RESULTS & DISCUSSION

Table 4 gives the fatty acid composition of raw as well as thermally oxidised sunflower oil used in the present study.

Table 4. Fatty acid composition of sunflower oil (Percentage of Fatty Acid/g Oil)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Fatty acid</th>
<th>Raw oil</th>
<th>Heated oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>9:03 OH</td>
<td>-</td>
<td>0.37±0.03</td>
</tr>
<tr>
<td>2.</td>
<td>10:00</td>
<td>1.15±0.07</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>3.</td>
<td>10:02OH</td>
<td>-</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td>4.</td>
<td>10:03OH</td>
<td>-</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>5.</td>
<td>11:00</td>
<td>-</td>
<td>5.27±0.29</td>
</tr>
<tr>
<td>6.</td>
<td>12:00</td>
<td>6.74±0.35</td>
<td>2.30±0.17</td>
</tr>
<tr>
<td>7.</td>
<td>13:00</td>
<td>1.76±0.05</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>14:00</td>
<td>1.31±0.05</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>9.</td>
<td>15:00</td>
<td>0.79±0.02</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>10.</td>
<td>16:00</td>
<td>18.93±1.74</td>
<td>15.98±1.49</td>
</tr>
<tr>
<td>11.</td>
<td>16:10</td>
<td>-</td>
<td>0.38±0.02</td>
</tr>
<tr>
<td>12.</td>
<td>17:00</td>
<td>-</td>
<td>0.69±0.04</td>
</tr>
<tr>
<td>13.</td>
<td>17:0cyclo</td>
<td>-</td>
<td>0.62±0.04</td>
</tr>
<tr>
<td>14.</td>
<td>18:00</td>
<td>8.87±0.45</td>
<td>9.99±0.67</td>
</tr>
<tr>
<td>15.</td>
<td>18:20</td>
<td>67.22±6.74</td>
<td>53.89±3.65</td>
</tr>
<tr>
<td>16.</td>
<td>18:12OH</td>
<td>-</td>
<td>2.12±0.14</td>
</tr>
<tr>
<td>17.</td>
<td>19:00</td>
<td>3.17±0.14</td>
<td>0.85±0.05</td>
</tr>
<tr>
<td>18.</td>
<td>20:00</td>
<td>0.51±0.04</td>
<td>1.44±0.06</td>
</tr>
<tr>
<td>19.</td>
<td>20:10</td>
<td>-</td>
<td>6.77±0.55</td>
</tr>
</tbody>
</table>

Values are mean± S. D. of six values
**Phytochemical screening of wheatgrass**

**Qualitative analysis of WG**

In our study, aqueous extract of WG was screened for the presence of bioactive compounds. The outcome showed the presence of alkaloids, flavonoids, saponin, tannins, amino acids, proteins, carbohydrates, coumarin, phenols, alkaloids, terpenoids and cardiac glycosides. But, sterol, steroids and quinone were absent in the aqueous extract (Table 5).

**Quantification of total phenolic and flavanoid contents of WG**

Aqueous extract of WG was evaluated quantitatively for the percentage composition of total phenols and flavonoids. The total phenolic content was estimated as 210.15± 2.41 µmol of GAE/g equivalent of WG. The total flavonoid content was found to be 160.25± 2.17 µmol of Quercetin/g equivalent of WG (Table 6).

**GC-MS analysis of WG**

The Gas Chromatography-Mass Spectrometry (GC-MS) study of WG aqueous extract showed the presence of many bioactive compounds. It contained various pharmacologically important compounds like gamma sitosterol, squalene, caryophyllene and amyrins (Table 7).

**Discussion**

Traditionally, WG has been used since ages for treating a number of diseases. However, not enough scientific experiments have been done in order to study the effect and mechanism of WG on various disorders and metabolic parameters. Detailed study of the constituents of the WG and its effects on different pharmacological parameters could help us to screen novel compounds some of which could potentially be lead molecules. Phytomedicines have multi-constituents that give multi-targeting effect and cause minimum side effects compared with synthetic drugs (Briskin, 2000).
Table 5. Qualitative analysis of WG aqueous extract

<table>
<thead>
<tr>
<th>PARTICULARS</th>
<th>AQUEOUS EXTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>++</td>
</tr>
<tr>
<td>Amino acid and Protein</td>
<td>++</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>+</td>
</tr>
<tr>
<td>Cardioglycoside</td>
<td>++</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Coumarin</td>
<td>++</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>++</td>
</tr>
<tr>
<td>Sterol And Steroids</td>
<td>—</td>
</tr>
<tr>
<td>Quinone</td>
<td>—</td>
</tr>
</tbody>
</table>

(“+” present; “—” absent).

Table 6. Quantification of phenols and flavonoids in WG aqueous extract

<table>
<thead>
<tr>
<th>Total Phenolic Content (µmol of GAE/g of WG powder)</th>
<th>Total Flavonoid Content (µmol of Quercetin /g of WG powder)</th>
</tr>
</thead>
<tbody>
<tr>
<td>210.15±2.41</td>
<td>160.25±2.17</td>
</tr>
</tbody>
</table>
GC-MS Chromatogram
Table 7. GC-MS analysis of WG aqueous extract

<table>
<thead>
<tr>
<th>S.NO</th>
<th>COMPOUND NAME</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Caryophyllene (sesquiterpene)</td>
<td>0.91</td>
</tr>
<tr>
<td>2.</td>
<td>Caryophyllene oxide (sesquiterpene)</td>
<td>1.88</td>
</tr>
<tr>
<td>3.</td>
<td>Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, 1.alpha,2.beta,5.alpha)-(cis-Pinnane)</td>
<td>2.41</td>
</tr>
<tr>
<td>4.</td>
<td>n-Hexadecanoic acid</td>
<td>5.33</td>
</tr>
<tr>
<td>5.</td>
<td>Phytol (acyclic diterpene alcohol)</td>
<td>0.98</td>
</tr>
<tr>
<td>6.</td>
<td>9,12,15-Octadecatrienoic acid, (Z,Z,Z)</td>
<td>12.57</td>
</tr>
<tr>
<td>7.</td>
<td>Ethyl 9,12,15-octadecatrienoate (ethyl ester)</td>
<td>1.33</td>
</tr>
<tr>
<td>8.</td>
<td>syn-9-Hydroxybicyclo[4.2.1]nonane (alcohol)</td>
<td>1.33</td>
</tr>
<tr>
<td>9.</td>
<td>1,4-Benzenediol, 2-methyl-(alcohol)</td>
<td>1.02</td>
</tr>
<tr>
<td>10.</td>
<td>1-Nonadecene (unsaturated hydrocarbon)</td>
<td>0.97</td>
</tr>
<tr>
<td>11.</td>
<td>2,4,6-Trimethyl-1,3-phenylenediamine (diamine)</td>
<td>3.21</td>
</tr>
<tr>
<td>12.</td>
<td>N,N-Tetramethylene-.alpha.-(aminomethylene)glutaconic anhydride</td>
<td>22.58</td>
</tr>
<tr>
<td>13.</td>
<td>1,2-Pentanediol, 5-(6-bromodecahydro-2-hydroxy-2,5,5a,8a-tetramethyl-1-naphthalenyl)-3-methylene-</td>
<td>4.54</td>
</tr>
<tr>
<td>14.</td>
<td>4-Fluorobenzyl alcohol, tert-butyldimethylsilyl ether</td>
<td>1.33</td>
</tr>
<tr>
<td>15.</td>
<td>Silane, chlorodiethyl(dodec-9-ynyloxy)-(Ester)</td>
<td>0.98</td>
</tr>
<tr>
<td>16.</td>
<td>Naphthalene, decahydro-2,6-dimethyl-(cyclohydrocarbon)</td>
<td>1.14</td>
</tr>
<tr>
<td>17.</td>
<td>2-methyloctacosane(hydrocarbon)</td>
<td>2.52</td>
</tr>
<tr>
<td>18.</td>
<td>Squalene(polyunsaturated hydrocarbon)</td>
<td>3.81</td>
</tr>
<tr>
<td>19.</td>
<td>Nonacosane(hydrocarbon)</td>
<td>3.78</td>
</tr>
<tr>
<td>20.</td>
<td>Triacontane(hydrocarbon)</td>
<td>3.64</td>
</tr>
<tr>
<td>21.</td>
<td>dl-.alpha.-Tocopherol(methylated phenol)</td>
<td>3.86</td>
</tr>
<tr>
<td>22.</td>
<td>Eicosane, 10-heptyl-10-octyl-</td>
<td>1.01</td>
</tr>
<tr>
<td>23.</td>
<td>Octadecane, 9-ethyl-9-heptyl-</td>
<td>3.70</td>
</tr>
<tr>
<td>24.</td>
<td>gamma.-Sitosterol</td>
<td>3.71</td>
</tr>
<tr>
<td>25.</td>
<td>4, 4, 6a, 6b, 8a, 11, 11, 14b – Octamethyl- 1, 4, 4a, 5, 6a, 6b, 7, 8, 8a, 9, 10, 11, 12, 12a, 14, 14a, 14b-octadecahydro-2H-picen-3-one</td>
<td>2.43</td>
</tr>
<tr>
<td>26.</td>
<td>beta.-Amyrin</td>
<td>0.99</td>
</tr>
<tr>
<td>27.</td>
<td>alpha.-Amyrin</td>
<td>4.02</td>
</tr>
</tbody>
</table>
The analysis of total flavonoids and phenolic contents showed that significant quantity of polyphenols and other flavonoids are observed in the WG extract. This was confirmed by GC-MS analysis. GC-MS analysis showed the presence of diverse class of organic compounds in varying percentage in the WG aqueous extract, ranging from fatty acids (eg. n-hexadecanoic acid and octadecatrienoic acid), alcohols (eg. 2-methyl benzenediol and phytol), and terpenes (such as alpha and beta amyrin, caryophyllene and caryophyllene oxide). Majority of the compounds extracted belong to hydrocarbon class such as octadecene, nonadecene, 2-methyl octacosane, squalene, nonacosane and tricontane. Sterols such as gamma sitosterol are also reported in GC-MS analysis. Each of these compounds influence metabolism in some way or the other.

