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Research Article

GREEN SYNTHESIS OF SILVER NANOBIOCONJUGATES FROM *PIPER BETLE* LEAVES AND ITS ANTICANCER ACTIVITY ON A549 CELLS

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ABSTRACT

Objective: This study focused on the anticancer effect of silver nanobioconjugates synthesis from betel leaf extract and its major component eugenol (EU) and its non-conjugated form.

Methods: The silver nanobioconjugates were synthesized from betel leaf extract and its active compound EU under sunlight exposure. The anticancer activity of nanobioconjugates was tested and reported in lung adenocarcinoma (A549 cell line). The non-toxicity of the conjugates was further affirmed in human peripheral blood lymphocytes as non-cancerous cells. The anticancer activity was analyzed by cell viability (3(4,5-dimethly-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay) and staining techniques (acridine orange/ethidium bromide), which were carried out in both the cancerous and non-cancerous cells. The phase of cell death was analyzed by flow cytometry.

Results: The activity of nanobioconjugates was mediated by apoptosis as an evidence of low viability and nuclear fragmentation in lung cancer cell line. The cell cycle analysis also confirmed the efficiency of silver nanobioconjugates; the data reveal that the phenolic compound of betel leaves has potent anticancer activity against the cancer cells in the form of nano, when compared with the non-conjugates.

Conclusion: The study demonstrated the synthesized silver nanobioconjugates have potent anticancer activity than their non-nano form.

Keywords: Betel leaves, Eugenol, Silver nanoparticles, Anticancer, A549 cells.

INTRODUCTION

Nanostructured materials are known as "intelligent" or "smart" materials, 1-100 nm in size, used for constructing controlled drug release systems and have the ability to control drug dosing in terms of quantity, location, and time for drug delivery system. The controlled drug release systems are responsive to circumstances such as temperature, pH, applied magnetic or electrical field, ultrasound, light or enzymatic action [1]. Employing nanotechnology, along with the natural products, such as phenolic-enriched plants and fruits and their derivatives, is expected to provide tremendous potential and a new way to find a path to fight against cancer.

Plants have been a rich source of therapeutic compounds for various traditional medicines worldwide for thousands of years, due to the presence of active phyto-components, which help in preventing various oxidative stress-associated diseases [2]. One of the most important groups of such active compound is phenol. Several researchers have shown interest in working with these compounds because of their therapeutic performance. More than 8000 phenolic phytochemicals have been reported [3], which exist in various fruits and vegetables. Polyphenols are considered as secondary metabolites of plants, with various chemical structures and activities. Moreover, several studies have reported that polyphenols correlate with the prevention of degenerative diseases such as cardiovascular diseases and cancer. They also possess anti-inflammatory, anticarcinogenic, antidiabetic, and antiulcer properties [4].

Most of the phenolic compounds are present in the herbs, which is widely used in traditional medicine to cure various diseases. However, the major disadvantages of the phenolic compound are susceptible when exposed to adverse environment, low oral bioavailability and poor stability, especially in the environment of gastrointestinal tract [5]. The biological activity might vary depending on the nature of the phenolic compound. A way to tackle the above problem is by conjugation of phenolic compounds with the nanoparticles to improve the bioavailability, absorption, and potential biological activity.

Among the various metal nanoparticles synthesized (such as silver, gold, zinc, palladium, or platinum), nowadays, silver nanoparticles (AgNPs) are one of the promising nanoparticles in the nanotechnology field, because of their applications in various disciplines such as biomedical, catalysis, energy, and materials [6]. Many researchers have demonstrated the cytotoxicity of AgNPs in cancer cells, which affects the membrane integrity and induces various apoptotic signaling genes, leading to programmed cell death [7].

