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Physicochemical Characterization, Cytotoxic Activity, and Caspase-9 Expression of Nanogold-Parijoto (*Medinilla Speciosa Reinw* .Ex, Bl) in Hela Cell Lines

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Abstract

Parijoto (*Medinilla speciosa*, Reinw. ex. Bl.), a tropical plant native to Southeast Asia, contains flavonoids, tannins, and saponins, which have the potential as an anticancer. Gold nanoparticle-based drug formulations are applied to increase the anticancer effectiveness of herbal medicines. The compounds in the stalk of parijoto have the potential to be bioreductor in the biosynthesis of gold nanoparticles. This study aims to determine the physicochemical characterization, cytotoxic activity, and expression of protein caspase-9 after treatment with nanogold parijoto (AuNPs-PR) on HeLa cell. The nanogold biosynthesis process was done by reacting 1 mM HAuCl4 with parijoto aqueous extract (EP). Physicochemical characterization measure of particle size, Polydisperse Index (PdI), and zeta potential of AuNPs-PR was carried out using a particle size analyzer. The cytotoxic effect and viability cell of AuNPs-PR were carried out using the MTT assay. The expression of caspase-9 was observed by immunocytochemistry assay. Physicochemical characterization of AuNPs-PR shows that the particle size value is 160.8 nm with PdI and zeta potential values of 0.430 and -4.56 mV respectively. In the MTT assay, both AuNPs-PR and EP demonstrated a reduction in the viability of Hela cells after 24 h in a dose-dependent manner, yielding IC_{50} values of 3.28 µg/mL and 19.22 µg/mL, respectively. AuNPs-PR and EP showed low cytotoxic activity against Vero normal cells, with IC_{50} values of over 500 µM. Further, the immunocytochemistry assay indicated that there was upregulation of caspase-9 by their expression. These results indicate that AuNPs-PR could effectively induce apoptosis in HeLa cells by upregulating caspase-9.

Keywords: *caspase-9, HeLa cells, MTT, nanogold, parijoto.*

INTRODUCTION

Cervical cancer is a type of cancer that Indonesian women suffer with an incidence rate of 9.3% and a death rate of 8.8% (WHO,

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2020). Chemotherapy using cisplatin is the most frequently used method for cancer treatment. However, cisplatin can cause side effects such as nausea and vomiting, neurotoxicity, and nephrotoxicity. Therefore, currently, many other alternatives have been developed, one of which is using herbal plants as anticancer agents (Romani, 2022). Several studies have shown that parijoto (*Medinilla speciosa* Reinw. Ex. Bl) is one of the Indonesian herbs that have cytotoxic activity against HeLa, WiDr, HepG2, and 4T1 cancer cells so it has the potential to be used as an anticancer agent (Sasikirana, *et al.*, 2021; Artanti, *et al.*, 2021). The methanolic and ethanolic extracts of parijoto fruit have cytotoxic activity against HeLa cells with IC_{50} values of 209.64 µg/ml and 233.43 µg/ml, respectively (Artanti, *et al.*, 2020). The ethyl acetate and n-hexane extract of parijoto has cytotoxic activity against HeLa cells with IC_{50} values of 352.9 µg/ml and 904.7 µg/ml, respectively (Winanta, *et al*., 2021). The combination of parijoto methanol extract (PME) with cisplatin (Cisp) demonstrated a strong synergistic effect, with a combination index value of less than 1. Flow cytometry test results revealed that the combined treatment of PME and Cisp significantly enhanced apoptosis induction compared to PME treatment alone (Artanti, *et al.*, 2022).

