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Silver nanoparticle biogenically synthesised by Psychotria malayana Jack: Physicochemical, cytotoxic and antimicrobial characterisations

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ABSTRACT

Introduction: Silver nanoparticles are targeted for antimicrobial and cytotoxic properties to combat antimicrobial resistance and chemoresistance. Green synthesis of silver nanoparticle method is widely used because it is environmental-friendly using biological substances as reducing and stabilising agents. *Psychotria malayana* Jack is rich with a wide range of phytochemicals that able to synthesise silver nanoparticle. **Methods:** The leaves of P. malayana Jack was extracted with ethanol-water solvent via ultrasound assisted extraction and the extract was analysed using liquid chromatography- mass spectrometry (LC-MS). The extract was then added to silver nitrate solution for 24 hours. The formation of AgNPs-PM was analysed using UV-visible spectrophotometry, scanning electron microscopy, zeta particle size and zeta potential analysis. The synthesised AgNPs-PM were tested for their cytotoxicity on human colorectal adenocarcinoma cells (Caco-2) and human epithelial breast adenocarcinoma cells (MCF-7) using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay. For antibacterial activity, the nanoparticles were tested on Gram-negative Escherichia coli and Pseudomonas aeruginosa and Gram-positive Bacillus subtilis and Staphylococcus aureus using disc diffusion method. **Results:** AgNPs-PM were successfully synthesised using P. malayana Jack extract. LC-MS analysis showed the presence of flavonoids, amino acids and heterocyclic compounds . An attempt in cytotoxic activity test showed that at concentrations between 12.5 µg/ml to 400 µg/ml of AgNPs-PM, no cytotoxic activity was observed. Whereas, in antibacterial assay, 2 mg/ml AgNPs-PM tested on the bacterial strains showed weak inhibition on their growth. **Conclusion:** AgNPs-PM has been successfully synthesised and characterised. However, the AgNPs-PM possess low bioactivities of cytotoxic and antibacterial activities.

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Introduction

In the last few decades, research on nanotechnology is being widely adapted worldwide. Nanoparticles refer to particles within the size range of 1 nm to 100 nm and they have many potential applications in various areas such as biotechnology, medicine, pharmaceutics, industries, biology and agriculture (Song & Kim, 2009; Susanti, Haris, Taher, & Khotib, 2022). In medical study, nanoparticles assist in more rapid diagnosing of diseases and more efficient disease treatments because functional molecules can be attached selectively to the metallic nanoparticles such as silver, platinum and gold, which allow the transportation of the molecules to the target site under the influence of magnetic field (Ahmed *et al.*, 2022; Muhamad, Ab.Rahim, Wan Omar, & Nik Mohamed Kamal, 2022). In other field such as nanotechnology, metallic nanoparticles are being widely utilised due to their high reactivity and high surface area to volume ration (Muhamad *et al.*, 2022; Susanti *et al.*, 2022).

Silver nanoparticles (AgNPs) are gaining attention among other metallic nanoparticles as they have remarkable biological and physicochemical properties due to their distinct surface plasmon resonance (Muhamad *et al.*, 2022). This surface plasmon resonance refers to electronic oscillations of the conduction electrons on nanoparticle's surfaces due to the interaction with the electromagnetic radiation (Eze, Tola, Nwabor, & Jayeoye, 2019). In synthesising the metallic nanoparticles such as AgNPs, a few methods have been introduced: physical, chemical and biological (Susanti *et al.*, 2022). Physical method renders a few disadvantages which include small number of AgNPs yield, a long completion period of the whole process and the utilisation of high energy which can cause a release of excessive heat to the surroundings. With chemical method, the production yield may be high, but the production of the toxic by-products is a big downturn of this process (Muhamad *et al.*, 2022).

Due to disadvantages presented *via* physical and chemical methods, the synthesis of nanoparticles using biological method such as bacteria, fungi and plant mediated synthesis is now being widely adapted (Susanti *et al.*, 2022). From the economical factor, green synthesis is cost-effective as the biological component of the biological agents which include bacteria, fungi, yeast, plant, viruses and by-products of these agents can act as the reducing agents (Alarjani, Huessien, Rasheed, & Kalaiyarasi, 2022; Susanti *et al.*, 2022). They also function as capping agents which are crucial for the stability and biocompatibility of nanoparticles (Susanti *et al.*, 2022).

Figure 1: The leaves and fruit bunch of P. malayana Jack.

The mature plant possesses dark-green leaves with a fruit bunch (Figure 1). The phytochemicals presence in the leaves extract of *P. malayana* Jack, or also known as "*meroyan sakat*" or "*salung*" in Malaysia can be utilised as the reducing and stabilising agents for the synthesising process of AgNPs in this study. Plants from genus *Psychotria* are rich with alkaloids as their major compounds such as indole, quinoline, isoquinoline, monoterpene indole, flavonoids, cyclic peptides, terpenoids and coumarins (Calixto *et al.,* 2016). Among these phytochemicals, flavonoids are well known for its role as reducing and capping agent, as a replacement for the use of toxic chemical products (Ahmad *et al.*, 2022). In this study, the leaves extract of *P. malayana* Jack was utilised to produce AgNPs. Other advantages of using green synthesis method include environmentally friendly as toxic chemicals are not being used with no application of high pressure, temperature, and energy. It is also stated that the use of plant mediated synthesis in nanoparticles production is simple as it can be produced in a single step at a room temperature, easy to manage commercial-scale processes and highly stable in storage (Alarjani *et al.*, 2022; Susanti *et al.*, 2022).

Studies reported that the emergence of multidrug-resistant bacterial strains due to misuse of antibiotics has become a major concern to the human health all over the world (Murray *et al.*, 2022; Wang, Hu, & Shao, 2017). A predictive statistical models stated that the deaths associated with antimicrobial resistance in the year 2019 was estimated to be 4.95 million deaths (Murray *et al.*, 2022). It was proven in many previous literatures that silver ions and silver-modified inorganic materials such as AgNPs exhibit multiple antimicrobial activity. This includes their ability to interfere with the bacterial DNA activity and generation of free radicals which induce the reactive oxygen species leading to bacterial cell death (Ahmad *et al.*, 2022). These mechanisms are different to most antibiotics which commonly work by targeting the cell wall synthesis, translation machinery and DNA replication machinery. These unique properties of AgNPs make them a comparable choice to antibiotic in the treatment of microbial infections as antibiotic's resistance mechanisms are not applicable to them (Ahmad et al., 2022; Susanti et al., 2022).