Eugenol (EU) (4-allyl-2-methoxyphenol) is the major phenolic compounds possess the anticancer activity, which was extracted from betel (*Piper betel*) leaf belonging to Piperaceae family. Majorly cultivated in most South East and South Asia. Betel leaves are generally used as a stimulant, an antiseptic and a breath-freshener pann [8]. Earlier studies conducted in our laboratory proved *Piper betle* leaf extract to be inherently rich in phenols, and to have antioxidant and hepatoprotective activity [9]. The leaves have also been shown to be antimutagenic and anticarcinogenic [8,10]. With this continuation the *P. betle* leaf extract and its pure compound, EU was used for the synthesis of silver nanobioconjugates in nanoform and to determine their medicinal properties, which is the novel criteria of the present research work. Fresh leaves of *P. betle* (Athur variety) were collected from local market and used in this study. The method of synthesis was also optimized which resulted sunlight exposure is the best method for the rapid synthesis, which have a potent antimicrobial effect than the non-conjugated raw material (extract/compound). The synthesized silver nanobioconjugates falls with 8-35 nm which had good biocompatibility properties. The results obtained encouraged as to focused on the anticancer activity of silver nanobioconjugates synthesized from betel leaf extract and its component, EU, which is commercially available.

METHODS

Synthesis of silver nanobioconjugates

The silver nanobioconjugates were synthesized using 10 ml of methanolic extract of *P. betle* extract (PE) or diluted 100 µg of EU with 90 ml of 1 mM silver nitrate (Sigma) solution. The mixture was kept under sunlight at 12500-13500 lux for 20 minutes. After the exposure period, the synthesized silver nanobioconjugates were centrifuged at 3500 g for 20 minutes and washed thrice with deionized water. The centrifuged nanobioconjugates were dried and dissolved in the dimethyl sulfoxide for the experiment.

Cell culture

The lung cancer cell line (A549) was obtained from National Center for Cell Sciences (NCCS), India. The cell line was grown in DMEM (Gibco) with 10% fetal bovine serum (FBS) (PAA) and 1X penicillin-streptomycin (MP Biomedicals), and the cells were incubated at 37°C at CO_2 incubator (Innova, UK) with 95% humidity and 5% CO_2. The confluent cells were trypsinized using trypsin-EDTA (PAA) and transferred to a fresh complete medium.

Lymphocyte cell culture

To determine the toxicity level of synthesized silver nanobioconjugates, human peripheral blood lymphocytes were used. For this study, the collection of non-cancerous cells from human volunteers were approved by the Institutional Human Ethics Committee (AUW/IHEC-14-15/XPD-08).

The blood (2 ml) was drawn from healthy human volunteers by venipuncture method and transferred into siliconized glass tube containing 10 µl of sterile heparin. The blood was immediately diluted with phosphate-buffered saline (PBS) or RPMI medium in 1:1 ratio. Lymphsep (4 ml) was taken into 15 ml siliconized glass tubes and 2 ml of diluted blood was layered carefully on the top of the lymphosep medium without mixing it. The sample tube was centrifuged for 30 minutes at 400 ×*g* at 18-20°C. After centrifugation, the gray layer of lymphocytes formed at the interface of blood plasma and lymphsep medium was aspirated without disturbing the separating medium. The aspirated lymphocytes layer was dispensed in one-third volume of PBS. The lymphocytes mixture was then centrifuged at 100 ×*g* for 15 minutes. The supernatant was discarded and the lymphocytes were washed thrice with PBS solution. Finally, the cells were suspended in fresh RPMI medium supplemented with 10% FBS, 1% penstrep, and 1% phytohaemagglutinin-P. The cells were cultured in sealed sterile cell culture flask and incubated at 37°C in an incubator with moderate shaking (Heraeus, Germany).

The cell count of isolated lymphocytes was determined using 10 µl of cell suspension using trypan blue dye in a hemocytometer. The healthy live cells (10^5 cells/ml) were seeded in sterile cell culture flask with complete medium, supplemented with 10% FBS, 1% penstrep and 1% PHAP. Then, the flask was closed tightly and incubated at 37°C for 48 hrs under mild shaking condition. After the incubation period, the cells were centrifuged at 100 ×*g* for 10 minutes and the cultured lymphocytes were washed thrice with PBS. The cells were resuspended in complete medium and treated with the silver nanobioconjugates and their respective plant extract/compound for 24 hrs at 37°C. The treated cells were then centrifuged at 100 ×*g* for 5 minutes and washed thrice with PBS. The harvested cells were then used for the viability assay and staining analysis.

