CHAPTER 3

ANTIOXIDANT ACTIVITY OF
Emblica officinalis
3.1. INTRODUCTION

Reactive oxygen species (ROS) are produced by cellular metabolic reactions and have been implicated in several diseases such as inflammatory conditions, atherosclerosis, cancer etc. and are also implicated in the aging process (2). Moreover, free radicals have been involved in the action of several xenobiotics and compounds that can scavenge free radicals have great potential in ameliorating these disease processes (279).

The human body has inherent mechanisms to reduce free-radical induced injury by the action of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase etc. and by non-enzymatic means involving ascorbic acid, vit. E, selenium etc. (28,280). At certain times these natural protective mechanisms may not be sufficient when compared to the insult against the body. Supplimentation with nontoxic antioxidants may have a chemoprotective role in the body under these conditions (29).

*Emblica officinalis* (E.O) is a major ingredient in several indigenous drug preparations. The fruit pulp is used against a variety of disease conditions such as liver injury (40, 41) and atherosclerosis (221, 222). *Chyavanaprash* (CHY), a drug preparation, which contains E.O as a major ingredient, has been used as a health tonic in India from time immemorial to rejuvenate the body system. The presence of ascorbic acid (52), gallotannins (18) and ellagic acid (232) have been detected in the fruit of E.O. Eventhough these materials have antioxidant activity, a real appreciation of the antioxidant activity of E.O and CHY have not been realized. A comparative study of antioxidant activities of E.O and CHY extracts both in *vitro* and *in vivo* were elaborated in this chapter.
3.2. MATERIALS AND METHODS

*Emblica officinalis* (E.O) fruits were purchased locally. *Chyavanaprash* (CHY), a drug preparation containing E.O was purchased from Vaidyaratnam Oushadhasala, Ollur, India. The extracts were prepared as described in chapter II.

3.2.1. Determination of superoxide scavenging activity

3.2.1.1. Riboflavin photo reduction method

Superoxide scavenging activity of the compounds was determined by the light induced superoxide generation by riboflavin and the corresponding reduction of NBT. The procedure has been described in chapter II. The inhibition produced by E.O and CHY extracts on superoxide production were measured using various concentrations of E.O (10-200 µg) and CHY extracts (10-100 µg) in the reaction mixture. Concentration needed for 50% inhibition was obtained from the graph. Inhibitory effects of various known antioxidants were also determined.

3.2.1.2. Xanthine - xanthine oxidase method

Superoxide anion was generated by Xanthine -xanthine oxidase and measured by the reduction of NBT. The procedure has been described in chapter II. The inhibition produced by E.O extract was determined using various concentrations (10 - 200 µg) of E.O in the reaction mixture and concentration needed for 50% inhibition was obtained from the graph.

3.2.2. Determination of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of E.O and CHY extracts were measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe³⁺/ascorbate / EDTA / H₂O₂
system. The hydroxyl radicals attack deoxyribose, which eventually results in TBARS formation. Deoxyribose degradation was measured by reaction with TBA. The procedure has been described in chapter II. The inhibition produced by E.O and CHY extracts were determined using various concentrations (0.1 - 1 mg) of the extracts in the reaction mixture and concentration needed for 50% inhibition was obtained from the graph.

3.2.3. Determination of lipid peroxidation inhibiting activity

3.2.3.1. Induction by Fe²⁺/ascorbate system

Lipid peroxidation was induced in liver homogenate in presence of different concentrations (10 - 200 μg) of E.O and CHY extracts in the reaction mixture. Malonaldehyde formed during peroxidation was measured by reaction with TBA. The method has been described in chapter II. The percentage inhibition was calculated and concentration needed for 50% inhibition was calculated from the graph.

The time course of lipid peroxidation induced by Fe²⁺/ascorbate was also studied in presence and absence of 200 μg of E.O extract and was measured by reaction with TBA.

