CHAPTER 3

PHYTOCHEMICAL ANALYSIS AND IN VITRO ANTIOXIDANT ACTIVITIES OF VITIS VINIFERA PEEL AND SEED AQUEOUS EXTRACT

3.1 INTRODUCTION

In cellular degeneration, free radicals seems to be an important factor involved in aging process. Hydrogen peroxide, nitric oxide, hydroxyl radicals, superoxide anion and peroxynitrate are chemical components of reactive oxygen species (ROS) that are implicated over many diseases in humans (Bagchi et al. 2000, Nohl et al. 2005). These harmful oxygen radicals accumulates during aging process and causes cellular changes that results in loss of organ function and homeostatic control (Harman 1981). Many disease conditions in humans are owing to "oxidative stress" that are due to the disparity between the capacity of the antioxidant defense and the production of reactive oxygen species resulting in damage to cell constituents such as lipids, DNA and proteins (Halliwell 1991). During continued or severe oxidative stress, antioxidants from external sources are required continuously to maintain sufficient level of antioxidants to balance free radicals (Li et al. 2008). According to several studies, the risk factor for incidence of cancer and other degenerative diseases are due to non-healthy diet (Lampe 1999). Increased consumption of vegetables and fruits contributes to well-being and improved health. Vegetables and fruits are the best sources of a number of nutrients and can improve antioxidant status (Mulholland et al. 1996, Borek 2006). Depletion of antioxidants can occur due to diminished intake of antioxidants, environmental pollution, limited absorption of these antioxidants and many other reasons. Therefore, health implication of antioxidants and substitution of synthetic antioxidants by natural antioxidants has fostered the growing interest on vegetables and fruits sources (Žitňanová et al. 2006) and to screen for identifying antioxidants.
Phenolics are main secondary metabolites of plants possessing many biological effects including antioxidant, anti-cancer, anti-allergic, anti-inflammatory and anti-microbial activity (de Sousa et al. 2007, Kähkönen et al. 2009). Phenolic phytochemicals, vitamin E and C are naturally occurring antioxidants which act as free radical scavengers and are known to function as chemopreventive agents against oxidative stress mediated disease (Takao et al. 1994, Wang et al. 1996). Compounds that delay or inhibit oxidation of other molecules by inhibiting the initiation of oxidizing chain reactions are called as antioxidants. Natural antioxidants can be flavonoids, tocopherols, phenolic acids, chlorophyll derivatives, alkaloids, amino acids, ascorbic acids as well as carotenoids (Larson 1988, Hudson 1990, Hall and Cuppett 1997). Several literature has showed the ability of phenolic compounds to act as antioxidants and they exhibit biological functions such as anti-carcinogenicity, anti-mutagenicity and antiaging (Huang et al. 1992, Cook and Samman 1996).

The main sources of exogenous antioxidants comes from food consumption, thereby, fruits, vegetables, cereals, legumes, teas and other food products are rich in antioxidants. A recent study has shown that antioxidants are higher in plant based products than animal or mixed food product. Hence, fruits, herbs and food from plant sources can be important contributors for antioxidant intake (Carlsen et al. 2010). Antioxidants such as catechins, anthocyanins, beta-carotene, flavonoids, lutein, lipoic acid, lycopene, vitamins C and E are abundant in fruits and vegetables and numerous antioxidants are available as dietary supplements (Franco et al. 2008, Lin et al. 2009, Chun et al. 2010, Jerome-Morais et al. 2011). Many research studies have identified wide array of plants like *Allium cepa*, *Aegle marmelos*, *Allium sativum*, *Aloe vera*, *Azadirachta indica*, *Curcuma longa*, *Ocimum sanctum*, *Withania somnifera*, *Sygium cumini*, *Piper nigrum*, *Zingiber officinalis* with antioxidant activity which exerts antioxidant mechanism (Devasagayam et al. 2004, Gupta et al. 2006, Kratchanova et al. 2010, Narendhirakannan and Subramanian 2010).
Grapes (Vitis vinifera L.) are considered the most prevalent fruit crop and one of the widely grown fruit throughout the world. Several studies have reported their composition, properties and presence of phenolic compounds (Oszmianski and Lee 1990, Macheix et al. 1990). The phenolic substances mainly reside in seeds and peels of grape berries. In grape peels, flavonols are the most abundant phenolic compounds while monomeric phenolic compounds, such as (-)-epicatechin, (+)-catechins, (-)-epicatechin-3-O-gallate, dimeric, trimeric and tetramerich procyanidins are rich in grape seeds. Grape seeds contain 40% fiber, 16% essential oil, 11% protein, 7% complex phenolic compounds like sugars, tannins, minerals and other constituents (de Campos et al. 2008) and these compounds act as antiviral and antimutagenic agents (Kammerer et al. 2004, Rodriguez et al. 2006). Grape seed extract has high content of proanthocyanidins (OPCs), made up of proanthocyanidin monomers and they represent flavanol-3-ol such as epicatechin and catechin. Grape seed proanthocyanidins are antioxidants and they exert effects such as chemoprevention, anti-tumor, anti-cancer and inhibitory effects against hypercholesterolemia and atherosclerosis (Bagchi et al. 2000, Carini et al. 2000, Peng et al. 2001). They can protect cells from free radicals and oxidative stress and they have significantly higher antioxidant activity than vitamin C and E (Dulundu et al. 2007). Recent studies have shown that proanthocyanidins can inhibit carrageenin-induced inflammation, improves visual performance in humans, stabilizes the capillary wall, and also it has shown many other valuable health benefits (Facino et al. 1996, Zafirov et al. 1990, Corbe et al. 1988, Halpern et al. 1998).

