexity of the plant, the mechanism of AgNPs synthesised is yet to be understood. Therefore, further evaluation is needed to explore the actual mechanism of AgNPs synthesis.

2.3 The Plant: *Clinacanthus nutans* (*C. nutans*)

C. nutans (Figure 2.1) is a small shrub belongs to the *Acanthaceae* family. They are commonly found in tropical Asian countries, mainly Malaysia, Thailand and Indonesia (Roeslan *et al.,* 2012; Shim *et al.,* 2013). *C. nutans* is commonly known as 'Belalai gajah' in Malaysia, Phaya yo or Phaya plongtong in Thailand and Dandang gendis in Indonesia (Tuntiwachwuttikul *et al.,* 2004; Roeslan *et al.,* 2012; Shim *et al.,* 2013). The taxonomy of *C. nutans* is shown in Table 2.4.

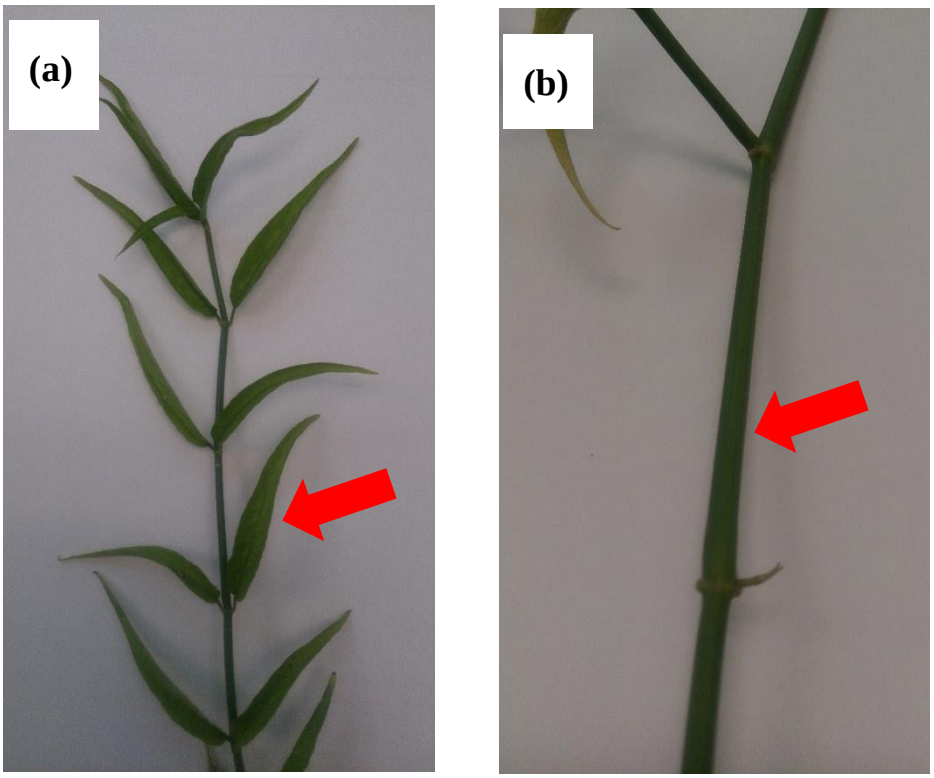

Figure 2.4: *C. nutans* (a): leaves and (b): stems

Table 2.2: The taxonomy of *C. nutans* plant

Kingdom	Plantae
Phylum	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Lamiales*
Family	*Acanthaceae*
Genus	*Clinacanthus*
Species	*nutans*

2.3.1 Bioactivities of *C. nutans*

C. nutans is widely used and possesses promising medicinal properties for human diseases. It has been extensively studied for various applications. Traditionally, *C. nutans* have been used to treat herpes infection, skin rashes, burn, fever and snakebite

(Sangkitporn *et al.,* 1995; Zulkipli *et al.,* 2017). In the early studies, *C. nutans* leaf extracts have been reported to possess anti-viral activity on varicella-zoster virus (Thawaranantha *et al.,* 1992). Direkbusarakom *et al.* (1998) reported the effects of ethanol extract of *C. nutans* leaves inhibited yellow-head rhabdovirus (YRV) in black tiger shrimp (*Panaeus monodon*) at 1 µg/mL.