3.2.3.2. Induction by Fe³⁺-ADP/ascorbate system

Lipid peroxidation was induced in liver homogenate by Fe³⁺-ADP/ascorbate system in presence of different concentrations of (10-200 μg) E.O extract in the reaction mixture. The lipid peroxide generated was estimated by reaction with TBA and percentage inhibition was calculated. Detailed procedure has been described in chapter II. Concentration needed for 50% inhibition was calculated from the graph.
3.2.4. Inhibition of superoxide generation by macrophages activated with PMA

Balb/c mice were divided into four groups. Group I was treated as control. Group II - IV were treated with different concentrations of E.O. extract (200 - 500 mg / kg b.wt.) orally (5 doses / mice). PMA (100 ng / animal) was administered i.p. to all groups after the 5th dose of drug administration. In another experiment dried fruit extract corresponding to the above concentration was also administered similarly. Inhibition of superoxide generation by the macrophages was measured by the reduction of NBT. Detailed procedure has been described in chapter II.

3.2.5. Determination of antiinflammatory activity

The antiinflammatory activity was measured by the carrageenan induced rat paw oedema method which has been described in chapter II. Male wistar rats were divided into groups of four and oedema was induced by injecting carrageenan (200 μg / 20 μl, in saline) into the subplanter region of left hind paw on 5th day to all groups. Group I was treated with carrageenan alone. Group II-IV were treated with different concentrations of E.O extract (125 mg, 250 mg and 500 mg / kg b.wt respectively) orally (5 doses / rat). Dried fruit extract corresponding to the above concentration was also administered similarly in a separate group. The thickness of the paw was measured before and after injection initially, and continued for 6 h at 30 min intervals. The increase in the thickness of the paw was compared at each time interval for the control and experimental rats.

3.2.6. Inhibition of phase-I enzyme activity in vitro

3.2.6.1. Aniline hydroxylase activity

Aniline hydroxylase was assayed using aniline as substrate and the activity was determined by the rate of formation of p-aminophenol formed by hydroxylation
of aniline. The procedure has been described in chapter II. The percentage inhibition produced by various concentrations (0.1-1 mg) of E.O extract in the reaction mixture was plotted on a graph and concentration needed for 50% inhibition was calculated.

3.2.6.2. Aminopyrene - N-demethylase activity

Aminopyrene demethylase was assayed using aminopyrene as substrate and the activity was determined by measuring the formation of dealkylated product, 4-aminoantipyrine. The procedure has been described in chapter II. The percentage inhibition produced by various concentrations (0.1-0.75 mg) of E.O. extract in the reaction mixture was plotted on a graph and concentration needed for 50% inhibition was calculated.

3.2.7. Determination of effect of E.O. and CHY extracts on lipid peroxidation and hepatic enzymes in normal rats.

Male Wistar rats weighing 150-200g were divided into four groups (6/group). Group I was kept as normal control. Group II and IV were treated with 250 mg/kg b. wt. of E.O extract and 1000 mg/kg b. wt. of CHY extract for one month and group III was treated with 500 mg/kg b. wt. of E.O extract for one week (5 doses). The animals were sacrificed by chloroform anaesthesia 24 h after the last dose of treatment. Blood and liver were collected. The following biochemical parameters were estimated as per the methods given in the parenthesis. Detailed procedure was given in chapter II.

3.2.7.1. Biochemical Analysis

Serum and liver lipid peroxides (thiobarbituric acid method)
Liver protein (Lowry's method)
Liver Glutathione (DTNB method)
Superoxide dismutase (SOD) (NBT reduction method)
Glutathione peroxidase (GPX) (Hafemann et al)
Glutathione reductase (GR) (Racker)
Glutathione - S-transferase (GST) (Habig et al)
Aniline hydroxylase (AH) (Mazel)
Aminopyrene - N-demethylase (AD) (Mazel)

3.2.8. Determination of effect of E.O and CHY extracts on phenobarbital induced hepatic enzymes

Male Wistar rats weighing 150-200 g were divided into four groups (6 / group). Group I was kept as normal control. Phenobarbital (80 mg / kg b. wt. / dose) was administered to group II to IV orally (5 doses). Group III and IV were also treated with 500 mg / kg b. wt. of E.O extract and 2.5 g / kg b. wt. of CHY extract. Drug administration was started 3 days prior to phenobarbital administration and continued till the end of the experiment. Animals were sacrificed 24 h after the last dose. Liver tissues were collected and levels of aniline hydroxylase and aminopyrene N-demethylase activities were determined.

