

Original Article

ANTICANCER ACTIVITY OF SILVER NANOBIOCONJUGATES SYNTHESISED FROM *PIPER BETLE* LEAVES EXTRACT AND ITS ACTIVE COMPOUND EUGENOL

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ABSTRACT

Objective: Our present study deals with the anticancer property of silver nano bio conjugates synthesized from the methanolic extract of *Piper betle* leaves and the pure compound eugenol.

Methods: The anticancer activity of the nano bio conjugates synthesized from the extract and pure compound, in comparison with their respective non-conjugated raw material, were studied using cancer cell lines. The anticancer activity was analyzed by cell viability and staining techniques. The cell viability was determined by the MTT assay and the apoptosis was evaluated by the AO/EtBr staining method, which was carried out in both the cancerous (oral carcinoma (KB) cell lines) and non-cancerous cells (primary cultured human buccal cells). The phase of cell death was assessed by the flow cytometry followed by the cytotoxicity assessment.

Results: The results revealed that *Piper betle* leaves as well as their active phenolic compound eugenol exhibited strong anticancer activity in KB oral carcinoma cells. The anticancer effect of the extract was attributable to the component phenolics, eugenol respectively in betel leaves, as the phenolics also exhibited strong cytotoxicity in cancer cells. The anticancer activity increased by a marked magnitude when the extract/compound was administered as nano-bio conjugates. The significant observation was the differential effect evoked by the AgNPs, which was non-toxic to non-cancerous buccal cells while evoking a strong cytotoxicity in the cancer cells.

Conclusion: The results proved that the phytoconstituents like phenols in the betel leaves play a major role in the reduction of nano bio conjugates. The anticancer properties of silver nano bio conjugates were higher in the conjugated nanoform than the non-conjugated form.

Keywords: *Piper betle*, Eugenol, Nano bio conjugates, Anticancer, KB cells

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INTRODUCTION

Currently about 50% of cancers in men and 20% of cancers in women are related to tobacco use, which has led to oral cancer death in the age group of 35-64 y. Developing countries of the South Asian region have a higher incidence of oral cancer compared to developed countries [1, 2]. The standard treatment followed all over the world is radiation therapy, or a combination of radiation therapy, chemotherapy and surgical removal of cancer. In spite of such interventions, cancer continues to take lives. Researchers are also intensifying their efforts on various therapy and trails on immunotherapy, antiangiogenic therapy, gene and viral therapy, cancer stem cell therapy and targeted therapy [3].

Over the last few decades, about 85,000 compounds have been screened for cancers, but only a few could be considered as effective and promising anti-cancer drugs [4]. Recently, the field of cancer therapies has been improved by many diverse scientific disciplines in order to fight better against cancer. Nanotechnology, which involves the creation, manipulation, and application of structures in the nanometer size range, has started revolutionizing cancer diagnosis and therapy [5]. Nanomedicine represents an innovative and multidisciplinary field that exploits nanotechnology to utilize in disease diagnosis and treatment. The main feature of these nanosystems is that their surfaces can be functionalized, exploiting reactive terminal groups, with specific proteins, peptides or monoclonal antibodies that are able selectively to bind a site of action or a particular target tissue, without interacting with other cells. They also improve the stability of drugs and release the drug in a constant, uniform concentration and in a controlled system, leading to reduced side effects [6]. Nowadays nanotechnology-based approaches to overcome the problem occur in the conventional system for treatment and the diagnosis of cancer [7].

The green production of nanoparticles using naturally occurring agents such as plant extract, microorganisms and biodegradable

sources like reductant and capping agents, have made a remarkable attraction toward nanotechnology. *Piper betle* (betel leaves) have been used in our Indian traditional medicine system for digestive and pancreatic lipase stimulant activities [8]. The leaves of *Piper betle* have been employed in traditional herbal remedies. Active phenolic compounds such as chavibetol, piperol, piperbetol, hydroxychavicol and eugenol are enriched in the betel leaves, among which eugenol is major phenolic possess remarkable chemotherapeutic and chemopreventive potential, and it is inexpensive and non-toxic [9-11].