The aim of the present study was to investigate the qualitative and quantitative analysis of the phytochemicals and its relative in vitro anti-oxidative properties of seed and peel aqueous extracts of purple black Vitis vinifera.
3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Folin-Ciocalteu’s phenolic reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide and ascorbic acid were obtained from Sigma, India. Other reagents and fine chemicals were obtained from SRL chemicals, Mumbai, India.

3.2.2 Collection of *Vitis vinifera* seeds and peels

For the present study, samples of *Vitis vinifera* L. (Muscat family of grapes) were collected from the farms of Grape growers association, Thondamoothur, Coimbatore, Tamil Nadu, India. The plant sample was authenticated BSI/SRC/5/23/2014-15/Tech.63 at Botanical Survey of India (BSI), Tamil Nadu Agricultural University campus (TNAU), Coimbatore, Tamil Nadu, India.

*Vitis vinifera*, uninfected and germ-free berries were collected at their fruit maturity and approximately 500g berries were arbitrarily cut from bunches. They were washed with Milli Q water to remove pesticide residues. The berries were finger pressed to remove pulp and juice. Grape seeds were separated from peel and pulp; peel was removed from the rest of the grape manually. The remaining traces of pulp were washed with distilled water several times. Then the seeds and peels were dried in oven at 50°C for 72 hrs. The dried seeds and peels were pulverized to fine powder using a sterile electric blender and stored for further analysis.

3.2.3 Preparation of aqueous extracts from *Vitis vinifera* peels and seeds

10 g each of grape seed and peel powder were taken separately and boiled in 200mL of distilled water and were further heated at 60-70°C to get a concentrated solution. The extract was filtered using muslin cloth and then by Whatman no 1 filter paper. The extract was then concentrated using rotary vacuum evaporator; residues were collected, dried and used for experiment. The extract obtained was then evaluated for the quantitative and qualitative phytochemical screening and studied for their *in vitro* antioxidant activities.
3.2.4 Phytochemical screening of *Vitis vinifera* seed and peel extracts

3.2.4.1 Qualitative analysis of phytochemicals (Harborne 1998, Kokate 2001)

The aqueous extracts were screened for their phytoconstituents and the following tests to analyse the presence of phytochemicals were done.

3.2.4.1.1 Test for alkaloids (Mayer’s test): 2 mL of HCl was added to 5 mL of the extract and to this acidic solution, 1 mL of dragendorff’s reagent was added. The presence of alkaloids is confirmed by the formation of orange or red precipitate immediately.

3.2.4.1.2 Test for flavonoids (Alkaline reagent test): Few drops of dilute NaOH was added to 1 mL of the extract. An intense yellow color was produced which turns colourless on addition of few drops of dilute acid which indicates the presence of flavonoids.

3.2.4.1.3 Test for carbohydrates (Molisch’s test): 2 to 3 drops of 1% alcoholic α-napthol solution was added to 2 mL extract and 2 mL of Conc. H₂SO₄ was added along the sides of the test tubes. At the junction of two liquids a brown ring is formed which confirms the presence of carbohydrates.

3.2.4.1.4 Test for glycosides (Legals test): Few drops of HCl is added to the extract to hydrolyse it for few hours in water bath. To the hydrolysate, few drops of sodium nitroprusside solution and 1 mL of pyridine was added and then NaOH is added to make the solution alkaline. Pink or red color appearance shows the presence of glycosides.

3.2.4.1.5 Test for saponins: 20 mL of distilled water was added to the extract to dilute it and agitated for 15 mins in a graduated cylinder. Formation of foam of 1 cm layer shows the presence of saponins.

3.2.4.1.6 Test for tannins: A few drops of 1% lead acetate is added to 2 mL of the extract which shows the formation of yellow precipitate indicating the presence of tannins.
3.2.4.1.7 **Test for amino acids** (Ninhydrin test): Appearance of purple color indicates the presence of amino acids when few drops of ninhydrin reagent was added to 1 mL of the extract.