Aside from that, reports state that AgNPs also demonstrate antitumourigenic activity on tumour models (Muhamad *et al.*, 2022). Based on the research, AgNPs which is concentration dependent can induce apoptosis or the programmed cell death in *in vitro* studies. Furthermore, AgNPs can also induce the alterations in the cell morphology, reduce cell viability and its metabolic activity and causing an increase in oxidative stress which lead to mitochondrial damage. This then leads to significant DNA damage and cell death. Thus, there is a potential usage of the AgNPs in cancer treatment (Zhang, Ma, Gu, Huang, & Zhang, 2020). Therefore, this study was done to investigate the antimicrobial activity and cytotoxicity of AgNPs-PM for their potential application in human use.

Methodology

1. Plant sample collection and extraction

Fresh leaves of *P. malayana* Jack, voucher specimen (PIIUM 0008-1) was collected at Kuantan, Pahang, Malaysia. The leaves were air dried at a controlled temperature drier (40 °C) for three days. The leaves were ground mechanically into fine powder using a mechanical grinder. 50g of the leaves powder were mixed with 500 ml ethanol-water (Ethanol 95%, GENE Chemicals) (80:20) in a 500 ml Erlenmeyer flask. The extraction was done via ultrasound-assisted extraction method at a temperature of 48 °C for 40 minutes using a probe sonicator (Qsonica Ultrasonic Sonicator Converter Model CL-334). The leaves extract was then filtered using filter papers (NICE Qualitative 102) and stored in a refrigerator at 4 °C (Bimakr, Ganjloo, Zarringhalami, & Ansarian, 2017).

2. Liquid Chromatography Mass Spectrometry Quadrupole of Flight (LC-MS-QTOF) Analysis

Sample preparation: P. malayana Jack leaves extract was dried via rotary evaporator (IKA HB 10 basic) at a speed of 130 rpm and a temperature of 50°C. The dried extract was reconstituted with methanol to a final concentration of 10 mg/ml, then it was diluted to a concentration of 1 mg/ml with methanol. Prior to analysis via LC-MS-QTOF (6520 Agilent Techologies, SA, USA), the extract was filtered using a 0.22 µm pore of PVDF membrane size syringe filter. LC-MS method: The chromatographic separation was operated using Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT (2.1 x 100 mm) 1.8 µm with (A) 0.1% formic acid in distilled water and (B) 0.1% formic acid in acetonitrile for positive mode. The gradient elution programme was 0.00 – 18.00 min, 5 – 95% (B); 18 – 23 minutes; 95% (B); 23.0 minutes; 5% (B). It was run for 30 minutes. Prior to new injection, re-equilibration of LC condition was conducted for 2 minutes. The sample injection volume and the flow rate of mobile phase was set at 2 µl and 0.25 ml/min, respectively. The mass spectrometer was operated in positive electrospray ionisation (ESI) mode with optimum gas temperature at 325°C, gas flow at 11 L/min and nebuliser at 35 psi. Data analysis: The chromatographic profiles were analysed using Agilent Mass Hunter Qualitative Analysis B.05.00 software (Agilent Technologies, Santa Clara, CA, USA) based on the accurate mass data identified and the predicted compounds were annotated using METLIN database (Al-Abd et al., 2015).

3. Preparation of silver nitrate ($AgNO_3$) solution

1 mM and 5 mM of $AgNO_3$ solutions were prepared by dissolving 0.0153g and 0.0764g of $AgNO_3$ powder (EMSURE, Macedonia) into 90 ml deionised water, respectively.

4. Synthesis of silver nitrate ($AgNO_3$) solution

10 ml of *P. malayana* Jack leaves extract was added slowly into a 90 ml $AgNO_3$ solution of two different concentrations (1 mM and 5 mM) under continuous stirring (100 rpm) using a magnetic stirrer to ensure the 1:9 ratio of the plant extract to $AgNO_3$ solution. The synthesis process was continued for 24 hours. After 24 hours, the synthesised solution was centrifuged at 7500 rpm for 15 minutes at 4 °C to purify it (Supra 22K, Korea). The resulting pellet was resuspended with a small amount of deionised water and was dried in an oven at 40 °C. The resulting powder of AgNPs-PM was left at room temperature for future use.

5. Characterization of silver nanoparticles (AgNPs-PM)

The characterisation of the synthesised AgNPs-PM was carried out by Ultraviolet and Visible spectrophotometer (UV-Vis), scanning electron microscopy (SEM), zetasizer and zeta potential analysis.

UV-Vis Spectroscopy analysis: The green synthesised AgNPs-PM were sampled at 1, 2, 4, 8 and 24 hours for analysis *via* UV-Visible double beam spectrophotometer (SHIMADZU UV-1800, Japan). Deionised water was used as a blank and reference solution. The spectrum was then recorded in the scanning range of 350 nm to 800 nm.

Morphological analysis: The image of the biosynthesised AgNPs-PM, the nanoparticles were analysed under Zeiss EVO-50X Scanning Electron Microscopy (SEM) instrument. Then, on a non-conduction carbon tape that functions as the stabiliser, AgNPs-PM powder was prepared by simply sprinkling 2 mg of the powder sample on the tape on the sample holder. After being fixed, SEM analysis was performed and the AgNPs-PM powder was analysed at room temperature.

Zetasizer and Zeta Potential analysis: The analysis sample was an aqueous solution of biosynthesised AgNPs-PM that was placed inside a Malvern Zetasizer instrument. The instrument then identified the electrical potential of ions surrounding a particle at its border as well as ions adsorbed in the diffuse layer at 25°C that was run 12 times.

