Chapter 6

Anticancer activity of silver nanobioconjugates
Anticancer activity of silver nanobioconjugates

6.1 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 years. Developing countries of South Asian region have a higher incidence of oral cancer compared to developed countries (Khan et al., 2015; Nair et al., 2015). It has been reported that over one-third of cancers are preventable and one-third of cancers are curable if they are diagnosed earlier. Lung cancer is one of the commonest cancers and causes cancer-related deaths all over the world. India constitutes about 6.9% of lung cancer cases, the highest incidences reported in Mizoram in both males and females (around 28 per 100,000 population in both the sexes). The incidence of lung cancer has also increased significantly in Delhi, Chennai and Bengaluru (Malik and Raina, 2015).

Cancer can affect the living cells in the body at all ages and the disease process differs due to various factors such as tobacco, alcohol, diet, occupation, pollution, geophysical factors and reproductive and sexual behaviour. 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 (Locatelli et al., 2014).

Over the last few decades, about 85,000 compounds have been screened against cancers, but only a few could be considered as effective and promising anti-cancer drugs (Kura et al., 2014). To overcome the disadvantages, over the last two decades, the propagation of knowledge in cancer research has led to the quest for new therapy or development of
novel therapeutic approaches in cancer management, especially in nanodrug-based therapy.

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 (Panzarini et al., 2013). 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 terminal reactive groups, with specific proteins, peptides or monoclonal antibodies that are able to selectively 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 (Herrmann, 2015).

Nanocarriers have been developed from organic materials of plant origin to stimulate the performance of medicine, which reduces systemic side effects and enhances therapeutic efficiency. In our study, the synthesized nanobioconjugates were biocompatible in nature and showed good druggability, with sustained release of the conjugated components. Hence, we were interested in studying their anticancer activity.

### 6.2 Experimental procedure

It was felt imperative to analyze the anticancer activity of the nanobioconjugates synthesized from betel leaves extract, grape seed extract and their respective pure compounds, in comparison with their respective non-nano, raw material, using cancer cell lines. Two cancer cell lines of different tissue origin were selected for this purpose, namely oral carcinoma (KB) cell line and lung carcinoma (A549) cell line. The WHO reported that the both oral and lung cancers are the most prevalent cancers around the world, with oral cancer having a high incidence in India, and lung cancer, all over the world. Both these cancer types are considered to be preventable,
as they arise predominantly due to lifestyle-associated causes like the use of tobacco. While oral cancer is predominantly occurring externally, within the oral cavity, lung cancer is internal (http://preventcancer.org; www.cancer.org; http://www.who.int).

Based on these reasons, the two cancer cell lines, which were procured from National Center for Cell Sciences (NCCS), Pune, were used in this study. As a non-cancerous control group for each of these cell lines, primary cultured buccal cells and peripheral blood lymphocytes from healthy human volunteers were used (Plate 6.1 and 6.2). 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).

Plate 6.1

a) Human buccal cells
b) Human oral carcinoma (KB) cells
6.2.1 Culturing of KB cell line and A549 cell line

The two cancer cell lines 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 healthy and confluently grown cancer cells in the tissue culture flasks were trypsinized using Trypsin-EDTA (PAA) and suspended in 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 haemocytometer and 10⁵ live cells/per millilitre were seeded into sterile 6-well and 96-well plates. For staining, a sterile cover slip was placed in the 6-well plate and the cells were seeded on the cover slip. Both the well plates were incubated for 24 hours, after which the medium was replaced with the fresh medium and treated with silver nanoconjugates or non-nano raw material.
6.2.2 Culturing of buccal cells

The buccal cells are an easily accessible tissue for sampling cells through a minimally invasive technique, which can be used to monitor early genotoxic events due to the ingestion or inhalation of potential carcinogens (Bortoluzzi et al., 2014). The usage of buccal cells has been very much cost-effective and safe to carry out various biological experiments. Various methods are available for buccal cell collection like mouth wash, spit type cards and cytobrush (Livy et al., 2012). In our study, we isolated and cultured the buccal cells to study the toxic effect of silver nanobioconjugates in comparison with oral cancer cells (KB cell line).

Reagents

- Listerine mouthwash solution
- Phosphate buffered saline (PBS)
- Saline (0.9% NaCl)
- DMEM
- Fetal bovine serum
- Penstrep (1X concentration)
- Flucanazole (12µl/ml)

Procedure

The buccal cells were collected from healthy human volunteers, who were not on any medication in the age group of 20-30 years. Before the collection of buccal cells, 5ml of listerine mouthwash solution was used to rinse the mouth for about 2-3 minutes. Then 5ml of sterile saline was used to thoroughly rinse the mouth, and this process was repeated twice. Once the mouth was rinsed, sterile soft wood scrapers were used to scrape the buccal cells from the buccal cavity. About 15ml of sterile saline was used to rinse the mouth, which was collected in sterile beaker, the scraper was also washed with 10ml of sterile saline into the same sterile beaker. The collected cells were transferred into centrifuge tubes and spun at 300g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1ml of
sterile PBS. The suspended cells were treated with 1X penstrep (Penicillin-Streptomycin, from a 100X stock (MP Biomedicals, USA)), 12µl/ml flucanazole (Cipla) and incubated for 30 minutes to remove any bacteria and fungi contaminating the cells. The cells were centrifuged at 300g for 5 minutes 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 flucanazole) in the cell culture flask. The cells were incubated in a CO\textsubscript{2} chamber (Napco, UK) for 24 hours.