3.2.4.1.8 **Test for proteins** (Biuret test): To 2 mL of the extract, equal volumes of copper sulphate and 5% NaOH solution were added. Appearance of pink color confirms the presence of proteins.

3.2.4.1.9 **Test for phytosterol** (Salkowski test): Solution of alcoholic potassium hydroxide was added to the extract for refluxing until complete saponification takes place. The mixture was extracted with ether and the ether layer was evaporated to get the residue. It was then tested for the presence of phytosterol. To the residue, few drops of diluted acetic acid and 3 mL of acetic anhydride was added; then followed by few drops of Conc. H₂SO₄. A bluish green color appearance show the presence of phytosterol.

3.2.4.1.10. **Test for triterpenoids** (Liebermann Burchard test): To 10 mg of the extract, 1 mL of chloroform was added to dissolve and then 1 mL of acetic anhydride was added followed by addition of 2 mL of Conc. H₂SO₄. Presence of triterpenoids was indicated by the formation of reddish violet color.

3.2.4.2 **Quantitative analysis of phytochemicals**

The total phenol content was determined using Folin-ciocalteau reagent (Singleton et al. 1999) and the total flavonoid content was determined using aluminium chloride method (Zhishen et al. 1999). Estimation of Ascorbic acid was performed according to the method of Sadasivam and Manickam (1987). Estimation of total carbohydrates was performed by following the method of Yemm and Willis (1954). Estimation of protein in seed and peel was done by the method of Bradford (1976).
3.2.5 *In vitro* Antioxidant Activity

### 3.2.5.1 DPPH scavenging assay

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the aqueous extracts in ethanol. Different aliquots of aqueous extract of *Vitis vinifera* peel and seed (10-50 µg/mL) were diluted with ethanol and to this, 3 mL of DPPH (4 mg dissolved in 100 mL ethanol) and 500 µL of 0.05M Tris-Hcl solution (pH 7.3) was added. The reaction mixture was mixed thoroughly and incubated in the dark for 25 mins. After the incubation time, the decrease in the absorbance was measured at 517 nm and ethanol was kept as blank whereas DPPH solution was kept as control. Lower absorbance of the reaction mixture indicates higher free radical-scavenging activity. Positive control was prepared by replacing ascorbic acid (Vitamin C) for the extract and under same conditions. The experiments were carried out in triplicates and calculated for the percentage inhibition using the equation.

\[
\text{Effect of scavenging (\%)} = \frac{A_{\text{control}} - A_{\text{sample}} (517\text{nm})}{A_{\text{control}} (517\text{nm})} \times 100
\]

Where \(A_{\text{control}}\) was calculated as the initial absorbance of DPPH with extract in ethanol. \(IC_{50}\) values were calculated according to the samples showing 50% inhibition (\(IC_{50}\)) from the graph plotting inhibition percentage against extract concentration.

### 3.2.5.2 Hydrogen peroxide scavenging assay (Gülcin et al. 2006)

The ability of aqueous extracts to scavenge hydrogen peroxide radicals were determined with a little modification. Hydrogen peroxide solution (2 mM/L) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the aqueous extract of *Vitis vinifera* peel and seed were mixed with 600 µL of hydrogen peroxide (H\(_2\)O\(_2\)) solution which was prepared by dissolving H\(_2\)O\(_2\) solution (2 mM) in phosphate buffer (0.05 M) (pH 7.3). After 10 mins of incubation, decrease in the H\(_2\)O\(_2\) concentration was measured at 230 nm. Phosphate buffer solution without H\(_2\)O\(_2\) was kept as blank while positive control was prepared by adding different concentrations of ascorbic acid along with 400 µL of phosphate buffer and 600 µL
of H₂O₂ solution and measured at 230 nm. The percentage inhibition of H₂O₂ by the extracts were calculated by

\[
\% \text{ Radical scavenged} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where, A₁ is the absorbance of extract or standard and A₀ is the absorbance of the control.

3.2.5.3 Assay of Reducing Power (Koleva et al. 2002)

Increasing concentrations of \textit{Vitis vinifera} peel and seed extracts (10-50 µg/mL) were added with 2 mL of phosphate buffer (200 mM) (pH 6.5) and 2 mL of potassium ferricyanide (1%), vortexed and incubated for 15 mins at 50º C. After 15 mins, 2 mL of trichloroacetic acid (1 g/10 mL) was then mixed with the mixtures and centrifuged at 4000 rpm for 15 mins. 2 mL of the obtained supernatant was added to 2 mL of distilled water and 500 µL of ferric chloride solution; then vortexed thoroughly. Absorbance at 700 nm was measured by keeping phosphate buffer as blank and ascorbic acid as positive control. Increased absorbance were observed with more reducing power of the extracts. The experiments were carried out in triplicate.