6. Cytotoxic assay

Cell culture: Human colorectal adenocarcinoma cells (Caco-2) and human epithelial breast adenocarcinoma cells (MCF-7) were cultured at 37°C in a 5% CO_2 incubator (Thermo Scientific Heraeus BB15) in Eagle's minimal essential medium, EMEM (ATCC 30-2003, Manassas, VA) supplemented with 10% fetal bovine serum, FBS (Gibco, Brazil) and 1% (v/v) penicillin-streptomycin (Nacalai Tesque, INC., Kyoto, Japan). After the cells reached confluency at 80%, trypsinisation process was applied to detach and subculture the cells. The cells were then seeded into a 96-well plate at a density of 15,000 cells per well. Cell viability assay: The evaluation of the cytotoxic activity of the synthesised AgNPs was done via 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay. The seeded cells were treated with various concentrations (400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µ/ml and 12.5 µg/ml) of AgNPs-PM and an anticancer drug Tamoxifen as positive control. The cells were incubated for 24 hours. After 24 hours, the cells were treated with 20 µg of MTT (5 mg/ml) and re-incubated for 30 minutes at 37°C. The formed crystals of formazan were dissolved using 200 µL of dimethyl sulfoxide, DMSO (EMPLURA, USA) and re-incubated for another 30 minutes at 37°C. Using a microplate reader, the difference in colour intensities (absorbance) was recorded at 630 nm.

7. Antimicrobial assay

Two Gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus* and two Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* were cultured in nutrient broth medium. Then, they were placed in the incubator for 18 hours at 37 ± 1°C. Disc diffusion assay was employed to screen for antimicrobial activity of AgNPs-PM. The grown microbes were sub-cultured on petri dishes and the discs were treated with 10 µl of 2 mg/ml AgNPs-PM, 10% *P. malayana* plant extract, 2 mg/ml $AgNO_3$ solutions and deionised water (negative control). Amoxicillin discs were used as the positive control. Then, the petri dishes were put in a CO_2 incubator (Binder) at 37°C for 24 hours. The zone of inhibition around the discs was measured in mm and was compared with the negative control.

8. Statistical study

Statistical analysis was done *via* Statistical Package for Social Sciences (SPSS). The tests were conducted in sets of three and the data were reported as mean ± standard deviation (SD) using one-way ANOVA test. The individual correlations were obtained *via* Duncan's technique. If $P < 0.05$, the value will be considered as significantly different (Anbumani *et al.*, 2022).

Results and Discussion

1. LC-MS-QTOF analysis of P. malayana Jack leaves extract

The preliminary compound identification was performed using LC-MS-QTOF analysis by comparing the m/z spectra belonging to each compound to the mass spectra database of METLIN (Figure 2). The analysis provided that there was a total of 80 compounds detected. Information on the chemical composition of the 10 major compounds from the analysis such as name, molecular formula, m/z, mass and classification of each compound were listed in Table 1. Among the compounds analysed were categorised as phospholipid, steroid, ketone, amino acids and flavonoids (flavans, falavanols and leucoanthocyanidin) (Table 1). Although, there were some unknown compounds as the data of the compounds are not available in the METLIN database.

The available literatures studying genus Psychotria reported that among chemical compounds found were indole alkaloids, cyclic peptides or cyclotides, quinoline and isoquinoline alkaloids, flavonoids, terpenoids, coumarins and tannins (Calixto *et al.*, 2016). This is in line with the findings from LC-MS analysis of *P. malayana* Jack (Figure 2 and Table 1). Many literatures also reported that among the phytochemicals that were responsible for synthesis of AgNPs were flavonoids, amino acids, tannins, polyphenols, sterols, heterocyclic compounds, triterpenoids, terpenoids, alkaloid, etc. due their ability to

act as the reducing, capping and stabilising agents (Ahmad *et al.*, 2022; Alarjani *et al.*, 2022; Nadaf *et al.*, 2022; Patil & Kim, 2017). Thus, attributable to the biological activity of *P. malayana* Jack, it can be utilised in the synthesis of AgNPs-PM.

2. Green synthesis of silver nanoparticles, AgNPs-PM

For the synthesis, 1 mM and 5 mM $AgNO_3$ solutions were added to the plant extract and changes were observed every 1, 2, 6, 8 and 24-hours (Figure 3A-3B). The changes in colour intensity when synthesising nanoparticles with 1 mM concentration were minimal, but darkest after 24 hours incubation. When the process was done with 5 mM, the colour of the solutions turned from green to dark brown after 1 hour incubation. As cited in many literatures, the formation of dark brown solution which is due to the excitation of AgNPs' surface plasmon resonance confirms the successful synthesis of AgNPs (Ahmad *et al.*, 2022). The yield of the AgNPs-PM was weighed after high-speed centrifugation process. The findings provided that 1 mM yielded the lowest and 5 mM the most (Figure 3C). The development of AgNPs-PM synthesised using 5 mM $AgNO_3$ solution was further observed using other characterisation methods.

2.1 Proposed mechanism for synthesis of AgNPs

A general mechanism for the formation of AgNPs include three main phases which are activation phase, growth phase and termination phase (Makarov *et al.*, 2014). During the activation phase, there will be reduction of metal ions and nucleation of the reduced metal atoms. Phytochemicals such as flavonoids in its keto form will convert into enol form. This will in turn release reactive hydrogen. However, due to the presence of two hydroxyl groups on the same carbon, the enol form is deemed as unstable, and thus, will convert back to its keto form. At this stage, the liberated reactive hydrogen causes the reduction of metal ions to metal atom, specifically Ag^+ to Ag^0, which then combine with each other forming small AgNPs. In addition to flavonoids, tannins also act as reducing agents (Ahmad *et al.*, 2022). In the growth phase, the small adjacent AgNPs coalesce spontaneously into larger particles. This process continues until the particles assume a stable shape and size. Finally, in the termination phase, AgNPs will acquire the most favourable conformation due to the influence of the phytochemicals that function as stabilising agents (Makarov *et al.*, 2014).