Anticancer effect of silver nanobioconjugates

The anticancer activity of silver nanobioconjugates on lung cancer (A549) was evaluated by 3(4,5-dimethly-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay [11]. Which was followed by the acridine orange/ethidium bromide (AO/EtBr) staining method to evaluate the apoptosis process [12]. The apoptotic index was calculated by the flowing formula:

$$\text{Apoptotic ratio} = \frac{\text{Number of apoptotic cells}}{\text{Number of normal cells}}$$

These experiments were also carried out in non-cancerous cells (blood lymphocytes) to determine the toxicity level in comparison with cancer cells. To determine the phase of cell cycle arrest cell cycle analysis was carried out by flow cytometry (BD FACSVerse, USA), to determine the phase of cell cycle arrest [13].

RESULTS

Proliferation assays like MTT are the typical example of functional assessment used in toxicity studies. It is a rapid and convenient method for determining viable cell proportion. This method was used to evaluate the effect of silver nanobioconjugates on the viability of A549 lung cancer cells in comparison with non-cancerous peripheral blood lymphocytes. The dose range, about 2.5 µg to 50 µg/ml of silver nanobioconjugates synthesized from *P. betle* leaf extract (PAgNP), EU (EAgNP) and their non-conjugates, betel leaf extract (PE)/pure compound EU alone were used for the study. Based on the available literature, 50 µM concentration of pure compound EU [14] was involved in the study. Initially, a time dependent and dose-dependent study was carried at 24 hrs exposure.

The extents of viability observed with the silver nanobioconjugates and their non-nano counterparts, at the same dose level in A549 cells are presented in Fig. 1. The non-nano extract and compound and their silver nanobioconjugates showed a decreased dose-dependent in the viability of cancer A549 cells. The extent of viability was much lower in the nanobioconjugates-treated groups compared to their respective non-nano forms at all the dose levels, in both the extract- and EU-treated groups (Fig. 1). These results indicated the high anticancer effect of both *P. betle* leaf extract and its active phenolic component, which increased the anticancer effect to several fold by treating them as nano form of silver nanobioconjugates.

The results of MTT assay showed that the influence of silver nanobioconjugates on lung cancer was high during the period of 24 hrs. To determine the process of apoptosis and phase of cell death, shorter duration of 16 hrs was planned to study using silver nanobioconjugates synthesized from leaf extract and compound. Similar results were obtained as of 24 hrs exposure results, both the AgNPs showed more

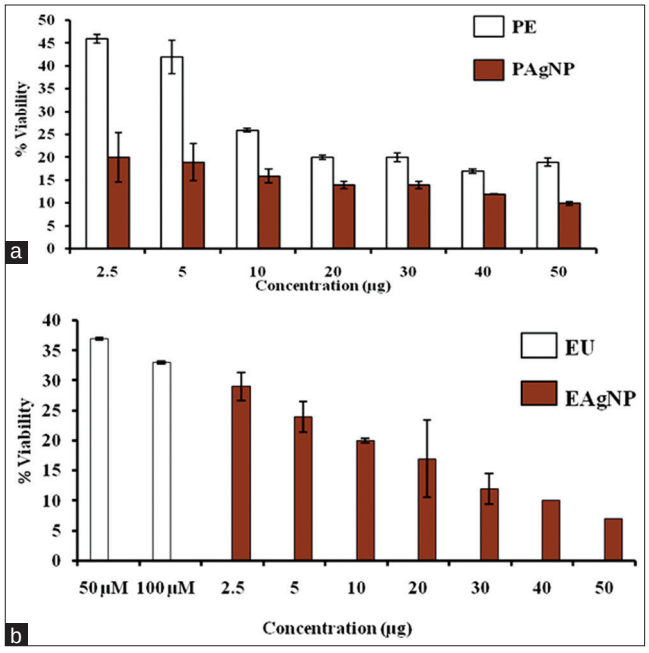

Fig. 1: Effect of silver nanobioconjugates from *Piper betle* leaves and their active component eugenol on the viability of A549 cells (24 hrs treatment). (a) *Piper betle* silver nanoparticles, (b) eugenol silver nanoparticles. The values are mean±standard deviation of triplicates

cell death in A549 cancer cells from the dose range (Fig. 2). Therefore, 2.5 µg and 5 µg of silver nanobioconjugates were tested for 12 hrs period, based on the results (Fig. 2) obtained 2.5 µg concentration of silver nanobioconjugates from betel leaf and EU was consider for the further experiments.

