6.1 INTRODUCTION:

To augment the present cancer therapy such as radiation and chemotherapy, enhancing the immune system is necessary as it shows disadvantages like side effects and suppression of the immune system (Sunila and Kuttan 2004). Immune system has a vital role in maintaining balance in the human body by adopting innate and adaptive immunity. They act as a defense mechanism to fight against foreign materials with the help of phagocytic cells and antigen presenting cells (APCs) wherein mostly B cells and T cells are responsible. Accordingly, enhancing the immune response for the treatment of any disease is crucial, but the abnormal level of immunosuppression or immunostimulation can also lead to negative effects (Jiao et al. 2014). Changes in immune response by altering, inducing or inhibiting the immune response can be termed as immunomodulation whereas the agents which modulates the immune response are termed as immunomodulators. They act by stimulating or suppressing the immune system (Mubashir et al. 2014).

Apart from the chemotherapy drugs that are used conventionally, there is an increase in demand of newer drugs with less side effects. Nanoparticles or nanomedicines when tested in vivo, has either induced immune response or has immunosuppressed when tested (Dobrovolskaia and McNeil 2007). Immunomodulation through nanoparticles can result in two sides i.e. can act as immunomodulatory agent or can result in adverse effects by altering the immune response in cancer therapy (Zogovic et al. 2009). Gold has been used as nanoparticles or as colloidal solution ever since the modern medicine has proved its valuable importance in treating arthritis and immunosuppressive disorders as it has immunomodulatory and anti-inflammatory activities (Souza-Fagundes et al. 2003,
Antibodies are produced when gold nanoparticles-antigen complex interacts with the immune cells resulting in induced immune response or immunomodulation. Gold nanoparticles can induce the phagocytic activity of macrophages as they have adjuvant like properties while colloidal gold can stimulate immune response by activating lymphocytes especially T cells (Sharma 2012). Gold nanoparticles have been used in various fields such as targeted drug delivery in cancer therapy due to their non-toxic effect on normal cells, tunable properties and biocompatibility (Cheng et al. 2008). The immunomodulating response can be affected by factors such as composition, surface properties, size, protein binding and exposure routes on entering the in vivo system (Jiao et al. 2014). Hence, synthesizing gold nanoparticles using natural medicinal plants can result in biocompatible particles which are capped by the reducing biomolecules that are present within the plant system.

For years medicinal plants have been used as immunomodulators as they are considered to possess immunomodulating properties and enhance humoral and cellular responses (Sunila and Kuttan 2004). Many medicinal plants and their isolated components are considered to possess immunomodulatory, anti-inflammatory, antioxidant and anticancer activities (Sharififar et al. 2009). *Vitis vinifera* (grapes) is a polyphenols rich fruit that are contained mainly in seeds and peels. Many studies have proven that the polyphenols from seeds and peels show antioxidant, chemoprevention, anticancer and immunomodulatory effects (Xia et al. 2010). When grape seed proanthocyanidin was given to irradiated mice, it induced immune response by showing significant hypersensitivity response and inhibited immunosuppression. Healthy individuals showed increase in T cells in their blood on consuming grape juice for ten weeks indicating the importance of fruits and vegetables in diet (Percival 2009). Various reports showed that proanthocyanidins of grape seeds showed inhibition of immune suppression, induced the production of cytokines, stimulated nonspecific immunity and activated cell and humoral mediated immune responses (Li et al. 2010, Vaid et al. 2011).
In the present study, *Vitis vinifera* seed and peel polyphenols coated gold nanoparticles as well as peel and seed aqueous extracts were tested for their immunomodulatory effects on the immune system in Swiss albino mice.
6.2 MATERIALS AND METHODS:

6.2.1 Animals

Male Swiss albino mice of 20-25 gms (6-8 weeks old) were procured from College of Veterinary and Animal Sciences, Thrissur, Kerala, India, and were kept under continuous 12-hour light and 12-hour dark cycle at 25±3ºC 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).

6.2.2 Administration of peel and seed gold nanoparticles and aqueous extracts

The Swiss albino mice were divided into five groups and studied for immunomodulatory activity on the hematological parameters and relative organ weights by administering peel and seed gold nanoparticles and aqueous extracts intraperitoneally for seven days.