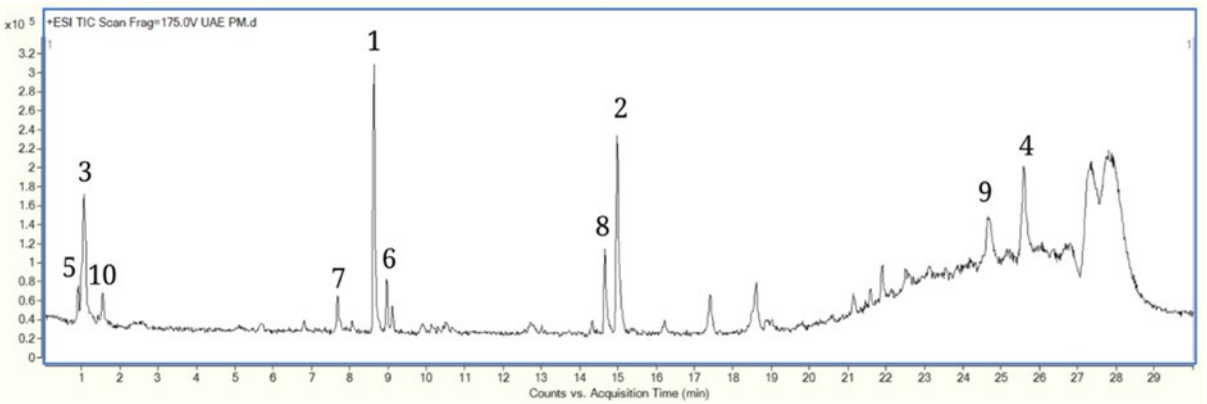
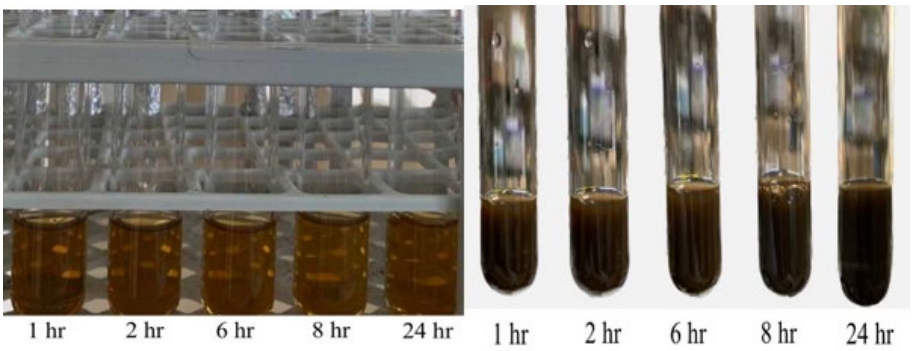


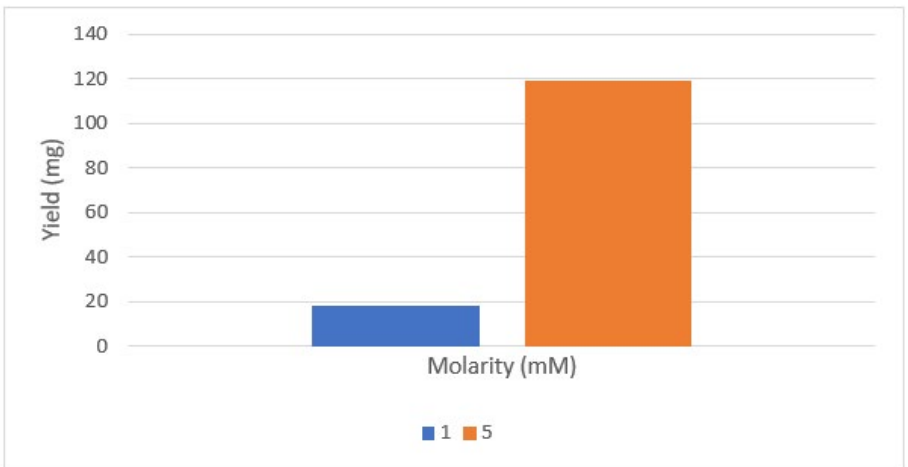
Figure 2: LC-MS analysis of *P. malayana* Jack. The sample was reconstituted with methanol to the final concentration of 10 mg/mL, then diluted to the concentration of 1 mg/mL with methanol. The sample was then filtered using a 0.22 µm pore size syringe filter before analysis. Chromatographic separation was performed at 40°C using Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT (2.1 x 100 mm) 1.8 µm with (A) 0.1% formic acid in dH20 and (B) 0.1% formic acid in acetonitrile for positive mode. Mass spectrometer was operated in positive electrospray ionisation (ESI) mode with optimum gas temperature at 325°C, gas flow at 11 L/min and nebuliser at 35 psi, respectively.

Table 1: 10 major compounds of *P. malayana* Jack analysed by LC-MS-QTOF analysis

No	Compound name	Molecular formula	m/z	Mass (g/mol)	Classification
1	LysoPE(0:0/18:4(6Z,9Z,12Z,15Z))	$C_{23}H_{40}NO_7P$	491.286	473.252	Lipid (Phospholipid)
2	Eplerenone	$C_{24}H_{30}O_6$	415.2116	414.2043	Lipid (Steroid)
3	4-(o-Carboxybenzamido) glutaramic acids	$C_{13}H_{14}N_2O_6$	295.0926	294.085	-
4	N-Cyclohexanecarbonyloentadecyl amine	$C_{22}H_{43}NO$	338.3421	337.3347	Ketone
5	2-Amino-3-methyl-1-butanol	$C_5H_{13}NO$	104.1069	103.0997	Amino acids
6	Unknown	-	533.3315	532.3242	-
7	Oritin-4beta-ol	$C_{15}H_{14}O_6$	291.0862	290.079	Flavonoids (Flavans, Flavanols and Leucoanthocyanidin)
8	Unknown	-	393.2862	375.2525	-
9	Unknown	-	568.4265	567.4192	-
10	Purine	$C_5H_4N_4$	121.0509	120.0473	Heterocyclic aromatic organic compound



(A) (B)



(C)

Figure 3: AgNPs-PM incubated at 1, 2, 6, 8 and 24hours incubation, respectively using (A) 1 mM $AgNO_3$ solution (B) 5 mM $AgNO_3$ solution (C) Yield of AgNPs-PM obtained from two different molarity of $AgNO_3$

3. Characterisation of silver nanoparticles, AgNPs-PM

3.1 UV-Visible spectrophotometer analysis

The formation of the synthesised AgNPs-PM was confirmed *via* UV-Visible spectroscopy analysis by measurement of surface plasmon resonance (Patra & Baek, 2014). UV-Vis absorption spectra of *P. malayana* Jack leaves extract showed the hypsochromic and hyperchromic shift of the UV-Visible spectra of the leaves extract, and AgNPs-PM at different incubation time (Figure 4). In this study, the collected absorption spectra within 350 nm to 800 nm showed maximum absorption at 412.0 nm (1 hr), 416.1 nm (2 hr), 422.2 nm (6 hr), 437.2 nm (8 hr) and 449.0 nm (24 hr). It was also observed that the absorption and intensity of the peak increased as incubation time increased. The broad peak in the range of 550 nm to 650 nm were attributed to the presence of flavonoids with aromatic benzene conjugated at C-2 and C-3. Another broad band that was centred at 520 nm suggested a conjugated benzene with electron donating group such as NH and OH, also suggesting flavonoids (Ahmad *et al.*, 2022). The data from LC-MS-QTOF analysis provided that one of the major phytoconstituents of *P. malayana* Jack extract was flavonoids. The observations on this analysis provided that flavonoids were the major constituent of *P. malayana* Jack that was responsible for the reduction and stabilisation of AgNPs-PM (Patil & Kim, 2017). The observation on the AgNPs-PM bands from this analysis provided that the hypochromic shift moved towards lower wavelength. These new maximum absorption peaks of AgNPs range from 410 nm to 450 nm represent the surface plasmon resonance of AgNPs-PM.