3.2.5.4 Total Antioxidant Capacity (Preito et al. 1999)

Known concentrations (10-50 µg/mL) of \textit{Vitis vinifera} peel and seed extracts were added with 2 mL of solution containing 600 mM of sulphuric acid, 0.028 M of sodium phosphate and 5 mM of ammonium molybdate in a test tube. It was then vortexed and incubated for 95 mins at 90º C. The absorbance was measured at 695 nm with ascorbic acid as positive control and solution without extracts are treated as blank. Increased antioxidant activity is indicated by increased absorbance of the extracts. The total antioxidant capacity is expressed as equivalents of ascorbic acid in mg per grams of the extract and the experiments were conducted in triplicates.
3.2.6 Gas Chromatography Mass Spectroscopy (GCMS) analysis

_Vitis vinifera_ peel and seed extracts were analysed for compounds using GCMS at the South India Textile Research Association (SITRA), Coimbatore, Tamil Nadu, India. The analysis was done by using Thermo GC Trace Ultra Version 5.0 equipment (Thermo Fisher Scientific Limited, India) equipped with DB-5MS non-polar silica capillary column (0.25 mm outside diameter x 0.25 µm internal diameter x 30 m length) and GC interfaced to a Mass Selective Detector Thermo MS-DSQ-II equipment was used with run time of 35:31 mins. Electron ionization system with ionization energy of -70eV was used for mass spectra of compounds in samples. Injector port temperature was set at 250 °C and Ion source temperature was set at 200 °C with interface temperature temperature kept at 250 °C. The temperature of the oven was set as 70 °C for 2 mins to 260 °C at 10 °C per min. The carrier gas used was helium at 1 mL/min. The compounds are identified based on the molecular mass, molecular structure and calculated fragmentations. The standard mass spectral database of National Institute Standard and Technology (NIST4) and WILEY9 were used for interpretation of mass spectrum and it was compared with the spectrum of known component (Dool and Kratz 1963).

3.2.7 Statistical analysis

All the results were expressed as mean ± S.D. for three experiments in each. The results are given as mean ± standard deviation (SD). Student’s t-test was used for comparison between the means of samples and standards.
3.3 RESULTS

3.3.1 Collection and processing of *Vitis vinifera* seeds and peels

The present study for phytochemical analysis and *in vitro* antioxidant activities were analysed on *Vitis vinifera* seed and peel aqueous extracts (Fig 3.1 and 3.2). The yield of the aqueous extracts for *Vitis vinifera* seed and peel extracts were 10.8% and 9.9%.

Fig 3.1 Collection and separation of *Vitis vinifera* peels and seeds

Fig 3.2 Aqueous extracts of *Vitis vinifera* peels and seeds
3.3.2 Qualitative analysis of phytochemicals

The results of qualitative phytochemical analysis of *Vitis vinifera* peel and seed aqueous extracts showed the presence of alkaloids, flavonoids, glycosides, saponins, tannins, carbohydrates, proteins, aminoacids, phytosterol and triterpenoids (Table 3.1).

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Grape peel</th>
<th>Grape Seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phytosterol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

‘+’ – Presence
3.3.3 Quantitative estimation of phytochemicals

Total phenolic content, flavonoid content, protein and ascorbic acid content were determined and the results are shown in Table 3.2. Quantitative estimation showed high amount of phenols, flavonoids, protein and ascorbic acid in *Vitis vinifera* seed extracts compared to *Vitis vinifera* seed extracts (Table 3.2). Grape seeds contained 18.5% of carbohydrates whereas peels had 16.2%. From these results it was also observed that grape seeds had high amount of phenols followed by grape peels. Similarly, grape seed extract had more flavonoids when compared with peel extract. Ascorbic acid content was comparatively higher in seeds than in peels.

3.3.4 GCMS analysis of *Vitis vinifera* seeds and peels extracts

Various compounds present in the *Vitis vinifera* seed and peel extracts were identified through GCMS analysis. The identification of some of the important phytoconstituents were based on the peak area, retention time and molecular weight. Major and pharmaceutically important compounds from the GC fractions of *Vitis vinifera* seed and peel aqueous extracts were identified through GC-MS analysis. Various compounds present in aqueous extracts of grape peel and seed were listed with their pharmacological activity (Table 3.3 and 3.4) and Fig 3.3 and 3.4 represents the chromatogram of aqueous extracts of *Vitis vinifera* peel and seed.
Table 3.2 Quantitative analysis of phytochemicals in *Vitis vinifera* seed and peel extract

<table>
<thead>
<tr>
<th>Extract</th>
<th>Phenols (mg gallic acid equivalent/g dry material)</th>
<th>Carbohydrates (% of dry weight)</th>
<th>Ascorbic acid (mg/100g)</th>
<th>Flavonoids (mg catechin equivalent/g dry material)</th>
<th>Protein (% of dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grape seed</td>
<td>196±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.5</td>
<td>2.4±0.22</td>
<td>96±4.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82</td>
</tr>
<tr>
<td>Grape peel</td>
<td>150±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.2</td>
<td>1.1±0.09</td>
<td>84±3.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.57</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total phenolics is expressed as gallic acid equivalent (GAE) and values are expressed as mean ± standard deviation (n = 3);
<sup>b</sup> The level of total flavonoids is expressed as catechin equivalent (CE) and values are expressed as mean ± standard deviation (n = 3); Values are expressed as Mean ±SD, n=3
Figure 3.3 GCMS chromatogram of *Vitis vinifera* seed extract