Gamma sitosterol has been reported to influence cholesterol synthesis in liver and intestinal cell lines (Ho and Pal, 2005). Squalene is a polyunsaturated hydrocarbon that has been reported to prevent oxidative damage and control the toxicity of 6-hydroxydopamine (Kabuto et al., 2013). Octadecane, another hydrocarbon, is present in significant amount in aqueous extract. It has been reported that it reduces pathophysiology consequences in Plasmodium berghei infected animals when treated with extracts that contain this particular compound in significant concentrations (Nahrevanian et al., 2010). Caryophyllene and its oxides are one of the most important biological compounds that influence diverse metabolism. Caryophyllene has been reported to have significant anti-cancer properties. It induces apoptosis through suppression of multiple pathways such as PI3-Kinase, AKT, mTOR and S6K1 (Park et al., 2011). Many more effects like anti-bacterial, anti-fungal, immunomodulatory and anti-inflammatory have been reported for caryophyllene oxides (Astani et al., 2011). It is also reported to possess anti-platelet aggregation activity (Lin et al., 2003).

Alpha and beta amyrins are the two biologically active pentacyclic triterpenes that influence wide physiological parameters such as anti-inflammatory, antioxidant, gastro protective and hepatoprotective effects at non-toxic concentrations (Oliveira et al., 2005; Holanda Pinto et al., 2008 and Aragao et al., 2007). Anti-hyperglycaemic effect and hypolipidemic effect of amyrin have been recently investigated with positive conclusion suggesting that this compound is a potential candidate for diabetes and atherosclerosis (Santos et al., 2012). Thus, the GC-MS analysis of WG
aqueous extract clearly shows the presence of some biologically functional active principles that are already reported to possess numerous effects in regulating the general physiological and biochemical parameters.

**EVALUATION OF *in vitro* ANTIOXIDANT POTENTIAL**

The antioxidant activity of WG extract was determined by measuring its ability to reduce ferric to ferrous ion. The reducing power was confirmed by the change of yellow colour to green and blue shades. WG extract had higher antioxidant activity compared to BHA (Butylatedhydroxyanisole), the reference compound (Figure 9a).

The metal chelating property of the plant extract was similar to that of Butylatedhydroxytoluene (BHT) (Figure 9b). WG aqueous extract quenched hydroxyl radical in a concentration dependent manner and a comparable quenching activity was observed between the extract and the standard drug (BHT) (Figure 9c). WG aqueous extract significantly reduced the Hydrogen peroxide with increase in concentration. The percentage inhibition of the Hydrogen peroxide correlates with results of BHT (Figure 9d). The scavenging property of the WG against Nitric Oxide released by Sodium Nitroprusside was checked with the help of standard BHA (Figure 9e). WG extract showed high inhibitory behavior at all the concentrations checked and the total antioxidant property of WG extract was comparable to the standard compound ascorbic acid used in the our study (Figure 9f).
Figure 9a. Ferric reducing power activity of WG aqueous extract

Values are mean ± SD of triplicate observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

Figure 9b. Metal chelating activity of WG aqueous extract

Values are mean ± SD of triplicate observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 9c. Hydroxyl radical scavenging activity of WG aqueous extract

Values are mean ± SD of triplicate observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

Figure 9d. Hydrogen peroxide radical scavenging activity of WG aqueous extract

Values are mean ± SD of triplicate observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 9e. Nitric oxide radical scavenging activity of WG extract

Values are mean ± SD of triplicate observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

Figure 9f. Total antioxidant activity of WG aqueous extract

Values are mean ± SD of triplicate observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Discussion

Free radicals such as superoxide anion radicals, hydroxyl radicals, hydrogen peroxide and other reactive oxygen species are highly reactive and effectively destroying transient radicals generated in metabolic process. They are known for playing a double role as both harmful and helpful species, in view of the fact that they can be either deleterious or helpful to living organism (Halliwell and Gutteridge, 1999).

The *in vitro* radical scavenging activities of WG were estimated by a panel of antioxidant assays including OH•, metal chelation, hydrogen peroxide radicals, NO radical scavenging assays as well as assay for reducing power. In all the assays WG effectively scavenged the free radicals almost in all doses in increasing order. The greater radical trapping efficacy of WG may be attributed to various effective compounds like flavonoids, phenols, phytol, sitosterol, squalene that are present in the WG aqueous extract.

**Dose dependent study**

**Hepatoprotective effect of WG**

Figure 10 shows the changes in the activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT). Marked increases in plasma hepatic markers were observed in Alcohol + ΔPUFA administered rats. Co-administration of WG, 50 mg, 75 mg and 100 mg/ kg b.Wt considerably reduced the activities of liver marker enzymes. Treatment with 75 mg/ kg b.Wt was identified as more effective dose than 50 mg and 100 mg/ kg b.Wt. WG control group showed no significant alterations in the levels of these liver markers enzymes when compared to normal.
Figure 10. Effect of WG on the activities of AST, ALT, ALP and GGT in the plasma

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤ 0.05
Figure 11. Effect of WG on the level of TBARS in the plasma, liver, kidney and heart

Figure 11a. Plasma

Figure 11b. Liver

Figure 11c. Kidney

Figure 11d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey’s test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 12. Effect of WG on the level of HP in the plasma, liver, kidney and heart

Figure 12a. Plasma

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Changes in the levels of lipid peroxidative markers

Figure 11 and 12 shows the amount of TBARS and HP in plasma, liver, heart and kidney of Alcohol + ∆PUFA administered rats when compared to normal. Co-administration of WG, 50 mg, 75 mg and 100 mg/ kg b.Wt significantly reduced level of TBARS and HP. But 75 mg/ kg b.Wt was found to be the more effective dose when compared to 50mg and 100 mg/ kg b.Wt.

Antioxidant effects of WG

Figure 13 a, b, c, d shows the activities of SOD in hemolysate, liver, kidney and heart. Catalase activity in hemolysate, liver, kidney and heart are given in the figure 14 a, b, c, d. Figure 15 a, b, c, d shows the activity of glutathione peroxidase in hemolysate, liver, kidney and heart. Figure 16 a,b,c shows the levels of vitamin C and vitamin E in plasma, liver and kidney. Figure 17 shows histopathology of liver.

The reduced glutathione level was considerably reduced in Alcohol + ∆PUFA groups. The activities of the enzymatic antioxidants superoxide dismutase, catalase and glutathione peroxidase and vitamin C and E were decreased significantly in Alcohol + ∆PUFA groups when compared to normal. Treatment with WG 50 mg,75 mg and 100 mg/ kg b.Wt significantly increased their activities. Treatment with 75 mg/ kg b.Wt was found to be more effective than 50mg and 100 mg/ kg b.Wt. WG control group showed no noteworthy change in the activities of these enzymatic and non enzymatic antioxidant levels when compared to normal.

Marked pathological changes were observed in liver sample of Alcohol + ∆PUFARats. Mild portal inflammation and increased micro vesicular fatty acid changes were seen in Alcohol + ∆PUFA groups. Treatment with WG (all three doses) effectively reduced the pathological abnormalities and only inflammatory cell infiltrations were seen in the treated liver. Normal histology was unaltered in WG control rats.
Figure 13. Effect of WG on the activities of SOD in the hemolysate, liver, kidney and heart

Figure 13a. SOD in Hemolysate  Figure 13b. SOD in Liver

Figure 13c. SOD in Kidney  Figure 13d. SOD in Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

*Unit-Enzyme reaction which gives 50% inhibition of NBT reduction/min.
Figure 14. Effect of WG on the activities of catalase in the hemolysate, liver, kidney and heart

Figure 14a. Hemolysate

Hemolysate

Figure 14b. Liver

Liver

Figure 14c. Kidney

Kidney

Figure 14d. Heart

Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

*Unit-µmoles of hydrogen peroxide liberated/min
Figure 15. Effect of WG on the activities of glutathione peroxidase in the hemolysate, liver, kidney and heart

Figure 15a. Hemolysate

Figure 15b. Liver

Figure 15c. Kidney

Figure 15d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

*Unit -µ moles of glutathione utilized/min
Figure 16. Effect of WG on the levels of vitamin C and vitamin E in the plasma, liver and kidney

Figure 16a. Plasma

![Plasma](image1)

Figure 16b. Liver

![Liver](image2)

Figure 16c. Kidney

![Kidney](image3)

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 17. Histopathology of liver (20X)

Normal liver showing normal histology with central vein

Alcohol + ΔPUFA Liver showing microvesicular changes, fibrosis

Alcohol + ΔPUFA + WG Liver (50mg) showing lymphocyte infiltration, microvesicular changes

Alcohol + ΔPUFA + WG Liver (75mg) showing mild lymphocyte infiltration

Alcohol + ΔPUFA + WG Liver (100mg) showing lymphocyte infiltration

WG liver showing normal histology with portal tract
Discussion

Dose dependent study was carried out to assess the effective dose of WG. The effect of WG on plasma liver marker enzymes, lipid peroxidative markers and antioxidant status in Alcohol+ ∆PUFA administered rats were assessed.

