SYNTHESIS AND CHARACTERISATION OF SILVER NANOPARTICLES USING *Vernonia cinerea* AQUEOUS EXTRACT AND THEIR CYTOTOXIC ACTIVITY AGAINST KASUMI-1 CELL LINE

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LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
CO_2	Carbon dioxide
DLS	Dynamic Light Scattering
DMSO	Dimethyl sulphoxide
hr	Hour
min	Minute
ROS	Reactive Oxygen Species
RPMI-1640	Roswell Park Memorial Institute-1640
SPR	Surface Plasmon Resonance
V. cinerea	*Vernonia cinerea*
V. cinerea-AgNPs	*Vernonia cinerea* silver nanoparticles
TEM	Transmission Electron Microscope
XRD	X-Ray Diffraction Analysis

LIST OF SYMBOLS / UNITS

%	percentage
g	gram
IC_{50}	Inhibitory Concentration at 50 %
kV	kilovolt
µg	microgram
µL	microlitre
µg/mL	microgram per millilitre
mg	milligram
mL	millilitre
mg/mL	microgram per millilitre
mM	milliMolar
mV	millivolt
M	Molar
nm	nanometer
OD	Optical Density
rpm	revolutions per minute

SINTESIS DAN PENCIRIAN NANOPARTIKEL PERAK MENGGUNAKAN EKSTRAK AKUES POKOK *Vernonia cinerea* DAN AKTIVITI SITOTOKSIK MELAWAN SEL KASUMI-1

ABSTRAK

Nanoteknologi telah mendapat perhatian kerana semakin banyak digunakan dalam beberapa aplikasi seperti diagnosis dan rawatan kanser. Biosintesis nanopartikel perak (AgNPs) menggunakan ekstrak tumbuhan adalah lebih selamat dan jimat kos berbanding teknik kimia dan fizik. Kajian ini menghasilkan AgNPs dengan menggunakan nisbah berbeza iaitu 1:5, 1:10 dan 1:20 di antara ekstrak *V. cinerea* penyelesaian ekstrak dan perak nitrat. Campuran kedua-dua bahan ini telah diinkubasi di dalam air bersuhu 40 ˚C selama 24 jam. Pembentukan AgNPs telah dipantau untuk perubahan warna dan sifat AgNPs telah ditentukan menggunakan UV-Vis spektroskopi diikuti oleh mikroskop elektron penghantaran (TEM) dan analisis potensi zeta. UV-Vis spectra AgNPs telah menunjukkan maksima penyerapan pada 450 nm manakala gambar TEM menunjukkan bentuk AgNPs adalah bulat dengan purata saiz 15.29 nm. Nilai zeta *V. cinerea*-AgNPs ialah – 29.50 mV dengan kawasan puncak intensity 100 %. Keputusan ini menunjukkan bahawa *V. cinerea*-AgNPs adalah agak stabil. Seterusnya, aktiviti proliferatif *V. cinerea*-AgNPs secara *in vitro* telah dikira menggunakan MTS assay. Nilai-nilai IC_{50} *V. cinerea*-AgNPs terhadap sel Kasumi-1 adalah 24.42 µg/mL, 25.39 µg/mL dan 21.49 µg/mL masing- masing pada 24, 48 and 72 jam inkubasi. Sebaliknya, nilai IC_{50} ekstrak *V. cinerea* terhadap sel Kasumi-1 adalah 210.53 µg/mL dan 131.58 µg/mL pada 48 dan 72 jam tetapi tiada kesan sel Kasumi-1 dilihat pada 24 jam inkubasi. Kesimpulannya, hasil kajian menunjukkan bahawa *V. cinerea*-AgNPs mempunyai potensi untuk menghasilkan AgNPs dan boleh digunakan sebagai agen anti-kanser terutama pada sel Kasumi-1.

SYNTHESIS AND CHARACTERISATION OF SILVER NANOPARTICLES USING *Vernonia cinerea* AQUEOUS EXTRACT AND THEIR CYTOTOXIC ACTIVITY AGAINST KASUMI-1 CELL LINES

ABSTRACT

Nanotechnology has gained attention due to their wide applications in various areas such as cancer diagnosis and therapy. Production of silver nanoparticles (AgNPs) using plant extracts is safer and cost-effective compared to chemical and physical methods. In this study, AgNPs were produced using 1:5, 1:10 and 1:20 ratios of *Vernonia cinerea* (*V. cinerea*) aqueous extract and silver nitrate solution. The mixtures were incubated for 24 hour at 40 ˚C water baths. The formation of silver nanoparticles was monitored for colour changes and characterized by UV-Vis spectroscopy followed by transmission electron microscopy (TEM) and zeta potential analyses. The UV-Vis spectrum of AgNPs showed absorption maxima at 450 nm while TEM images showed that the shape of AgNPs is spherical in shape with an average size of 15.29 nm. The zeta value of synthesised *V. cinerea*-AgNPs is – 29.50 mV with a peak area of 100 % intensity. This indicates that the *V. cinerea*-AgNPs are fairly stable. The cytotoxicity activity was tested *in vitro* against Kasumi-1 cell line where the proliferative activity of *V. cinerea*-AgNPs was measured using MTS assay. The IC_{50} values of *V. cinerea*-AgNPs on Kasumi-1 cells were 24.42 µg/mL, 25.39 µg/mL and 21.49 µg/mL at 24, 48 and 72 hours of incubations. On the other hand, the IC_{50} values of *V. cinerea* aqueous extract on Kasumi-1 cells were 210.53 µg/mL and 131.58 µg/mL at 48 and 72 hours respectively but no significant difference on the Kasumi-1 cells at 24 hours incubation. Therefore, it is concluded that *V. cinerea* extract has potential in producing AgNPs extracellularly and used as anticancer agents particularly on Kasumi-1 cells.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nanooncology has been a new breakthrough in the field of research because of its potential in the diagnosis and treatment of cancer (Jacob *et al.*, 2012; Sahayaraj *et al.*, 2014). Nanoscale treatment is preferable than chemotherapeutic drugs because of its high specificity, long shelf life, limited dose requirement, and stability (Chen *et al.*, 2014; Riggio *et al.*, 2011). Metallic nanoparticles such as gold, platinum and silver are extensively used in nanomedicine as in targeted drug delivery systems, invasive imaging techniques and also in the development of engineered organs (Barkalina *et al.*, 2014). Nanoparticles are preferred because of their capability to interact strongly with functional biomolecules and flow easily through cellular barriers. This is all due to their size, solubility, large surface area per volume ratio, and bioavailabilty (Gnanasekar *et al.*, 2014).

