

SYNTHESIS AND CHARACTERISATION OF SILVER NANOPARTICLES USING *VERNONIA CINEREA* AQUEOUS EXTRACT AND THEIR CYTOTOXICITY ACTIVITY AGAINST KASUMI-1 CELL LINE

Article history

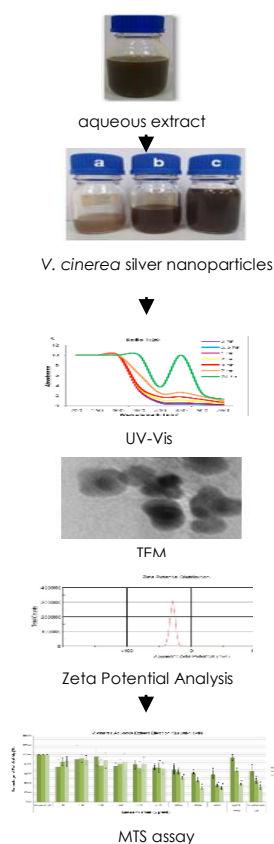
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Graphical Abstract



Abstract

Green synthesis is preferable for the synthesis of silver nanoparticles (AgNPs) due to its rapid and reliable process. Previous studies have demonstrated that active compounds present in *Vernonia cinerea* (*V. cinerea*) are responsible for bioreduction during synthesis of AgNPs. Hence, in this present study, aqueous extract of *V. cinerea* was used to synthesise AgNPs. The *V. cinerea* aqueous extract was added to AgNO_3 solution (25 mL, 50 mL and 100 mL) to produce mixture with ratio of 1:5, 1:10 and 1:20. The formation of *V. cinerea*-AgNPs was monitored for colour changes, and characterised by UV-Vis spectroscopy, transmission electron microscopy (TEM) and zeta potential analyses. The UV-Vis spectrum of *V. cinerea*-AgNPs showed absorption maxima at 450 nm while TEM images showed that the *V. cinerea*-AgNPs were spherical in shape with an average size of 15.29 nm. The zeta value of synthesised *V. cinerea*-AgNPs was -29.50 mV with a peak area of 100 % intensity. The cytotoxicity of *V. cinerea*-AgNPs were tested *in vitro* on Kasumi-1 cells using MTS assay. The MTS assay revealed that the *V. cinerea*-AgNPs with the concentration of 31.25 $\mu\text{g}/\text{mL}$ at 72 hr produced highest cytotoxicity effects on Kasumi-1 cells. These results indicate that AgNPs can be biologically synthesised from *V. cinerea* and has potential to be used as an anticancer agents on acute myeloid leukaemia cell line.

Keywords: Silver nanoparticles; vernonia cinerea; cytotoxicity; kasumi-1

Abstrak

Biosintesis menggunakan tumbuhan adalah pilihan yang baik bagi menghasilkan nanopartikel perak (AgNPs) disebabkan oleh prosesnya yang cepat dan dipercayai. Kajian terdahulu menunjukkan bahawa sebatian aktif di dalam *Vernonia cinerea* (*V. cinerea*) berperanan sebagai bioreduksi semasa proses penghasilan AgNPs. Oleh itu, dalam kajian ini, ekstrak akua *V. cinerea* telah digunakan untuk menghasilkan AgNPs. Ekstrak akua *V. cinerea* ditambahkan kepada larutan AgNO_3 (25 mL, 50 mL dan 100 mL) untuk menghasilkan campuran dengan nisbah 1:5, 1:10 dan 1:20. Pembentukan *V. cinerea*-AgNPs telah dipantau dengan melihat perubahan warna, dan sifatnya dinilai menggunakan spektroskopi UV-Vis, mikroskop elektron penghantaran (TEM) dan analisis potensi zeta. UV-Vis spectra telah menunjukkan maksima penyerapan *V. cinerea*-AgNPs pada 450 nm manakala analisa TEM menunjukkan bentuk *V. cinerea*-AgNPs yang bulat dengan purata saiz 15.29 nm. Nilai zeta *V. cinerea*-AgNPs ialah -29.50 mV dengan kawasan puncak intensiti sebanyak 100 %. Kesitotoksian *V. cinerea*-AgNPs telah diuji secara *in vitro* ke atas sel-sel Kasumi-1 menggunakan ujian MTS. Ujian MTS telah menunjukkan kesan kesitotoksian yang tertinggi terhadap sel-sel Kasumi-1 dengan kepekatan *V. cinerea*-AgNPs sebanyak 31.25 $\mu\text{g}/\text{mL}$ pada 72 jam. Hasil kajian ini menunjukkan bahawa AgNPs boleh

dihasilkan secara biologi menggunakan *V. cinerea* dan berpotensi untuk digunakan sebagai agen anti-kanser terutama pada titisan sel leukemia mieloid terakut.

