This chapter deals with assessment of free radical scavenging / antiperoxidative potential of different fruit peels using in vitro studies, where different chemical methods such as inhibition of DPPH and NO radicals, beta-carotene bleaching and biochemical models including $\text{H}_2\text{O}_2$ induce lipid peroxidation in rat erythrocytes and liver tissue have been considered. Dose and method specific radical scavenging properties of all the test peel extracts have been studied which provide the informations on the varying degree of anti-peroxidative activity of the extracts.
3.1 Introduction

Dietary antioxidants, including polyphenolic compounds, vitamin C and carotenoids are believed to act as effective supplements in the prevention of some oxidative stress related diseases (Ames et al., 1995; Kaur and Kapoor, 2001). While on one hand, various epidemiological studies have established an inverse correlation between the intake of fruits and the occurrence of health related problems such as cardiovascular diseases, cancer, diabetes and ageing (Leontowicz et al., 2003; Tiwari, 2004; Takachi et al., 2008), on the other hand, fruit peels are gradually emerging as potential source of antioxidants with possession of rich amount of flavonoids, polyphenolics, ascorbic acid, dietary fibers and dopamine (Kanazawa and Sakakibara, 2000; Higashi-Okai et al, 2002; Leontowicz et al., 2003). In fact, a good number of investigations have been made on the antiperoxidative nature of the plant extracts (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). However, on this aspect, peels from many commonly available fruits were not tried earlier. Particularly, using in vitro study which is commonly considered as a prerequisite before proceeding for in vivo investigations, practically very limited attempts were made (Gorinstein et al., 2001; Singh et al., 2002; Leontowicz et al., 2002, 2003).

There are many methods for total antioxidant determination, and every one has its limitations (Gorinstein et al., 2001; Ou et al., 2002; Yu et al., 2002). Some in vitro methods are thought to be nonspecific, while some others are more specific for the determination of antioxidative potential, particularly in fruits and peels (Gorinstein et al., 2001; Leontowicz et al., 2003). Previous work of our laboratory had indicated dose specific
antiperoxidative effects of some other herbal extracts in, *in vivo* (Panda and Kar, 1998, 2003a; Popat et al., 2001; Oh and Small, 2002; Githiori et al., 2006; Parmar et al., 2006). It was therefore expected that the test fruit peel extracts may possess some dose dependent antioeroxidative effects. Therefore, an investigation was made considering different concentrations of different fruit peel extracts, selected on the basis of their ethno-pharmacological properties, chemical constituents and available reports on various other biological properties on the officinal parts of the test plants, as mentioned in general introduction. Thus, the *in vitro* study was carried out primarily to evaluate the direct radical scavenging / antiperoxidative effects of the peel extracts of *Citrus sinensis* (CS), *Punica granatum* (PG), *Musa paradisiaca* (MP), *Citrullus vulgaris* (CV), *Cucumis melo* (CM) *Mangifera indica* (MI) and *Carica papaya* (CP) by using some recommended in vitro methods to understand dose specificity and method specific trends in the radical scavenging activities, if any.

3.2. Experimental Design

*Chemical in vitro analyses*

Three independent experiments were carried out to evaluate the direct free radical scavenging activities in the peel extracts, following the specific methods for the evaluation of the antiperoxidative activities of fruits/ fruit peels (Leontowicz et al., 2003). These include (1) *Radical scavenging activity (RSA) using the 1, 1 Diphenyl-2-picryl hydrazyl (DPPH) method*; (2) *Antioxidant assay using β-carotene bleaching* and (3) *NO radicals scavenging activity*. Three different doses of each peel extract (25, 50 and 100 ppm concentration) were used each in five sets (Leontowicz et al., 2003). Butylated hydroxyl anisole (BHA) was considered as a standard antioxidant for comparison.
Biochemical in vitro analysis:

In this study, lipid peroxidation was induced by H$_2$O$_2$ in rat RBCs and in chopped liver tissue. Erythrocytes were considered because they contain high amount of PUFA (polyunsaturated fatty acids), ferrous ions and molecular oxygen that make them vulnerable to oxidative stress (Clemens and Waller, 1987; Yadav et al., 1997) and liver was considered as a primary drug target site (Ganong, 2005). Four different doses of the test peel extracts were used for this purpose (0.25, 0.5, 1.0 and 2.0 μg/ml) each in five sets. Here also BHA was used as a standard antioxidant at respective doses.

