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Research Article

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Phyllanthus niruri leaf extract and its mediated silver nanoparticles as anticancer agent against neuroblastoma cancer (SH-SY5Y) cell lines

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ABSTRACT

Silver nanoparticles (AgNPs) have enormous potential in numerous medical uses, such as molecular imaging, drug delivery, and treatment of different diseases, including cancer. Among many AgNP syntheses, the eco-friendly production of AgNPs utilising plants has many advantages including, sustainable and inexpensive process. Further, it provides safer and more effective treatment of numerous diseases. In this research, *Phyllanthus niruri* (PN) leaf extract was utilised for the biological synthesis of AgNPs. UV-Vis spectroscopy, ATR-FTIR spectroscopy, FESEM-EDX, and TEM were applied to analyse the synthesised *P. niruri* - mediated silver nanoparticles (PN-AgNPs). The images from FESEM and TEM analysis show the AgNPs are in spherical shapes with surface sizes of 11 to 63 nm and 12 to 24 nm, respectively. The EDX spectrum indicates that the nanoparticles contain 88.46% silver. PN and PN-AgNPs were further investigated to see whether they have any anticancer properties against the SH-SY5Y neuroblastoma cell line. Both PN and PN-AgNPs were effective in killing cancer cells, but the results indicated that PN-AgNPs had higher cytotoxic effects than PN.

KEYWORDS: *Phyllanthus niruri*, Silver nanoparticles, Anticancer study, SH-SY5Y Neuroblastoma cell line

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INTRODUCTION

Advancements in nanoscience and nanotechnology in every science sector have made life simpler in recent decades. Nanoscience is the field that works at the nanoscale and deals with molecules or shapes with dimensions ranging from 1 to 100 nanometres; meanwhile, the technology that utilises this knowledge in our daily lives, such as devices, is called nanotechnology. Nanotechnologies represent one of the most promising 21st century innovations because they can transform nanoscience theory into usable applications (Bayda *et al.*, 2019). Almost all the areas of study, ranging from chemistry, biology, physics, materials, and computer science/engineering, depend on the application of nanotechnology. Nanotechnologies have also been applied in the medical field to enhance human health, especially in cancer treatment (Bayda *et al.*, 2019).

Materials that have undergone size reduction to a range of 1 to less than 100 nanometres are known as nanomaterials and are one of the main products of nanotechnologies. Nanomaterials are being applied in many industries, such as agriculture, healthcare, wastewater treatment, energy preservation, and catalysis (Gajanan & Tijare, 2018). Besides, it is different from the general macro-materials because they exhibit special features of nanoparticles such as volume, surface and quantum effect. These characteristics cause nanomaterials to exhibit superior or unique mechanical, thermal, magnetic, electronic, optical and catalytic capabilities.

The grains will become somewhat more refined when common materials are mixed with nanoparticles. This will result in the formation of either an intergranular or an intragranular structure (Wu *et al.*, 2020). Among the metal nanoparticles,

AgNPs are known to be one of the most effective and attractive nanomaterials in many sectors, including food, healthcare, and industry, due to their special features like good electrical conductivity, optical, thermal conductivity, and biological aspects. Additionally, they were previously employed as antimicrobial agents and have enhanced the anticancer medications' ability to kill cancer cells (Zhang *et al*., 2016). Nowadays, AgNPs are widely utilised in textiles, medical equipment, keyboards and wound dressings. The formation of AgNPs involves a variety of reduction agents, which are essential for determining their cytotoxicity. Other factors that affect AgNPs' biological activity include surface chemistry, variation in size, morphology, arrangement, coating or capping, accumulation, and dissolving rate, as well as particle reaction in solution, ion release effectiveness, and the kind of cell (Zhang *et al*., 2016).