Kata kunci: Nanopartikel perak; *vernonia cinerea*; kesitotoksian; kasumi-1

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1.0 INTRODUCTION

There are increasing number of cytotoxic drugs developed for cancer treatment. However, these drugs have several undesirable side effects such as genotoxic, carcinogenic and teratogenic effects especially in non-tumour cell [1, 2]. The problems arise from the conventional cancer treatment require scientist to explore a new approach in order to increase the effectiveness of the treatment. Nanotechnology has been a new breakthrough in the field of research due to its wide applications especially in biomedical field [3, 4]. The rapid development of nanotechnology has improved the ability of nanoparticles as medicinal values in diagnosis and treatment of cancer. Due to their unique characteristics, including large surface area, structural properties and long shelf life, nanomaterials has the potential to revolutionise the diagnosis and treatment of several diseases especially in invasive imaging techniques and delivering bioactive agents to biological targets [5].

Gold, platinum and silver nanoparticles have been widely used in products with direct contact to the human body [4, 6]. Among all, silver is the main particle of interest because of its medicinal values and anticancer ability [7]. 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* [4]. Due to their proven antibacterial properties, AgNPs have been widely applied in medical products like catheters, dental fillers, wound dressing and other medical devices [5].

Nanoparticles can be synthesised using 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 which will potentially give negative impact on human health [8]. Previous study has reported that sodium borohydride is not environmental friendly, and long term exposure towards sodium borohydride could affect human nervous system which can lead to breathing difficulty [9]. Hence, biological method for AgNPs synthesis using plant, fungi or bacteria has been suggested as one of eco-friendly alternatives as compared to chemical synthetic procedures and physical methods [10].

AgNPs can be biologically synthesised from bacteria, fungi, yeast and plant extracts [11]. This includes the fungal species such as *filamentous fungus Penicillium*

[12] and bacteria like *Bacillus cereus* [13]. However, synthesis of AgNPs from plant extracts is preferable because plants are easily available, cost-effective and safe to handle [4, 14, 15]. Moreover, the rate of synthesis of AgNPs by plants is faster than microorganism [16, 17]. Plant extract such as *Rosa rugosa* [18], *Chenopodium album* [19], *Nicotiana tobaccum* [20], *Stevia rebaudiana* [21], *Catharantus roseus* [22] and *V. cinerea* [4] have been reported to contain bioreduction agents for the synthesis of AgNPs. According to Sahayaraj *et al.*, *V. cinerea* aqueous extract can be used to produce AgNPs [30].

The *V. cinerea* is an erect annual, branched and slender-stemmed herb can be found mainly in Asian countries such as India, Bangladesh, Sri Lanka and Malay island [23]. The leaves are dark-green alternate spiral or elliptic. The flowers are white, pink, violet reddish or purple in rounded or flat-topped corymbs [24]. The plant is traditionally used for smoking cessation and relief of asthma, cough, fever, malaria, urinary calculi and arthritis [23]. The whole plant is traditionally combined with small dose of quinine to treat malaria fevers [25]. The plant also provides remedy for diarrhea, stomach ache, cough and bronchitis in the decoction form [26]. According to Pratheeshkumar *et al.* Vernolide-A sesquiterpene lactones present in *V. cinerea* is responsible for biological activity such as cytotoxicity and antimetastatic action towards cancer cells [27]. It had been reported that there is presence of apoptotic bodies, cytoplasmic shrinkage and nuclear condensation when B16F-10 melanoma cells treated with vernolide-A [27]. The sesquiterpene lactones from *V. cinerea* extract play significant role in regulating inflammatory response and as an anticancer activity against various cell types such as colon adenocarcinoma, lung large carcinoma and *Hela* tumor cell lines [27]. Despite numerous studies tested on various type of cancer cells, little or no study has conducted on leukemia cancer cells using *V. cinerea*.

In the present study, AgNPs were synthesised through reduction of aqueous extract of *V. cinerea* and characterised by UV-Vis spectroscopy, transmission electron microscopy (TEM) and zeta potential analyses. The synthesised *V. cinerea*-AgNPs were tested on Kasumi-1 cell line to investigate anti-proliferative effects of *V. cinerea*-AgNPs on Kasumi-1 cancer cell line.