Silver is the main particle of interest among other nanoparticles because of its medicinal values and anticancer ability (Mollick *et al.*, 2015). Silver nanoparticles (AgNPs) have been applied in pharmaceutical and biomedical fields as an antimicrobial, cytotoxic, antioxidant and antibacterial agents towards many different types of bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Sahayaraj *et al.*, 2014). Due to their proven antibacterial properties, AgNPs have been widely applied in medical products like catheters, dental fillers, wound dressing and other medical devices (Sangiliyandi *et al.*, 2013b). Unfortunately, the potential of AgNPs as anticancer agent is still new and yet to be discovered.

Production of AgNPs can be achieved through different methods such as chemical and physical methods. However, these methods involve the use of toxic and hazardous chemicals such as sodium borohydride, potassium bitartrate, methoxypolyethylene glycol and hydrazine in their synthesis protocol which will potentially give impact on human health (Sukumaran & Eldho, 2012). According to Durai *et al.* (2014), sodium borohydride is not environmental friendly and expensive. Long term exposure towards sodium borohydride could give effects on the nervous system and lead to difficulty in breathing. Hence, biological methods for AgNPs synthesis using plant, fungi or bacteria have been suggested as one of eco-friendly alternative as compared to chemical synthetic procedures and physical methods (Satyavani *et al.*, 2012). Among these methods, plant-synthesised AgNPs have been shown to be faster in production of AgNPs as compared to microbial synthesis method (Rajan *et al.*, 2015). Besides that, this method is less toxic, low cost as well as environmentally friendly (Aruna *et al.*, 2014).

Previous study done by Khay *et al.* (2012) has shown that that *V. cinerea* aqueous extract has a significant inhibitory effect on HT29 (colon adenocarcinoma) and HepG2 (hepatoma) cancer cell lines. Leukaemia is known to be the seventh most prevalent cancer among adult age and it is also common among children (Meng *et al.*, 2013). National Cancer Registry (NCR) of Malaysia reported that in 2007, 4.1 % from 741 cancer cases are leukaemia where 490 cases which is 66.1 % were among patients of 50 years and above while the rest 251 cases which is 33.9 % were among children aged of 0-14 years old (Zainal & Nor Saleha, 2011). However, there is no report the efficacy of *V. cinerea* aqueous extracts or *V. cinerea*-AgNPs on Kasumi-1 (human acute myeloid leukaemia) cell line *in vitro*. Hence, there is emerging need to develop therapeutic drugs that are biocompatible and effective by seeing the several of limitations and severe side effects of

current anticancer drugs. Thus, this study is initiated to investigate the anticancer effects of *V. cinerea*-AgNPs on Kasumi-1 cancer cell lines.

1.2 Research Objectives

This study embarks on the following objectives:

i. To synthesis and characterise the *V. cinerea*-AgNPs synthesised by *V. cinerea* aqueous extract.

ii. To evaluate the effects of *V. cinerea*-AgNPs on the proliferation of Kasumi-1 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Nanotechnology

Nanotechnology is a vast developing field of nanoscience which is expected to be the latest advancement of technology in recent years. The term nanotechnology can be defined as the creation, exploitation and synthesis of materials for at least one dimension of smaller than 100 nanometers (Gholami-Shabani *et al*., 2014). Nanobiotechnology is a field resulting from the application of nanotechnology in the field of life science technology or biotechnology. Nanobiomedicine is one of the applications of nanobiotechnology that specially relates with fundamental studies in biology as well as its application to the fields of medicine (Jayachandra *et al.*, 2014).

Nanooncology is an important area of nanomedicine and it is related to diagnosis and treatment of cancer. The example of nanooncology applications includes nanoparticles conjugated with monoclonal antibody for detection of cancer and quantum dots to diagnose cancer (Jain, 2010). A nanomaterial has large surface area to volume ratio due to their tiny size and that enables the material to pass through cellular barriers while carrying conjugated compounds such as nucleic acids, antibodies, drugs, probes and proteins (Thakor & Gambhir, 2013).

2.2 Silver Nanoparticles (AgNPs)

2.2.1 Introduction to AgNPs

Silver metal is classified as an inert inorganic metal element that exists naturally and the products from ionisation of silver metal are silver ions (Barillo & Marx, 2014). In recent years, development of nanotechnology has allowed the production of silver metal ion into nanoscale particles with sizes less than 100 nm (Muthu *et al.*, 2010). Catalytic activity, good electrical conductivity and high antibacterial activity are among the unique properties that make AgNPs has potential applications as therapeutic agents (Aruna *et al*., 2014). The nanoscale size of nanoparticles allows them to be chemically active results in the production of reactive oxygen species (ROS) and free radicals leading to inflammation and damage to the cells. Various studies have shown that AgNPs are able to penetrate sarcoma cells, primary neural cells and carcinoma cells and causing oxidative stress and damage to the genetic materials and induce the production of ROS.

AgNPs in vitro anticancer activities has been recorded that they interfere with the proliferation of several cancer cell lines. This include cervical carcinoma cell lines (Karunamoorthy *et al.*, 2014; Panchanathan *et al.*, 2013), human lung carcinoma A549 cell lines (Balaji *et al.*, 2014; Renu *et al.*, 2013), MCF-7 cell lines (Gnanasekar *et al*., 2014; Khatiravan *et al.*, 2014), Dalton's ascites lymphoma (DAL) cell lines (Muthu *et al*., 2010) and human glioblastoma cell lines (Rajan *et al.*, 2015).

2.2.2 Synthesis of AgNPs

The AgNPs can be synthesised by various methods such as chemical and physical methods (Ahmed *et al*., 2015). However, these methods are relatively expensive and require high energy and pressures for the synthesis of nanoparticles (Rajan *et al.*, 2015).

Chemical method is the most commonly applied method for the synthesis of AgNPs using aqueous solution or organic solvents (Kholoud *et al*., 2010). A large amount of nanoparticles can be produced using chemical in a short period of time (Iravani *et al.*, 2014). However, this procedure requires the use of chemicals that are toxic and produced the non-environmentally friendly by-products (Sahayaraj *et al.*, 2014).