When cells that are abnormal develop and spread out of control due to a confluence of genetic, environmental, internal, and external causes, it is known as cancer, a complex and multifactorial disease. Options for cancer treatment include immunotherapy, targeted therapy, exposure to radiation, hormonal treatment, and chemotherapeutics. Significant problems with current cancer treatments include inadequate selectivity, undesirable side effects, and drug resistance (Takáč *et al*., 2023). AgNPs exhibit intrinsic antiproliferative activity, garnering increasing interest in cancer research due to their unique physical and chemical features. According to recent studies, AgNPs have anticancer properties due to their ability to slow down the growth of tumour cells. This may be a result of their inhibition of many signalling processes involved in the development and growth of cancer. Therefore, AgNPs are useful to prevent this problem because they can inhibit the growth of tumours and cause cytotoxicity to cancerous cells while remaining harmless to healthy cells (Gomathi *et al*., 2020).

The synthesis of AgNPs has often been accomplished using three distinct methodologies, *viz*., physical, chemical, and biological. The physical technique involves evaporation-condensation in a tube furnace that operates at ambient pressure to produce nanoparticles (Zhang *et al*., 2016). Physical approaches are faster, utilize radiation as a reducing agent, and do not require the use of harmful substances; however, they have negative aspects involving high use of energy, low yield, uneven distribution, and chemical contamination (Elsupikhe *et al*., 2015). On the other hand, AgNPs, which may be synthesised using chemical approaches, can use water or organic solvents in the manufacturing process. Three basic components are often used in this process reduction agents, capping agents or stabilizer, and metal precursors.

In general, the reduction of silver salts comprises two phases, which are nucleation and subsequent development. Two broad techniques can be used to obtain silver nanomaterials; 'top-down' and 'bottom-up' (Zhang *et al*., 2016). Chemical approaches are more efficient meanwhile, physical approaches are less effective, however, chemical reducing substances used in chemical approaches pose a threat to living things.

The ease of use, economical, reliability, and environmental friendliness of biological technologies make them a viable substitute to overcome the disadvantages of chemical techniques. There is now close attention to the use of nanotechnology in the biological system, for instance, fungal and bacterial, plant extracts, and other significant bioagents like minerals and proteins in producing AgNPs of specific sizes and high yield output. This approach can be applied to produce various nanoparticles, including gold and graphene (Gurunathan *et al*., 2014). Numerous studies have confirmed that biological techniques are cost-effective and biocompatible for producing AgNPs using green chemistry without toxic chemicals. Extracts from plants like *Allophylus cobbe*, *Artemisia princeps* and *Typha angustifolia*, as well as a number of bacteria and fungi, including *Pseudomonas stutzeri* AG259, *Lactobacillus* strains, *Bacillus licheniformis*, *Escherichia coli*, *Brevibacterium casei*, *Fusarium oxysporum*, *Ganoderma neo-japonicum* Imazeki have been applied to the green chemical technique (Zhang *et al*., 2016). One of the primary benefits of the biological technique is that the presence of proteins or secondary metabolites simplifies the synthesis of AgNPs by eliminating further procedures to avoid particle aggregation and utilising biological substances for AgNPs production is eco-friendly and non-polluting. Then, biological approaches also offer regulated particle dimensions and shape, which are significant when applied in the biomedical field.

Using plant extracts or bacterial proteins as reduction agents can influence the shape, dimensions, and monodispersity of the obtained nanoparticles (Gurunathan *et al*., 2014). Plant-based green synthesis is gaining popularity due to its safety, ecologically friendly, simplicity, and cost-effectiveness (Singh *et al*., 2016). Proteins, enzymes, tannins, phenols, sugars and flavonoids are plant metabolites that reduce, capping, or stabilising agents for producing nanometals (Duan *et al*., 2015). According to certain research, these substances show potential as bio-reducing agents to synthesis metal nanoparticles (Kartini *et al*., 2020) within the Phyllanthaceae family, Phyllanthus represents one of the largest genera with 11 subgenera and contains more than 700 different species that have been widely distributed throughout the tropics and subtropics worldwide (Abdel-Sattar *et al*., 2023). Recently, Phyllanthus species have emerged as prospective sources of natural anticancer drugs. *Phyllanthus niruri* (PN) is an herbal plant that can work both for reducing and capping agents during the production of AgNPs owing to the presence of the phenolic and flavonoid compounds (Anantharaman *et al*., 2020). In addition, PN has been shown to exhibit anticancer activities against hepatocellular carcinoma, prostate cancer, breast cancer, and lung cancer. Because of its inhibitory effects on cancer cells by altering several cell signalling pathways, studies have shown that it is harmless for normal cells (Paul *et al*., 2019).