To determine the cytotoxicity levels, as a comparative control for the internalized lung cancer cells, primary cultured peripheral blood lymphocytes were used. The low (2.5 µg), intermediate (20 µg), and the high (50 µg) dose levels of the silver nanobioconjugates were used for the experiment. The lymphocytes cells were exposed to both PAgNP and EAgNP for 24 hrs. The results confirmed that all the three dose range of silver nanobioconjugates were non-toxic to the human peripheral blood lymphocytes (Fig. 3). From the results obtained, it was clear that AgNPs of both have a differential effect which is toxic to cancer cells and non-toxic to non-cancerous human blood lymphocytes.

The AO/EtBr staining was used to determine the apoptotic index of A549 cells influenced by both PAgNPs and EAgNP in comparison with extract and compound, respectively. Using the strain trough microscopic study, the results clearly indicate the apoptosis caused by silver nanobioconjugates. The apoptotic index was calculated and presented in Table 1. The apoptotic ratio was increased significantly in the AgNPs treated cancer cells than the betel leaf extract and its active compound EU. Based on the viability assay and staining technique results, it confirmed that the nanoform of betel extract and pure compound have more effect than the non-nanoform (Table 1 and Fig. 4).

The arrest of cell cycle in the A549 cancer cells was identified by cell cycle analysis using cytometry with PI. The proportion of cells in each phase was quantified using the software in flow cytometry (Table 2). The results exhibited that cell cycle arrest found in S phase and G_2/M phase, when compare with the non-conjugate betel extract and EU. The effect was significantly more in the nanobioconjugates treated group (Fig. 5). The earlier results obtained from viability assay and staining method rendered supported to flow cytometry results.

Table 1: Effect of extract/compound/silver nanobioconjugates in A549 cells and human blood lymphocytes cells (AO/EtBr staining)

Treatment groups	Number of Apoptotic cells/100 cells			
	A549 cells		Human peripheral blood lymphocytes	
	Extract/ compound alone	AgNP	Extract/ compound alone	AgNP
Control	11±2		11±2	
Piper betle	37±5[a]	45±5[a,b]	12±2	12±2
Eugenol	47±3[a,c]	59±2[a,b,c]	9±2	14±2

The values are mean±SD of triplicates. Sample size per 100 cells. [a]Statistically significant ($p<0.05$) compared to untreated control, [b]Statistically significant ($p<0.05$) compared to corresponding unconjugated extract/ compound, [c]Statistically significant ($p<0.05$) compared to the corresponding extract/compound/group. SD: Standard deviation, AO/EtBr: Acridine orange/ ethidium bromide, AgNP: Silver nanoparticles

Table 2: Cell cycle analysis of extract/compound/silver nanobioconjugates in A549 cells

Groups	G0/G1	S	G2/M
PE	36.13	4.31	35.69
PAgNP	31.20	3.31	42.60
EU	59.58	5.29	23.70
EAgNP	37.60	7.81	41.28

EAgNP: Eugenol silver nanoparticles, PAgNP: *Piper betle* silver nanoparticles, EU: Eugenol, PE: *Piper betle* extract

Overall, the results exhibited that the silver nanobioconjugates of betel leaves and EU have more effect when it was administered in the nanoform to the lung cancer cells. The major component present in the betel leaves is responsible for the synthesis of silver nanobioconjugates, which have the similar effect as like the nanobioconjugates synthesized from EU.

