Chapter - 2

In Vitro Antioxidant Activity Studies

Sorrow and silence are strong, and patient endurance is god-like.

- H. W. Longfellow
Ayurveda and other traditional medicines have gained momentum due to their totalitarian approach. Potential application of *A. paniculata*, *T. cordifolia* and *T. foenum-graecum* extracts in Ayurveda and other traditional medicines to reduce oxidative stress along with other health benefits is well documented. Several synthetic antioxidants such as Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT) have restricted use in foods as they are suspected to be carcinogenic (Basniwal *et al*., 2009). In this context, natural, multifunctional, stable, non-toxic and natural compounds from plants which are pharmacologically effective or with low or no side effects are preferred for use in preventive medicine and in food industry (Sati *et al*., 2010).

Based on the extensive literature review it was observed that aerial parts of *A. paniculata* and roots of *T. cordifolia* were used for antioxidant studies. Earlier reports on *T. foenum-graecum* are on the antioxidant activity of its seeds and not the leaves. Hence, we have carried out antioxidant activity studies of these plants by taking only the leaves and extracting them in different solvents. All the extracts were tested for antioxidant activity in various *in vitro* antioxidant models *viz.*, Ferrous reducing antioxidant power assay, Total reducing power, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, Lipid peroxidation inhibitory activity and Superoxide scavenging activity in order to choose a solvent extract which shows potent antioxidant activity. The details of the work carried out are presented in this chapter.
Material and Methods

Plant material

Fresh and healthy leaves of *A. paniculata* and *T. cordifolia* were obtained from local growers of Mysore. *Trigonella foenum-graecum* leaves were obtained from the local market, Mysore. The sample specimen was identified based on the taxonomical characteristics. The leaves were washed thoroughly in distilled water and the surface water was removed by air drying under shade. The leaves were subsequently dried in a hot air oven at 40°C for 48 h, powdered to 100-120 mesh in an apex grinder [Apex Constructions, London] and used for extraction.

Chemicals

Solvents *viz.*, chloroform, hexane, methanol and ethanol were of AR grade from Merck (Mumbai, India). BHT, BHA, quercetin, rutin and DPPH were from Sigma Chemicals, USA. Trichloroacetic acid, potassium ferricyanide, Tris HCl, L-ascorbic acid, 2, 4, 6-tripyridyl-s-tri-azine (TPTZ), HCl, ferric chloride and thiobarbutyric acid were from Sisco Research Laboratories (Mumbai, India). Nitroblue tetrazolium (NBT), Nicotinamide adenine dinucleotide (NADH), Phenazine methosulphate (PMS), phenol reagent, sodium carbonate, gallic acid and potassium acetate were from SD Fine Chemicals (Mumbai, India).

Preparation of aqueous extract

Fifty grams each of powdered leaves of *A. paniculata*, *T. cordifolia* and *T. foenum graecum* were macerated with 100 ml sterile distilled water in a blender for 10 min. The macerate was first filtered through double layered muslin cloth and centrifuged at 4000 rpm for 30 min. The supernatant was filtered through Whatman No.1 filter paper and heat sterilized at 120°C for 30 min. The extracts were preserved aseptically in brown bottles at 4°C until further use.

Preparation of solvent extract

Extraction was carried out according to the method of Okigbo *et al.* (2005). Fifty grams each of the powdered material was extracted initially with 300 ml of chloroform, hexane, methanol and ethanol separately for 24 h at 23 ± 2°C. The extract
was filtered with sterile Whatman No. 1 filter paper into a clean conical flask. Second extraction was carried out with same amount of solvent for another 24 h at 23 ± 2°C and filtered. The extracts were later pooled and transferred into the sample holder of the rotary flash evaporator [Buchi Rotavapor R-124, Switzerland] for the evaporation of the solvents. The evaporated extracts were preserved at 4°C in airtight bottles until further use.

