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Biosynthesis, Cytotoxicity and Antimicrobial Effect of Silver Ganoparticle from Polysaccharide Extract of *Ganoderma lucidum*

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***Ganoderma species are one of the most widely studied medicinal mushrooms due to their potent bioactive properties among which* edible mushroom *Ganoderma lucidum* (Reishi) is being widely used for the promotion of health and longevity in Asian countries. The present paper reports the rapid synthesis of silver nanoparticles (Ganoparticles) using *Ganoderma lucidum* polysaccharide (GLP) extract. Biosynthesis process was effective and silver ganoparticles were formed after 120 h. MTT cell proliferation assay and trypan blue exclusion test were used to study the antitumor activity of ganoparticles on human lung cancer cell lines (A549 and NCIH520). Cell growth inhibition was found to be increasing with increasing concentration of ganoparticles, while cell viability was found to be decreasing with increasing concentration of ganoparticles. Ganoparticles and crude extract inhibited the cell proliferation in dose and time dependent manner. However, efficacy of ganoparticles was found to be higher as compared to GLP crude extract. Ganoparticles were also found to possess potent antibacterial activity against both gram negative and gram positive bacterial strains. *In-vitro* inhibitory and apoptotic effect of ganoparticles and GLP crude extract on human lung cancer cell lines indicate that GLP may potentially serves as a chemopreventive agent for cancer therapy.**

Keywords: Ganoparticles, Lung cancer, *Ganoderma lucidum*, cytotoxicity, antimicrobial.

Ganoderma lucidum is a basidiomycetous fungus which has been used as a medical remedy in China and Japan for centuries (Kim *et al*., 1990). Traditionally known as the God of fungi, it has been used for almost everything and said to work for everything. *G. lucidum* has properties often associated with health and healing and is considered to preserve the human vitality to promote longevity (Shiao *et al*., 1994). *Ganoderma* has a large amount of bioactive molecules and there is no single molecule in this mushroom that can be said to be the main bioactive component (Borchers *et al*., 1999). The components of this mushroom reported to date are triterpenoids, polysaccharides, proteins, minerals, phenols, nucleotides and their derivatives, glycoproteins and Sterols (Chang, 1996). It contains 1.8% ash, 26–28% carbohydrate, 3–5% crude fat, 59% crude fiber and 7–8% crude protein (Hobbs, 1995).

Mushroom proteins contain all the essential amino acids and are especially rich in lysine and leucine. The low total fat content and high proportion of polyunsaturated fatty acids relative to the total fatty acids of mushrooms are considered significant contributors to the health value of mushrooms (Chang, 1996).

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Polysaccharides, Peptidoglycans and triterpenes are three major physiologically active constituents in *G. lucidum* (Boh *et al*., 2007). Different compounds that includes Polysaccharides, triterpenoids etc with various biological activities are extracted from mycelia, the fruiting bodies or spores of *G. lucidum.* However, more than 100 types of polysaccharides isolated from this medicinal mushroom till date, comprise the major source of its biological activity and are linked to possible therapeutic effects (Boh *et al., 2007) (*Lindequist *et al*., 2005).

G. lucidum is a popular remedy to treat conditions like chronic hepatitis, hypertension, arthritis, insomnia, bronchitis, asthma, gastric ulcer, diabetes and cancer. It possesses anti-tumor activity and has also been found to inhibit platelet aggregation and to lower blood pressure, cholesterol and blood sugar (Borchers *et al*., 2004). *Ganoderma* may affect different stages of cancer development: by inhibition of angiogenesis (formation of new tumor-induced blood vessels, created to supply nutrients to the tumor), by inhibiting migration of the cancer cells called metastasis or by inducing and enhancing apoptosis of tumor cells (Paterson *et al* .,2006). In clinical trials conducted on humans over the last 40 years, *G. lucidum* has been used to treat a wide variety of disorders (Sanodiya *et al*, 2009), including: 1) Nervous system disorders, dizziness, insomnia, anxiety and stress-related concerns. 2) Respiratory tract conditions and asthma. 3) Duodenal ulcers, leukopenia, progressive muscular dystrophy. 4) Mental disease caused by environmental stress and Alzheimer's disease. 5) Hyperlipidemia, diabetes, liver disease and hepatitis.

