CHAPTER 5

EVALUATION OF IN VITRO ANTICANCER ACTIVITY OF GOLD NANOPARTICLES SYNTHESIZED USING VITIS VINIFERA SEED AND PEEL EXTRACTS

5.1 INTRODUCTION

Skin carcinogenesis represents growing major public health problem and almost one-third of cancer originates in the skin among all new cancers diagnosed in the world annually (Greenlee et al. 2001). Environmental toxins play a vital role in the initiation of skin cancer (Boukamp 2005). Oxidative stress can lead to DNA damage in the skin and uncontrolled release of reactive oxygen species can be the cause of skin cancer and it is involved in pathogenesis of number of human skin disorders (Black 2004, van Berlo et al. 2010). Enhanced therapeutic efficacy with minimal side effects is the primary goal of cancer treatment (Arvizó et al. 2010). Chemoprevention with distinct molecular mechanisms of dietary agents has received much interest as a means to achieve potency with reduced toxicity and higher efficacy (Ohigashi and Murakami 2004).

Defects of apoptosis pathway are associated with the development of cancer and therefore, anticancer drugs are intended to specifically target signalling molecules of survival pathways and cell death (Wong 2009, Qiao and Wong 2009). Apoptosis is measured by a distinct set of morphological changes and biochemical phases including translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane, the activation of caspases, chromatin condensation and fragmentation of the cell into many cellular fragments which are called as “apoptotic bodies” and it is called as “programmed cell death” (Leist and Jäättelä 2001, Kroemer et al. 2009). Cells cultured in vitro undergoes secondary necrosis and apoptotic cells shut down metabolism, lose membrane integrity and they release
cytoplasmic contents into the medium when incubated for extended period of time (Riss and Moravec 2004).

Usually, cancer cells will be under augmented stress related with changes in metabolic activity, oncogenic transformation and increased production of reactive oxygen species (ROS). This increased amounts of ROS leads to substantial consequences such as promotion of mutations, stimulation of cellular proliferation and alterations in cellular sensitivity to anticancer agents. Hence, ROS which can inflict severe cellular damage and are chemically active could possibly deliver a distinctive chance to kill the cancer cells by increasing the susceptibility to still more ROS insults and this unique feature possibly can have significant therapeutic implications (Pelicano et al. 2004). Mitochondria plays a distinctive role in ROS production in cancer cells as they are designated as the main basis of cellular ROS (Richter et al., 1995; Halliwell and Gutteridge, 1999).

More than 60% of anticancer agents currently used are derived from natural sources (Cragg and Newman 2003). Phytochemicals exhibit anticancer activity by induction of apoptosis and antiproliferative activity in cancer cells (Alias et al. 2009). Thus far, cancer chemotherapy has employed natural products like flavones, flavonoids, alkaloids, isoterpenoids etc., that shows promising effects in both in vivo and in vitro studies. Thus, it's obvious that in cancer prevention naturally occurring plant compounds plays a vital role (Patel et al. 2011). Vitis vinifera (grapes) are one of the most extensively consumed fruits with enormous health benefits. They are the potent source of proanthocyanidins, anthocyanins, resveratrol, flavonols and phenolic acids. Flavonoids in grape seeds and peels are (-) epicatechin, (+)catechins and procyanidin polymers (Xia et al. 2010). Proanthocyanidins in grape seeds have shown promising anticancer and chemopreventive efficacy in various animal models and cell cultures (Surh 2003).

Many studies has shown that grape seed extracts reduce the incidence of carcinogen induced mammary and skin tumors in rat and mice and they inhibit progress of human tumour cells in vivo and in vitro (Ye et al. 1999, Kim et al.
2004). It also exhibits cytotoxicity towards breast (Sharma et al. 2004), lung (Ye et al. 1999), skin (Meeran and Katiyar 2007), colon (Engelbrecht et al. 2007) and prostate cancers (Agarwal et al. 2002) while it enhances the viability and growth of normal cells (Bagchi et al. 2000). Grape seed extract induces apoptosis and G1 phase cell cycle, mitochondrial damage leading to apoptosis-inducing factor and cytochrome c release and activation of p53 (Agarwal et al. 2002, Kaur et al. 2005, Kaur et al. 2006). Grape peel extracts were able to target mitogen-activated protein kinase survival and phosphatidylinositol 3-kinase–Akt pathways in cell apoptosis (Hudson et al. 2007). Kuwajerwala et al. (2002) studied that resveratrol induced apoptosis and antiproliferative activity in prostate cancer cell lines at ≥ 15 µmol/L.

Developing nanotechnology for targeted drug delivery can be a possible method (Arvizo et al. 2010). Drug carriers embedded on nanoparticles have developed as new anticancer carrier vehicles and it has proven to be more powerful than other methods. Delivery of the drug to cancer cells is achieved by using nanoparticles as the delivery vehicle (Xu et al. 2006, Soppimath et al. 2007). Development of proficient nanoparticle delivery vehicles with low side effects is in advance when likened with the conventional drugs for treatment of malignancies (Wang et al. 2009). Gold nanoparticles seems to have a high possibility as drug delivery carriers. Gold nanoparticles exhibit physico-chemical properties and have been utilized for many applications such as diagnosing, imaging and therapeutic uses. Specific control of size and shape, ease of synthesis and surface chemistry provides multifunctionality to gold nanoparticles which facilitates attachment of a diverse class of drugs and compounds to the surface (Connor et al. 2005, Jain et al. 2007, Xiao and Qi 2011). Various studies shows that gold nanoparticles can inhibit proliferation of some kind of cells and can interact with heparin-binding glycoproteins resulting in inhibition of proliferation and angiogenesis (Fritsche et al. 1993). Chithrani et al. (2006) experimented that spherical shaped gold nanoparticles had best internalization when compared to other nanoparticles and it exhibited increased uptake over rod shaped nanoparticles in the cells. According to
recent literature, gold nanoparticles when synthesized biologically can be used as carriers in drug delivery systems and this functionalized gold nanoparticle core is essentially nontoxic and inert (Han et al. 2007a; Kim et al. 2009). Studies shows that nanoparticles encapsulated with other biological agents may have potential for cancer control (Muthuirulappan and Francis 2013).

Tumour-derived cell lines have been used as drug discovery tools for many years. Investigators have begun to appreciate the genomic heterogeneity with the human cancer patient population and thereby across cell lines derived from tumor and this diversity shows variable clinical response to treatment. Accordingly, cancer cell lines are used to predict the clinical efficacy of new agents that is already affecting the course of drug development. In the development and discovery of new cancer therapeutics, human tumor derived cell lines plays crucial role (Sharma et al. 2010).

The present study was aimed to evaluate the anti-carcinogenic and antiproliferative effects of gold nanoparticles synthesized biologically using Vitis vinifera peel and seed extracts. A431 human epidermoid carcinoma cells (skin cancer) was used as an in vitro model to study the anti-carcinogenic effects and induction of apoptosis. The nanoparticles were also tested on normal human epidermal keratinocytes (HaCaT) to check cell viability.
5.2 MATERIALS AND METHODS

5.2.1 Chemicals

Phosphate buffered saline (PBS), Dulbecco’s Modified Eagle Medium (DMEM), Foetal Bovine Serum (FBS), Trypsin-EDTA (0.25%, 0.02%) solution, penicillin and streptomycin were purchased from Gibco, Life technologies, India. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DMSO (dimethyl sulfoxide) were obtained from Himedia, India. Annexin V-FITC Apoptosis Detection kit was purchased from Sigma, USA. Rhodamine 123, 2’,7’-dichlorodihydro fluorescein diacetate (DCFH-DA), ethidium bromide and acridine orange were purchased from Sigma Aldrich, India. All other solvents and chemicals were of analytical grade.