**Estimation of total phenol content**

Total soluble phenol content was estimated by Folin-Ciocalteau method based on the procedure of Malick and Singh (1980). Different aliquots of plant extracts from the stock solution (1 g/ml) were mixed with distilled water to reach a final concentration of 3 ml. To this, 0.5 ml of freshly prepared Folin-Ciocalteau reagent was added and allowed to stand for 3 min at room temperature after which 2 ml of 20% sodium carbonate was added. The mixture was kept in boiling water for a minute, cooled and checked for absorbance at 650 nm in a spectrophotometer against a reagent blank. The concentration of the total phenol compounds in the extracts was obtained by extrapolating the absorbance of gallic acid on standard gallic acid graph. The concentration of total phenols was expressed as mg/g of dry extract.

**Estimation of total flavonoid content**

The total soluble flavonoid content was estimated according to the method of Woisky and Salatino (1998). Stock solution of 0.5 ml (1g/ml) of the extract, 1.5 ml methanol, 0.1 ml potassium acetate (1M) was added to reaction tubes and volume was made up to 5 ml with distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. Total flavonoid content was calculated by extrapolating the absorbance of reaction mixture on a standard curve of rutin. The total flavonoid content was expressed as equivalent to rutin in mg/g of the extracts.

**Antioxidant activity assays**

**Ferrous reducing antioxidant power assay (Total antioxidant activity assay)**

The FRAP assay was carried out according to the method of Benzie and Strain (1996) with slight modifications. The FRAP solution contained 25 ml acetate buffer (300 mM, pH 3.6), 2.5 ml 2, 4, 6-tripyridyl-s-tri-azine (10 mM solution in 40 mM HCl) and 2.5 ml ferric chloride (20 mM). The temperature of the solution was raised
to 37°C before use. Stock solution of 0.15 ml (1g/ml) of the extract was allowed to react with 2.85 ml of the FRAP solution for 30 min in dark condition. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm in a spectrophotometer [UV-160A, Shimadzu Co. Japan]. Results were expressed as µM Fe (II)/g dry mass using the standard curve constructed for different concentrations of ascorbic acid.

**Total reducing power**

The determination of reducing power was performed as described by Yen and Duh (1993). Test samples at different concentrations (0.1 - 0.9 mg/ml) were mixed with 0.5 ml of phosphate buffer (20 mM, pH 6.6) and 0.5 ml of 1% potassium ferricyanide and incubated at 50°C for 20 min. To these mixtures 0.5 ml of 10% trichloro acetic acid was added and centrifuged at 2500 rpm for 10 min. The supernatant was mixed with 1.5 ml of distilled water and 0.3 ml of 0.1% ferric chloride and the absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

**DPPH radical scavenging activity**

The DPPH scavenging assay was a modification of the procedure of Moon and Terao (1998). Briefly, 0.1 ml of test sample at different concentration (0.1 - 0.9 mg/ml) was mixed with 0.9 ml of Tris-HCl buffer (pH 7.4) and 1 ml of DPPH (500 µM in ethanol). The mixture was shaken vigorously and left to stand for 30 min. The absorbance of the resulting solution was measured at 517 nm and compared with that of BHA. Radical scavenging potential was expressed as IC₅₀ values, which represents the sample concentration at which 50% of the radicals are scavenged. The percentage of DPPH scavenging was calculated using the following formula:

Per cent scavenging = \[\left(\frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{sampleblank}})}{A_{\text{control}}}\right) \times 100\]

**Lipid peroxidation inhibitory activity**

The lipid peroxidation inhibitory activity was determined according to the method of Duh and Yen (1997). In brief, egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated using ultraschallprozessor [dr. Hielscher GmbH, UP 50H, Germany] for 10 min to ensure proper liposome formation. Test samples (0.1 ml) at
different concentrations (0.1 - 0.9 mg/ml) were added to 1 ml of liposome mixture and the control was without the test sample. Lipid peroxidation was induced by adding 10 µl of ferric chloride (400 mM) and 10 µl of L-ascorbic acid (200 mM). After incubation for 1 h at 37°C the reaction was stopped by adding 2 ml of hydrochloric acid containing 15% trichloroacetic acid and 0.375% of thiobarbituric acid. The reaction mixture was subsequently boiled for 15 min, cooled, centrifuged at 1000 rpm for 15 min and the absorbance of the supernatant was measured at 532 nm and compared with that of BHA. Inhibitory activity was expressed as IC$_{50}$ value, which represents the sample concentration at which 50% lipid peroxidation inhibition takes place. Percentage of lipid peroxidation inhibition was calculated using the following formula:

Per cent inhibition = \[(A_{\text{control}} - (A_{\text{sample}} - A_{\text{sampleblank}})) / A_{\text{control}}\] × 100

**Superoxide radical scavenging activity**

The superoxide scavenging ability was assessed according to the method of Nishikimi et al. (1972) with slight modifications. The reaction mixture contained nitroblue tetrazolium (0.1 mM) and nicotinamide adenine dinucleotide (0.1 mM) with or without sample to be assayed in a total volume of 1 ml of tris-HCl buffer (0.02 M, pH 8.3). The reaction was started by adding phenazine methosulphate (10 µM) to the mixture, and change in the absorbance was recorded at 560 nm every 30 sec for 2 min. The per cent scavenging was calculated against a control without test sample. Radical scavenging potential was expressed as IC$_{50}$ value, which represents the sample concentration at which 50% of the radicals are scavenged. The results were compared with that of quercetin. The percentage scavenging of superoxide anion was calculated using the following formula:

Per cent scavenging = \[(A_{\text{control}} - (A_{\text{sample}} - A_{\text{sampleblank}})) / A_{\text{control}}\] × 100

**Statistical analysis**

The experimental results are expressed as mean ± standard deviation (SD) of triplicate measurements. The data was subjected to One Way Analysis of Variance (ANOVA) and the significance of differences between the sample means was calculated by Turkeys test. Data was considered statistically significant at P value ≤ 0.05. Statistical analysis was performed using Graph Pad statistical software.
Results and discussion

Total phenol and flavonoid content of leaf extracts

The result of total phenol and flavonoid content of *A. paniculata* leaves in different solvent extracts is summarized in Table 2.1. Ethanol extract had the highest phenol and flavonoid contents of 64.82 mg/g and 0.87 mg/g respectively followed by methanol. The lowest phenol and flavonoid contents were found in the chloroform extract (14.96 mg/g and 0.11 mg/g).

Table 2.1 : Polyphenol and flavonoid content of *Andrographis paniculata* leaves in different solvent extracts

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Total phenols (mg/g)</th>
<th>Flavonoid content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>17.53 ± 0.68a</td>
<td>0.13 ± 0.01ab</td>
</tr>
<tr>
<td>Ethanol</td>
<td>64.82 ± 1.35d</td>
<td>0.87 ± 0.06d</td>
</tr>
<tr>
<td>Methanol</td>
<td>47.84 ± 2.05c</td>
<td>0.49 ± 0.02c</td>
</tr>
<tr>
<td>Hexane</td>
<td>23.23 ± 2.27b</td>
<td>0.21 ± 0.03b</td>
</tr>
<tr>
<td>Chloroform</td>
<td>14.96 ± 1.19a</td>
<td>0.11 ± 0.02a</td>
</tr>
</tbody>
</table>

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)

The result of the total phenol and flavonoid contents of *T. cordifolia* leaf extracts in different solvents is given in Table 2.2. The present study revealed that the level of polyphenols in the ethanol extract (5.1 mg/g) was higher when compared to methanol, hexane, chloroform and aqueous extracts. Ethanol extract of the leaves had a flavonoid content of 0.52 mg/g which was followed by methanol (0.42 mg/g). The flavonoid content of other extracts tested was lower than the ethanol and methanol extracts. Aqueous extract had the least polyphenol and flavonoid contents.
Table 2.2: Polyphenol and flavonoid content of *Tinospora cordifolia* leaves in different solvent extracts