DISCUSSION

The ancient knowledge on ayurvedic medicine based on metal, mineral and herbal preparations have laid the foundation for modern drug formulation and delivery. In the recent times, the metal nanoparticles are becoming more useful and popular, especially for biomedical applications [15]. The research related to toxicity of nanoparticles reported a negative perception of their use. However, toxicity itself can

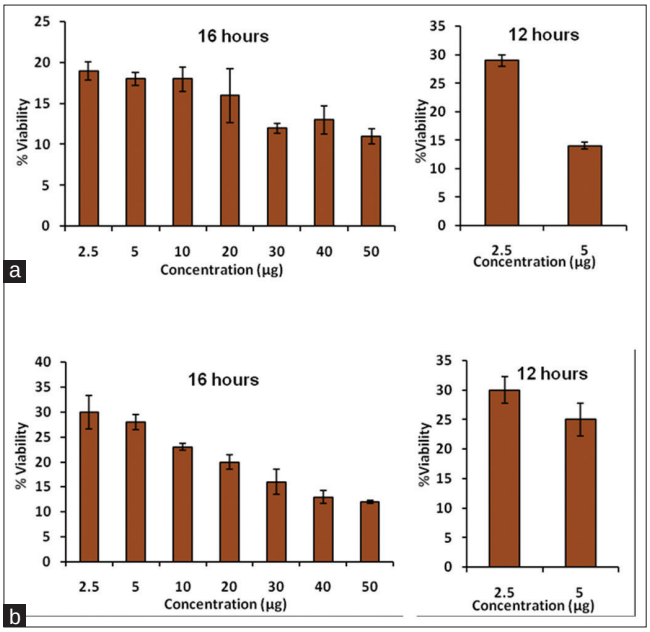

Fig. 2: Effect of silver nanobioconjugates synthesized from *Piper betle* leaves and their active component eugenol on the viability of A549 cells (16 and 12 hrs treatment). (a) *Piper betle* silver nanoparticles, (b) eugenol silver nanoparticles. The values are mean±standard deviation of triplicates

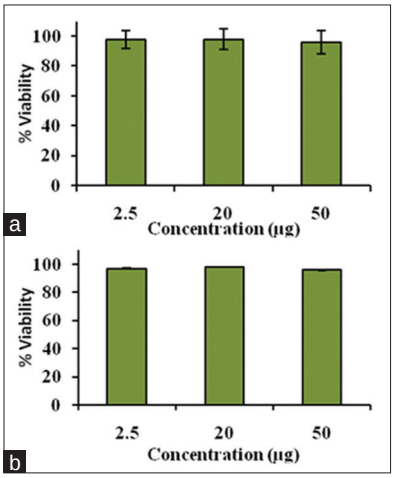

Fig. 3: Effect of silver nanobioconjugates on human peripheral blood lymphocytes. (a) *Piper betle* silver nanoparticles, (b) eugenol silver nanoparticles. The values are mean±standard deviation of triplicates

