CHAPTER 7

CHEMOPREVENTIVE POTENTIAL OF GOLD NANOPARTICLES SYNTHESIZED USING VITIS VINIFERA PEEL AND SEED EXTRACTS

7.1 INTRODUCTION:

Carcinogenesis involves multistage process such as tumor initiation, promotion and progression that are closely linked (Surh 2003). Initiation, a rapid and irreversible process includes the initial uptake or exposure to a carcinogen, its distribution and transport to tissues where detoxification and metabolic activation occurs leading to genotoxic damage. Tumor promotion is a lengthy and reversible process where actively proliferating neoplastic cells accumulate. The final stage of neoplastic transformation is progression involving growth of a tumor (Parmar et al. 2010). In human body, skin is the largest organ that protects from UV-radiation, chemicals and other organisms (Armstrong and Kricker 2001). Skin cancers are uncommon malignancies worldwide and there has been a progressive increase in the incidence of skin cancers (Howe 2001, American Cancer Society 2015). Squamous cell carcinoma (SCC), basal cell carcinoma (BCC) together called as nonmelanoma skin cancers (NMSC) and melanoma are the frequent skin cancers (Leiter and Garbe 2008). The incidence of skin cancer in India is about 1-2% of all diagnosed cancers and various cancer registries in India has shown incidence of skin cancer and SCC as the most predominant skin carcinoma (ICMR 2003). In US, SCC is the second most common skin cancer diagnosed annually and is a biologically aggressive tumor believed to largely represent a neoplasm of interfollicular epidermis (Green and Khavari 2004).

Risk factors such as chemicals, tobacco, radiation, mutations, immune status and hormones can cause cancer which may act together to initiate or promote carcinogenesis (Madan and Esmaeili 2012). The major cause of skin melanoma and squamous cell carcinoma are due to ultraviolet radiation and high-frequency
ionizing radiation (Moan et al. 2008). Many types of chemical exposures can increase the occurrence of cancer in humans (Wogan et al. 2004). 7, 12-dimethylbenz[a]anthracene (DMBA) is generally employed as a chemical carcinogen to initiate and promote neoplastic transformation and is preceded by hyperplasia, dysplasia and carcinoma in experimental animals (Letchoumy et al. 2006). DMBA forms DNA adducts, generates excess reactive oxygen species and produces chronic inflammation leading to carcinogenesis. Moreover, DMBA mediated biochemical, molecular and histopathological changes were similar to those observed in humans and therefore used as a model to study the chemopreventive potential of gold nanoparticles and medicinal plants (Miyata et al. 2001, Manoharan et al. 2013).

Chemoprevention has evolved as a valuable and promising strategy to suppress or inhibit the incidence of carcinogenesis by using natural or synthetic agents and these agents act by scavenging excess reactive oxygen species (ROS), improving antioxidant defense system and inhibition of carcinogen activation by enhancing DNA repair and by activation of tumor suppressor genes (Sharma et al. 1994, Steele 2003). Agents that have significant ability to delay tumor onset, reduce tumor incidence and prevent tumor progression can be called as the most useful chemopreventive agents. Therefore, medicinal plants that are rich in bioactive phytochemicals and antioxidants are considered as possible chemopreventive agents that have got more attention for the past few years (Alias et al. 2009). Nutritional deficiencies might also can cause cancer and these factors are implicated in the prevention and pathogenesis of carcinogenic process. These nutritional factors can function as antioxidants, antimutagens, tumor growth suppressors and chemical inactivators (Kohlmeier et al. 1995, Shukla and Pal 2004).

Unfortunately, chemoprevention has led to very limited success owing to the ineffective bioavailability and systemic delivery of chemopreventive agents and hence novel approaches are needed to increase the bioavailability of potential agents. Therefore, nanoparticle facilitated delivery could be beneficial to increase the bioavailability and limit the toxicity of promising agents (Siddiqui et al. 2009).
Gold compounds have been revealed to have antioxidative, immunomodulatory activities in rats (Norton 2008). Gold nanoparticles (AuNPs) have been used in recent years in various areas of nanomedicine for therapeutic uses as they serve to deliver drugs for the treatment of various disorders as well as tumors (Khlebtsov and Dykman 2011). Nanoparticles were able to deliver therapeutic agents through a mechanism called enhanced permeation and retention (EPR) by accumulating in the tumors without getting eliminated through any organs. So gold nanoparticles between 30 nm to 100 nm were able to effectively accumulate inside the tumor and this accumulation can also be controlled by altering nanoparticle’s size. Long term distribution in organs and toxicity studies are still under concern (Albanese et al. 2012). Leonaviciene et al. (2012) has evaluated the effect of gold nanoparticles (13 nm and 50 nm) on the arthritis induced rats and it was found to show significant increase in level of antioxidant enzymes and reduced joint swelling and inflammation. Therefore, several studies suggests that surface modifications by capping agents or surface coatings of gold nanoparticles can play an important role in biological systems and for site directed delivery (Fraga et al. 2014). *Cassia fistula* gold nanoparticles were also shown to possess antidiabetic activity (Daisy and Saipriya 2012) and increased antioxidant enzyme level in diabetes induced rats (Barathmanikanth et al. 2010).

Consumption of fruits and vegetables in high amount is linked to reduced risk of cancers (Krishnaswamy and Polasa 1995) and lower intake of vitamin C, vitamin E, vitamin D, β-carotene and retinal have been related with increased risk of squamous cell carcinogenesis in animal studies (de Luca et al. 1996, van Dam et al. 2000). Grapes (*Vitis vinifera* L.), a phenolics rich fruit contains nutrient components such as phytochemicals, vitamins, carbohydrates, minerals and edible fibers which possess many health promoting and biological activities. Grape seeds and peels contains resveratrol, flavanols, phenolic acids, flavonols, proanthocyanidins and anthocyanins. Zhao et al. (1999) studied the anti-tumor activity of grape seed proanthocyanidins on a skin two-stage carcinogenesis by
applying 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol 13-acetate (TPA) as initiator and promoter in SENCAR mouse and found that due to the high antioxidant activity of procyanidins it showed high anti-tumor activity. Consumption of *Vitis vinifera* juice significantly prevented DMBA-DNA formation of adduct in mammary carcinogenesis by 34% in animals that were fed with 346 mg/dL phenolics compared to the controls (Jung et al. 2006). Parmar et al. (2010) had assessed the anticarcinogenic effect of *Syzium cumini* in Swiss albino mice after inducing with DMBA. They found that *Syzium cumini* was able to alter the antioxidant status in the mice and also reduced the number of papillomas. Grape seed extract also prevented tumor multiplicity and tumor formation when administered as a diet in DMBA induced mammary cancer in rats (Kim et al. 2004).

In this chapter, studies were performed to assess the chemopreventive effect of a gold nanoparticles synthesized using *Vitis vinifera* seed and peel aqueous extracts on 12-O-tetradecanoylphorbol 13-acetate (TPA) tumor promotion in the 7, 12-dimethylbenz[a]anthracene (DMBA) tumor initiated Swiss albino mice.
7.2 MATERIALS AND METHODS:

7.2.1 Chemicals and Reagents

7,12-dimethylbenza(α)anthracene (DMBA) and 12-α-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma Aldrich, Bangalore, India. Thiobarbituric acid (TBA), Nitroblue tetrazolium (NBT), 2,4-dinitrophenyl hydrazine (DNPH), Trichloroacetic acid (TCA), 5,5-Dithiobis-2-nitrobenzoic acid (DTNB) and reduced glutathione (GSH) were purchased from Himedia laboratories, Mumbai, India. Analytical grade acetone was used and all other reagents and chemicals were bought from Qualigens, India with highest purity grade.