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Total phenols (mg/g)</th>
<th>Flavonoid content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>1.13 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.10 ± 0.20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.52 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methanol</td>
<td>4.36 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.42 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexane</td>
<td>2.13 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.66 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)

The total phenol and flavonoid content of *T. foenum-graecum* leaves in different solvent extracts is represented in Table 2.3. The results obtained in the present study revealed that the amount of polyphenols in the ethanol extract was 4.9 mg/g which was higher when compared to other tested solvents extracts. Ethanol extract of the leaves had a flavonoid content of 0.47 mg/g. The flavonoid content of other extracts tested was lower than the ethanol extract. Aqueous extract had the least polyphenol and flavonoid contents of 1.5 mg/g and 0.18 mg/g respectively.

Table 2.3: Polyphenol and flavonoid content of *Trigonella foenum-graecum* leaves in different solvent extracts

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Total phenols (mg/g)</th>
<th>Flavonoid content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>1.5 ± 0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.18 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>4.9 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methanol</td>
<td>4.2 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexane</td>
<td>2.6 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.7 ± 0.26&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.16 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)
Medicinal plants are an important source of antioxidants (Rice-Evans, 2004). Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases (Prior and Cao, 2000). Natural antioxidants mainly come from plants in the form of polyphenols such as flavonoids, phenolic acids and tocopherols (Ali et al., 2008; Demiray et al., 2009). The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites that possess an aromatic ring bearing one or more hydroxyl constituents (Singh et al., 2007). Phenolic compounds are widely found in the secondary products of medicinal plants, as well as in many edible plants (Hagerman et al., 1998). A number of studies have focused on the biological activities of phenolic compounds, which are the potential antioxidants and free radical scavengers (Rice-Evans et al., 1995; Cespedes et al., 2008; Chanda and Dave, 2009; Annegowda et al., 2010). It is reported that the phenols are responsible for the variation in the antioxidant activity of the plant (Cai et al., 2004). Flavonoids are phenolic acids which serve as an important source of antioxidants found in different medicinal plants and related phytomedicines (Pietta, 1998). Flavonoids contain several phenolic hydroxyl groups in their ring structure. Many flavonoids are found to be strong antioxidants capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups (Cao et al., 1997). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role as free radical scavengers, reducing agents, quenchers of singlet oxygen and complexes of pro-oxidant metals (Mohan et al., 2008; Butkhup and Samappito, 2011). They exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Pokorny 2001; Pitchaon et al., 2007). The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports (Moller et al., 1999; Aziz et al., 2007; Chinedu et al., 2011). Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Cakir et al., 2003). Due to the important physiological functions of phenolic compounds, the total polyphenol content and flavonoid content of the leaves of A. paniculata, T. cordifolia and T. foenum-graecum were measured in different solvent extracts.

The result from the present study indicates that the phenolic compounds and flavonoids are better extracted with ethanol than with other solvents. The result obtained from the estimation of total polyphenol and flavonoid contents in our study
is in agreement with the study by Tsao and Deng (2004) which showed that phenolic acids and flavonoids are generally better extracted using alcohols, water or a mixture of water and alcohols. Eventhough, methanol has been reported by various workers as the most suitable solvent for the extraction of polyphenolic compounds from plant tissues (Siddhuraju and Becker, 2003; Yao et al., 2004; Suhaj, 2006), the result of our study is contrary to the earlier done studies. The result from our study is in agreement with Karadeniz et al. (2005) and Koffi et al. (2010) who showed that ethanol is preferred for the extraction of antioxidant compounds mainly because of its low toxicity. Syeda et al. (2008) have shown greater extraction of phenols in fenugreek seeds by using ethanol. Similar results were observed in the present study with fenugreek leaves in ethanol extract. Earlier reports on extracts of A. paniculata aerial parts have shown lower phenolic contents (Tanwer et al., 2010; Tewari et al., 2010). The results obtained from our study vary from the earlier reports on T. cordifolia stem and root extracts, which have shown higher concentrations of phenols and flavonoids (Singh et al., 2010; Vaghasiya et al., 2011). Vaghasiya et al. (2011) have shown higher concentration of phenols and flavonoids in methanolic stem and leaf extract of T. foenum-graecum. The difference between the phenol and flavonoid contents of the extracts from our study and earlier done studies are likely due to genotypic and environmental differences within species, choice of parts tested, time of taking samples and determination methods. In plants, polyphenol synthesis and accumulation is generally stimulated in response to biotic/abiotic stresses (Shan et al., 2005). Lisiewska et al. (2006) have shown that secondary metabolites distribution might fluctuate between different plant organs.