5.2.2 Cell culture

A431 cancer cell line (skin carcinoma, human) and HaCaT (Normal, Human immortalized keratinocyte cell line) were obtained from National Centre for Cell Science (NCCS), Pune, India. A431 skin cancer and HaCaT cell line were maintained as a monolayer in tissue culture petridishes in DMEM medium supplemented with 10% heat inactivated foetal bovine serum (FBS), 100 IU mL\(^{-1}\) penicillin, 100 µg mL\(^{-1}\) streptomycin and 2 mM L-glutamine. The cultured cells were passaged twice each week, seeding at a density of about 2x10\(^3\) cells mL\(^{-1}\) and the medium was replaced every 2 days. The cultures were maintained in a humidified atmosphere with 5% CO\(_2\) at 37ºC. Cell viability was determined by the trypan blue dye exclusion method. In all experiments, 70–85% confluent cultures were used.

5.2.3 Cell cytotoxicity assay

To measure the cytotoxic effects of a particular drug and to quantify cell growth and viability in 96-well microtiter plate, a conventional method of cytotoxicity analysis is followed. Mitochondrial dehydrogenases in live cells are capable of converting yellow colored MTT salt [3-(4, 5-dimethyl-2-thiazolyl)-2,5-
diphenyl-2H-tetrazolium bromide] to purple formazan, a water-insoluble crystals. Hence, this method might be useful to assess the inhibitory concentration of gold nanoparticles on cancer cells.

Cell cytotoxicity was measured by experimenting MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide) assay (Mosmann, 1983). A431 cells and HaCaT cells (5 x 10³ cells per well) were plated in 96-well plates with 100 µL of medium and incubated for 24 hrs. After 24 hrs, the cells reached confluency and were incubated with the presence or absence of series of increasing concentrations of Vitis vinifera seed and peel AuNPs and Vitis vinifera seed and peel extracts dissolved in 0.1% DMSO for 24 hrs at 37°C in a final volume of 100 µL per well. 5- Fluorouracil was used a standard drug at an increasing concentration to test cytotoxicity on the A431 cells. At the end of the treatment, 20 µL of MTT (5 mg mL⁻¹) dissolved in PBS was added to each well by diluting with the medium and was incubated with the plates wrapped in aluminium foil for 4 hrs at 37° C. The purple formazan crystals formed by the live cells were dissolved in 100 µL of dimethyl sulfoxide (DMSO, Sigma) replaced by media and the optical density was measured at 570 nm on microplate reader (BIO-RAD microplate reader-550, Japan). Each concentration was tested in three different experiments run in triplicates. Cells cultured with the same volume of DMEM without extracts served as positive control (OD+) and DMSO without any cells served as negative control (OD-). The results are presented in bar graph plotting the percentage of cell death or inhibition (Y-axis) and the concentrations of sample (X-axis). The inhibitory concentration (IC₅₀) i.e the drug concentration inhibiting 50% of cell growth was elucidated from the graph.

5.2.3.1 Calculation for the percentage of growth inhibition and cell viability

\[
\text{Percentage inhibition (100%)} = 100\% - \left( \frac{A_p - A_{cm}}{A_{cc} - A_{cm}} \right) \times 100\% 
\]
Where $A_p$ is the absorbance value of the well containing cells treated with the extracts and nanoparticles at increasing concentration, $A_{cm}$ is the absorbance of the wells without cells (control of the medium), $A_{cc}$ is the mean absorbance in wells containing untreated cells (Nevozhay 2014). The percentage of viability for HaCaT cells were calculated by the following formula,

\[
\% \text{ viability} = 100 - \% \text{ cytotoxicity}
\]

5.2.4 UV-Visible spectroscopy of the cells treated with gold nanoparticles

The A431 cells were incubated with the *Vitis vinifera* seed and peel synthesized gold nanoparticles at the IC$_{50}$ concentrations of grape seed AuNPs (24.2 µg/mL) and grape peel AuNPs (23.6 µg/mL) for 24 hrs. The absorbance of culture medium with the untreated cells, culture medium with nanoparticles without A431 cells and A431 cells treated with gold nanoparticles at 545 nm and 534 nm respectively for *Vitis vinifera* seed and *Vitis vinifera* peel AuNPs were measured (Kalpana et al. 2013).

5.2.5 Morphological changes in A431 cells

A431 cells were grown in a 35-mm sterile cell culture petridishes at 37°C in a CO$_2$ incubator and were exposed to *Vitis vinifera* peel and seed gold nanoparticles, *Vitis vinifera* peel and seed extracts and fluorouracil at the IC$_{50}$ concentrations. Morphology of A431 cells were visualized using inverted phase contrast microscope (Nikon TS100F, Japan). Untreated cells were kept as control and the morphological changes were visualized with the extent of the cell roundedness (Patra et al. 2007).

5.2.6 Assessment of reactive oxygen species by DCFH-DA

Reactive oxygen species (ROS) were induced in cancer cells with *Vitis vinifera* seed and peel AuNPs and *Vitis vinifera* seed and peel extracts. A fluorescence probe, 2’, 7’- dichlorodihydro fluorescein diacetate (DCFH-DA) is
used to determine the ROS production in cytosol. DCFH-DA is converted to H₂DCF which is then changed to DCF by esterase in the cytoplasm. The intensity shown by the fluorescent component (DCF) is then captured by fluorescent microscope and the level of ROS is assessed.

The fluorescence intensity is proportionate to the level of ROS formed inside the cells. Cells were incubated with grape seed extract (111.11 µg/mL), grape seed AuNPs (24.2 µg/mL), grape peel extract (319.14 µg/mL), grape peel AuNPs (23.6 µg/mL) for 24 hrs and H₂O₂ for 30min to induce free radical damage to the cells with or without N-acetyl cysteine (NAC). NAC was used as an antioxidant at a concentration of (100 µM/mL) which was also tested along with the test compounds. Fluorouracil was used as a standard drug at the IC₅₀ concentration of 23.43 µM with or without NAC. A determined amount of the cell suspension (150 µl) was grown in a 24-well plate for 24 hrs. The test compounds (10 µl) were added onto the 24-well plate after the growth of the cells and incubated for 24 hrs. PBS was used as a negative control and H₂O₂ (20 µl) was used as positive control and also to induce free radical damage in the A431 cells, and then after incubation, DCFH-DA (10 µl, 5 mM) was added in all the wells. The intensity of the fluorescence (DCF) was measured using inverted fluorescent microscope (Olympus IX71, USA) (Rastogi et al. 2010). Images were analyzed using ImageJ software, National Institute of Health, USA.

5.2.7 Annexin V-FITC apoptosis assay (TUNEL)

Annexin V-FITC kit was used to measure the amount of apoptotic cells after treatment with Vitis vinifera seed and peel extracts and Vitis vinifera seed and peel gold nanoparticles treated A431 cells. Annexin V binds to the phosphatidylinerine (PS) when PS gets translocated from the inside of the membrane to the exterior in early apoptotic cells. Annexin V-FITC will be bound to early apoptotic cells while propidium iodide binds to late apoptotic and necrotic cells and the viable cells remained unstained.
The percentage of apoptotic cells were determined by analyzing with Annexin V-FITC apoptosis detection kit (Sigma Aldrich, USA) as per the instructions given by the manufacturer. Equal amount of cells per well were grown overnight in a 24-well culture plate for 24 hrs and then treated with IC_{50} concentrations of *Vitis vinifera* seed and peel extracts and *Vitis vinifera* seed and peel AuNPs and the cells were grown for another 24 hrs. The cells were treated with Staurosporine (0.1 nM), treated with fluorouracil (23.43 µM), treated with grape peel (319.14 µg/mL), treated with grape seed (111.11 µg/mL), treated with grape peel AuNPs (23.6 µM), treated with grape seed AuNPs (24.2 µM). The cells were washed three times with dulbecco’s phosphate buffer (0.1 M, pH 7.4) and 500 µl of 1X binding buffer was added, after which Annexin V FITC (5 µl) and propidium iodide (10 µl) was added and then kept in dark for 10 min at room temperature. Staurosporine was taken as a positive control (Cheng et al. 2004). Then it was viewed under fluorescence microscope (Olympus IX71, USA). Images were analyzed using ImageJ software, National Institute of Health, USA.