Figure 3.4 GCMS chromatogram of *Vitis vinifera* peel extract
### Table 3.3 GCMS analytical report for major phytoconstituents present in *Vitis vinifera* seed extract

<table>
<thead>
<tr>
<th>RT</th>
<th>Name of the Compound</th>
<th>Molecular formula</th>
<th>Molecular Weight</th>
<th>Peak Area (%)</th>
<th>Structure</th>
<th>Pharmacological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.61</td>
<td>Dibutyl phthalate</td>
<td>C_{16}H_{22}O_{4}</td>
<td>278</td>
<td>23.74</td>
<td><img src="structure1.png" alt="Structure" /></td>
<td>Antifungal, Antimicrobial agent, antimalarial and antifungal (Elija et al. 2012)</td>
</tr>
<tr>
<td>24.58</td>
<td>9-Octadecenoic acid (Z)-, methyl ester</td>
<td>C_{19}H_{36}O_{2}</td>
<td>296</td>
<td>0.33</td>
<td><img src="structure2.png" alt="Structure" /></td>
<td>Antioxidant activity, exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligand (Hema et al. 2011). Anticarcinogenic activity (Yeong et al. 1989)</td>
</tr>
<tr>
<td>13.44</td>
<td>Octadecanoic acid</td>
<td>C_{18}H_{36}O_{2}</td>
<td>284</td>
<td>0.24</td>
<td><img src="structure3.png" alt="Structure" /></td>
<td>Antifungal, antitumor activity, antibacterial (Hsouna et al. 2011)</td>
</tr>
<tr>
<td>21.48</td>
<td>2-[4-(2-methylpropyl) phenyl]propanoic acid</td>
<td>C_{13}H_{18}O_{2}</td>
<td>206</td>
<td>9.42</td>
<td><img src="structure4.png" alt="Structure" /></td>
<td>Anti-inflammatory agent used in the therapy of rheumatism and arthritis, Analgesic, antipyretic, and platelet-inhibitors. Otherwise called as ibuprofen. (Bradley et al. 1991)</td>
</tr>
<tr>
<td>22.37</td>
<td>1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester</td>
<td>C_{20}H_{36}O_{4}</td>
<td>334</td>
<td>37.43</td>
<td><img src="structure5.png" alt="Structure" /></td>
<td>Antineoplastic Agents, Antiviral Agents (otherwise called as delta-12-Prostaglandin J2). (Kato et al. 1986)</td>
</tr>
<tr>
<td>22.86</td>
<td>2,6-diter- butyl-4-methylphenol</td>
<td>C_{15}H_{24}O</td>
<td>220</td>
<td>3.25</td>
<td><img src="structure6.png" alt="Structure" /></td>
<td>Antioxidant activity, anti-viral effects against herpes family viruses. (Babu and Wu 1998)</td>
</tr>
</tbody>
</table>
Table 3.4 GCMS analytical report for major phytoconstituents present in *Vitis vinifera* peel extract

<table>
<thead>
<tr>
<th>RT</th>
<th>Name of the Compound</th>
<th>Molecular formula</th>
<th>Molecular Weight</th>
<th>Peak Area (%)</th>
<th>Structure</th>
<th>Pharmacological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.06</td>
<td>2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one</td>
<td>C₆H₈O₄</td>
<td>144</td>
<td>4.11</td>
<td><img src="image1" alt="Structure" /></td>
<td>Antimutagenic activity, Anti-proliferate and pro-apoptotic effects, anticancer and antioxidant activities (Yumiko et al. 1998)</td>
</tr>
<tr>
<td>9.70</td>
<td>2-Furancarboxaldehyde, 5-(hydroxymethyl)-(CAS)</td>
<td>C₆H₆O₃</td>
<td>126</td>
<td>18.10</td>
<td><img src="image2" alt="Structure" /></td>
<td>Inhibitor of adipogenesis and enhancer of osteoblastogenesis. Antioxidant and anti-proliferative activities. (Zhao et al. 2013)</td>
</tr>
<tr>
<td>22.66</td>
<td>ethyl stearate</td>
<td>C₂₀H₄₀O₂</td>
<td>312</td>
<td>11.92</td>
<td><img src="image4" alt="Structure" /></td>
<td>Anticancer activities (Mustafa et al. 2004)</td>
</tr>
<tr>
<td>25.64</td>
<td>Linoleic acid ethyl ester</td>
<td>C₂₀H₃₆O₂</td>
<td>308</td>
<td>8.70</td>
<td><img src="image5" alt="Structure" /></td>
<td>Attenuates lipopolysaccharide-induced pro-inflammatory cytokine production, Antioxidant and anti-inflammatory activity. (Chan et al. 2013, Park et al. 2014)</td>
</tr>
<tr>
<td>32.56</td>
<td>Hexadecanoic acid, ethyl ester (CAS)</td>
<td>C₁₈H₃₆O₂</td>
<td>284</td>
<td>2.36</td>
<td><img src="image6" alt="Structure" /></td>
<td>Anti-inflammatory activity. (Saeed et al. 2012) Antioxidant, Hypcholesterolemic, Nematicide, Pesticide, Lubricant, Antiandrogenic, Flavor. (Hema et al. 2011)</td>
</tr>
</tbody>
</table>
3.3.5 *In vitro* antioxidant activities of *Vitis vinifera* seed and peel extracts