In order to assess the possible reason of the radical scavenging activities in the test peel extracts, total polyphenols, total flavonoids and ascorbic acid contents of the peel extracts were also determined (Omaye et al., 1979; Higashi-Okai et al., 2002; Leontowicz et al., 2003). For details of the analysis "general materials and methods" section may be seen.

Statistics

Data were analyzed by non parametric analysis of variance (ANOVA) followed by post hoc Newman-Keuls multiple comparison test, using a trial version of Graph pad prism version 4 for Windows (GraphPad, San Diego, CA).

3.3. Results

Although peel extracts showing more than 50% free radical scavenging or antioxidative potential were considered to be highly effective, almost all peels were found to be antiperoxidative either in one or in other chemical method.

DPPH radical scavenging activity (Fig. 1)

Observations by radical scavenging assay revealed that at 25 ppm, the highest activity was exhibited by CS, MP, and CV (95.65, 61.37, and 18.02 % respectively); while at 50 ppm it was 152.07, 19.31, and 17.96 for PG,
CM, and MI respectively. Of course, 100 ppm could not exhibit highest activity by any of the test peel extract. However, the higher (50 % or more) radical scavenging activity was observed only in 25 ppm of CS and MP extracts and in all three concentrations of PG, maximum being at 50 ppm of the later one. Interestingly, all the studied doses of CP were found to be pro-oxidative.

\( \beta \)-carotene bleaching activity (Fig. 2)

Results of \( \beta \)-carotene bleaching revealed a greater activity at 100 ppm of PG, CM and CP (120.21, 149.53 and 148.39 % respectively), while for MP, MI and CV it was observed at 50 ppm (121.48, 17.96 and 165.19 % respectively) and only CS could exhibit higher activity at 25 ppm (67.68 %). In fact, 50 % or more was exhibited by all the peel extracts, except MI, while the maximum activity was noticed at 50 ppm of CV.

NO-radical scavenging activity (Fig. 3)

NO radical scavenging assay indicated some what positive activity of CS, PG, MP and CV at 25 ppm (33.47, 52.06, 51.38 and 31.38 %, respectively) and at 50 ppm of CM, MI and CP (20.31, 17.11 and 38.98 %, respectively). Thus more than 50 % NO-radical scavenging activity was observed at 25 ppm of PG and MP only.

Inhibition of \( H_2O_2 \)-induced LPO in erythrocytes (Fig. 4)

With respect to the inhibition of \( H_2O_2 \)-induced LPO in erythrocytes, all the test peels were found to be effective in one or the other doses. The inhibition was greater at a dose of 0.5 \( \mu g /ml \) for CS, PG, MI and CP (119.67, 140.33, 95.12 and 82.62 %, respectively) and at 0.25 \( \mu g /ml \) for MP, CM and CV (147.31, 89.45 and 111.18 % respectively). However, maximum being at 0.25 \( \mu g /ml \) of MP.
Inhibition of $\text{H}_2\text{O}_2$-induced LPO in liver tissue (Fig. 5)

$\text{H}_2\text{O}_2$-induced LPO in chopped liver tissue was inhibited by all the peel extracts at one or the other doses. Although it was reduced to a greater extent (> 50 %) at a dose of 0.5 μg /ml of CS, MP and CM (123.12, 160.19 and 107.91 %, respectively), at 1.0 μg /ml of PG and MI (121.78 and 90.94 % respectively), and at 0.25 μg /ml of CV and CP (131.0 and 111.78 % respectively). The maximum activity was observed at 0.5 μg /ml of MP.

Determination of total polyphenols in peel extracts (Fig. 6)

High content of total phenolic compounds was found in most of the test peel extracts except in MP and CP extracts, where it was only 87 and 265 mg / 100 gm of dry weight respectively.

Determination of total flavonoids in peel extracts (Fig. 7)

Very rich amount of total flavonoids was measured in the test peel extracts of CS, PG, and MI and the highest was observed in PG.

Determination of ascorbic acid content (Fig. 8)

Determination of total ascorbic acid contents revealed its presence in very rich amount invariably in all the test peel extracts.

3.4 Discussion

From the results, it was evident that different fruit peel extracts exhibit varying degree of free radical scavenging / antiperoxidative potential depending on their concentrations and on the type of assay system used. Particularly, the DPPH assay method exhibited a different trend, where, unlike other test peels, CP extract was found to be pro-oxidative. In fact, marked inhibition (more than 50%) of DPPH radicals was observed only by CS, PG and MP peel extracts. These observations are similar to that of some
other fruit peels (Higashi-Okai et al., 2002; Singh et al., 2002; Leontowicz et al., 2002, 2003). Similar to CP also toxic or pro-oxidative effects are reported by some other herbal extracts (Alebiowu et al., 2007; Saini et al., 2007; Yu et al., 2007).