Nanoparticles are one of the novel drug delivery systems, which can be of potential use in controlled and targeted drug delivering. Due to limited selectivity accompanied by toxicity to normal cells, targeted drug delivery systems can be a suitable solution for most anticancer drugs (Zhao *et al*., 2007). Nanoparticle approaches to targeted drug delivery for malignant tumours offer new opportunities to improve patient care and quality of life by reducing toxicity related issues (Karwa *et al*., 2011). Receptor mediated cellular uptake of nanoparticle could be achieved by attaching suitable ligands that recognize tumour associated antigens. The current knowledge on *Ganoderma* can be exploited to design polymer-*Ganoderma* conjugate as a tool for sustained and targeted drug delivery. So *Ganoderma* based nanoparticle (ganoparticles) can be designed for its targeted and sustained release at tumour sites.

Cancer is a worldwide leading cause of death and despite of comprehensive advances in the early diagnosis of the disease and chemotherapy, it remains a major clinical challenge (The cancer cure foundation, 1976). Chemotherapy using cytotoxic anticancer drugs is being practiced but has a lot of side effects and offers little survival benefits for patients. So there is a need to use alternative natural medicine with lesser side effects to cure Cancer. For that reason there has been a search for new chemopreventive and chemotherapeutic agents for which hundreds of plant species including Mushrooms have been evaluated. This has resulted in the isolation of thousands of bioactive molecules that have shown to be having antitumor activity from numerous mushroom species including *Ganoderma* species. *Ganoderma* extract is an active constituent of medicinal fungus *Ganoderma lucidum* and has proved to have numerous pharmacological activities and can act as a potential anticancer agent (Sliva, 2003). Keeping the above fact in view, the aim of the present study was to generate nanoparticles (named as ganoparticles) using *Ganoderma lucidum* extract and to evaluating its antitumor activity.

MATERIALS AND METHODS

Microorganism and culture maintenance

G. lucidum strain used in the present study was procured from "National Research Centre for Mushrooms", Solan, H.P, India. The stain was aseptically transferred to fresh Potato Dextrose Agar (PDA) slants followed by incubation at 25^0C until confluent growth was achieved. Potato Dextrose Agar media was used for the growth of *G. lucidum*. These slants were maintained in the active stage by transferring mycelial disks (plugs) aseptically on fresh plates at regular time interval and were stored at 4^0C.

Biomass Production

A 6mm Culture plug was taken from the growing edge of a 3 day old culture and were

transferred to a conical flask containing 100 ml of PD Broth followed by incubation for a period of 7 days under sterile conditions (Vahabi *et al*., 2011) [20]. After the growth as in a disk form above the PD Broth, the mycelial biomass was separated from the culture medium by filtration through Whatmann's filter paper. The mycelial biomass was then rinsed with water until the water ran clear and the resulting biomass was dried in an oven at 60^0C for 24 hrs.

Hot water aqueous Extraction

The dried mycelial disk was weighed and grinded to a fine powder using a mortar and pestle. Five grams of dried mycelial powder was treated with hot water for polysaccharide extraction (Chang *et al*., 2004). Dried biomass powder was transferred into a 250 ml beaker,followed by addition of 100ml water, the mixture was heated at 95 to 100^0C for 2 hours with continuous stirring. After 2 h the filtrate was separated from the mycelial biomass by filtration using Whatmann's filter paper.

Generation of Ganoparticles

Biosynthesis of silver ganoparticles was carried out by taking 10g wet biomass of *G. lucidum* fungus in 100 ml aqueous solution of 1 mM silver nitrate ($AgNO_3$) (Ahmad *et al*., 2003). The mixture was placed in an orbital Shaker with a temperature of 28^0C for an incubation period of 120 h with a agitation speed of 100 RPM. Silver ganoparticles were produced through reduction of the silver ions to metallic silver. The pale yellow colour of the fungus cells in the reaction mixture was changed to brownish colour, the well known yellowish-brown color of silver nanoparticles arises due to excitation of surface plasmon vibrations (essentially the vibration of the group conduction electrons) in the silver nanoparticles. The appearance of a yellowish brown color in solution containing the biomass is a clear indication of the formation of silver nanoparticles (ganoparticles) (Duran *et al*., 2005).