The SK-N-SH neuroblastoma cell line is the source of the three-subcloned cell line known as SH-SY5Y. Because it can proliferate in culture for extended periods of time and is free of contamination, it is used as an in vitro model system for neuronal research. Furthermore, the SH-SY5Y cell line was extensively applied in neurological research laboratories, involving analyses of the growth of neurons, metabolism and functions relating to neurodegenerative disorders, neurotoxicity

and neuroprotection (Park *et al.*, 2017). This research examined the effectiveness of the synthesis of AgNPs using a *P. niruri* extract. Then, the cytotoxicity properties of the PN and AgNPs were investigated.

MATERIALS AND METHODS

Phyllanthus niruri Leaf Extract Preparation

The *P. niruri* leaves is a tropical and subtropical plant.The leaves were collected from Kepala Betas, Penang, Malaysia, washed 3-4 times using distilled water and were left to dry at ambient temperature for 10 days. The well-dried leaves were ground into powder. The powdered leaves were extracted with methanol (solvent) for 24 hours in the ratio of weight: solvent, 1:10. After being filtered using a separating funnel, the same volume of fresh solvent was used to extract the residue. The process was repeated for three straight days. Then, each extract was combined, and a rotary evaporator was used to dry the extract.

Green Synthesis of AgNPs using *P. niruri* Extract

The 1000 ppm stock solution was prepared by weighing 0.1 g $AgNO_3$ and diluting it with distilled water in a 100 mL volumetric flask. *P. niruri* (0.1 g) extract was dissolved in 2 mL of methanol and made up to 100 mL using double-distilled water. Further, 50 mL of $AgNO_3$ solution and 50 mL of *P. niruri* solution were mixed, and the resulting mixture was placed on a magnetic stirrer, vigorously stirring for 48 hours at 25 °C. The solution's colour changed from yellow to dark brown, indicating AgNP formation. The AgNPs solution was centrifuged at 6000 rpm for 20 minutes. The aqueous solution (upper layer) was removed, and the colloidal (bottom layer) AgNPs were collected for further study.

Characterisation of AgNPs Synthesis

UV-Vis spectroscopy

A UV-2600 spectrophotometer (Shimadzu, Japan) was used to perform the UV-Vis spectroscopic absorption analysis to verify the AgNPs' 48-hour formation. AgNPs. The UV-Vis absorbance of AgNPs samples was measured at various time intervals between 200 nm and 700 nm.

ATR-FTIR spectroscopy

The PN and PN-AgNPs solutions were analysed using the ATR-FTIR spectroscopy model Shidmadzu to analyse the chemical groups in the samples. The ATR crystal surface was cleaned using a solvent such as ethanol to remove any residual contaminants. Samples were applied thinly to the ATR crystal surface. The spectra were obtained for both PN and PN-AgNPs with a wavelength in the 4000-400 cm^{-1} region.

FE-SEM/EDX analysis

The AgNPs were analysed for their chemical composition and morphological characteristics using a Hitachi Regulus 8220+Oxford EDX Windowless 100 mm. The samples were prepared by placing a drop of colloidal solution on a glass slide wrapped with aluminium foil and dried at room temperature.

TEM analysis

The Zeiss Libra 200 FE (TEM) model was utilised to determine the AgNPs' average size and structure. On top of a copper plate, a drop of AgNP solution was dropped. Images of the specimen were captured after the material was left to dry at ambient temperature.