Gold (Au), a noble metal, is renowned for its resistance to corrosion and oxidation. These qualities have been recognized for centuries, as demonstrated by its extensive history of use in medicinal applications. Nanoparticles are a method that can be used to increase selectivity, drug delivery, effectiveness, and safety, thereby reducing dosage regimens and increasing patient compliance (Patil, *et al*., 2019). Recently, scientists have discovered that green-synthesized metallic nanoparticles from medicinal plants exhibit remarkable anti-cancer properties. These nanoparticles have gained significant attention in the medical field. Current research indicates that certain nanoparticles possess therapeutic properties, making them an excellent alternative to traditional metal-supported nanoparticles, antibacterial agents, and especially anticancer drugs (Shaneza, *et al*., 2018). Biosynthesis of gold nanoparticles using plant extracts is an eco-friendly method that leverages biomolecules from plants to reduce gold ions into gold nanoparticles. Plants contain various bioactive compounds such as alkaloids, flavonoids, terpenoids, polyphenols, and enzymes that can reduce metal ions and stabilize the formed nanoparticles. Recent advancements in the multi-functional design of gold nanoparticles (Au-NPs) enable the localized generation of heat near cancer tissues and facilitate the controlled, targeted delivery of multiple drugs. Au-NPs offer several advantages for photothermal cancer treatment, including the ability to be administered directly into the tumor area, minimizing non-specific distribution, activation by near-infrared (NIR) laser light, allowing for deep tissue penetration, the potential to be engineered for multifaceted photothermal therapy (PTT) and drug delivery systems (Vines, *et al.*, 2019).

Green-synthesized gold nanoparticles from medicinal plants, a distinct type of well-known metallic nanoparticles, have recently been utilized in the treatment of various tumors and cancers. The necessity of formulating the extract with Au-NPs lies in its potential to enhance the extract's bioavailability, stability, and targeted delivery. Au-NPs can improve the solubility and cellular uptake of the active compounds, increasing their therapeutic effectiveness. Au-NPs biosynthesized using cassava leaf extract were proven to have cytotoxic activity against T47D cells with an IC_{50} value of 42.47 µg/ml (Aprilia, *et al*., 2018). The best results of cytotoxicity and anti-ovarian cancer properties were seen in the case of Au-NPs of *Curcumae kwangsiensis* leaf aqueous extract. They had very low cell viability and high anti-ovarian cancer activities dose-dependently against PA-1, SW-626, and SK-OV-3 cell lines without any cytotoxicity on the normal cell line (HUVEC) (Chen, *et al*., 2021).

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Cancer is a highly prevalent disease marked by the uncontrolled growth of abnormal cells. One of the defining characteristics of cancer is the suppression of apoptosis (Hanahan & Weinberg, 2011). Abnormalities in apoptotic function are linked to cervical cancer and its resistance to chemotherapy and radiotherapy. Disruption in the regulation of apoptosis can enhance tumorigenesis and contribute to cervical cancer resistance (Kumar, *et al.,* 2018). Caspase is a pro-apoptotic protein marker for apoptosis involving the mitochondria. Cytochrome-c (Cyt-c) interacts with apoptosis protease-activating factor 1 and caspase-9 to form an apoptosome complex. The activation of caspase-9 and caspase-8 leads to the cleavage of caspase-3, the activation of endonucleases, and ultimately, nuclear DNA fragmentation, which is a hallmark of apoptosis (Redza-Dutordoir & Averill-Bates, 2016).

In this research, we aimed to investigate the effects of gold nanoparticles (Au-NPs) formulated using an aqueous extract from the stalk of *Medinilla speciosa* on ovarian cancer cell lines (HeLa cells) on caspase-9 expression.

MATERIALS AND METHODS

Synthesis of *Medinilla speciosa* Stalk Green Synthesized Au Nanoparticles (AuNPs-PR)

Parijoto stalk aqueous extract is made by filtering parijoto stalks that have been sorted and washed using distilled water at a temperature of 90˚C for 15 minutes. Biosynthesis and characterization of nanogold using gold solution ($HAuCl_4$) is made by dissolving gold metal with aquaregia ($HCl:HNO_3$=3:1) on a hotplate at a maintained temperature (60˚C–80˚C) until the gold is completely dissolved. Biosynthesis of nanogold was carried out by reacting the Parijoto stalk aqueous extract with 1 mM gold solution. The indicator for the formation of nanogold is a color change from yellow to a purplish tinge. Nanogold characterization was carried out using Particle Size Analyzer (PSA) (Malvervn,UK) to determine the size and distribution of nanogold.

