Chapter 7

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Nanotechnology is a fast-growing field, which has been implemented in most of the areas of science and technology due to the significant chemical and physical properties of material in the nanoscale. The recent focus and development of nanotechnology in medicine is countless, which involves diagnostics, therapeutic and preventive systems for various diseases. Now-a-days, the trend of nanotechnology is also shifted towards the nature-based systems, as they are eco-friendly. Among the various nanoparticles available, metal nanoparticles like silver, gold and iron have gained much interest towards biology, due to their well recognized bioactivities.

From ancient times, plants and fruits have played an important role in all our lives as food and medicine. India is a rich source of medicinal plants and fruits, widely utilized and practiced in the traditional medicine to control, cure and prevent various diseases. In the recent years, interest in synthesizing nanoparticles from the extracts and active components present in the plant and fruits, especially as metal nanoparticles, has gained much attention. The synthesis of nanoparticles using this biological route has been shown to have more advantages than the chemical and physical synthesis patterns, with low cost and non-toxic nature.

The present study focussed on the green synthesis of silver nanobioconjugates from two phenolic-rich plant sources, namely Piper betle leaves and Vitis vinifera seeds, and their major component phenolics, eugenol and resveratrol respectively. The method of synthesis was optimized and the antimicrobial effect, biocompatibility and anticancer properties of the synthesized silver nanobioconjugates were evaluated.

As the initial phase of the study, the presence of the phenolics in the plants was confirmed by HPLC. The HPLC profiles proved that the selected
plant sources contained considerable amounts of the phenolics under study. Based on the HPLC profiles, the leaves of *Piper betle* and the seeds of the pink grapes were taken for further study, along with commercially available eugenol and resveratrol. For both the plant sources, methanolic extracts were prepared as they contained the maximum phenol content. From all these four sources (*Piper betle* leaf extract, *Vitis vinifera* seed extract, eugenol and resveratrol), silver nanobioconjugates were synthesized.

Four different methods, namely microwave heating, heating in water bath (60°C), exposure to bright sunlight and incubation at 37°C, were used for the synthesis of silver nanobioconjugates. The synthesis of nanoparticles was monitored by a change in colour (qualitatively) and an increase in absorbance (quantitatively). All the four methods were quite efficient in the synthesis of silver nanobioconjugates using *Piper betle* leaf and *Vitis vinifera* seed extracts. Among the four different methods, there was a notable colour change, yield and increase in the intensity of colour from 5 to 20 minutes by the sunlight exposure, than the other methods. These observations confirmed that the sunlight exposure for 20 minutes was the best method for the rapid synthesis of silver nanoparticles using *Piper betle* leaf and *Vitis vinifera* seed extracts. So the respective major phenolic compounds of betel leaf and grape seed extract, namely eugenol and resveratrol, were employed in the synthesis of silver nanobioconjugates only under sunlight exposure for 20 minutes. Both the compounds also showed the colour changes and confirmed the synthesis of silver nanobioconjugates.

The silver nanobioconjugates synthesized from betel leaf extract and grape seed extract using the four different methods showed distinct peaks at 420nm and 440nm respectively, which is the characteristic of AgNPs. The peaks were more pronounced in the sunlight-exposed samples than the other methods, reiterating that this was the best among the methods tested.

Following the successful synthesis of silver nanobioconjugates, their bioactivity was determined as antibacterial effect against the clinical isolates of one Gram positive (*Staphylococcus aureus*) and one Gram negative
(Shigella flexneri) organisms. The synthesized silver nanobioconjugates from betel leaf and grape seed extracts and their respective active compounds, eugenol and resveratrol, showed potent antimicrobial activity against both Gram positive and Gram negative microorganisms. The silver bioconjugates were more effective in inhibiting the growth of the organisms than their non-conjugated counterparts, showing that the antimicrobial efficiency of the biomaterials used can be improved significantly by preparing nanobioconjugates from them.

Based on the yield and bioactivity, only the nanobioconjugates synthesized using sunlight exposure for 20 minutes were taken for further detailed characterization. TEM images showed that the nanobioconjugates synthesized from Piper betle leaf extract, Vitis vinifera seed extract and their active compounds (eugenol and resveratrol) showed spherical shape, with a size range of 16-35nm, 14-28nm, 16-28nm and 8-21nm respectively. All the nanobioconjugates were also very well dispersed, without aggregation. These observations suggested that the nanobioconjugates synthesized in the present study, possessed properties ideally suited for biomedical applications.

The EDX profile of all nanobioconjugates showed the presence of typical peaks of silver. Additional peaks were also observed which indicated the presence of carbon and oxygen, representing the existence of organic compounds in the silver nanobioconjugates. The X-ray diffraction profiles confirmed the presence of silver in the nanobioconjugates and the highly crystalline nature of the particles. The XRD patterns of the nanobioconjugates synthesised from betel leaf and grape seed extracts showed additional peaks, indicating the presence of other organic molecules in the extract. These results proved the successful and stable conjugation of the extracts and pure active phenolics to silver to form nanostructures.

