STANDARDIZATION OF DOSE AND DURATION OF TREATMENTS

This chapter involves the in-vivo studies performed to evaluate the changes with respect to hepatic lipid peroxidation. Emphasis has been given on the response of different concentrations and durations of treatments for different extracts.
4.1 Introduction

It is a general notion that plants are safe and free from any side effects. However, this does not always hold true, as quite a good number of reports have indicated possible toxic effects of some plant extracts (De Smet, 1993; Malik, 1995; Ernst, 1996; Drew and Myers, 1997; Thompson, 1997; Kar and Panda, 2004, 2005; Adebiyi et al., 2005; Adedapo et al., 2005; Alebiowu et al., 2007; Bottenberg et al., 2007; Bacchini et al., 2008). Therefore, it was also crucial to reveal the safe nature of all the test peel extracts proposed in the present study. For this, hepatic lipid peroxidation (LPO) was chosen as the end parameter. Since, liver is the major target organ of a drug and is primarily responsible for the detoxification processes inside the body, any chemical, if hazardous/toxic by nature, normally exhibits its first impact on the liver. Obviously, any drastic alteration in basic structure and physiology of the liver is reflected by an increase in peroxidation process ultimately causing degeneration of the cells. It is also understood that lipid peroxidation/oxidative stress is the root cause of various health ailments including that of thyroid problems, cardiovascular problems, diabetes mellitus, cancer and Alzheimer’s disease (Tiwari, 2004).

The importance of antiperoxidative potential in naturally occurring compounds/herbal extracts is clearly indicated by the vast availability of literature (Ferguson, 2001; Surh et al., 2001; Kar and Panda, 2004, 2005; Lee et al., 2005; Parmar et al., 2006; Gundermann and Muller, 2007; Asl and Hosseinzadeh, 2008). Even various fruits including orange, pomegranate, mango, banana, watermelon, melon, apple and pear are also known for their antiperoxidative properties (Sivarajan and Balchandran, 1994; Kanazawa and Sakakibara, 2000; Higashi-Okai et al., 2002; Leontowicz et al., 2002, 2003; Vouldoukis et al., 2004; Naito et al., 2005; Rodriguez et al., 2006; Rocha Ribeiro et al., 2007). From the results of the
previous chapter (in-vitro studies), it was revealed that all the test peels exhibit varying degree of antiperoxidative activities in dose dependent manner. On the other hand when this work was started, no detailed investigation was made on the test peel extracts using in vivo model, except on *Punica granatum* peel extract (Chidambara-Murthy et al., 2002).

Dose dependent harmful effects of phytomedicines are also documented, particularly on increased oxidative stress (Panda and Kar, 1998c; Fraunfelder, 2005; Parmar et al., 2006; Starakis et al., 2006; Goldstein et al., 2006; Greenberg et al., 2007; Elmer et al., 2007; Baccsini et al., 2008), indicating, that, dose and period of treatment of the drug (s) are some of the factors of prime importance, to decide whether any compound / substance is a drug or toxicant (Satoskar et al., 1999). In fact, duration of the therapy is a major concerning factor for drug’s safety evaluation and also necessary to be investigated for the desirable effects of any drug (FDA, 1999). It is expected that drug should be maximally effective at minimum possible dose and duration of the therapy. The best medical practice should be based on the fact that the desired effects are observed after the treatment for a short duration (Liu et al., 1997, 2007; Sungnoon and Chattipakorn, 2005; Kawahara et al., 2007; Zu et al., 2007).

In the light of the aforesaid evidences, in this chapter studies were based on the dose and duration effects of the peel extracts in *in vivo* by using mouse as a working model and considering hepatic LPO as the end parameter. In this section four different doses (50-300 mg / kg) of all the test peels such as *Citrus sinensis* (CS), *Punica granatum* (PG), *Musa paradisiaca* (MP), *Mangifera indica* (MI), *Citrullus vulgaris* (CV), *Cucumis melo* (CM) and *Carica papaya* (CP) were administered for 15 days, based on only available reference of *in vivo* study (Chidambara-Murthy et al., 2002).
Selected doses were further used for the standardization of treatment, where 15 days and 10 days were considered. Lower doses were also explored for two peels where the minimum concentration (50 mg / kg) was also found to be toxic.