Cell Culture

Hela (ATCC® CCL-2) cervix cancer cells were acquired from the Medicine Faculty at Universitas Sebelas Maret, Indonesia. The cells were grown in RPMI medium containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, Missouri, USA), 1 % Penicillin-Streptomycin (Sigma), 0.5% Fungizone (Sigma), maintained at 37°C in an atmosphere of 5% CO_2. Vero (ATCC® CCL-81) normal kidney cells were acquired from the Medicine Faculty at Universitas Gadjah Mada, Indonesia. The cells were grown in DMEM High Glucose medium containing 10% FBS (Sigma), 1 % Penicillin-Streptomycin (Sigma) and 0.5% Fungizone (Sigma), maintained at 37°C in an atmosphere of 5% CO_2. Both cells were subcultured by employing trypsin-EDTA (Gibco, Invitrogen, USA) to facilitate their detachment. The substance utilized in the study was Cisplatin 50 mg/50 mL (Kalbe, Indonesia).

Cytotoxicity Using 3-(4,5-dimethylthiazol-2-yl)-2,5-2,5-diphenyltetrazolium (MTT) Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-2,5-diphenyltetrazolium bromide (MTT) assay was employed to evaluate cytotoxicity. MTT is a yellow tetrazolium compound. Living cells absorb it, and mitochondrial dehydrogenases convert it into an insoluble, pigmented substance known as formazan. This transformation occurs in metabolically active cells (Ghasemi, *et al*., 2021). Initially, a total of 1x104 Hela cells were seeded into each well of a 96-well plate. The following day, the cells were exposed to concentration 100; 50; 25; 12.5; 6.1; 3.5 and 1.7 of AuNPs-PR, EP, gold solution, and cisplatin for a duration of 24 h. Subsequently, 100 µL of 5 mg/mL MTT reagent was added to the cells and incubated for 2-4 h. Following incubation, a stopper solution containing SDS and 0.01N HCl was added

to each well. These solvents assist in dissolving the formazan crystals formed within the cells, resulting in a colored solution. After the formazan crystals dissolve, the absorbance of the resulting colored solution is measured using a spectrophotometer, usually at a wavelength of approximately 595 nm, and the cell viability percentage is subsequently measured using an ELISA reader, and the cell viability percentage was calculated based on the absorbance data (Mosmann, 1983). The IC_{50} value was established by using linear regression analysis to correlate the sample concentration with the cell viability percentage (Reynold & Maurer, 2005).

Observation of Caspase-9 Protein Expression Using Immunocytochemistry

Hela cells ($5x10^4$ cells/well) were plated on coverslips in 24-well plates and incubated for 24 h at 37°C with 5% CO_2. Subsequently, AuNPs-PR at concentrations of 2 µM and 1 µM were added to the cells and incubated for another 24 h under 5% CO_2. The cells were then washed with PBS and fixed on glass slides for 5 minutes, followed by treatment with H_2O_2 to block non-specific binding sites for 10–15 minutes at room temperature. After two washes with PBS, the cells were incubated with caspase-9 (Santa Cruz Biotechnology, dilution 1:500) antibody for 1 h at room temperature. Following washing three times with PBS, a secondary antibody dilution 1 of 500 (Biotinylated universal secondary antibody number catalog sc-133109) (Santa Cruz, USA) was added and incubated for 10 minutes at room temperature, followed by washed two times with PBS. 3,3-diaminobenzidine was added as a chromogen and incubated for 3–8 minutes. The cells were then washed with distilled water, treated with hematoxylin solution, and incubated for 5 minutes at room temperature. The expression of caspase-9 was observed and documented under a light microscope in 10 fields of view for each treatment group. Cells expressing caspase-9 exhibited a brown color, while cells lacking caspase-9 showed a purple color.

Data Analysis

The data were presented in the format of mean ± Standar Deviation (SD) and analyzed using SPSS 21.0 software. One-way ANOVA was used to assess the statistical significance of differences between the untreated group and various treatment groups. The respective *p*-values (*$p<0.05$; **$p<0.01$) have been provided within each figure in the experiments.