2.0 METHODOLOGY

2.1 Preparation of *V. cinerea* Aqueous Extract

The *V. cinerea* was collected from Pokok Sena, Kedah, Malaysia. The whole plant was washed several times using tap water to remove dirt and rinsed with distilled water. Then, they were cut into small pieces and dried in an oven (Daihan Labtech, Korea) at 40 °C overnight [Figure 1 (B)]. The aqueous extract was prepared by mixing 40 g of coarse powder with 600 mL of distilled water in 1000 mL glass beaker. The mixture was boiled for 30 min [Figure 1 (C)]. The extracts were cooled down at room temperature and centrifuged (Heraeus multifuge 3SR+ centrifuge, Thermo Scientific) to separate supernatant and pellet. The supernatant was collected and filtered using Whatmann filter paper No 1. The extract obtained was stored at 4 °C for further use.

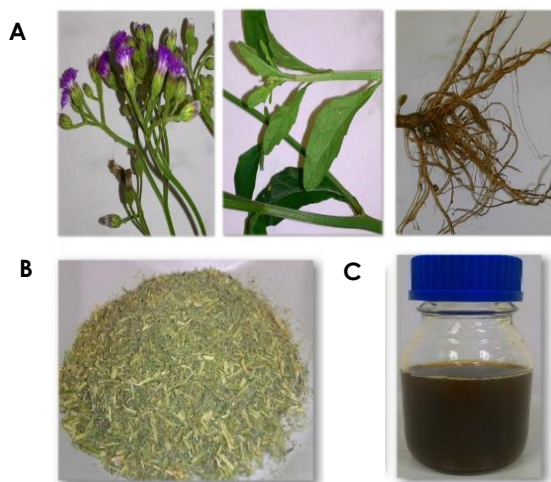


Figure 1 (A) *V. cinerea* consisting of flower, leaves and root (B) Coarse powder and (C) Aqueous extract of whole plant of *V. cinerea*.

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 and different ratios as showed in Table 1. The mixture was incubated in a water bath at 40 °C at different incubation times. The color changes was observed during incubation period.

Table 1 Different ratios and incubation times for the synthesis of *V. cinerea*-AgNPs

<i>V. cinerea</i> aqueous extract (ml)	AgNO_3 (ml)	ratio	incubation time (hr)
5	25	1:50	0.5, 1, 2, 4,
	50	1:10	9,24
	100	1:20	

2.3 Ultraviolet-Visible (UV-Vis) Spectroscopy Analysis

The formation of *V. cinerea*-AgNPs was monitored by observing the colour changes at 0.5, 1, 2, 4, 9 and 24 hr, followed by characterisation using 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 to 550 nm.

2.4 Transmission Electron Microscope (TEM) Analysis

TEM analysis was carried out to visualise the size and shape of synthesised *V. cinerea*-AgNPs. 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 minute and the grid was removed before screening using Transmission Electron Microscope (Zeiss Libra 120 Transmission Electron Microscope, USA).

2.5 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 per sample.

2.6 Cell Proliferation Analysis

The Kasumi-1 cells were plated at a density of 5×10^4 cells per well in a 96-well plate. Next, the cells were treated with the *V. cinerea*-AgNPs, *V. cinerea* aqueous extract and AgNO_3 solution at 500 $\mu\text{g/mL}$, 250 $\mu\text{g/mL}$, 125 $\mu\text{g/mL}$, 62.5 $\mu\text{g/mL}$, 31.25 $\mu\text{g/mL}$, 15.63 $\mu\text{g/mL}$, 7.81 $\mu\text{g/mL}$, 3.91 $\mu\text{g/mL}$ and 1.96 $\mu\text{g/mL}$ in 96-well plates for 72, 48 and 24 hr. Untreated cells were used as negative control while camptothecin at 1.25 $\mu\text{g/mL}$ was used as a positive control. Each treatment was done in triplicate. The cells were incubated in 37 °C in a humidified 5 % CO_2 incubator for 24, 48 and 72 hr. Following the incubation time interval, 20 μL of MTS/PMS mixture 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 absorbance was measured at 490 nm using an ELISA plate reader (Bio Tek, USA). 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. 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:

Percentage of cell viability (%) =

$$\frac{\text{Mean OD of treated cells}}{\text{Mean OD of untreated (control) cells}} \times 100$$

2.7 Statistical Analysis

All statistical analyses were performed using Microsoft Excel 2010 and IBM SPSS Statistics 22.0 and presented as means \pm 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 < 0.05.