Antibacterial assay

Anti-Bacterial activity was analyzed by agar diffusion well variant Method on four of the bacterial species: *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis* and *E.coli*.

Agar well diffusion method

The zone of inhibition of silver ganoparticles was measured by the agar well diffusion assay (Burns *et al*., 2000). The inoculum suspensions of the test microorganisms were prepared by using 16 h old cultures adjusted to 108 cfu/mL by referring the 0.5 McFarland standards. Total 20 mL of Nutrient agar medium was poured into each petri plate, and then plates were swabbed with 100 µL inoculum of the test microorganisms and kept for 15 min for adsorption. Wells were bored into the seeded agar plates using a sterile cork borer of diameter (8 mm), and these were loaded with a 100 µL volume with concentration of 5.0 mg/mL of ganoparticles. The incubation of all theplates was carried at 37 °C for 24 h. antimicrobial activity against the selected organisms was evaluated by measuring the zone of inhibition with zone reader (Hi antibiotic zone scale).

Anti-Tumor Assays

MTT Assay

Two lung cancer cell lines A549 & NCIH520 were used to examine the characteristics of MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) which is reduced by metabolically active cells by the action of dehydrogenase enzymes to generate reducing equivalents such as NADH and NADPH. The resulting purple formazan can be solubilized and quantified by spectrophotometer (Panchanathan *et al*., 2013).

For most tumor cell lines 5,000 cells per well is required to perform proliferation assays, hence viable cells of cell lines A549 and NCIH520 were seeded in 96-well plates in RPMI-1640 Medium and were kept for incubation overnight. When cells reached more than 80% confluence, the medium was replaced and cells were incubated with different concentrations 5,10, 15, 20, 25, 30, 35 and 40¼g/100¼l of Ganoparticle solution and Crude polysaccharide extract taken from the Stock solution 5mg/ml. The ganoparticle solution and crude polysaccharide extract, at a concentration of stock (5mg/ml) were added as the positive control and cells containing only media were used as blank wells. After 24hrs, the supernatants were removed and cell layers were incubated with MTT Reagent for about 5 h at 37 °C and 25 µl of the Detergent Reagent was added to dissolve the Formazan Crystals formed (Guoqiang *et al*., 2012).

The Optical Density (OD) of each well was quantified at 570 nm wavelength by an ELISA

Reader. The absorbance of untreated cells was considered as 100%. Each polysaccharide extract and ganoparticle treated well including control wells were assayed in triplicate. percent cell viability and percent cytotoxicity (percent inhibition) of cells exposed to treatments was calculated as:

% Viability = Absorbance of test wells/ Absorbance of control wells x100

(Sample Absorbance/ Control Absorbance x100)

% Cytotoxicity = 100- %Viability

Effects of Tryphan Blue on Cell Viability in Human Lung Cancer cells

Cell suspension of 10^6 cells/ml was prepared in serum free media to be assayed and 1:1 dilution of the suspension was prepared using a 0.4% trypan blue solution. Three wells were prepared, the first was kept as control contaning only cells , Second well containing cells was treated with ganoparticles and the third well containing cells treated with polysaccharide extract, the mixing was performed in microtitre plate (Masters *et al*., 2000a).

The solution with cells and trypan blue was added to the counting chambers of a haemocytometer, followed by counting of stained cells and total number of cells. The calculated Percentage of unstained cells was represented as the Percentage Viable cells (Masters, 2000b). Cell viability was calculated as the Number of viable cells divided by the total number of cells within the grids on the heamocytometer.

% Viability = Total number of viable cells per ml/ Total no. of cells per ml x 100.

(No. of live cells/ Total cell count x 100)

Live Cell Count (Average live cell per large square) = $1^{st}+2^{nd}+3^{rd}+4^{th}$ outer squares live cell count/ 4.

Cell Density (cells/ml) = Average live cells x Dilution factor/ Volume of Square (ml)

As 1:1 Dilution, dilution factor is 2.