**Ferrous reducing antioxidant assay**

The ferrous ion reducing ability of A. paniculata leaf extract in different solvents is summarised in Table 2.4. An antioxidant capable of donating a single electron to the ferric-TPTZ (Fe (III)-TPTZ) complex would cause the reduction of the complex into the blue ferrous TPTZ (Fe (II)-TPTZ) complex which absorbs strongly at 593 nm. The ethanol extract showed a total antioxidant activity of 59.01 µM Fe (II)/g which was higher than the other solvent extracts tested.
### Table 2.4: Total antioxidant activity assay of *Andrographis paniculata* leaf extracts

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Total antioxidant activity (µM Fe (II)/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>15.71 ± 0.59(^a)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>59.01 ± 1.06(^d)</td>
</tr>
<tr>
<td>Methanol</td>
<td>43.70 ± 1.71(^c)</td>
</tr>
<tr>
<td>Hexane</td>
<td>21.83 ± 0.77(^b)</td>
</tr>
<tr>
<td>Chloroform</td>
<td>16.10 ± 1.49(^a)</td>
</tr>
</tbody>
</table>

Values are means of three independent replicates. Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05).

The FRAP assay results of *T. cordifolia* is shown in Table 2.5. Among the extracts tested, ethanol extract had a total antioxidant activity of 41.13 µM Fe (II)/g followed by methanol 33.36 µM Fe (II)/g. Aqueous extract had the least reducing ability of 4.96 µM Fe (II)/g.

### Table 2.5: Total antioxidant activity assay of *Tinospora cordifolia* leaf extracts

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Total antioxidant activity (µM Fe (II)/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>4.96 ± 0.15(^a)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>41.13 ± 1.38(^e)</td>
</tr>
<tr>
<td>Methanol</td>
<td>33.36 ± 1.85(^d)</td>
</tr>
<tr>
<td>Hexane</td>
<td>20.96 ± 1.15(^c)</td>
</tr>
<tr>
<td>Chloroform</td>
<td>11.36 ± 1.58(^b)</td>
</tr>
</tbody>
</table>

Values are means of three independent replicates. Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05).

The ability of *T. foenum-graecum* leaf extracts to reduce ferric ions was determined by FRAP assay (Table 2.6). Among the solvent extracts tested, ethanol extract had a reducing ability of 47 µM Fe (II)/g followed by methanol 31 µM Fe (II)/g. Aqueous extract had the least reducing ability.
Table 2.6: Total antioxidant activity assay of *Trigonella foenum-graecum* leaf extracts

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Total antioxidant activity (µM Fe (II)/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>6.5 ± 0.5(^{ef})</td>
</tr>
<tr>
<td>Ethanol</td>
<td>47 ± 1.0(^{b})</td>
</tr>
<tr>
<td>Methanol</td>
<td>31 ± 1.0(^{c})</td>
</tr>
<tr>
<td>Hexane</td>
<td>22.2 ± 0.85(^{d})</td>
</tr>
<tr>
<td>Chloroform</td>
<td>11.2 ± 0.47(^{e})</td>
</tr>
</tbody>
</table>

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (\(P \leq 0.05\))