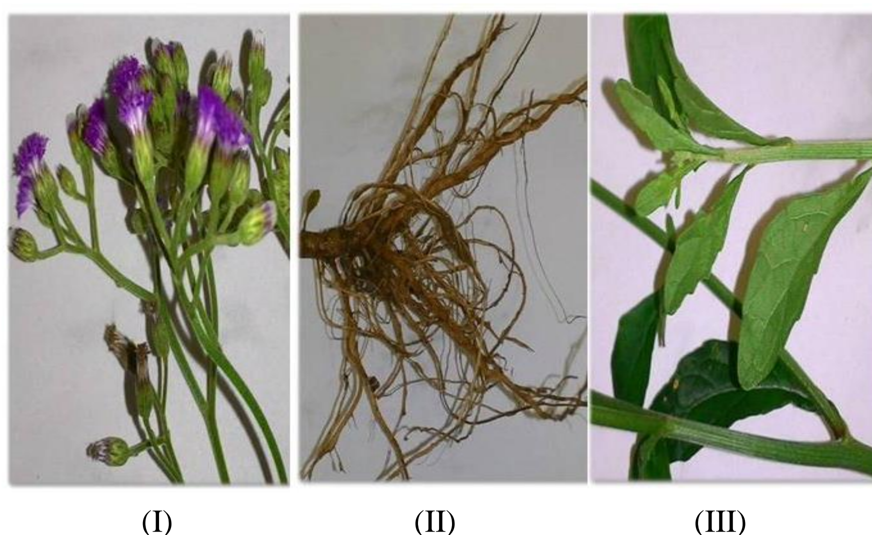
In recent years, biological methods using bacteria, fungi, yeast or plant extracts have been implemented to synthesis AgNPs (Silambarasan & Abraham, 2013). Many research works employed plant leaves extract such as *Ficus benghalensis* (Saxena *et al.*, 2012), *Rosa rugosa* (Dubey *et al.*, 2010), *Stevia re*baudiana (Yilmaz *et al.*, 2011), *Chenopodium album* (Dwidevi & Gopal, 2010), *Nicotiana tobaccum* (Prasad *et al.*, 2010) to produce AgNPs. The production of AgNPs using plant aqueous extracts is preferable because plants are easily available, safe to handle and have many active compounds which potentially act as reducing agent and capping agent. In addition, the cost of culture media and microorganism isolation can be minimised with the use of plant extracts (Prasad, 2014; Sahayaraj *et al*., 2014).

2.3 *Vernonia cinerea*

2.3.1 Introduction to *V. cinerea*

Vernonia is the largest genus in Vernonieae tribe which has about 1000 species of shrubs. It belonged to the family of Asteraceae. The genus was named after an English Botanist, William Vernon in late 1600 (Toyang & Verpoorte, 2013). The species are widely distributed in Africa, Europe and Asia (Old World) and North and South America (New World) but mostly are found in tropical and subtropical regions (Sangeeta & Venkatarathinakumar, 2011). This genus is edible and there are several species that have

been used for food, medicine and industries purposes. For example, folk used *Vernonia* species such as *Vernonia amygdalina* and V*ernonia cinerea* are used for treating diseases. However, there is still lack of studies on *Vernonia cinerea* species compared to *Vernonia amygdalina*.



(I) (II) (III)

Figure 2.1: *Vernonia cinerea* plant collected from Pokok Sena, Kedah. The flowers (I), root (II) and leaves (III) of *V. cinerea* are shown in the above figure.

The *V. cinerea* as shown in Figure 2.1 is an erect annual, branched and slender-stemmed herb (Shelar *et al*., 2014). This plant is normally called as "little ironweed" in English, "joanbeer", "kukshim" in Bengali, "puvamkurunnel" in Malayalam and "sahadevi" in Sanskrit and Hindi. This species can be found mainly in Asian countries such as India, Bangladesh, Sri Lanka and Malay island (Abirami & Rajendran, 2012). Its natural habitat is at the roadside, open waste places and dry grassy areas (Md. Ahsanul *et al*., 2012). It can grow up to 12 – 75 cm high (Shelar *et al*., 2014). The stems are cylindrical solid hairy and basal region of branches greenish-brown (Md. Ahsanul *et al*., 2012). The leaves are dark-green alternate spiral or elliptic with characteristics odour and has bitter

taste. The flowers are white, pink, violet reddish or purple in rounded or flat-topped corymbs (Shelar *et al*., 2014).

2.3.2 Ethno-medicinal uses of *V. cinerea*

V. cinerea is tropically used as traditional herbal medicine in the area where it is historically grown like grassy areas of Southest Asia and Hawaii (Sahayaraj *et al*., 2014). All parts of the *V. cinerea* including roots, leaves and flowers have their own therapeutic values. The whole plants have been recognized in the Ayurvedas (Asha & Abraham, 2015) and use for treatments of intermittent fever, filariasis, blisters, boils and vaginal discharges in Indian Ayurvedic medicine (Arivoli *et al.*, 2011). In traditional Chinese herbal system, the whole plant has been used as a tranquilliser and sedative and for the treatment of fever, cold, sore throat, headache, eczema, mastitis, bleeding, swelling, dysentery, dyspepsia, pus, infection and injuries (Chen *et al*., 2006; Hsu *et al*., 2011; Lai & Wu, 2013).

It is also reported that the plant can be used to treat cancer and various gastrointestinal disorders (Choudhary *et al*., 2013) Abirami and Rajendran (2012) has reported that in traditional medicine, the practitioners use the plant as smoking cessation and relief of asthma, cough, fever, malaria, urinary calculi and arthritis. The whole plant is traditionally combined with small dose of quinine to treat malaria fevers (Asha & Abraham, 2015). The plant also provides remedy for diarrhoea, stomach ache, cough and bronchitis in the decoction form (Rajamurugan *et al*., 2011).

2.3.3 Anticancer activities of *V. cinerea*

V. cinerea have been documented contain phytochemicals flavonoid and sesquiterpene lactones that have important effects on cancer activity. (Patel *et al*., 2010; Toyang & Verpoorte, 2013). According to Pratheeshkumar and Kuttan (2011), Vernolide-A sesquiterpene lactones present in *V. cinerea* is responsible for cytotoxicity and antimetastatic activity towards cancer cells. It has been reported that there are presence of apoptotic bodies, cytoplasmic shrinkage and nuclear condensation when B16F-10 melanoma cells were treated with vernolide-A (Pratheeshkumar & Kuttan, 2011). According to Chadwick *et al*. (2013), sesquiterpene lactones can inhibit over-expression of NF-κB that is usually found in various cancers. The sesquiterpene lactones from *V. cinerea* extract play significant role in regulating inflammatory response and act as an anticancer against various cell types such as colon adenocarcinoma, lung large carcinoma and Hela tumor cell lines (Latha *et al*., 2010; Pratheeshkumar & Kuttan, 2011).