RESULTS AND DISCUSSION

Extracellular Synthesis of AgNPs

In the Present study, *G. lucidum* extract was used for the Synthesis of AgNPs (Fig 1). The mycelial polysaccharide extract was treated with silver nitrate, It was observed that the extract had a Pale-yellow color before reaction with the silver ions, which changed to a brownish color on completion of the reaction (Ahmed *et al*., 2003). The appearance of a yellowish-brown color in solution containing the extract was a clear indication of the formation of AgNPs in the reaction mixture and was due to the excitation of surface plasmon vibrations in the NPs (Sastry *et al*., 1997). The color change indicates that *G. lucidum* mycelial extract could be used as a reducing and stabilizing agent for AgNPs synthesis (Patil *et al*., 1998). The color formation is similar to the results found by (Vahabi *et al*., 2011) and (Hemath *et al*., 2010).

Effect of AgNPs on human lung cancer cells

The Cell Viability assay is one of the important parameters for toxicology analysis that explains the cellular responses to toxic materials and can provide information on cell death, survival and their metabolic activities (AshaRani *et al*., 2009). Human Lung cancer cell lines A549 and NCIH520 were treated with AgNPs and Crude Polysaccharide extract for 24 hrs. To examine the effect of AgNPs and crude polysaccharide extract on mitochondrial activity, cells were treated with various concentrations of test compounds ranging from 5µg/100µl to 40µg/100µl. The absorbance was measured at 570nm in a Microtitre plate Reader, %

Table 1. Percentage cell viability, cell density and live cell count

Cell Lines	Samples	Live Cellcount	% Viability	Cell Density
1.**A549** Lung	a) Control well	12.25	100%	2.45×10^5Cells/ml
Cell Line Cancer	b) Nanoparticle treated well.	6.75	55%	1.4×10^5 Cells/ml
	c) Polysaccharide Extract treated well.	8.25	67.34%	1.7×10^5 Cells/ml
2. **NCIH 520** Lung	a) Control well	10.5	100%	2.10×10^5Cells/ml
Cancer Cell Line	b) Nanoparticle treated well.	5.25	50%	1.1×10^5Cells/ml
	c) Polysaccharide Extract treated well.	6.5	61.9%	1.3×10^5Cells/ml

Viability and % Inhibition was calculated for each Concentration using Control. The results are graphically represented in Fig. 2-4. The Percentage growth inhibition was found to be increasing with increasing concentration of ganoparticles and percentage cell viability was found to be decreasing with increasing concentration of ganoparticles and crude polysaccharide extract. however, ganoparticles treated cells showed much decreased metabolic activity than crude polysaccharide extract treated cells. Our results are in agreement with the previous report of Park *et al*. (2011). AgNPs and Crude polysaccharide extract inhibited the proliferation of A549 and NCIH520 following dose and time dependent manner. However, A549 was found to be more resistant than NCIH520, Ganoparticles, when compared to polysaccharide extract, were found to be more effective in suppressing the growth of cancer cells in comparison to crude polysaccharide extract. Our results are in agreement with the previous report of anti tumor effect of *ganoderma neo-japonicum* in suppressing the growth of breast cancer cells (Gurunathan et. al, 2013). Guoqiang et.al (2012) also reported synthesis of silver nanoparticles and their antiproliferation against human lung cancer cells.

Tryphan Blue Assay

Percentage of viable cells can be obtained by performing trypan blue dye exclusion technique. Tryphan Blue is an essential dye, used in estimating the number of viable cells present in a population (Phillips *et al*., 1957). Table 1 shows the percent cell viability of A549 and NCIH520 cell line. Ganoparticle treated cells lines shows 50-55% viability (1.1-1.4x10^5cells/ml), whereas crude polysaccharide extract treated cells were found to be 62-67% viable (1.3-1.7x10^5 cells/ml). In a similar study, Patel et.al (2009) reported the cytotoxicity activity of *Solanum nigrum* extract against HeLa cell line and Vero cell line

Fig. 1. Synthesis of silver nanoparticles (AgNPs) using *G. lucidum* extract. (a) *G. lucidum* mycelial polysaccharide extract. (b) Polysaccharide extract treated with $AgNo_3$