3.0 RESULTS AND DISCUSSION

3.1 Visual Observation

Synthesis of *V. cinerea*-AgNPs can be observed according to colour changes of the mixture. Figure 2 shows the colour changes of *V. cinerea* aqueous extract added into AgNO₃ solution at different ratios. After addition of *V. cinerea* aqueous extract to the AgNO₃ solution, the solution changed from colourless to pale yellow. The solution changed to yellowish-brown within 1 hr at ratio 1:20. In contrast, the solutions with ratio of 1:5 and 1:10 show no colour changes after 1 hr of incubation. After 24 hr, the solution with ratio of 1:20 turned to dark brown-greyish indicates the formation of AgNPs resulting from the reduction of Ag⁺ ions into AgNPs.

The observed colour change in the solution is similar to previous report [28]. The colour change occurred due to excitation of surface plasmon resonance (SPR) in AgNPs [28, 29]. The surface of AgNPs is surrounded with free electrons in conduction band and positively charged nuclei. The collective oscillation of electrons of the AgNPs in resonance with the light wave results in the formation of SPR absorption band [30, 31]. This absorption depends on the particle size, dielectric medium and chemical surroundings [30]. Therefore, the findings are parallel to previous study that indicated that *V. cinerea* aqueous extract has potential to synthesise AgNPs [4].

3.2 UV-Vis Spectroscopy Analysis

UV-Vis spectroscopy is one of the characterisation methods to determine nanoparticles formation. Their properties and sizes based on SPR band exhibit at different wavelength. Figure 3 shows the UV-Vis spectra recorded from the *V. cinerea* aqueous extract with different ratios of AgNO₃ solution at different time intervals of reaction. The spectrum showed maximum absorption sharp peak at 450 nm at incubation time of 24 hr for the solution with ratio 1:20 [Figure 3 (C)]. The highest absorbance peaks in the UV-Vis spectra

obtained indicates the level of formation of AgNPs. However, no distinction peaks were observed for the solutions at ratios 1:5 and 1:10 [Figure 3 (A, B)]. It is found that intensity of SPR increases along with the incubation times. Similar results were previously reported by which resonance peak of AgNPs appears at wavelength in the range 400 to 450 nm [32, 33]. Another study reported that the maximum absorbance of AgNPs synthesised using *V. cinerea* aqueous extract was approximately at 430 nm [4].

It has been suggested that the increase of intensity could be either due to the increased number of nanoparticles formed or due to generation of silver nano crystals as a result of reduction of Ag⁺ ions present by the phytochemicals present in *V. cinerea* whole plant extract [34]. Some AgNPs dispersions exhibited unique peaks because of the excitation of SPR of interband transitions [4].

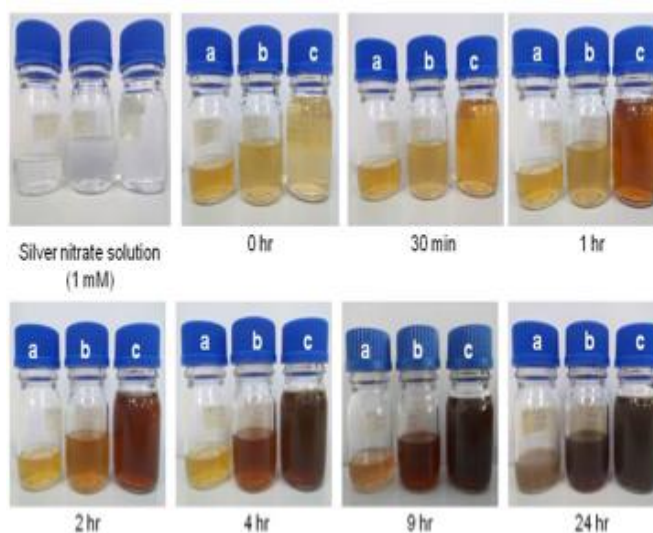


Figure 2 Synthesis of *V. cinerea*-AgNPs. The *V. cinerea* aqueous extract was added into AgNO₃ solution at (a) 1:5 (b) 1:10 (c) 1:20 ratios. The colour of the mixture changes from pale yellow at 0 hr to dark brown-greyish at 24 hr.