7.2.2 Experimental design

Animals : Swiss albino mice (Random-bred).
Weight : 20-25 gms (6-8 weeks old)
Sex : Male.
Feed : Standard mice chow diet.

The animals were procured from College of Veterinary and Animal Sciences, Thrissur, Kerala, India, and were kept under a continuous 14-hour light and 10-hour dark cycle at 25±3°C with a humidity of 50±5%. The mice were kept in sanitized polypropylene cages in the animal house and were adapted to the laboratory condition for nearly one week and then were set in groups for experiments. The animals were fed with normal laboratory food pellets (Saifeeds, Bangalore, India) and water ad libitum. The present protocol was approved by the Institutional animal Ethics Committee (IAEC/KU/BT/12/005).

7.2.3 Acute toxicity studies

The acute toxicity was studied on healthy Swiss albino mice that were starved overnight and separated into groups. Biosynthesized gold nanoparticles using Vitis vinifera seed and peel and Vitis vinifera peel and seed aqueous extracts were injected intraperitoneally according to the dosage level. Groups of 6 mice each
were injected with 1, 2, 3 and 4 mg/kg/b.wt./animal/day of *Vitis vinifera* seed and peel gold nanoparticles and 1, 2, 3 and 4 mg/kg/b.wt./animal/day of *Vitis vinifera* seed and peel aqueous extracts intraperitoneally, daily for 30 days. At the end of the 30<sup>th</sup> day, the mice were checked for skin and fur color, motor activity and mortality of the animals. Weight of the animals were recorded daily. The doses were selected according to the pilot experiment for 30 days (Chen et al. 2009).

**7.2.4 Tumor induction**

Using electrical hair clipper the hair on the dorsal side of the mice were removed (2x2 cm). The mice were left untreated and was checked for hair growth for 2 days and mice with the resting phase of growth cycle were used for further experiments.

The inhibition of tumor by gold nanoparticles synthesized using *Vitis vinifera* seed and peel extracts were evaluated on two-stage skin carcinogenesis, induced by a single application of DMBA (initiator), and two weeks later, 12-o-tetradecanoylphorbol 13-acetate (Promotor) thrice per week was applied topically on the dorsal side, thus promoting tumor formation. The experiment was carried out for 16 weeks following the protocol (Slaga et al. 1996).

- **Group I** - normal control mice were topically applied acetone 0.1 mL thrice a week on the shaven skin (n=6)
- **Group II** –a single dose of DMBA (25µg/ 0.1 mL of acetone) over the shaven area of the skin were applied topically. Two weeks later, TPA (50 µg/100 µl acetone) were applied topically three times per week until the end of experiment (i.e.16 weeks). These animals were served as carcinogen control group. (n=6)
- **Group III** - *Vitis vinifera* peel extract (2.5mg/kg/b.wt./animal/day) were applied topically starting from 7 days before and 7 days after DMBA application and served as treated group. (n=6)
• **Group IV** - *Vitis vinifera* seed extract (2.5mg/kg/b.wt./animal/day) were applied topically starting from 7 days before and 7 days after DMBA application and served as treated group. (n=6)

• **Group V** - *Vitis vinifera* peel AuNPs (2.5mg/kg/b.wt./animal/day) were applied topically starting from 7 days before and 7 days after DMBA application and served as treated group. (n=6)

• **Group VI** - *Vitis vinifera* seed AuNPs (2.5mg/kg/b.wt./animal/day) were applied topically starting from 7 days before and 7 days after DMBA application and served as treated group. (n=6)

### 7.2.5 Tumor related parameters

During the 16 weeks of study, mice were weighed and examined for papillomas each week and recorded. The animals were examined for the presence of skin papillomas and papillomas which persisted for more than a week and having a diameter of >1 mm were considered and evaluated.

The animals were sacrificed at the end of the 16th week by cervical dislocation. Serum was collected after centrifuging the blood at 1500 x g for 10 mins for biochemical assays i.e. liver and kidney function tests. Dorsal skin tissues from all the groups of mice were dissected, washed carefully with PBS (pH 7.4), dried by blotting, weighed and then 10% homogenate was prepared after homogenising using 0.15 M Tris-KCl (pH 7.4) followed by centrifugation at 10000 rpm for 10 min. The supernatant was collected and assayed for antioxidant enzymes such as catalase, reduced glutathione, superoxide dismutase, glutathione peroxidase, vitamin E and also for lipid peroxidation. For histopathological evaluation, skin tissues were collected, processed and then stored.

The following assessments were made in all the groups (Parmar et al. 2010, Roslida et al. 2011)

1. **Tumor incidence**: The number of mouse carrying at least one tumor expressed as a percentage incidence.
2. **Tumor yield:** The average number of papillomas per mouse.

3. **Tumor burden** was calculated by multiplying tumor volume and the number of tumors per animal.

4. **Tumor volume** was measured by the formula

\[ n = \frac{4}{3} \pi \left[ \frac{D_1}{2} \right] \left[ \frac{D_2}{2} \right] \left[ \frac{D_3}{2} \right] \]

where \( D_1, D_2 \) and \( D_3 \) are the three diameters (mm) of the tumors. Number in parenthesis indicates total number of animals bearing tumors.

5. **Tumor weight:** The weight of each tumor was measured at the end of the experiment.

6. **Body weight:** The weights of the mice were measured each week and recorded.

7. **Average latent period:** The average latent period was calculated by multiplying the number of tumors appearing each week (\( F \)) by the number of weeks (\( X \)) after the application of the promoting agent and dividing the sum by total number of tumors (\( N \)) by the formula

\[ \sum FX/N \]

8. **Inhibition of tumor multiplicity** is calculated by

\[
\frac{(\text{Total number of papillomas in carcinogen control}) - (\text{Total number of papillomas in treated}) \times 100}{\text{Total no of papillomas in carcinogen control}}
\]

7.2.6 Hematological Parameters

The haemoglobin concentration in the blood was estimated by cyanomethaemoglobin method of Alexander and Griffith (1993). Erythrocyte sedimentation rate (ESR) was estimated by the method of Bain et al. (2006). The packed cell volume (PCV) was estimated by the method of Alexander and Griffith (1993). Total white blood cell count (WBC) and red blood cell count (RBC) were enumerated according to the method of Bain et al. (2006). Platelets in blood were counted using the improved Neubauer chamber method of Bain et al. (2006). Differential blood count was estimated using the method of Osim et al. (2004).
7.2.7 Biochemical assays

Skin tissues from the mice of all the groups that were homogenized to get the supernatant were used for antioxidant assays.