The antimicrobial activity have been studied on *C. nutans* extract. Arullappan *et al.* (2014) reported that the ethyl acetate fraction of *C. nutans* leaf extract possessed antimicrobial activity against *Bacillus cereus, E. coli, Salmonella enterica typhimurium* and *Candida albican* with minimum inhibitory concentration (MIC) at 1.39 mg/mL. While, the methanolic leaf extract of C. *nutans* possess significant (p<0.05) antimicrobial effects with MIC value of 12.5 mg/mL against *Staphylococcus aureus* and *E. coli* (Yang *et al.,* 2013). However, insignificant antimicrobial activity was shown against acne-inducing bacteria even at high concentration (Chomnawanga *et al.* 2005)

The ethanol extract of *C. nutans* leaves has been reported to possess antioxidant activity based on the DPPH assay (Pannangpetch *et al.,* 2007). Studies by Yong *et al.* (2013) indicated that chloroform extract of *C. nutans* leaves showed higher antioxidant activity compared to methanol and aqueous extracts. Study conducted by Lee *et al.* (2014) on methanol extract of stems *C. nutans* showed higher antioxidant compared to methanol extract of leaves *C. nutans.* In other studies, leaves and stems of petroleum ether, ethyl acetate and methanol extracts reported 70% of DPPH radical scavenging activity within the concentrations of 4-10 mg/mL (Arullappan *et al.,* 2014). Wong *et al.* (2014) reported that aqueous extract with the concentration of 16 mg/mL was

needed to reach 60% of the DPPH radical scavenging activity. Table 2.3 shows the *C. nutans* extract with antioxidant activity.

Cases have been reported that cancer patients in Malaysia (Farooqui *et al.,* 2016) and Singapore (Siew *et al.,* 2014; Poonthananiwatkul *et al.,* 2015) hospitals consumed *C. nutans* as complementary therapy to slow down the cancer progression. Nevertheless, the adjunct cancer therapy needs to be verified with pre-clinical and clinical studies before it is ready for consumption. Based on the *in vitro* anti-cancer studies, chloroform extract of *C. nutans* leaves showed anti-proliferative effect against human erythroleukemia (K-562) and human Burkitt's lymphoma (Raji) cell lines (Yong *et al.,* 2013). Also, the methanol, chloroform and aqueous extract of leaf *C. nutans* (3.125-100 µg/mL) did not show any cytotoxic activity on normal cell (HUVEC cells) (Yong *et al.,* 2013). Cytotoxicity effect was exhibited in petroleum ether extract on HeLa cell lines (leaves, IC_{50} 20 µg/mL and stems, 28 µg/mL) after 24 hr incubation, while after 72 hr on incubation against K-562 with IC_{50} values of 18 and 20 µg/mL, respectively (Arullappan *et al.,* 2014). Studies also showed that leaf (Sulaiman *et al.,* 2015) and root (Teoh *et al.,* 2017) of ethyl acetate extract possessed antiproliferative activity against MCF-7. Further to this, polysaccharide-peptide complex (CNP-1-2) has been isolated from the hot aqueous extraction of the leaves and reported to have antiproliferative activity against human gastric cancer cells (SGC-7901) (Huang *et al.,* 2016).

In *in vivo* study, ethanolic leaf extract of *C. nutans* reduced the hepatoma size in mice with dose dependent manner (8.2 % and 58.6 % inhibition at doses of 3 and 10 mg/kg), which was higher compared to fluorouracil (positive control, 37.1% inhibition at 20 mg/kg) for treatment with 10 mg/kg. Apart from that, *C. nutans* also has shown various

bioactivities such as anti-inflammatory (Wanikiat *et al.*, 2008), antidiabetic (Khoo *et al.*, 2015), anti-papillomavirus (Sookmai *et al.*, 2011), herpes simplex virus type-1 and type-2 (Thongchai *et al.*, 2008; Vachirayonstien *et al.*, 2010; Kunsorn *et al.*, 2013). *C. nutans* have been studied within various biological applications, however there still no studied conducted for synthesised AgNPs.