### 5.2.8 Mitochondrial membrane potential by Rhodamine 123

In this study, Rhodamine 123 (RH-123) was used to quantify Δψm (membrane potential) in intact cells as a microscopic fluorescent stain after it electrophoretically accumulates in mitochondria (Johnson et al, 1980, Johnson et al. 1981). Emaus et al. (1986) first showed that energization prompted a red shift and extensive quenching of RH-123 fluorescence can lead to dye accumulation and can be recommended as a cationic voltage-sensitive sensitive probe of Δψm (Scaduto and Grotyohann 1999).

Exponentially grown A431 cells (2 x 10^5 cells/mL/well) seeded in 24 well plates for 24 hrs were treated with *Vitis vinifera* seed and peel extracts and *Vitis vinifera* seed and peel AuNPs for 24 hrs. A431 cells were treated with grape seed extract (111.11 µg/mL), grape seed AuNPs (24.2 µg/mL), grape peel extract (319.14 µg/mL) and grape peel AuNPs (23.6 µg/mL). Rhodamine 123 (10 µg/mL)
was added to the cells in cell medium and incubated for 30 min at 37°C. Cells were detached from the plate after washing with ice cold PBS, and the cells were analysed by fluorescent microscope (Olympus IX71, USA). Data is expressed in percentage of cells stained with Rhodamine 123 (Dash et al. 2013).

5.2.9 Apoptotic morphological changes by acridine orange (AO) and ethidium bromide (EtBr)

Morphological analysis of apoptosis by AO/EtBr dual staining procedure was performed (Ribble et al. 2005). AO is a vital dye that stains both live and dead cells; EtBr stains cells that have lost membrane integrity. Early apoptotic cells stain green and contain bright dots in the nuclei. Late apoptotic cells show condensed, often fragmented nuclei because it incorporates EtBr. Necrotic cells stains orange, but the nuclear morphology resembles that of viable cells.

For apoptotic morphological changes, cells were seeded in 24-well plates for 24 hrs and then treated with IC50 concentrations of Vitis vinifera seed and peel extracts and Vitis vinifera seed and peel AuNPs for 24 hrs. A431 cells were treated with Grape seed extract (111.11 µg/mL), Grape seed AuNPs (24.2 µg/mL), Grape peel extract (319.14 µg/mL) and Grape peel AuNPs (23.6 µg/mL). After harvesting by trypsinisation, cells were washed with 1X PBS once or twice. Twenty-five microlitres of the cell suspension was then mixed with 1 µL of the dyes, containing 100 mg mL⁻¹ of acridine orange and 100 mg mL⁻¹ of ethidium bromide in 1X PBS. After staining, cells were visualised immediately under a fluorescence microscope (Olympus IX71, USA). Approximately, 200 cells were counted and the quantitative estimations were carried out by ImageJ software, National Institute of Health, USA.
5.2.10 Statistical analysis:

Results are expressed as mean ± SD for each experiment performed. All *in vitro* data were obtained from at least three independent experiments. Comparisons were made between the control and the treated groups by one-way ANOVA test (SPSS 20) (SPSS Inc., Chicago, IL, USA) followed by Duncan’s test. *P* values less than 0.05 (*P*<0.05) were considered statistically significant.
5.3 RESULTS

5.3.1 Cytotoxic effect of *Vitis vinifera* seed and peel AuNPs and extracts

The percentage growth inhibition was found to be increasing with increasing concentration of *Vitis vinifera* seed and peel extract (Fig 5.1a and b). The A431 cells were exposed to *Vitis vinifera* seed aqueous extracts at 50, 100, 150, 200 and 250 µg/mL and peel extract at 100, 200, 300, 400, 500 µg/mL for 24 hr in the logarithmic growth phase and cytotoxicity was measured by MTT assay. The IC$_{50}$ values were recorded to be 111.11 µg/mL for *Vitis vinifera* seed and 319.14 µg/mL for *Vitis vinifera* peel extract. In fig 5.1a and b, the percentage of inhibition is plotted against the seed and peel aqueous extracts concentration.

In fig 5.2, the percentage growth inhibition was found to be increasing with increasing concentration of *Vitis vinifera* seed and peel bioconjugated AuNPs. The A431 cells were exposed to peel and seed AuNPs of size 50±5nm at 5, 10, 15, 20, 25 µM concentration for 24 hr in the logarithmic growth phase and cytotoxicity was measured by MTT assay. The IC$_{50}$ values were recorded to be 24.2 µM for *Vitis vinifera* seed AuNPs and 23.6 µM for *Vitis vinifera* peel AuNPs. The IC$_{50}$ value was recorded to be 3.04 µg/mL (23.43 µM) for fluorouracil which was used as standard drug. In fig 5.2, the percentage of inhibition is plotted against the seed and peel AuNPs concentration and in fig 5.3, the percentage of inhibition is plotted against the fluorouracil concentration. The percentage inhibition of cell growth by the gold nanoparticles were calculated based on difference in inhibition between respective controls and AuNPs treated cell lines. Control was taken as 100% cell proliferation.
Figure 5.1 Inhibitory effect of *Vitis vinifera* seed and peel extract on the A431 cells after 24 hrs post incubation. (a) *Vitis vinifera* seed extract; (b) *Vitis vinifera* peel extract. Each result represents the mean ± standard deviation (SD) and each of these were performed in triplicate. The results were considered statistically significant at ‘*’ *p*<0.05 compared to the untreated control taken as 0% inhibition.
Figure 5.2 Inhibitory effect of *Vitis vinifera* peel and seed AuNPs on A431 cells after 24 hrs post incubation. Each result represents the mean ± standard deviation (SD) and each of these were performed in triplicate. The results were considered statistically significant at ‘*’ p<0.05 compared to the untreated control taken as 0% inhibition.

Figure 5.3 Inhibitory effect of fluorouracil on A431 cells after 24 hrs post incubation. Each result represents the mean ± standard deviation (SD) and each of these were performed in triplicate. The results were considered statistically significant at ‘**’ p<0.05 compared to the untreated control taken as 0% inhibition.
Figure 5.4 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on cell proliferation of HaCaT cells. Values are expressed as mean ± SD of three independent experiments.

![Graph showing effect of *Vitis vinifera* peel and seed AuNPs and extracts on cell viability](image)

Figure 5.5 Visible spectroscopic analysis of gold nanoparticles in the cell culture medium with A431 cells. (A1, B1) – Absorbance of gold nanoparticles in the culture medium without A431 cells, (A2, B2) – Absorbance of culture medium with A431 cells without treatment of gold nanoparticles, (A3, B3) – Absorbance of gold nanoparticles in the cell culture medium with A431 cells. (A1, A2, A3) corresponds to *Vitis vinifera* seed gold nanoparticles treated or untreated group. (B1, B2, B3) corresponds to *Vitis vinifera* peel gold nanoparticles treated or untreated group. Absorbance was measured at 534 and 545 nm for (B1, B2, B3) and (A1, A2, A3) respectively. A431 cells without treatment of gold nanoparticles were kept as controls and absorbance were measured. Values are expressed as mean ± S.D for three experiments.

![Graph showing visible spectroscopic analysis](image)
5.3.2 Effect of *Vitis vinifera* seed and peel AuNPs and extracts on cell viability

HaCaT cells were treated at an increasing concentrations of *Vitis vinifera* peel and seed AuNPs as well as aqueous extracts for 24 hrs to check for cell viability. Fig 5.4 clearly illustrates that peel and seed gold nanoparticles at concentrations of 10, 20, 30, 40, 50 µM did not show any cytotoxicity to the normal immortalized keratinocyte cell line (HaCaT). It was observed that even at higher concentrations *viz.*, 50 µM of grape seed and peel AuNPs, 91.9% and 93.3 % of the cells remained viable after incubation for 24 hrs. In fig 5.4, the percentage of inhibition is plotted against the seed and peel AuNPs and seed and peel aqueous extracts concentration.

5.3.3 Accumulation of *Vitis vinifera* peel and seed AuNPs and extracts in A431 cells

A431 cells were treated with IC$_{50}$ concentrations of *Vitis vinifera* peel and seed gold nanoparticles for 12 hrs and were checked for their absorption wavelength and were observed at 534 and 545 nm. The absorbance of *Vitis vinifera* peel and seed gold nanoparticles in the culture medium treated for 12 hrs against A431 cells were found to completely decrease as shown in Fig 5.5 compared to the gold nanoparticles suspended in the medium without cells. The absorbance of A431 cells grown in the culture medium was also measured without the treatment of gold nanoparticles and it showed negative absorbance which indicated that the absorbance of gold nanoparticles does not interfere with the culture medium.
Figure 5.6 Morphological changes in A431 cells after treatment with *Vitis vinifera* peel and seed extract and AuNPs (20X).