2.4 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is cancer of myeloid blood cells and is characterised by accumulation of immature myeloid progenitors in the bone marrow and peripheral blood (Chen *et al*., 2014). There are several chromosomal translocations present in AML and the most common chromosomal abnormalities is t (8; 21) (q22; q22). The t (8; 21) (q22; q22) translocation resulted from the fusion of the AML1 gene on chromosome 21 with ETO gene on chromosome 8 (Jiang *et al.*, 2013).

In recent years, cytosine arabinoside (Ara-C) based chemotherapy is one of the common treatment options for AML patients (Chen *et al.*, 2014). Since Ara-C has several undesirable side effects, this nucleoside analog cannot be given to patients in high dosage.

Moreover, high dosage chemotherapy is also not suitable for elder patients. Thus, it is very important to develop alternative therapy for the purpose of combating cancer by targeting the genetic aberration of leukemia cells. Plants may provide rich resources for cancer treatment. *V. cinerea* is one of the plant that have been shown to contain active compounds capable of inhibiting the growth of breast cancer (Yuan *et al.*, 2014), However, no research has been done to investigate the effect of *V. cinerea* on the growth of leukemia cancer cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Before use in this study, all glasswares were sterilized and autoclaved for 1 hr at 121 ˚C at a pressure of 100 kPa.

3.2 Methods

3.2.1 Preparation of *V. cinerea* aqueous extract

The whole plant of *V. cinerea* was collected from Pokok Sena, Kedah. The whole plant was washed few times using tab water to remove dirt, rinse with distilled water before cut into small pieces. Then, the samples were dried in an oven (Daihan Labtech, Korea) at 40 ˚C for overnight. After that, the dried samples were grounded into coarse powder using blender and the powder was stored in the Schott bottles at – 20 ˚C until used.

The aqueous extract was prepared by mixing 40 g of coarse powder with 600 mL distilled water in 1000 mL glass beaker. The mixture was boiled for 30 min until the colour of the aqueous solution change from light yellowish to dark green. The extracts were cooled down at room temperature and centrifuged at 2000 rpm (Heraeus multifuge $3SR_{+}$ centrifuge, Thermo Scientific) for 15 min at 25 ˚C to separate supernatant and pellet. Then, the supernatant was collected and filtered using Whatmann filter paper No 1 (Sartorius, Germany) and stored at 4 °C in Schott bottles until further used.

A **B**

Figure 3.1: Coarse powder (A) and a Aqueous extract (B) of whole plant of *V. cinerea*.

3.2.2 Synthesis of *V. cinerea*-AgNPs

The AgNPs were prepared by mixing *V. cinerea* aqueous extract with 1 mM silver nitrate ($AgNO_3$) solution (Qrec, New Zealand). In this study, several incubation periods were selected at 0, 0.5, 1, 2, 4, 9 and 24 hr. The 5 mL of *V. cinerea* aqueous extract were added to $AgNO_3$ solution at 25 mL, 50 mL and 100 mL to produce mixture with ratio of 1:5, 1:10 and 1:20. The mixtures were incubated in a water bath at 40 ˚C for 24 hr until the colour of the solution turned from light yellow to dark brown.

3.2.3 Characterisation of the *V. cinerea*-AgNPs

The *V. cinerea*-AgNPs synthesised at different ratios and incubation time were characterised by Ultraviolet-Visible (UV-Vis) Spectroscopy. Based on the UV-Vis Spectroscopy analysis, samples that are showing maximum absorption peak around 430 – 450 nm were selected and further characterised by Transmission Electron Microscopic (TEM) Analysis and Zeta Potential Analyses.

3.2.3.1 Ultraviolet-Visible (UV-Vis) Spectroscopy Analysis

The formation of *V. cinerea*-AgNPs were monitored by observing the colour changes at 0.5, 1, 2, 4, 9 and 24 hr and the samples were characterised by UV-Vis spectroscopy (Varian Cary 50 UV-Vis Spectrophotometer). The reduction of the silver ions in solution was monitored by measuring the absorbance values of 1 mL of *V. cinerea*-AgNPs solution at wavelength range between 200 – 550 nm. Distilled water was used as blank. Based on result obtained from UV-Vis, samples at 24 hr incubation time was selected for further characterisation. Before analysis, the solution was centrifuged at 6 000 rpm for 15 min. After that, the supernatant was discarded and the pellet was re-suspended with sterile distilled water before dried for 48 hr at 40 ˚C in the oven (Daihan Labtech, Korea).

3.2.3.2 Transmission Electron Microscopic (TEM) Analysis

TEM analysis was carried out to visualise the size and shape of synthesised *V. cinerea*-AgNPs. The sample was sent to School of Biological Sciences, USM for analysis. Briefly,

the sample was suspended in 95 % alcohol followed by 15 min ultrasonication using ultrasonic water bath Elmasonic S 80H (Elma, Germany). Then, a small drop of suspension was placed on a "staining mat". A carbon coated copper grid was inserted into the drop with the coated side grid placing upwards. The thin film suspensions on the grid were dried for 10 min and the grid was removed before screened in Transmission Electron Microscope (Zeiss Libra 120 Transmission Electron Microscope, USA).

3.2.3.3 Zeta Potential Analysis

The synthesised *V. cinerea*-AgNPs were sent to Analytical Lab, School of Chemical Engineering, USM for characterisation of colloidal stability in dispersion using Zetasizer Nano-ZS90 System (Malvern, UK). Stock solution at 0.1 mg/mL was prepared by mixing 1.5 mg of *V. cinerea*-AgNPs powder with 15 mL distilled water. The suspension was placed in ultrasonic bath for 20 min. The suspension was then transferred into the measurement cell. The measurements were performed for three times for each sample.