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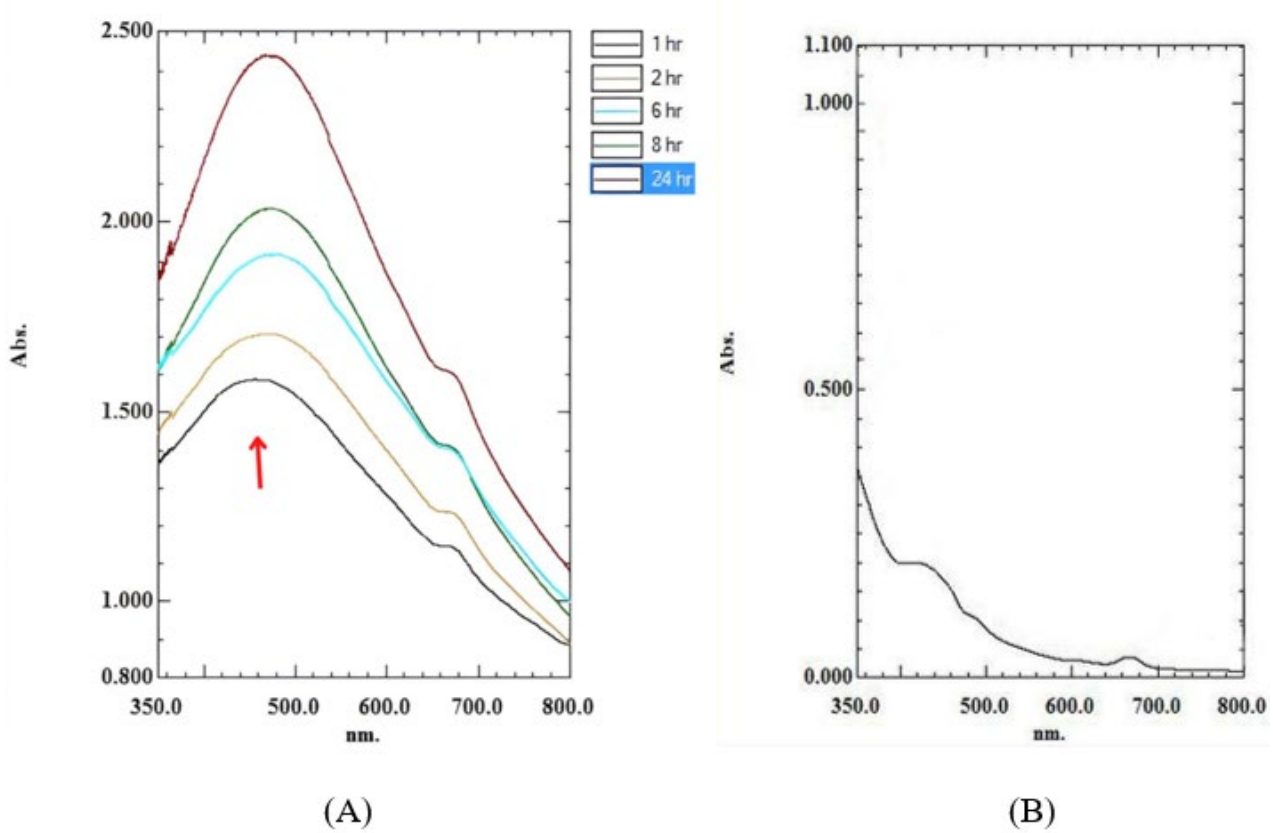

Figure 4: UV-Vis absorption spectra of (A) the green synthesized AgNPs-PM after incubation at 1, 2, 6, 8 and 24 hours (B) P. malayana Jack leaves extract measured between 350-800 nm. An arrow indicated a specific band for silver nanoparticles.

3.2. Scanning electron microscopy (SEM) analysis

The morphological characteristics of the synthesised AgNPs-PM such as size and shape were directly viewed using SEM *via* electron scanning. These characteristics were associated with the toxicity, drug and tissue targeting, drug release and the biological fate of AgNPs-PM (Patra & Baek, 2014). The SEM image indicated that the AgNPs-PM formed were mostly aggregated, which is attributed to the function of the phytochemicals of *P. malayana* Jack leaves extract (Figure 5). The formed AgNPs-PM were having a hexagonal cluster and the particle sizes of AgNPs-PM analysed ranged from 75 nm to 145 nm, which was magnified under 4000x. Some factors could have an influence in the formation of the size and shape of AgNPs-PM such as temperature of the reaction medium, time of the synthesising reaction, exposure to light and the storage conditions (Patra & Baek, 2014; Ye et al., 2022). Aggregation of nanoparticles may occur during the storage, in which they might shrink or grow, thus affecting their potential activities. However, the information provided by SEM about the size distribution and the true population average is limited (Patra & Baek, 2014).