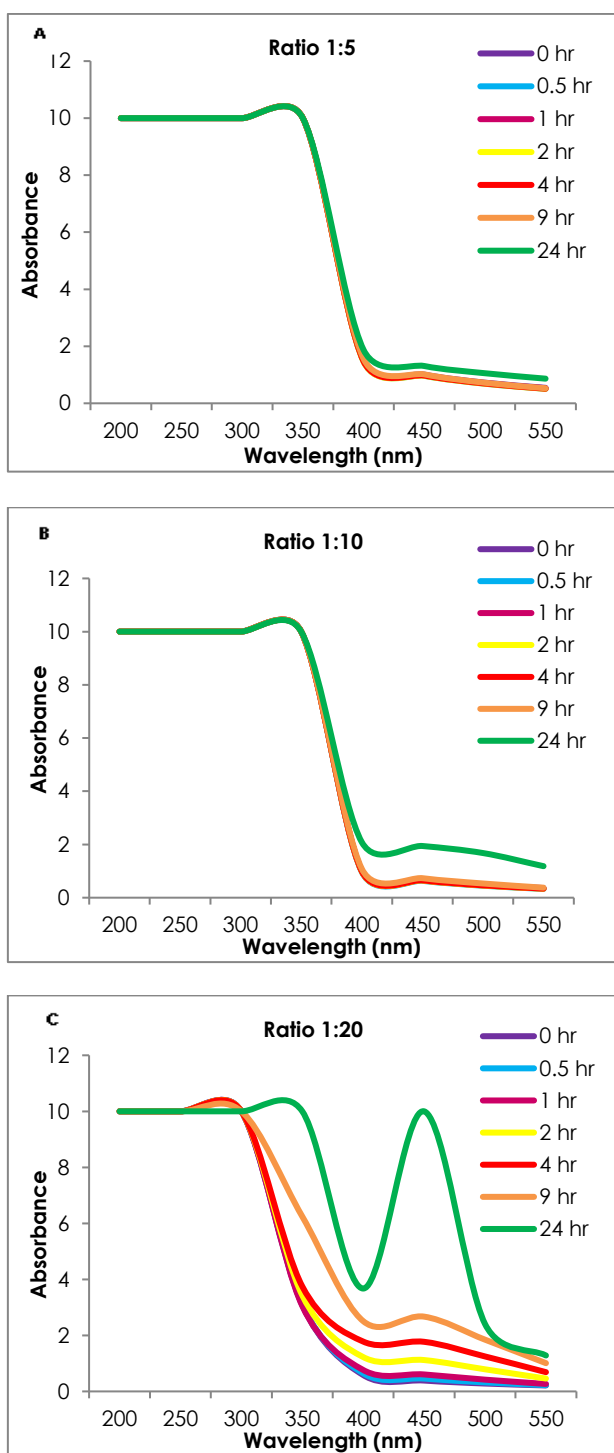


Figure 3 UV-Visible spectra recorded for mixture of *V. cinerea* aqueous extract with aqueous $AgNO_3$ at (A) ratio 1:5 (B) ratio 1:10 (C) ratio 1:20 incubated at different time intervals.

3.3 TEM Analysis

Size and shape of *V. cinerea*-AgNPs were investigated using TEM analysis. Figure 4 (A) and (B) show TEM images of synthesised *V. cinerea*-AgNPs are spherical in shape. The AgNPs are in the form of agglomerate or single

particles. The single particles of *V. cinerea*-AgNPs were selected to measure the size of particle. Figure 4 (C) represents the frequency of TEM size distribution of *V. cinerea*-AgNPs ranging from 5.83 nm to 53.50 nm. TEM average diameter was calculated by measuring 51 particles of AgNPs in random area of TEM view. The average TEM diameter of *V. cinerea*-AgNPs was 15.29 ± 6.16 (mean \pm SD) nm. Similarly, Sahayaraj *et. al.* also reported that they successfully synthesised AgNPs in spherical shape with the size range 5 to 50 nm using *V. cinerea* extract [4].

