Section 4

Antioxidant constituents and antioxidant properties of natural products
Exposure to gamma radiations can lead to free radical or ROS formation by aqueous radiolysis in the cellular milieu. In the event of excessive production of ROS, the host antioxidant defence mechanisms may be overwhelmed, resulting in oxidative damage of cellular constituents (Weiss and Kumar, 1998). The antioxidants may specifically quench free radicals, chelate redox metals or interact with other antioxidants and regenerate them (Chan, 1993). Many synthetic antioxidants are used to retard the radiation mediated oxidative damages but the use of these synthetic antioxidants is restricted due to their high toxicity (Park et al., 2009). Natural and herbal extracts usually contain a large number of bioactive molecules, each with different properties, which render protection to most of the vital organs of living systems against oxidative stress (Jagetia et al., 2002). The literature strongly suggests that naturally occurring polyphenols (especially phenolics and flavonoids) and triterpenoids exhibit antioxidative, neuroprotective, radioprotective and anti-inflammatory effects (Chang and Lin, 2011; Zhang et al., 2011).

In view of the present understanding about the role of ROS mediated oxidative stress in pathogenesis of multiple diseases and beneficial role of antioxidants, attempts have been made to examine the antioxidant status of selected herbal and natural products i.e., TCE, PE, WSE and ARE. Therefore, in the present study total phenol, flavonoid and triterpenoid content of TCE, PE, WSE and ARE were analyzed. Since, antioxidant potential of a product is an index of the quantitative and qualitative profile of various antioxidants present in it, various in vitro antioxidant parameters including tendency to inhibit lipid peroxidation (LPO), free radical scavenging activity (FRSA), reducing power (RP) and metal chelating ability (MCA) in radical generating system were performed, to assess the antioxidant potential of TCE, PE, WSE and ARE.

**Total phenol, flavonoid and triterpenoid content**

The results of total phenols, flavonoids and triterpenoids contents of TCE, PE, WSE and ARE are presented in Table 5. Results showed that the total phenol content was maximum in TCE (395.5 ± 4.4 GAE mg/g) followed by PE (54.7 ± 40.8 GAE mg/g), while very low total phenol content was observed in WSE and ARE. Similarly, total flavonoid content was maximum in TCE (80.25 ± 5.9 QE mg/g) followed by PE (56.0 ± 3.2 QE mg/g), while
Table 5. Evaluation of total phenolic, total flavonoid and triterpenoid content of herbal and natural extracts

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TCE</th>
<th>PE</th>
<th>WSE</th>
<th>ARE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenol content¹</td>
<td>395.50 ± 4.4</td>
<td>54.7 ± 0.8</td>
<td>1.38 ± 0.1</td>
<td>1.70 ± 0.2</td>
</tr>
<tr>
<td>Total flavonoid content²</td>
<td>80.25 ± 5.9</td>
<td>56.0 ± 3.2</td>
<td>2.13 ± 0.2</td>
<td>1.85 ± 0.4</td>
</tr>
<tr>
<td>Total triterpenoid content³</td>
<td>10.62 ± 0.7</td>
<td>96.2 ± 3.3</td>
<td>12.53 ± 0.6</td>
<td>71.80 ± 4.2</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 4 replicates.
1= GAE mg/g of the test sample. The test sample (50 µl) was used for the estimation of polyphenols. Propyl gallate (1 mg/ml) in concentration range 10-50 µg was used as standard.
2= QE mg/g of the test sample. The test sample (100 µl) was used for the estimation of flavonoids. Quercitrin hydrate (1 mg/ml) in concentration range 20-100 µg was used as standard.
3= UAE mg/g of the test sample. The test sample (100 µl) was used for the estimation of triterpenoids. Ursolic acid (1 mg/ml) in concentration range 10-50 µg was used as standard.
WSE and ARE showed considerable low amount of flavonoids. Total triterpenoid content was maximum in PE (96.2 ± 3.3 UAE mg/g) followed by ARE (71.80 ± 4.2 UAE mg/g), WSE (12.53 ± 0.6 UAE mg/g) and TCE (10.62 ± 0.7 UAE mg/g).