Cytotoxicity Activity

Cell culture conditions

Human neuroblastoma cell, SH-SY-5Y, was purchased from ATCC. In an incubator with 5% CO_2 and 90% relative humidity, the cells were cultivated in DMEM/F12 media supplemented with 10% FBS and 1% penicillin-streptomycin solution at 37 °C.

Cytotoxicity of formulation

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) technique was used to examine the samples' cytotoxicity. SH-SY5Y cells were seeded at a cell density of 20,000 cells per well in 96-well plates and treated with samples at concentrations ranging from 0.49 to 250.00 µg/mL for 48 hours. Wells with or without 0.1% DMSO were used as a control. The MTT solution (5 mg/mL) was added to the wells and incubated for an additional three hours after the corresponding incubation period. When the corresponding incubation period had passed, the medium was taken out and replaced with DMSO. The assay was carried out in the absence of light. Using a Multiskan Go UV microplate reader, the absorbance was measured at 570 and 650 nm wavelengths. This experiment was performed in triplicate using two separate assays. The following formula was used to determine the cells' viability (Md Zin *et al.*, 2022; Chia *et al.*, 2023).

$$\text{Percentage viable cell (\%)} = \frac{\text{Absorbance (Treated)} - \text{Absorbance (Blank)}}{\text{Absorbance (Untreated)} - \text{Absorbance (Blank)}} \times 100\%$$

In addition, an inhibitory concentration 50% (IC_{50}) for both samples were determined based on a dose-response graph plotted using GraphPad Prism (Md Zin *et al.*, 2022; Chia *et al.*, 2023).

RESULTS AND DISCUSSION

Synthesis of Silver Nanoparticles

P. nururi leaf extract was utilised as a reducing agent, and the colour change was observed visually, which confirmed the formation of silver nanoparticles. When PN leaf extract (Figure 1b) was added to the colourless silver nitrate solution,

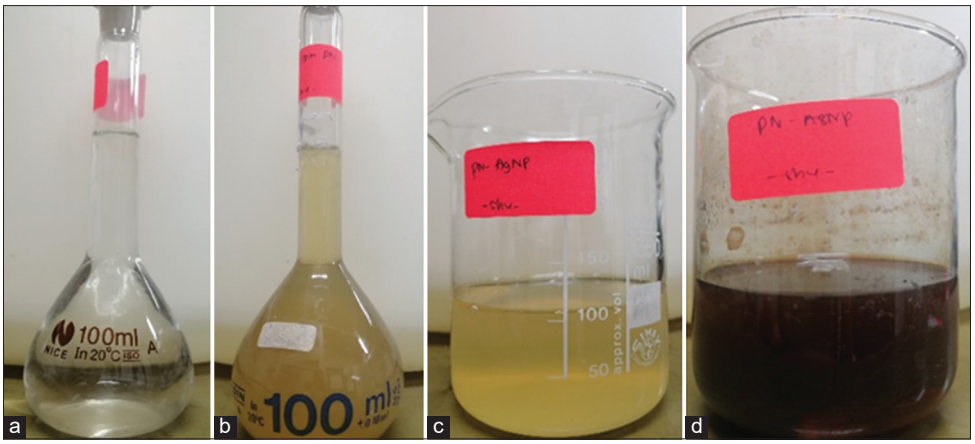

Figure 1: Solution of a) silver nitrate ($AgNO_3$), b) *Phyllanthus niruri* leaf extract, c) mixture of silver nitrate and *Phyllanthus niruri* - 0 min and d) silver nanoparticle (PN-AgNPs) - 24 hr

$AgNO_3$ (Figure 1a), it changed to light-yellow (Figure 1c). Figure 1d shows that the reaction mixture's light-yellow colour changed to a dark brown solution, indicating that the Ag^+ was reduced to Ag^0. This results from the synthesised AgNP's SPR being excited (Devi *et al.*, 2020).