Based on the preliminary study conducted in our laboratory, the method of synthesis of silver nanobioconjugates was optimized using the methanolic extracts of *Piper betle* leaves as well as their active phenolic ingredient eugenol (commercially available). The synthesized bioconjugates were also bioactive, as reflected by their antibacterial effect due to their nano size which was confirmed by the various characterization techniques [12]. Encouraged by these results. Therefore, the present study deals with the anticancer effect of silver nano-bio conjugates synthesis from *Piper betle* leaf extract and its active phenolic component, eugenol.

MATERIALS AND METHODS

Synthesis of silver nano-bio conjugates

Based upon the earlier research conducted in our laboratory, the method for nanobioconjugates synthesis was optimized and characterized, which confirmed that the sunlight exposure was rapid in synthesis [12]. The nanobioconjugates were synthesized by adding methanolic extract of *Piper betle* extract (PE) or diluted purified 100µg of eugenol (EU) to 1 mmol silver nitrate (Sigma) solution in the ratio of 10:90. The mixture of extract or compound and silver nitrate solution was exposed to bright sunlight (12500-13500 lux) for 20 min. After the exposure period, the mixture was centrifuged at 3500g for 20 min and washed thrice with deionized water. The nanobioconjugates were dried and dissolved in the DMSO for the further study.

Culturing of KB cells

The oral cancer cell line (KB) (Plate 1) were procured from National Center for Cell Sciences (NCCS), Pune, and used in this study. The cell lines were cultured and maintained in DMEM (Gibco) with 10% FBS (PAA) and 1X penicillin-streptomycin (MP Biomedicals) as per standard procedures and maintained in a CO₂ incubator (Innova, UK). The confluent grown cells were trypsinized using Trypsin-EDTA (PAA) and suspended in a fresh complete medium. The flasks were incubated in a CO₂ incubator (Innova, UK) with 95% humidity and 5% CO₂. For the experiments, the cells were counted using hemocytometer and 105 live cells/per milliliter were seeded for the further experiments.

Culturing of buccal cells

The protocol of collection and the use of non-cancerous cells from healthy human volunteers was scrutinized and approved by the Institutional Human Ethics Committee (AUW/IHEC-14-15/XPD-08).

The buccal cells (Plate 1) were collected from healthy human volunteers, who were not on any medication in the age group of 20-30 y. The mouth was rinsed with 5 ml of Listerine; then sterile soft wood scrapers were used to scrape the buccal cells from the buccal cavity. About 15 ml of a sterile saline was used to rinse the mouth, which was collected in a sterile beaker, the scraper was also washed with 10 ml of sterile saline into the same sterile beaker. The cells were centrifuged at 300g for 5 min and the pellet was resuspended in 1 ml of sterile PBS. The suspended cells were treated with 1X pen-strep (Penicillin-Streptomycin, from a 100X stock (MP Biomedicals, USA)), 12 µl/ml fluconazole (Cipla) and incubated for 30 min to remove any contamination. The cells were centrifuged at 300g for 5 min and washed twice with sterile PBS. Then the buccal cells were resuspended in complete DMEM (supplemented with 2% FBS, 1X Penstrep and 1.2µg/ml fluconazole) in the cell culture flask. The cells were incubated in a CO₂ chamber (Napco, UK) for 24 h.

After 24 h of incubation, the buccal cells were transferred into a complete medium with 5% FBS. Again, the buccal cells were incubated for another 24 h and the cells were collected by trypsinization, centrifuged as earlier and the cell count was determined using a hemocytometer. The live healthy cells (105 cells/ml) were seeded in a 6-well plate with complete medium and with nano bio conjugates synthesized from betel leaf extract, their respective pure compound, and their respective non-nano raw material. The exposure was done at 37 °C for 24 h. The cells were then harvested and centrifuged at 4000 rpm for 5 min, washed twice with sterile PBS and resuspended in PBS for the viability and staining analysis.