6.2.2.1 Experimental design

6.2.2.2 Grouping of animals

- Group I : control (normal)
- Group II : treated with grape seed extract (2.5 mg/kg body weight)
- Group III : treated with grape peel extract (2.5 mg/kg body weight)
- Group IV : treated with grape seed gold nanoparticles (2.5 mg/kg body weight)
- Group V : treated with grape peel gold nanoparticles (2.5 mg/kg body weight)
6.2.2.3 Determination of the effect of *Vitis vinifera* peel and seed AuNPs on hematological parameters and relative organ weight

Five groups of Swiss albino mice (6 mice/group; 6–8 weeks old) were treated with doses of *Vitis vinifera* peel and seed gold nanoparticles and aqueous extracts as explained earlier. Blood was collected from the caudal vein and parameters such as total white blood cell count (WBC) was enumerated according to the method of Bain et al. (2006), Differential blood count was estimated using the method of Osim et al. (2004), hemoglobin concentration (Hb) in the blood was estimated by the cyanomethaemoglobin method of Alexander and Griffith (1993). These parameters were recorded prior to the administration of nanoparticles and extracts and continued every third day for 30 days after administration. Relative organ weights were recorded at the end of the last dose.

6.2.3 Experimental design

6.2.3.1 Grouping of animals

- **Group I**: control untreated
- **Group II**: SRBC injected- treated with grape seed extract (2.5mg/kg bodyweight)
- **Group III**: SRBC injected- treated with grape peel extract (2.5mg/kg bodyweight)
- **Group IV**: SRBC injected- treated with grape seed gold nanoparticles (2.5mg/kg bodyweight)
- **Group V**: SRBC injected- treated with grape peel gold nanoparticles (2.5 mg/kg bodyweight)

6.2.3.2 Antigen

6.2.3.2.1 Preparation of Sheep Red Blood Cells (SRBC)

Blood was collected from healthy sheep in the animal house and mixed thoroughly with sterile Alsever’s solution (1:1). It was then centrifuged at 3000 rpm for 5 min. SRBC pellets were collected after discarding the supernatant and it was washed with sterile phosphate buffer saline (PBS) (pH 7.2) for 3 times. Then SRBC pellet was collected in PBS and the SRBC’s were counted using a hemocytometer.
Therefore, 1x10^9 SRBCs (0.1ml) were injected intraperitoneally for immunizing and challenging the mice (Patel et al. 2010).

**6.2.3.3 Delayed Type Hypersensitivity (DTH) response**

Groups of six animals each from control and treated with peel and seed gold nanoparticles and extracts (i.p for 7 days) were immunized on day 0, with intraperitoneal administration of 0.1 mL of 1 x 10^9 SRBC/mice. It was then challenged by subcutaneous injection of 0.05x10^8 SRBC/ml into the right hind foot paw on 14th day while left hind footpad was kept as control and injected equal volume of saline. After 24 hrs the foot thickness volume was measured using a plethysmometer and the difference between the left and right footpad volume was calculated and expressed as percentage inhibition of paw edema (Puri et al. 1993). Percentage of paw edema inhibition was calculated by

\[
\% \text{ inhibition} = \frac{\text{Mean edema of the control} - \text{Mean edema of the treated group}}{\text{Mean edema of the control}} \times 100
\]

**6.2.3.4 Circulating antibody titre**

The groups were treated with *Vitis vinifera* gold nanoparticles and extracts for 7 days (i.p) according to the experimental design and all the animals were immunized with SRBC (1 X 10^8 cells/animal) intraperitoneally on the final day of the drug treatment. Blood was collected on every third day; Serum was separated from the blood drawn from tail vein and it was inactivated by heat at 56 °C. Antibody levels were measured by hemagglutination in a 96 well microtitre plate by adding 0.1% SRBC and serum in 1:1 ratio and kept for incubation for 1 hr. The maximum dilution that gave hemagglutination was calculated for antibody titre (Singh et al. 1984).