Thiobarbituric acid reactive substances (TBARS) in skin tissues were determined by the method of Ohkawa et al. (1979) to measure the lipid peroxidation (LPO) level. The reduced glutathione (GSH) level in skin tissues were determined by the method of Beutler and Kelly (1963). Superoxide dismutase (SOD) activity in skin tissues were assayed by the method of Kakkar et al. (1984). The activity of catalase (CAT) skin tissues was assayed by the method of Sinha (1972). The activity of glutathione peroxidase (GPx) in skin tissues were determined using the method of Rotruck et al. (1973). Vitamin E was estimated by the method of Desai, (1984). Protein estimation was studied by using bovine serum albumin as the standard (Bradford 1976).

In order to examine the effect of *Vitis vinifera* peel and seed AuNPs on the regulation of biochemical parameters of the skin cancer mice, serum glucose (Sasaki et al. 1972), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) (King 1965) and Alkaline phosphatase (ALP) (Walter and Schutt 1974) levels were determined to evaluate the hepatic functions, while creatinine (Owen et al. 1954) and uric acid (Caraway 1963) concentrations were studied to assess the renal functions.

7.2.8 Histopathological evaluation

Skin tissues from normal mice, tumor tissues from cancer control group and skin tissues from all the treated groups were removed after sacrificing the mice and were immediately fixed in fixative (10% formalin) for 24 hrs. All the tissues were then processed for dehydration in series of alcohol and were embedded in paraffin wax. The embedded tissues were then cut at 5 μm thickness and finally stained with hematoxylin and eosin. The single sections were then viewed under the normal
microscope (Alias et al. 2009). The sections were viewed for their epidermal changes such as (i) hyperkeratosis - an increase in the superficial cornified layer, (ii) hyperplasia - epidermal thickness without dysplastic cells, (iii) dysplasia, (iv) Squamous cell carcinoma - invasion of epithelial cells (Pindborg et al. 1997).

7.2.9 Evaluation of immunoexpression of p53, BcL-2 and pan cytokeratin in *Vitis vinifera* seed and peel gold nanoparticles treated mice.

Skin tissues from control and gold nanoparticles treated animals from each group were fixed in buffered formalin solution (10%), made to embed in paraffin wax and sections were cut at 2-3µm using rotary microtome. The sections were then picked on glass slides and dried at room temperature and analyzed further for immunohistochemical studies. These sections were then deparaffinized at 60º C and processed through ethanol for hydration. It was then incubated in citrate buffer (pH 6) for 10 mins to retrieve antigen in a microwave oven and washing with tris-buffered saline (pH-7.5). It was then brought down to normal temperature and washed with hydrogen peroxide (3%) in distilled water for 10 mins, thus blocking endogenous peroxidase activity. Normal serum was added to the sections to block non-specific binding of antibody incubated for 30 mins. Skin tissue sections were then incubated with primary antibodies of p53, BcL-2 and pan cytokeratin (Dako p53 –DO-7, Dako BcL-2/124, Dako pan cytokeratin AE1/AE3, CA, USA) at 4 º C and incubated overnight. The slides were washed and incubated with secondary antibody (Dako, CA, USA) followed by horse radish peroxidase (BioGenex, CA, USA) for 30 min at normal room temperature. 3,3’-diaminobenzidine (sigma) was then added to the already bound secondary antibody which acts as a substrate after washing with TBS (Tris buffered saline). After the color intensity developed the slides were washed and counterstained with haematoxylin. Primary antibody were not added in the negative control but the washed with TBS. The expression level was recorded as grading I, II, III, IV for p53 and BcL-2, Grade I and II for pan cytokeratin expression level and 100 positively stained cells were counted to grade the expression level (Chandra Mohan et al. 2006).
7.2.10 Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 20.0 for windows using one way analysis of variance (ANOVA). The values were considered statistically significant, if the $p$-value was < 0.05.
7.3 RESULTS

7.3.1 Oral toxicity induced by *Vitis vinifera* peel and seed AuNPs and extracts

Gold nanoparticles synthesized using *Vitis vinifera* seed and peel aqueous extracts of size ~ 50±5 nm and *Vitis vinifera* seed and peel aqueous extracts were tested for their toxicity in swiss albino mice. The peel and seed gold nanoparticles and peel and seed aqueous extracts were intraperitoneally injected at a dose of 1, 2, 3 and 4 mg/kg/b.wt./animal/day for 30 days. The peel and seed gold nanoparticles of ~ 50±5 nm when injected did not exhibit any toxicity until 30 days. The animals behaved normally and did not show any signs of loss of weight, fur color change or skin color change. All the mice in the treated groups for all the dosages survived throughout the 30 experiment days. *Vitis vinifera* peel and seed gold nanoparticles treated mice did not show any abnormal behaviour and henceforth, for further experiments the mice were treated with 2.5 mg/kg/b.wt./animal/ day of peel and seed gold nanoparticles and peel and seed aqueous extracts.

7.3.2 Chemopreventive potential of *Vitis vinifera* peel and seed AuNPs and extracts in DMBA induced skin cancer in mice

In the present study the chemopreventive potential of topically painted *Vitis vinifera* seed and peel gold nanoparticles were evaluated in DMBA-induced mouse skin carcinogenesis by monitoring the percentage of tumor-bearing animals, cumulative number of papillomas, tumor yield, tumor volume, average latent period (week), inhibition of tumor multiplicity and tumor burden. Table 7.1 and 7.2 represents the inhibitory potential of *Vitis vinifera* seed and peel gold nanoparticles as well as *Vitis vinifera* peel and seed aqueous extracts on DMBA initiated and TPA promoted skin papillomagenesis in Swiss albino mice. A significant gradual increase in the body weight of mice was noted in the experimental groups at the end of the experiment treated with the topical application of *Vitis vinifera* peel and seed AuNPs (27.5±1.8g and 27.8±1.2g) (*p*<0.01), while decrease in the body weight was evident in carcinogen treated control animals (19.6±2.1g). The body weight of *Vitis vinifera* peel and seed aqueous extract treated mice increased significantly (*p*<0.05).
and were 24.4±1.5g and 25.1±2.2g, respectively whereas the body weight of normal mice was 29.5±2.9g. The topical application of peel and seed gold nanoparticles did not affect the body weight, the morphological appearance of mice or the skin color, thus confirming non-toxic effects of the biosynthesized nanoparticles.

Topical application of *Vitis vinifera* peel and seed AuNPs during the initiatitional stages of DMBA and promotional stages of TPA induced skin papillomagenesis (Fig 7.1), significantly reduced the tumor burden to 22.60±1.0 mm³ and 8.37±0.01 mm³ (*p*<0.001) in the experimental groups when compared to 1156.6±96.3 mm³ of the carcinogen control group, and it was 141.1±20.66 mm³ and 75.06±15.22 mm³ in *Vitis vinifera* peel and seed treated group (*p*<0.01). The development of tumors commenced at 4.5 weeks and it was evident in the all carcinogen treated mice by 8th week, whereas in the *Vitis vinifera* peel and seed AuNPs the onset was delayed by 14.2 and 15.3 weeks. The group treated with *Vitis vinifera* peel and seed extracts had started formation of tumors earlier at 8.2 to 9.6 weeks. Topical application of ~50nm *Vitis vinifera* peel and seed gold nanoparticles were able to delay tumor formation latency period and also reduced tumor burden to a significant level when compared to the aqueous extracts treated groups.