3.2.9. Isolation of active principle from Emblica officinalis

3.2.9.1. Preparation of extract

250 g of dried Emblica powder was extracted with 1 litre methanol by overnight stirring. Supernatant was collected and the residue was again extracted twice with 250 ml methanol. Methanolic extracts were pooled and evaporated in a water bath. It was resuspended in 10% methanol and a small fraction was kept for comparison and treated as original extract.
Methanolic extract was taken in a separating funnel and extracted with solvents of different polarity in the order of petroleum ether, chloroform, ether, ethyl acetate and butanol. Fractions were evaporated, weighed and the antioxidant activity was measured by the inhibition of superoxide generation by photoreduction of riboflavin. Fractions obtained were spotted on thin layer chromatography which was developed using the solvent system, Toluene: ethylacetate: formic acid (50:40:10).

The spots were separated into different bands and were detected by
1. Treatment with iodine vapour
2. FeCl₃ reaction

Rf values were noted and superoxide scavenging activity was compared. Active fraction obtained having maximum scavenging activity was then subjected to column chromatography using silica gel. Column was eluted with solvents (3 bed volume) of different polarity. Different fractions were collected and compared superoxide scavenging activity and Rf values by spotting on TLC.

3.3. RESULTS
3.3.1. Effect of E.O and CHY extracts on superoxide generation in vitro

E O and CHY extracts were found to scavenge the superoxide generated by photoreduction of riboflavin. Concentration of E.O extract needed for 50% inhibition of superoxide was found to be 10.7 µg / ml. E.O extract at a concentration of 66.7 µg / ml produced 95.5% inhibition of superoxide generation (Fig 3.1). Concentration needed for 50% inhibition of known antioxidants such as ellagic acid, curcumin and bixin were found to be 7.05, 9.21 and 19.73 µg / ml respectively. α- tocopherol upto 28.7 µg/ ml and ascorbic acid upto 1,173 µg / ml did not show any activity by this method (Table 3.1).
Superoxide scavenging activity was done by NBT – photo reduction method in presence and absence of various concentrations of antioxidants.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration for 50% inhibition (µg / ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagic acid</td>
<td>7.05</td>
</tr>
<tr>
<td>Curcumin</td>
<td>9.21</td>
</tr>
<tr>
<td>Bixin</td>
<td>19.73</td>
</tr>
<tr>
<td>(\alpha)-tocopherol</td>
<td>&gt;25.00</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>E.O</td>
<td>10.7</td>
</tr>
</tbody>
</table>
CHY extract was also found to scavenge superoxide generation by this method. Concentration of CHY extract needed for 50% inhibition was found to be 243 µg/ml (Fig 3.5).

E.O extract was found to scavenge superoxide generation by Xanthine-xanthine oxidase method. Concentration needed for 50% inhibition was found to be 23.2 µg/ml (Fig 3.1). E.O extract at a concentration of 66.7 µg/ml produced 5% inhibition of superoxide generation by this method.

3.3.2. Effect of E.O and CHY extracts on hydroxyl radical generation

Hydroxyl radicals generated by the Fe³⁺/ascorbate/EDTA/H₂O₂ system were found to be inhibited by the E.O and CHY extracts. This was evident from the inhibition of degradation of deoxyribose to malonaldehyde, mediated by hydroxyl radicals. The concentration of E.O extract needed for 50% inhibition was 340 µg/ml (Fig 3.2). E.O extract at a concentration of 1000 µg/ml produced 91.4% inhibition of deoxyribose degradation. Concentration needed for 50% inhibition of CHY extract was found to be 3,200 µg/ml by this method (Fig 3.5).

3.3.3. Effect of E.O and CHY extracts on lipid peroxidation

Lipid peroxides generated by Fe²⁺/ascorbate system, in rat liver homogenate were found to be inhibited by the addition of E.O and CHY extracts. The concentration of E.O extract needed for 50% inhibition was found to be 100 µg/ml (Fig 3.3). The time course of lipid peroxidation induced by Fe²⁺/ascorbate as shown by TBARS in the presence and absence of E.O extract is shown in fig. 3.4. E.O extract at a concentration of 400 µg/ml was found to be effective in completely inhibiting lipid peroxidation under these conditions.
Fig. 3.1. Effect of Emblica officinalis extract on superoxide generation

Fig. 3.2. Effect of Emblica officinalis extract on hydroxyl radical formation
Fig. 3.3 Effect of *Emblica officinalis* extract on lipid peroxide formation