DPPH is synthetic, relatively stable nitrogen radical and this assay system is based on electron transfer, where antioxidant compound reduces the oxidant by the donation of electron which results in changes of color and subsequently in absorbance (Huang et al., 2005; Letelier et al., 2007). Using DPPH radicals several investigations have been made earlier considering some other natural products, and is believed to act as a suitable model compound for free radicals, originating in lipids (Brand Williams et al., 1995; Chen et al., 1999; Sanchez-Moreno et al., 1999). Thus, it appears that in our test peel extracts, particularly in CS, PG and MP, considerable amount of free radical scavenging compounds (primarily responsible for direct electron donation) are present. As only few reports are available on the DPPH radicals scavenging activity of fruit peels (Higashi-Okai et al., 2002; Singh et al., 2002; Leontowicz et al., 2002, 2003), present findings are certainly significant indicating their possible potential to inhibit other free radicals as well as lipid peroxidation. However, only one peel extract (CP) appeared to be negatively involved as it increased the color intensity of the solution, suggesting that it might be possessing some compounds similar to DPPH radicals or compounds having similar absorption maxima (517 nm). Other three test peels (MI, CV and CM) did not quench the DPPH radicals substantially. For these, it can be suggested that they may not work through electron donation.

Singlet oxygen produced during photosensitized oxidations can cause cellular damage by reacting with DNA and proteins, or by inducing lipid peroxidation (Steven et al., 1993) and β-carotene commonly protects photosensitized tissue injury by scavenging free radicals/ quenching singlet
oxygen. Because of this, β-carotene is very often employed clinically to prevent photosensitized tissue damage in humans with erythropoietic porphyria (Sies and Stahl, 2004). Because of the significance of singlet oxygen radicals in biological systems, β-carotene bleaching assay is considered as an elegant model to evaluate the singlet oxygen scavenging potential of various natural products (Steven et al., 1993; Huang et al., 2005).

In the present study results of the β-carotene bleaching assay showed that except MI, all the test peel extracts exhibit marked antiperoxidative or radical scavenging activities, suggesting their potential for scavenging singlet oxygen, evolved in the reaction (Huang et al., 2005). Similar observations using β-carotene bleaching assay have also been made by earlier authors (Ebermann et al., 1996; Saija et al., 1999). However, on peel extracts, only limited investigations have also been made (Singh et al., 2002; Leontowicz et al., 2002, 2003). The present observations therefore indicate that most of the test peels have the potential to inhibit oxyradicals and may act as antioxidants in in vivo system.

NO radicals are known to be involved in cytotoxicity and can also interact with superoxide anions resulting in the formation of peroxynitrite (ONOO-), which is the most reactive nitrogen species (RNS) (Lisu et al., 2006). When the NO-radical scavenging activity of the experimental peels were evaluated, results revealed that only PG and MP peel extracts are capable to reduce NO radicals substantially (>50 %). However, CS, CV and CP peel extracts could also inhibit NO radical generation to appreciable extent. Although literature on the NO radical scavenging activity of fruit peels is meager (Leontowicz et al., 2002, 2003), some reports are already available on other natural products (Marcocci et al., 1994; Saija et al., 1999; Lisu et al., 2006), suggesting that most test peels have the potential to scavenge NO radicals. It appears that the NO scavenging activity in the test
peels could be due to the presence of bioactive components, as suggested in some other fruit peel extracts (Leontowicz et al., 2002, 2003).