RESULT

Particle Size Analyzer of *Medinilla speciosa* Stalk Green-Mediated Au-NPs

The resulting nanogold showed good homogeneity and size. The polydispersity index (PdI) indicates the uniformity of particle size. The smaller the polydispersity Index (PdI) value, the better the homogeneity of particle size (Hajrin, *et al*., 2021). The polydispersity index (PdI) value reflects the uniformity of the size of gold nanoparticles in a liquid medium. A lower PdI value indicates a better particle size distribution within the sample, as the PdI value represents the size distribution of nanoparticles dispersed in the gold nanoparticle sample. In this study, the gold nanoparticles synthesized from the infusion of parijoto leaves (*Medinilla speciosa* Folium) exhibited a homogeneous particle size distribution, indicated by a PdI value close to 0. Conversely, a particle size distribution is considered highly heterogeneous if the PDI value exceeds 0.5 (Avadi, *et al.*, 2010). Nanoparticles with a uniform size distribution (low PdI) tend to have more consistent sizes, which can influence how they interact with cell membranes and endocytosis mechanisms. An optimal particle size is usually more effective for cellular penetration, and a uniform size distribution ensures that most nanoparticles are of the right size for penetration (Lee & Kim, 2018).

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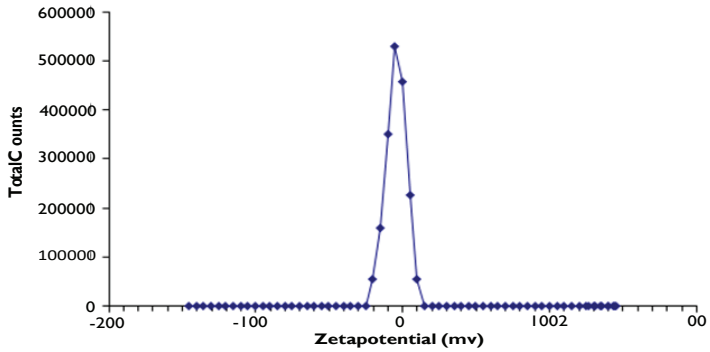

Figure 1. Profile zeta distribution data.

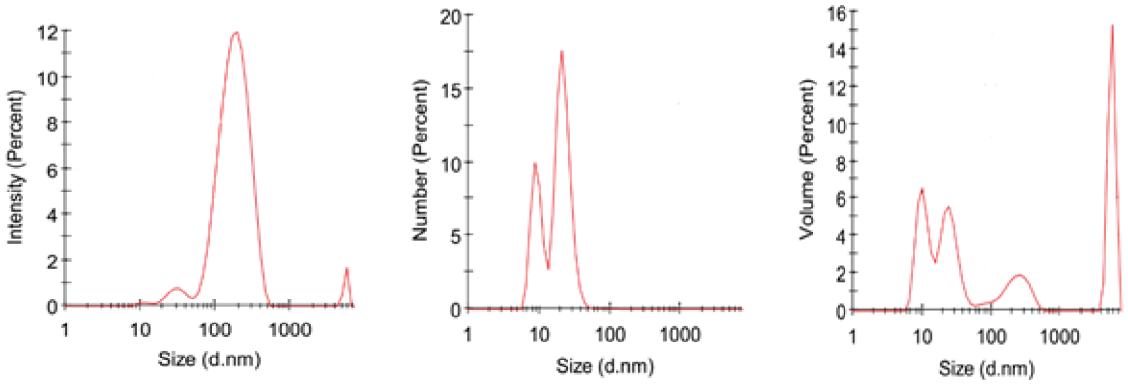

Figure 2. Profile of size distribution by intensity, size distribution by number, size distribution by volume.

Table 1. Characterization data using the *particle size analyzer*.

Parameter	Value Range Based on Literature	Results
Z-Average	1-1000 nm (Shaneza, *et al*., 2018)	160.8 nm
PdI	< 0.5 (Vines, *et al*., 2019)	0.430
Zeta Potential	±30 mV (Aprilia, *et al*., 2018)	-4.56±6.72 mV