In our study, the activities of liver marker enzymes and the levels of lipid peroxidative markers were significantly increased in Alcohol + ∆PUFA administered rats and the antioxidant status was significantly decreased. Administration of three different doses (50 mg, 75 mg and 100 mg/Kg b.Wt) of WG significantly altered the above mentioned parameters.

The lesser dosage 50 mg/Kg b.Wt was not effective, because its concentration might not be sufficient to control the oxidative stress. While, the high dose 100 mg/Kg b.Wt was also not so effective when compared to 75 mg/Kg b. Wt, because at elevated concentration, the WG might react with some ligands in the system and thus might not be entirely accessible for controlling the oxidative stress. Quality of an antioxidant is that it should be effective at lowest possible concentration. WG proved its antioxidant property through its action and the effective dose was 75 mg/Kg b.Wt. Since WG at 75 mg/Kg b.Wt dose gave a maximum effect, it was fixed as the optimum dosage for further study.

Phase II studies
Changes in body weight gain and food intake

Both Alcohol + ∆PUFA are known to influence body weight and food intake. Alcohol has substantial energy value (7.1 kcal/ kg) and is perceived to act exclusively as 'empty calories'. A considerable use of alcohol has greater effect on dietary status (Lieber, 1992). Alcoholic consumption may cause primary malnutrition by cutting down other nutrients in the diet. Alcohol also increases nutrient degradation or impaired activation. Such primary and secondary malnutrition can affect virtually all nutrients. Thus the mixture of reduced nutrient intake, reduced absorption, consumption and storage, elevated excretion as well as the elevated requirement affects the nutrient status during chronic alcohol intake, leading to decreased body weight gain (Lieber, 2001). Previous reports have also confirmed that higher doses of alcohol produce a decrease in weight gain (Aruna et al., 2002).
Reports suggest that large consumption of frying oils is detrimental to health. During deep frying, oxidative and heating effects end up in the production of many volatile and non-volatile products some of which are potentially lethal depending on their ingestion (Durak et al., 1999). Intake of byproducts formed as a result of repeated heating and oxidation of frying oils are identified to guide to a diversity of signs including liver toxicity (Warner, 1999), which may ultimately lead to decreased weight gain. Previous reports also suggest that body gains weight in proportion to food intake and normal growth (Eder, 1999). These findings corroborate the decrease in body weight gain and food intake in diseased group in our study.

**Table 8. Effect of WG on body weight**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Groups</th>
<th>Initial Weight (g)</th>
<th>Final Weight (g)</th>
<th>Weight gain (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal</td>
<td>144.99±2.78</td>
<td>257.03±5.20</td>
<td>107.39±6.43</td>
</tr>
<tr>
<td>2.</td>
<td>Alcohol+ΔPUFA</td>
<td>154.06±2.98</td>
<td>199.72±8.99</td>
<td>44.99±3.72</td>
</tr>
<tr>
<td>3.</td>
<td>Alcohol+ΔPUFA+WG</td>
<td>154.17±1.02</td>
<td>230.54±4.24</td>
<td>76.49±4.24</td>
</tr>
<tr>
<td>4.</td>
<td>WG</td>
<td>146.45±2.78</td>
<td>257.28±3.18</td>
<td>111.14±3.98</td>
</tr>
</tbody>
</table>

**Table 9. Effect of WG on the food intake**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups</th>
<th>Food Given (g/day)</th>
<th>Food left (g/day)</th>
<th>Food consumed (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal</td>
<td>20.0</td>
<td>Nil</td>
<td>20.00</td>
</tr>
<tr>
<td>2.</td>
<td>Alcohol+ΔPUFA</td>
<td>20.0</td>
<td>3.98±0.99</td>
<td>16.02±0.99</td>
</tr>
<tr>
<td>3.</td>
<td>Alcohol+ΔPUFA+WG</td>
<td>20.0</td>
<td>1.61±0.527</td>
<td>18.38±0.52</td>
</tr>
<tr>
<td>4.</td>
<td>WG</td>
<td>20.0</td>
<td>Nil</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
In our study, initial body weights were in the range of 140-50g. Our results showed a considerable reduction in weight gain of Alcohol + ΔPUFA group. Reports suggest that supplementation of ΔPUFA increases gastric injury induced by ethanol, which may probably account for the decreased food intake and decreased weight gain due to malabsorption and malnutrition. Moreover the combined ingestion of Alcohol + ΔPUFA causes severe liver damage, leading to organ dysfunction, thus causing weight loss. Previous studies have also shown that the reduction in body weight linked with alcohol intake is more remarkable when high fat diet is consumed (Rukkumani et al., 2002).

WG enhanced the weight gain and food intake when compared to diseased group. Dietary supplement WG is rich in phenolic and flavonoidic compounds (Varalakshmi et al., 2014). WG directly interact with metabolites of Alcohol + ΔPUFA and nullify their harmful effects on liver and thus improve body weight. Hence we observed improvement in weight gain and food intake in WG treatment groups in our study. The increased food intake in WG treatment group suggests that its potent antioxidant property decreases damage to gastric mucosa and increases food intake. It fights against malnutrition and improve weight gain. Hence it can be a promising hepatoprotective agent (Varalakshmi et al., 2014). Thus, by protecting the liver against Alcohol + ΔPUFA administered liver toxicity; it restores the functional capacity of the liver and improves body weight.

Hepatoprotective effect of WG

Figure 18 shows the changes in the activities of GGT, ALP, AST and ALT. Marked increase in plasma hepatic markers was observed in Alcohol + ΔPUFA administered rats. Co-administration of WG75 mg/Kg b.Wt significantly reduced the activities of liver marker enzymes. WG control group showed no significant change in the activities of these liver markers enzymes when compared to normal.
Figure 18. Effect of WG on the activities of GGT, ALP, AST and ALT

Figure 18a. Activity of GGT

Figure 18b. Activity of ALP

Figure 18c. Activity of enzyme AST

Figure 18d. Activity of ALT

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Discussion

Liver marker enzyme like GGT, AST, ALT and ALP are hallmark of hepatic injury caused due to alcohol (Mirunalini et al., 2010; Tsai et al., 2012). When the liver gets damaged after consumption of heavy alcohol, the cellular enzymes leak into the plasma, due to enhanced permeability, injury and necrosis of hepatocytes (Hidirogloou and Madere, 1999). However, the membranes are made up of lipid particularly PUFA, which are highly susceptible for lipid peroxidation. Therefore, excess consumption of PUFA in diet in turn increases oxidative stress and lipid peroxidation (Shireen et al., 2008; Datta et al., 2012). Moreover during deep frying process, the repeated heating modifies thenutritional properties of PUFA, which exacerbate the alcoholic liver injury by inducing oxidative stress (Patere et al., 2011).

ALT is a cytoplasmic enzyme found in increased concentrations in the liver. AST is located in mitochondria and is less specific than ALT as an indicator of hepatic damage. The elevated levels of serum enzymes such as AST and ALT have been observed in Alcohol + ΔPUFA given rats, which indicate the enhanced permeability, destruction and/or necrosis of hepatocytes (Goldberg and Watts, 1965).

ALP and GGT are membrane bound enzymes, which are released inequitably depending on the pathological condition. ALP is eliminated by liver through bile and therefore when liver is affected, the plasma enzyme level increases due to defective excretion. In alcoholics GGT is more frequently elevated than either of the transaminases and ALP. Serum GGT is generally considered auseful laboratory marker for high alcohol consumption (Sillanaukee, 1996) and its estimation is a precious ‘screening test’ with an elevated negative predictive value for liver disease (Sillanaukee, 1996). Thus, in our study, the combined ingestion of Alcohol + ΔPUFA might have damaged the membrane of the hepatic cell and released the biomarker enzymes in the plasma. Reduced expression of these enzymes on WG co-administration pointed out the hepatoprotective effect of WG.
ANTIOXIDANT EFFECT OF WG

Figure 19 shows the changes in the levels of thiobarbituric acid reactive substances (TBARS). Figure 20 shows the changes in the levels of hydroperoxides (HP). The activities of these lipid peroxidative indices were increased significantly in plasma of Alcohol + ΔPUFA group when compared to normal. Administration of WG significantly reversed these effects and decreased the levels of TBARS, HP. WG control group showed no significant change in the levels of TBARS, HP when compared to normal.

Figure 21 a, b c d shows the changes in the levels of reduced glutathione in plasma, liver, heart and kidney. Treatment with WG significantly increased their levels.

Figure 22 a, b, c shows the changes in the levels of vitamin C and vitamin E in plasma, liver and kidney. The levels of these non enzymatic antioxidants were reduced significantly in plasma of Alcohol + ΔPUFA group when compared to normal. Treatment with WG significantly increased their levels. WG control group showed no significant change in the activities of these non enzymatic antioxidants when compared to normal.