4.2 Experimental Design

Total of nine experiments (Exp. 9-17) were carried out in healthy male mice of similar age group (Body wt. 28 ±2 gm) using all the seven peel extracts. In the first four experiments effects of four different doses (50, 100, 200 and 300 mg / kg) of each peel extract were studied for 15 days.

In expt 9, sixty three mice were randomly divided into nine groups of seven each. While, group I animals receiving vehicle (D.W.) only served as controls, animals of group II, III, IV and V received 50, 100, 200 and 300 mg / kg of CM respectively. Similarly, animals of group VI, VII, VIII and IX received the equivalent doses (50, 100, 200 and 300 mg / kg) of CS peel extract. Treatment was continued for 15 consecutive days. At the end of the experiments overnight fasted animals were sacrificed after mild anesthesia and liver was removed immediately from each animal, cleaned with 0.1 M PBS (pH 7.4), cleaned off the blood clots, homogenized in PBS and then homogenates of each animal were processed for the study of hepatic LPO (See Material and Methods section for further details). Similarly, experiments (10, 11 and 12) were performed using PG and MP, CV and CP, and MI respectively.

As CS and CP extracts appeared to be toxic in all the four doses used earlier, another experiment (Exp 13) was conducted for these two peels considering three lower doses (6.25, 12.50 and 25 mg / kg) for the same duration of 15 days.

In order to study the duration dependent effects four more experiments (experiment 14 to 17) were performed considering 10 days and 15 days of treatments. However, in these experiments only the selected dose
of each peel extract was used, which was otherwise found to be maximally effective in reducing hepatic LPO.

In experiment fourteen, forty two mice were divided into three groups of 14 each. While, group I animals receiving vehicle (D.W.) only served as controls, animals of group II and III were treated with CS (25 mg / kg) and CP (6.25 mg / kg), respectively. After 10 days of treatment 07 animals from each group were sacrificed and hepatic LPO was studied in the same manner as described earlier. Rest of the animals continued to receive the similar treatments for five days more before they were sacrificed and effects were seen on the hepatic LPO. Similar to this experiment, three more experiments were performed (experiment 15 to 17) using five other peel extracts considering their most effective doses (100 mg / kg of CM, CV, MP and 200 mg / kg of MI and PG, as observed in first four experiments).

### 4.3 Results

**Effects of different peel extracts on hepatic LPO: Dose standardization (Figs. 9-13)**

Administration of CM, CV, MP, MI and PG peel extracts for 15 days to animals at 100 mg / kg (for previous three) and 200 mg / kg (the last two) were found to inhibit hepatic LPO maximally significantly \( P<0.01 \) for CM and \( P<0.001 \) for rest all), while the two higher doses (200 and 300 mg / kg) of CM were found to be prooxidative \( P<0.05 \) and \( P<0.01 \) respectively). PG extract at 300 mg / kg and 200 mg / kg of MP were also found to be significantly peroxidative \( P<0.01 \) for both). The highest percent inhibition in LPO was calculated to be 19.90, 40.02, 48.02, 35 and 56 % for CM, CV, MP, MI and PG, respectively (either at 100 or 200 mg / kg).

Administration of CS and CP peel extracts at all the four studied doses (50-300 mg / kg) enhanced hepatic LPO significantly \( P<0.001 \) at all 4 doses and both the peels). Results of another experiment at lower doses of
CS and CP revealed CS as antiperoxidative at 25 mg / kg ($P<0.001$), with 37.03 % inhibition; while CP was still found to be toxic at 12.5 and 25 mg / kg ($P<0.01$ and $P<0.001$, respectively).