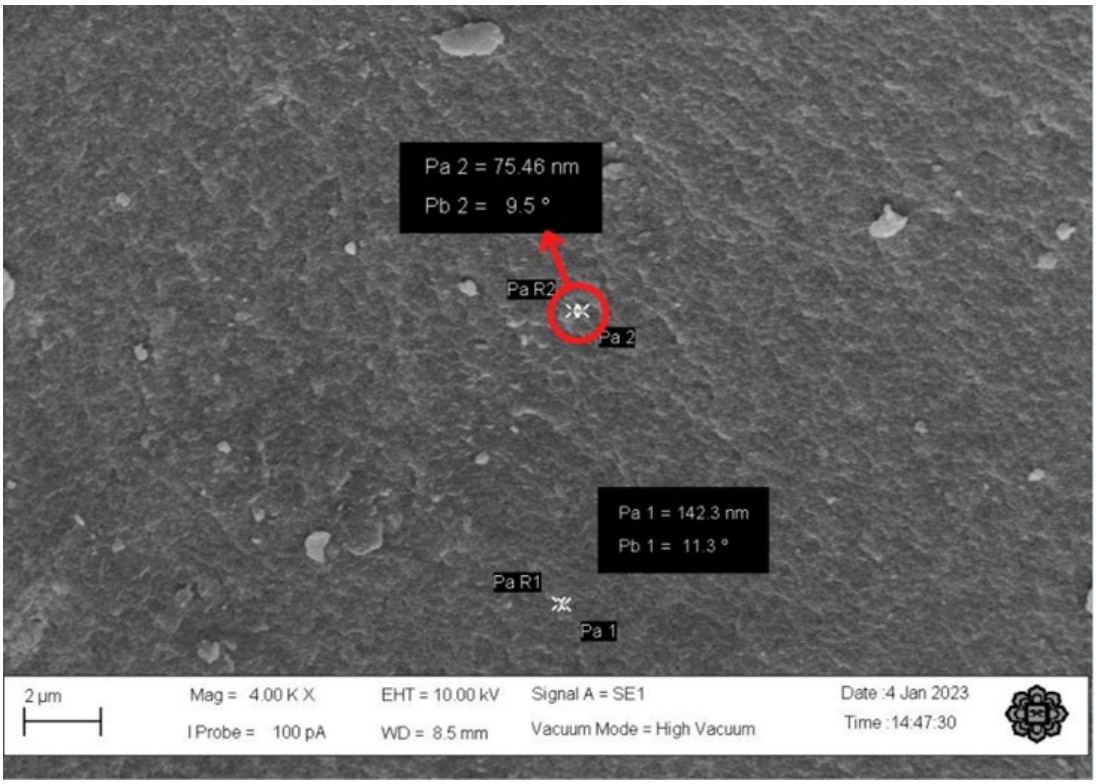

Figure 5: SEM analysis of AgNPs-PM. Characteristic morphology of silver nanoparticles produced in this study.

3.3 Zeta particle size and zeta potential analysis

Dynamic Light Scattering (DLS) analysis provided information on the particle hydrodynamic size, zeta potential and polydispersity index (PDI) of AgNPs-PM (Suriyakala *et al.*, 2022). In this study, the average size distribution of the synthesised AgNPs-PM as analysed *via* zetasizer nanomachine was 110.1 ± 64.66 nm (Table 2). Particle size and their morphology are the most important parameters in determining their properties. It had been proven in many studies that nanoparticles are more efficient in delivering drugs than microparticles due to nanoparticles having larger surface areas, thus more drug interactions can be observed (Patra & Baek, 2014). Many literatures accepted the descriptive size of nanoparticles to be between 1 nm to 100 nm (Susanti *et al.*, 2022). In pharmaceutical field, those particles having a diameter of 10 nm to 1000 nm are also regarded as nanoparticles (Mazayen, Ghoneim, Elbatanony, Basalious, & Bendas, 2022).

Zeta potential measurement can provide predictive data on the surface charge, which affects the storage stability of the colloidal dispersion (Patil & Kim, 2017; Suriyakala *et al.*, 2022). The maximum zeta potential value of the formed AgNPs-PM (Table 2) was approximately -117 ± 15.2 mV. This high negative value indicated that there were electrostatic repulsion between the synthesised AgNPs-PM, making them stable. The negative value also indicated that there were presence of negatively charged functional groups from the *P. malayana* Jack leaves extract (Suriyakala *et al.*, 2022).

In addition, zeta potential at ± 35 mV suggested a formation of stable particles (Buszewski *et al.*, 2018). Previous findings also stated that in order to avoid particle aggregation and ensuring its stability, either a high positive or a high negative value of zeta potential must be achieved (Patra & Baek, 2014).

Table 2. Average size distribution and zeta potential of AgNPs-PM analysed using zetasizer instrument.

Particle size analysis	
Particle size average (d.nm)	110.1 ± 64.66
PdI	0.287
Zeta potential analysis	
Zeta potential (mV)	-117 ± 15.2
Zeta deviation	18.2
Conductivity (mS/cm)	0.551

4. Cytotoxic Assay

Cancer is one of the most leading death-causing diseases and 1 out of 3 people has the possibility to get cancer (Alyami, Alyami, & Almeer, 2022; Zhang et al., 2020). Different types of cancer therapies being offered include chemotherapy, surgery, radiation, hormonal, targeted and immunotherapy. However, the therapies are challenging due to induction of enormous side effects, which include neuropathies, alopecia and gastrointestinal and skin disorders. In addition, high rate of recurrence and multi-drug resistance against common chemotherapeutic drugs are other factors that limit the therapeutic efficacy (Alyami *et al.*, 2022; Gavas, Quazi, & Karpiński, 2021). Thus, to avoid the systemic side effects and drug resistance, many researchers are focusing on the development of nanomaterials as an alternative formulation that can specifically target tumour cells (Zhang *et al.*, 2020).

Cytotoxic assay was used to measure the ability of tested compounds in killing cell lines (Shelembe, Mahlangeni, & Moodley, 2022). In this study, the cytotoxic effect of AgNPs-PM at different concentrations of 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml was investigated against Caco-2 and MCF-7 cell lines using MTT cell viability assay. The formation of purple formazan crystals is dependent on NADPH and oxidoreductase enzymes of the cancer cells. Thus, the intensity of purple colour is directly proportional to the cell viability (Shelembe *et al.*, 2022). The absorbance of the dissolved formazan crystals was measured *via* microplate reader at 570 nm. The number of viable cells is proportional to the absorbance and percentage viability (%) was calculated using the following formula:

$$\% \text{ Cell viability} = \frac{A_{sample}}{A_{untreated}} \times 100 \qquad \text{(Eq. 1)}$$

Many studies from *in vitro* assays reported that AgNPs possess cytotoxic activity on several human cell lines, which include human peripheral blood mononuclear cells, human bronchial epithelial cells, red blood cells, liver cells (HepG2), human colorectal cells (Caco-2) and human epithelial breast cells (MCF-7) (Liao, Li, & Tjong, 2019; van der Zande *et al.*, 2016). In this study, the percentage viability of Caco-2 and MCF-7 cell lines was reduced after interaction against various concentrations of AgNPs-PM **(Figure 6)**. In Caco-2 cells, after incubation with 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml, the cell viability calculated was 89.2%, 84.6%, 82.2%, 88.6%, 80.18% and 81.675, respectively. However, previous study reported that at a concentration of 5 µg/ml of AgNPs, 50% of cell will be inhibited after exposure for 48 hours (Zein, Alghoraibi, Soukkarieh, Salman, & Alahmad, 2020). In MCF-7 cells, the cell viability calculated against the increasing concentration of AgNPs-PM was 97.5%, 96.7%, 90.2%, 95.14%, 90.97% and 89.7%, respectively. Whereas the IC_{50} of AgNPs against MCF-7 in a study conducted by Fard, Tafvizi, and Torbati (2018) was found to be 9.85 µg/ml.