After 24 hours of incubation, the buccal cells were transferred into fresh complete medium, which was supplemented with 5% FBS, antibiotics and fungizone. Again, the buccal cells were incubated for another 24 hours. At the end of the incubation period, the cells were collected by trypsinization, centrifuged as earlier and the cell count was determined using 10µl of cell suspension by trypan blue dye exclusion in a haemocytometer. The healthy live cells (10\textsuperscript{5} cells/ml) were seeded in 6-well plate with complete medium and with nanobioconjugates synthesized from betel leaf extract, grape seed extract, their respective pure compounds and their respective non-nano raw material. The exposure was done at 37ºC for 24 hours. The cells were then harvested and centrifuged at 4000 rpm for 5 minutes, washed twice with sterile PBS and resuspended in PBS for the viability and staining analyses.

6.2.3 Culturing of human peripheral blood lymphocytes

Human peripheral blood is often studied by the research and clinical laboratories. The methods for collection, storage and preparation of peripheral blood will vary depending on the cell lineage to be examined as well as the type of assay to be performed (Dagur and McCoy, 2015). To evaluate the toxic effect of silver nanobioconjugates, the in vitro cytotoxic effect on the human peripheral blood lymphocytes was carried out.

**Reagents**

- RPMI medium
- Fetal bovine serum
Penstrep
1% Phytohaemagglutinin-P (PHAP)
Phosphate buffered saline (PBS)
Lymphocyte separation medium (Lymphosep)

Procedure
Isolation of lymphocytes

About 2ml of fresh blood was drawn from healthy human volunteers by venipuncture method and transferred into siliconized glass tube containing 10µl of sterile heparin. The blood was immediately diluted with PBS or RPMI medium in 1:1 ratio. Lymphsep (4ml) was taken into 15ml siliconized glass tubes and 2ml 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 400xg at 18-20ºC. After centrifugation, the grey coloured 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 100xg 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% PHAP. The cells were cultured in sealed sterile cell culture flask and incubated at 37ºC in an incubator with moderate shaking (Heraeus, Germany).

The cell count of isolated lymphocytes was determined using 10µl of cell suspension using trypan blue dye in a haemocytometer. 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 37ºC for 48 hours under mild shaking condition. After the incubation period, the cells were centrifuged at 100xg 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
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24 hours at 37°C. The treated cells were then centrifuged at 100xg for 5 minutes, and washed thrice with PBS. The harvested cells were then used for the viability assay and staining analysis.

6.2.4 Evaluating the anticancer effect of the nanobioconjugates

Apoptosis is a type of programmed cell death. The process of apoptosis can be monitored by the morphologic changes (chromatin condensation, fragmentation, membrane blebbing and the reduction of cell volume) and biochemical changes (membrane surface modifications and breakdown of protein and DNA). Current anticancer treatments, like chemotherapeutic agents and ionizing radiation therapy induce the apoptosis mechanism to kill the cancer cells (Fulda, 2015a; Yang et al., 2015). The regulation of apoptosis and their dysregulation in human cancers is expected to provide opportunities for exploiting this cellular program for cancer therapy (Fulda, 2015b). In our study, the anticancer effect of the nanoconjugates and their respective extract / pure compound were assessed in both the cell lines, as well as the primary cultured cells.

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 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. betel*
- Cells treated with eugenol / silver nanobioconjugates of eugenol
- Cells treated with seed extract / silver nanobioconjugates of *V. vinifera*
- Cells treated with resveratrol / silver nanobioconjugates of resveratrol
6.2.4.1 Cell viability assay

MTT dye reduction assay

The MTT [3(4,5-dimethly-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay, described by Igarashi and Miyazawa (2001), was used to evaluate the cell viability.

Principle

The MTT cell viability assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, there is reduction in cell viability. This is a colorimetric assay that determines the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase in actively growing cells to produce a purple formazan derivative. This derivative is dissolved in a suitable solvent like isopropanol and measured in an ELISA reader.

Reagents

+ PBS
+ MTT (3g/ml in PBS)
+ Acid-Propanol (Isopropanol containing 0.4% of 0.04N HCl)
+ HCl (0.04N)

Procedure

The individual 96 well plates seeded with the cancer cells KB / A549, or their respective primary cells, namely buccal and primary blood lymphocytes, were centrifuged at 1800 rpm for 10 minutes in a well-plate rotor (Plastocraft, Inida). The supernatant was discarded and the cells were treated with 50µl of MTT and incubated at 37°C for 3 hours. At the end of the incubation period, the plates were centrifuged and the medium was removed. Then, the cells were washed with PBS carefully. The purple precipitate was solubilized using 200µl of acid-propanol and incubated
overnight in the dark. The absorbance values were recorded at 595nm in an ELISA reader (Bio-rad, USA). Absorbance values that were lower than the control cells indicated a reduction in the rate of cell proliferation. The viability of the control cells were fixed as 100% viability and the percent viability of cells in the other treatment groups were calculated relative to the control group.

6.2.4.2 Acridine orange / Ethidium bromide staining

Acridine orange and ethidium bromide can be used in combination as a dual stain, which can discriminate the live cells and evaluate the proportion of apoptotic cells. The apoptotic cells were evaluated by the method described by Parks et al. (1979).

**Principle**

Acridine orange is a nucleic acid-selective fluorescent cationic dye that stains both live and dead cells. Ethidium bromide is a DNA intercalator, which will stain only cells that have lost membrane integrity and inserts itself between the base pairs in the double helix, indicating the apoptotic cells.

**Reagents**

+ Phosphate buffered saline
+ Stock stain solution (100x): Acridine orange (15mg) and ethidium bromide (50mg) were dissolved in 1ml of 95% ethanol and 49ml of distilled water. The solution was aliquoted in 1ml cryovials and frozen.
+ Working solution: 1ml aliquot of the stock solution was thawed and diluted 100 times in PBS. This was mixed well and stored at 4ºC upto one month, protected from light.