The maximum inhibition of multiplicity of papillomas occurred in *Vitis vinifera* peel and seed AuNPs treated groups with 94.59 % and 97.29 %. *Vitis vinifera* peel and seed extracts alone treated groups had 81.08 % and 89.18 % inhibition of multiplicity of papillomas. The peel and seed gold nanoparticles were completely able to inhibit the tumor formation even in the promotional stages of papillomagenesis. Single application of DMBA followed by TPA in mice were able to show tumor formation (100%) in carcinogen control group with mean tumor volume (156.3±12.2 mm³), while in *Vitis vinifera* peel and seed AuNPs treated groups, the tumor formation was reduced significantly to 33.3% and 16.6% with remarkable decrease in the mean tumor volume (12.56±0.42 and 8.37±0.11 mm³) (*p*< 0.001). The *Vitis vinifera* peel and seed extracts were also able to decrease incidence of tumor to 50% and 33.3% with decrease in the mean tumor volume of
61.33±10.5 mm³ and 37.53±1.5 mm³. Carcinogen control group showed tumor size of ≥ 2 mm while the gold nanoparticles treated mice showed tumor size range of ≤ 1 mm. The cumulative number of papillomas that were present by the end of the 16th week of experiment was 37 in the carcinogen control group while for *Vitis vinifera* peel and seed AuNPs, it was greatly reduced to 2 and 1 respectively. The *Vitis vinifera* peel and seed extracts treated group showed 7 and 4 cumulative number of papillomas. The percentage of survival was 100% in gold nanoparticles treated as well as aqueous extract treated group till the end of the experiment while only 83.3 % of mice survived in the carcinogen treated group. The topical application of *Vitis vinifera* peel and seed AuNPs also showed significant chemopreventive potential by inhibiting tumor yield in the treated groups (1.8±0.16 and 1.0±0.16) (*p* < 0.001) when compared to the DMBA treated group (7.4±0.4).
7.1a Skin papillomas in DMBA + TPA alone induced

7.1b DMBA + TPA induced animals treated with Grape peel

7.1c DMBA+ TPA induced animals treated with Grape seed

Contd……
Figure 7.1 (a-e) Effect of *Vitis vinifera* peel and seed AuNPs and extracts in DMBA induced skin papillomagenesis in mice. Arrows in red indicates the presence or absence of papilloma.
Table 7.1 Inhibitory Potential of *Vitis vinifera* seed and peel AuNPs against DMBA-induced Skin Papillomagenesis

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups</th>
<th>Cumulative no of papillomas</th>
<th>Tumor incidence</th>
<th>Tumor yield</th>
<th>Tumor volume (mm$^3$)</th>
<th>Tumor burden (mm$^3$)</th>
<th>Average latent period (week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (Normal)</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>DMBA induced</td>
<td>37</td>
<td>100% (6/6)</td>
<td>7.4±0.4</td>
<td>156.3±12.2</td>
<td>1156.6±96.3</td>
<td>4.5±0.2</td>
</tr>
<tr>
<td>3</td>
<td>DMBA+Grape peel</td>
<td>07</td>
<td>50% (3/6)</td>
<td>2.3±0.09**</td>
<td>61.33±10.5**</td>
<td>141.1±20.66**</td>
<td>8.2±0.3*</td>
</tr>
<tr>
<td>4</td>
<td>DMBA+Grape seed</td>
<td>04</td>
<td>33.3% (2/6)</td>
<td>2.0±0.10**</td>
<td>37.53±1.5**</td>
<td>75.06±15.22**</td>
<td>9.6±0.75**</td>
</tr>
<tr>
<td>5</td>
<td>DMBA+Grape peel nanoparticles</td>
<td>02</td>
<td>33.3% (2/6)</td>
<td>1.8±0.16***</td>
<td>12.56±0.42***</td>
<td>22.60±1.0***</td>
<td>14.2±0.2***</td>
</tr>
<tr>
<td>6</td>
<td>DMBA+Grape seed nanoparticles</td>
<td>01</td>
<td>16.6% (1/6)</td>
<td>1.0±0.16***</td>
<td>8.37±0.11***</td>
<td>8.37±0.01***</td>
<td>15.3±0.1***</td>
</tr>
</tbody>
</table>

Data are represented as mean ± S.D. Significance level compared between carcinogen control and treated groups at *$p<$ 0.05, **$p<$ 0.01, *** $p<$ 0.001. n=6
Table 7.2 Effect of *Vitis vinifera* seed and peel AuNPs on body weight, tumor weight and inhibition of tumor

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Tumor weight (g)</th>
<th>Inhibition of tumor multiplicity</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control (Normal)</td>
<td>26.2±3.5</td>
<td>29.5±2.9</td>
<td>Nil</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>DMBA induced</td>
<td>27.0±2.2</td>
<td>19.6±2.1</td>
<td>0.12±0.02</td>
<td>83.3</td>
</tr>
<tr>
<td>3</td>
<td>DMBA+Grape peel</td>
<td>26.5±1.8</td>
<td>24.4±1.5*</td>
<td>0.08±0.01*</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>DMBA+Grape seed</td>
<td>26.1±2.0</td>
<td>25.1±2.2*</td>
<td>0.07±0.01*</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>DMBA+Grape peel nanoparticles</td>
<td>27.1±2.12</td>
<td>27.5±1.8**</td>
<td>0.052±0.02**</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>DMBA+Grape seed nanoparticles</td>
<td>26.8±2.1</td>
<td>27.8±1.2**</td>
<td>0.050±0.01**</td>
<td>100</td>
</tr>
</tbody>
</table>

Data are represented as mean ± S.D. Significance level compared between carcinogen control and treated groups at *p* < 0.05, **p** < 0.01. n=6
7.3.3 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the hematological parameters in DMBA induced skin cancer in mice

Table 7.3 and 7.4 shows a significant decrease in the levels of Hb, RBC count, WBC count, platelet count and PCV count in DMBA induced control mice (Group II) when compared with normal mice (Group I). Topical application of *Vitis vinifera* seed and peel AuNPs (2.5mg/kg bodyweight) to mice resulted in an increase in WBC count (7.1±0.15 and 6.9±0.25×10³ /mm³) significantly (*p*<0.05) to near normal levels. There was no significant difference between the gold nanoparticles treated group when compared to the *Vitis vinifera* seed and peel aqueous extract treated group that showed a significant (*p*<0.05) increase in the WBC count (6.6±0.2 and 6.3±0.21×10³ /mm³). The increase in levels of ESR were suppressed by the *Vitis vinifera* seed and peel AuNPs significantly on applying gold nanoparticles on the dorsal side of the mice while *Vitis vinifera* seed and peel extracts brought them to a near normal level.