2.3.2 Phytochemical

Phytochemical are secondary metabolites act as active compound in plants. The most common bioactive compounds in plants are alkaloids, flavonoids, tannins and phenolic compounds (Harborne 1985; Karthik *et al*., 2014). The phytochemicals obtained from extraction of plant material such as isoflavones and organic acid can act as natural reducing agent as substitute of chemical reducing agent (J. Lee *et al*., 2014).

Phenolic is one of the secondary metabolites that give the taste, flavour and healthy properties in a plant (Ghasemzadeh and Ghasemzadeh 2011). In addition, flavonoid, which is the subgroup of the phenolic compounds also common in plants. Both phenolic and flavonoid are very well known for the antioxidant (Sulaiman *et al*., 2015), anticancer (Inbathamizh, Ponnu and Mary, 2012) and antimicrobial activity (Yang *et al*., 2013)

2.3.3 Chemical constituents of *C. nutans*

Several bioactive compounds from *C. nutans* extract have been isolated and studied. Lupeol and β-sitosterol have been isolated from the stem and leaves extracts of *C. nutans* (Dampawan *et al.,* 1977) besides isoorientin, orientin, isovitexin, schaftoside and vitexin (Figure 2.5) (Teshima *et al.,* 1997; Chelyn *et al.,* 2014). Other bioactive groups also have been isolated from *C. nutans* such as sulphur-containing glucosides and chlorophyll derivatives (Teshima *et al.,* 1998; Ayudhya *et al.,* 2001; Sakdarat *et al.,* 2008). Saponin, phenolics, flavonoids, diterpenes and phytosterols were identified in methanol extract of *C. nutans* leaves (Yang *et al.,* 2013). Gas chromatography-mass spectra analysis conducted by Yong *et al.* (2013) on chloroform leaf extract identified fourteen compounds with 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester as the major compound.

Sulphur-containing compounds such as clinamides A, clinamides B, clinamides C, 2-cis-entadamide A and entadamide A were also isolated from *C. nutans* (Figure 2.6). LC-MS-MS analysis of ethanolic extract identified shaftoside, orientin, vitexin, isoorientin, isovitexin and 6,8-apigenin-C-α-L-pyranarabinoside (Huang *et al.,* 2015). A novel polysaccharide peptide complex composing of rhamnose, arabinose, mannose, glucose and galactose was isolated from the leaves extract of *C. nutans* (Huang *et al.,* 2016). Apart from leaves extract, ethyl acetate root extract yielded phytosterols and terpenoids from GC-MS analysis (Teoh *et al.,* 2017). Other parts like stems extract possess phenolic acid, stigmasterol, terpenoid, inositol, cyclitol, and fatty acid by GC-Q-TOF-MS (Alam *et al.,* 2017).