3.2.4 Cell Culture

The AML cell line, Kasumi-1 cell line was a gift from Professor Norazah Mohd Yusof (USM RUI grant 1001CIPPT/812095). The cells were cultured in complete Roswell Park Memorial Institute-1640 (RPMI 1640) (GIBCO, USA) supplemented with sterile 10 % heat-inactivated fetal bovine serum (GIBCO, USA), 1% of 10 units/mL penicillin-streptomycin (GIBCO, USA) and 1% of 200 mM-L-Glutamine Stock Solution (GIBCO, USA). To culture the cells, frozen cells from – 80 ˚C freezer were thawed immediately in a

water bath at 37 ˚C until the cells becomes semi-fluid. The semi-fluid cells suspension were diluted in pre-warmed complete RPMI-1640 growth medium (GIBCO, USA) and centrifuged at 1800 rpm for 7 min. The DMSO containing supernatant was then discarded and the pellet was re-suspended with pre-warmed complete RPMI-1640 growth medium before transferred into 25 cm^2 tissue culture flask. The cells suspension was then incubated in the CO_2 incubator (Thermo Scientific, USA) at 37 ˚C in 5 % CO_2. The cells were routinely observed under inverted microscope (Carl Zeiss Axiovert 40 CFL, USA) and the culture medium was changed every 3 days. Cells were grown until it reached 70 to 80 % confluency before used for cell proliferation assay.

3.2.5 Cell Proliferation Assay

The cytotoxicity effect of *V. cinerea* aqueous extract and *V. cinerea*-AgNPs on Kasumi-1 cell was determined using Cell Titer 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, USA) which composed of [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carbonxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium/phenazine methosulphate; MTS] and (phenazine methosulfate; PMS). The assay was performed according to manufacturer's protocol. The Kasumi-1 cells at 70 % to 80 % confluency were plated at a density of 5×10^4 cells in each well of 96-well plate. Following that, the cells were treated with *V. cinerea*-AgNPs, *V. cinerea* aqueous extract and $AgNO_3$ solution in a serial dilution manner at 1.96 µg/mL, 3.91 µg/mL, 7.82 µg/mL, 15.63 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL and 500 µg/mL. Untreated cells were used as negative control and camptothecin at 1.25 µg/mL was used as a positive control. Each treatment was performed in triplicate. The cells were incubated in 37 ˚C in a humidified 5 % CO_2 incubator (Thermo

Scientific, USA) for 24, 48 and 72 hr. Following the incubation time interval, 20 µL of mixture of MTS/PMS solution were added to the cultured cells. The cells were then incubated for 3 hr at 37 ˚C in a humidified 5 % CO_2 incubator. The plates were read at 490 nm using an ELISA plate reader (Bio Tek, USA). The half maximal inhibitory concentration (IC_{50}) which means the sample concentration that caused 50% cell death were determined from the graph.

The cell viability (%) was calculated as follows:

$$\text{Percentage Cell Viability (\%)} = \frac{\text{Mean OD of treated cells}}{\text{Mean OD of untreated (control) cells}} \times 100$$

Where, OD is the optical density.

3.2.6 Statistical Analysis

All statistical analyses were performed using Microsoft Excel 2010 and IBM SPSS Statistics 22.0 and expressed as means ± S.D. Treatment effects were determined using one-way ANOVA followed by Bonferroni post-hoc analysis. Values were considered as statistically significant if the *p*- value is less that 0.05 ($p < 0.05$).

CHAPTER 4

RESULTS

4.1 Characterisation of Synthesised *V. cinerea*-AgNPs

4.1.1 UV-Vis Spectroscopy Analysis

The formation of *V. cinerea*-AgNPs can be observed according to the colour changes of the mixture. Figure 4.1 shows the colour changes observed in mixture of *V. cinerea* aqueous extract and $AgNO_3$ at different ratios. For each incubation period, the $AgNO_3$ solution at 25 mL, 50 mL and 100 mL were mixed with 5 mL of *V. cinerea* aqueous extract to produce mixture with ratio of 1:5, 1:10 and 1:20. Upon addition of *V. cinerea* aqueous extract to the $AgNO_3$ solution, the solution changed from colourless to pale yellow. The solution changed to yellowish-brown within 1 hr at 1:20 ratio. In contrast, the solutions with ratio of 1:5 and 1:10 show no colour changes within 1 hr of incubation. At 24 hr of incubation, the solution with ratio of 1:20 turned to dark brown while mixture at ratio 1:5 and 1:10 showed light brown.

Figure 4.2 shows the UV-Vis spectra recorded from the *V. cinerea* aqueous extract reaction at different time intervals. The spectrum showed maximum absorption peak at 450 nm for solution at 1:20 ratio (Figure 4.2 C). However, no distinction peaks were observed for the mixture at ratios of 1:5 and 1:10.