Significant increase (*p*<0.05) in the level of PCV and RBC level were evident when treated with seed and peel AuNPs than the carcinogen control group which showed peel and seed gold nanoparticles activity on the hematological parameters. There was no significant changes in the hemoglobin content when treated with gold nanoparticles while *Vitis vinifera* seed and peel extracts treatment exhibited significant (*p*<0.05) changes in increasing the Hb content. It is obvious from the table that *Vitis vinifera* seed and peel gold nanoparticles were able to decrease % neutrophils, % eosinophils and increase % lymphocytes and % monocytes and the values were comparable to the group treated with *Vitis vinifera* seed and peel extracts.
Table 7.3 Effect of *Vitis vinifera* seed and peel extracts and AuNPs on the hematological parameters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WBC (\times 10^3 /\text{mm}^3)</th>
<th>RBC (\times 10^6/\text{mm}^3)</th>
<th>DC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>N</strong></td>
</tr>
<tr>
<td>Normal control</td>
<td>6.9±0.1</td>
<td>4.2±0.19</td>
<td>61.1±1.8</td>
</tr>
<tr>
<td>Cancer control</td>
<td>3.5±0.42*a</td>
<td>3.3±0.16*a</td>
<td>71.1±1.9*a</td>
</tr>
<tr>
<td>Grape seed treated</td>
<td>6.6±0.2*b</td>
<td>4.17±0.2*b</td>
<td>61.8±2.4*b</td>
</tr>
<tr>
<td>Grape peel treated</td>
<td>6.3±0.21*b</td>
<td>4.1±0.24*b</td>
<td>64.1±2.1*b</td>
</tr>
<tr>
<td>Grape seed nano treated</td>
<td>7.1±0.15*b</td>
<td>3.7±0.16*b</td>
<td>55.8±1.2*b</td>
</tr>
<tr>
<td>Grape peel nano treated</td>
<td>6.9±0.25*b</td>
<td>3.59±0.11*b</td>
<td>55.9±0.9*b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±S.D. for five animals. Comparisons are made between \*a, Group I vs. Group II, \*b, Group II vs. Group III and Group IV. The letters \*a and \*b represents the statistical significance at \(p<0.05\) and \*\*a and \*\*b indicates the statistical significance at \(p<0.01\).
Table 7.4 Effect of *Vitis vinifera* seed and peel extracts and AuNPs on the Hematological parameters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hb (g/dl)</th>
<th>PCV (%)</th>
<th>Platelet Count (Lakhs/ml)</th>
<th>ESR (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>Normal control</td>
<td>12.6±0.63</td>
<td>38±2.2</td>
<td>2.9±0.27</td>
<td>05±0.12</td>
</tr>
<tr>
<td>Cancer control</td>
<td>10.5±0.7*</td>
<td>31±2.3*</td>
<td>1.3±0.19*</td>
<td>20±0.203*</td>
</tr>
<tr>
<td>Grape seed treated</td>
<td>12.6±0.8*</td>
<td>39±1.6*</td>
<td>2.6±0.13*</td>
<td>06±0.12*</td>
</tr>
<tr>
<td>Grape peel treated</td>
<td>12.3±0.6*</td>
<td>38±1.9*</td>
<td>2.5±0.09*</td>
<td>08±0.5*</td>
</tr>
<tr>
<td>Grape seed nano treated</td>
<td>11.5±0.54ns</td>
<td>35±0.9*</td>
<td>1.91±0.04*</td>
<td>11±0.2*</td>
</tr>
<tr>
<td>Grape peel nano treated</td>
<td>11.3±0.34ns</td>
<td>34±1.7ns</td>
<td>1.68±0.07*</td>
<td>12±0.2*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±S.D. for five animals. Comparisons are made between *a, Group I vs. Group II, *b, Group II vs. Group III and Group IV. The letters *a and *b represents the statistical significance at \( p < 0.05 \) and ns indicates non-significance.
7.3.4 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the serum liver and kidney enzymes levels

Fig 7.2 shows the effect of *Vitis vinifera* seed and peel AuNPs on serum markers enzymes and glucose level in DMBA induced skin cancer mice. Topical application of *Vitis vinifera* seed and peel gold nanoparticles before and after DMBA and TPA application resulted in a significant \( p<0.001, p<0.001, p<0.01 \) decrease in the ALT, AST and ALP enzyme level to a near normal level showing the protective effect of gold nanoparticles. The carcinogen control group showed marked significant increase \( p<0.001, p<0.001, p<0.01 \) in the ALT, AST and ALP enzymes level which on treatment with gold nanoparticles significantly reverted the enzyme levels to normal state. The *Vitis vinifera* seed and peel aqueous extracts also showed significant \( p<0.001, p<0.001, p<0.01 \) decrease in the liver enzyme levels when compared to the carcinogenic control. It is also obvious to note that the glucose levels had significantly \( p<0.01 \) decreased to a normal level when treated with *Vitis vinifera* seed and peel gold nanoparticles topically. And *Vitis vinifera* seed and peel extracts also showed comparable results \( p<0.01 \) with the gold nanoparticles treated group. This shows the hepatoprotective nature of the peel and seed gold nanoparticles and the activity of the polyphenols that are capped onto the gold nanoparticles.

Fig 7.3 depicts the effect of *Vitis vinifera* seed and peel gold nanoparticles on the kidney enzyme levels which showed significant \( p<0.01, p<0.05 \) decrease in the creatinine and uric acid levels in the peel and seed gold nanoparticles treated group when compared to the DMBA treated carcinogen group. Treatment of *Vitis vinifera* seed and peel aqueous extracts before and after DMBA treatment in the mice had significantly \( p<0.01, p<0.05 \) reduced the creatinine and uric acid levels and thus showing the protective nature of the extracts on the kidney function level when tested on the serum.
Figure 7.2 Effect of *Vitis Vinifera* seed and peel AuNPs on the AST, ALT, ALP and Glucose levels. Values are the mean ± SD for 6 animals in each group. Values are statistically significant at *p* < 0.05, **p** < 0.01, ***p*** < 0.001 and statistical significance was compared within a carcinogenic control vs treated groups and b normal control vs Carcinogenic control.

Figure 7.3 Effect of *Vitis Vinifera* seed and peel AuNPs on the kidney enzyme levels. Values are the mean ± SD for 6 animals in each group. Values are statistically significant at *p* < 0.05, **p** < 0.01, ***p*** < 0.001 and statistical significance was compared within a carcinogenic control vs treated groups and b normal control vs Carcinogenic control.
Figure 7.4 Effect of *Vitis vinifera* seed and peel extracts on Catalase, Vit-E, SOD and LPO levels in normal and experimental mice. CAT-micromoles of H$_2$O$_2$ decomposed/mt/mg protein, Vit E- mg/dl, SOD- 50% inhibition of epinephrin autooxidation/ml, LPO- nmol/ml. Values are the mean ± SD for 6 animals in each group. Values are statistically significant at *p< 0.05, **p< 0.01, ***p< 0.001 and statistical significance was compared within a carcinogenic control vs treated groups and b normal control vs Carcinogenic control.

Figure 7.5 Effect of *Vitis vinifera* seed and peel extracts on GSH, GPx levels in normal and experimental mice. GSH- mg/dl, GPx- micromoles of glutathione oxidized/mt/mg protein. Values are the mean ± SD for 6 animals in each group. Values are statistically significant at *p< 0.05, **p< 0.01, ***p< 0.001 and statistical significance was compared within a carcinogenic control vs treated groups and b normal control vs Carcinogenic control.
7.3.5 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the enzymatic and non-enzymatic antioxidant levels

Fig 7.4 and 7.5 elucidates the levels of SOD, GSH, GPx, TBARS, catalase and vit-E in the skin tissues of the carcinogenic control and gold nanoparticles and extracts treated swiss albino mice. Carcinogenic group showed significant \((p<0.01)\) elevated levels of TBARS when treated with the carcinogen for 16 weeks whereas *Vitis vinifera* peel and seed AuNPs treated group showed significant \((p<0.01)\) lower levels of TBARS and *Vitis vinifera* peel and seed extracts also exhibited antioxidant activities in the skin tissues of mice. Topical application of gold nanoparticles treated group showed better antioxidant activity than the extracts. The levels of catalase and non-enzymatic antioxidant vitamin E were found to be lower in the carcinogen treated control group. Application of *Vitis vinifera* peel and seed AuNPs before 7 and after 7 days of DMBA treatment to the mice exhibited significant \((p<0.01)\) increase in the antioxidant enzymes level, thus proving the effect of peel and seed gold nanoparticles in scavenging the free radicals and increasing the enzyme level in the skin tissue. *Vitis vinifera* peel and seed extracts also enhanced the catalase and vitamin E level in the skin tissue significantly \((p<0.05)\) when compared to the carcinogen control which showed significant \((p<0.05)\) decrease in the levels of catalase and vitamin E in comparison to normal control groups.