Effect of AuNPs-PR, Cisp, and EP on Cell Viability of Hela cells and Vero Cells

Initially, we assessed the cytotoxic effects of AuNPs-PR, Cisp, and EP on both HeLa and Vero cells. HeLa cells served as a model for cervical cancer, whereas Vero cells were used to represent normal kidney cells. The potential cytotoxicity from AuNPs-PR, Cisp, and EP on Hela and Vero cells was measured by using the MTT method. The parameter used is the IC_{50} value, which shows the concentration value of a compound that results in 50% inhibition of cell proliferation and the potential for oxidation of a compound into cells. HeLa cells were observed using an inverted microscope with 10x magnification to observe differences in morphology before and after treatment. In general, HeLa cells that have not been treated have a polygonal or round shape and are clustered with widened nuclei, while dead HeLa cells have an irregular round shape and are not clustered (Figure 3). AuNPs-PR was applied at concentration1.72 to 100 µg/ml leading to a dose dependent, y=13.274x+43.149 with coefficient of correlation (R2)=0.9701. The calculated IC_{50}

value for this effect was 3.28 µg/ml, indicating that it exhibit a high level of cytotoxicity (Prayong, *et al*., 2008). Interestingly, when administered at concentrations up to 500 µM, it did not cause cytotoxicity in Vero cells, exhibiting an IC_{50} value >500 µM. The Selectivity Index (SI) is a measure to assess the safety and specificity of a compound. It is calculated as the ratio of the cytotoxic concentration that adversely affects normal cells to the effective concentration that inhibits or eliminates cancer cells (van Loggenberg, *et al*., 2022).

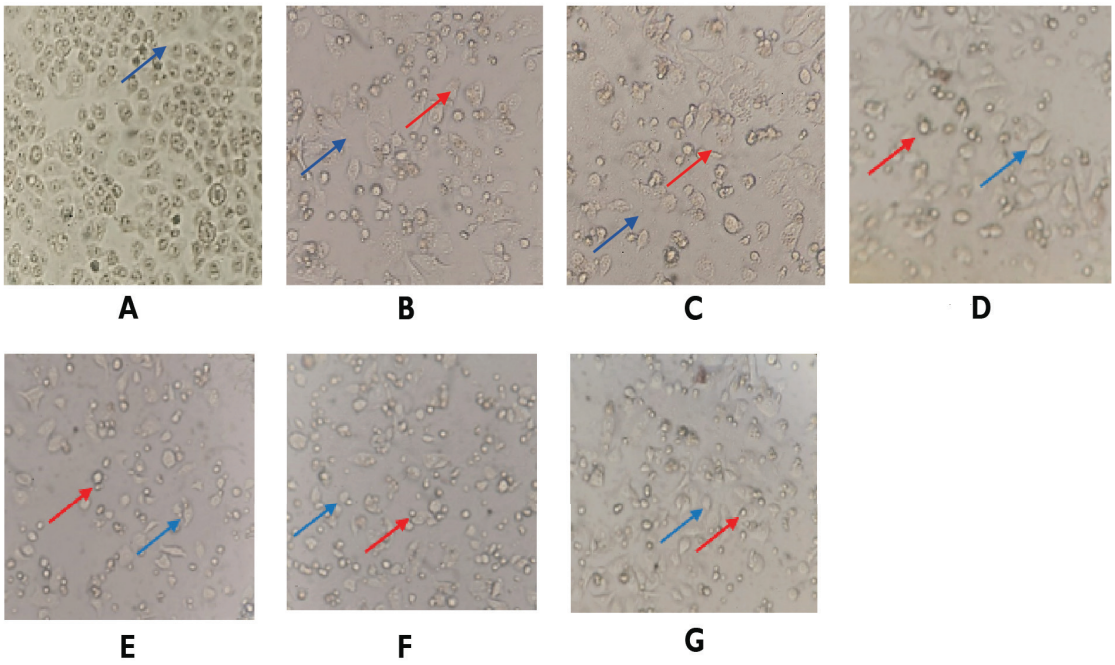

Figure 3. Profile of HeLa cells observed with an inverted microscope at 10x magnification (A) control cells (B) 25 µg/ml AuNPs-PR (C) 50 µg/ml AuNPs-PR (D) 25 µg/ml EP (E) 50 µg/ml EP (F) Cisp 25 µg/ml (G) Cisp 50 µg/ml Description: (——▶) : living cells; (——▶) : dead cells.