Fig 3.5 depicts the hydrogen peroxide scavenging activity of *Vitis vinifera* seed and peel extracts. Hydroxyl radicals were scavenged more at higher concentrations of the extracts and showed more percentage of inhibition. The IC$_{50}$ values were recorded to be 25.48 µg/ml for grape peel extract and 28.38 µg/ml for grape seed extract when related to ascorbic acid as standard showing 21.18 µg/ml. Grape peel extract showed more inhibition of hydroxyl radicals than seed extracts. Fig 3.6 shows the free radicals scavenging activity of *Vitis vinifera* seed and peel extracts. Peel and seed extracts possess antioxidant which can scavenge DPPH, a stable radical that changes them to a colorless product. With the increase in concentration, the absorbance decreased which confirmed the scavenging capacity. In the present study, the grape seed extracts exhibited appreciable scavenging activity when compared with grape peel extracts. The IC$_{50}$ values of grape peel extract was 21.11 µg/ml and for grape seed extract was 19.42 µg/ml when compared to ascorbic acid having 11.19 µg/ml.

Fig 3.7 represents the total antioxidant capacity of *Vitis vinifera* peel and seed extracts. Total antioxidant capacity depends on the conversion of Mo (VI) to Mo (V) by the peel and seed extracts and thus green phosphate/Mo (V) complex is formed at acid pH. The results showed higher total antioxidant capacity (expressed as equivalents of ascorbic acid) of the extracts is evident even at low concentrations and possess antioxidant capacity equivalent to ascorbic acid standard. The total antioxidant capacity of *Vitis vinifera* peel and seed extracts were 76.24 µg/mL and 78.86 µg/mL ascorbic acid equivalents at 49.5 µg/mL extract concentrations. Fig 3.8 elucidates the reducing capabilities of *Vitis vinifera* seed and peel extracts as the concentration increases. The extracts were able to reduce Fe$^{3+}$ to Fe$^{2+}$ confirming the presence of reductones having antioxidant activity. Grape seed extracts showed more reducing power compared to grape peel extracts which were equivalent to ascorbic acid standard. The reducing power of *Vitis vinifera* seed and peel extracts ranged from 0.254 and 0.159 absorbance value for 50 µg/mL of extracts when compared to 0.272 absorbance for ascorbic acid.
Figure 3.5 Free radical scavenging effect of *Vitis vinifera* seed and peel extracts on H$_2$O$_2$ radicals. Values are expressed as Mean ±SD, n=3

Figure 3.6 Scavenging effect of *Vitis vinifera* seed and peel extracts on DPPH free radicals. Values are expressed as Mean ±SD, n=3
Figure 3.7 Effect of *Vitis vinifera* seed and peel extracts on total antioxidant activity. Values are expressed as Mean ±SD, n=3.

Figure 3.8 Reducing power assay of *Vitis vinifera* seed and peel extracts. Values are expressed as Mean ±SD, n=3.
3.4 DISCUSSION

Plant phenolics are secondary metabolites that are major group of compounds having free radical scavenging potential and this medicinal values of plants have reached an important dimension in the past few decades (Žitňanová et al. 2006, Afaq and Katiyar 2011). Increased attention has been paid to phytochemicals because of their biological activities (Cho et al. 2003). The qualitative analysis of grape seed and peel extracts revealed the presence of alkaloids, flavonoids, carbohydrates, saponins, tannins, triterpenoids, glycosides and phytosterols. The phytochemicals such as alkaloids, flavonoids, saponins and tannins are implicated as important bioactive agents and might be involved in the therapeutic use of *Vitis vinifera*. According to previous reports, phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids has showed significant anti-inflammatory effects (Manach et al. 1996, Orhan et al. 1997, Akindele and Adeyemi 2007) while glycosides, flavonoids, tannins and alkaloids showed antihyperglycemic activities (Oliver 1980, Cherian and Augusti 1995) and steroids and triterpenoids had shown analgesic properties (Rupasinghe et al. 2003).