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Figure 5.6 Morphological changes in A431 cells after treatment with *Vitis vinifera* peel and seed extract and AuNPs (20X). Phase contrast images of (a) – Control, (b) – Fluorouracil, (c) - *Vitis vinifera* seed extract, (d) - *Vitis vinifera* peel extract, (e) - *Vitis vinifera* seed AuNPs and (f) - *Vitis vinifera* peel AuNPs treated at the IC$_{50}$ concentrations. Arrow head shows the morphological changes induced by peel and seed gold nanoparticles and extracts in (c), (d), (e), (f) images respectively.
5.3.4 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the morphological changes of A431 cells

A431 cells incubated with the IC\textsubscript{50} concentrations of *Vitis vinifera* peel and seed gold nanoparticles were observed under the phase contrast microscope to detect the morphological changes occurred and the images are presented in Fig 5.6. In the control group, no changes in the morphology of the cells were found while the cells treated with the nanoparticles showed roundedness, a characteristics of stressed cells, irregular shaped, cytoplasmic vacuolation and enlarged cells due to the AuNPs induced stress showing significant morphological changes when observed under phase contrast microscope. *Vitis vinifera* seed aqueous extract treated A431 cells also showed significant changes in the morphology when compared with the peel extract treated group and gold nanoparticles treated group. Apoptotic cells were clearly visible with the seed gold nanoparticles and seed extract treated cells. Morphological changes like roundedness, irregular shape of cells and stressed cells were evident in nearly all the fluorouracil drug treated cells and this can be comparable with the *Vitis vinifera* seed and peel gold nanoparticles treated cells.
5.3.5 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the measurement of reactive oxygen species (ROS)

The effect of *Vitis vinifera* peel, seed extracts and *Vitis vinifera* peel, seed gold nanoparticles on generation of ROS such as H$_2$O$_2$, O$_2^-$ and peroxynitrite were examined by using 2',7'- dichlorodihydro fluorescein diacetate (DCFH-DA) which shows enhanced fluorescence when oxidative stress is generated intracellularly. A431 cells were exposed to peel and seed gold nanoparticles resulting in significant increase ($p<0.01$) in the ROS generation at the IC$_{50}$ concentrations (Fig 5.7 and 5.8). The intensity of ROS production was more significantly increased ($p<0.001$) when incubated with *Vitis vinifera* seed extracts when compared to the control group. The cells treated with hydrogen peroxide showed more ROS production and was comparable with the cells treated with *Vitis vinifera* seed and peel nanoparticles as well as *Vitis vinifera* seed extracts treated cells. The fluorescence pixel intensity for the control group was 10.1 while for hydrogen peroxide treated cells it was 131.5. Fluorouracil was used as standard drug to compare with the test compounds and it showed 73.62 whereas *Vitis vinifera* seed extracts showed high fluorescence intensity with total ROS to be 100.18 showing increased ROS production similar to H$_2$O$_2$ treated cells and *Vitis vinifera* seed and peel nanoparticles showed intensity of 75.6 and 67.65.

Fluorescence intensity increased continuously with the intracellular ROS content until the end of 24 hrs. This increase in ROS and subsequent fluorescence was abrogated significantly in cells treated with N-acetylcysteine suggesting the role of ROS in gold nanoparticles mediated cell death. NAC inhibited ROS generation which was evident from the fluorescence intensities. To determine whether apoptosis was related to ROS, the cancer cells were incubated with NAC with gold nanoparticles. NAC showed complete abrogation of gold nanoparticles induced apoptosis in cancer cells. In the presence of H$_2$O$_2$, elevation of intracellular ROS was exponentially potentiated. The results also showed that gold nanoparticles were able to generate ROS and nanoparticle induced apoptotic cell death in A431 cells can be mediated by ROS signalling.
Figure 5.7 Effect of *Vitis vinifera* seed and peel extracts and AuNPs on production of ROS in A431 cell lines

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Figure 5.7 Effect of *Vitis vinifera* seed and peel extracts and AuNPs on production of ROS in A431 cell lines. (a) – Control, (b) – Treated with H$_2$O$_2$ (10 µL/mL), (c) – Treated with NAC (100 µM/mL), (d) – Treated with fluorouracil (23.43 µM), (e) – Treated with fluorouracil + NAC, (f) – Treated with Grape peel (319.14 µg/mL), (g) – Treated with Grape peel + NAC, (h) – Treated with Grape peel AuNPS (23.6 µM), (i) – Treated with Grape peel AuNPs + NAC, (j) – Treated with Grape seed (111.11 µg/mL), (k) – Treated with Grape seed + NAC, (l) – Treated with Grape seed AuNPS (24.2 µM), (m) – Treated with Grape seed AuNPs + NAC. Experiments were performed with or without NAC. NAC – N-acetyl cysteine, ROS is an important parameter and it can be capable of producing free radicals and induced severe cell death compared to normal cells. Intracellular ROS levels were detected by fluorescent H$_2$DCF-DA dye. Treated cells show bright fluorescence indicating production of ROS by inducing stress to the cancer cells.
Figure 5.8 Quantification of fluorescence intensities in A431 cell lines

ROS production was determined by DCFH-DA staining using fluorescent microscopy indicating increased ROS production. Experiments were performed in triplicates. The results are expressed as mean ± SD. ‘**’ and ‘***’ represents statistically significant compared to control group ($p<0.01$) and ($p<0.001$) respectively.
5.3.6 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the identification of apoptosis by Annexin V-FITC staining

Treatment of A431 skin cancer cells at the IC$_{50}$ concentrations of *Vitis vinifera* peel and seed extracts as well as gold nanoparticles were able to induce apoptosis and were stained with Annexin V-propidium iodide that were visualized and quantified (Fig 5.9 and 5.10). After 24 hrs treatment, the cells entered apoptotic stage and some cells were necrotic. More necrotic cells were found in staurosporine and fluorouracil treated cells while *Vitis vinifera* seed extracts ($p<0.001$) and seed nanoparticles ($p<0.01$) showed significant necrotic cells when compared to the control whereas seed extracts showed significant apoptotic cells ($p<0.01$) and seed and peel gold nanoparticles also showed significant apoptotic cells when compared to the control ($p<0.05$). The percentage of apoptotic cells treated with seed and peel gold nanoparticles were 11.69% and 10.02% while the necrotic cells were 31.71% and 55.5%. More apoptotic and secondary necrotic cells were found with seed and peel extracts treated cells with 16.32% and 3.12% apoptotic and 80.6% and 52.75% secondary necrotic cells and IC$_{50}$ concentrations of *Vitis vinifera* seed extracts were able to induce the cells to enter secondary necrosis. Control untreated cells showed intact cells and A431 cells were stained with Annexin V-FITC conjugate which is a fluorescent probe that binds to cells of early apoptosis where the phosphatidylserine is translocated to the external portion of membrane.
Figure 5.9 Effect of *Vitis vinifera* seed and peel extracts and AuNPs on A431 cell lines by inducing apoptosis

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Figure 5.9 Effect of *Vitis vinifera* seed and peel extracts and AuNPs on A431 cell lines by inducing apoptosis. (a) - Control cells, (b) - treated with Staurosporine (0.1 nM), (c) – treated with fluorouracil (23.43 µM), (d) – treated with Grape peel (319.14 µg/mL), (e) – treated with Grape seed (111.11 µg/mL), (f) – treated with Grape peel AuNPs (23.6 µM), (g) – treated with Grape seed AuNPs (24.2 µM). Apoptosis was detected by fluorescence staining and observed under fluorescence microscope after 24 hrs of treatment with extracts and gold nanoparticles. Apoptotic and necrotic cells were marked with white arrow on the image.
Figure 5.10 *Vitis vinifera* seed and peel nanoparticles induce apoptosis in A431 cells.