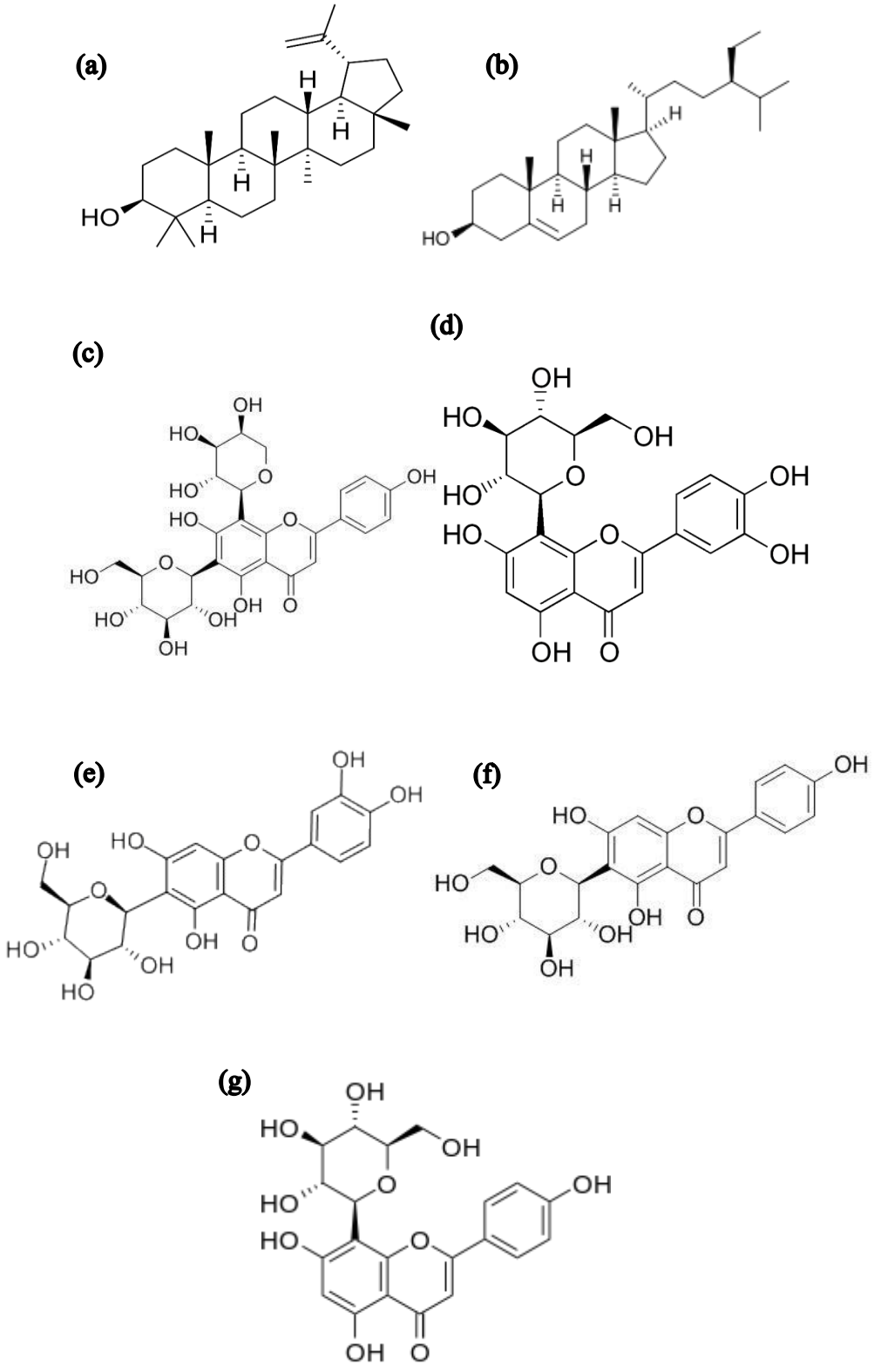

Figure 2.5: Bioactive compounds identified from *C. nutans* (a): lupeol, (b): β-sitosterol, (c): schaftoside, (d): orientin, (e): isoorientin, (f): isovitexin and (g): vitexin.