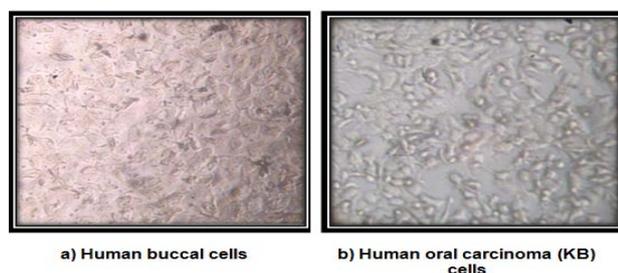


Plate 1

Anticancer effect of nano bio conjugates

The anticancer activity was analyzed by cell viability and staining techniques. The cell viability was determined by the MTT [3(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay [13] and the apoptosis was evaluated by the AO/EtBr staining method [14] using a fluorescent microscope (Nikon, Japan) using B2A filter at 40X magnification. Live and apoptotic cells were counted and discriminated to calculate the apoptotic ratio. Live cells appeared uniformly green. Apoptotic cells incorporate the ethidium bromide and show the chromatin condensation and nuclear fragmentation. The apoptotic ratio was calculated using the following formula.

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

The assay and staining method were carried out in both the cancerous and non-cancerous cells. The following treatment groups were set up:

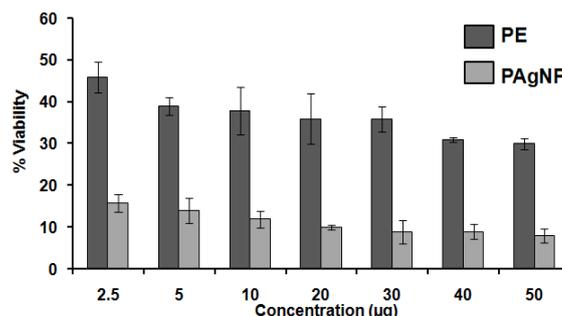
- Untreated (negative) control cells
- Cells treated with leaf extract/silver nanobioconjugates of *P. betle*
- Cells treated with eugenol/silver nanobioconjugates of eugenol

The parameters analyzed in this study were subjected to statistical analysis using SigmaStat (Version 3.1) statistical software. Statistical significance was determined by one-way ANOVA and the standard deviation value falls within ± 1 to ± 7 .

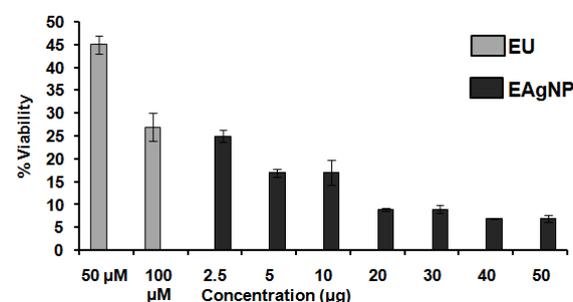
Cell cycle analysis was carried out by flow cytometry, to determine the phase of cell cycle arrest [15]. The cells were analyzed for the populations of sub-G₀, G₀/G₁, S and G₂/M phases of the cell cycle by the software of flow cytometry (BD FACSVers, USA).

RESULTS

The MTT assay was used to determine the influence of silver nanobioconjugates on the viability of oral cancer cells along with the non-transformed buccal cells. A dose and time dependent effect of silver nanobioconjugates synthesized from *Piper betle* leaf extract (PAgNP) and eugenol (EAgNP) was studied. A wide range of both silver nanobioconjugates (2.5µg to 50µg/ml) and their extract alone. Based on the literature reports 50µM/ml of the active component, eugenol [16] was used in the study, at the exposure period of 24 h. The *Piper betle* leaf extract, eugenol, and their nanobioconjugates showed a dose-dependent decrease in the viability of KB cells. The results showed that the viability was lower in the treatment of silver nanobioconjugates groups when compared with the extract and compound alone (fig. 1(i), 1(ii)). These observations show that the anticancer effect of both *Piper betle* leaf extract and its active phenolic component, eugenol, can be increased several folds by administering them as silver nanobioconjugates.