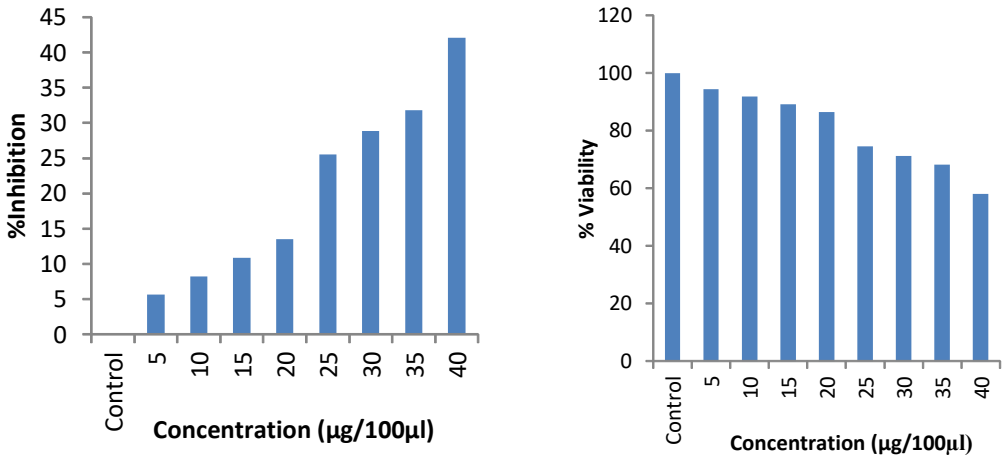

Fig. 2. Effect of *G. lucidum* mycelial polysaccharide extract on A549 cell line. a) A549 cell line inhibition b) A549 cell line viability

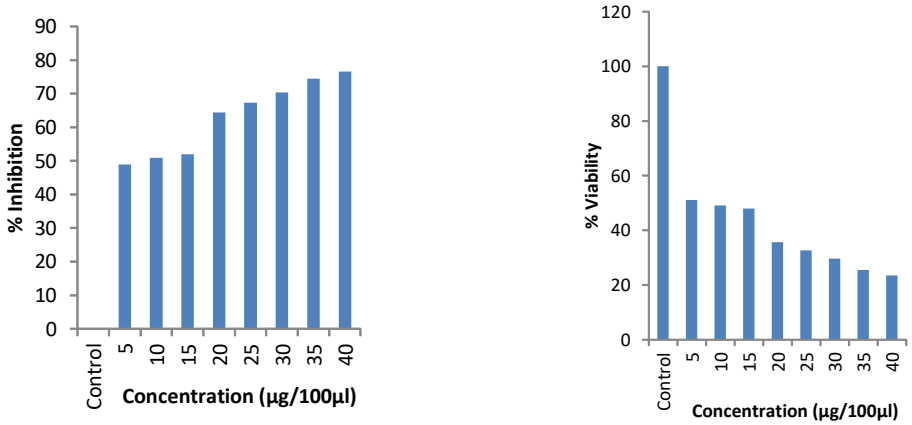

Fig. 3. Effect of silver ganoparticle (AgNPs) on A549 cell line. a) A549 cell line inhibition b) A549 Cell line viability

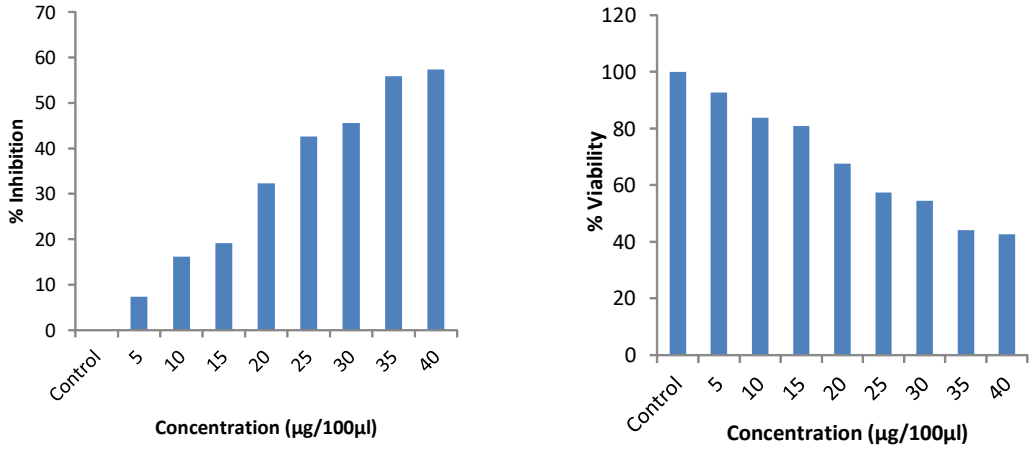

Fig 4. Effect of *G. lucidum* mycelial polysaccharide extract on NCIH 520 cell line. a) NCIH 520 cell line inhibition b) NCIH 520 cell line viability