Expression of Caspase-9 protein treated AuNPs-PR, Au-NPs, EP, and Cisp on Hela cells

Caspase is a type of enzymes that break down crucial proteins within cells, triggering the process of apoptosis. As illustrated in Figure 5, the treated cells displayed a high-intensity of brown color when exposed to AuNPs-PR, showing caspase-9 expression. In the control group, caspase-9 expression was relatively low, indicating minimal apoptosis under normal conditions. In the cisp, AuNPs and EP groups, moderate caspase-9 expression was observed compared to the control. This suggests that the compounds may induce some degree of apoptosis, but it is less effective than AuNPs-PR. The AuNPs-PR 2 µg/ml group, showed the highest levels of caspase-9 expression. This significant increase indicates that AuNPs-PR can induce apoptosis in Hela cell lines by increasing the levels of caspase-9 protein.

DISCUSSION

In the recent research, Au-NPs were prepared using an aqueous extract of parijoto (*Medinila speciosa*). Phytochemical compounds found in plants, such as polyphenols, flavonoids, sugars, enzymes, and proteins, can be used as reducing and stabilizing agents in the biosynthesis

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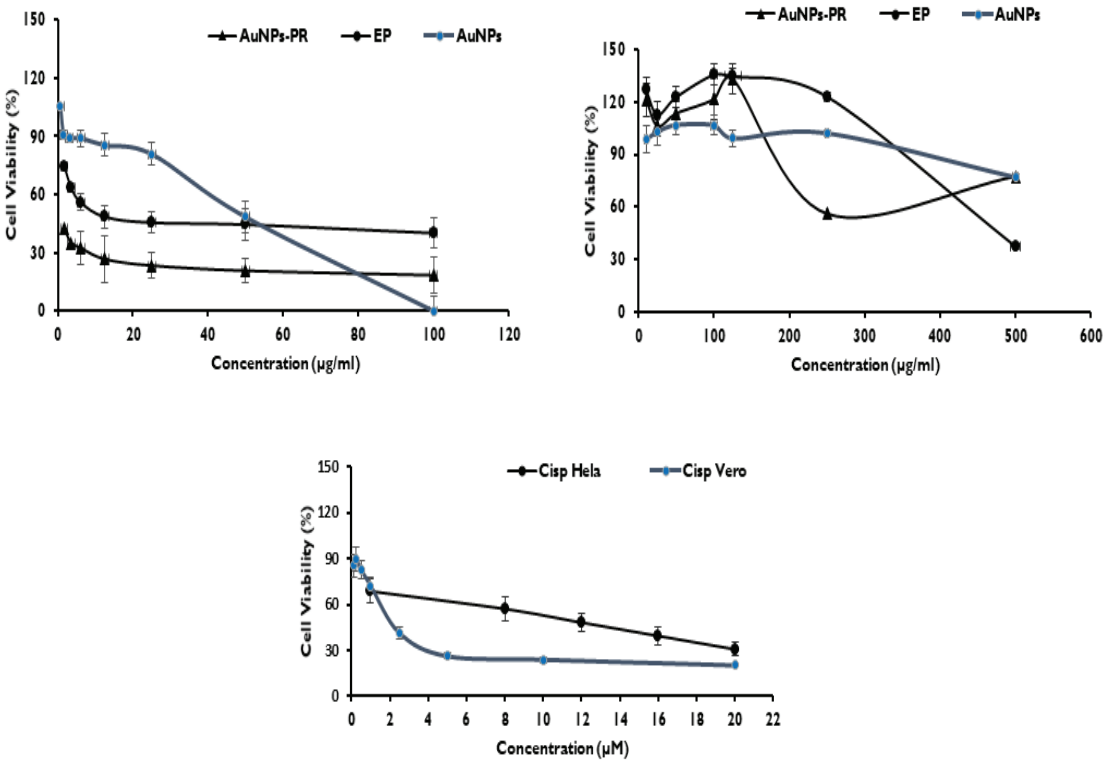

Cell line	IC_{50} value and Selectivity Index (SI)				
		AuNPs-PR (µg/ml)	AuNPs (µg/ml)	EP (µg/ml)	Cisp (µM)
Hela	IC_{50} value	3.28	36.18	19.22	10.85
	SI	>2	>2	>2	0.28
Vero	IC_{50} value	>500	>500	>500	3.1