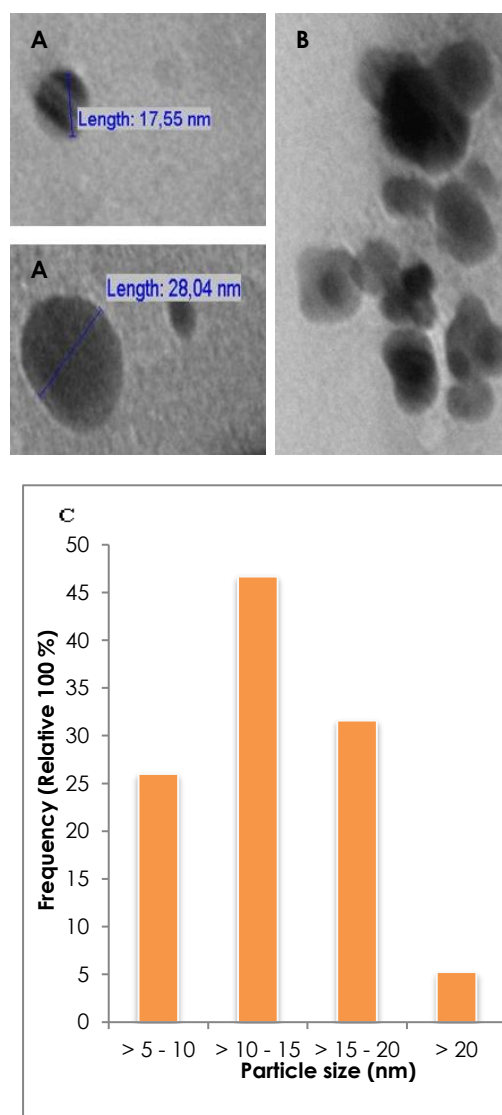


Figure 4 (A) Single particles of *V. cinerea*-AgNPs analysed by TEM (B) Agglomerate particles of *V. cinerea*-AgNPs analysed by TEM (C) Frequency of size distribution of *V. cinerea*-AgNPs.

3.4 Zeta Potential Analysis

The stability of *V. cinerea*-AgNPs in solution was determined using zeta potential analysis by measuring electrical charges on surface of *V. cinerea*-AgNPs. As shown in Figure 5 and Table 2, the *V. cinerea*-AgNPs obtained possess a negative zeta potential value. According to Padalia *et al.*, value of the zeta potential is higher than + 30 mV or lesser than - 30 mV indicated as stable nano suspension [35]. The metal nanoparticles with higher zeta potential produce strong repulsive force between particles, which prevent from

aggregation [36]. As shown in the present study, the zeta potential value of *V. cinerea*-AgNPs was - 29.50 mV with a peak area of 100% intensity. So, this result clearly indicated that the particles are fairly stable due to the electrostatic repulsion. This might be the reason why AgNPs obtained were single particles and agglomerated as shown by TEM analysis. The photochemical present in extract like flavonoids, alkaloids and tannins could be responsible for stabilising the synthesised AgNPs [35]. The AgNPs synthesised using *V. cinerea* aqueous extract were stable up to more than 6 months [4].

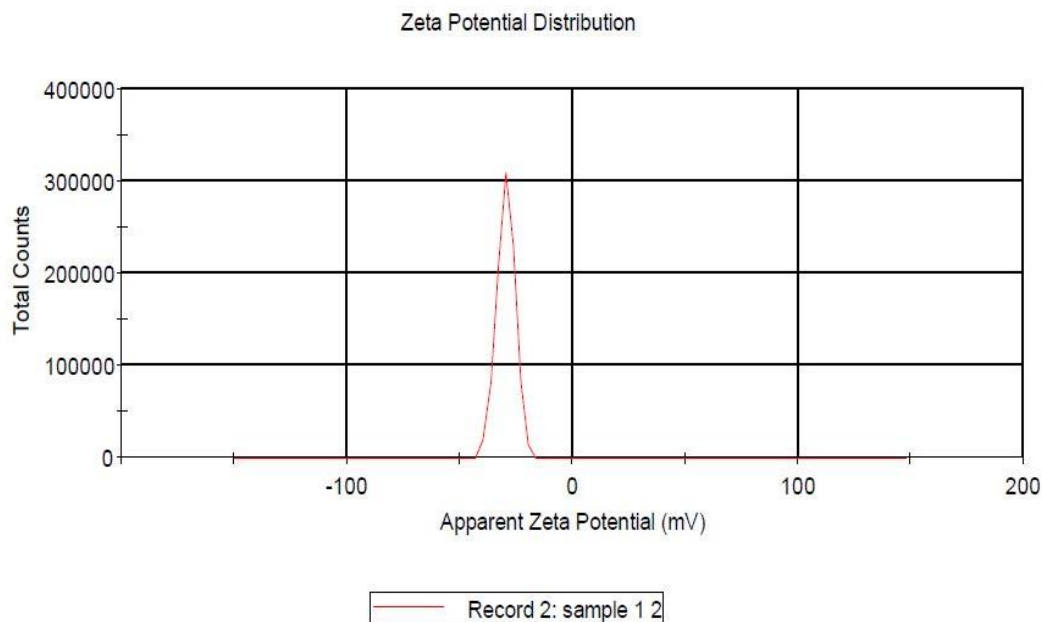


Figure 5 Zeta potential distribution of AgNPs synthesised by using *V. cinerea* aqueous extract.