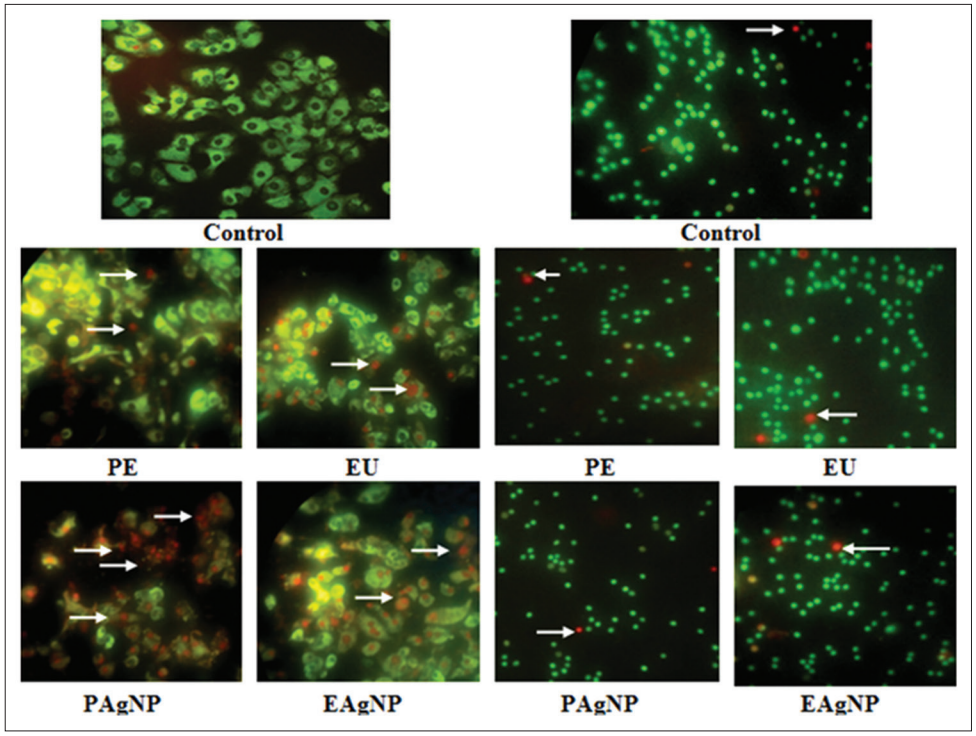

Fig. 4: Effect of extract/compound/silver nanobioconjugates on the A549 cancer cells and non-cancerous lymphocytes cells

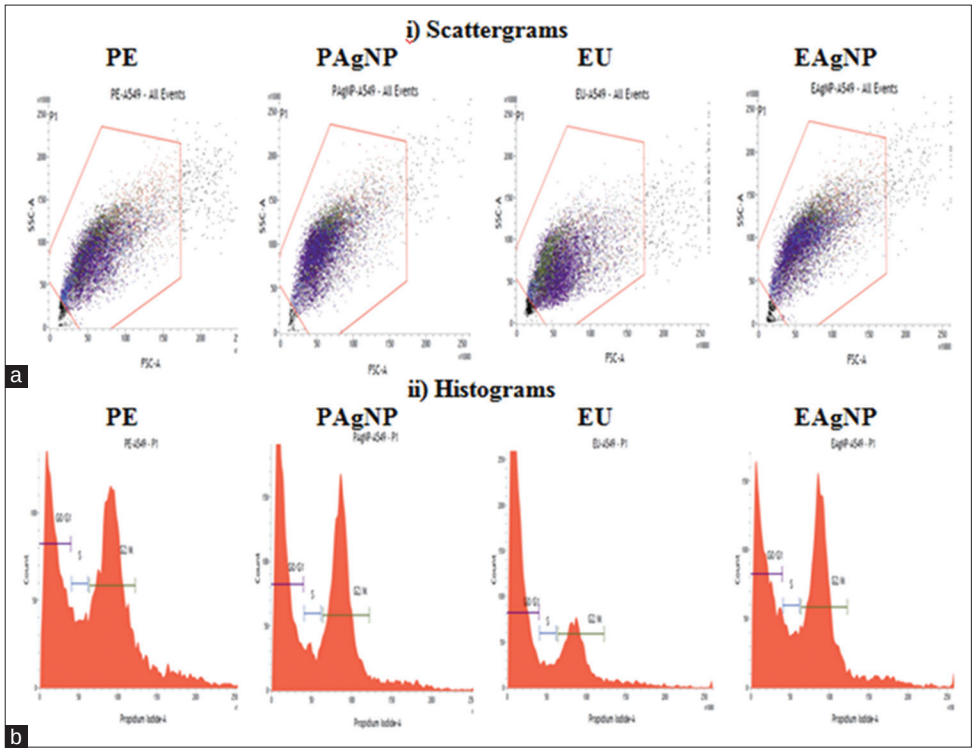

Fig. 5: Effect of *Piper betle* leaf extract and its silver nanobioconjugates on cell cycle events in A549 cells. (a) Scattergrams, (b) histograms

be useful for cancer therapies. Successful outcomes have been achieved when incorporating AgNPs into cancer treatments. They can not only passively interact with cells but also actively mediate molecular processes to regulate cell functions [16].

In this study, the anticancer activity of the nanobioconjugates synthesized from the betel leaf extract and pure compound, in comparison with their respective non-conjugated raw material, was studied using lung cancer cell line. The MTT assay and AO/EtBr dual staining method were used to determine the influence of silver nanobioconjugates on the viability and apoptosis process of A549 cells and the non-transformed cells. The results revealed that *P. betle* as well as their active phenolic, exhibited strong anticancer activity to A549 lung adenocarcinoma cells. Another significant observation was the differential effect evoked by the AgNPs, which was non-toxic to non-cancerous lymphocytes, while evoking a strong cytotoxicity in the cancer cells.