The biosynthesised silver nanobioconjugates were studied for their stability and dispersion by zeta potential and poly dispersity index analysis respectively. The zeta potential values of the synthesised nanobioconjugates
from *Piper betle* leaf extract, *Vitis vinifera* seed extract, eugenol and resveratrol were -19mV, -7.17 mV, -13.6mV and -14.3mV respectively. The polydispersity index of all the four nanobioconjugates recorded values within 2, proving the stability of the nanoparticles synthesized. The net negative charge on the bioconjugates indicated that all the four nanobioconjugates are well-dispersed without aggregation, which was also shown in the TEM analysis. The FTIR spectra of the nanobioconjugates synthesized in the present study revealed the typical functional groups of phenolics (hydroxyl groups), aldehyde, nitro, aliphatic groups, aromatic ring, alkanes and alkenes, which are probably engaged in the synthesis of AgNPs from the extracts and compounds.

Having characterized the formation of the ideally suited structures, their ability as drug carriers and biosafety were assessed. This was done by tracing the drug release profile of the synthesized nanobioconjugates and by testing their biocompatibility with human cells.

The drug release profiles of the nanobioconjugates prepared from the extracts of the *Piper betle* leaves and *Vitis vinifera* seeds, were recorded from 0 hour upto 48 hours at one-hour intervals. The profiles showed that the nanobioconjugates released the conjugated material steadily upto 14 hours, after which a plateau of release was observed. These profiles confirmed that the silver nanobioconjugates synthesized in the present study are capable of a steady and sustained release of the medicinal components conjugated, proving their druggability.

Following this, the toxicity of all the four silver nanobioconjugates was tested on red blood cells from healthy human volunteers, to determine their biocompatibility. The results showed that the synthesized nanobioconjugates from the extracts and the compounds did not cause hemolysis of the human red blood cells, showing that they can be safely administered to humans. The non-toxicity of the synthesized AgNPs was further investigated based on the changes in the morphology of RBC, wherein the observations further
proved that the AgNPs were absolutely biocompatible in nature, and are safe for human use.

All the four silver nanobioconjugates showed no significant differences in blood clotting, confirming that they effectively prevent cell clumping and clotting, reflecting their safety and biocompatibility. From the results of these biocompatibility studies, it can be clearly understood that they were not toxic and act as very good biocompatible materials, which can be applied in the biomedical applications such as anticancer activity.

In the last phase of the study, the anticancer activity of the nanobioconjugates synthesized from the extracts and pure compounds, in comparison with their respective non-conjugated raw material, was studied 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. 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.

The MTT assay was used to determine the influence of silver nanobioconjugates on the viability of KB cells, A549 cells and the non-transformed cells. The results revealed that both *Piper betle* and *Vitis vinifera*, as well as their active phenolics, 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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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. The leaf extract of *Piper betle*, seed extract of *Vitis vinifera* as well as their active components, eugenol and resveratrol respectively, by themselves, possessed marked anticancer effect, which was mediated via the induction of apoptotic pathway, as shown by AO/EtBr staining. When these bioagents were conjugated with silver to form nanoscale particles, the extent of apoptosis in the cancer cells increased significantly.

Followed by this, the events of the cell cycle in the exposed cancer cells was analyzed by flow cytometry to understand the cellular events. Treatment with the silver nanobioconjugates of all the test materials included in the present study caused a shift of more 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. These observations rendered further support and proof for the increased anticancer effect of the silver nanobioconjugates, compared to their unconjugated raw materials.

The findings of the present study, thus, validate and strengthen the method of synthesis of silver nanobioconjugates from *Piper betle* leaves, *Vitis vinifera* seeds and their major polyphenols (eugenol and resveratrol) using a rapid, inexpensive and eco-friendly method. All the synthesized nanobioconjugates exhibit the ideal characteristics of nanoscale material, which can be used in biomedical applications as drug carriers in the targeted drug delivery system. The anticancer properties of silver nanobioconjugates 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 nanobioconjugates.
Suggestions for future research

The outcome of the present study has opened up a number of avenues for future research. Some of them that can be suggested for active research are given below.

- Nanoparticles of other metals like gold and iron can be synthesized using the *Piper betle* leaves, *Vitis vinifera* seeds and their major polyphenols (eugenol and resveratrol) and their bioactivity compared with silver nanobioconjugates.

- The genomic and proteomic profiles of silver nanobioconjugates treated cancer cells can be investigated to understand the mechanism of the anticancer effect at the gene and protein levels.

- *In vivo* studies using animals can be done to test the effect of silver nanobioconjugates.

- The mechanism of apoptosis induced by the silver nanobioconjugates can be assessed by studying caspase activities and other molecules involved in apoptosis.

- The interaction of silver nanobioconjugates to the biological target molecules can be determined using *in silico* docking analysis.