The activities of GPx and GSH were reduced significantly \((p<0.05)\) in the DMBA control mice when compared to normal control animals. Peel and seed gold nanoparticles when applied topically on the dorsal side of the mice resulted in increase in the levels of GSH and GPx in the skin tissue significantly \((p<0.05)\) and it was comparable to the *Vitis vinifera* peel and seed extracts treated mice which also showed significant \((p<0.05)\) increase in the antioxidant enzymes. *Vitis vinifera* seed gold nanoparticles and seed extracts revealed significant \((p<0.01)\) antioxidant activity by augmenting the levels of SOD in the skin tissues of DMBA treated mice. *Vitis vinifera* peel gold nanoparticles and peel extracts also increased the SOD levels in the skin tissue to a significant \((p<0.05)\) levels. Thus, *Vitis vinifera* peel and
seed gold nanoparticles exhibited significant antioxidant activity on the DMBA induced skin papillomagenesis in swiss albino mice showing chemopreventive potential of the peel and seed gold nanoparticles.

7.3.6 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the histopathological features of the DMBA induced skin papillomagenesis in Swiss albino mice

The histopathological analysis of the normal control, carcinogen control and treated animals in the skin tissues are presented in figure 7.6. Carcinogen control group which was initiated with DMBA and promoted by TPA for 16 weeks showed malignant skin papillomas on all the mice. It was characterized by keratinous pearls with hyperkeratosis and epithelial dysplasia leading to squamous cell carcinoma. The pre and post treated animals with *Vitis vinifera* peel gold nanoparticles showed mild hyperkeratosis and mild acanthosis while the group treated with topical application of *Vitis vinifera* seed gold nanoparticles showed mild hyperkeratosis with intact epithelial layer confirming the chemopreventive potential of the peel and seed gold nanoparticles. The skin tissues of the mice treated with *Vitis vinifera* seed aqueous extracts showed mild thickening of the skin (epithelial layer) whereas mice pre and post treated with *Vitis vinifera* peel aqueous extracts showed mild acanthosis on the skin tissue of the mice. The cells did not penetrate the basement membrane and if tumors were formed it was benign.
Figure 7.6 Representative photomicrographs of the histopathological observations in skin tissues of control and experimental animals. A- Normal control, B- DMBA control showing well differentiated squamous cell carcinoma with dysplastic epithelium and keratin pearls, C- Grape peel extract treated showing acanthosis, D- Grape peel AuNPs treated showing mild hyperkeratosis and mild acanthosis, E- Grape seed extract treated showing Mild acanthosis, F- Grape seed AuNPs treated showing mild hyperkeratosis with an intact epithelial layer.
Figure 7.7 Representative photomicrographs of immunohistochemical staining of immunoexpression of p53 in skin tissues of control and experimental mice (10X) (n=6)
Figure 7.8 Representative photomicrographs of immunohistochemical staining of immunoexpression of BcL-2 in skin tissues of control and experimental mice (10X) (n=6)
Figure 7.9 Representative photomicrographs of immunohistochemical staining of immunoexpression of pan cytokeratin in skin tissues of control and experimental mice (10X) (n=6)
7.3.7 Evaluation of immunoexpression of p53, BcL-2 and pan cytokeratin in *Vitis vinifera* peel and seed AuNPs and extracts treated Swiss albino mice.

Fig 7.7, 7.8 and 7.9 illustrates the immunoexpression level of p53, BcL-2 and pan cytokeratin on the DMBA induced papillomagenesis of the mice treated with *Vitis vinifera* peel and seed gold nanoparticles and extracts as well as carcinogenic control. Topical application of peel and seed gold nanoparticles suppressed the nuclear expression of p53 significantly when compared to the carcinogenic control. The positive staining of p53 was significantly increased in DMBA treated control group compared to the skin tissue of the normal mice which showed negative staining. More than 70% of the cells showed positive nuclear staining. Treatment with *Vitis vinifera* peel and seed extracts also reduced the p53 immunoexpression to a significant level.

Skin tissues treated with DMBA alone showed positive staining for BcL-2 and the cytoplasmic expression level was significantly increased while the expression was suppressed in the *Vitis vinifera* peel and seed gold nanoparticles treated group. The control skin tissue showed negative staining but showed staining of the keratinocytes and dendritic cells. *Vitis vinifera* peel and seed extracts treated skin tissue showed lower expression of BcL-2 in comparison with the carcinogenic control. The animals treated topically with DMBA and TPA showed strong significant expression of pan cytokeratin levels but treatment of *Vitis vinifera* peel and seed gold nanoparticles decreased the cytokeratin expression level significantly. Staining of normal cells for cytokeratin showed no expression of cytokeratin while tumor cells showed increased immunoexpression of cytokeratin levels.
7.4 DISCUSSION

Carcinogenic chemicals like polyaromatic hydrocarbons, UV light exposure and chronic wounding can result in regenerative cell proliferation. Hence stimulation of cell proliferation and maintaining continuous hyperplasia along with inhibition of apoptosis can become crucial factors for skin tumor promotion. During tumor promotion, inflammatory cells are recruited into the dermis and they produce free radicals leading to oxidative stress. Thus, tumor promoters produce reactive oxygen species (ROS) in the cells and 12-O-tetradecanoylphorbol-13-acetate (TPA) is one such promotor increasing hydroperoxides and ROS in the dermal cells (Rundhaug et al. 2010). But even before carcinogenesis promotion can occur, human beings are exposed to large numbers of tumor initiating agents. Mouse skin carcinogenesis is considered as a model system to study tumor initiation and promotion model which can induce papillomas in animals and also to investigate the chemopreventive efficacy of dietary vegetables and fruits to reduce the risk of epithelial carcinogenesis (Arya and Kumar 2011, Hamizah et al. 2012). When the efficacy of the chemotherapy drug influences the treatment effects by showing reduced cell specificity, high toxicity and reduced availability of suitable drug, delivery vectors can assist in this method by reducing adverse effects and improving efficacy (Surendiran et al. 2009).