Characterisation of Silver Nanoparticles

UV-Vis spectroscopy

UV-Vis spectroscopy was employed to further monitor the PN-AgNPs at the 200-700 nm wavelength region. The synthesised PN-AgNPs' spectra showed high absorbance within the 450-550 nm region with the increased time from 0 min to 48 hrs (Figure 2). Following 6 hr of continuous stirring, the maximum absorbance for converting silver ions to silver nanoparticles began. The free electrons in AgNPs simultaneously vibrate in resonance with the light wave to produce Surface Plasmon Resonance (SPR) absorption band (Kotaru & Korimelli, 2023). The formation of SPR bands depends on the size, shape, type of morphology, chemical composition and dielectric environment of the produced nanoparticles (Amalorpavamary *et al.*, 2019). The absorbance peak representing PN-AgNPs, seen at 500 nm in Figure 2, confirms the synthesis of AgNPs.

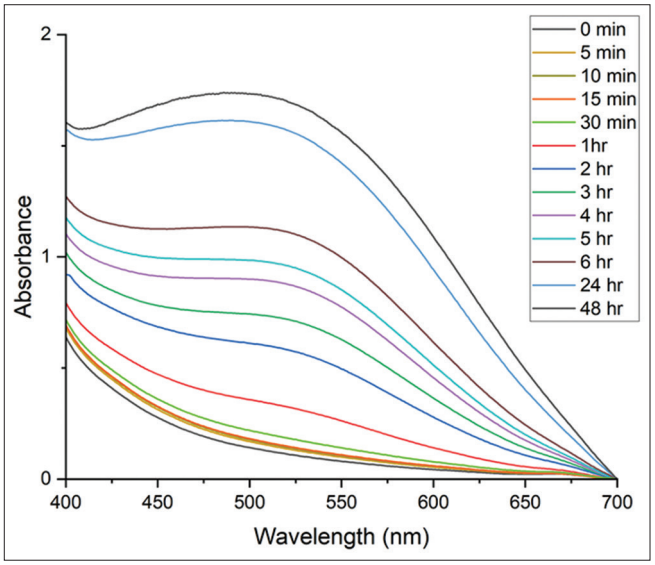

Figure 2: UV-Vis spectra of synthesised PN-AgNPs at different time intervals

ATR-FTIR spectroscopy

PN leaf extract has a functional group that contributes to the bio-reduction of silver ions and increases the stability of AgNPs. This functional group was identified using ATR-FTIR spectra. Figure 3 represents the spectrum of *P. niruri* leaf extract and synthesised AgNPs with absorbance bands between 500 cm^{-1} to 4000 cm^{-1}. The spectra showed broad absorption peaks at 3328 cm^{-1} and 3337 cm^{-1} in the PN leaf extract and PN-AgNPs, respectively, which correlate to O-H stretching with H-bond, indicating the presence of OH group (Amalorpavamary *et al.*, 2019).

Research conducted by Kaur and colleagues found that the chemical test enabled quick detection of phenolic compounds like phenols and flavonoids, alkaloids, and terpenoids in PN leaf extract. The presence of phenols can be detected using the ferric chloride test by observing the appearance of a blue colour, and the ammonia test revealed that the yellow colour of PN leaf extract had disappeared, indicating the presence of flavonoids. Then, Hager's test can be used to determine the presence of alkaloids, the formation of a yellow precipitate is observed, and the Salkowski test to determine the presence of terpenoids, the yellow colour of PN leaf extract changes to reddish brown (Kaur *et al.*, 2017).