**Effects of different peel extracts on hepatic LPO: Duration standardization (Figs. 14-17)**

The results of duration dependent effects of test peel extracts on hepatic LPO at selected doses indicated that, 10 days administration of all the test peel extracts except CP significantly reduced the hepatic LPO ($P<0.001$ for all). Similarly, the treatment of 15 days with all the six peel extracts (CS, PG, MP, CV, CM and MI) inhibited the hepatic LPO ($P<0.001$ for first three and $P<0.01$ for last three). However, the percent inhibition of LPO was calculated out to be 49.04, 64.11 and 50 % for CS, PG and MP, respectively after 10 days of treatment, and nearly similar inhibition was also observed (38.13, 59.03 and 48.06 % respectively) after 15 days. In case of CM, CV and MI peel extracts maximum inhibition was observed only after 10 days treatment where the reduction was found to be 39.07, 63.01, and 56.16 % respectively.

**4.4 Discussion**

From the results of this study it was evident that different peel extracts were effective at different concentrations and at different duration of the treatments. Out of all the concentrations, 25 mg / kg of CS; 200 mg / kg of PG and MI; 100 mg / kg of MP, CV and CM were found to be most effective to inhibit hepatic LPO, when treated for 15 days. Duration dependent study indicated that both 15 and 10 days of treatments although appear to be equally effective for CS, PG and MP peel extracts. % inhibition analyses indicated that 10 days duration is more effective for all the peels. However, all the studied doses of CP peel extract were found to be either toxic or ineffective. Therefore, no further experimentation on CP is justified.
The observed dose and duration dependent effects on the peel extracts are not unexpected as, it is a well established phenomenon that the effect of drug depends upon different pharmacologic variables including absorption, distribution, half life, elimination of the drug, saturation concentration (a dose beyond which no further increment in effects can be achieved), sensitivity of the target organs and the difference in the physiological conditions (Katzung, 1998). In fact, similar dose and duration dependency for the efficacy of the herbal extracts have also been reported by earlier workers (Panda and Kar, 1998; Kar and Panda, 2004, 2005; Parmar et al., 2006).

In this study, CS, PG and MP peel extracts were found to be more or less equally effective after both 10 and 15 days of treatment, while MI, CV and CM were found to be highly effective when administered for 10 days (and not after 15 days). This duration dependent differential effect is in accordance with the earlier reports on herbal medicines that also indicate the equipotent nature of the same drug in different durations of treatments (Liu et al., 1997, 2007; Sungnoon and Chattipakorn, 2005; Kawahara et al., 2007; Zu et al., 2007). Thus, the present findings suggest that longer duration of treatment may not necessarily bring better effect always.

So far, dose dependency is concerned, it might be mediated through the concentration dependent action of the ascorbic acid and naturally occurring polyphenolic compounds present in the studied peel extracts (Laughton et al., 1989; Stadler et al., 1995; Yoshino and Murakami, 1998; Sakagami et al., 1999; Racek et al., 2000; Pinelo et al., 2004; Joubert et al., 2005). It is also well known that these compounds exhibit dual behaviour in dose dependent manner, as at one concentration antiperoxidative and on the other one pro-oxidative in nature (Laughton et al., 1989; Stadler et al., 1995; Yoshino and Murakami, 1998; Sakagami et al., 1999; Racek et al.,
2000; Pinelo et al., 2004; Joubert et al., 2005). In fact, dual and reverse effects of some herbal extracts have been revealed earlier from our laboratory (Panda and Kar, 1997, 1998). Therefore, the increase in the hepatic LPO by higher doses of CS and CM could be the result of this prooxidative effects.

Whatever may be the mode of actions, findings of the present study, reveal differential effects for different peel extracts, depending on the concentrations and durations of treatment used. Therefore, it appears that, some of the peel extracts (MI, CV and CM) exhibit better antiperoxidative activities following 10 days of treatment as compared to that of 15 days. Even in case of PG, CS and MP, although the effects of both 10 and 15 days treatments did not have much differences, 10 days of treatment proved to be good enough for their antiperoxidative activities, suggesting the consideration of this duration for further experimentations.
Table 1: Relative percentage of increase (+) or decrease (-) in hepatic lipid peroxidation as compared to the values of respective control animals following the administration of different concentrations (6.25 to 300 mg / kg, presented in top raw) of the peel extracts of *C. sinensis* (CS), *P. granatum* (PG), *M. paradisiaca*, *M. indica* (MI), *C. vulgaris* (CV), *C. melo* (CM) and *C. papaya* (CP) for 15 days to normal healthy mice:

<table>
<thead>
<tr>
<th>Peel extract</th>
<th>6.25</th>
<th>12.50</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>-2.19</td>
<td>-3.29</td>
<td>-37.03*</td>
<td>+52.17</td>
<td>+79.63</td>
<td>+85</td>
<td>+94</td>
</tr>
<tr>
<td>PG</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-1.31</td>
<td>-16.05</td>
<td>-56*</td>
<td>-29</td>
</tr>
<tr>
<td>MP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-21.05</td>
<td>-48.02*</td>
<td>-26</td>
<td>-16</td>
</tr>
<tr>
<td>MI</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-10</td>
<td>-1.19</td>
<td>-35*</td>
<td>-7.1</td>
</tr>
<tr>
<td>CV</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-22</td>
<td>-40.02*</td>
<td>-3.1</td>
<td>-8.0</td>
</tr>
<tr>
<td>CM</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>-0.457</td>
<td>-19.90*</td>
<td>+24</td>
<td>+39</td>
</tr>
<tr>
<td>CP</td>
<td>+6.59</td>
<td>+34.06</td>
<td>+53.54</td>
<td>+150</td>
<td>+220</td>
<td>+37</td>
<td>+600</td>
</tr>
</tbody>
</table>

NA: Not Applicable; *, Maximum inhibition.
Table 2: Relative percentage of increase (+) or decrease (-) in hepatic lipid peroxidation (LPO) as compared to that of respective control values, following the administration of 25 mg / kg of *C. sinensis*, 200 mg of *P. granatum* and *M. indica*, 100 mg / kg of *C. vulgaris*, *C. melo* and *M. paradisiaca* and 6.25 mg / kg of *C. papaya* peel extracts for 10 and 15 days durations to normal healthy male mice:

<table>
<thead>
<tr>
<th>Peel extract</th>
<th>10 days duration</th>
<th>15 days duration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. sinensis</em></td>
<td>-49.04</td>
<td>-38.13</td>
</tr>
<tr>
<td><em>P. granatum</em></td>
<td>-64.11</td>
<td>-59.03</td>
</tr>
<tr>
<td><em>M. paradisiaca</em></td>
<td>-50.0</td>
<td>-48.06</td>
</tr>
<tr>
<td><em>M. indica</em></td>
<td>-56.16</td>
<td>-31.88</td>
</tr>
<tr>
<td><em>C. vulgaris</em></td>
<td>-63.01</td>
<td>-42.02</td>
</tr>
<tr>
<td><em>C. melo</em></td>
<td>-39.07</td>
<td>-22.10</td>
</tr>
<tr>
<td><em>C. papaya</em></td>
<td>+3.10</td>
<td>+6.77</td>
</tr>
</tbody>
</table>

Note: Higher inhibition was there after 10 days of treatment.
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Legends to the figures

**Figure 9**: Effects of the different concentrations (50, 100, 200 and 300 mg / kg) of *Cucumis melo* (CM) and *Citrus sinensis* (CS) peel extracts for 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, $P<0.001$; b, $P<0.01$; c, $P<0.05$ as compared to the respective control values (vehicle treated).

**Figure 10**: Effects of the different concentrations (50, 100, 200 and 300 mg / kg) of *Punica granatum* (PG) and *Musa paradisiaca* (MP) peel extracts for 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, $P<0.001$; b, $P<0.01$; c, $P<0.05$ as compared to the respective control values (vehicle treated).

**Figure 11**: Effects of the different concentrations (50, 100, 200 and 300 mg / kg) of *Citrullus vulgaris* (CV) and *Carica papaya* (CP) peel extracts for 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, $P<0.001$; b, $P<0.01$; c, $P<0.05$ as compared to the respective control values (vehicle treated).

**Figure 12**: Effects of the different concentrations (50, 100, 200 and 300 mg / kg) of *Mangifera indica* (MI) peel extract for 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, $P<0.001$; b, $P<0.01$; c, $P<0.05$ as compared to the respective control values (vehicle treated).
Figure 13: Effects of the different concentrations (6.25, 12.50 and 25 mg / kg) of *Citrus sinensis* (CS) and *Carica papaya* (CP) peel extracts for 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, *P* <0.001; b, *P* <0.01; c, *P* <0.05 as compared to the respective control values (vehicle treated).