Flavonoids are free radical scavenger and potent water soluble antioxidant and shows strong anticancer activity. Terpenoids are known to possess immunomodulatory, anti-cancer, anti-inflammatory, anti-viral and antimicrobial properties. Steroids help in reducing cholesterol levels and it regulates the immune response (Salah et al. 1995, Rio et al. 1997, Wagner and Elmadfa 2003, Sultana et al. 2008, Rabi and Bishayee 2009). Plants having more glycosides and carbohydrates are known to exert beneficial action by increasing strength and hence boosting immune system (Theis and Lerdau 2003). Proteins and carbohydrates are present more in grape seed than in grape skin wherein proteins are the building blocks of life and our body needs proteins for repair and to maintain itself (Ojala et al. 2000).

High phenolic contents in *Vitis vinifera* peel and seed aqueous extracts indicated high antioxidant activity of the extracts as it was able to react with active
oxygen radicals such as DPPH radicals (Cho et al. 2003), hydrogen peroxide radicals, hydroxyl radical (Husain et al. 1987) and superoxide radical (Afanaslev et al. 1989). In the present study, grape seed extracts revealed the presence of more amount of phenols and flavonoids compared to grape peel extracts. Various other studies shows correlation between high phenolic content and more antioxidant activity. Free radicals have been involved in the pathogenesis of many diseases and our body has number of mechanisms to minimize free radical induced damage. Normally polyphenols, vitamins A, E and vitamin C act as antioxidants and play a key role in defense mechanism (Aruoma 2003, Dasgupta and De 2004, Bahar et al. 2013). Total flavonoids and phenolics possess a broad spectrum of biological and chemical activities including radical scavenging properties and phenolic compounds are powerful chain breaking antioxidants and the activity is due to the presence of hydroxyl group (Hatano et al. 1989, Shahidi and Wanasundara 1992, Miliauskas et al. 2004)

The mechanism involved for oxidation is proton radical-scavenging action. *Vitis vinifera* peel and seed extracts were able to scavenge radicals due to its proton donating ability. The in vitro antioxidant activities of grape seed and peel extracts were assessed by antioxidant assays like scavenging assay of DPPH radicals, total antioxidant capacity, assay of scavenging hydrogen peroxide and assay of reducing capacity. The free radical scavenging activity of grape seed and peel extracts were determined by the DPPH method. Antioxidant molecules can quench DPPH free radicals and convert them to a colourless product, resulting in a decrease in absorbance at 517 nm (Arokiyaraj et al. 2008). DPPH is a proton, free radical and its purple color fades when it encounters radical scavenger suggesting antioxidant activity of the peel and seed extracts even at 10 µg/mL (Yamagushi et al. 1998, Chang et al. 2002). This suggests that the phenolic content of *Vitis vinifera* seed and peel extract contributed to scavenging of free radicals. In the present study, the grape seed extracts exhibited appreciable free radical scavenging activity compared to grape peel extracts. The results were similar with the studies of Li et al. (2008)
showing appreciable scavenging activity of Cabernet Sauvignon *Vitis vinifera* seed extracts at 3.35 mg/mL and the activity was dependent on the amount of polyphenols present in the extract. Red grape cultivar Shiraz showed significant DPPH radical scavenging effect from 0.47 to 2.10 µg/mL and this activity is attributed to the presence of high phenols, flavonoids and anthocyanin content in peel and seed extracts which were par with the present study (Butkhup et al. 2010).

The present study indicated that the total antioxidant capacity of the grape seed and peel extracts were high even at low concentrations of the extracts. Total antioxidant capacity is determined from both fat-soluble and water soluble antioxidant capacity. Therefore, it is known that *Vitis vinifera* seed and peel extracts have as much quantity of antioxidant compounds as equivalents of ascorbic acid that effectively reduce the oxidant in the reaction mixture. Banerjee and Narendhirakannan (2011) had evaluated the total antioxidant potential of *Syzygium cumini* seed which showed high antioxidant capacity at 100 µg/mL compared to ascorbic acid standard.

The reducing power depends on the antioxidant capacity to reduce the free radicals by sharing hydrogen atoms. Studies have proven the direct association of presence of polyphenols, its antioxidant activity and reducing power. The reducing power is largely related to the presence of reductones in inhibiting the formation of free radicals (Gordon 1990). Aliyu et al. 2013 have obtained similar reducing power capacity with the roots of *Anchomanes difformis* extracts which exhibited higher absorbance even at lower concentrations of the root extracts acting as reducing agent and scavenging single oxygen radical. The extracts were able to transform Fe³⁺ to Fe²⁺ showing reductive ability and reducing properties that are generally attributed to the presence of reductones (Oyaizu 1986, Gordon 1990, Duh et al. 1999). The reducing capacity of the extracts may serve as indicator of its significant antioxidant activity.