Therapeutics based on nanoparticles can efficiently deliver them into tumors by enhanced permeability and retention effect (EPR) and therapeutic substances can be attached or adsorbed onto the surface of the nanoparticles. Recently, gold nanoparticles are considered as delivery vehicles significant for their effective distribution inside tumors and they act as chemotherapeutic agents (Chen et al. 2008). In the process of passive targeting, gold nanoparticles can accumulate inside the tumor tissues as it has leaky vasculatures and compromised lymphatic drainage and by controlling the size of the nanoparticles to be less than 100nm the targeting capability and circulation time can be improved (Nie et al. 2007).
Chemopreventive agents can be of natural or synthetic products that can arrest or stop progression of cancerous cells, induce apoptosis and inhibit mutagenesis. Fruits, vegetables and medicinal plants rich in phytochemicals have been studied for their chemopreventive potential and these phytochemicals are considered as chemopreventive agents as they are able to inhibit or reverse progression of epithelial carcinogenesis and have been the attractive strategy for controlling malignant cells. Researchers have proven the chemopreventive potential of many plant extracts like *Triticum aestivum* (Arya and Kumar 2011), *Apium leptophyllum* (Sahoo et al. 2014), *Annona muricata* (Hamizah et al. 2012), *Ardisia crispa* (Roslida et al. 2011), *Syzygium cumini* (Parmar et al. 2010), *Aloe vera* (Chaudhary et al. 2007), *Phyllanthus niruri* (Sharma et al. 2009), *Vitis vinifera* (Zhao et al. 1999) and plant compounds such as ferrulic acid (Alias et al. 2009), *S. libanotica* oil (Gali-Muhtasib and Affara 2000), proanthocyanidins (Roy et al. 2004) and berberine (Manoharan et al. 2011) in DMBA induced skin carcinogenesis.

*Vitis vinifera* (grapes) possess phenolic antioxidants which are distributed in peels and seeds. Grape phenolics are known for their potential anticancer activities and various studies have demonstrated the *in vivo* anticancer and antioxidant activities of grapes as they act as free radical scavengers (Zhou et al. 2012). In the present study the chemopreventive potential of topically painted *Vitis vinifera* seed and peel gold nanoparticles as well as extracts were evaluated in DMBA-induced mouse skin papillomagenesis by monitoring the percentage of tumor-bearing animals, inhibition of tumor multiplicity, tumor volume and burden as well as lipid peroxidation and antioxidants enzymes levels. Topical application of *Vitis vinifera* seed and peel gold nanoparticles to DMBA-painted mice completely prevented the formation of skin tumors.

Topical application of *Vitis vinifera* seed and peel gold nanoparticles (~50 nm) on the DMBA initiated and TPA promoted skin papillomagenesis in mice revealed chemopreventive potential either by preventing or inhibiting the formation of tumors. The present study shows the anti-tumor promoting activity of the peel and
seed gold nanoparticles. The gold nanoparticles might have acted either by penetrating the skin and inhibiting the formation of papillomas or accumulated inside the tumors to prevent further progression of the growth of papillomas. Topical application of peel and seed gold nanoparticles before and after DMBA treatment effectively reduced the number of papillomas, tumor burden and tumor volume and the average latent period for the formation of tumor was more than 14 and 15 weeks thus showing inhibitory activity of the nanoparticles on the abnormal proliferation of cells. The treatment of gold nanoparticles also delayed the initiation and promotional stages of tumor formation when compared to the carcinogen control. The size and weight of the tumor was reduced when treated with the 2.5 mg/kg of peel and seed gold nanoparticles that may be due to the activity of the gold nanoparticles acting as a blocking agent to prevent initiation stage or suppressing agent by preventing abnormal cell proliferation in both the stages of cancer (Hamizah et al. 2012). Treatment of *Vitis vinifera* seed and peel extracts before and after the carcinogen exposure has also prevented the formation of tumor and tumor multiplicity. The suppressing or inhibiting activity of gold nanoparticles may also be attributed to the presence of phytochemicals in the *Vitis vinifera* seed and peel that are capped onto the surface of the nanoparticles. Similar results have been obtained by Qiblawi et al. (2012) on evaluating the chemopreventive potential of cardamom on the DMBA and croton oil treated skin papillomagenesis in mice.

The tumor volume and weight of the tumor were recorded lower in the treated group. Significant reduction in the tumor burden, tumor incidence and cumulative number of papillomas were recorded in the cardamom treated group. *Daucus carota* oil extract was experimented against the DMBA-TPA promoted mouse skin carcinogenesis model. The administration of oil extract inhibited tumor incidence, tumor yield, reduced tumor volume and delayed tumor onset showing the significant antitumor activity of the oil extract which were in par with our results (Zeinab et al. 2011).
These phytochemicals may have exerted the chemopreventive potential even at a low dose (Russo 2007) and the nanoparticles localised at the site of the tumor tissue. Various studies have shown the uptake of gold nanoparticles in the tumor tissue when PEGylated with a diameter of 20, 40, 60, 80 and 100 nm (Perrault et al. 2009). The nanoparticles also accumulated depending on the size and larger particles show better amassing (Dreher et al. 2006). Colon tumor was targeted with gold nanoparticles loaded with tumor necrosis factor (TNF) through intravenous administration and found that TNF when bound to nanoparticles were more efficient and nanoparticles acted as therapeutic drug when compared to TNF alone (Paciotti et al. 2004). Chemotherapeutic drug paclitaxel (PTX) when conjugated with gold nanoparticles (26 nm) accumulated in the solid tumors (Paciotti et al. 2006). Previous reports have proved the nontoxic nature of gold nanoparticles at a concentration of 2 mg/kg whereas 20 mg/kg and 40 mg/kg caused lethality and toxicity also depends on the molecules present on the surface of the gold nanoparticles (Chen et al. 2006).

*Vitis vinifera* seed and peel gold nanoparticles showed no toxic effects like decreased body weight or change in skin or fur color when applied on the skin of the mice and showed increase in body weight indicating non-toxic nature of the gold nanoparticles. The obtained results were in agreement with Chen et al. (2009) who had studied the toxicity of gold nanoparticles according to size like 3, 5, 50 and 100 nm which showed no toxicity or harmful effects in Balb/c mice when injected intraperitoneally. Studies have shown the nontoxic effect of gold nanoparticles for 50 nm diameter in a diabetic induced mice model proving the therapeutic nature of the gold nanoparticles (Barathmanikanth et al. 2010).

DMBA metabolism can result in accumulation of reactive oxygen species (ROS) on the applied sites and on tumor formation can diffuse to other nearby cells. Continuous exposures to tumor promoters can also generate free radicals and result in oxidative stress. This free radicals can damage DNA and can produce lipid peroxidation. Hence, generation of free radicals and ROS is mediate an important
role in DMBA and TPA promoted tumorigenesis as well as skin tumor promotion (Rundhaug and Fischer 2010) and there is considerable evidence of the involvement of ROS in the initiation and promotion of chemical carcinogenesis (Shvedova et al. 2004). Topical application of *Vitis vinifera* peel and seed gold nanoparticles also reverted back the antioxidant level in the treated mice showing the antioxidant potential of the gold nanoparticles. The imbalance of antioxidant enzymes have been restored back to a substantial level by the peel and seed gold nanoparticles suggesting that nanoparticles were able to detoxify the carcinogens, prohibited the metabolic stimulation of carcinogens and blocked the reaction of macromolecules with the carcinogens (Mohan et al. 2006). GSH, an antioxidant acts by maintaining the integrity of the organ during insults of ROS. CAT and SOD scavenges the free radicals and decomposes reactive oxygen species like H$_2$O$_2$ and O$_2^-$ in the process of chemical carcinogenesis (Lu 1999, Li et al. 2000, Dasgupta et al. 2004). Vitamin E is an antioxidant that acted as a chemopreventive agent in various studies including skin cancer (Rahman et al. 2008).