**6.2.4 Statistical analysis:**

Values are expressed as mean ± standard deviation (SD). Statistical analysis was analysed with 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.
6.3 RESULTS

6.3.1 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on the hematological parameters

Administration of *Vitis vinifera* peel and seed gold nanoparticles increased the total WBC count in Swiss albino mice (Fig 6.1). In the animals treated with *Vitis vinifera* seed extract the maximum WBC count was $8517\pm234.7$ cells/mm$^3$ obtained on 15th day while for *Vitis vinifera* peel extract it was $8314\pm204.2$ cells/mm$^3$. The total WBC count for *Vitis vinifera* peel AuNPs was $8676\pm212.1$ cells/mm$^3$ obtained on 15th day whereas the total WBC count for *Vitis vinifera* seed AuNPs was $9012\pm245.8$ cells/mm$^3$. Prior to the *Vitis vinifera* treatment the total WBC count for *Vitis vinifera* seed was only $6112\pm194.3$ cells/mm$^3$ and for *Vitis vinifera* peel it was $6012\pm204.2$ cells/mm$^3$. The untreated control animals had the normal total WBC count during the experimental time. *Vitis vinifera* seed and peel AuNPs treated animals exhibited significant ($p<0.05$) increase in the total WBC count between control and treated groups.

6.3.2 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on hemoglobin content

Hemoglobin content was significantly increased by the administration of *Vitis vinifera* gold nanoparticles when compared to the control which showed 14.9±0.12 gm/Hb. It was significantly ($p<0.05$) increased to 15.6±0.1 and 15.5±0.09 gm/Hb in *Vitis vinifera* seed and peel AuNPs treated group on the 12th day. *Vitis vinifera* seed and peel aqueous extracts also showed significant ($p<0.05$) increase in the haemoglobin content 15.6±0.09 and 15.4±0.11 gm/Hb on the 12th day of treatment (Fig 6.2). There was no appreciable change in the differential count and body weight after the administration of *Vitis vinifera* seed and peel gold nanoparticles.
Figure 6.1. Effect of *Vitis vinifera* seed and peel gold nanoparticles on total WBC Count. Values are expressed as mean±S.D. Significance level compared between untreated control vs treated group *p < 0.05, ‘#’ denotes non significance.

Figure 6.2. Effect of *Vitis vinifera* seed and peel gold nanoparticles on
**Hemoglobin content.** Values are expressed as mean±S.D. Significance level compared between untreated control vs treated group *p < 0.05.

Table 6.1 Effect of *Vitis vinifera* on the relative lymphoid organ weight (g/100 g body weight)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Organ</th>
<th>Weight of the organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Untreated</td>
<td>Spleen</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>0.013±0.001</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>1.76±0.03</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>0.254±0.01</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.13±0.02</td>
</tr>
<tr>
<td>Grape peel extract</td>
<td>Spleen</td>
<td>0.36±0.03*</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>0.016±0.001*</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>1.95±0.05*</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>0.254±0.02ns</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.14±0.01ns</td>
</tr>
<tr>
<td>Grape seed Extract</td>
<td>Spleen</td>
<td>0.38±0.01*</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>0.018±0.002*</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>1.98±0.08*</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>0.253±0.03ns</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.15±0.02ns</td>
</tr>
<tr>
<td>Grape peel AuNPs</td>
<td>Spleen</td>
<td>0.38±0.01*</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>0.019±0.002*</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>2.04±0.07*</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>0.253±0.02ns</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.17±0.02*</td>
</tr>
<tr>
<td>Grape seed AuNPs</td>
<td>Spleen</td>
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<tr>
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<td>Thymus</td>
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<tr>
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<td>Liver</td>
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<td>Lungs</td>
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</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.18±0.031*</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± S.D. Significance level compared between untreated control vs treated group *p < 0.05, ‘ns’ denotes non significance.
6.3.3 Effect of *Vitis vinifera* peel and seed AuNPs and extracts on relative organ weight

Table 6.1 shows the effect of *Vitis vinifera* peel and seed gold nanoparticles and aqueous extracts on the relative organ weight of liver, lungs, kidney, thymus and spleen. *Vitis vinifera* peel and seed AuNPs treated group of animals showed marked increase in the weight of the thymus, spleen and liver compared to the untreated control showing statistical significance (*p* < 0.05). Slight increase was seen in organ weight of kidney treated with peel and seed nanoparticles whereas the organ weight of lungs showed no significant change on treatment with gold nanoparticles as well as aqueous extracts. *Vitis vinifera* peel and seed extracts treatment also showed significant (*p* < 0.05) increase in the organ weights of spleen, liver and thymus compared to the control but was not significant when compared to the gold nanoparticles treated groups. *Vitis vinifera* peel and seed gold nanoparticles treated animals revealed significant increase in spleen, liver and thymus organ weights when compared to the aqueous extracts group.