The presence of phenolic OH can act as a reducing agent to reduce Ag^+ into Ag^0 to form AgNPs (Amalorpavamary *et al.*, 2019). The spectral bands of PN at 1637 cm^{-1} corresponded to C=C stretching (conjugated alkene). Besides, the PN-AgNPs synthesised from the PN leaf extract also showed peaks at 2924 cm^{-1} (C-H stretching, alkane), 2852 cm^{-1} (O-H stretching, carboxylic acid), 1625 cm^{-1} (C=C stretching, aromatic), 1445 cm^{-1} (CH_2 bending), 1358 cm^{-1} (O-H bending, phenol), 1206 cm^{-1} (C-O stretching vibration, alcohols, carboxylic acid, ester, and ether) and 1040 cm^{-1} (C-N stretching, aliphatic amines)

(Amalorpavamary *et al.*, 2019; Nandiyanto *et al.*, 2019). These bands are related to compounds such as alkaloids and terpenoids and are accountable for the effective stabilising and capping agent of obtained PN-AgNPs (Amalorpavamary *et al.*, 2019).

FE-SEM/EDX

FE-SEM research was performed to better comprehend the morphology of silver nanoparticles. As shown by the SEM pictures of PN-AgNPs in Figure 4, nanoparticles exhibited a range of sizes and shapes, from 11 to 63 nm at 100 k magnification. Nanoparticles were spherical in shape, with most of them having a size of less than 100 nm. Thus, the resulting particle size indicates that the silver ions have been converted into AgNPs. Additionally, EDX evaluation was carried out to verify that the particles were composed of Ag elements. Figure 5 shows the EDX spectrum of PN-AgNPs. It exhibits a distinct signal for the Ag element at 3 keV, which is caused by SPR. The small peaks belong to carbon, nitrogen, and oxygen molecules ranging from 0.2 to 0.5 keV, most likely corresponding to the surface biomolecules (capping structures) found in the *P. niruri* leaf extract (Kumar *et al.*, 2023). According to Table 1, silver has the highest weight percentage (88.46%) compared to other elements such as carbon (8.37%), nitrogen (0.85%) and oxygen (2.32%).

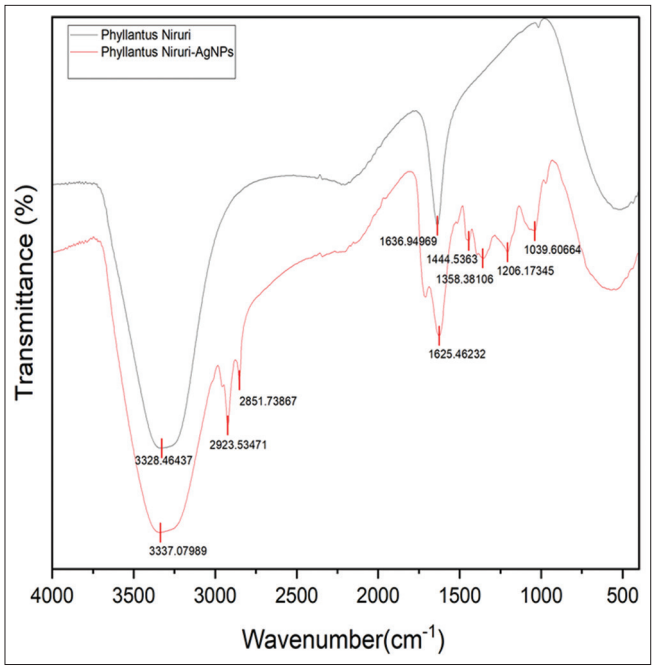

Figure 3: ATR-FTIR spectra of *Phyllanthus niruri* leaf extract and synthesised AgNPs

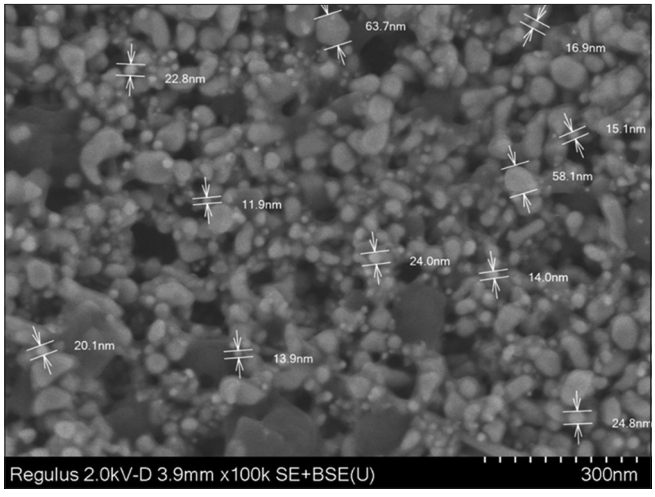

Figure 4: SEM images of silver nanoparticles mediated by *Phyllanthus niruri* leaf extract