Figure 23 shows the changes in the activity of superoxide dismutase, Figure 24 shows the changes in the activity of catalase and Figure 25 shows the changes in the activity of glutathione peroxidase in hemolysate, liver, heart and kidney respectively. The activities of these enzymatic antioxidants, superoxide dismutase, catalase and glutathione peroxidase were decreased significantly in hemolysate, liver, heart and kidney of Alcohol + ΔPUFA group when compared to normal. Treatment with WG significantly increased their activities. WG control group showed no significant change in the activities of these enzymatic antioxidant levels when compared to normal.

Discussion

Liver is the vital organ involved in the metabolism of ethanol so it is directly affected by excessive ethanol consumption (Faremi et al., 2008). In liver, alcohol is metabolized to acetaldehyde by an enzyme alcohol dehydrogenase. Acetaldehyde is then oxidized to acetate by aldehyde oxidase, generating free radicals via cytochrome
P (CYP2E1). This is a central pathway for the generation of free radical. Oxidative stress is considered to be a significant factor for the development of alcohol related liver diseases (Tilg et al., 2011).

Figure 19. Effect of WG on the levels of TBRAS in the plasma, liver, kidney and heart

Figure 19a. Plasma

Figure 19b. Liver

Figure 19c. Kidney

Figure 19d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 20. Effect of WG on the levels of HP in the plasma, liver, kidney and heart

Figure 20 a. Plasma

Figure 20 b. Liver

Figure 20 c. Kidney

Figure 20 d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05.
Figure 21. Effect of WG on the level of reduced glutathione in the plasma, liver, kidney and heart

Figure 21 a. Plasma

Figure 21 b. Liver

Figure 21 c. Kidney

Figure 21 d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 22. Effect of WG on the levels of vitamin C and vitamin E in the plasma, liver, kidney

Figure 22 a. Plasma

Figure 22 b. Liver

Figure 22 c. Kidney

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 23. Effect of WG on the activities of SOD in the hemolysate, liver, kidney and heart

Figure 23a. Hemolysate

Figure 23b. Liver

Figure 23c. Kidney

Figure 23d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

*Unit-Enzyme reaction which gives 50% inhibition of NBT reduction/min.
Figure 24. Effect of WG on the activities of catalase in the hemolysate, liver, kidney and heart

Figure 24a. Hemolysate

Figure 24b. Liver

Figure 24c. Kidney

Figure 24d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey’s test. Values sharing a common superscript do not differ significantly at P ≤ 0.05

*Unit-µmoles of hydrogen peroxide liberated/min
Figure 25. Effect of WG on the activities of glutathione peroxidase in the hemolysate, liver, kidney and heart

Figure 25a. Hemolysate

Figure 25b. Liver

Figure 25c. Kidney

Figure 25d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

*Unit - µ moles of glutathione utilized/min
In normal biological systems, lipid peroxidation increases the membrane fluidity, degrades the membrane-bound protein and increases the aldehyde production. Lipid hydroperoxide (LH), which are water soluble, further generate other free radicals and increase the membrane damage (Maheshwari et al., 2011). Reactive oxygen species, during oxidation of unsaturated fatty acids, produces a breakdown intermediate product called TBARS; they are usually used as an indicator for oxidative stress (Seghrouchni et al., 2002). In our study, increased levels of TBARS and LH in Alcohol + ΔPUFA treated rats indicate the oxidative damage to the hepatocytes. This was reversed on WG treatment. This shows that WG may act by decreasing the production of ROS, thus decreasing the lipid peroxidation and the formation of intermediate product.

To protect from the oxidative stress, human cells contain enzymatic antioxidants like SOD, CAT, GPx and non-enzymatic antioxidants like ascorbic acid, tocoheroler, GSH (Hazra et al., 2008). Alcohol consumption not only increases the oxidative stress but also decreases the antioxidant defense system (Faremi et al., 2008). In our study, Alcohol + ΔPUFA treated group showed diminished expression of enzymatic antioxidants like SOD, CAT and GPx. It could be due to oxidative modification of these enzymatic proteins because of the excessive free radical generation (Faremi et al., 2008). Oxidative modification of protein reflects the excess formation of protein carbonyls. Free radicals react with side chain of lysine, arginine, proline, threonine and glutamic acid and forms protein carbonyls. Moreover, aldehyde produced during lipid peroxidation also reacts with lysine and sulphhydryl group and incorporate in the enzymes which also forms carbonyl protein (Maheshwari et al., 2011). The enhanced utilization of antioxidants to counteract the excess free radicals formed during Alcohol + ΔPUFA ingestion could also have contributed for the decreased levels.

In the liver, GSH plays an imperative role in protecting the cell from the deleterious effect of ROS by effectively scavenging them. The level of GSH was decreased in Alcohol + ΔPUFA group, which may be due to rapid utilization of GSH in scavenging ROS and detoxification of acetaldehyde during chronic alcohol
consumption (Faremi et al., 2008). In addition to GSH, we have also observed depletion in the level of vitamin C and E in Alcohol + ΔPUFA groups.

During some pathological conditions, the endogenous system is not enough to protect the cells from oxidative stress, so antioxidant supplement from natural source are vital to combat the oxidative damage to the cell (Hazra et al., 2008). WG is shown to have good antioxidant properties, thus could have effectively neutralized the ROS generated in Alcohol + ΔPUFA rats. WG contains vitamin C and E, β carotene, ferulic acid, vanilic acid (Kulkarni et al., 2006). Vitamin E is a fat soluble antioxidant, responsible for termination of chain reactions that results from oxidation of ΔPUFA. In addition to that, Vitamin C is a natural antioxidant that helps in regenerating the vitamin E from its tocopheroxyl radical form (Shireen et al., 2008). Vitamin E and C which is present in WG, may help in the prevention of lipid peroxidation and thereby restore free radical chelating capacity.

WG has been reported to contain good amount of phenols and flavonoids (Kulkarni et al., 2006). Phenolics are potent antioxidants and their activity could be due to several mechanisms such as free radical scavenging activity, their ability to transfer hydrogen to free radicals, chelate metal catalyst involved in free radical generation and activation of antioxidant enzymes (Barreira et al., 2008). WG also contains ferulic acid, which is a natural phenol found to protect the liver from lipid peroxidation as reported by Rukkumani et al. (2004).

In our study, WG extract is observed to be effectively protecting the liver by countering the oxidative stress and improving the host cellular antioxidant enzyme response. Thus, WG decreases lipid peroxidation and supplements the antioxidant machinery. Therefore from the results obtained in our study, we conclude that WG is an effective hepatoprotective agent, against Alcohol + ΔPUFA administered oxidative stress.

**Antihyperlipidemic effect of WG**

Figure 26 a, b, c, d shows the cholesterol levels in plasma, liver, heart and kidney. The levels were high in Alcohol + ΔPUFA administered rats which were significantly reduced after WG treatment. Figure 27 and 28 a, b c, d shows the changes in the levels of triglycerides (TG) and free fatty acids (FFA) in plasma, liver,
heart and kidney respectively. The levels were appreciably raised in Alcohol + ΔPUFA groups, which were decreased after WG treatment.

Figure 29 a, d depicts the phospholipid levels in plasma and heart. The levels of phospholipids were increased in Alcohol+ ΔPUFA treated rats, which were significantly decreased after WG treatment. Figure 29 b, c depicts the phospholipids levels in liver and kidney. The levels of phospholipids were decreased in liver and kidney of Alcohol + ΔPUFA administered rats, which were significantly increased after WG treatment.
Figure 26. Effect of WG on the levels of cholesterol in the plasma, liver, kidney and heart

Figure 26a. Plasma

Figure 26b. Liver

Figure 26c. Kidney

Figure 26d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05.
Figure 27. Effect of WG on the levels of triglycerides in the plasma, liver, kidney and heart

Figure 27a. Plasma

![Plasma Graph]

Figure 27b. Liver

![Liver Graph]

Figure 27c. Kidney

![Kidney Graph]

Figure 27d. Heart

![Heart Graph]

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤ 0.05
Figure 28. Effect of WG on the levels of free fatty acid (FFA) in the plasma, liver, kidney and heart

Figure 28a. Plasma

Figure 28b. Liver

Figure 28c. Kidney

Figure 28d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 29. Effect of WG on the levels of phospholipids (PL) in the plasma, liver, kidney and heart

Figure 29a. Plasma

Figure 29b. Liver

Figure 29c. Kidney

Figure 29d. Heart

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Discussion

Universally, chronic consumption of alcohol remains a major health problem (Gramenziet al., 2006). Heavy alcohol consumption primarily affects the liver (Datta et al., 2012). Liver plays an imperative role in lipid metabolism and therefore, an abnormal lipid profile is expected with severe liver dysfunction (Ghadiret al., 2010).

It is well known that the earliest response to the chronic alcohol consumption is the development of fatty liver (Datta et al., 2012). During metabolism of excess ethanol, high NADH is generated which alters the redox status of the hepatic cell. This causes disturbed citric acid cycle, increased hepatic lipogenesis, decreased hepatic release of lipoproteins, increased lipolysis of peripheral fat, and increased fat uptake, thus predisposing fatty liver (Maralla et al., 2012). Triglycerides are transported to peripheral tissues by very low density lipoprotein (VLDL). Heavy ethanol consumption blocks the secretion of VLDL, thus suppressing the transport of triglycerides and release of free fatty acid from lipoprotein (Yang et al., 2012). Conversely, high intake of dietary fats leads to increased storage of triglycerides not only in adipose tissues but also in liver (Perez-Martinez et al., 2010). Heavy alcohol consumption causes accumulation of fatty acids and triglycerides in the liver by inducing the expression of key enzyme of lipid synthesis like hepatic glycerol-3-phosphate acyltransferase, fatty acid synthase, and malic enzyme (You and Crabb, 2004). Hence the levels of TG and FFA would have increased during Alcohol+ΔPUFA ingestion in our study. Several reports have suggested that phenolic antioxidants possess hypocholesterolemic, hypolipidemic, antidiabetic, antihypertensive and anticancer activities (Chon et al., 2009). WG treatment significantly reduced the plasma lipid levels. This could be due to the presence of phenolics in WG, which might have decreased the expression of key enzymes involved in lipid synthesis.