**Procedure**

The control and treated cells on the cover slip were removed carefully without disturbing the cells and placed on the glass slides, 10µl of AO / EtBr was added on the cells and spread by placing a fresh cover slip. The slides were incubated at room temperature for 5 minutes and viewed under upright
fluorescent microscope (Nikon, Japan) using B2A filter at 40X magnification. Live and apoptotic cells were counted and discriminated. Live cells appeared uniformly green. Apoptotic cells incorporate the ethidium bromide and show the chromatin condensation and nuclear fragmentation. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin. The apoptotic ratio was calculated using the formula.

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

6.2.5 Cell cycle analysis

Cell cycle analysis was carried out by flow cytometry, to determine the phase of cell cycle arrest (Krishan, 1975).

Reagents

+ PBS
+ PI reagent - 100ml

  I) Sodium citrate - 100mg
  II) Propidium iodide - 2.5mg
  III) RNAase - 4mg
  IV) Tween 20 - 30µl

Procedure

Cells (1x10^6 cells/ml) treated with silver nanobioconjugates/non-conjugates as explained above were collected and washed with PBS. The incubation period was for 12 hours, as the cytotoxicity observed at 24 hours was quite high. Then the cells were incubated with 1ml of the prepared mixture of PI reagent, and were stained for 30 minutes in the dark. After incubation, the cells were subjected and analyzed for the populations of sub-G₀, G₀/G₁, S and G₂/M phases of the cell cycle by flow cytometry (BD FACSVers, USA).
6.3 Results

The effect and influence of nanobioconjugates in transformed cells (KB oral cancer cell line and A549 lung cancer cell line) and non-transformed cells (buccal cells and peripheral blood lymphocytes) were studied using cytotoxicity assay, staining technique and flow cytometry.

6.3.1 Effect of silver nanobioconjugates on the viability of KB cells, A549 cells, buccal cells and peripheral blood lymphocytes

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. The MTT assay was used to determine the influence of silver nanobioconjugates on the viability of KB and A549 cancer cells, along with the non-transformed cells (buccal cells and peripheral blood lymphocytes). A dose- and time-dependent effect of nanobioconjugates synthesized from Piper betle leaf extract, eugenol, Vitis vinifera seed extract and resveratrol was evaluated. A dose-response curve was constructed using a wide dose range (2.5µg to 50µg/ml) of all the nanobioconjugates and their extract alone. Based upon the literature reports 50µM and 100µM of active components, eugenol (Jaganathan and Supriyanto, 2012) and resveratrol (Udenigwe et al., 2008), were taken for the study. Initially, an exposure interval of 24 hours was used.

The extents of viability observed with the nanobioconjugates and their non-nano counterparts, at the same dose level, in KB cells are presented in Figures 6.1 and 6.2 for Piper betle and Vitis vinifera respectively. Piper betle leaf extract, eugenol and their nanobioconjugates caused a dose-dependent decrease in the viability of KB cells. These results showed that the extract and its components possess strong anticancer activity against the oral cancer 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-treated and eugenol-treated groups. These observations show that the anticancer effect of both
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*Piper betle* leaf extract and its active phenolic component, eugenol, can be increased several fold by administering them as silver nanobioconjugates.

In a similar trend, *Vitis vinifera* seed extract, resveratrol and their nanobioconjugates also caused dose-dependent death of KB cells (Figure 6.2). The extent of death was markedly more pronounced in the silver nanobioconjugates than their respective non-conjugated forms. These results reiterate that the anticancer effect of the plant extracts as well as their components can be increased in magnitude by preparing the silver nanobioconjugate forms.

From among the dose range tested (2.5µg to 50µg), three doses representing low (2.5µg), intermediate (20µg) and high (50µg) levels, were chosen for testing the cytotoxicity in the primary cultured buccal cells. This effect was studied using the nanobioconjugates of both the plant extracts and the pure phenolic components. Figure 6.3 represents the results obtained. As can be observed from the results, all the four nanobioconjugates did not affect the viability of the buccal cells, at all the three doses tested. These results clearly demonstrate the differential effect of *Piper betle* leaf extract, *Vitis vinifera* seed extract and their phenolic components on cancerous and non-cancerous cells of the oral cavity.

Following this, the effect of the synthesized nanobioconjugates and their respective non-conjugated biomaterial were studied on A549 lung cancer cells at the same dose range and 24-hours exposure time. The results obtained (Figure 6.4 for *Piper betle* extract and eugenol, and Figure 6.5 for *Vitis vinifera* extract and resveratrol) showed a similar trend as KB cells, confirming the anticancer effects of the test materials on lung cancer also. As observed in the oral cancer cells, the nanobioconjugates induced a higher cytotoxicity than their respective non-conjugated forms, in lung cancer cells.

As a comparative control for the internalized lung cancer cells, primary cultured peripheral blood lymphocytes were used. The low (2.5µg),
intermediate (20µg) and the high (50µg) dose levels of the silver nanobioconjugates were tested for their cytotoxicity at 24 hour interval in these cells. The results (Figure 6.6) showed that, at all the three doses, the bioconjugates were non-toxic to the lymphocytes, providing without ambiguity, the differential effect of the nanobioconjugates.