TEM

The synthesised PN-AgNPs' morphology was further analysed in detail using TEM. Figure 6 shows the images captured by TEM at 195 k magnification. Based on the results, PN-AgNPs that were biologically synthesised have a spherical shape and range in diameters between 12 and 24 nm. The same pattern is also discernible in the SEM analysis, where the particle sizes vary from 11 to 63 nm. Furthermore, analysis of the data obtained from the TEM indicated that the formed PN-AgNPs have a mean diameter of 17.23 nm with spherical shapes. The AgNPs synthesised from *P. niruri* leaf extract had a shape and size similar to those of the AgNPs synthesised from acacia lignin

Table 1: Elemental composition of ***Phyllanthus niruri*** leaf extract mediated silver nanoparticles

Element	C	N	O	Ag	Total
Wt%	8.37	0.85	2.32	88.46	100.00

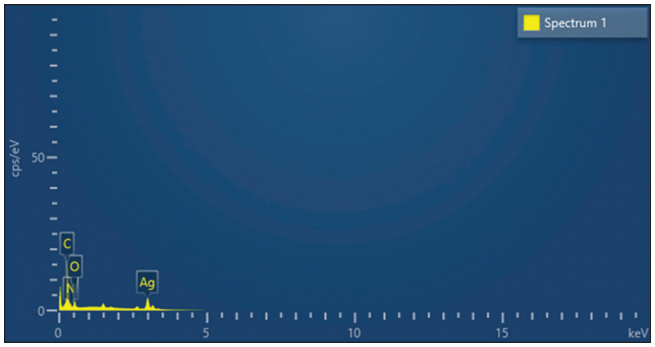

Figure 5: EDX spectrum of *Phyllanthus niruri* leaf extract mediated silver nanoparticles

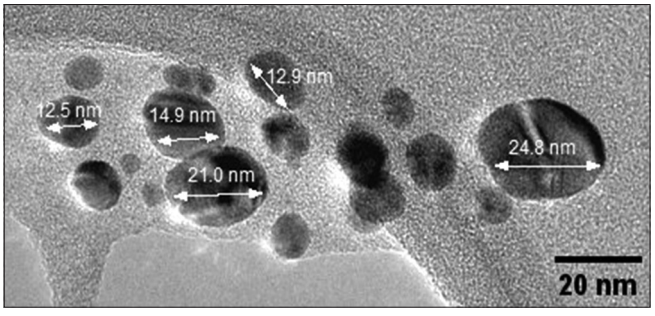

Figure 6: TEM image of *Phyllanthus niruri* leaf extract mediated silver nanoparticles

that had been previously reported (Aadil *et al.*, 2019). The study also found that the particles were unique and stable due to the absence of nanoparticle aggregation (Aadil *et al.*, 2019).

Anticancer Activity

In this study, a cytotoxicity test was applied to determine whether PN leaf extract and synthesised PN-AgNPs effectively killed cancer cells. The anticancer effect of PN and PN-AgNPs was tested against neuroblastoma cancer (SH-SY5Y) cell lines. PN leaf extract and PN-AgNPs were investigated for their cytotoxicity at concentrations between 0.49 µg/mL to 250 µg/mL for 48 hrs incubation time. By referring to the cell viability in Figure 7, both samples showed a concentration-dependent manner in cytotoxicity. PN leaf extract and PN-AgNPs showed a killing effect in the range of 7.81-250 µg/mL. However, adding silver to the extract enhanced the killing effect on SH-SY5Y cancer cell lines. The effect can be observed at 15.63-250 µg/mL.