Alcohol increases the anabolism and decreases the degradation of cholesterol ensuing in hypercholesterolemia. Various studies have shown that oil rich in PUFA, increases the circulating cholesterol (NechiforandDinu, 2010; Otunola et al., 2010). These reports are in agreement with our finding where cholesterol levels were
increased in Alcohol+ ΔPUFA treated rats. This may be attributed to increase in HMG CoA reductase activity by ethanol, which is a regulating enzyme involved in cholesterol biosynthesis. Moreover increased oxidative stress due to alcohol induces the synthesis of cholesterol and fatty acid and controls their degradation (Nechifor and Dinu, 2010). Fatty acid, in turn increases the production of other lipids. Hence we observed increased cholesterol and other lipids in our study. WG protects the liver, preserve the functional activity andalso increase fecal excretion of cholesterol and bile acids because of its rich flavonoids content. Reports have suggested that flavanoids in green tea reduce triglycerides and total cholesterol (Rehrah et al., 2007). So the compounds in WG help in regulating the enzymes which are responsible in triglycerides and cholesterol metabolism. Herbal products have antioxidant activity and can reduce LDL oxidation. Phytosterols present in the plants slowdown cholesterol absorption and this finding also support our data. Reports suggest that many compounds present in herbs are able to reduce plasma triglycerides and cholesterol levels and elevate high density lipoprotein (Joshi, 2005; Patel, 2008). So clearly, compounds like phytosterols and flavanoids are the reason for the hypolipidemic effect of WG.

Phospholipids are the major structural components of cellular membranes. Chronic alcoholism alters the lipid bilayer and its composition by interacting with phospholipids. Alcohol increases the fluidity of the hepatic membrane and increased PUFA changes the PUFA content of the membrane. In our study, the levels of phospholipids were markedly decreased in Alcohol + ΔPUFA-fed rats. The main metabolite of ethanol, acetaldehyde, is more lipid-soluble and produces membrane derangement by increasing the free radical formation, lipid peroxidation and initiating the immunologic reactions resulting in decreased phospholipids (Ingolfsson and Olaf, 2011; Zima, 1993). Reports have shown that consumption of deep-fried food items prepared with repeatedly heated oil rich in PUFA leads to lipid peroxidation, because of the increased susceptibility of PUFA to oxidation (Jaarin et al., 2011; Kirpich et al., 2012; Aoun et al., 2012). Our findings are in agreement with their study that the repeatedly heated PUFA increased the lipid peroxidation, altered the membrane integrity resulting in the decreased phospholipids content of the membrane.
The products of lipid peroxidation are reactive molecules and thus are the potent damagers of cellular molecules (Rukkumani et al., 2004). The peroxidation products are the reason for the chemical susceptibility of individual fatty acids to oxidation which ultimately results in differences in membrane composition (Hulbert, 2005). In WG treatment group, we observed increased phospholipids level. This may be due to the antioxidant property of WG which might have reduced the free radicals and prevented lipid peroxidation, and membrane damage, and thus preserved the phospholipids content of the membranes (Garima et al., 2012a, b; Sethi et al., 2010).

Thus in our study, WG effectively modulated the lipid profiles. This could be either by enhanced protection against free radical attack, or by augmentation of fatty acid turnover, or perhaps both.

**Changes in the activities of membrane modeling enzymes**

Figure 30 indicates the activities of phospholipase A (PLA) phospholipase C (PLC). The activities of both PLA and PLC were significantly increased in liver of Alcohol + ΔPUFA groups. Treatment with WG considerably decreased their activities when compared to normal. WG control group showed normal activities of PLA and PLC.

**Figure 30. Effect of WG on the levels of phospholipases A and C in liver**

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Discussion

Phospholipases are a group of enzymes that catalyze phospholipids cleavage. Processing of phospholipids’ by these membrane-modeling enzymes converts these molecules into lipid mediators or secondary messengers. In our study, we observed the increased activities of phospholipase A and phospholipase C (PLA, PLC) in Alcohol + ∆PUFA-fed group rats. Previous studies have shown that alcohol modulates PLA activity by increasing intracellular Ca\(^{2+}\) ion concentration (Oide et al., 2000). PLA is an important tool in regulation of phospholipids acyl turnover for membrane repair and production of inflammatory mediators. The increase in PLA activity has been suggested to reduce the proportion of unsaturated acyl composition of selected membrane phospholipids and help in developing resistance to the disordering of ethanol (Kode et al., 2007). An increase in PLA activity after exposure to ethanol has been reported by Aruna et al. (2005a, b). This increased PLA activity results in excessive release of arachidonic acid (AA), which then enters into CYP450, cyclooxygenase, and lipoxygenase pathway and acts as a precursor of the eicosanoid complexes, leukotrienes, thromboxanes, and prostaglandins resulting in the production of various inflammatory mediators during chronic ethanol toxicity, which are known to be associated with ethanol-induced liver injury (Balsinde et al., 2002).

Cytochrome P450 exacerbates PLA2 and AAdependent injury and promotes lipid peroxidation or production of metabolites that alter Ca\(^{2+}\) homeostasis (Caro and Cederbaum, 2006). Ca\(^{2+}\)-sensitive domain is homologous among PLC isoenzymes, and hence ethanol ingestion increases the PLC activity. PLC normally cleaves phospholipids and produces phosphatidyl inositol 4, 5-bisphosphate and subsequently, diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP3). DAG binds to the membrane and IP3 diffuses through the cytosol and bind to IP3 receptors, specifically, calcium channels, in the smooth endoplasmic reticulum. This causes the cytosolic intracellular concentration of calcium to increase. Calcium and DAG together activate protein kinase C, which phosphorylates other molecules, and affect a cascade of signaling pathways leading to the repair of the lipid bilayer (Ellis et al., 1998; Aruna et al., 2004). Hence, the increased PLC in Alcohol +ΔPUFA group may be a defense mechanism to combat the membrane damage caused by Alcohol +ΔPUFA. In our
study, WG treatment decreased the activation of PLA, C. This may be because of the reduction in lipid peroxidation and associated membrane modifications.

**Changes in the phospholipid fatty acid composition in the liver**

Figure 31 gives the changes in the phospholipid fatty acid composition in the liver. In membrane fatty acid composition analysis, 16:0, 16:1,18:0,18:1,18:2 fatty acids were increased significantly in the Alcohol + ∆PUFA treated group which was controlled on treatment with WG. 20:4 fatty acid levels were decreased in Alcohol + ∆PUFA group but raised on treatment with WG.

**Figure 31. Effect of WG on the levels of phospholipid fatty acid composition in liver**

![Graph showing changes in fatty acid composition](image)

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

**Discussion**

Penetration of alcohol into the lipid bilayer results in disordering of the composition of fatty acids in phospholipid bilayer. In our study, the levels of 16:0 (palmitic acid), 16:1 (palmitoleic acid), and 18:0 (stearic acid), 18:1 (oleic acid), 18:2
(linoleic) were increased in Alcohol +ΔPUFA-fed groups, but 20:4 arachidonic acid (AA) was significantly reduced in this group. Ethanol disturbs the fatty acid metabolism by inhibiting 5, 6 delta desaturase activity. Inhibition of desaturases leads to accumulation of 18:2 (linoleic acid) and results in the increased levels of monounsaturated fatty acid (MUFA), such as 16:1 and 18:1. Ethanol blocks the anabolic pathway of arachidonic acid, and moreover AA is diverted for the formation of inflammatory markers during Alcohol + ΔPUFA ingestion (Aruna et al., 2005a, b). Blockage in anabolic pathway of AA and utilization for inflammatory markers production may be the reason for the depleted level of AA, observed in Alcohol+ΔPUFA-ingested group in our study. The increased PUFA intake increases the degree of unsaturation of the membrane (Rajakrishnan and menon, 2002), and as a compensating effect, the levels of saturated fatty acids, like 16:0 (palmitic acid) and 18:0 (stearic acid), are increased for maintaining the fluidity of phospholipids bilayer. Apart from this, interaction of linoleic with nine delta desaturase boosts up the production of palmitic and stearic acids. Hence, we observed the increased levels of palmitic acid and stearic acid in our study as reported in earlier studies (Aruna et al., 2005a, b). The fatty acid composition was near normal when WG was given along with Alcohol +ΔPUFA.