The results showed that TCE was rich in phenols and flavonoids while, PE contained moderate amounts of phenol, flavonoid and relatively higher triterpenoid content. ARE contained moderate amount of triterpenoid content while, WSE contained low amount of phenol, flavonoid and triterpenoid content.

Inhibition of in vitro LPO

The ability of TCE, PE, WSE and ARE to inhibit iron induced LPO in rat liver homogenate was measured (Fig. 17). Control in presence of FeCl₃ (0.5 ml of 10 µM) without extract in the reaction mixture showed maximum (13.7 ± 0.3 nmol of MDA formed/mg of protein) MDA equivalents. However, the presence of the extracts TCE, WSE, PE and ARE in the radical generating system significantly inhibited LPO (P < 0.001) in presence of FeCl₃ as compared to control. The inhibition of iron induced LPO was found to be maximum in PE followed by TCE, ARE and WSE as compared to control in presence of FeCl₃.

Free radical scavenging activity (FRSA) using DPPH

The FRSA of TCE, PE, WSE and ARE was measured by reduction of DPPH (Table 6). All the four extracts showed significant DPPH radical scavenging activity. The results showed that maximum FRSA was found in TCE (93.97 ± 0.8 %) followed by PE (92.67 ± 1.4 %), while WSE and ARE showed 24.69 ± 1.9 % and 16.0 ± 1.7 % DPPH radical scavenging activity respectively. It is emphasized that both TCE and PE have higher FRSA than the positive control i.e. BHT.

Total antioxidant potential using FRAP

The total antioxidant potential of TCE, PE, WSE and ARE was assessed by FRAP values (Table 6). The results showed that maximum FRAP value was observed for TCE (730.43 ± 9.6 µM/g) followed by PE (33.99 ± 2.2 µM/g), while FRAP values for WSE and ARE were low (1.01 ± 0.2 µM/g and 0.73 ± 0.1 µM/g respectively).
Fig. 17. In vitro inhibition of iron induced LPO in presence of extracts. LPO in presence of FeCl$_3$ (0.5 ml of 10 µm) for 1 hr without extract was 13.7 ± 0.3 nmol of MDA formed/mg of protein.

Values are mean ± SE of 4 replicates.

***P < 0.001 as compared to LPO in presence of FeCl$_3$. 

[Graph showing MDA formed (nmol/mg protein) for different extracts, with FeCl$_3$, TCE, PE, WSE, and ARE labeled.]
Table 6. Free radical scavenging activity and total antioxidant potential of herbal and natural extracts

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TCE</th>
<th>PE</th>
<th>WSE</th>
<th>ARE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRSA&lt;sup&gt;1&lt;/sup&gt;</td>
<td>93.97 ± 0.8</td>
<td>92.67 ± 1.4</td>
<td>24.69 ± 1.9</td>
<td>16.0 ± 1.7</td>
</tr>
<tr>
<td>TAP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>730.43 ± 9.6</td>
<td>33.99 ± 2.2</td>
<td>1.01 ± 0.2</td>
<td>0.73 ± 0.1</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 4 replicates.

1= % of DPPH radical scavenging activity. The test sample (100 µl) was used for the estimation of FRSA. BHT (1-5 mM) as a positive control showed proportionate increase in % DPPH radical scavenging activity. BHT (5 mM) showed 52.3 % DPPH radical scavenging activity.

2= µM/g of the test sample. The test sample (100 µl) was used for the estimation of FRAP values. FeSO₄•7H₂O in a concentration range 0.015-0.075 µM (10-50 µl) was used as the standard.
Reducing power

The reducing ability of the TCE, PE, WSE and ARE was assessed by the reduction of ferric ions and it served as an indicator of their potential antioxidant activity. The RP of TCE, WSE and PE increased with the increasing concentrations of the extract, while RP of ARE was not altered in concentration dependent manner (Fig. 18-22). The results showed that TCE and PE showed higher reducing ability at a very low concentration range (0.25 – 1.00 mg) and in a concentration dependent manner (Fig. 18 and 19), while WSE and ARE showed RP at higher concentration range (2.5 – 10.0 mg) (Fig. 20 and 21). RP of ARE showed no variations with increasing concentrations but was still higher than WSE at relatively all concentrations of the extract. When compared at the same concentration (1mg) of the extracts, reducing power was maximum for TCE followed by PE, ARE and WSE (Fig. 22).