**Figure 6.1**

Effect of silver nanobioconjugates synthesized from *Piper betle* leaves and their active component eugenol on the viability of KB cells

(24 hours treatment)

i) Silver nanobioconjugates from *Piper betle* leaf extract

![Graph showing viability vs concentration for PE and PAgNP](image)

ii) Silver nanobioconjugates from eugenol

![Graph showing viability vs concentration for EU and EAgNP](image)

The values are Mean ± S.D. of triplicates
Figure 6.2
Effect of silver nanobioconjugates synthesized from *Vitis vinifera* seeds and their active component resveratrol on the viability of KB cells (24 hours treatment)

i) Silver nanobioconjugates from *Vitis vinifera* seed extract

![Graph showing the effect of silver nanobioconjugates from *Vitis vinifera* seed extract on KB cell viability. The graph displays concentration vs. % viability, with concentrations ranging from 2.5 to 50 µg for both VE and VAgNP.]

The values are Mean ± S.D. of triplicates

ii) Silver nanobioconjugates from resveratrol

![Graph showing the effect of silver nanobioconjugates from resveratrol on KB cell viability. The graph displays concentration vs. % viability, with concentrations ranging from 50 µM to 50 µg for R and RAgNP.]

The values are Mean ± S.D. of triplicates
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Figure 6.3
Effect of silver nanobioconjugates on human buccal cells

i) PAgNP

ii) EAgNP

iii) VAgNP

Green synthesis of biologically active silver nanobioconjugates using eugenol and resveratrol and their plant sources, 
Piper betle leaves and Vitis vinifera seeds
Figure 6.4

Effect of silver nanobioconjugates synthesized from *Piper betle* leaves and their active component eugenol on the viability of A549 cells (24 hours treatment)

i) Silver nanobioconjugates from *Piper betle* leaf extract

![Graph showing the effect of silver nanobioconjugates from *Piper betle* leaf extract on cell viability.](image)

ii) Silver nanobioconjugates from eugenol

![Graph showing the effect of silver nanobioconjugates from eugenol on cell viability.](image)

The values are Mean ± S.D. of triplicates.
Figure 6.5

Effect of silver nanobioconjugates synthesized from *Vitis vinifera* seeds and their active component resveratrol on the viability of A549 cells (24 hours treatment)

i) Silver nanobioconjugates from *Vitis vinifera* seed extract

![Graph showing % viability vs concentration for silver nanobioconjugates from Vitis vinifera seed extract.](image)

The values are Mean ± S.D. of triplicates

ii) Silver nanobioconjugates from resveratrol

![Graph showing % viability vs concentration for silver nanobioconjugates from resveratrol.](image)
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Figure 6.6
Effect of silver nanobioconjugates on human peripheral blood lymphocytes

i) PAgNP

ii) EAgNP

ii) VAgNP

iii) RAgNP

Green synthesis of biologically active silver nanobioconjugates using eugenol and resveratrol and their plant sources, *Piper betle* leaves and *Vitis vinifera* seeds.
The extent of cell death induced by the nanobioconjugates was very high in both KB and A549 cells at 24 hour exposure period. Therefore, in order to optimize the duration of exposure for flow cytometry studies, a shorter exposure time of 16 hours was studied. The results (Figure 6.7 and 6.8 for *Piper betle* and *Vitis vinifera* extracts and phenolics respectively) obtained with KB cells showed a high cytotoxicity, at all the dose levels tested. Therefore, the two lower dose levels (2.5µg and 5µg) of all the four bioconjugates were tested at 12 hour exposure period. These results are also presented in Figures 6.7 and 6.8. From the results obtained, it can be inferred that pronounced anticancer effect is induced by all the nanobioconjugates synthesized in the present study in KB oral cancer cells.

The dose levels and durations of exposure were also studied in the A549 cells lung cancer cells. The results (Figures 6.9 and 6.10) exhibited the same trend observed in KB oral cancer cells, reiterating the strong anticancer effect of the silver nanobioconjugates.

The shorter exposure time was not tested on the primary cultured buccal cells and lymphocytes, because, even at the longer duration (24 hours exposure), all the nanobioconjugates did not exhibit toxicity in these cells. Thus, the extent of viability observed in the present study revealed that both *Piper betle* and *Vitis vinifera* exhibited strong anticancer activity to both KB oral carcinoma cells and A549 lung adenocarcinoma cells. The anticancer effect of the extract was attributable to the component phenolics, eugenol and resveratrol respectively in betel leaves and grape seeds, as the phenolics also exhibited strong cytotoxicity in both the types of cancer cells. The anticancer activity increased by a marked magnitude when the extracts / compounds were administered as nanobioconjugates. Another significant observation was the differential effect evoked by the AgNPs, which were non-toxic to non-cancerous buccal cells and lymphocytes, while evoking a strong cytotoxicity in the cancer cells.
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Figure 6.7

Effect of silver nanobioconjugates synthesized from *Piper betle* leaves and their active component eugenol on the viability of KB cells (16 and 12 hours treatment)

i) PAgNP

![Graph showing viability of KB cells treated with PAgNP](image)

The values are Mean ± S.D. of triplicates

ii) EAgNP

![Graph showing viability of KB cells treated with EAgNP](image)
Figure 6.8

Effect of silver nanobioconjugates synthesized from *Vitis vinifera* seeds and their active component resveratrol on the viability of KB cells (16 and 12 hours treatment)

i) VAgNP

![Graph showing the effect of VAgNP on KB cell viability.](image)

The values are Mean ± S.D. of triplicates

ii) RAgNP

![Graph showing the effect of RAgNP on KB cell viability.](image)
Figure 6.9

Effect of silver nanobioconjugates synthesized from *Piper betle* leaves and their active component eugenol on the viability of A549 cells (16 and 12 hours treatment)

i) PAgNP

![Graph showing % viability vs. concentration (µg) for 16 hours and 12 hours treatment with error bars indicating Mean ± S.D. of triplicates.]

ii) EAgNP

![Graph showing % viability vs. concentration (µg) for 16 hours and 12 hours treatment with error bars indicating Mean ± S.D. of triplicates.]