Hepatoprotective effects of plants against ethanol-induced hepatotoxicity have been reported previously by several investigators (Bhawna and Upendra, 2009; Gujrati et al., 2007; Pramyothin et al., 2007; Jaishree and Badami, 2010). WG contains alkaloids, tannins, saponins, and sterols (Garima et al., 2012a, b; Kothari et al., 2008) and is also rich in antioxidants such as polyphenols and flavonoids. WG has shown higher free radical scavenging activities, higher elemental content (Kulkarni et al., 2006) and higher oxygen radical absorbance capacity (Lachnicht et al., 2002; Kulkarni et al., 2006). The various phytochemicals present in WG could have scavenged the free radicals produced during the metabolism of ethanol and PUFA and thus decreased the membrane alteration. Study from our lab also confirmed the presence of phenolics, flavonoids and other compounds having the reducing activities, such as squalene, phytol, and α, βamyrin to be present in the WG. WG can also interact with acetaldehyde; thereby reducing the latter’s toxicity effects on membrane
phospholipids. Hence, in our study, WG maintained the membrane fatty acid's composition by regulating the phospholipases.

We conclude that WG can effectively prevent the oxidative stress-induced changes in the phospholipid's fatty acid composition of the membrane and restore the membrane integrity. The above study suggests that WG shows direct hepatoprotective effect by inhibiting lipid peroxidation in phospholipids bilayer, controls the activity of PLA, C and reduces the changes in the fatty acid composition of membrane. Thus, it maintains and preserves the membrane integrity against Alcohol + ΔPUFA induced liver toxicity in rats.

**Histopathology of liver**

Histopathological changes in the liver are given in Figure 32. Mild portal inflammation and increased micro vesicular fatty acid changes were seen in Alcohol + ΔPUFA groups. Treatment with WG effectively reduced the pathological abnormalities and only inflammatory cell infiltrations were seen in the treated liver. Normal histology was unaltered in WG control rats.

**Histopathology of kidney**

Histopathological changes in kidney are given in Figure 33. Enhanced pathological changes were observed in histology of kidney during Alcohol + ΔPUFA treatment. Alcohol +ΔPUFA rats' kidney sections showed thick vessel walls, extensive fatty infiltration and lymphocyte aggregation. WG treated group showed lymphocytic infiltration. Normal rats and WG control rats kidney showed normal glomeruli and tubules.
Figure 32. Histopathology of Liver (20X)

- **A**: Normal Liver (20X) Liver showing normal histology with central vein (↓)
- **B**: Alcohol + ΔPUFA Liver (20X) Mild portal inflammation (↓) and microvesicular fatty changes (↑)
- **C**: Alcohol + Δ PUFA + WG Liver (20X) Fatty changes and inflammatory cell infiltration around central vein (↑)
- **D**: Wheatgrass Liver (20X) Liver showing normal histology with portal trial (↓)
Figure 33. Histopathology of Kidney

- Normal
  Kidney normal showing glomeruli and tubules

- Alcohol + ΔPUFA
  Thickened wall, fatty infiltrate and adipocyte aggregation

- Alcohol + ΔPUFA + WG
  Lymphocyte infiltration

- WG
  Kidney normal showing glomeruli and tubules
Changes in the levels of collagen

Figure 34 shows the levels of collagen of liver. It was significantly increased in Alcohol + ΔPUFA group when compared to normal, but appreciably reduced on WG treatment. WG control group showed no significant modification in the levels of collagen in comparison to normal.

Figure 34. Effect of WG on collagen in Liver

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

Total activities of matrix metalloproteinases (MMPs)

Changes in the levels of total MMPs Activity

Figure 35 a shows multiwell zymogram, the total MMPs activity in liver. The total MMPs activity decreased considerably in Alcohol + ΔPUFA group compared to normal and treatment with WG considerably increased the total MMPs activity. The WG control group showed no considerable change in MMP activity compared to control group. Figure 35 b gives the densitometric analysis of total MMPs activity. The activities of MMPs were reduced in Alcohol + ΔPUFA group which were increased in WG treatment group.
Figure 35a. Effect of WG aqueous extract on multiwell zymogram in the liver

Control   Normal   Treatment   Disease   WG

Figure 35b. Densitometry analysis of multiwell zymogram

Control is the well without any sample addition.
Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

Changes in the levels of individual MMPs Activity

Figure 36. Effect of WG on MMP zymogram activity in the liver tissue

Lane 1   Lane 2   Lane 3   Lane 4

Lane 1: Normal,   Lane 2: Alcohol + ΔPUFA,   Lane 3: Alcohol + Δ PUFA + WG,   Lane 4: WG
Individual activities of MMPs as by zymogram are given in Figure 36. Four types of MMPs (45 kDa, 72 kDa, 92 kDa and 130kDa) were expressed in liver during Alcohol + ΔPUFA induction and subsequent action of WG. There was a significantly decreased MMP activity in Alcohol + ΔPUFA fed group. Administration of WG appreciably improved the activities of matrix metalloproteinase. WG control showed no noteworthy change in MMPs activities when compared to normal.

**Changes in the levels of tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2)**

Figure 37 shows the level of TIMP 1 and TIMP 2 in liver. They were considerably increased in Alcohol + ΔPUFA group in comparison with normal. Co administration of WG considerably decreased their levels. WG control group showed no considerable change in the levels of TIMP1 and TIMP2 compared to the normal.

**Figure 37. Effect of WG on the levels of TIMP-1 and TIMP-2**

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

**Discussion**

The general pathological result of Alcohol + ΔPUFA induced oxidative insult is fibrosis. Fibrosis results from chronic injury to the cells in combination with the accumulation of ECM proteins and alteration in levels of ECM regulating enzymes.
(Groblewska et al., 2010), which is characteristic of most types of chronic conditions. Elevated expression of MMPs and TIMPs were found to play an important role in the development of chronic liver diseases (Badra et al., 2010).

In our study, we estimated the levels/activities of collagen, MMPs and TIMPs in Alcohol + ΔPUFA induced toxicity and subsequent treatment with WG. The collagen levels were significantly increased in Alcohol + ΔPUFA treated rats. During ingestion of ethanol, Kupffer cells generate ROS either via NADPH oxidase, xanthine oxidase in mitochondria, or possibly by CYP2E1, and may enhance HSC activation and collagen I synthesis (Urtasun and Neito, 2007).

Normal liver has small quantity of collagen types I, III and IV. In pathological conditions, the total collagen content increases. It has been reported that type III collagen increases in the initial fibrotic process, whereas type I collagen predominates in advanced fibrosis and cirrhosis (Joon-seung et al., 1991). Due to increased susceptibility of membrane to lipid peroxidation and the toxic metabolites produced during heating of PUFA, there is an increased membrane damage leading to fibrosis during ΔPUFA ingestion (Jethmalani et al., 1989). HSCs show significant morphological and functional changes during stress conditions and the star-shaped HSCs are modified to myofibroblastic cells with increased expression of α-smooth muscle actin and reduced retinoid storage.

The normal ECM in the space of Disse rich in basement membrane-like matrices is switched to fibrillar, contractile ECM in order to facilitate proteolytic degradation. All of these factors result in the activation of HSCs leading to increased fibrosis and collagen deposition (Benyon and Arthur, 2001). In our study, MMPs were reduced in Alcohol + ΔPUFA treated rats. It has been reported that the MMPs are increased during early phase of liver injury, maximal during inflammation and are diminished at the end fibrotic stages (Arthur, 2000). Hence the reduced MMP expression in Alcohol + ΔPUFA in present study indicate advanced fibrosis. Moreover, hepatic stellate cells are an important source of TIMP-1 and 2 in fibrotic liver. In Alcohol + ΔPUFA group, the activities of MMPs might have declined in response to increased TIMPs levels (Surya Narayanan et al., 2011). Expression of TIMPs had been demonstrated in liver fibrosis. TIMPs 1 and 2 were shown to
promote progression of hepatic fibrosis through inhibition of matrix degradation (Roderfeld et al., 2007).

TIMPs are co-expressed with the MMPs and hold back the action of collagen degrading enzymes. Also TIMPs expression appears to enhance as acute fibrosis becomes chronic (Surya Narayanan et al., 2011). An increase of TIMP over MMP expression has been discussed as one of the pathological condition of liver fibrosis (Roderfeld et al., 2007; Durairaj et al., 2014). This explains why there was an increased expression of TIMPs in Alcohol + ΔPUFA fed groups in our study.

Treatment with WG considerably reduced the fibrotic changes by reducing the levels of collagen and TIMPs and increasing the expression of MMPs in Alcohol + ΔPUFA groups. This can be attributed to the valuable antioxidant property of WG (Varalakshmi et al., 2015).

Being a natural compound, rich in flavonoids, phenols and other important antioxidants such as vitamin C, E, β carotene, ferulic acid, vanilic acid, WG could have terminated the lipid peroxidation and prevented the damage to the tissue (Varalakshmi et al., 2015). It might also have decreased the activation of HSCs and thus controlled advancement of fibrosis (Wang et al., 2011; Alena et al., 2003). From the results obtained, we state that WG acts as an anti-fibrotic agent primarily by deregulating collagen and TIMPs. It also promotes matrix degradation during severe stage of injury. These qualities are ideal for the fibrosis reversing agents. Hence WG can be used as effective anti-fibrotic agent.

**Effect of WG on inflammatory markers**

Figure 38 shows the changes in the levels of cytokine markers. The levels of Interleukin-1 (IL-1), Tumor Necrosis Factor-α (TNF-α), Transforming Growth Factor – β (TGF-β) and Interleukin-6 (IL-6) were markedly increased in Alcohol + ΔPUFA fed rats. Treatment with WG significantly reduced the levels of these cytokine markers.
Figure 38. Effect of WG on IL-1, TNF-α, TGF-β and IL-6 in serum

Values are mean ± SD of 6 observations in each group. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

Discussion

The liver carry out an important role in the inflammatory reactions. It is a potential site of chronic inflammatory condition (e.g., ALD) and acts as a synthetic site for inflammatory agents. Alcoholism raises the permeability of the intestine, allowing certain endotoxin transport via the intestinal wall into the circulation. The endotoxin stimulates the KC to generate cytokines expression. It leads to liver inflammation (Craig et al., 1997).