i) Silver nanobioconjugates from *Piper betle* leaf extract



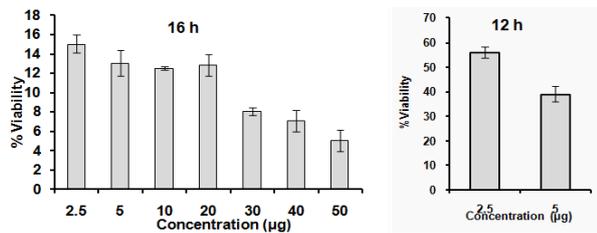
ii) Silver nanobioconjugates from eugenol

Fig. 1: Effect of silver nanobioconjugates synthesized from *Piper betle* leaves and their active component eugenol on the viability of KB cells (24 h treatment)

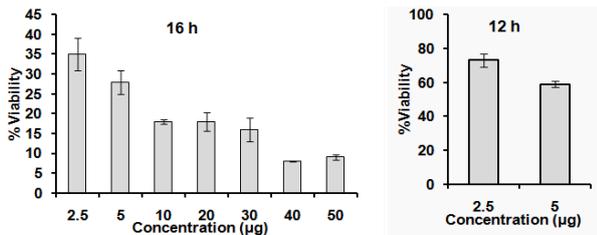
The bars are mean \pm Standard deviation of triplicates, (PE-*Piper betle* leaf extract, PAgNP-Silver nanobioconjugates from betle leaves, EU-Eugenol, EAgNP-Silver nanobioconjugates from eugenol)

The extent of cell death induced by the nanobioconjugates was very high at 24 h exposure period, therefore, the shorter exposure period

of 16 h was studied. Similarly, as 24-h exposure results were obtained, KB cells showed high toxicity against all the dose levels (fig. 2). Again the silver nanobioconjugates with the dose range of 2.5µg and 5µg/ml were tested for 12 h period. The results showed about 50% toxicity level, therefore, the 2.5µg concentration of silver nano bio conjugates was taken for the further study.



i) Silver nano bio conjugates from *Piper betle* leaf extract



ii) Silver nano bio conjugates from eugenol

Fig. 2: Effect of silver nano bio conjugates synthesized from *Piper betle* leaves and their active component eugenol on the viability of KB cells (16 and 12 h treatment)

The values are mean±Standard deviation of triplicates

From the wide range of doses 2.5µg, 20µg and 50µg/ml of silver nano bio conjugates synthesized from leaf extract and eugenol were selected for testing the cytotoxicity in the primary cultured buccal cells. The results (fig. 3(i), 3(ii)), revealed that both the nano bio conjugates did not affect the viability of the buccal cells at the various dose level tested, which confirmed the differential effect of *Piper betle* leaf extract and its phenolic component on cancerous and non-cancerous cells of the oral cavity.