The values are Mean ± S.D. of triplicates
Figure 6.10

Effect of silver nanobioconjugates synthesized from *Vitis vinifera* seeds and their active component resveratrol on the viability of A549 cells (16 and 12 hours treatment)

i) VAgNP

![Graph showing the effect of VAgNP on A549 cell viability](image)

The values are Mean ± S.D. of triplicates

6.3.2 Microscopic study of KB cells, A549 cells, buccal cells and peripheral blood lymphocytes exposed to silver nanobioconjugates

Having established the anticancer activity of the prepared nanobioconjugates, an attempt was made to observe the type of cell death induced in the cancer cells, using AO/EtBr dual staining method. AO/EtBr is
a convenient differential staining method that can differentiate between normal, apoptotic and necrotic cells. The cancerous (KB and A549) cells and non-cancerous (buccal and lymphocytes) that were exposed to the nanobioconjugates or their non-conjugated component were subjected to AO/EtBr staining and counted under the fluorescent microscope. Apoptotic and necrotic cells (stained orange) were discriminated by the nuclear morphology, while the normal cells stained green and were easily differentiated.

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. The results obtained are presented in Tables 6.1 and 6.2. The apoptotic indices calculated are represented in Figures 6.11 and 6.12. The photomicrographs of the stained cells in each group are presented in Plates 6.3, 6.4, 6.5 and 6.6.

From the results, it can be clearly inferred that the leaf extract of *Piper betle*, seed extract of *Vitis vinifera* as well as their active components, eugenol and resveratrol respectively, by themselves, possess marked anticancer effect, which is mediated via the induction of apoptotic pathway. When these bioagents were conjugated with silver to form nanoscale particles, the extent of apoptosis in the cancer cells increased significantly. Our results clearly demonstrate that the anticancer effect of the two candidate plants and their active components can be improved significantly by converting them into silver nanobioconjugates.
Table 6.1
Effect of extract / compound / silver nanobioconjugates in KB cells and human buccal cells (AO/EtBr staining)

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>KB cells</th>
<th>Human Buccal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract / Compound alone</td>
<td>AgNP</td>
</tr>
<tr>
<td>Control</td>
<td>7 ± 2</td>
<td>9 ± 2</td>
</tr>
<tr>
<td><em>Piper betle</em></td>
<td>34 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47 ± 5&lt;sup&gt;a, b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Eugenol</em></td>
<td>47 ± 3&lt;sup&gt;a, c&lt;/sup&gt;</td>
<td>55 ± 2&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Vitis vinifera</em></td>
<td>25 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36 ± 3&lt;sup&gt;a, b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Resveratrol</em></td>
<td>49 ± 1&lt;sup&gt;a, c&lt;/sup&gt;</td>
<td>57 ± 6&lt;sup&gt;a, b, c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are mean ± S.D of triplicates

- <sup>a</sup> – Statistically significant (P<0.05) compared to untreated control
- <sup>b</sup> – Statistically significant (P<0.05) compared to corresponding unconjugated extract/compound
- <sup>c</sup> – Statistically significant (P<0.05) compared to the corresponding extract/compound/group

Figure 6.11
Effect of extract / compound / silver nanobioconjugates on apoptotic ratio in KB cells and human buccal cells as determined by AO/EtBr staining
Table 6.2
Effect of extract / compound / silver nanobioconjugates in A549 cells and primary peripheral blood lymphocytes (AO/EtBr staining)

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Number of Apoptotic cells/100 cells</th>
<th>A549 cells</th>
<th>Human peripheral blood lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract / Compound alone</td>
<td>AgNP (12 hours)</td>
<td>Extract / Compound alone</td>
</tr>
<tr>
<td>Control</td>
<td>13 ± 2</td>
<td>10 ± 2</td>
<td></td>
</tr>
<tr>
<td><em>Piper betle</em></td>
<td>37 ± 5 <em>a</em></td>
<td>45 ± 5 <em>a, b</em></td>
<td>12 ± 2</td>
</tr>
<tr>
<td><em>Eugenol</em></td>
<td>47 ± 3 <em>a, c</em></td>
<td>59 ± 2 <em>a, b, c</em></td>
<td>9 ± 2</td>
</tr>
<tr>
<td><em>Vitis vinifera</em></td>
<td>38 ± 3 <em>a</em></td>
<td>42 ± 3 <em>a</em></td>
<td>12 ± 2</td>
</tr>
<tr>
<td><em>Resveratrol</em></td>
<td>48 ± 6 <em>a, c</em></td>
<td>56 ± 1 <em>a, b, c</em></td>
<td>13 ± 1</td>
</tr>
</tbody>
</table>

The values are mean ± S.D of triplicates

- *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

Figure 6.12
Effect of extract / compound / silver nanobioconjugates on apoptotic ratio of KB cells and human buccal cells as determined by AO/EtBr staining
Chapter 6: Anticancer studies of silver nanobioconjugates

Plate 6.3
Effect of extract / compound / silver nanobioconjugates on KB cells

Control

PE - Piper betle leaf extract, EU - Eugenol, VE - Vitis vinifera seed extract, R - Resveratrol, PAgNP - Silver nanobioconjugates from betle leaves, EAgNP - Silver nanobioconjugates from eugenol, VAgNP - Silver nanobioconjugates from Vitis vinifera seeds extract, RAgNP - Silver nanobioconjugates from resveratrol