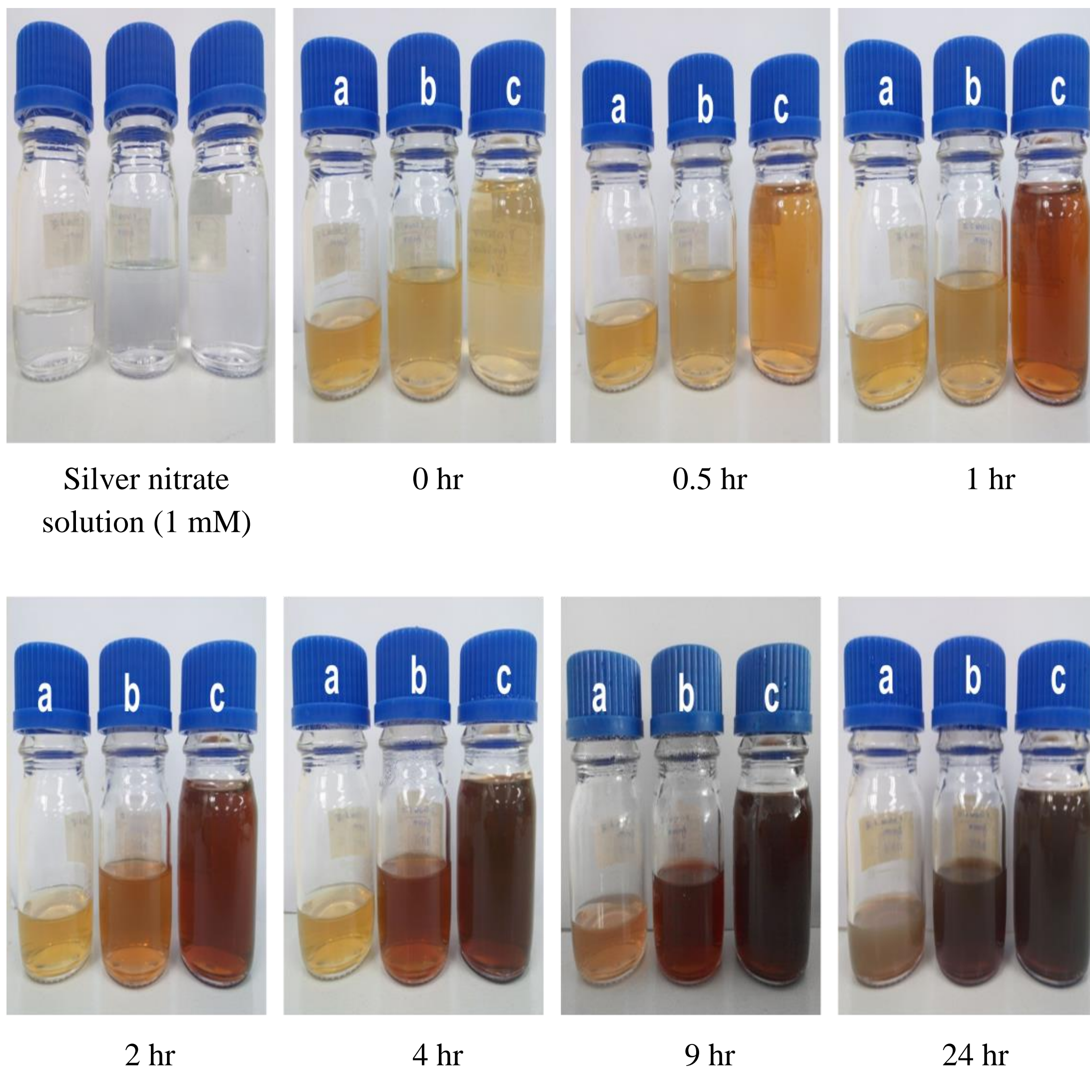


Figure 4.1: Synthesis of *V. cinerea*-AgNPs. The *V. cinerea* aqueous extract was added into $AgNO_3$ solution at (a) 1:5 (b) 1:10 (c) 1:20 ratios. The colour of the mixture changes from pale yellow at 0 hr to dark brown at 24 hr.

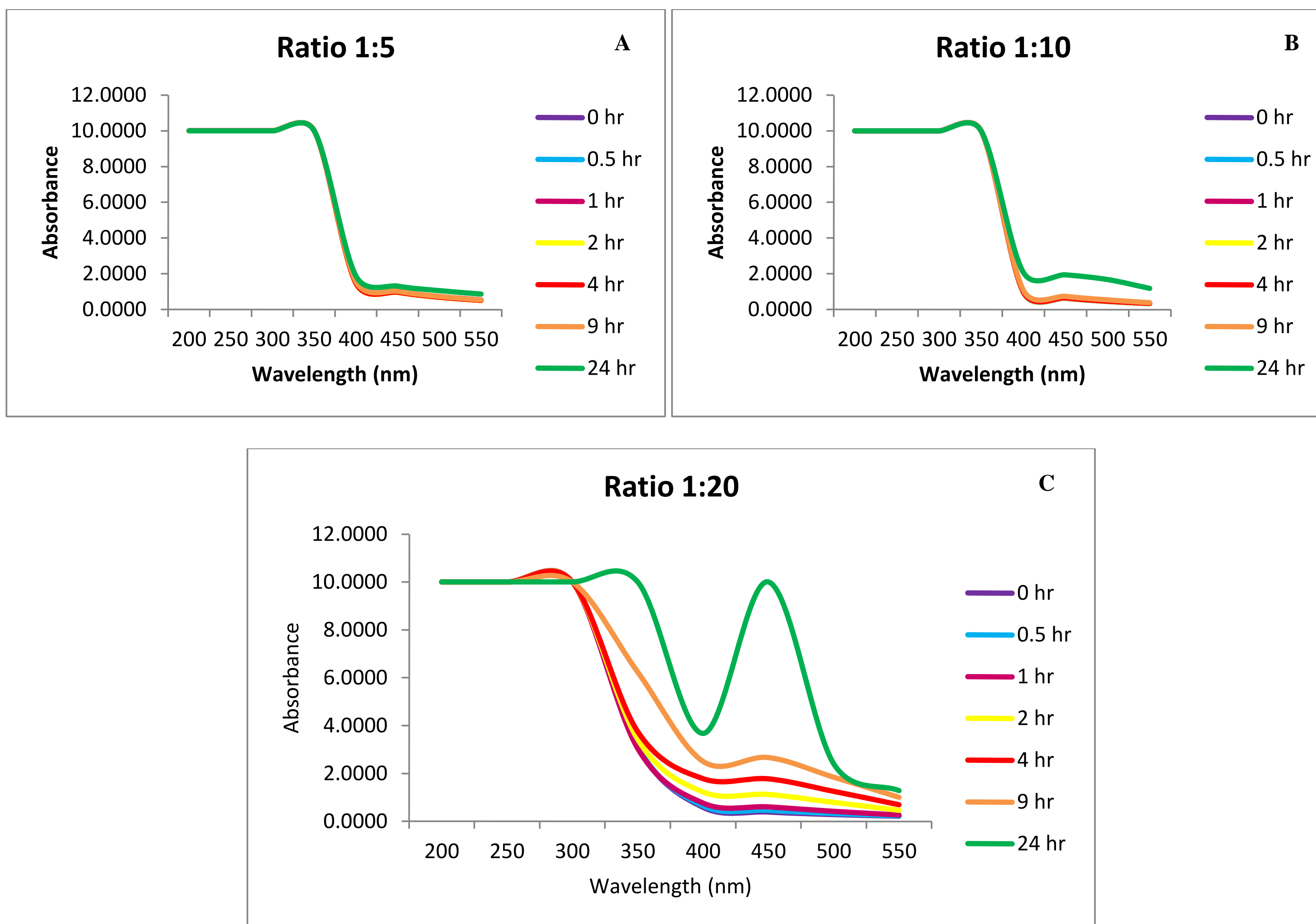


Figure 4.2: UV–Visible spectra recorded for mixture of *V. cinerea* aqueous extract with aqueous silver nitrate at (A) ratio 1:5 (B) ratio 1:10 (C) ratio 1:20 incubated at different time intervals.