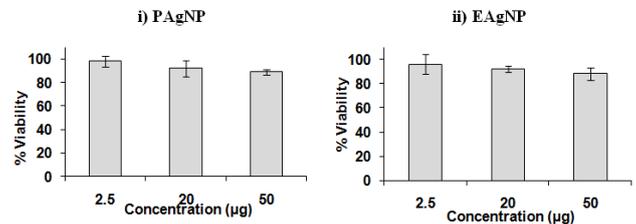


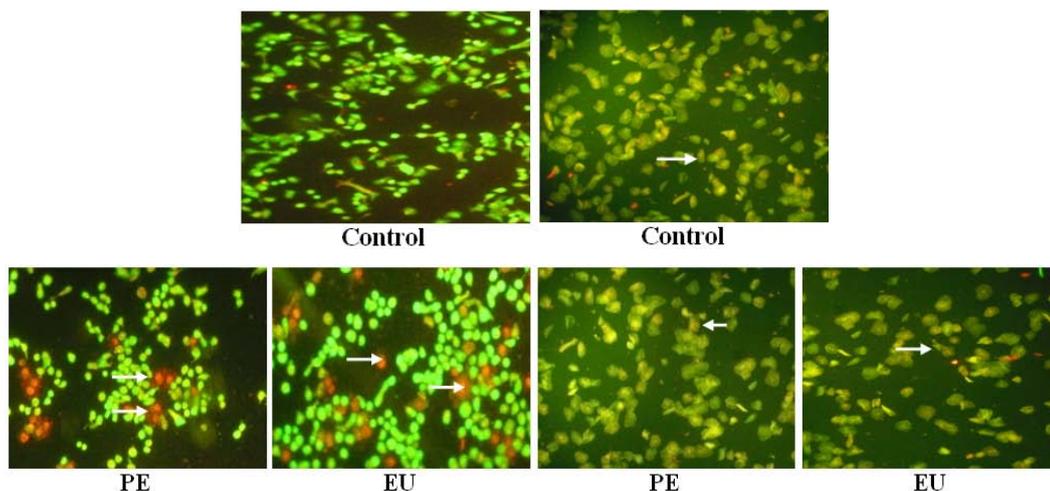
Fig. 3: Effect of silver nano bio conjugates on human buccal cells the values are mean±Standard deviation of triplicates

Following with the results of MTT assay, the type of cell death occurs in the cancer cells was studied by the AO/EtBr staining using a fluorescent microscope. From the microscopic study, it could be observed that the type of cell death observed was predominantly apoptosis. The number of apoptotic cells per 100 cells was counted in each treatment group, and the apoptotic index was calculated. From the results, it was observed that the leaf extract and its active component eugenol mediate the apoptosis process, whereas in the nanoform the apoptotic ratio was increased significantly (table 1 and Plate 2) The arrow marks in the plate 2 indicating the apoptotic cells.

Table 1: Effect of extract/compound/silver nanobioconjugates in KB cells and human buccal cells (AO/EtBr staining)

Treatment groups	Number of apoptotic cells/100 cells			
	KB cells		Human Buccal cells	
	Extract/Compound alone	AgNP	Extract/Compound alone	AgNP
Control	7±2		9±2	
<i>Piper betle</i>	34±5 ^a	47±5 ^{a,b}	8±2	11±2
Eugenol	47±3 ^{a,c}	55±2 ^{a,b,c}	11±1	10±2

The values are mean±Standard deviation of triplicates, ^aStatistically significant (P<0.05) compared to untreated control, ^bStatistically significant (P<0.05) compared to corresponding unconjugated extract/compound, ^cStatistically significant (P<0.05) compared to the corresponding extract/compound/group