Figure 4. Effect of AuNPs-PR, EP, AuNPs, and Cisp on the viability of Hela and Vero cells. Cells were cultured in increasing concentrations of AuNPs-PR, EP, and AuNPs (1.7-100 µg/ml) on Hela cells (A) and (10-500 µg/ml) on Vero cells (B). The cell viability was assessed by MTT assay in triplicate (n=3). The IC_{50} value and Selectivity Index (SI) of AuNPs-PR, EP, AuNPs, and Cisp. Data represent the means ±SD.

of metal nanoparticles, replacing chemicals that potentially cause environmental hazards and damage (Avadi, *et al*., 2010). Secondary metabolites present in the infusion of parijoto stems, such as tannins, saponins, and flavonoids, have shown a function to reduce Au^{3+} ions in $HAuCl_4$ to neutral Au ions (Au^0) (Lestari & Cahyadi, 2022). The reduction of Au^{3+} to Au^0 is necessary because Au^+ in its ionic form can cause repulsive reactions due to the influence of the same ionic charge. After the reduction reaction, Au ion charge becomes neutral (Au^0), allowing Au atoms to interact with each other to form nano-sized clusters through metallic bonds (Vifta & Advistasari, 2018).

The zeta potential results showed that there was an aggregation potential of Au nanoparticles as it had a zeta potential value far from the value (+/-) 30 mV (Table 1). The aggregation of nanoparticlesallows possible precipitation and flocculation to occur during long-term storage (Wahab, *et al.*, 2020). However, it needs to be seen whether the aggregation potential affects the

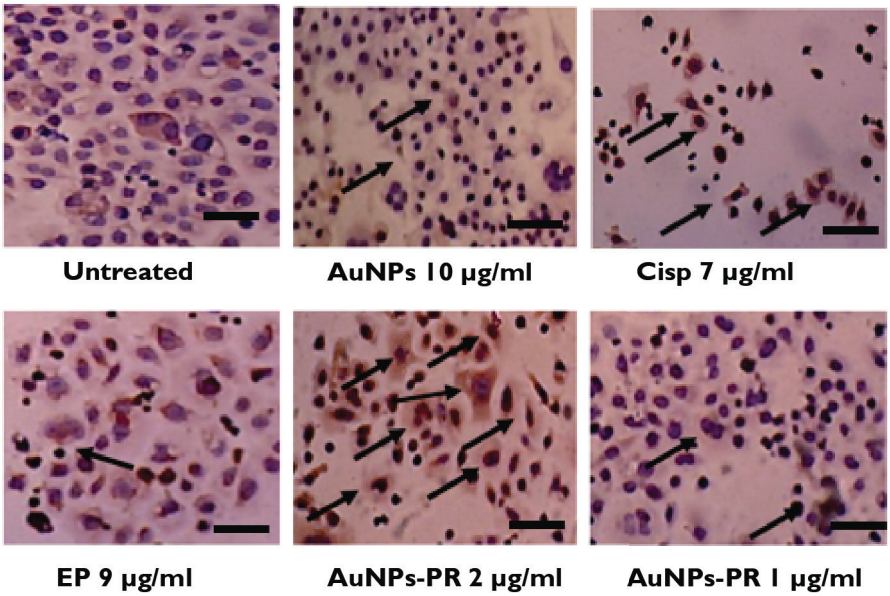

Figure 5. Expressions of caspase-9 after treatment with AuNPs-PR, Au-NPs, EP, and Cisp observed by immunocytochemistry assay in HeLa cells (magnification 200x).