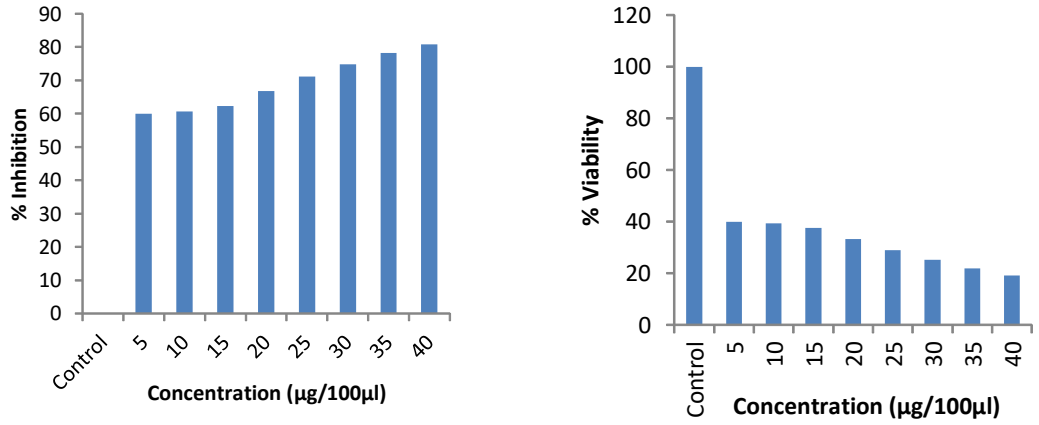

Fig 5. Effect of silver ganoparticles (AgNPs) on NCIH 520 cell line. a) NCIH 520 cell line inhibition b) NCIH 520 cell line viability

Antibacterial effect of AgNPs and crude extract

Table 2 show the antibacterial effect of ganoparticles and crude polysaccharide extract.. Antibacterial potential of the polysaccharide extract and silver ganoparticles was estimated by agar well diffusion method (Irshad *et al*., 2012) and the zone of inhibition was observed against all test organisms with largest zone of inhibition observed against *Staphylococcus aureus* (22mm) followed by *Bacillus subtilis (20mm) Escherichia coli (19mm)* and *Pseudomonas aeruginosa*. A larger zone of inhibition was observed in the presence of ganoparticles than Crude polysaccharide extract suggesting that *G. lucidum* AgNPs are more effective against bacterial strains than its extract.

Table 2. Antibacterial effect of *G.lucidum* polysaccharide crude extract and silver ganoparticles (AgNPs)

Test Organism	Zone of Inhibition (Dia. In mm)	
	G.lucidum polysaccharide crude extract	*G.lucidum* Ganoparticles
Staphylococcus aureus	19	22
Bacillus subtilis	18	20
Escherichia coli	16.5	19
Pseudomonas aeruginosa	15	18

CONCLUSION

Ganoderma extract is an active constituent of medicinal fungus *Ganoderma lucidum* & has proved to have numerous pharmacological activities and it can act as a potential anticancer agent. As tumor growth and progression require angiogenesis, an agent which acts as anti-angiogenic can arrest tumor growth to a defined location. It could further inhibit metastasis of the tumor tissue to other part. Further effort should be made to enhance the bioactivity of *Ganoderma* so as to increase its potency. This promising research about generation of *Ganoparticles* using *G. lucidum* extract & evaluation of its anti-proliferatory effect and antitumor properties would be considered as a good research in study and development of a new anti-cancer drug. We demonstrated the synthesis of AgNPs and its pharmaceutical importance using *G. lucidum* mycelial extract. Toxicity studies confirmed the potential Cytotoxic effects of biologically synthesized AgNPs in A549 and NCIH520 Lung cancer cells. AgNPs and Crude extract treated cells exhibited dose-dependent cell death. This study demonstrates the possibility of using AgNPs and polysaccharide extract to inhibit the growth of cancer cells and their cytotoxicity for potential therapeutic treatment. Application of AgNPs based on these findings may lead to valuable discoveries in pharmaceutical industries in developing new antitumor and antibacterial drugs.

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