TNF-α is a cytokine involved in programmed inflammation and is a part of cytokine family that activates acute inflammation. TNF-α is synthesized by a variety of cells in the body. In liver, TNF-α is mainly produced by Kupffer cells. It is also an important mediator of various physiological processes, such as inflammation, cell proliferation, and apoptosis (McClain et al., 2004). The role of TNF-α as a critical inflammatory cytokine in the progression of ALD is now well understood (Kitazawa et al., 2003). In alcoholic hepatitis (AH), inflammatory cytokines, such as TNF-α or IL-6 or IL-1, induce liver injury (Hansen et al., 1994). Elevated serum levels of TNF-α
inducible cytokines or chemokines, including IL-6, IL-8, IL-1 and IL-18 have also been reported in subjects with AH (Hill et al., 1992).

KC are triggered by IL-1, discharged from damaged hepatocytes (Luedde and Schwabe, 2011; Kawaratani et al., 2013). NFκB dependent cytokine that is produced by triggered KC is IL-6. Various studies supported our results of IL-1, IL-6 and STAT-3 elevation during liver damage (Tilg et al., 1992; Trikha et al., 2003). Chronic alcohol consumption also causes activation of complement system, followed by the induction of TNF-α and liver damage (Gao et al., 2011; Szabo et al., 2011).

ROS production and a mild acidic environment are able to induce TGF-β activation. TGF-β activates monocytes, inducing synthesis of a diversity of cytokines (such as IL-1 and TNF-α) and certain growth factors (Letterio and Roberts, 1998). TGF-β is markedly upregulated in experimental models of liver disease. The level of TGF-β is increased with the severity of liver fibrosis in cell (Szuster-Ciesielska et al., 2013). During injury, TGF–β stimulates HSCs. Normally they are fat storing cells. Activation results in collagen production, the important content of scar tissue. It develops fibrosis in subjects with alcoholic steatohepatitis (Bedossa and Paradis, 1995). Activation of TGF–β via alcohol initiates programmed cell death (Neuman et al., 2001; Katz et al., 2001).

Hence in the present study, IL-1, IL-6, TGF-β and TNF-α in Alcohol +ΔPUFA fed rats were increased. On treatment with WG the above mentioned inflammatory markers were decreased indicating the anti-inflammatory and hepatoprotective role of WG.

**Analysis of DNA damage by comet assay**

DNA damage has been identified at the beginning of a lot of diseases and could be a helpful biomarker of the oxidative status. It has been calculated that the quantity of oxidative injury even under normal physiological state, may be fairly wide, which estimates as high as one base modification per 1,30,000 bases in nuclear DNA. There are variety of DNA damage, such as strand breaks, exchange of sister chromatid, DNA-DNA and DNA-protein interlinks and base modifications (Simic et
al., 1989; Teebor et al., 1988). Several studies have attempted to correlate oxidative DNA damage with hepatotoxicity (Castelli et al., 1999, Fedeli et al., 2003).

Figure 39a and b shows the alterations in the levels of DNA damage by comet assay (% head and tail DNA and tail length) in the hepatocytes. In Alcohol + ΔPUFA fed rats there was a noteworthy raise in percentage of tail DNA, tail length and a reduced percentage of head DNA. WG administration considerably diminished the levels of DNA damage.

**Figure 39a. Effect of WG on DNA damage by comet assay in the hepatocytes.**
Discussion

The result of ethanol intake on lymphocytes and hepatocytes has been well described in human alcoholics and experimental animals (Castelli et al., 1999, Fedeli et al., 2003). DNA damage was amplified in Alcohol +ΔPUFA administered rats as compared to control. This clearly suggests a relationship between alcohol consumption and DNA damage. Our finding is in correlation with the other reports (Navasumrit et al., 2000 and 2001). Ingestion of repeated heated oil resulting in genotoxicity has also been reported by Srivastava et al. (2010). Ethanol combined with acetaldehyde, the major metabolite of ethanol, induces DNA cleavage in rat hepatocytes (Rajasinghe et al., 1990). Acetaldehyde is reported to participate in the pathogenesis of ethanol mediated genotoxicity (Singh and Khan, 1995). The DNA damaging effect of acetaldehyde may be mediated by generation of ROS. Other possible mechanisms by which acetaldehyde could exert its toxic effects includes generation of DNA cross-links and acetaldehyde protein adducts. The significance of
ROS in genetic toxicity induction is broadly accepted and has been extensively studied in the past decade (Simic, 1994). Superoxide radicals (O$_2^-$) can directly or indirectly damage DNA whereas hydrogen peroxide (H$_2$O$_2$) mediates DNA damage by the production of hydroxyl radical (OH$^-$) via events such as the Fenton's reaction (Imlay et al., 1988). In addition, chronic exposure to active oxygen radicals caused by ethanol may decrease an individual’s DNA repairing capacity (Topinka et al., 1991).

WG co-administered rats showed decreased DNA damage. The capability of WG to bring down the ethanol mediated DNA damage to normal level reveals the beneficial effect of WG against genotoxicity. Several investigations have proved the capability of antioxidants/ROS quenching compounds to defend cellular DNA against injury caused by chemicals (Blasiak and Kowalik, 2001; Gabbianelli et al., 2004). WG is one of the most effective liver cleansers.

The defensive effect of WG may be by hunting the ROS prior to they cause lethal effect to DNA. This may be because of the rich natural antioxidants in WG that have the capacity for free radical scavenging. WG was already reported to scavenge free radicals (Varalakshmi et al., 2014). It reduces ROS formation and increases natural antioxidant mechanism (Varalakshmi et al., 2014). Thus the antioxidant effect of WG could be accountable for their defensive effect against Alcohol + ∆PUFA mediated liver toxicity.

**Changes in the levels of molecular markers**

**Effect of WG on protein and gene expression of PPAR-α**

Figure 40a depicts the representative western blot expression of PPAR-α. Protein expression of PPAR-α was decreased in Alcohol + ∆PUFA fed rats which were considerably raised in WG administrated rats. WG control group showed no noteworthy change compared to normal. Densitometry analysis of western blot is given in figure 40b.

Figure 41a shows mRNA gene expression of PPAR-α. Expression of PPARα was decreased in Alcohol + ∆PUFA fed rats which increased significantly in WG treated rats. WG control group showed no major change. Histogram analysis of mRNA is given in figure 41b.
Figure 40. Effect of WG on PPAR-α protein expressions in the liver tissue

**a. Western blot analysis**

Lane 1: Normal, 2: Alcohol + ΔPUFA, 3: Alcohol + Δ PUFA + WG, 4: WG

**b. Band intensity scanned by densitometer**

Histogram depicts quantification of three independent experiments (means ± S.D), with data normalized by defining the control group with PPAR-α as 1 unit. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05.
Figure 41. Effect of WG on PPAR-α mRNA expression in the liver tissue

a. mRNA expression by qRT-PCR

b. Histogram showing relative PPAR-α mRNA expression

Histogram depicts quantification of three independent experiments (means ± S.D). The PPAR-α mRNA expression was normalized with the expression level of the GAPDH mRNA expression. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05.
Discussion

PPAR-α plays a vital role in alcoholic liver toxicity (Nakajima et al., 2004). Increased expression of PPAR-α is observed in liver and adipose tissue, followed by heart and kidney (Braissant et al., 1996). PPAR-α exerts a hypolipidemic effect (Isserlin and Green, 1990) and defends the liver from alcohol-induced damage (Nakajima et al., 2004). In liver, beta-oxidation genes are transcriptionally regulated by PPAR-α. Several articles suggest the importance of PPAR-α in lipid metabolism, and in the development of hepatic steatosis, steatohepatitis, and liver cancer (Reddy and Hashimoto, 2001). In our study, hepatic PPAR-α expression was down-regulated in Alcohol + ΔPUFA administered rats. The dysfunction of PPAR-α could be one of the reasons for the development of hepatic steatosis (Nanjee et al., 2004). PPAR-α is an effective sensor for free fatty acid metabolism. Apart from regulating the gene expression of fatty acid β-oxidation, it controls fatty acid synthesis, trafficking and maintain lipid and glucose homeostasis (Evans et al., 2004; Crabb et al., 2004). Thus, induction of PPARs regulatory pathways modulates cholesterol metabolism, and inflammatory responses indirectly (Evans et al., 2004).

PPAR-α also directly controls the inflammatory process by regulating the production of cytokines and inhibiting the transcription of NFκB. In our study, it was observed that PPAR-α expression was down-regulated in Alcohol + ΔPUFA group and up-regulated in WG treated group. PPAR-α induced-control of steatosis can be because of the down-regulation of TNF-α and TGF-β, thus, suppressing inflammation. Inhibition of PPAR-α by Alcohol + ΔPUFA leads to induction of hepatotoxicity (Kong et al., 2011). PPAR-α expression was modified by WG through anti-inflammatory and hypolipidemic effect, thus, protecting the hepatocytes from steatosis. WG significantly attenuated hepatosteatosis (Chou et al., 2002; Kim et al., 2003) by inducing the appearance of PPAR-α.