![Graph showing the effect of Emblica officinalis extract on lipid peroxide formation.](image)

- **Fe²⁺/ascorbate**
- **Fe³⁺/ADP ascorbate**

Fig. 3.4 Time course of inhibition of lipid peroxidation by *Emblica officinalis* (E.O) extract

![Graph showing the time course of inhibition of lipid peroxidation.](image)

- **Untreated**
- **Treated with 400ug of E.O**

Time in minutes: 0, 15, 30, 60

Absorbance: 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45
Fig. 3.5 Antioxidant activity of *chyavanaprash* (CHY).
CHY extract was also found to inhibit lipid peroxides generated by this method. Concentration of CHY extract needed for 50% inhibition of lipid peroxidation was found to be 320 μg / ml (Fig 3.5).

E.O extract was also found to inhibit Fe$^{3+}$ / ADP - ascorbate induced lipid peroxidation (Fig. 3.3). Concentration needed for 50% inhibition was found to be 64 μg / ml. E.O extract at a concentration of 200 μg /ml showed 82.3% inhibition of lipid peroxide generation.

3.3.4. Effect of E.O extract on the inhibition of PMA induced superoxide production

Different concentrations of fresh and dried E.O extracts were found to inhibit the production of superoxide generation by peritoneal macrophages. Administration of 500 mg / kg b. wt. of fresh E.O extract and corresponding dried E.O extract showed 72.96% and 88.4% inhibition of PMA induced superoxide production (Table 3.2) indicating that the oxygen radical scavenging activity of E.O did not lose in biological conditions.

3.3.5. Antiinflammatory activity of E.O extract

Since the oxygen radicals are directly involved in the inflammatory process, the antiinflammatory activity was determined using fresh and dried fruits of E.O. It was found that fresh and dried fruit extracts of E.O showed antiinflammatory activity in a dose dependent manner. Difference in thickness of paw oedema was increased (0.03 - 0.22 mm) during the time interval of 150 min. in control group. After 24 h paw oedema was decreased to normal level in both groups (Fig 3.6 and 3.7).
Table 3.2: Effect of E.O extract on the inhibition of superoxides produced by peritoneal macrophage

<table>
<thead>
<tr>
<th>Concentration of E.O (mg / kg b. wt.)</th>
<th>% Inhibition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh E.O</td>
<td>Dried E.O</td>
</tr>
<tr>
<td>200</td>
<td>35.85</td>
<td>54</td>
</tr>
<tr>
<td>400</td>
<td>56.92</td>
<td>80</td>
</tr>
<tr>
<td>500</td>
<td>72.96</td>
<td>88</td>
</tr>
</tbody>
</table>

E.O extract (200-500 mg / kg b.wt.) was given orally (5 doses / mice). PMA (100 ng / animal) was administered i.p after 5" dose. Peritoneal macrophages were collected after 3h.
Fig. 3.6 Antiinflammatory activity of *Emblica officinalis* (E.O) extract (fresh fruit)

Fig. 3.7. Antiinflammatory activity of *Emblica officinalis* (E.O) extract (Dried fruit)
3.3.6. Effect of E.O extract on aniline hydroxylase (AH) and aminopyrene-N-demethylase (AD) \textit{in vitro}

Role of P-450 enzymes in carcinogen metabolism has been known. Since E.O extract was found to inhibit the carcinogen metabolism (chapter 5), effect of E.O on P-450 enzymes such as AH and AD were also determined. E.O extract showed a dose dependent inhibition of AH and AD activity \textit{in vitro} (Fig 3.8). Concentration needed for 50\% inhibition of AH and AD activities were found to be 430 $\mu$g / ml and 301 $\mu$g / ml respectively.

3.3.7 Effect of E.O and CHY extracts in normal rats

3.3.7.1 Effect of E.O and CHY extracts on lipid peroxidation

Treatment of E.O (250 and 500 mg / kg. b. wt.) and CHY (1000 mg / kg b. wt.) extracts did not show significant effect on serum lipid peroxidation in normal rats (Table 3.3). Liver lipid peroxide was remained without any significant change in E.O treated group, while CHY treated group produced a reduction in liver lipid peroxide ($p<0.2$) compared to the untreated group.