Various reports on the anticancer activity of AgNPs of plant base are available. The AgNPs synthesized from AgNPs *Moringa oleifera* [17], olive leaf [18], *Albizia adianthifolia* leaf [19], and *Melia azedarach* leaf extract [20] showed toxicity to various cancer cells tested. AgNPs induce cell responses often specific to cell types, resulting in varying degrees of toxicity. Depending on nanoparticle concentration and exposure time, AgNPs induced different degrees of toxicity *in vitro* in human breast cancer cells [21], skin epithelial A431 and lung epithelial A549 cells [22]. Similarly, Nakkala *et al.* [23], Palaniappan *et al.* [24], and Rathi Sre *et al.* [14] also observed the cytotoxicity of AgNPs synthesized from different plants in lung cancer cells (A549). Our results were supported by the mentioned reports.

Increased cytotoxicity, genotoxicity, and reactive oxygen species were induced by the colloidal suspensions of AgNP prepared from sodium citrate in human lung cancer (A549) cells, while only a slight toxicity was observed in human dermal fibroblasts [25]. The AgNPs capped with polyvinylpyrrolidone caused high cytotoxicity in triple-negative breast cancer cells but were nontoxic to non-cancerous breast and other cells derived from liver, kidney and monocyte lineages [26]. Similarly, in our studies also, the results showed that the synthesized silver nanobioconjugates possess differential effect, which was non-toxic in nature to human cells.

Various studies have suggested that AgNPs accumulated inside the cells and enhanced stress by GSH depletion reduced mitochondrial potential and increased the formation of ROS, which typically include the hydroxyl radical and hydrogen peroxide [27-30]. Guo *et al.* [31] reported that both the size and surface area of silver coated with polyvinylpyrrolidone nanoparticles played a major role in the cytotoxicity, with the smaller nanoparticles having bigger surface area and higher reactivity, with stronger cytotoxic effect. Another possible mechanism proposed by Sathishkumar *et al.* [32] was that AgNPs of *Dendrophthoe falcata* extract induced toxicity by the cellular uptake through clathrin-dependent endocytosis and macropinocytosis. Our results showed the strong anticancer activity of the silver nanobioconjugates synthesized.

The results of AO/EtBr staining method was used to confirmed the apoptosis. Similarly, a study illustrated that the AgNPs from *Achillea biebersteinii* showed the morphological changes in MCF-7 breast cancer cells [33]. In another study, AgNPs of *Butea monosperma* bark extract shoed the apoptotic cells in KG-1A cell line [34]. The cytotoxic effect AgNPs from *Indigofera aspalathoids* using AO/EtBr staining indicated the induction of apoptosis [35]. The extent of the anticancer effect was significantly more in the nanobioconjugates than their non-conjugated forms, proving that the anticancer activity of the test materials used can be enhanced markedly by preparing silver nanobioconjugates. The results of cell cycle analysis showed the maximum number cell cycle arrest at G_2/M phase. A study reported the cell cycle arrest in G_2/M phase in MCF-7 cells treated with rhamnolipid-functionalized AgNPs [36]. Human colon cancer cells treated with the AgNPs synthesized from the leaf extract of *Vitex negundo* showed cell cycle arrest in both G_0/G_1 and G_2/M phase [37]. Similarly, the silver nanopartciles synthesized from plants *Phytolacca decandra*, *Gelsemium sempervirens*, *Hydrastis canadensis,* and *Thuja occidentalis* mediated the cell cycle arrest through G_2/M phase [38]. Our results are in agreement with these reports. When these bioagents were conjugated with silver to form nanoscale particles, the extent of apoptosis in the cancer cells increased significantly.

CONCLUSION

The findings of this study, exposed that the synthesis of silver nanobioconjugates from *P. betle* leaves and their major polyphenol (EU) possess strong anticancer activity in the nanoform. By using an inexpensive and eco-friendly sunlight exposure method, the nanobioconjugates can be synthesized from the betel plants, which is enriched with the medicinal properties and easily available and medicinal rich. This method can be adopted for the synthesis of silver nanobioconjugates which can be applied in the field of biomedical applications.

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