FRAP assay is based on the ability of antioxidants to reduce ferric (Fe\(^{3+}\)) to ferrous (Fe\(^{2+}\)) in the presence of 2, 4, 6-tri (2-pyridyl)-s-triazine (TPTZ) forming an intense blue Fe\(^{2+}\)-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH dependent (optimum pH 3.6). From the results obtained, it was found that the ethanol leaf extracts of *A. paniculata, T. cordifolia* and *T. foenum-graecum* had the highest antioxidant activity which may be due to the increased concentration of polyphenols. The antioxidant activity was proportional to the polyphenol content of the solvent extracts. Antioxidant compounds such as polyphenols are more efficient reducing agents for ferric iron (Wong *et al.*, 2005). According to Oktay *et al.* (2003), a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species. It is generally believed that plants which are having more phenolic content show good antioxidant activity and there is a direct correlation between total phenols and antioxidant activity (Velioglu *et al.*, 1998; Brighente *et al.*, 2007; Biglari *et al.*, 2008; Salazar *et al.*, 2008; Saravana *et al.*, 2008). Differences in antioxidant activities of the extracts could be due to the different polarities of the solvents and thus different extractability of the antioxidative compounds (Maisuthisakul *et al.*, 2007). Antioxidant properties of single compounds within a group can vary remarkably, so that the same levels of phenolics do not necessarily correspond to the same antioxidant responses (Parejo *et al.*, 2002).
Total reducing power

The reducing ability of *A. paniculata* leaves in different solvent extracts is shown in Figure 2.1. Ethanol extract showed better reducing ability when compared to methanol, hexane, chloroform and water. All the extracts exhibited concentration dependent activity. Ethanol extract showed an increase in the absorbance at 700 nm from 0.38 at 0.1 mg/ml to 1.39 at 0.9 mg/ml. Both aqueous and chloroform extracts showed least reducing abilities.

**Fig. 2.1 : Total reducing power of *Andrographis paniculata* leaf extracts**

![Graph showing total reducing power of Andrographis paniculata leaf extracts](image)

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)

The reducing power of different solvent extracts of *T. cordifolia* leaves using the potassium ferricyanide method is shown in Figure 2.2. The result indicates that the reducing ability of the extracts increased with the concentration. Among all the extracts tested for their reducing abilities, ethanol extract showed better reducing power with an increase in the absorbance at 700 nm from 0.19 at 0.1 mg/ml to 0.90 at 0.9 mg/ml which was followed by methanol.
Fig. 2.2: Total reducing power of *Tinospora cordifolia* leaf extracts

![Graph showing total reducing power of *Tinospora cordifolia* leaf extracts](image1)

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)

The reducing power of *T. foenum-graecum* leaves in different solvent extracts is shown in Figure 2.3. The result indicates that the reducing ability of all the extracts increased with the concentration. Among all the extracts tested for their reducing abilities ethanol extract showed better reducing power with an increase in the absorbance at 700 nm from 0.24 at 0.1 mg/ml to 1.03 at 0.9 mg/ml. Aqueous extract showed least reducing ability with an absorbance of 0.06 at 0.1 mg/ml and 0.40 at 0.9 mg/ml.

Fig. 2.3: Total reducing power of *Trigonella foenum-graecum* leaf extracts

![Graph showing total reducing power of *Trigonella foenum-graecum* leaf extracts](image2)

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)
The reducing capacity of a compound from Fe\(^{3+}\) to Fe\(^{2+}\) form may serve as a significant indicator of its antioxidant capacity (Meir et al., 1995; Oktay et al., 2003; Hazra et al., 2008; Rachh et al., 2009). The leaf extracts of *A. paniculata*, *T. cordifolia* and *T. foenum-graecum* exhibited concentration dependent activity and showed a positive correlation with the polyphenol content. Accordingly, ethanol extract of all the three plants showed better reducing ability when compared to other solvent extracts. The presence of compounds with hydroxyl groups in the extracts may be responsible for reducing power (Policegoudra et al., 2007; Vedpriya and Yadav, 2011). It appears that antioxidant activity may have a mutual correlation with the reducing effect. The reducing properties are generally associated with the presence of reductones. Gordan (1990) reported that the antioxidant activity of reductones is believed to break radical chains by donation of a hydrogen atom, indicating that the antioxidative properties are concomitant with the development of the reducing power. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995).