The antioxidants has the capacity to reduce the hydrogen peroxide radicals and this method can be utilized to quantify the H₂O₂ scavenging activity of the
medicinal plant extracts. Increased amount of hydrogen peroxide can cause damage to the cells as it is converted to hydroxyl radical and H$_2$O$_2$ alone is not reactive. Many studies have experimented the correlation between the presence of high amount of phenols and flavonoids to the high free radicals scavenging and antioxidant activities (Czochra and Widensk 2002, Gulcin 2006, Naskar et al. 2011). Another study by Khan et al. 2012 have reported that extracts of *Sonchus asper* have exhibited substantial hydroxyl radical scavenging activity at 67 μg/mL of extract.

Grape peels and seeds are good sources of phytochemicals such as catechin, epicatechin and gallic acid for the production of antioxidant dietary supplements. According to other literatures, antioxidant properties and phenolic profile of Cabernet Franc clone1, Norton (*Vitis aestivalis*) and Cabernet Franc clone 313 (*Vitis vinifera*) grape were evaluated and their antioxidant activities were assessed. Cabernet Franc clone1 showed strongest DPPH radicals scavenging activity (8.8 μmol TE/g) while Cabernet Franc clone313 grape extracts showed activity at 5.4 μmol TE/g with Norton at 7.9 μmol TE/g. Higher flavonoid content, total phenolic and anthocyanins were found in Norton grape than Cabernet Franc grapes. Gallic acid was the major phenolic acids in all the grape varieties (Shelly et al. 2009). Highest content of total phenolics (TP), total flavonoids (TF) and total antioxidant activity has been found in Medas beli variety. TP and TF contents were 238.3 to 420.6 mg gallic acid equivalent per litre of wines and they found high correlation between antioxidant and total phenolic content in white wines (Mitić et al. 2010). Bartolome et al. (2004) investigated the *in vitro* antioxidant activity of red grapes (*Vitis vinifera*) peels. They showed that radical scavenging activity against DPPH was very high (3.2–11.1 mg dried skin/mg DPPH) in correlation to other foodstuffs and this can be influenced by grape variety, vintage and grape ripening. They also showed that grape peels in wines have high antioxidant potential and they found significant correlation between grape phenolic content and antioxidant activity.
Butkhup et al. (2010) has determined the TP, TF and total monomeric anthocyanin content in red grape cultivar Shiraz seed, skin and whole grape extract. They evaluated the total phenolics to be 48.04 to 116.73 g GAE/100g db (dry weight base), total flavonoids to be 74.82 to 258.69 mg CE/g db, and anthocyanin content to be 5.06 to 55.45 mg/100g db. Catechin, epicatechin and gallic acid contents were 52.30–231.92 mg/100g db, 27.92–174.10 mg/100g db and 4.42–27.80 mg/100g db, flavonols that were present in grape seed. DPPH radical-scavenging assay showed significant activity ranging from 0.47 to 2.10 µg/mL. Guendez et al. 2005 found that there is a significant correlation between DPPH scavenging activities of grape seed extracts and total phenolic content. Many previous studies have proven the antioxidant activity of grape cultivars particularly in seeds and peels (Rababah et al. 2008, Li et al. 2008, Choi and Lee 2009, Chedea et al. 2010 and Sharl et al. 2010).

Alcohols, esters, acids, long chain and branched chain hydrocarbons and volatile matter constituents can be identified by GCMS analysis. Qualitative analysis with more precise information can be obtained by gas chromatography coupled with mass spectroscopy (Zhang et al. 2007). The GC-MS analysis of aqueous extracts of *Vitis vinifera* seed and peel extracts revealed the presence of various secondary metabolites. The presence of these compounds exhibited different pharmacological activities like anti-cancer, anti-proliferative, antioxidant, anti-inflammatory, anti-microbial, antimutagenic and anti-viral activities.

The present study suggests that the aqueous extracts of *Vitis vinifera* seed and peel has potent *in vitro* antioxidant activities. Most of the biologically active phytochemicals such as flavonoids, tannins, alkaloids, triterpenoids, phytosterols and glycosides were present in the aqueous extracts of *Vitis vinifera* seed and peel and the antioxidant properties may be attributed to the presence of these polyphenols. High amounts of phenols, ascorbic acid and flavonoids present in seeds and peels may also be responsible for the *in vitro* antioxidant activity and thus they can be used as a free radical scavengers to protect against various damages.
initiated by free radicals. *Vitis vinifera* seed aqueous extract showed high amount of polyphenols and *in vitro* antioxidant activities when compared to *Vitis vinifera* peel extract. *Vitis vinifera* seed and peel extracts were studied for their *in vivo* and *in vitro* anticancer activity along with the gold nanoparticles synthesized using *Vitis vinifera* seed and peel aqueous extracts.