Metal chelating activity

The MCA of the TCE, PE, WSE and ARE was measured by the ability of the extract to chelate ferrous ions and thereby inhibiting the formation of Fe$^{2+}$-Ferrozine complex. The MCA of TCE and ARE increased with the increasing concentrations of the extract, while MCA of PE and WSE was not altered in concentration dependent manner (Fig. 23-27). TCE and PE showed higher MCA at a very low concentration range (0.25 – 1.00 mg) (Fig. 23 and 24) while WSE and ARE showed MCA at higher concentration range (2.5 – 10.0 mg) (Fig. 25 and 26). At the same concentration (1 mg) of the extracts, MCA was maximum for TCE followed by PE, WSE and ARE (Fig. 27).

Discussion

Phenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds (Chen et al., 2002; Djeridane et al., 2006; Luximon-Ramma et al., 2005). The antioxidant activity is believed to be mainly due to their redox properties, which plays an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Yingming et al., 2004).
Fig. 18. Reducing power of different concentrations of TCE. Values are mean ± SE of 4 experiments. All experimental values were significantly higher (P < 0.05) as compared to control.

Fig. 19. Reducing power of different concentrations of PE. Other details are as shown in Fig. 18.
Fig. 20. Reducing power of different concentrations of WSE. Other details are as shown in Fig. 18.

Fig. 21. Reducing power of different concentrations of ARE. Other details are as shown in Fig. 18.
Fig. 22. Comparison of reducing power of different herbal and natural extracts at same concentration (1 mg). Values are mean ± SE of 4 experiments.
Fig. 23. Metal chelating effect of different concentrations of TCE. Values are mean ± SE of 4 experiments. All experimental values were significantly higher (P < 0.05) as compared to control.

Fig. 24. Metal chelating effect of different concentrations of PE. Other details are as shown in Fig. 23.
Fig. 25. Metal chelating effect of different concentrations of WSE. Other details are as shown in Fig. 23.

Fig. 26. Metal chelating effect of different concentrations of ARE. Other details are as shown in Fig. 23.
Fig. 27. Comparison of metal chelating activity of different herbal and natural extracts at same concentration (1 mg). Values are mean ± SE of 4 experiments.
The results showed that total phenol and flavonoid content was higher in TCE followed by PE, while triterpenoid content was higher in PE followed by ARE. These extracts were also further examined for their ability to neutralize the stable free radicals such as DPPH and for ferric reducing ability (FRAP assay) in order to evaluate their antioxidant potential. FRSA and FRAP was significantly high with TCE followed by PE while WSE and ARE showed lesser FRSA and FRAP values. From the above results it can be suggested that high phenols and flavonoids content in TCE and PE might be responsible for their high radical scavanging and antioxidant activity than WSE and ARE. The extracts showed significant reducing power as measured by Fe\(^{3+}\)/Fe\(^{2+}\) transformation in presence of the extracts. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. All the extracts significantly (P < 0.001) inhibited the iron induced LPO. Since, LPO is a consequence of cellular injury (Spiteller, 1996), the ability to inhibit iron catalyzed peroxidation may represent one of the mechanism by which these extracts can inhibit cellular damage. The ability to significantly inhibit LPO might be attributed to the metal chelating property of the extracts.

Antioxidant activity may be related to the polyphenols and flavonoids content, since it has been reported that phenolic compounds can break the chain reaction of lipid peroxidation by scavenging several ROS and inhibiting chemiluminesence reactions (Marquele et al., 2005). The presence of the potent antioxidants (phenolics, flavonoids and triterpenoids) at significant levels in the extracts may be responsible for the overall antiradical and antioxidant activity through different mechanisms such as metal chelation ability, reducing capacity, radical scavanging activity etc.

In conclusion, TCE and PE have significant levels of phenols, flavonoids and triterpenoids, while WSE and ARE showed lower content of these compounds. The antioxidant potential of the extracts was considerably high as measured by inhibition of LPO, FRSA, RP and MCA. The variation in the antioxidant potential of the extracts might be due to the variation in the proportion of these active constituents.