As reactive oxygen species (ROS) are believed to be involved in the pathogenesis of many of the diseases including thyroid, cardiac problems, diabetes and cancer (Kar and Panda, 2004; Tiwari, 2004), it was of great importance to evaluate the ROS scavenging potential of the test peel extracts. In fact, H$_2$O$_2$ induced lipid peroxidation is believed to be the results of ROS formation. Therefore, treating RBCs and chopped liver tissue was earlier found to be a suitable biochemical in vitro model to evaluate the antiperoxidative potential of herbal extracts (Stocks and Dormandy, 1971; Clemens and Waller, 1987; Yadav et al., 1997; Barillari et al., 2006). Erythrocytes are considered suitable, because they contain high concentration of polyunsaturated fatty acids (PUFA), ferrous ion and molecular oxygen, which make them vulnerable to oxidative stress (Clemens and Waller, 1987), while liver is considered as it is a major drug target site (Ganong, 2005). In the present investigation, H$_2$O$_2$ induced lipid peroxidation in RBCs and in liver slices was inhibited by all the test peel extracts at one or the other concentrations, suggesting their potential to scavenge peroxy radicals generated by H$_2$O$_2$ (Huang et al., 2005) as reported by some earlier workers (Yadav et al., 1997; Lisu et al., 2006; Yadav and Bhatnagar, 2007). One of the possible reasons for this is that, the test peel extracts might be acting through the enzymatic and / or non enzymatic cellular oxidative defense system or through the direct quenching of peroxy radicals as suggested earlier for some herbal extracts, which also inhibit LPO (Parmar et al., 2006; Yadav and Bhatnagar, 2007; Panda and Kar, 2007b).

Reviewing all these findings it appears that antiperoxidative activities of test peel extracts exhibit method specific trends, as was thought earlier
(Ou et al., 2002). It could be due to the differences in the principles upon which assay systems are based and/or different mechanisms of action and the radical specific scavenging activity of studied peel extracts (Huang et al., 2005; Lisu et al., 2006). We further emphasize that the observed radical scavenging activity of the test peel extracts might have been mediated through the presence of polyphenolic components as correlated by earlier workers (Leontowicz et al., 2003; Pinelo et al., 2004). This possibility is further supported by high contents of total phenolics, flavonoids and ascorbic acid contents in the test peel extracts (Fig. 6, 7 and 8). Interestingly, some dose specific / dependent activities were observed similar to our earlier reports, based on in vivo studies (Panda and Kar, 2003a; Kar and Panda, 2004, 2005; Parmar et al., 2006). This could be the result of the variety of polyphenols and their varying concentrations present in the test peel extracts. This is further supported by some earlier findings that the radical scavenging capability of phenolic compounds are due to their hydrogen donating ability/ number of hydroxyl groups present, which in turn is closely related both to the chemical structure and spatial conformation, that can modify the reactivity of the molecules (Ariga and Hamano, 1990; Chen and Ho, 1995). In fact, it is also well established that naturally occurring polyphenolic compounds and ascorbic acid exhibit dual behavior in dose dependent manner as their antiperoxidative and/or pro-oxidative nature depends upon the dose at which it is used (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 conclusion the present findings clearly suggest that the test peels may have the potential to quench free radicals and also the tissue lipid peroxidation indicating their possible use as antioxidants. However, they exhibit varying degree of antiperoxidative activities in method specific and dose dependant manner. Therefore, it is suggested that the drug efficacy
evaluations by *in vitro* methods may not be considered as conclusive without further *in vivo* studies. Obviously later investigation is a must for each herbal extract.
Legends to the figures

**Fig. 1**: DPPH radical scavenging activity (RSA), expressed in % inhibition, as compared to that of standard antioxidant, Butylated hydroxy anisole (BHA) by different fruit peel extracts such as CS (*Citrus sinensis*), PG (*Punica granatum*), MP (*Musa paradisiaca*), MI (*Mangifera indica*), CV (*Citrullus vulgaris*), CM (*Cucumis melo*) and CP (*Carica papaya*) at 25, 50 and 100 ppm concentrations. Values are means ± SE of five measurements. Means in bars for the same concentration (25 or 50 or 100 ppm) without common letters differ significantly (*P* ≤ 0.05). Inverted bars represent the % increase in DPPH radicals by CP.

**Fig. 2**: Inhibition in β-carotene bleaching (%) as compared to that of standard antioxidant Butylated hydroxy anisole (BHA) by the fruit peel extracts of CS (*Citrus sinensis*), PG (*Punica granatum*), MP (*Musa paradisiaca*), MI (*Mangifera indica*), CV (*Citrullus vulgaris*), CM (*Cucumis melo*) and CP (*Carica papaya*) at 25, 50 and 100 ppm concentrations. Values are means ± SE of five measurements. Means in bars for the same concentration (25 or 50 or 100 ppm) without common letters differ significantly (*P* ≤ 0.05).