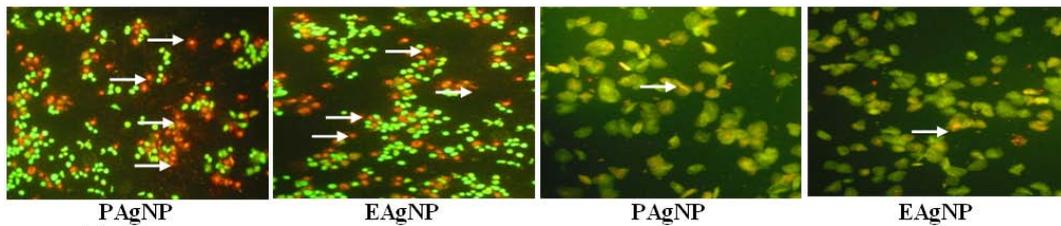
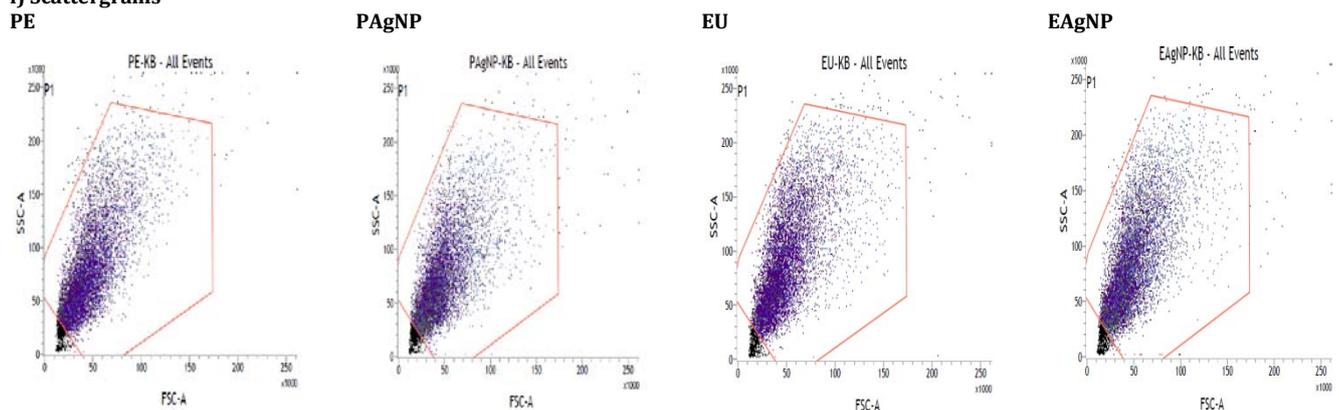


Plate 2: Effect of extract / Compound / silver nano bio conjugates on the KB cancer cells and non-cancerous buccal cells, PE - Piper betle leaf extract, EU - Eugenol, PAgNP - Silver nano bio conjugates from betel leaves, EAgNP - Silver nano bio conjugates from eugenol

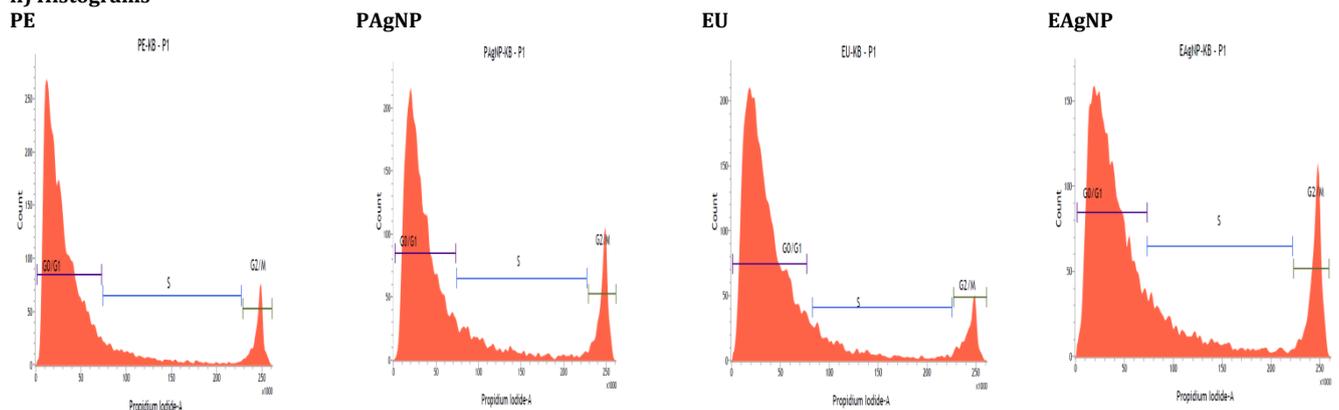
The events of the cell cycle in the exposed cancer cells was analyzed by flow cytometry. The results showed that the nanobioconjugates exhibited the anticancer effect by shifting the cells into S phase, and G2/M phase compares with the non-conjugate betel extract and

eugenol (fig. 4). The results obtained supported the earlier results obtained by MTT assay and staining technique, which proved that the effect of betel leaf extract and eugenol was more when they were administered as silver nano bio conjugates in the oral cancer cells.