activity of the Au nanoparticles. In general, the zeta potential value is influenced by several factors such as the pH of the medium, the type of functional groups present in the reductant, and the molecular weight of the desired material (Pertiwi, *et al*., 2018). The addition of buffers and pH adjustments can be considered to increase the stability of parijoto stem-infused nanogold (Ostolska & Wisniewska, 2014. Therefore, optimizing the nanogold formula by adding a buffer to minimize flocculation is necessary. Zeta potential analysis assesses the surface charge of metallic nanoparticles following synthesis. A negative zeta potential measurement suggests potential for molecular binding, indicating a stable structure in AuNPs. The average zeta potential values for AuNPs-PR was -4.56 mV. Nanoparticles with lower negative charges can more readily penetrate cells and engage with cellular components, offering distinct advantages in studies involving pathogenic cancer cells. The efficacy of synthesized Au-NPs in anticancer activities hinges significantly on their surface charge (Chen, *et al*., 2016). Zeta potential is a key parameter in understanding the surface charge of nanoparticles and plays a crucial role in determining their stability, interaction with biological membranes, and overall effectiveness in cellular penetration. Nanoparticles with a positive zeta potential can interact more strongly with negatively charged cell membranes through electrostatic attractions, enhancing their uptake. Conversely, negatively charged nanoparticles might experience repulsion but can still penetrate cells via specific uptake mechanisms like endocytosis (Sweeney & Martin, 2014).

Cancer cells often have altered programmed endocytosis mechanisms that can allow negatively charged nanoparticles to enter more efficiently compared to normal cells (Sweeney & Martin, 2014). The gold nanoparticles synthesized from the parijoto leaf infusion demonstrated particle sizes within the nanometer range, specifically less than 1000 nm. Gold nanoparticles are generally considered biocompatible, meaning they do not cause toxic reactions in the cells being tested. This allows the use of gold nanoparticles as drug carriers or diagnostic agents without compromising cell health (Wang, *et al*., 2013). The nanometer size of gold nanoparticles enables them to penetrate cell

membranes and enter cells easily. This is crucial in cytotoxicity testing to ensure that the nanoparticles and their carried drugs can reach intracellular targets (Bhattacharya & Mukherjee, 2008).

In this study, we examined the cytotoxic potential of gold nanoparticles synthesized using parijoto (*Medinilla speciosa*). The impact of various concentrations (10-100 µg/mL) of AuNPs-PR on Hela and Vero cell lines was studied using an *in vitro* MTT assay. The results revealed that the biosynthesized AuNPs-PR exhibited cytotoxic activity against Hela cell lines. After 24 h, the data showed no toxic effect on normal Vero kidney cells. Gold nanoparticles are internalized by human cells without inducing acute cytotoxic effects (Connor, *et al*., 2005). AuNPs-PR with a concentration of 3.2 µg/mL reduced cancer cell viability by 52%. Despite increasing AuNPs-PR concentrations in Hela cells, the observed increase in cell viability percentage is attributed to the antiproliferative properties of AuNPs-PR for Hela cells (Keskin, *et al*., 2022).

The mechanism of cytotoxic effects caused by AuNPs-PR administration was explored by identifying the protein target of caspase-9. Caspase-9 triggers apoptosis via the intrinsic pathway, which can be activated by various stimulants such as chemotherapy and radiation. This process involves the formation of an apoptosome complex, which contains zymogen monomers that activate caspase-9. Caspases, a group of cysteine-aspartate proteases, are crucial in apoptosis, with caspase-9 acting as an executor of apoptosis. Caspases play essential roles in carcinogenesis, neurodegeneration, immunodeficiency, and autoimmunity (Li, *et al*., 2019). The administration of AuNPs-PR led to increased expression of caspase-9, indicating apoptotic induction via the mitochondrial pathway. Immunocytochemical tests demonstrated that administering 2 µg/mL of AuNPs-PR enhanced the expression of caspase-9 protein. The positive cells, which were stained brown, were observed in the cytoplasm but not in the nucleus. The increased presence of caspases may result from the anticancer effects of AuNP-PR, which induces apoptosis. AuNPs, known for their easy synthesis and biocompatibility, show promise as nanocarriers, enhancing the anticancer properties of the stalk of *Medinilla speciosa*.

CONCLUSION

In summary, AuNPs-PR demonstrate strong cytotoxicity against HeLa cell growth, while exhibiting low cytotoxicity towards the Vero normal kidney cell line.AuNPs-PR could be effective in reducing Hela cells by upregulation of caspase-9. Nevertheless, the underlying molecular mechanism of AuNPs-PR needs to be clarified. In conclusion, the overall findings suggest that AuNPs-PR has the potential to function as a co-chemopreventive agent and may also be an alternative option for maintaining health in normal kidney cells.

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