Table 2 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

3.5 Proliferative Effects of *V. cinerea*-AgNPs on Kasumi-1 Cells

In this study, *V. cinerea*-AgNPs shows significant cytotoxic activity in Kasumi-1 cells. The IC_{50} values were 23.44 $\mu\text{g}/\text{mL}$, 19.54 $\mu\text{g}/\text{mL}$ and 13.68 $\mu\text{g}/\text{mL}$ at 24, 48 and 72 hr of incubations, respectively as shown in Figure 6. The most significant concentration that causes the inhibition of the cell proliferation was found to be at the concentration of 31.25 $\mu\text{g}/\text{mL}$ at 72 hr with 71 % reduction compared to untreated cells. Other than

that, the effect of *V. cinerea* aqueous extract on Kasumi-1 cell line was also evaluated in this present study. The data revealed that the IC_{50} values of the aqueous extract on Kasumi-1 cells were 93.75 $\mu\text{g}/\text{mL}$ and 19.54 $\mu\text{g}/\text{mL}$ at 48 and 72 hr, respectively as shown in Figure 7. The highest concentration of *V. cinerea* aqueous extract that inhibit the proliferation of Kasumi-1 cell was found to be at 250.00 $\mu\text{g}/\text{mL}$ at 72 hr with cell viability of approximately 29 %.

The cytotoxicity of *V. cinerea*-AgNPs was higher in Kasumi-1 cell lines compared to *V. cinerea* aqueous extract. This may be due to their biokinetics properties that refer to the uptake rate of the nanoparticles into cells, intracellular distribution and exocytosis. The biokinetics of the nanoparticles is controlled by their characteristics such as their small size, large surface area to volume ratio and surface functionalisation [37]. Small size of AgNPs makes it easier to pass through the cellular barriers and will strongly interact with the biological micro molecule [38]. This is supported by a study done by Wei *et al.* which observed high number of AgNPs compared to silver micro particles presence

in mouse fibroblast cells [39]. This proves that AgNPs have shown higher cytotoxic effects on cells compared to micro particles because of the smaller size of particles with high surface area exposed to the cells. The AgNPs show their toxicity by the disruption of mitochondrial chain which induces ROS that further cause oxidative stress, which in turn leads to DNA

damage on cells [37, 40]. Damage in DNA will be increased by high exposure of AgNPs towards the DNA leading to change in cell cycles in G2/M phase, cell proliferation and eventually lead to cell death by apoptosis or necrosis in many types of cells [41].