Green synthesis of biologically active silver nanobioconjugates using eugenol and resveratrol and their plant sources, Piper betle leaves and Vitis vinifera seeds
Chapter 6: Anticancer studies of silver nanobioconjugates

Plate 6.4
Effect of extract / compound / silver nanobioconjugates on human buccal cells

Control

PE - Piper betle leaf extract, EU - Eugenol, VE - Vitis vinifera seed extract, R - Resveratrol, PAgNP - Silver nanobioconjugates from betle leaves, EAgNP - Silver nanobioconjugates from eugenol, VAgNP - Silver nanobioconjugates from Vitis vinifera seeds extract, RAgNP - Silver nanobioconjugates from resveratrol

Green synthesis of biologically active silver nanobioconjugates using eugenol and resveratrol and their plant sources, 
Piper betle leaves and Vitis vinifera seeds
Chapter 6: Anticancer studies of silver nanobioconjugates

Plate 6.5
Effect of extract / compound / silver nanobioconjugates on A549 cells

Control

PE - Piper betle leaf extract, EU - Eugenol, VE - Vitis vinifera seed extract, R - Resveratrol, PAgNP - Silver nanobioconjugates from betle leaves, EAgNP - Silver nanobioconjugates from eugenol, VAgNP - Silver nanobioconjugates from Vitis vinifera seeds extract, RAgNP - Silver nanobioconjugates from resveratrol

Green synthesis of biologically active silver nanobioconjugates using eugenol and resveratrol and their plant sources, Piper betle leaves and Vitis vinifera seeds
Chapter 6: Anticancer studies of silver nanobioconjugates

Plate 6.6
Effect of extract / compound / silver nanobioconjugates on human peripheral blood lymphocytes

Green synthesis of biologically active silver nanobioconjugates using eugenol and resveratrol and their plant sources, *Piper betle* leaves and *Vitis vinifera* seeds
6.3.3 Cell cycle analysis

Having ascertained that the cell death induced by the selected test substances (nanobioconjugates) was apoptosis, the events of the cell cycle in the exposed cancer cells was analyzed by flow cytometry to understand the cellular events. The cell cycle events were recorded by flow cytometry using propidium iodide, after exposing the oral / lung cancer cells for 12 hours to the nanobioconjugates. The proportions of cells arrested in $G_0/G_1$ phase, S phase and $G_2/M$ phase of the cell cycle were quantified. The scattergrams and histograms obtained in the different treatment groups are depicted in Figures 6.13, 6.14, 6.15, 6.16, 6.17, 6.18, 6.19 and 6.20. The proportion of cells in each cell cycle phase was calculated for each group as per cent values, which are listed in Tables 6.3 and 6.4 for KB and A549 cells respectively.

Table 6.3

<table>
<thead>
<tr>
<th>Groups</th>
<th>$G_0/G_1$</th>
<th>S</th>
<th>$G_2/M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>76.00</td>
<td>9.69</td>
<td>6.97</td>
</tr>
<tr>
<td>PAgNPs</td>
<td>68.66</td>
<td>13.81</td>
<td>10.81</td>
</tr>
<tr>
<td>EU</td>
<td>77.27</td>
<td>10.28</td>
<td>5.72</td>
</tr>
<tr>
<td>EAgNPs</td>
<td>63.81</td>
<td>16.24</td>
<td>12.54</td>
</tr>
<tr>
<td>VE</td>
<td>77.22</td>
<td>11.85</td>
<td>5.52</td>
</tr>
<tr>
<td>VAgNPs</td>
<td>61.81</td>
<td>17.97</td>
<td>12.60</td>
</tr>
<tr>
<td>R</td>
<td>70.37</td>
<td>15.49</td>
<td>8.81</td>
</tr>
<tr>
<td>RAgNPs</td>
<td>58.71</td>
<td>23.69</td>
<td>12.15</td>
</tr>
</tbody>
</table>
Table 6.4

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>G₀/G₁</th>
<th>S</th>
<th>G₂/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>36.13</td>
<td>4.31</td>
<td>35.69</td>
</tr>
<tr>
<td>PAgNPs</td>
<td>31.20</td>
<td>3.31</td>
<td>42.60</td>
</tr>
<tr>
<td>EU</td>
<td>59.58</td>
<td>5.29</td>
<td>23.70</td>
</tr>
<tr>
<td>EAgNPs</td>
<td>37.60</td>
<td>7.81</td>
<td>41.28</td>
</tr>
<tr>
<td>VE</td>
<td>47.28</td>
<td>7.90</td>
<td>29.47</td>
</tr>
<tr>
<td>VAgNPs</td>
<td>34.34</td>
<td>7.98</td>
<td>31.09</td>
</tr>
<tr>
<td>R</td>
<td>61.96</td>
<td>6.79</td>
<td>15.68</td>
</tr>
<tr>
<td>RAgNPs</td>
<td>10.15</td>
<td>6.10</td>
<td>67.66</td>
</tr>
</tbody>
</table>

Treatment with the silver nanobioconjugates of all the test materials included in the present study caused a shift of cells into the S phase and G₂/M phase compared to the non-conjugated test materials. This clearly suggests that more cells commit to apoptosis when the silver nanobioconjugates were administered. This trend was observed in both KB and A549 cells. The effect was more pronounced in the A549 cells. The significance of this observation evades us at present, and requires more studies. Thus, the flow cytometry studies rendered support to our earlier results, that the anticancer effect of *Piper betle* leaf extract, *Vitis vinifera* seed extract, and their active components (eugenol and resveratrol) was significantly enhanced when they were administered to the oral and lung cancer cells as silver nanobioconjugates.
Figure 6.13
Effect of *Piper betle* leaf extract and its silver nanobioconjugates on cell cycle events in KB cells

i) Scattergrams

![Scattergrams for PE and PAgNP](image)

ii) Histograms

![Histograms for PE and PAgNP](image)