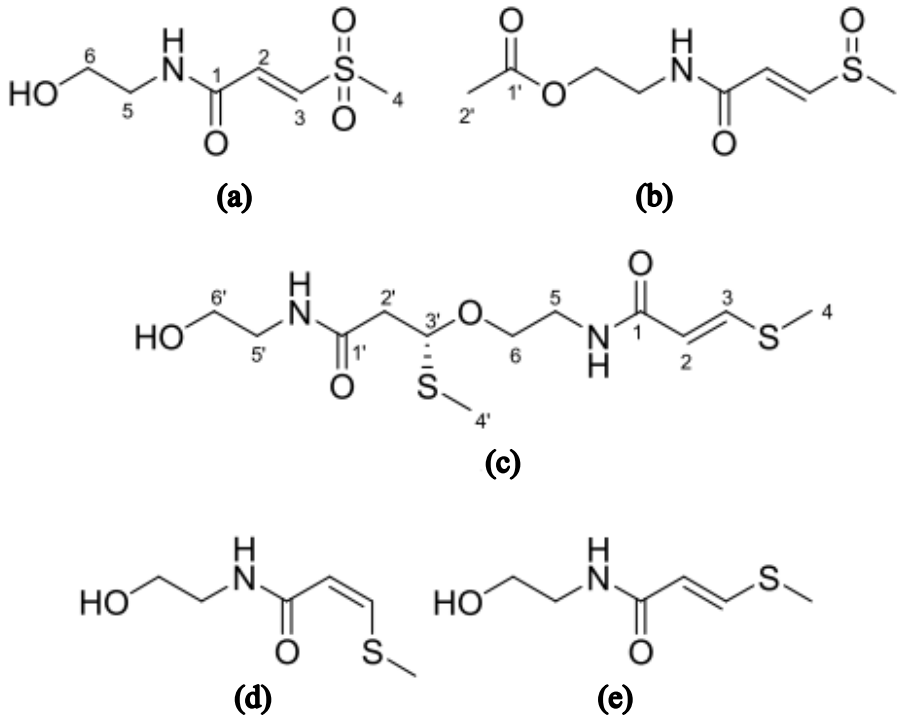

Figure 2.6: Sulphur-containing compounds identified from *C. nutans* (a): clinamides A, (b): clinamides B, (c): clinamides C, (d): 2-cis-entadamide A and (e): entadamide A.

2.4 Application of silver nanoparticles

2.4.1(a) AgNPs as antimicrobial agent

Antibacterial activity is either bacteriostatic or bactericidal. Bacteriostatic means inhibition of microbial growth while bactericidal means direct killing of the bacteria. There are various antimicrobial mechanisms such as inhibition of cell wall, inhibition of cell membrane function, inhibition of protein synthesis and inhibition of nucleic acid synthesis. AgNPs have gained interest in research and known to be an alternative antimicrobial agent (Kim *et al.,* 2007). AgNPs act in a broad spectrum against gram positive, gram negative bacteria as well as multi-resistant bacteria (Rai *et al.,* 2009; Lara *et al.,* 2010). Due to current medication with multi-resistant, pathogenic

microorganism has become a major problem. Therefore there is essential to develop a new antimicrobial agent.

There are many research on AgNPs due to their antimicrobial effects (Patil and Kim 2016) for different purposes such as against food pathogens (Antony *et al.*, 2016). Besides, for household; silver-treated cotton fabric showed antibacterial effects against *S. aureus*, *E. coli* and fungus (Zhang *et al.* 2009). The washed fabric showed reduction in fungus and useful in hospitals (Jung *et al.*, 2007). In medical, AgNPs are coated on bacterial cellulose (Maneerung *et al.*, 2008), cotton fabric (Hebeish *et al.*, 2014) and gelatine nanofibers (Xu and Zhou, 2009) as wound dressing to provide better healing. AgNPs from different parts of plant have been used for their antimicrobial activities against various type of microbes (Table 2.3).