3.3.7.2. Effect of E.O and CHY extracts on endogenous antioxidant levels

Administration of 250 mg / kg b. wt. of E.O and 1000 mg / kg b. wt. of CHY extracts for one month and 500 mg / kg b. wt of E.O for 5 days did not show any significant effect on liver SOD and GPX activity (Table 3.4).

3.3.7.3. Effect of E.O and CHY extracts on carcinogen metabolizing enzymes

Microsomal enzyme AD was found to be slightly increased by E.O treatment in normal animals but it was not much significant ($p<0.5$). CHY extract did not have any effect on AD activity. AH activity was found to be unaltered by E.O and CHY treatment. GST levels were found to be increased by E.O treatment. At a
Fig. 3.8 Effect of *Emblica officinalis* (E.O) extract on phase I enzymes *in vitro*
Table 3.3: Effect of E.O and CHY extracts on serum and liver lipid peroxides in normal rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Serum lipid peroxide nmol / ml</th>
<th>Liver lipid peroxide nmol / mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>1.93 ± 0.20</td>
<td>1.72 ± 0.20</td>
</tr>
<tr>
<td>II</td>
<td>E.O (250 mg)</td>
<td>1.91 ± 0.24</td>
<td>1.47 ± 0.64</td>
</tr>
<tr>
<td>III</td>
<td>E.O (500 mg)</td>
<td>1.90 ± 0.30</td>
<td>1.58 ± 0.72</td>
</tr>
<tr>
<td>IV</td>
<td>CHY (1000 mg)</td>
<td>1.98 ± 0.57</td>
<td>1.25 ± 0.43</td>
</tr>
</tbody>
</table>

Table 3.4: Effect of E.O and CHY extracts on antioxidant enzymes in normal rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Liver SOD U/mg protein</th>
<th>Liver GPX U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>24.1 ± 3.7</td>
<td>76.2 ± 9.7</td>
</tr>
<tr>
<td>II</td>
<td>E.O (250 mg)</td>
<td>19.8 ± 4.3</td>
<td>71.4 ± 12.3</td>
</tr>
<tr>
<td>III</td>
<td>E.O (500 mg)</td>
<td>21.0 ± 2.8</td>
<td>79.3 ± 25.0</td>
</tr>
<tr>
<td>IV</td>
<td>CHY(1000 mg)</td>
<td>20.5 ± 3.6</td>
<td>82.3 ± 10.2</td>
</tr>
</tbody>
</table>

E.O (250 mg / kg b. wt.) and CHY (1000 mg / kg b. wt) extracts given orally for one month to group II & IV. E.O (500 mg / kg b. wt) extract was given 5 days (5 doses / rat) to group III.
dose level of 500 mg / kg b. wt., the increase was found to be nearly 2 fold (p<0.001) GSH and GR levels were found to be unaltered by the treatment with the extract (Table 3.5).

3.3.8. Effect of E.O and CHY extracts on phenobarbital induced hepatic enzymes

Inhibition of microsomal enzymes by E.O and CHY extracts were studied in rats after induction with phenobarbital (PB). Phenobarbital administration increased microsomal enzymes AH and AD activities in the liver (Table 3.6) to 1.62 and 0.236 μmol / min / mg protein respectively compared to the normal value of 0.212 and 0.196. E.O extract (500 mg / kg b. wt.) was found to reduce AH level moderately (p<0.3), while CHY did not change AH levels. Both E.O and CHY did not have any effect on AD levels.

3.3.9. Antioxidant activity of isolated material from E.O

Comparison of superoxide scavenging activity of different fractions is shown in Table 3.7. Ether and ethylacetate fractions were found to have maximum activity and the concentration needed for 50% inhibition was found to be 4.67 and 5.3 μg / ml respectively. Thin layer chromatography showed well defined bands for ether or ethyl acetate fractions with similar Rf of 0.57, which showed maximum superoxide scavenging activity (Fig. 3.9). The bands obtained reacted with iodine and FeCl₃ solution.