6.3.4 Immunomodulatory effect of *Vitis vinifera* peel and seed AuNPs and extracts on circulating antibody titre and delayed type hypersensitivity

Evaluation on the immunostimulant activity of the AuNPs of *Vitis vinifera* seed and peel was done to check the specific immune response using sheep red blood cells (SRBC) as the antigen and circulating antibody titre and delayed type hypersensitivity were studied (Fig 6.3 and 6.4). A significant increase (*p* < 0.05) in the maximum antibody titre value of 170±2.3 and 201±2.7 was observed on 12th day in *Vitis vinifera* peel and seed AuNPs treated animals. The control animals showed the maximum antibody titre value of only 55±1.2 on the 12th day. *Vitis vinifera* peel and seed extracts treated animals showed 162±2.5 and 180±2.4 antibody titre levels. SRBC-induced delayed type hypersensitivity was used to assess the effect of the AuNPs on cell-mediated immunity. In cell mediated immune
response determination, the footpad volume after 24 h and 48 h by *Vitis vinifera* seed and peel gold nanoparticles and *Vitis vinifera* seed and peel extracts were determined. The percentage of inhibition of paw edema was found to be significantly (*p*<0.05) increased when treated with *Vitis vinifera* seed and peel gold nanoparticles with 83.2% and 87.5% inhibition when compared to control group showing 39% inhibition in 24hr treatment. *Vitis vinifera* seed and peel showed cell mediated response by inhibiting paw edema by 83.8% and 80.5% which was significant (*p*<0.05) when compared to the control. *Vitis vinifera* seed and peel gold nanoparticles exhibited more humoral and cell mediated immune response when compared to *Vitis vinifera* seed and peel aqueous extracts.
Figure 6.3 Effect of *Vitis vinifera* seed and peel gold nanoparticles on circulating antibody titre. Values are expressed in mean ± S.D. Significance level compared between untreated control vs treated group *p < 0.05

Figure 6.4 Effect of *Vitis vinifera* seed and peel gold nanoparticles on Delayed type hypersensitivity. Values are expressed in mean ± S.D. Significance level compared between untreated control vs treated group *p < 0.05
6.4 DISCUSSION

There needs to be a balance in maintaining the immunological reactions as a means of retaining a disease free state in the body. A decline in the regulation of the immune system *i.e.* between effector and regulatory cells can lead to pathogenicity. Conventional cancer therapies like chemotherapy and radiation therapy can suppress the immune system and immunity. Therefore, immunostimulating agents play an alternative vital role in the treatment of cancer by activating cells like B and T cells, NK cells, macrophages and lymphokines and preventing the growth of tumor cells. These agents act by boosting the body’s immunity and treat incapacitating health condition (Sakamaki et al. 1992, Yoon et al. 1998, Lu et al. 2007).

Immunomodulators can enhance or suppress the immune response according to their effects and these agents modulate the immune responses via humoral and cell mediated immunity which are part of the innate and adaptive immunity (Manu and Kuttan 2009). B cells which differentiates independently from the bone marrow are a part of humoral immunity by producing antibodies and other cells involves in antigen processing and immunization. The complex thus formed between the antigen and antibody can fight against infection caused by the pathogen (Han et al. 1998). Immune stimulators can also stimulate cytotoxic T cells, macrophages, natural killer cells, neutrophils and lymphocytes which are part of the cell mediated immunity. They have the ability to destroy tumor cells via producing toxins, phagocytosis and kills particular targeted cells (Han et al. 1998, Kang et al. 2001).