PE - *Piper betle* leaf extract
PAgNP - Silver nanobioconjugates from betle leaves
Chapter 6: Anticancer activity of silver nanobioconjugates

Figure 6.14
Effect of eugenol and its silver nanobioconjugates on cell cycle events in KB cells

i) Scattergrams

ii) Histograms

EU - Eugenol
EAgNP - Silver nanobioconjugates from eugenol
Figure 6.15
Effect of *Vitis vinifera* seed extract and its silver nanobioconjugates on cell cycle events in KB cells

- **i) Scattergrams**
  - VE (Vitis vinifera seed extract)
  - VAgNP (Silver nanobioconjugates from *Vitis vinifera* seeds extract)

- **ii) Histograms**
  - VE (Vitis vinifera seed extract)
  - VAgNP (Silver nanobioconjugates from *Vitis vinifera* seeds extract)
Chapter 6: Anticancer activity of silver nanobioconjugates

Figure 6.16
Effect of resveratrol and its silver nanobioconjugates on cell cycle events in KB cells

i) Scattergrams

R - All Events

RAgNP - KB - All Events

ii) Histograms

R - P1

RAgNP - KB - P1

R - Resveratrol

RAgNP - Silver nanobioconjugates from resveratrol

Green synthesis of biologically active silver nanobioconjugates using eugenol and resveratrol and their plant sources, *Piper betle* leaves and *Vitis vinifera* seeds

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Figure 6.17
Effect of *Piper betle* leaf extract and its silver nanobioconjugates on cell cycle events in A549 cells

i) Scattergrams

![Scattergrams](image)

PE - *Piper betle* leaf extract
PAgNP - Silver nanobioconjugates from betle leaves

ii) Histograms

![Histograms](image)
Figure 6.18
Effect of eugenol and its silver nanobioconjugates on cell cycle events in A549 cells

i) Scattergrams

EU

EU-A549 - All Events

EAgNP

EAgNP-A549 - All Events

ii) Histograms

EU

EU-A549 - PI

EAgNP

EAgNP-A549 - PI

EU - Eugenol,
EAgNP - Silver nanobioconjugates from eugenol
Figure 6.19

Effect of *Vitis vinifera* seed extract and its silver nanobioconjugates on cell cycle events in A549 cells

i) Scattergrams

![Scattergrams](image1)

 VE - *Vitis vinifera* seed extract  
 VAgNP - Silver nanobioconjugates from *Vitis vinifera* seeds extract

ii) Histograms

![Histograms](image2)
Chapter 6: Anticancer activity of silver nanobioconjugates

Figure 6.20
Effect of resveratrol and its silver nanobioconjugates on cell cycle events in A549 cells

i) Scattergrams

ii) Histograms

R - Resveratrol
RAgNP - Silver nanobioconjugates from resveratrol

Green synthesis of biologically active silver nanobioconjugates using eugenol and resveratrol and their plant sources, *Piper betle* leaves and *Vitis vinifera* seeds
6.4 Discussion

The ancient knowledge on Ayurvedic medicine based on metal, mineral and herbal preparations has 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. It has been stressed over the years that size reduction of nanoparticles plays a vital role in improving their bioavailability for therapeutical applications in treating diseases like cancer (Arachchige et al., 2015). In search of eco-friendly methods for the nanoparticle synthesis, plants possessing limitless active compounds are being exploited as key resources for the synthesis of nanoparticles. The rationale behind the use of plants for the synthesis of metal nanoparticles is due to the presence of active compounds, which conjugate with the metal and provide great potential in cancer management.

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 (Ceresa et al., 2014). 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 (Luo et al., 2014). The research related to toxicity of nanoparticles reported a negative perception of their use. However, toxicity itself can 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 (Wei et al., 2015).

The synthesized silver nanobioconjugates in the present study exhibited potent antibacterial activity. In this phase of the study, the samples were analyzed for their anticancer activity, which was determined by cell viability assay, staining and flow cytometry.

Our results showed the strong anticancer activity of the silver nanobioconjugates synthesized. 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. Additionally, the nanobioconjugates expressed a differential effect in cancerous and non-cancerous cells. That is, no lethality was observed in the primary cultured cells, while both oral cancer and lung cancer cells showed significant death at the same dose of nanobioconjugates.

Differential staining identified the cell death in the cancer cells as predominantly apoptosis. Cell cycle analysis by flow cytometry lend support to this observation, in that, more cells were arrested at the G2/M phase when exposed to the nanobioconjugates, implicating their commitment to the programmed cell death of apoptosis.

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 (Sumantran, 2011). 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 (Manikandan et al., 2015). Vasanth et al. (2014) showed that the AgNPs of stem bark extract of Moringa oleifera possessed potent anticancer activity in human cervical carcinoma cells. Phytochemicals, especially phenols, flavonoids and terpenoids present in the plant extracts of Cucurbita maxima (petals), Moringa oleifera (leaves) and Acorus calamus (rhizome) involved in the AgNPs synthesis, showed significant anticancer activity against A431 skin cancer cell line (Nayak et al., 2015). Cell viability assays suggested a dose-dependent toxic effect of AgNPs synthesized from oak fruit hull extract in human breast cancer cells (Heydari and Rashidipour, 2015).