**DPPH radical scavenging activity**

Figure 2.4 represents the DPPH radical scavenging activity of *A. paniculata* leaf extracts in different solvents. Ethanol extract showed the highest scavenging ability of 53% at 0.5 mg/ml (IC\(_{50}\) value). Methanol extract showed a scavenging ability of 53.86% at a higher concentration of 0.9 mg/ml (IC\(_{50}\) value). IC\(_{50}\) values could not be achieved with other solvent extracts tested in this assay.

**Fig. 2.4 : DPPH radical scavenging activity of *Andrographis paniculata* leaf extracts**

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)
The DPPH radical scavenging activity of *T. cordifolia* leaf extracts is shown in Figure 2.5. Among the extracts tested, ethanol extract had better scavenging activity of 50.23% (IC$_{50}$ value of 0.5 mg/ml). Methanol had a scavenging activity of 53.43% (IC$_{50}$ value of 0.9 mg/ml). The IC$_{50}$ values could be reached at a lower concentration with ethanol extract as compared to methanol. As with other extracts tested IC$_{50}$ values could not be reached even at higher concentrations.

**Fig. 2.5 : DPPH radical scavenging activity of *Tinospora cordifolia* leaf extracts**

![Graph showing DPPH scavenging activity of *Tinospora cordifolia* leaf extracts](image)

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)

The DPPH radical scavenging activity of *T. foenum-graecum* leaf extracts is shown in Figure 2.6. Among all the solvent extracts tested, ethanol extract had better scavenging activity (59.7%) followed by methanol (53.2%). When compared to BHA which had an IC$_{50}$ value of 0.0053 mg/ml, the IC$_{50}$ value of ethanol extract was quite high (0.7 mg/ml).

**Fig. 2.6 : DPPH radical scavenging activity of *Trigonella foenum-graecum* leaf extracts**

![Graph showing DPPH scavenging activity of *Trigonella foenum-graecum* leaf extracts](image)

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)
The stable radical DPPH has been used widely for the determination of primary antioxidant activity (Brand-Williams et al., 1995; Katalinic et al., 2004; Goncalves et al., 2005). DPPH is one of the compounds that possess a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers. The reaction of DPPH is measured by degree of decrease of absorbance at 517 nm, by an antioxidant (Brand-Williams et al., 1995; Conforti et al., 2002). The DPPH antioxidant assay is based on the ability of DPPH a stable free radical to decolourize in the presence of antioxidants (Singh et al., 2002; Ara and Nur, 2009). Antioxidants react with DPPH which is a stable free radical and convert it to 1, 1-diphenyl-2-picryl hydrazine.

It is well accepted that the DPPH radical scavenging by antioxidants is due to their hydrogen donating ability (Nikolaos et al., 2003; Singh and Rajini, 2004; Goncalves et al., 2005; Hou et al., 2005; Negi et al., 2005). The antioxidant activity may be directly correlated to the phenolic content in different solvent extracts (Chu et al., 2000; Singh et al., 2002; Cai et al., 2004; Kulkarni et al., 2004; Tawaha et al., 2007; Chinedu et al., 2011). The ethanol leaf extracts of A. paniculata, T. cordifolia and T. foenum-graecum recorded the highest phenol and flavonoid contents and also had the highest DPPH scavenging activity. This suggests that the phenolic compounds contributed significantly to the antioxidant capacity of the investigated plant species.