Moreover, the effect of adding silver also showed a lower IC_{50} value where IC_{50} of silver nanoparticles (15.92±2.16 µg/mL) decreased half-fold as compared to PN leaf extract (42.77±7.71µg/mL) as shown in Table 2. The US NCI states that a compound's cytotoxicity activity is classified as high if its IC_{50} is less than 20 µg/mL, moderate if it is between 21 and 200 µg/mL, weak if it is between 201 and 500 µg/mL, and non-cytotoxic activity if it is greater than 500 µg/mL (Damasuri *et al.*, 2020). Based on these parameters, it is reasonable to determine that PN-AgNPs exhibiting an IC_{50} value of 15.92±2.16 µg/mL had high cytotoxic properties, whereas the PN leaf extract exhibiting an IC_{50} value of 42.77±7.71 µg/mL exhibited moderate cytotoxic properties on SH-SY5Y cancer cell lines.

Similar results were found in earlier studies utilising PN leaf extract in breast cancer MDAMB-231 cells and AgNPs against the human lung epithelial adenocarcinoma cell line and SH-SY5Y cell, indicating the dose-dependent cytotoxicity response of PN leaf extract and AgNPs (Dayem *et al.*, 2018; Hermansyah *et al.*, 2023). The cell toxicity significantly increases in proportion to the exposure dose of PN leaf extract and AgNPs. When the synthesised AgNPs are incubated with the cancer cells, the released Ag^+ ions from the nanoparticles initiate the production of ROS, resulting in enhanced oxidative stress in the cells, subsequent damage to the mitochondria and DNA, ultimately resulting in apoptosis (cell death) (Kovács *et al.*, 2022). Therefore, PN leaf extract and PN-AgNPs induced cell death by forming reactive oxygen species (ROS) in neuroblastoma cancer (SH-SY5Y) cancer cells.

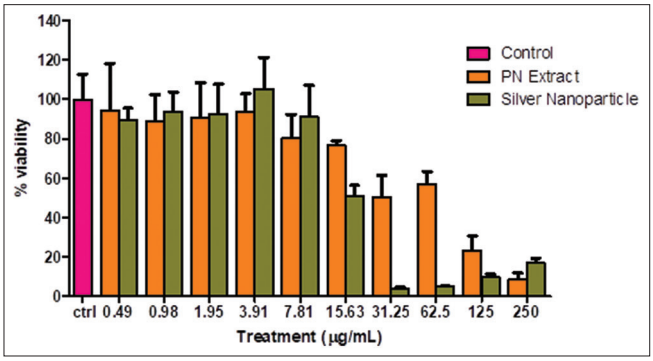

Figure 7: Cell viability of PN leaf extract and PN-AgNPs

Table 2: IC_{50} of PN leaf extract and PN-AgNPs towards SHSY-5Y cancer cells

Sample	IC_{50} (µg/mL)
PN leaf extract	42.77±7.71
PN-AgNPs	15.92±2.16

CONCLUSION

In this research, *P. niruri* mediated silver nanoparticles were successfully synthesised. The PN was effectively employed as a reducing agent to synthesise PN-AgNPs. The biosynthesised PN-AgNPs were assessed using UV-Vis, ATR-FTIR, FE-SEM/EDX and TEM analysis. SEM and TEM studies are evident that PN-AgNPs particles are in spherical shape with a size range of 11 to 63 nm and 12 to 24 nm respectively. The functional groups of alcohol function as a reducing agent and functional group of amine, carboxylic acid, ester, and ether serve as stabilising agent in synthesise AgNPs using PN leaf extract were also identified. The cytotoxicity analysis proves the PN-AgNPs have a substantially strong killing effect. In contrast, PN has a moderate killing effect when tested against neuroblastoma cancer (SH-SY5Y) cell lines, with IC_{50} values of 15.92±2.16 µg/mL and 42.77±7.71 µg/mL, respectively.

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