4.1.2 TEM Analysis

The size and shape of *V. cinerea*-AgNPs were determined using TEM. Figure 4.3 (A) and (B) shows the representative images of TEM analysis of *V. cinerea*-AgNPs with most of the AgNPs were roughly circular in shape with smooth edge. The sizes of the particles range from 5.83 nm to 31.44 nm. Figure 4.3 (C) represents the frequency of TEM size distribution calculated by measuring over 50 particles in random fields of TEM view. The particle size ranges from more than 10 to 15 nm (>10 to 15 nm) was observed at the highest level with 37 % while the particles at more than 20 nm was observed at lowest level with 7 %. The average diameter of AgNPs as observed by TEM was 15.29 ± 6.16 nm.

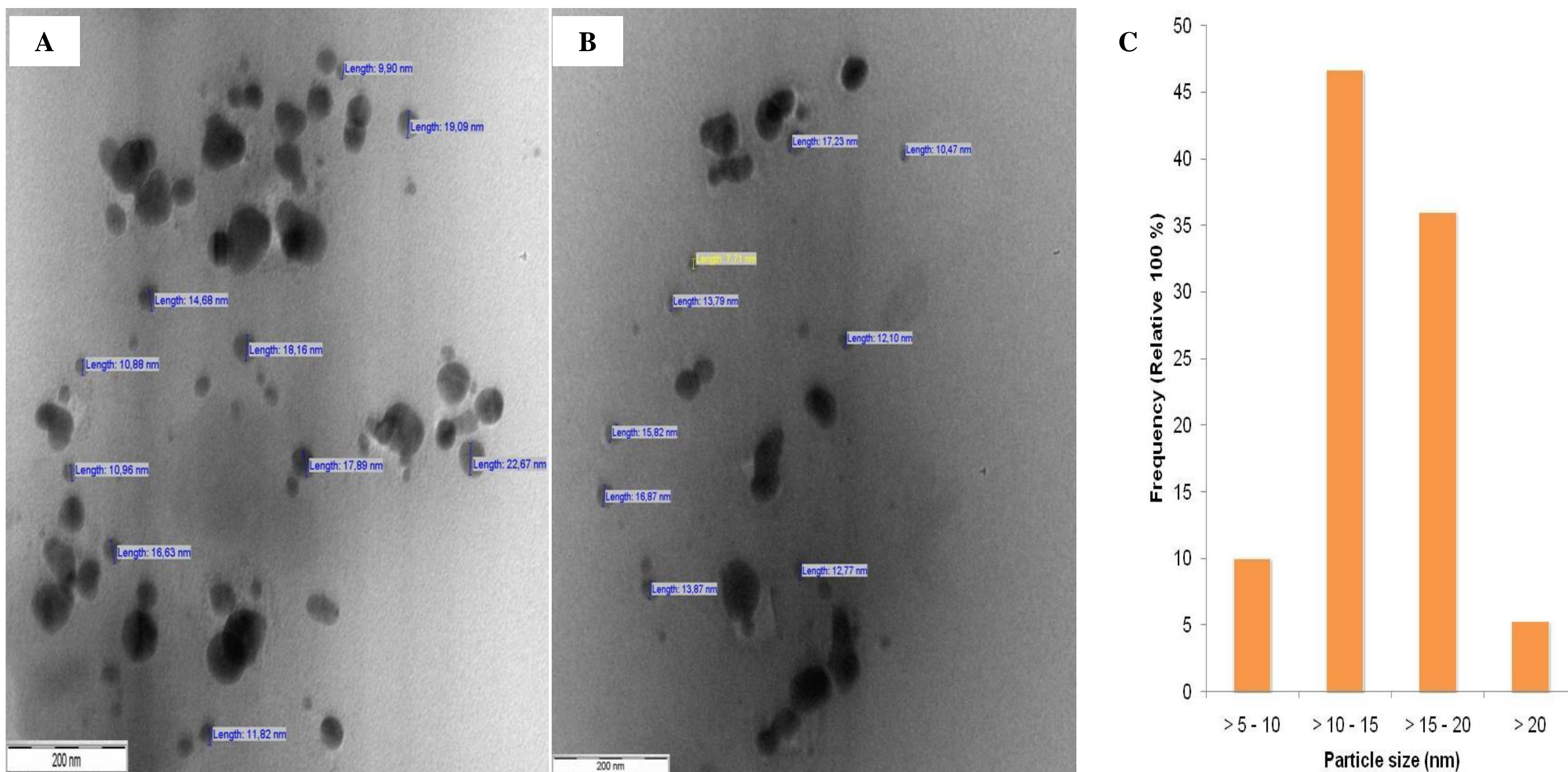


Figure 4.3: Representative images of TEM analysis of *V. cinerea*-AgNPs (A & B) with different particle size ranges from 5.83 nm to 31.44 nm observed at different angles. (C) Frequency of size distribution of *V. cinerea*-AgNPs.

4.1.3 Zeta Potential Analysis

The stability of *V. cinerea*-AgNPs in solution was determined using zeta potential analysis by measuring electrical charges on the surface of *V. cinerea*-AgNPs. As shown in Figure 4.4 and Table 4.1, the zeta potential value of *V. cinerea*-AgNPs was – 29.50 mV with a peak area of 100% intensity.

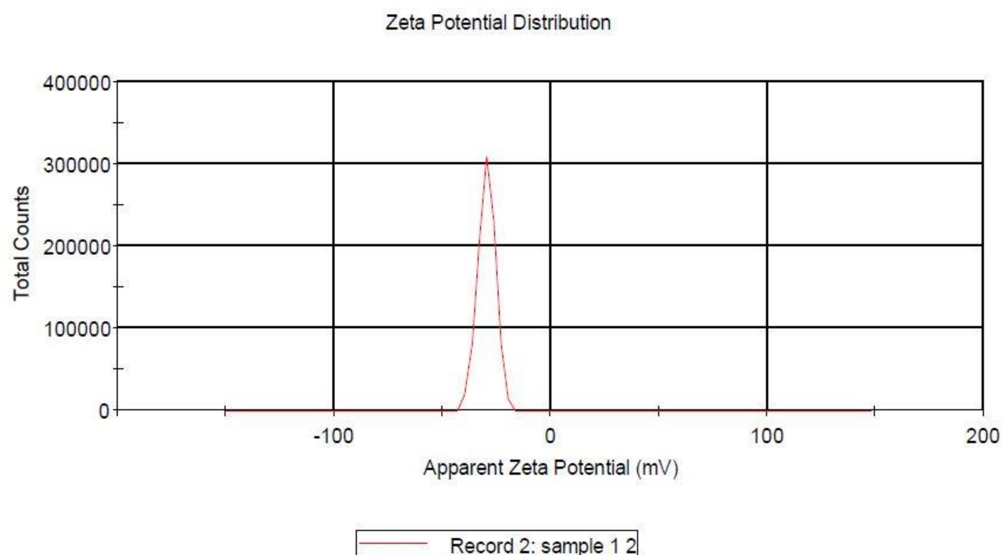


Figure 4.4: Zeta potential distribution of AgNPs synthesised from *V. cinerea* aqueous extract.