Cells, which are early in the apoptotic process, were stained with the Annexin V FITC (green) conjugate alone. Live cells showed no staining by either the propidium iodide solution or Annexin V FITC Conjugate. Necrotic cells were stained by both the propidium iodide (red) and Annexin V FITC Conjugate (green). Experiments were performed in triplicates. The results are expressed as mean ± SD. ‘*’, ‘**’ and ‘***’ represents statistically significant compared to control group (p<0.05), (p<0.01) and (p<0.001) respectively. The mean percentage of apoptotic and necrotic cells ± S.D for each treatment and the experiments were done in triplicates. Nearly 500 cells were counted in a blind manner to score the percentage of apoptosis in each treatment group.
5.3.7 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the nuclear morphological changes

A431 cells were treated with *Vitis vinifera* seed and peel gold nanoparticles and *Vitis vinifera* seed and peel extracts for 24 hrs and stained for nuclear morphological changes using Acridine orange/Ethidium bromide and visualized under fluorescent microscopy. The results obtained from the AO/EtBr staining are presented in fig 5.11 and 5.12. Staining of control cells showed nuclei having round and green nucleus however, early apoptotic cells had fragmented DNA appearing as green colored nuclei. Late apoptotic cells stained orange and necrotic cells stained red and DNA is fragmented. The results exhibited increase in early and late apoptotic cells when treated with seed and peel gold nanoparticles and was able to decrease the number of viable cells immensely. Treatment with seed nanoparticles significantly produced more necrotic cells (30.60%) ($p<0.01$) and the percentage of apoptotic cells were 43.48 ($p<0.01$) and showed more toxic to the cancer cells. *Vitis vinifera* peel nanoparticles showed more apoptotic cells (77.04%) ($p<0.001$) and 6.7% of necrotic cells. *Vitis vinifera* seed extracts treated cells showed more number of apoptotic as well as necrotic cells (48.89% and 48.25%) while *Vitis vinifera* peel extracts exhibited 82.16% and 11.65 of apoptotic and necrotic cells. *Vitis vinifera* seed and peel extracts as well as *Vitis vinifera* peel and seed nanoparticles treated A431 cells showed more nuclear morphological changes compared to the control cells.

Staurosporine was used to induce apoptosis and it showed 80.21% of apoptotic cells whereas fluorouracil which was used as standard drug showed 78.21% apoptotic and 15.4% necrotic cells. Most of the gold nanoparticles treated A431 cells showed typical characteristics like formation of apoptotic bodies and plasma membrane blebbing. Seed gold nanoparticles and seed extracts treated cells were largely stained red. Peel gold nanoparticles and peel extracts treated cells were mostly stained orange significantly. These results indicated that cell death in most of the cells occurred due to apoptosis and secondary necrosis.
Figure 5.11 Effect of *Vitis vinifera* seed and peel extracts and AuNPs on A431 cell lines by inducing apoptotic morphological changes
Figure 5.11 Effect of *Vitis vinifera* seed and peel extracts and AuNPs on A431 cell lines by inducing apoptotic morphological changes. A431 cells were treated with AO/EtBr and visualized under fluorescent microscope. (a) -Control cells, (b) - treated with Staurosporine (0.1 nM), (c) –treated with fluorouracil (23.43 µM), (d) – treated with Grape seed (111.11 µg/mL), (e) –treated with Grape seed AuNPs (24.2 µM), (f) – treated with Grape peel AuNPs (23.6 µM), (g) –treated with Grape peel (319.14 µg/mL).
Figure 5.12 Apoptotic morphological studies of A431 cells treated with *Vitis vinifera* seed and peel gold nanoparticles

Control cells showed green normal nucleus. Treated cells showed early apoptotic cells with green bright nucleus and late apoptotic cells showed condensed red nucleus. Experiments were performed in triplicates. The results are expressed as mean ± SD. Significant difference between treated and control group given as *(p<0.05), **(p<0.01) and ***(p<0.001).*
5.3.8 Effect of *Vitis vinifera* seed and peel gold nanoparticles and extracts on the mitochondrial membrane potential

A431 cells treated with *Vitis vinifera* seed and peel gold nanoparticles showed green fluorescence indicating apoptotic cells with low mitochondrial Δψm whereas untreated control cells showed healthy with high mitochondrial Δψm with intense red fluorescence due to its electrophoretic accumulation in mitochondria (Fig 5.13). Rhodamine-123, a lipophilic dye was used to assess the membrane potential which accumulates within mitochondria in a potential dependent manner. When *Vitis vinifera* peel and seed nanoparticles at 24.2 µM and 23.6 µM concentrations were added according to the IC<sub>50</sub> into the cells, disruption of membrane potential was evident as the fluorescence emission of Rhodamine-123 dye changes from red to green when compared with *Vitis vinifera* peel and seed extracts at 319.14 µg/mL and 111.11 µg/mL concentrations at 24 hrs.

The orange-red fluorescence intensity was more in the control when compared with the *Vitis vinifera* seed and peel gold nanoparticles treated A431 cells, as the fluorescence intensity was decreased to nearly 75% in the AuNPs treated group. Staurosporine was used as positive control which showed green fluorescence while fluorouracil also showed significant green fluorescence which was used as standard drug (Fig 5.14). These results proved that *Vitis vinifera* seed and peel coated gold nanoparticles when treated against A431 cells showed disruption and loss of mitochondrial membrane potential. Grape seed and peel nanoparticles were able to induce significant (*p*<0.05) loss of membrane potential in A431 skin cancer cells than with grape seed and peel aqueous extracts treatment.
Figure 5.13 Effect of *Vitis vinifera* seed and peel AuNPs and extracts on mitochondrial membrane potential of A431 cells

Contd…
Figure 5.13 Effect of *Vitis vinifera* seed and peel AuNPs and extracts on mitochondrial membrane potential of A431 cells. The cells were treated with (a) Control (untreated), (b) Fluorouracil (23.43 μM), (c) Staurosporine (0.1 nM), (d) Grape seed (111.11 μg/mL), (e) Grape seed AuNPs (24.2 μg/mL), (f) Grape peel (319.14 μg/mL), (g) Grape peel AuNPs (23.6 μg/mL) for 24 hrs. After washing with PBS, incubated with Rhodamine-123 dye (10 μg/mL) for 30 mins. Fluorescence images were captured in rhodamine filters and images are represented above. Control cells showed high mitochondrial membrane potentiality due to formation of aggregates inside the polarized cells. Depolarized mitochondria are marked by green fluorescence and polarized mitochondria are marked by orange-red fluorescence. Treatment with *Vitis vinifera* seed and peel AuNPs significantly resulted in loss of membrane potential compared with the control and rhodamine 123 was not absorbed onto the membrane; mitochondrial depolarization occurs in the early stages of apoptosis.
Figure 5.14 Percentage of cells showing fluorescence intensity after treatment with *Vitis vinifera* seed and peel AuNPs on A431 cells. The percentage of the cells that emit only green fluorescence indicates the depolarized mitochondrial membrane and loss of membrane potential and orange-red fluorescence indicates polarized mitochondrial membrane. The values are represented as mean ±S.D and three independent experiments were performed. *- represents significant difference compared with control (p<0.05).
5.4 DISCUSSION

In the present study, the cytotoxic effects of *Vitis vinifera* seed and peel AuNPs of size 55.1 nm and 52.1 nm were investigated and their possible effects on cell death in A431 cells were also determined. Significant cytotoxicity was observed in *Vitis vinifera* seed AuNPs treated A431 cells at higher molar concentrations when compared to seed extracts treated cells. *Vitis vinifera* peel AuNPs also showed significant cytotoxic effects in A431 cells when compared to seed and peel extracts alone treated group. An increase in the total activity of mitochondrial dehydrogenases in the cancer cells is the result of an increase in the number of viable cells. Notably, the nanoparticles were proved to be toxic as it accumulated outside the cell nucleus of the A431 cells. The cytotoxicity of the seed and peel nanoparticles were confirmed by MTT assay and the concentration that produced 50% of cell death of A431 cells was non-toxic to the normal cells (HaCaT). Bioconjugated gold nanoparticles synthesized using *Vitis vinifera* seed and peel extract were toxic even at 5 µM concentration and it inhibited the cancer cells growth with concentration dependent activity. Most of the A431 cells treated with *Vitis vinifera* seed and peel AuNPs were not viable and had floated, rounded and clumped dead cells after 24 hrs treatment but the HaCaT cells treated with the nanoparticles showed viability and adherence to the bottom of the wells.