Figure 14: Effects of 200 mg / kg of *Punica granatum* (PG) and 100 mg / kg of *Musa paradisiaca* (MP) peel extracts for 10 and 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, *P* <0.001; b, *P* <0.01; c, *P* <0.05 as compared to the respective control values (vehicle treated).

Figure 15: Effects of 25 mg / kg of *Citrus sinensis* (CS) and 6.25 mg / kg of *Carica papaya* (CP) peel extracts for 10 and 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, *P* <0.001; b, *P* <0.01; c, *P* <0.05 as compared to the respective control values (vehicle treated).

Figure 16: Effects of 200 mg / kg of *Mangifera indica* (MI) and 100 mg / kg of *Citrus vulgaris* (CV) peel extracts for 10 and 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, *P* <0.001; b, *P* <0.01; c, *P* <0.05 as compared to the respective control values (vehicle treated).

Figure 17: Effects of 100 mg / kg of *Cucumis melo* (CM) peel extract for 10 and 15 days on the alterations in hepatic lipid peroxidation (LPO, nM MDA formed/ hr/ mg protein) in male mice. Data are mean ± SEM. (n=7); a, *P* <0.001; b, *P* <0.01; c, *P* <0.05 as compared to the respective control values (vehicle treated).
Fig. 9

nM MDA/hr/mg protein

Groups
CM
CS

0 50 100 200 300

a a a a a

b b c b

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Fig. 10

The figure shows the levels of MDA (mg protein) over time, with different concentrations treated with groups PG and MP. The y-axis represents the MDA levels in nM/hr/mg protein, while the x-axis represents different concentration levels. The figure includes error bars indicating the standard deviation. The groups are labeled with distinct markers ('a' and 'b'), indicating significant differences between the groups at different concentrations.
Fig. 11

The graph shows the formation of nM MDA (thiobarbituric acid-reactive substances) in different groups over time. The x-axis represents the groups labeled CV and CP, while the y-axis represents the nM MDA formed per hour per mg protein. Different concentrations (0, 50, 100, 200, 300) are indicated on the y-axis. The graph includes error bars indicating the variability of the data. Significance is marked with 'a'.
Fig. 13

<table>
<thead>
<tr>
<th>Groups</th>
<th>nM MDA/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>0.6</td>
</tr>
<tr>
<td>CP</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Legend:
- □ 0
- □ 6.25
- □ 12.5
- □ 25

Significant differences:
- a compared to CS
- b compared to CP
Fig. 14

Durations

nM MDA/hr/mg protein

10 days 15 days

Ctrl PG MP

Legend

a

Note: The figure shows the levels of nM MDA/hr/mg protein for different durations and conditions.
Fig. 15

![Bar chart showing the effect of different durations on nM MDA/hr/mg protein. The chart compares Ctrl, CS, and CP groups.](chart.png)
Fig. 16

![Graph showing nM MDA/hr/mg protein over 10 and 15 days with different groups labeled Ctrl, MI, and CV, with markers indicating statistical differences.]

- Ctrl group shows a peak at 10 days followed by a decrease at 15 days.
- MI group has a relatively flat profile with a slight increase at 15 days.
- CV group shows a moderate increase from 10 to 15 days.

Statistical significance indicated by:
- a: Significant difference from Ctrl at 10 days.
- b: Significant difference from MI and CV at 15 days.
Fig. 17

![Graph showing nM MDA/hr/mg protein for 10 and 15 days for Ctrl and CM groups.](image)

- **Ctrl** group shows a decrease in nM MDA/hr/mg protein from 0.7 at 10 days to 0.6 at 15 days.
- **CM** group shows an increase in nM MDA/hr/mg protein from 0.6 at 10 days to 0.7 at 15 days.

- The graph indicates a statistically significant difference between the Ctrl and CM groups at both 10 and 15 days.
- The Ctrl group at 10 days is denoted by 'a' and the Ctrl group at 15 days is denoted by 'b'.