Column chromatography of ether or ethyl acetate fraction using different ratios of solvents in the order of increasing polarity further purified the active material present in the extract. Chloroform and ether in the ratio 75:25 and 50:50 were found to have maximum inhibition of superoxide generation by photo reduction
Table 3.5: Effect of E.O and CHY extracts on carcinogen metabolizing enzymes

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>GSH</th>
<th>GST</th>
<th>GR</th>
<th>AH</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(nmol / mg protein)</td>
<td>(μmol / min / mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Normal</td>
<td>10.6 ± 1.0</td>
<td>401.5 ± 61.4</td>
<td>52.1 ± 11.7</td>
<td>0.212 ± 0.18</td>
<td>0.196 ± 0.09</td>
</tr>
<tr>
<td>II</td>
<td>E.O (250 mg)</td>
<td>9.3 ± 1.9</td>
<td>423.1 ± 49.5</td>
<td>44.9 ± 11.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III</td>
<td>E.O (500 mg)</td>
<td>14.0 ± 2.0</td>
<td>**878.3 ± 128</td>
<td>44.1 ± 2.1</td>
<td>0.205 ± 0.15</td>
<td>*0.230 ± 0.02</td>
</tr>
<tr>
<td>IV</td>
<td>CHY (1000 mg)</td>
<td>9.3 ± 1.2</td>
<td>432.2 ± 71.2</td>
<td>45.4 ± 1.7</td>
<td>0.265 ± 0.09</td>
<td>0.181 ± 0.02</td>
</tr>
</tbody>
</table>

* p<0.5. ** p<0.001

ND – Not determined

E.O (250 mg / kg b. wt.) and CHY (1000 mg / kg b. wt) extracts were given orally for one month to group II & IV. E.O (500 mg / kg b. wt) extract was given 5 days (5 doses / rat) to group III.
Table 3.6: Effect of E.O and CHY extracts on phenobarbital induced enzyme levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>AH</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µmol / min / mg protein)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Normal</td>
<td>0.21 ± 0.18</td>
<td>0.196 ± 0.09</td>
</tr>
<tr>
<td>II</td>
<td>Phenobarbital alone (PB)</td>
<td>1.62 ± 0.42</td>
<td>0.236 ± 0.06</td>
</tr>
<tr>
<td>III</td>
<td>PB + E.O (500 mg)</td>
<td>* 1.30 ± 0.15</td>
<td>0.203 ± 0.14</td>
</tr>
<tr>
<td>IV</td>
<td>PB + CHY (2.5 g)</td>
<td>1.60 ± 0.22</td>
<td>0.244 ± 0.22</td>
</tr>
</tbody>
</table>

* p<0.3

Phenobarbital (PB) (80 mg / kg b. wt) was given orally to Group II - IV (5 doses). Group III and IV treated with 500 mg /kg b. wt of E.O and 2.5g/ kg b. wt. of CHY extracts respectively. Drug administration was started 3 days prior to phenobarbital treatment and continued till the end of the experiment.
Table 3.7: Superoxide scavenging activity of extracted materials

<table>
<thead>
<tr>
<th>Extracts used</th>
<th>Concentration for 50% inhibition (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>10.7</td>
</tr>
<tr>
<td>Pet. ether</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>-</td>
</tr>
<tr>
<td>Ether</td>
<td>4.68</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>5.3</td>
</tr>
<tr>
<td>Butanol</td>
<td>6.7</td>
</tr>
<tr>
<td>Methanol</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 3.8: Antioxidant activity of isolated material and Emblicanin

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration for 50% inhibition (µg / ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superoxide scavenging</td>
</tr>
<tr>
<td>Original</td>
<td>10.7</td>
</tr>
<tr>
<td>Chloroform : Ether (75:25)</td>
<td>0.175</td>
</tr>
<tr>
<td>Chloroform : Ether (50:50)</td>
<td>1.70</td>
</tr>
<tr>
<td>Emblicanin</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Superoxide scavenging activity was done by NBT- photo reduction method and inhibition of lipid peroxidation was done by Fe²⁺ / ascorbate system.

N.D – Not determined
of riboflavin. TLC of column fractions were compared with original methanolic, ether and ethylacetate extracts as well as with emblicanin, which was recently reported to be responsible for the antioxidant activity of E.O. Chloroform and ether in the ratio 75:25 showed single spot corresponding to the original extract (Fig. 3.10). This fraction had the maximum scavenging activity with Rf. 0.57. The spots obtained showed blue colour with FeCl₃ and was found to be different from Emblicanin. Concentration needed for 50% inhibition of superoxide generation by CHCl₃ and ether in the ratio 75:25, 50:50 and emblicanin were found to be 0.175, 1.70 and 2 μg/ml respectively (Table 3.8). Concentration needed for 50% inhibition of lipid peroxidation was found to be 55 and 375 μg/ml respectively for column fraction (CHCl₃: Ether, 75:25) and emblicanin.