In this study, *Vitis vinifera* seed and peel synthesized gold nanoparticles capped with the biomolecules such as phenols, alkaloids, flavonoids caused more than 50% cell death in A431 skin cancer cells and showed better cytotoxicity when compared to seed and peel aqueous extracts treated cells. The A431 cells were treated according to the IC$_{50}$ concentrations and it showed toxicity on the cancer cell line while it was non-toxic to the normal human keratinocyte cell lines which substantiates the chemotherapeutic activity of gold nanoparticles. This cytotoxicity of the gold nanoparticles can be attributed to the synergistic effects of the phenolic moieties assumed to have anti-proliferative activities (Kawaii et al. 1999). The IC$_{50}$ concentration strongly indicated that *Vitis vinifera* seed and peel AuNPs had a
potent cytotoxic effect on the skin cancer cell lines. Many studies report that cytotoxicity and quantitative uptake was dependent on their size and shape of the gold nanoparticles (Chithrani and Chan 2007). Patra et al. (2007) studied that gold nanoparticles synthesized via citrate reduction were able to induce death response in human carcinoma lung cell line A549 while other cell lines HepG2 and BHK21 were not affected by gold nanoparticles treatment implying that gold nanoparticles do not commonly target all cell types. A549 cells showed decrease in percentage of viability in the gold nanoparticle concentration range 0-30 nM with the average size of the nanoparticle to be 33 nm. Gold nanoparticles synthesized using red algae Corallina officinalis of 14nm were able to show potent cytotoxicity against MCF-7 cells causing necrosis. Their FTIR analysis showed that the gold nanoparticles were capped with phytochemicals (El-Kassas and El-Sheekh 2014) which is then confirmed by another study showing that gold nanoparticles induce apoptosis in MCF-7 breast cancer cell lines via p53, caspase and Bax/Bcl-2 pathways (Selim and Hendi 2012). Krishnaraj et al. (2014) evaluated the cytotoxicity of gold nanoparticles by MTT assay and found that it reduced the viability of MDA-MB-231 cells in a dose dependent manner at a concentration of 100 µg/mL. Gold nanoparticles synthesized using Cinnamomum japonicum was found to be toxic to the 3T3-L1 cell lines even at 10 µg/mL and 34.83% of cells were inhibited (Kalpana et al. 2013). Gold nanoparticles synthesized using sodium citrate as reducing agent reduced the viability of A549 and NCIH441 cell lines and also affected the cellular proliferation where citrate acted as the capping agent (Uboldi et al. 2009).

*Padina gymnospora*, a marine microalgae synthesized gold nanoparticles as Au clusters were highly toxic and concentration of 82.91 nM induced 50% decrease in cell viability of HepG2 liver cancer cells. The synthesized gold nanoparticles were also found to be mildly cytotoxic to A549 cell lines which can due to the presence of fucoxanthins, a kind of carotenoids with hydroxyl group on the algal cell wall that are adsorbed on the surface (Singh et al. 2014). Gold nanoparticles
conjugated with gallic acid inhibited the cancer cell proliferation by inducing apoptosis while it was non-toxic to normal Vero kidney cells even at high concentration (150µM) which was in par with our results that showed gold nanoparticles synthesized using Vitis vinifera seed and peel extracts were not toxic to normal keratinocyte (HaCaT) cells. Thus, phytochemically synthesized gold nanoparticles have the potential to be used as a delivery agents as an alternative cancer treatment to reduce side effects of chemotherapy (Daduang et al. 2015).

The results for visible spectroscopy analysis of cells treated with the gold nanoparticles showed that the seed and peel gold nanoparticles either entered into the cells or attached onto the cells. Decrease in absorbance maximum of cells treated with peel and seed gold nanoparticles also showed that nanoparticles were absent in the culture medium after treatment. Kalpana et al. (2013) evaluated the in vitro cytotoxicity of three plant extracts from the leaves of Cinnamomum japonicum, Torreya nucifera and Nerium indicum and found that that nanoparticles synthesized using these extracts were uptaken into the 3T3-L1 cells and showed a decrease in the culture medium with gold nanoparticles treated against 3T3-L1 cells. These findings were in par with our results after the gold nanoparticles treatment against A431 cells in the culture medium.

Morphology changes that occurred in the A431 cells is due to the toxicity of the gold nanoparticles that were suspended in the culture medium at the IC_{50} concentrations. Some of the cell lost its morphology as it entered apoptosis pathway and some cells were seen with roundedness. The results were proven by previous studies where gold nanoparticles prepared by citrate reduction method resulted in stressed cells when incubated for 48 hrs. A549 cells were visualized in the phase contrast microscope after incubation with the gold nanoparticles and were found to be round, a characteristics of stressed cells whereas BHK21 and HepG2 cells remained unaffected by gold nanoparticles treatment (Patra et al. 2007). Gold nanoparticles were synthesized through chemical reduction and stabilized using triphenylphosphine (TPP) which were of 1.5 nm were evaluated for the cell
morphology after exposure in HaCaT cells. The gold nanoparticles (10 µg/mL) showed changes in cell morphology of HaCaT cells on incubation and demonstrated a dose-dependent toxicity. The cells were rounded and exhibited a loss of actin expression. The monolayer of the cells were disrupted completely when compared to the control (Schaeublin et al. 2007).

Literatures till date suggests that cancer cells are under increased oxidative stress compared to normal cells which was associated with increased generation of reactive oxygen species (ROS) and alterations in metabolic activity (Hileman et al. 2003, Pelicano et al. 2004). ROS are chemically active molecules and can impose severe cellular damage and the fact that cancer cells are already under increased ROS stress, it is vulnerable for further ROS insults and this can also provide unique opportunity to kill the malignant cells and also can be a significant therapeutic implication. In biological systems, ROS such as hydrogen peroxide (H₂O₂), hydroxyl radicals (HO•) and superoxide (O₂•⁻) that contains unpaired electron are generally generated through many pathways such as non-enzyme and enzyme-catalysed reactions (Staniek et al. 2002, Pelicano et al. 2004). The major source of cellular ROS is mitochondria and it plays a significant role oxidative stress in cancer cells (Richter et al. 1995). Cancer cells require high level of ATP as they are metabolically active to maintain their active biochemical activities associated with proliferation and uncontrolled cell growth. This demand of energy places stress on the mitochondrial respiration chain and can probably contribute to increased ROS generation (Hlavata et al. 2003).

In cancer cells, the level of superoxide dismutase (SOD) seem to be reduced which compromises the cells ability to eliminate free radicals and causes accumulation of ROS in the cells (Van Driel et al. 1997). This increase in ROS stress results in biological responses ranging from increase in cellular proliferation, senescence, apoptosis and finally necrosis (Davies 1999). Previous in vitro studies reported by Szatrowski and Nathan (1991), showed that seven tumor cell lines were able to produce large amounts of H₂O₂ for atleast 4 hrs at a constant rate. Compared
to normal lymphocytes, primary leukemia cells showed significant increase of oxygen radicals in the malignant cells (Devi et al. 2000). Kong and Lillehei (1998) experimented that many chemopreventive agents exert their killing effect by production of free radicals in cancer cells. ROS generating compounds can be used as a therapeutic strategy to trigger apoptosis in cancer cells. Schumacker (2006) has speculated that chemotherapeutic agents can induce ROS toxicity and can be effective in eradicating malignant cancer cells. Due to high levels of ROS, the malignant cells would be dependent on antioxidant defense systems to eliminate oxidative stress. Accordingly, exposures to exogenous ROS insults and inhibition of antioxidant enzymes would cause more ROS insulted damage to the cancer cells than normal cells and thus therapeutic selectivity can be improved. Studies have demonstrated that human ovarian cancer cells and leukemia cells are more sensitive to inhibition of SOD than normal cells (Zhou et al. 2003, Hileman et al. 2004).