**Fig. 3**: NO radical scavenging activity (% inhibition) by fruit peel extracts of CS (*Citrus sinensis*), PG (*Punica granatum*) MP (*Musa paradisiaca*), MI (*Mangifera indica*), CV (*Citrullus vulgaris*), CM (*Cucumis melo*) and CP (*Carica papaya*) at 25, 50 and 100 ppm concentrations. Values are means ± SE of five measurements. Means in bars for the same concentration (25 or 50 or 100 ppm) without common letters differ significantly (*P* ≤ 0.05).
**Fig. 4:** Inhibition of H$_2$O$_2$ induced LPO in erythrocytes (in %) by fruit peel extracts of CS (*Citrus sinensis*), PG (*Punica granatum*), MP (*Musa paradisiaca*), MI (*Mangifera indica*), CV (*Citrullus vulgaris*), CM (*Cucumis melo*) and CP (*Carica papaya*) at 0.25, 0.50, 1.00 and 2.00 μg / ml concentrations. Values are means ± SE of five measurements. Means in bars for the same concentration (0.25 or 0.50 or 1.00 or 2.00 μg / ml) without common letters differ significantly (P ≤ 0.05).

**Fig. 5:** Inhibition H$_2$O$_2$ induced LPO in chopped liver tissue and (in %) inhibition by fruit peel extracts of CS (*Citrus sinensis*), PG (*Punica granatum*), MP (*Musa paradisiaca*), MI (*Mangifera indica*), CV (*Citrullus vulgaris*), CM (*Cucumis melo*) and CP (*Carica papaya*) at 0.25, 0.50, 1.00 and 2.00 μg / ml concentrations. Values are means ± SE of five measurements. Means in bars for the same concentration (0.25 or 0.50 or 1.00 or 2.00 μg / ml) without common letters differ significantly (P ≤ 0.05).

**Fig. 6:** Total polyphenolic contents present in the peels of CS (*Citrus sinensis*), PG (*Punica granatum*), MP (*Musa paradisiaca*), MI (*Mangifera indica*), CV (*Citrullus vulgaris*), CM (*Cucumis melo*) and CP (*Carica papaya*) respectively (in mg gallic acid equivalents / 100 gm dry wt. of peel extract). Values are means ± SE of five measurements.

**Fig. 7:** Total flavonoids contents present in the peels of CS (*Citrus sinensis*), PG (*Punica granatum*), MP (*Musa paradisiaca*), MI (*Mangifera indica*), CV (*Citrullus vulgaris*), CM (*Cucumis melo*) and CP (*Carica papaya*) respectively (in mg quercetin equivalents / 100 gm dry wt. of peel extract). Values are means ± SE of five measurements.

**Fig. 8:** Total ascorbic acid contents present in the peels of CS (*Citrus sinensis*), PG (*Punica granatum*), MP (*Musa paradisiaca*), MI (*Mangifera indica*), CV (*Citrullus vulgaris*), CM (*Cucumis melo*) and CP (*Carica papaya*) respectively in mg / 100 gm dry wt. of peel extract. Values are means ± SE of five measurements.
Fig. 2

[Bar chart showing Beta carotene bleaching (% inhibition) for different peel extracts at 25 ppm, 50 ppm, and 100 ppm. The bars are labeled with letters (a, b, c, d, e, f) indicating statistical significance.]
Fig. 3

Inhibition in NO radical generation (%)

Peel extracts

25 ppm
50 ppm
100 ppm
Fig. 4

Inhibition in erythrocytes LPO (%)

0.25μg/ml
0.5 μg/ml
1.0 μg/ml
2.0 μg/ml

Peel extracts

CS  PG  MP  MI  CV  CM  CP
Fig. 5

Inhibition in hepatic LPO (%)
Fig. 6

The figure shows the quantity of gallic acid equivalent (mg/100 gm) in various fruit peels. The y-axis represents the quantity in gallic acid equiv., ranging from 0 to 2000. The x-axis lists the peels: CS, PG, MP, MI, CV, CM, and CP. The graph indicates that PG has the highest quantity, followed by MI, CV, CM, and CP, with MP having the lowest.
Fig. 7

Quantity in quercetin equiv. (mg / 100 gm)

CS  PG  MP  MI  CV  CM  CP
Fig. 8

The bar chart shows the quantity of ascorbic acid (mg/100 gm) in different fruit peels. The quantities are as follows:

- CS: 750 mg/100 gm
- PG: 700 mg/100 gm
- MP: 750 mg/100 gm
- MI: 900 mg/100 gm
- CV: 950 mg/100 gm
- CM: 850 mg/100 gm
- CP: 800 mg/100 gm