Effect of WG on protein & gene expression of NFκB & STAT 3

Figure 42 a shows the western blot analysis of NFκB & STAT-3. Protein expression of NFκB & STAT-3 was increased in Alcohol + ΔPUFA rats which were significantly decreased on WG treatment. WG control group showed no significant
change compared to normal. Figure 34b shows the densitometry analysis of these markers.

Figure 43 (a and b), 44 (a and b) shows the mRNA expression of NFκB & STAT-3. The up regulated expression of NFκB & STAT-3 was seen in Alcohol + ΔPUFA fed rats which were significantly down regulated in WG treated rats. WG control group showed no significant change when compared to normal.

**Figure 42. Effect of WG on NFκB and STAT-3 protein expressions in the liver tissue**

a. Western blot analysis

Lane 1: Normal, 2: Alcohol + ΔPUFA, 3: Alcohol + ΔPUFA + WG, 4: WG

b. Band intensity scanned by densitometer

Histogram depicts quantification of three independent experiments (means ± S.D), with data normalized by defining the control group with NFκB and STAT-3 as 1 unit. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at $P \leq 0.05$
Figure 43. Effect of WG on NFκB mRNA expression in the liver tissue

a. mRNA expression by qRT-PCR

![Figure 43a: mRNA expression by qRT-PCR](image)

b. Histogram showing relative NFκB mRNA expression

![Figure 43b: Histogram showing relative NFκB mRNA expression](image)

Histogram depicts quantification of three independent experiments (means ± S.D). The NFκB mRNA expression was normalized with the expression level of the GAPDH mRNA expression. ANOVA followed by Tukey’s test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 44. Effect of WG on STAT-3 mRNA expression in the liver tissue

a. mRNA expression by qRT-PCR

b. Histogram showing relative STAT-3 mRNA expression

Histogram depicts quantification of three independent experiments (means ± S.D). The STAT-3 mRNA expression was normalized with the expression level of the GAPDH mRNA expression. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤ 0.05.
Discussion

Alcohol +ΔPUFA induce accumulation of ROS. ROS generation stimulates NFκB activation (Park et al., 2004). NFκB signalling produces a group of cytokines and growth factors, including IL-6 (Luedde and Schwabe, 2011; Maeda et al., 2005). IL-6 released by Kupffer cells activates STAT-3 in hepatocytes. However, STAT-3 has also been reported to contribute to activation of NFκB (Chen et al., 2001).

STAT-3/NFκB interaction has been reported in several articles (He and Karin, 2011; Lee et al., 2009). NFκB and STAT-3 collaboratively binds at a subset of gene promoters to cooperatively provoke their target genes expression (Yang et al., 2007). Many cytokines like IL-6 induced by NFκB or STAT-3 can allosterically provoke more STAT-3 and NFκB activation (Gao et al., 2007; Sansone et al., 2007). NFκB interacts with STAT-3 and their relationship elevates their transcriptional activity (Lee et al., 2009). NFκB and STAT-3 are likely to play pivotal roles in liver inflammatory responses (He and Karin, 2011). They together control the expression of genes responsible for cell proliferation, survival, oxidative stress and immune responses. Certain target genes for NFκB and STAT-3 overlap and therefore, are engaged in positive and negative crosstalk (Grivennikov and Karin, 2010; Atkinson et al., 2010).

NFκB and STAT-3 are highly interactive key regulators in tumor microenvironment also (Grivennikov and Karin, 2010). STAT-3 and NFκB are elevated in inflammatory cells (Yu et al., 2007). Our study suggests that up regulation of NFκB and STAT-3 observed in Alcohol + ΔPUFA could be for protecting the liver damage induced by Alcohol+ΔPUFA.

Our findings suggest that the WG effectively reduced the accumulation of ROS (Varalakshmi et al., 2014), reduced the level of cytokines by down-regulating the expression of NFκB and STAT-3 genes and protein expression in test rats. Thus, WG regulates the progression of inflammation.
Figure 45. Activation of WG

Effect of WG on protein and gene expressions of Bcl-2, Bax, Caspase-3

Figure 46a shows the western blot of Bcl-2, Bax and Caspase-3. Protein expression of Bcl-2 was increased in Alcohol + ΔPUFA rats which decreased significantly on WG treatment. Protein expressions of Bax, Caspase-3 were decreased in Alcohol + ΔPUFA rats which were significantly increased in WG treated rats. WG group showed no significant change in the levels of these parameters when compared to normal. Figure 46b shows densitometry analysis of Bcl-2, Bax and Caspase-3.

Figure 47a shows the mRNA expression of Bcl-2. Figure 48a shows Bax, and Figure 49a shows Caspase-3 mRNA gene expression. Gene expression of Bcl-2 was increased in Alcohol + ΔPUFA rats which decreased significantly on WG treatment. Gene expressions of Bax, Caspase-3 were decreased in Alcohol + ΔPUFA rats which were significantly increased in WG treated rats. WG control group showed no significant change compared to normal. Figure 47, 48 and 49b shows histogram of Bcl-2, Bax and Caspase-3.
Figure 46. Effect of WG on Bcl-2, Bax, Caspase-3 expressions in the liver tissues

a. Western blot expression of Bcl-2, Bax, Caspase-3.

Lane 1: Normal, Lane 2: Alcohol + Δ PUFA, Lane 3: Alcohol + Δ PUFA + WG, Lane 4: WG

b. Band intensity scanned by densitometer

Histogram depicts quantification of three independent experiments (means ± S.D) with datas normalized by defining the control group Bcl-2, Bax and Caspase-3 as 1 unit. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05
Figure 47. Effect of WG on Bcl-2 mRNA expression in the liver tissue

a. mRNA expression by qRT-PCR

b. Histogram showing relative Bcl-2 mRNA expression

Histogram depicts the quantification of three independent experiments (means ± S.D). The Bcl-2 was normalized with the expression level of the GAPDH mRNA expression. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05.
Figure 48. Effect of WG on Bax mRNA expression in the liver tissue

a. mRNA expression by qRT-PCR

![qRT-PCR graph](image)

b. Histogram showing relative BaxmRNA expression

![Histogram](image)

Histogram depicts quantification of three independent experiments (means ± S.D). The Bax was normalized with the expression level of the GAPDH mRNA expression. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at \( P \leq 0.05 \).
Figure 49. Effect of WG on Caspase-3 expression in the liver tissue

a. mRNA expression by qRT-PCR

![Graph showing mRNA expression by qRT-PCR](chart.png)

b. Histogram showing relative Caspase-3 mRNA expression

![Histogram showing Caspase-3 mRNA expression](chart.png)

Histogram depicts quantification of three independent experiments (means ± S.D). The Caspase-3 was normalized with the expression level of the GAPDH mRNA expression. ANOVA followed by Tukey's test. Values sharing a common superscript do not differ significantly at P ≤0.05

Discussion

Chronic liver disease has become a global health problem; it causes around 20,000 deaths every year (Garima et al., 2012a). Hepatocellular injury can be initiated by a wide array of intrinsic and extrinsic factors such as inborn errors of metabolism, malnutrition, viral infection, exposure to toxins, alcohol and diseases like non-alcoholic fatty liver disease. Apoptosis, cytolytic cell death, enhanced ROS generation
in mitochondria, leakage of ROS, activation of Kupffer cells and collagen production by stellate cells are the major signs of the pathophysiology of the disease condition (Saravanan et al., 2013). In our study, we evaluated the potential of WG on apoptosis in Alcohol + ΔPUFA induced rat model.

A remarkable feature of apoptosis is that its essential features are conserved in all cellular system. Caspases are synthesized as inactive zymogens that must be activated in order to function (Thornberry and Lazebnik, 1998). Caspases, cysteine proteases, are essential for apoptotis. Caspase-3 could be activated by free radicals (Schon and Manfredi, 2003). Recent articles have shown that enhanced Bcl-2 levels restrict the function of Bax and prevent the mitochondrial release of cyt C, thereby hold back the opening of caspase cascade and programmed cell death (Qian et al., 2008). In our study, we found that the elevation of Bcl-2 reduced the appearance of Bax and caspase-3 activity in the Alcohol + ΔPUFA rats. Similarly, elevated levels of Bcl-2 and low levels of Bax and caspase-3 have been reported in the disease-induced animal model and it was reversed upon the treatment with plant extract (Wang et al., 2014). In the present study also we observed an increase in Bax and caspase-3 and a decrease in Bcl-2 in WG treated rats. These findings suggest that WG regulates the expression of apoptosis-related proteins. It induces apoptosis via the intrinsic/mitochondrial pathway and thus, clears damaged cells by apoptosis.

We investigated the mRNA expression profiles of these markers of apoptosis in order to confirm the apoptotic effects of WG on Alcohol + ΔPUFA induced ALD rats. According to the results of qRT-PCR, WG treatment in rats induced the up-regulation of caspase-3 and Bax expression and down regulated the Bcl-2 in a dose-dependent manner. These results comply with the results of western blotting. Our observations confirm that WG significantly activates the caspase cascade via intrinsic apoptotic pathway and thereby leading to cell death. Thus, it could be concluded that WG possess potential apoptosis inducing effects. WG effectively removes the damaged cells through apoptosis and recovers natural mechanism. It also induces the regenerative property of liver. Further studies may throw a light on the potential use of WG as a candidate for hepatic cancer treatment as well.