H$_2$DCFDA, a cell permeable stain gives fluorescence upon oxidation by intracellular reactive oxygen species and it is employed to directly demonstrate ROS inside the cells. In the present study, *Vitis vinifera* seed and peel gold nanoparticles and *Vitis vinifera* seed and peel extracts induced accumulation of ROS in A431 cells suggesting that increased ROS production can lead to induction of apoptosis. The results also indicated an increase in fluorescence intensity when treated with seed and peel gold nanoparticles and seed extracts compared to control untreated cells suggesting that the cytotoxicity of the extract and nanoparticles are associated with increased ROS production. Thus, our study showed that ROS generation is due to the exhaustion of antioxidant defense systems and impairment of mitochondrial electron transport chains. Pajaniradje et al. (2014) had evaluated the anticancer therapeutics of leaves of *Sesbania grandiflora* on MCF-7, Hep-2, HepG2, HCT-15, and A549 cancer cell lines. The methanolic fraction was found to exert potent antiproliferative effects particularly A549 cancer cells and high levels of ROS intermediates were generated as seen by DCF-DA staining that played a role in apoptotic induction. Gold nanoparticles synthesized using *Abelmoschus*
esculentus (L.) extract of size ~14nm when treated against Jurkat cells showed elevation of intracellular reactive oxygen species indicating involvement of apoptosis in cell death. Killing cancer cells by ROS-mediated mechanism can be a therapeutic strategy in a variety of clinical settings and development of pharmacological agents that directly increase ROS production can be considered (Mollick et al. 2014). Effect of commercially available gold nanoparticles on oxidative stress was examined by utilizing the fluorescent dye H$_2$DCFDA exhibiting fluorescence in the presence of intracellularly synthesized oxidative stress. Three different sized gold nanoparticles (39nm, 41nm, 45nm) were exposed to AGS cells which resulted in significant increase of ROS at certain concentrations suggesting that ROS signalling played a role for apoptotic cell death in AGS cells while the nanoparticles did not induce increase of fluorescence in A549 cells (Chuang et al. 2013). Several chemopreventive and anticancer agents like cisplatin, bleomycin, and doxorubicin are used currently for cancer treatment and they are shown to increase cellular ROS. Thus, it generates increased ROS causing loss of mitochondrial membrane potential mediating mitochondrial damage and apoptosis in a p53-independent manner (Miyajima et al. 1997, Hug et al. 1997, Tsang et al. 2003). Thus, Vitis vinifera seed and peel gold nanoparticles exerted its mechanism of action for the anticancer effect through generation of ROS intermediates and probably, triggering apoptotic cascade for cell death.

The contact between gold nanoparticles and cells is believed to induce formation of reactive oxygen species (ROS) signalling cascades which controls inflammatory processes, cellular proliferation and cell death (Nel et al. 2006). Enzymes maintain normal redox state through constant input of metabolic energy and disturbances in the normal redox state would have caused toxic effects by producing free radicals and peroxides that can damage lipids, proteins and DNA. The production of ROS by the gold nanoparticles can also be from the chemical reactivity of the nanoparticle materials. The toxicity and increased ROS production can also affect the plasma membrane with enzymes such as NADPH oxidases and
mitochondrial electron leakage from the inner membrane. Many researchers have identified mitochondria as a potentially target organelle with regard to cellular effects of nanoparticles and various studies has showed that nanoparticles can elicit damage to the nuclear DNA (Unfried et al. 2007). In our study, seed and peel gold nanoparticles exerted toxicity and it can be due to the extent of DNA damage, induction of DNA cell cycle arrest and activation of signal transduction pathways which promote apoptosis.

Highly organized process of programmed cell death can be called as ‘Apoptosis’ and it removes unwanted cells from the body in the process of tissue remodelling, organ development and immune responses. When there is a defect in the apoptotic pathway, it can be implicated in the development of cancer and are linked to the resistance of tumors to chemotherapy (Fesik 2005, Qiao and Wong 2009). Apoptosis can be characterized by a set of morphological changes and biochemical steps including translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane and apoptotic bodies formed as a result of chromatin condensation and fragmentation of the cell (Kroemer et al., 2009). Early apoptotic cells are cleared by phagocytosis and are degraded within the macrophages particularly in the phagolysomes and are removed without any damage to the tissue. In contrast, another form of cell death is necrosis which accompanies the release of cellular contents into the extracellular space and promotes inflammatory response in the normal cells. Therefore, the primary goal of chemotherapy is to kill cancer cells by apoptosis so that they can be cleared by neighbouring phagocytic cells (Coussens and Werb 2002, Anderson et al. 2003, Grivennikov et al. 2010). During apoptosis, due to specific damage to mitochondria resulting in loss of function the electron transfer is stopped leading to loss of mitochondrial membrane potential and cytochrome c starts leaking out from the mitochondria (Gulbins et al. 2003). Apoptotic cells cultured in vitro undergoes secondary necrosis after extended incubation as it ultimately shut down metabolism,
they lose its membrane integrity and release the cytoplasmic contents in the cell culture medium (Riss and Moravec 2004).

In the present study Annexin V-FITC apoptosis staining was used to check for the early and late apoptotic cells through fluorescence microscopy. Annexins are homologous proteins and can bind to phospholipids whereas Annexin V-FITC is a fluorescent probe that binds to phosphatidylserine in the presence of calcium (Pigault et al. 1994, Trotter et al. 1995). During apoptosis, the cell loses its phospholipid symmetry in the process during the early stage of apoptosis and phosphatidylserine gets translocated from the internal part to the external portion of the membrane (Kuypers et al. 1996). This available phosphatidylserine then binds to the annexin V-FITC conjugate in the presence of calcium in the beginning of the apoptotic process and propidium iodide binds to cellular DNA where the cell membrane has compromised. Staurosporine was used to induce apoptosis by incubating with the cells (Martin et al. 1995). Live cells showed no staining either by Annexin V-FITC conjugate or propidium iodide (PI) while necrotic cells were stained by both annexin conjugate and PI.

Biologically synthesized *Vitis vinifera* seed and peel gold nanoparticles were treated against A431 cells and were stained with Annexin V-FITC and the morphological changes were immediately observed under fluorescence microscope. *Vitis vinifera* seed and peel gold nanoparticles treated and *Vitis vinifera* seed and peel extracts treated cells showed greenish orange for propidium iodide and Annexin V-FITC positive cells which indicated that A431 cells when treated has undergone apoptosis. There was a significant increase in propidium iodide positive cells i.e dead cells which showed that gold nanoparticles were able to induce secondary necrosis. The staining also confirmed the efficiency of bioconjugated gold nanoparticles causing apoptosis and secondary necrosis. Seed aqueous extracts was able to induce significant apoptotic and necrotic cells and the results were comparable to grape peel extracts. Gold nanoparticles induced more apoptotic cells and less necrotic cells and it produced more stressed cells. The results can be
compared with the increased production of ROS in A431 cells and loss of membrane potential. Investigation of gold nanoparticle and extracts induced apoptosis has also been reported, e.g., HepG2 cells treated with gold nanoparticles of 82.91 nM and A549 cells treated with 144.16 nM showed an increase in apoptotic cells and propidium iodide positive dead cells (Singh et al. 2014). Gold nanoparticles capped with triphenylphosphine monosulfonate with a diameter of 1.4nm were more cytotoxic and cells entered necrosis not apoptosis as a predominant pathway in HeLa cervical cancer cells (Pan et al. 2009). Grape seed proanthocyanidins inhibited cell proliferation in A431 cells and induced (1–48%) cell death in a dose (5–100 µg/ml) and time dependent manner (24, 48 and 72 hrs). Grape seed proanthocyanidins (20-80 µg/ml) also resulted in dose-dependent increase in apoptotic cell death (26–58%) and suggested that it induced apoptosis in caspase-3-dependent pathway (Meeran and Katiyar 2007). Thus seed and peel gold nanoparticles acted as apoptotic inducing agents and were able to trigger apoptosis in A431 skin cancer cells without causing any damage to normal cells.