Table 4.1: Zeta potential measurement of synthesised *V. cinerea*-AgNPs.

Zeta potential	– 29.50 mV
Zeta deviation	4.07 mV
Polarity	Negative
Conductivity	0.0220 mS/cm

4.2 Proliferative effects of *V. cinerea*-AgNPs on Kasumi-1 cells

The IC_{50} values were 24.42 μg/mL, 25.39 μg/mL and 21.49 μg/mL at 24, 48 and 72 hr of incubations, respectively as shown in Fig. 4.5. Figure 4.6 shows the effects of *V. cinerea*-AgNPs on the proliferation of Kasumi-1 cells. The *V. cinerea*-AgNPs have significantly ($p < 0.05$) inhibited the proliferation of Kasumi-1 cells from the concentration of 31.25 μg/mL to 500 μg/mL at 48 and 72 hr of incubations as compared to untreated cells. On the other hand, at 24 hr of incubations, the growth inhibition was only observed significantly ($p < 0.05$) at concentration ranges from 15.63 μg/mL to 250.00 μg/mL. The lowest percentage of cell viability following the treatment of *V. cinerea*-AgNPs was significantly ($p < 0.05$) observed with approximately 21 % at concentrations of 31.25 μg/mL. The data also revealed that there were no significant differences observed in cells treated with $AgNO_3$ at 24 hr of incubations. Camptothecin was used as a positive control in this study and has significantly ($p < 0.05$) inhibited the cell proliferation at all incubations times.

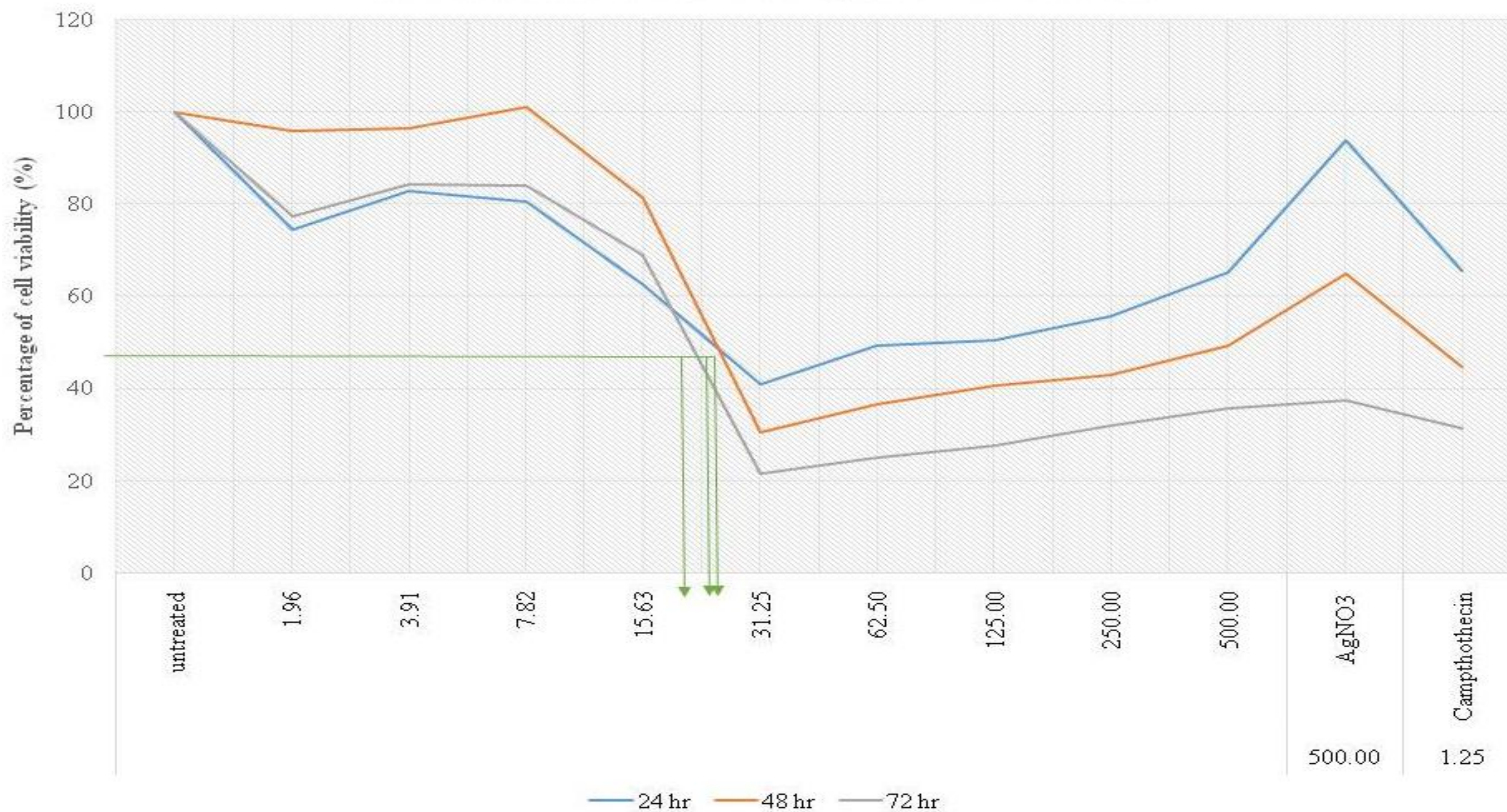


Figure 4.5: Growth inhibitory effects of *V. cinerea*-AgNPs on Kasumi-1 cells. The lines represent the IC_{50} values that inhibit cell proliferation at 24 hr, 48 hr and 72 hr of incubation times.