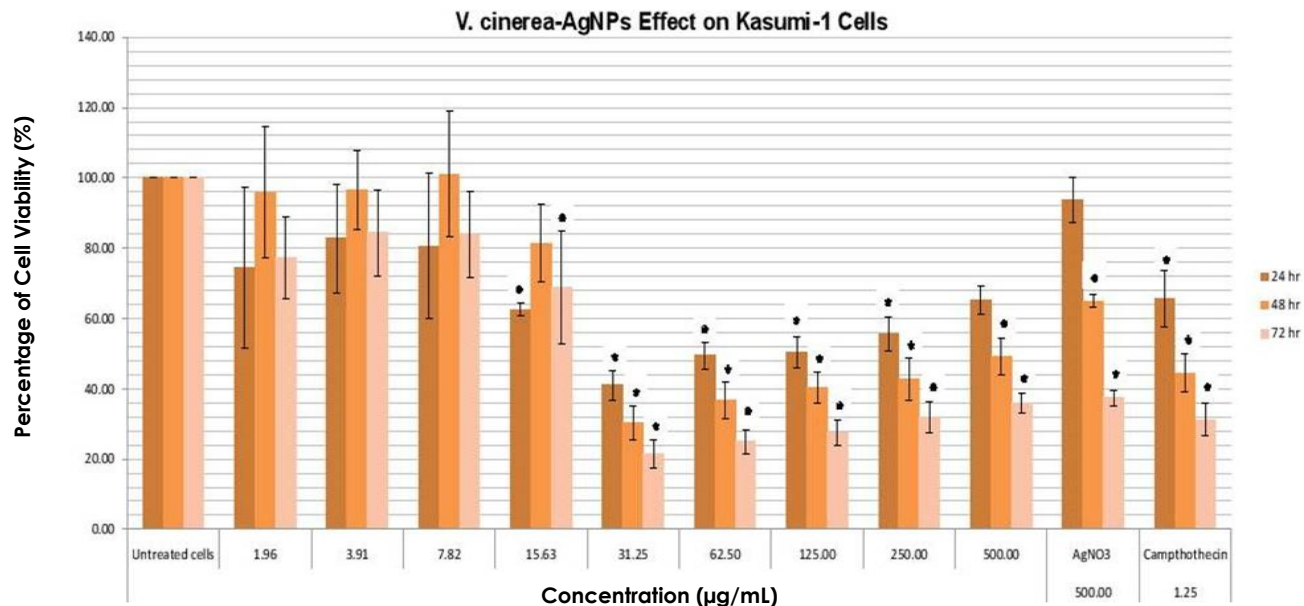


Figure 6 Proliferative effects of *V. cinerea*-AgNPs on Kasumi-1 cells. Kasumi-1 were treated with *V. cinerea*-AgNPs at double dilution manner ranging from 1.96 to 500 µg/mL for 24 hr, 48 hr and 72 hr. Untreated cells were used as negative control while camptothecin were used as positive control. The percentage of viable cells was determined by MTS assay. The values represent means \pm SD of the triplicates. * indicates significant differences ($p < 0.05$) with respect to untreated group.

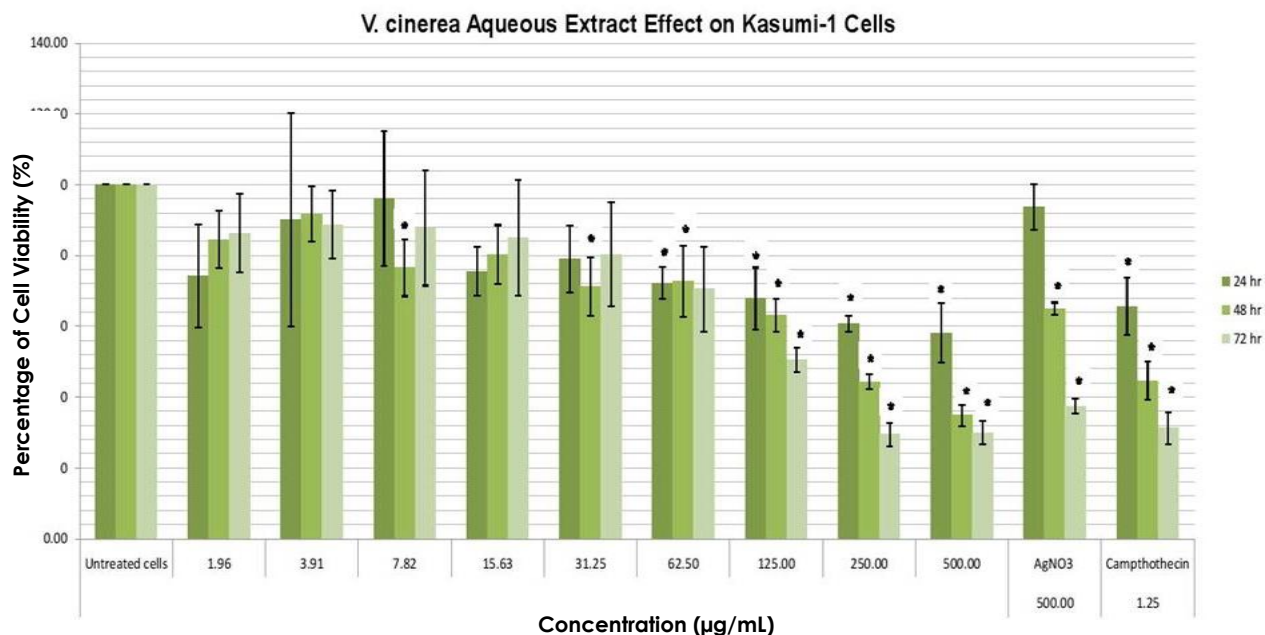


Figure 7 Proliferative effects of *V. cinerea* aqueous extract on Kasumi-1 cells. Kasumi-1 were treated with *V. cinerea* aqueous extract at double dilution manner ranging from 1.96 to 500 µg/mL for 24 hr, 48 hr and 72 hr. Untreated cells were used as negative control while camptothecin were used as positive control. The percentage of viable cells was determined by MTS assay. The values represent means \pm SD of the triplicates. * indicates significant differences ($p < 0.05$) with respect to untreated group.

4.0 CONCLUSION

V. cinerea aqueous extract have the ability to synthesise AgNPs where the synthesised AgNPs are fairly stable in solution. In the findings, *V. cinerea*-AgNPs produce high induction of cytotoxic effects on Kasumi-1 cell line compared to *V. cinerea* aqueous extract. The synthesised *V. cinerea*-AgNPs showed promising anti-proliferative activity on Kasumi-1 cell line, and may hold promise into development of cancer therapeutic agents specifically towards acute myeloid leukemia. However, further studies are necessary to determine the mechanism of cell death and also to compare with the normal cells counterpart.

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