During apoptosis the refractive index of the cell changes followed by nuclear condensation and cytoplasmic shrinkage. The cell membrane shows blebs and eventually the blebs separate from the dying cell and they form apoptotic bodies (Hengartner 1997). The morphological changes include membrane blebbing, shrinkage of cell, chromatin condensation and degradation of DNA characterized as apoptosis (Kabeer et al. 2013). Useful strategies for anticancer drug development is the induction of apoptosis in cancer cells (Hu and Kavanagh 2003). Acridine orange/ethidium bromide staining is done to observe cell death and cellular morphological changes involved in apoptosis. Therefore, it was examined whether Vitis vinifera seed and peel gold nanoparticles and Vitis vinifera seed and peel extracts induced cytotoxicity on A431 cells through apoptosis and nuclear morphological changes. A431 cells treated with seed and peel gold nanoparticles revealed morphological features such as membrane blebbing, shrinkage of cell and chromatin condensation after 24 hrs treatment. These findings are in agreement with
the other authors where Isodeoxyelephantopin isolated from chloroform extract of *Elephantopus scaber* has shown anticancer effects against KB cells. The morphological changes of apoptosis by AO/EtBr revealed chromatin condensation and nuclear fragmentation (Kabeer et al. 2013). Ho et al. (2009) experimented that Vanillin showed cytolytic and cytostatic effects and that it could be a useful colorectal cancer preventive agent. Apoptosis was induced by vanillin to the HT-29 cells and by AO/EtBr staining it exhibited morphological changes in the cancer cells at 400 μg/mL.

Pajaniradje et al. (2014) studied the anticancer activities of *Sesbania grandiflora* leaves on A549 cells and found that it showed fragmented apoptotic bodies, marginated and shrunken nuclei in contrast to the untreated cells which proves the apoptotic potential of the extract. Gold nanoparticles were investigated for the cell specific response and was reported to induce death response in A540 lung cancer cell line. Exposure of gold nanoparticles to A540 cells resulted in significant nuclear morphology changes and nuclear condensation and was observed after staining with 4',6-diamidino-2-phenylindole (DAPI) under fluorescence microscope proving cytotoxicity of nanoparticles (Patra et al. 2007). *Acalypha indica* synthesized gold nanoparticles were tested for their in vitro cytotoxic effect against MDA-MB-231. At 100 μg/ml concentration, gold nanoparticles exhibited significant cytotoxicity effects and apoptotic features such as membrane blebbing, condensed nuclei and apoptotic bodies and it was confirmed by acridine orange and ethidium bromide dual staining (Krishnaraj et al. 2014).

In living cells, mitochondria generates a proton gradient across the inner membrane when it respires resulting in pH gradient and a membrane potential or $\Delta \Psi_{\text{mit}}$. Using the fluorescent cation RH-123, the membrane potential can be measured and this lipophilic dye accumulates in the mitochondrial matrix driven by the electron gradient which follows the Nerst equation. More Rhodamine-123 is taken up into the matrix as $\Delta \Psi_{\text{mit}}$ increases and RH-123 shows a red shift and fluorescence quenching (Emaus et al. 1986, O'Connor et al. 1988, Nicholls and
Mitochondrial membrane permeability to water and small ions causing transmembrane potential signifies a sensitive parameter of the effectiveness of the mitochondrial function. But mitochondrial membrane impermeability is important for maintaining the proton gradient required for oxidative phosphorylation. The most important feature of the induction of the intrinsic apoptotic pathway is the loss of mitochondrial membrane potential. During apoptosis, mitochondrial membrane becomes permeable leading to release of apoptogenic factors into the cytosol. Many cationic dyes like Rhodamine-123 get electrophoretically distributed into the mitochondrial matrix in response to the electric potential and they are sensitive and reliable probe (Baracca et al. 2003, Singh 2007, Faddan et al. 2010). Even at extreme conditions like oxidative stress mitochondria undergoes drastic changes including de-energization, decrease in the mitochondrial potential, swelling and permeabilization of the inner membrane (Lemasters et al. 1999).

In this study, a decrease in the mitochondrial membrane potential in the A431 cells after treatment with *Vitis vinifera* seed and peel gold nanoparticles and *Vitis vinifera* seed and peel extracts were observed using fluorescent microscope indicating loss of membrane potential (fig 5.10). The percentage of membrane potential and fluorescence intensity decreased (nearly 75%) significantly (*p*<0.05) in the A431 cells when treated with *Vitis vinifera* seed and peel gold nanoparticles at their IC₅₀ concentration. This results were par with other studies which showed that gold nanoparticles (8.17 µg/mL) synthesized using *Abelmoschus esculentus* (L.) pulp extract showed significant elevation of intracellular ROS and decreasing mitochondrial membrane potential which indicates the involvement of apoptosis in cell death (Mollick et al. 2014). The green fluorescent staining clearly indicates significant induction of apoptosis, a process which eliminates hyperproliferating neoplastic cells and is a protective mechanism against cancer (Hickman 1992). This decrease in the ΔΨₘᵦᵦ is reflected in the loss of the ability to accumulate the Rhodamine 123 dye in the cell. This results confirmed that *Vitis vinifera* peel and
seed AuNPs were able to decrease membrane potential such that it follows mitochondrial pathway in the process of apoptosis which is considered as an important mediator of cell apoptosis (Zhang et al. 2010).

Various studies have shown that anticancer agents can increase the permeability of the inner mitochondrial membrane on interaction with mitochondria (Fulda et al. 1998). Wani et al. (2013) have evaluated the methanolic extract of *Gentiana kurroo* for antiproliferative activities and also induction of apoptosis in human pancreatic cancer cell line (MiaPaCA-2) and found that the extract induced potent apoptotic effects against the cancer cells and it induced a significant decrease in mitochondrial membrane potential which led to apoptosis.

Thus, our study had demonstrated that in vitro treatment of *Vitis vinifera* seed and peel gold nanoparticles can inhibit growth of A431 skin cancer cells by inducing cytotoxicity, generating reactive oxygen species followed by loss of mitochondrial membrane potential and inducing apoptosis by exhibiting morphological changes whereas in normal HaCaT cells it was found to be nontoxic. Apoptosis inducing agents have the potential to be developed as new anti-tumor drugs that specifically targets tumor cells as apoptotic cell death does not induce inflammatory response (Rahman et al. 2010). Hence based on the results obtained from the in vitro studies, it is quite evident that bioconjugated gold nanoparticles synthesized using *Vitis vinifera* have better therapeutic potentials compared to chemically synthesized nanoparticles. Chithrani et al. (2006) have concluded that gold nanoparticles enter cells in a shape and size dependent manner as they investigated the cellular level toxicity of gold nanoparticles. Smaller particles have greater surface area to volume ratio and its biological and chemical activity is said to be higher. Due to the large surface area, they absorb some of the macromolecules they encounter on their surface (Ramakrishna and Rao 2011) and with gold nanoparticles specific cell targeting is achieved using surface functionalization of gold nanoparticles (El-Sayed et al. 2006). Gold nanoparticles of synthesized using peel and seed extracts of *Vitis vinifera* were capped with
aromatic compounds, phenols, alkaloids, flavonoids and other polyphenols may or may not affect cell viability in short term but can affect cell proliferation and cause damage to DNA as the cell response was long lasting. The cells were able to internalize the gold nanoparticles and mount stress response in the nuclear level. Various researchers have shown that cause of the cell death is predominantly due to necrosis (Pan et al. 2009). Their results showed that gold nanoparticles at high doses led to necrosis and at lower doses it showed apoptosis (Kroemer 1995). Currently gold nanoparticles are also used in various biomedical applications such as chemotherapy and drug delivery (Han et al. 2007b, El-Kassas et al. 2014). It might be valuable to explore biosynthesized gold nanoparticles as a possible source of unique and novel anticancer drugs. A detailed study of gold nanoparticles that it is not a mere vehicle to carry specific antibodies or substances but it can have a direct interaction with the cellular or subcellular receptors in certain cells should be carried out as it can be an important step in gold nanomedicine. Therefore, *Vitis vinifera* synthesized gold nanoparticles were evaluated for their antitumor potential and immunomodulatory potential in *in vivo* studies.