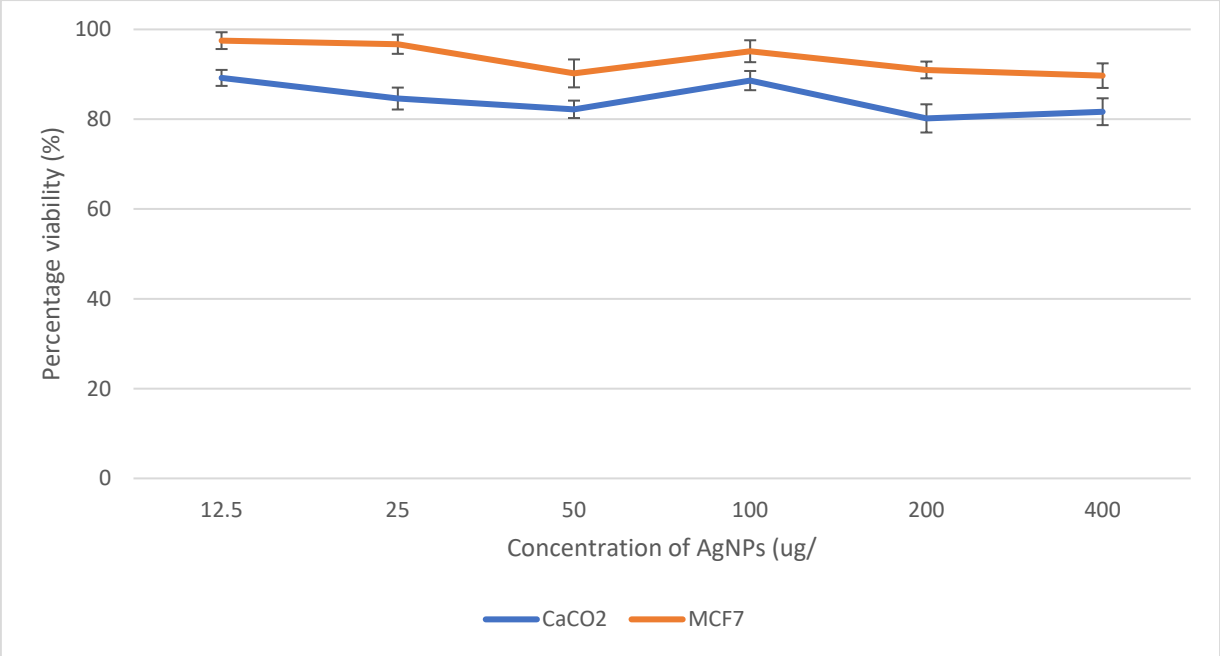

Figure 6: Percentage viability of AgNPs-PM on Caco-2 and MCF-7 against various concentrations ranging from 12.5 µg/ml to 400 µg/ml of AgNPs-PM

However, the findings from this study showed that the cytotoxicity of AgNPs-PM on Caco-2 cells and MCF-7 were both insignificant. It was also expected that the percentage viability of the two cell lines to decrease with increasing concentration of AgNPs-PM as AgNPs exhibit concentration-dependent cell death (Alyami *et al.*, 2022; Zhang *et al.*, 2020). However, in this study, the trend fluctuated. In addition to that, the standard errors of this assay were high, indicating that the results were not reliable, thus, the percent viability might not reflect a true value of the overall cytotoxic assay. This less reliable result might be due to variable density of cell in each well, unsuitable incubation time of cells in MTT, the wavelength at which the optical density was measured, and the type of culture media used (Ghasemi, Turnbull, Sebastian, & Kempson, 2021). In this assay, it was also observed that the percentage inhibition of AgNPs-PM against Caco-2 cells were more prominent than MCF-7 cells. This finding might be attributed to the higher sensitivity of Caco-2 cells to AgNPs-PM than MCF-7 cells (van der Zande *et al.*, 2016).

5. Antibacterial Assay

Many pathogenic bacteria are showing resistance to various antibiotics. To address this issue, new antibiotics are necessary (Ahmad *et al.*, 2022). AgNPs were proven to possess antimicrobial properties, making them suitable alternatives to antibiotics (Nguyen et al., 2021). This study investigated the activity of AgNPs-PM, $AgNO_3$ solutions and *P. malayana* Jack leaves extract on four bacterial strains *via* disc diffusion method. The tested bacteria were *B. subtilis, E. coli, P. aeruginosa* and *S. aureus*. In this study, the negative control discs were treated with deionised water, and the positive control discs were treated with antibiotic amoxicillin. For the sample discs, they were treated with 10% *P. malayana* plant extract, 2 mg/ml AgNPs-PM and 2 mg/ml $AgNO_3$ solutions

2 mg/ml AgNPs-PM and 2 mg/ml $AgNO_3$ solutions both showed antimicrobial activity on the microbial growth against all the tested bacterial strains (Figure 7). At a same concentration of 2 mg/ml, AgNPs-PM exhibited good inhibition on the growth of *P. aeruginosa,* followed by *S. aureus, B. subtilis* and *E. coli* at 3.00 ± 0.17 mm, 2.00 ± 0.28 mm, 1.75 ± 0.14 mm and 1.25 ± 0.26 mm, respectively. The activity of 2 mg/ml $AgNO_3$ solutions were almost comparable to AgNPs-PM, but with a larger zone of inhibition than AgNPs-PM. The zone of inhibition caused by $AgNO_3$ solutions was 2.00 ± 0.35 mm, 2.50 ± 0.41 mm, 4.00 ± 0.00 mm and 3.50 ± 0.22 mm on *P. aeruginosa,* followed by *S. aureus, B. subtilis* and *E. coli,* respectively. This is in line with the previous publications stating that silver ions and silver-modified materials like AgNPs possess multiple antimicrobial activity such as induction of reactive oxygen species (Ahmad *et al.*, 2022).