i) Scattergrams



ii) Histograms



PE-Piper betle leaf extract, PAgNP-Silver nano bio conjugates from betel leaves, EU-Eugenol, EAgNP-Silver, nano bio conjugates from eugenol

Fig. 4: Effect of Piper betle leaf extract and its silver nano bio conjugates on cell cycle events in KB cells

DISCUSSION

The past ten years of research using metal nanoparticles such as silver, gold, copper, iron, titanium and ruthenium in preclinical research has shown promising anticancer properties [17]. Among the metal nanoparticles, silver and gold have been an attractive frontier of nanoparticle research because of their unique physicochemical properties in biomedical applications, such as bioimaging, biosensing, antimicrobial agents and cancer therapy [18].

Our results showed the strong anticancer activity of the silver nano bio conjugates synthesized. The extent of the anticancer effect was significantly more in the nano bio conjugates than their non-conjugated forms, proving that the anticancer activity of the test

materials used can be enhanced markedly by preparing silver nano bio conjugates. The *in vitro* cytotoxicity assays have long been used for obtaining data on cell viability. Among these, MTT cell survival assay is used for analyzing and validating the cytotoxic potential of compounds [19]. Various reports on the anticancer activity of silver nanoparticles of plant-mediated extracts are available in the literature. The AgNPs synthesized from the ethanolic extract of rose petals showed a dose-dependent decrease in the viability of human colon cancer HCT 15 cells [20]. Vasanth *et al.* [21] showed that the AgNPs of stem bark extract of *Moringa oleifera* possessed potent anticancer activity in human cervical carcinoma cells.

Followed by the cell viability assay, AO/EtBr staining was used to confirm the type of cell death caused by the silver nano bioconjugates,

which was observed to be apoptosis in oral (KB). AO is a fluorescent dye that stains both dead and live cells, whereas EtBr stains only the dead cells, due to lost membrane integrity. Morphological changes have been reported in MCF-7 breast cancer cells treated with silver nanoparticles from *Achillea biebersteinii* using AO/EtBr staining [22]. Another study illustrated that nanosilver showed a stronger inhibitory effect on the liver cancer cells (HepG2) compared to the primary liver cells of mice, which was also observed using AO/EtBr staining [23]. In another study, silver nanoparticles of polyvinylpyrrolidone did not show any cytotoxicity in bone marrow cells, erythrocytes or human keratinocytes by AO/EtBr staining [24]. These reports lend further credibility to the results observed in the present study.

The silver nano bio conjugates showed a higher shift of cells from G0/G1 phase to the S phase and G2/M phase compared to the extracts/compounds in KB cells. These results clearly showed the induction of apoptosis by the silver nano bio conjugates, which was higher than the respective non-conjugated forms. The arrest of cells in G2/M phase has been recognized as representing a high level of cytotoxicity in several literature reports. The silver nanoparticles synthesized from ethanolic extracts of *Phytolacca decandra*, *Gelsemium sempervirens*, *Hydrastis canadensis* and *Thuja occidentalis* showed the highest peak in G2/M phase, which was suggested to indicate cytotoxic effect in human amelanotic melanoma (A375 cells) [25]. Similarly, the silver nanoparticles synthesized from mint, ginger, and coffee in HepG2 (hepatocellular liver carcinoma) and HeLa (human cervical cancer) caused cell cycle arrest in the G2/M phase [26]. Our results are in agreement with these reports. Thus, the silver nano bio conjugates synthesized from the plant source selected in this study exhibit stronger anticancer activity than the extracts and compound in their unconjugated form.

CONCLUSION

The findings of the present study, thus, validate and strengthen the method of synthesis of silver nano-bio conjugates from *Piper betle* leaves and their major polyphenol (eugenol) using a rapid, inexpensive and eco-friendly method. The anticancer properties of silver nano-bio conjugates were higher than their respective unconjugated forms, showing that the efficacy of their anticancer principles can be increased markedly by conjugating with silver to form nano bio conjugates.

CONFLICTS OF INTERESTS

Declared none

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