Table 2.3 AgNPs with antimicrobial activities

Plant species	Test microorganisms	Method	References
Bergenia ciliata (whole plant)	*M. luteus, S. aureus, B. bronchiseptica, E. aerogens, A. fumigatus, F. solani, A. niger* and *A.flavus*	Disc diffusion	(Phull *et al.*, 2016)
Azadirachta indica (leaf)	*E. coli* and *S. aureus*	Disc diffusion	(Ahmed, Saifullah, *et al.*, 2016)
Artemisia marschalliana (Sprengel aerial)	*S.aureus, B. cereus, A. baumannii* and *P. aeruginosa*	Disc diffusion	(Salehi *et al.*, 2016)
Coffea arabica (seed)	*S. aureus* and *E. coli*	Well diffusion	(Dhand *et al.*, 2016)
C. roxburghii (leaf)	*A. niger, A. fumigatus, A. flavus, Penicillium sp., C. albicans, R. solani, F. oxysporum and Curvularia sp*	Well diffusion	(Balashanmugam *et al.*, 2016)
Vigna radiata (seed)	*E. coli and S. aureus*	Well diffusion	(Choudhary *et al.*, 2015)
Momordica charantia (leaf)	*E. coli, Pseudomonas* spp., *Bacillus* spp., *A. niger, A. flavus* and *Penicillium* spp.	Well diffusion	(Ajitha *et al.*, 2015)
Vaccinium macrocarpon (Cranberry fruits)	*S. aureus, P. aeruginosa, C.albican*, methicillin-resistant *S. aureus* and multidrug resistant *P.aeruginosa*	Broth microdilution and killing curve	(Ashour *et al.*, 2015)

Table 2.3: Continued

Tagetes erecta (marigold flower)	*S. aureus, B.cereus, E. coli, P.aeruginosa, C. albican, C.glabarata* and *C. neoformans*	Disc diffusion	(Padalia *et al.,* 2015)
Withania somnifera (whole plant)	*E. coli, P.aeruginosa* and *A. tumefaciens*	Disc diffusion	(Marslin *et al.,* 2015)
Skimmia laureola (leaf)	*E. coli, K. pneumoniae, P. aeruginosa, P. vulgaris* and *S. aureus*	Disc diffusion	(Ahmed *et al.,* 2015)
Tea (leaf)	*E. coli*	Disc diffusion	(Sun *et al.,* 2014)
Piper longum (fruits)	*B. subtilis, B. cereus, S. aureus* and *P. aeruginosa*	Disc diffusion and growth curve studies	(Reddy *et al.,* 2014)
Phyllanthus amarus (whole plant)	*P. aeruginosa*	Disc diffusion	(Singh *et al.,* 2014)
Acacia leucophloea (bark)	*S. aureus, B. cereus, L. monocytogenes* and *S. flexneri*	Well diffusion	(Murugan *et al.,* 2014)
Ficus sycomorus (leaf and latex)	*S. typhimurium, S. typhi, S. flexneri, E.faecalis, E. cloacae, E. aerogenes, P. aeruginosa,, K. pneumoniae, E. coli* and *methicillin-resistant S. aureus*	Disc diffusion	(Salem *et al.,* 2014)
Mimusops elengi, Linn (leaf)	*K. pneumoniae, M. luteus* and *S. aureus*	Disc diffusion	(Prakash *et al.,* 2013)

2.4.1(a) Antimicrobial mechanism of AgNPs

The mechanism of AgNPs on antimicrobial activity is not fully known. However, there are various reported theories on the mechanism of action to cause the antimicrobial effect (Marambio-Jones and Hoek 2010). AgNPs are believed to kill the cell by damaging the membrane after being attached to it. Based on the result by transmission electron microscopy (TEM), AgNPs interact on the cell membrane and penetrate into the bacteria cell. This is further tested by using *E. coli* treated with 50 μg/mL of AgNPs completely destroy the membranes (Sondi and Salopek-sondi 2004; Li *et al.,* 2010) (Figure 2.7). Further this, AgNPs are found mobilising into the inner membrane of *P. aeruginosa*, claiming that AgNPs are also working on the inner side of the bacteria cell (Figure 2.8) (Xu *et al.*, 2004; Morones *et al.*, 2005).

The damage of cell membrane can be due to small size of AgNPs contributing to high surface area to volume (Raza *et al.*, 2016). Size affects the membrane permeability and cell destruction. A size-controlled AgNPs with 5 nm showed the lowest minimum inhibitory concentration compared to 100 nm against *E.coli*, *B. subtilis* and *S. aureus* (Agnihotri *et al.,* 2014). Apart from size, the shape of AgNPs also influence the antimicrobial activity. According to Pal *et al.* (2007) triangular shape of AgNPs showed higher antimicrobial activity against *E. coli* compared to spherical and rod-shape nanoparticles.