AgNPs induce cell responses often specific to cell types, resulting in varying degrees of toxicity. Depending on nanoparticle size, concentration and exposure time, silver nanoparticles induced different degrees of toxicity.
in vitro in human breast cancer cells (Jeyaraj et al., 2013), skin epithelial A431 and lung epithelial A549 cells (Kaur and Tikoo, 2013). Similarly, Nakkala et al. (2014), Palaniappan et al. (2015) and Rathi Sre et al. (2015), also observed the cytotoxicity of AgNPs synthesized from different plants in lung cancer cells (A549). Foldbjerg et al. (2011) also reported the cytotoxicity by disturbing the mitochondrial function of human alveolar cancer (A549) cells, wherein it was shown that AgNPs are taken up by the cells, leading to increased production of ROS and ultimately apoptotic and necrotic cell death. Suman et al., (2013) also reported the cytotoxicity against HeLa cell lines using the silver nanoparticles fabricated from Morinda citrifolia root extract.

Several researchers have also observed a differential effect of nanobioconjugates between non-cancerous and cancerous cells. Patra et al. (2015) demonstrated the delivery of anticancer components to the cancer cells using biosynthesized gold and silver nanoparticles from Butea monosperma leaf extract. Both the nanoparticles showed significant inhibition of B16F10, MCF-7 cancer cells proliferation and biocompatibility towards normal endothelial cells (HUVEC, EVC-340). 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 (Hatipoglu et al., 2015). The silver nanoparticles 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 (Swanner et al., 2015).

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 (Jacob et al., 2012; Faedmalekia et al., 2014; Swamy et al., 2014; Ramar et al., 2015). Guo et al. (2013) 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. (2014) was that silver nanoparticles of Dendrophthoe falcata extract induced toxicity by the cellular uptake through clathrin-dependent endocytosis and macropinocytosis.

The results of the present study are in accordance with these studies. The size of the nanoparticles synthesized in the present study ranged between 8nm and 35nm, as shown in the earlier phase of the study (Chapter 4). This small size could very well be a contributing factor to the high cytotoxicity observed in both the cancer cell types.

Followed by the cell viability assay, AO/EtBr staining was used to confirm the type of cell death caused by the silver nanobiointerconjugates, which was observed to be apoptosis in both oral (KB) and lung (A549) cancer. 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 (Baharara et al., 2015). Another study illustrated that nanosilver showed 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 (Faedmaleki et al., 2014). In another study, silver nanoparticles of polyvinylpyrrolidone did not show any cytotoxicity in bone marrow cells, erythrocytes or human keratinocytes by AO/EtBr staining (Huk et al., 2014).

The cytotoxic effect of silver nanoparticles from Frangipani (Plumeria alba) flower in COLO 205 colon cancer cells using AO/EtBr staining indicated the induction of apoptosis (Mata et al., 2015). Morphological analysis of AO/EtBr on Hela and MCF7-cells in the presence of silver nanoparticles synthesized from Guignardia mangiferae showed the induction of apoptosis (Balakumaran et al., 2015). Song et al. (2014) observed a high cytotoxicity of silver and zinc oxide nanoparticles on human
epithelial colorectal adenocarcinoma (Caco-2) cells. Silver nanoparticles synthesized from *Phyllanthus emblica* showed higher cell death in Hep2 laryngeal carcinoma cells, than the extract of amla (Rosarin *et al*., 2013). The AgNPs from *Ficus religiosa* exhibited toxicity in Dalton’s ascites lymphoma tumour cells through apoptosis, as shown by AO/EtBr staining with permeabilization of cytoplasmic membrane (Antony *et al*., 2013). These reports lend further credibility to the results observed in the present study.

Following the MTT assay and AO/EtBr staining results, the events of cell cycle in the both the cancer cells was analyzed by flow cytometry to understand the cellular events. All the four nanobioconjugates were administered to the KB and A549 cell line for 12 hours. The silver nanobioconjugates showed a higher shift of cells from G0/G1 phase to the S phase and G2/M phase compared to the extracts / compounds in both KB and A549 cells. These results clearly showed the induction of apoptosis by the silver nanobioconjugates, 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) (Das *et al*., 2013). 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 (Chunyan and Valiyaveettil, 2013).

The cell cycle arrest of HepG2 liver cancer cells in G2/M phase was shown to result in a significant increase in the apoptosis rate (Xue *et al*., 2015). Austin *et al*., (2011) reported the cell cycle arrest in G2/M of HSC-3 oral cancer cells when treated with silver nanoparticles of sodium citrate. Similarly, the data on cell cycle analysis of human cervical cancer cells
treated with platinum nanoparticles synthesized from tea polyphenols also indicated G2/M phase arrest, which was attributed to DNA damage (Alshatwi et al., 2015).

Another study also reported the cell cycle arrest in G2/M phase in MCF-7 cells treated with rhamnolipid-functionalized AgNPs (Dwivedi et al., 2015). Human colon cancer cells treated with the silver nanoparticles synthesized from the leaf extract of *Vitex negundo* showed cell cycle arrest in both G0/G1 and G2/M phase (Prabhu et al., 2013). Our results are in agreement with these reports.

Thus, all the silver nanobioconjugates synthesized from the plant sources selected in the present study (*Piper betle* leaf extract, eugenol, *Vitis vinifera* seed extract and resveratrol) exhibit stronger anticancer activity than the extracts and compounds in their unconjugated form.

Our study, thus, optimized the method of green synthesis of silver nanobioconjugates. All the nanobioconjugates, when characterized for their properties, showed that they possess the ideal size, surface charge and other characters needed for druggability, including the drug release profile. All the nanobioconjugates also possessed good bioactivity. The results of the study are summarized and the conclusions drawn therein are presented in the next chapter.