Since the activity of AgNPs-PM was both greatest on *P. aeruginosa* and least on *E. coli,* it is unknown

whether AgNPs work better on Gram-negative bacteria or Gram-positive bacteria. In addition, the true mechanism of how AgNPs exhibit antimicrobial activity is still unclear (Ahmad *et al.*, 2022). The proposed mechanisms are grouped into three main actions which are induction of oxidative stress, release of metal ion and non-oxidative mechanism. These actions can either act independently or simultaneously. This can lead to denaturation of the proteins and leaking of the cell contents (Goyal, Verma, Kharewal, Gahlaut, & Hooda, 2022).

However, when the bacterial strains were tested against the 10% *P. malayana* plant extract, no zone of inhibition was seen and measured, indicating that the extract does not possess antimicrobial activity. From this assay, it can be concluded that the antimicrobial activity of AgNPs-PM was caused by the synthesised AgNPs solely (Nguyen *et al.*, 2021). There is no significant difference of the antibacterial activity between the samples (AgNPs-PM and $AgNO_3$ solutions) and Amoxicillin against *B. subtilis* and *E. coli.* However, testing on *P. aeruginosa* and *S. aureus* showed that there is a large significant difference observed between the samples (AgNPs-PM and $AgNO_3$ solutions) and Amoxicillin.

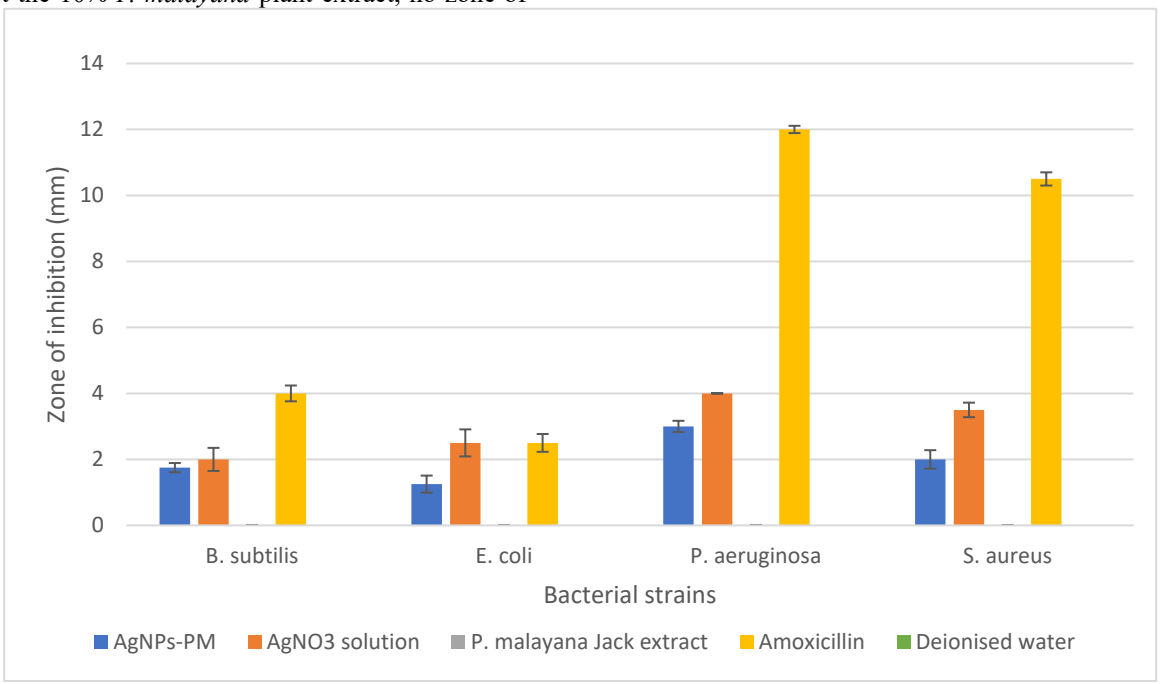

Figure 7: Zone of inhibition (mm) of four bacterial strains against AgNPs-PM, $AgNO_3$, *P. malayana* Jack extract. The test was conducted by disc diffusion method using amoxicillin disc as a positive control and deionised water as negative control.

Conclusion

The study was conducted by synthesising AgNPs-PM from *P. malayana* leaves extract *via* biological route which is known as green synthesis method. The major phytochemicals of *P. malayana* Jack as analysed by LC-MS-QTOF, namely flavonoids, amino acids and heterocyclic aromatic organic compound which were possible for the formation of AgNPs-PM as they act as the reducing and stabilising agents for the process. Characterisations were done on the synthesised AgNPs-PM to study their characteristics as the nature of the AgNPs play an important role in their application. The investigations proposed that the formed AgNPs-PM were having hexagonal cluster with the size of around 75 nm to 145 nm as viewed under electron microscope. Another analysis also confirmed that the size distribution of AgNPs-PM was 110.1 ± 66.64 nm with a zeta potential of approximately -117 ± 15.2 mV, indicating that stable AgNPs-PM were successfully synthesised using green synthesis method. Their cytotoxicity was also determined *via* MTT colourimetric assay and it was found out that at concentrations of 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml of AgNPs-PM, the cell viability of both Caco-2 and MCF-7 cell lines were over 80%. This finding indicated that the percentage inhibition was low and insignificant. However, in this study, a few confounding factors may have an effect on the result, thus, making it unreliable. AgNPs-PM were also studied for antimicrobial activity against *B. subtilis, E. coli, P. aeruginosa* and *S. aureus* using disc diffusion method, and

it was observed that AgNPs-PM inhibited the growth of the four bacterial strains, with the highest inhibition on *P. aeruginosa* and the lowest inhibited was *E. coli.* Thus, the biogenic synthesis of AgNPs-PM might be a promising process for the production of other metallic nanoparticles which could have potential applications in various fields. However, the improvement on this study for cytotoxic and antimicrobial assays can be made by adjusting the size and formulation of nanoparticles.

Author contributions

MT and DS design the study. MT, DS, TK supervise the works. NA conduct the research and collect the data. NA wrote the manuscript. MT review the manuscript. All authors have read the manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest in the writing of this manuscript.

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