Decrease in lipid peroxidation and increase in antioxidant enzymatic and non-enzymatic level such as SOD, CAT, GPx, GSH and vitamin E were found as a part of defense mechanism when treated topically with peel and seed gold nanoparticles as well as extracts showing the free radical scavenging effect which are well correlated with the results obtained in the DMBA induced carcinogenesis in mice treated with *Apium leptophyllum* extracts (Sahoo and Santani 2014). Barathmanikanth et al. (2010) has also reported the antioxidant potential of gold nanoparticles in diabetic mice model showing the control of AuNPs over the antioxidant enzymes. Yakimovich et al. (2008) have also demonstrated the antioxidant activities of gold nanoparticles which was in par with our results.

Detoxification and biotransformation process of carcinogens is mediated by liver and measuring the status of these markers helps to identify the chemopreventive potential of therapeutic agents (Rastogi et al. 2007). Markers of liver damage such as AST, ALT and ALP were studied on the DMBA induced
control as well as gold nanoparticles treated animals. These enzyme markers in serum reveals the tumor response to peel and seed gold nanoparticles therapy, mechanism of neoplastic formations and the level of cellular damage (McIntyre and Rosalki 1992). The decrease in the level of ALT, AST, ALP suggests the protective role of peel and seed gold nanoparticles in the DMBA induced skin papillomagenesis. It also exhibits the non-toxicity of the gold nanoparticles that are applied topically. Increase in levels of transaminases and ALP enzymes correlates well with the carcinogenesis development, toxicity of the therapeutic agent and are related to proliferation of cells in cancer conditions. Peel and seed gold nanoparticles have well-acted as protective agent and inhibited tumor formation compared to peel and seed extracts alone when treated on DMBA and TPA promoted skin papillomagenesis and also suggests that gold nanoparticles have aided the excretion of DMBA metabolites or inhibited the metabolic activation. Our results can be substantiated well with the studies of Ali and Dixit (2015) where increased level of aminotransferase enzymes and ALP enzyme were found in the skin cancer conditions and treatment with quercetin reverted back the enzymes to near normal levels in mice. Sahoo et al. 2014 have studied that tumor marker enzymes were decreased in serum on treatment with Apium leptophyllum extract suggesting the chemopreventive potential of the extract in the DMBA induced skin cancer model.

Decrease in the creatinine and uric acid in the peel and seed gold nanoparticles treated group shows the regulated renal function of the nanoparticles. Creatinine and uric acid are considered as the renal markers in serum or plasma levels. Elevated levels of these markers in the DMBA treated animals indicates the renal dysfunction and peel and seed gold nanoparticles were able to restore the function or prevented renal damage caused by the metabolites and it can also suggest the regenerative capabilities of renal tubules (Kissane 1985). The results were in agreement with Daisy and Saipriya (2012) who reported that Cassia fistula gold
nanoparticles were able to restore liver and kidney function enzymes to near normal levels in albino Wistar rats.

Significant increase in WBC, RBC and platelet count were seen in the nanoparticles treated mice while non-significant increase in Hb content were evident. This shows that the phytochemicals present in the gold nanoparticles as well as extracts were able to induce the development and stimulation of cells in bone marrow. The ~50 nm gold nanoparticles were not toxic to the animals and the results can be substantiated with the reports of Zhang et al. (2010) where 10 nm AuNPs have increased WBC production and Bovine serum albumin capped gold nanoparticles also increased WBC and Hb content in mice. A similar correlation is found between the increase in RBC, PCV and Hb content as variation in one parameter affects the other (Ikpi and Nku 2008).

Histopathological analysis of the normal, carcinogenic control, peel and seed gold nanoparticles and extracts treated skin of the animals showed the chemopreventive potential of the nanoparticles as well as extracts. Treatment with gold nanoparticles restored the skin with normal histology through scavenging free radicals, balancing antioxidant enzymes and control over lipid peroxidation thus showing the therapeutic properties of gold nanoparticles biosynthesized using Vitis vinifera peel and seed (Daisy and Saipriya 2012).

Topical application of peel and seed gold nanoparticles for 16 weeks on DMBA induced skin papillomagenesis resulted in decreased expression of p53, Bcl-2 and cytokeratins while DMBA application on the skin of the carcinogen control group resulted in upregulated expression of p53, Bcl-2 and cytokeratins. In epithelial tumors stable increased expression of cytokeratins which are epithelial tumorigenesis marker and also intermediate filament proteins are found in the infiltrating tumors. In the present study decreased expression of pan cytokeratins was found in the gold nanoparticles treated groups. This result was well in correlation with Chandra Mohan et al. (2006) as they report increased expression of cytokeratins, p53 and Bcl-2 in buccal pouch carcinogenesis. Up regulated
expression of Bcl-2 and p53 was found in skin tumorigenesis and in almost all skin cancers p53 mutations is evident (Basset-Seguin et al. 1994, Manojprabhakar 2010). Mutant p53 overexpression was found in DMBA treated control mice while the expression was down regulated in the treated mice. This mutant p53 also increases the expression of Bcl-2 and inhibits the process of apoptosis (Khan et al. 2010). Topical application of gold nanoparticles down regulated expression of mutant p53 and Bcl-2 and may have facilitated the process of apoptosis in the chemical carcinogenesis process. Gold nanoparticles also mediated p53 dependent arrest of cell proliferation when compared to peel and seed extracts alone. Administration of rosmarinic acid in DMBA induced skin tumorigenesis in mice decreased the expression of p53 and Bcl-2 in the treated group which were par with our results (Sharmila and Manoharan 2012).

Nanoparticles of size >30nm and <200 nm can accumulate inside tumors and circulation time is longer when compared to the smaller nanoparticles and delivers the therapeutic substances (Jain and Stylianopoulos 2010). Studies have suggested that gold nanoparticles are cleared by reticuloendothelial system (RES) in the biological system and also by drainage in the permeable endothelium. But still the biodistribution of gold nanoparticles depends on the size of the nanoparticles. Most of the nanoparticles that enters the circulation ends up in spleen and liver (Papasani et al. 2012). Nanoparticles of size 50 nm mostly accumulates in the spleen and liver and therefore *Vitis vinifera* peel and seed gold nanoparticles of size ~50nm would have accumulated in the tumour and thus may have delivered the therapeutic potential of *Vitis vinifera* peel and seed extracts. Surface coating of gold nanoparticles also plays a major factor for undetection of RES and thus delaying the circulation time (Owens and Peppas 2006). Phytochemicals from *Vitis vinifera* peel and seed extracts that formed the coating on gold nanoparticles may also have offered therapeutic potential to the cancer cells.

The beneficial action of *Vitis vinifera* peel and seed AuNPs is probably due to its ability to stimulate the antioxidant enzymes in the cells. Our results thus
suggested that *Vitis vinifera* peel and seed AuNPs and extracts suppressed abnormal skin cell proliferation occurring during DMBA-induced skin papillomagenesis and demonstrated significant chemopreventive potential of peel and seed gold nanoparticles in DMBA induced skin carcinogenesis than seed and peel aqueous extracts.