The results indicated that most of the superoxide scavenging activity of the extract is due to a ferric chloride reacting material (poly phenol) and that the activity was increased upon purification on silica gel column. The actual chemical structure of the compound is not known at present.

3.4. DISCUSSION

The close relationship between free radical activity and malignancy has been well documented. The basic assumption appears to be that free radical damage of cellular materials would result in triggering or transforming normal cells to malignant ones (10,11). Increased consumption of fruits and vegetables reduces the risk of cancer. A large part of the protective ability of fruits and vegetables were derived from their content of the antioxidant nutrient or its ability to act by stimulating the activity of detoxifying enzymes (29, 32-34).

The present study indicated a strong antioxidant activity of E.O, which may
Fig. 3.9 Thin layer chromatography of different fractions from methanolic extract of E.O 1. Petroleum ether 2. Chloroform 3. Ether 4. Ethylacetate 5. Butanol 6. Methanol 7. Crude Methanolic extract

be partially responsible for many of the biological properties manifested by this fruit. The concentration of E.O extract needed for the in vitro inhibition of the oxygen radicals such as superoxides, hydroxyl radicals and lipid peroxides were relatively low and comparable or more than the activity manifested by known antioxidants such as ellagic acid, curcumin, \(\alpha\)-tocopherol, bixin and ascorbic acid. Reducing activity was found to be heat stable. CHY extract also showed a remarkable antioxidant activity inspite of the high heat used in its preparation provided a scientific explanation for the observed medicinal properties of the preparation.

In the in vivo system E.O extract was found to inhibit PMA induced superoxide generation. There is a strong correlation between inflammation and superoxide generation (16). In fact administration of E.O could inhibit the superoxides and reduce the incidence of cancer in animals and phorbol esters have been reported to promote tumour formation by generating the superoxide radicals and causing subsequent DNA damage.

E.O extract maintained the normal level of lipid peroxides in serum and liver tissue while CHY extract reduced tissue lipid peroxide level. The key enzymes of in vivo antioxidant system, GPX and SOD remained without change by E.O and CHY treatment.

E.O extract was found to inhibit phase I enzymes AH and AD in vitro, which are microsomal P-450 enzymes and are implicated in the activation of carcinogens (135). In the in vivo system AH level was remained unaltered while AD level was increased by E.O treatment, but the increase was not significant. CHY treatment maintained normal level of AH and AD activity. E.O and CHY extracts also maintained normal level of reduced glutathione, which is probably the most
important cellular antioxidant. GR level was also unaltered by E.O and CHY treatment. E.O extract was found to induce the levels of GST, which is a major detoxification pathway. Phenobarbital induced AH and AD levels were moderately reduced by E.O treatment. CHY treatment unaltered phenobarbital induced AH and AD levels.

Components present in E.O include ascorbic acid (52), tannins (18), trigalloyl glucose (232), flavonoids (229) etc. Ascorbic acid present in plants was associated with polyphenols. Polyphenols present in fruits are ellagic acid (232) and phyllembic acid (230). The antioxidant activity was found to be dialyzable and heat stable, associated with ether fraction, indicating that the antioxidant activity is mainly due to the presence of polyphenolic compounds present in the fruit. Although E.O has been reported to contain ascorbic acid the results of the present study indicates that ascorbic acid has only a minor role in the biological activity of E.O. This is further substantiated by a recent work, which questions the presence of ascorbic acid in *Emblica officinalis* (231). Ghosal et al established that potent vitamin C like activity located in the low mol. wt (<1000) hydrolysable tannins, emblicanin A and B, punigluconin and pedunculagin. The isolated material associated with ether fraction showed different Rf on TLC compared to emblicanin indicating that antioxidant activity may be due to some other poly phenolic compound. Concentration needed for inhibition of Lipid peroxidation and superoxide generation was lower than that of emblicanin. The present results indicate that the fruit and the preparation (Chyavanaprash) have potential antioxidant activity and may thus be medicinally useful in the case of oxygen radical - mediated injuries. Chyavanaprash, which showed a remarkable antioxidant activity, provided a scientific explanation for the observed medicinal properties of the preparation.