Nanoparticles can stimulate or suppress the immune response in the host system as they act as foreign materials. First, they can be deleterious to the health as they disturb the immune system and second, they can show immunomodulating effects by preventing diseases and thus, used as a drug in treating arthritis. Therefore, surface properties, size and other physicochemical characteristics of the nanomedicine plays a vital role on interaction with the immune cells. Surface modified gold nanoparticles can induce immunogenicity in living systems. They interact with immune cells of innate and adaptive immunity and can cause

*Vitis vinifera* peel and seed extracts are known to exhibit immunomodulating properties in various earlier studies. Polyphenols from grapes are considered to be rich antioxidants by scavenging the free radicals and also exhibited chemopreventive and protective effect on normal cells when toxicity is induced by chemotherapeutic agents treating cancers (Bagchi et al. 1997, Bagchi et al. 2001, Bagchi et al. 2003). Proanthocyanidins from grape seed showed significant stimulation of cell mediated and humoral immune responses in a immunomodulatory study by Tong et al. (2011).

Evaluation on the immunostimulant potential of the grape seed and peel AuNPs was done to evaluate the specific immune response using sheep red blood cells (SRBC) as the antigen while circulating antibody titre and Delayed type hypersensitivity were investigated. Administration of *Vitis vinifera* peel and seed AuNPs was found to increase total WBC count and haemoglobin content significantly ($p<0.05$) indicating that the gold nanoparticle were able to induce the hematopoetic system and provide immunity. The significant increase in spleen, liver and thymus when treated with the peel and seed gold nanoparticles may be due to the immunostimulatory activity of the gold nanoparticles capped with the polyphenols of *Vitis vinifera* peel and seed on the immune cells while increase in liver size did not indicate toxicity.

The peel and seed gold nanoparticles (~50 nm) at 2.5 mg/ kg body weight were found to increase the production of B cells through stimulating the immune response. The gold nanoparticles also showed significant increase in the antibody synthesis by inducing the humoral immune response. Nanoparticles also augmented the increased response of B lymphocytes in synthesizing antibodies against SRBC antigen. *Vitis vinifera* peel and seed extracts at 2.5 mg/kg body weight was also able to enhance the production of antibodies and gold nanoparticles showed enhanced synthesis of antibodies when compared to extracts treated alone groups.
Vitis vinifera peel and seed AuNPs significantly increased the cell mediated immune response by stimulating the response of sensitized T-lymphocytes. They secrete lymphokines on the site of inflammation and stimulate the infiltrating cells for its defensive mechanism (Gabhe et al. 2006, Sharififar et al. 2009). Thus, gold nanoparticles showed more stimulatory response on the impaired hypersensitivity reactions when compared to the peel and seed extracts treated group alone. The results were comparable with several other studies that have evaluated the immunomodulating potential of gold nanoparticles and plant extracts. Gold nanoparticles on formulating with alum and coated with merozoite surface protein 1 (MSP-1_{19}) was able to increase antibody production. Gold nanoparticles when capped with a polysaccharide from Tamarindus indica was found to possess immunomodulatory activities and anticancer effects which was in par with our results (Parween et al. 2011, Joseph et al. 2013).

Gold preparations at 12.5 and 25 mg/kg body weight showed increase immunological responses when compared to 50 mg/kg as it revealed suppressed level of immune activity (Bajaj et al. 1999). Vitis vinifera peel and seed extracts also showed significant immunomodulatory activity due to the presence of flavonoids, alkaloids, proanthocyanidins and resveratrol. The results were in par with other literatures which showed immunomodulatory and antitumor activity (Zhang et al. 2005, Tong et al. 2011). PEG coated gold nanoparticles were able to augment immune responses through increasing antibody production (Simpson et al. 2010) and 40 nm AuNPs were able to enhance the level of macrophages and neutrophils and was comparable to our results (Hussain et al. 2011).

Vitis vinifera peel and seed gold nanoparticles showed immunomodulatory activity in swiss albino mice when treated at 2.5 mg/kg stimulating the humoral and cell mediated immune responses. The activities can thus be attributed to the presence of capped polyphenols on the surface of the gold nanoparticles inducing immune responses. Hence the gold nanoparticles were used to evaluate the chemopreventive potential of Vitis vinifera peel and seed gold nanoparticles in DMBA induced skin cancer mice.