The amount of sample required to decrease the initial DPPH concentration (IC\textsubscript{50}) by 50% is a parameter widely used to measure the antioxidant activity. The lower the IC\textsubscript{50} value, the higher the antioxidant activity. Earlier study on DPPH scavenging ability of A. paniculata aerial parts by Arash et al. (2010) had shown 86.87% scavenging of DPPH radicals at a concentration of 10 mg/ml of ethanol extract. But, the present study has shown the inhibition at a lower concentration. This may be due to the difference between antioxidant capacity of different varieties and cultivars of the same plant grown in different regions (Kedage et al., 2007; Henriquez et al., 2009). A study carried out by Hasan et al. (2009) has shown the DPPH radical scavenging activity of T. cordifolia aerial parts with an IC\textsubscript{50} value of 0.02 mg/ml. The difference in the IC\textsubscript{50} value can be attributed to the distribution of secondary metabolites that may fluctuate between different plant organs (Lisiewska et al., 2006).
Lipid peroxidation inhibitory activity

Figure 2.7 represents the lipid peroxidation inhibitory activity of leaf extracts of *A. paniculata* in different solvents. Ethanol extract had an IC$_{50}$ value as low as 0.1 mg/ml and showed a lipid peroxidation inhibition of 58.73% which was followed by methanol with an IC$_{50}$ value of 0.7 mg/ml (% lipid peroxidation of 51.56). As with other extracts tested, 50% inhibition could not be reached even at higher concentrations.

**Fig. 2.7:** Lipid peroxidation inhibitory activity of *Andrographis paniculata* leaf extracts

The lipid peroxidation inhibitory activity of *T. cordifolia* leaf extracts is shown in Figure 2.8. Ethanol extract had an IC$_{50}$ value of 0.1 mg/ml which showed a lipid peroxidation inhibition of 57.5%, whereas methanol extract showed an inhibition of 50.43% at a higher concentration of 0.7 mg/ml (IC$_{50}$ value). As with other extracts, 50% inhibition could not be achieved even at 0.9 mg/ml.

**Fig. 2.8:** Lipid peroxidation inhibitory activity of *Tinospora cordifolia* leaf extracts
The antioxidative action of *T. foenum-graecum* leaf extracts in the liposome model, induced by ferric chloride plus ascorbic acid and determined by thiobarbituric acid method is shown in Figure 2.9. Ethanol extract had an IC$_{50}$ value of 0.3 mg/ml which showed a lipid peroxidation inhibition of 62.3%. As with other solvent extracts, 50% inhibition of lipid peroxidation could not be achieved even at higher concentrations. BHA showed a very strong lipid peroxidation inhibitory activity with an IC$_{50}$ value 0.012 mg/ml.

**Fig. 2.9 : Lipid peroxidation inhibitory activity of *Trigonella foenum-graecum* leaf extracts**

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)

To evaluate the lipid peroxidation inhibitory activity of the leaf extracts, the liposome model system was used. Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm. MDA is a major product of lipid peroxidation and is used to study the lipid peroxidation process (Singh *et al.*, 2002; Dok-Go *et al.*, 2003).

Polyunsaturated fatty acids (PUFA) present in cell membranes are oxidized by both enzymatic and by free radical chain reactions (Torel *et al.*, 1986). An over abundance of free radicals can lead to uncontrolled chain reactions of lipid peroxidation resulting in pathological conditions that may include liver injury, atherosclerosis, kidney damage, ageing and susceptibility to cancer (Rice-Evans and Burdon, 1993). Lipid peroxidation proceeds in three stages: initiation, propagation
and termination. In the initiation stage of lipid peroxidation, free radicals obstruct hydrogen from PUFA to form the lipid radical. In the propagation stage, the lipid radical breaks down to generate more free radicals thus maintaining the chain of reactions. In the termination stage, the free radical species react together or with antioxidants to form inert products. Lipid peroxidation can be suppressed by enzymatic inactivation of free radicals by antioxidants that inhibit the initiation stage and/or accelerate the termination stage (Shimada et al., 1992). Thus, lipid peroxidation can be prevented at the initiation stage by free radical scavengers and singlet oxygen quenchers, and peroxyl-radical scavengers which break propagation of chain reaction (Cook and Samman, 1996).

The result of lipid peroxidation inhibitory activity of different solvent extracts of *A. paniculata*, *T. cordifolia* and *T. foenum-graecum* showed that ethanol leaf extract of all the three plants showed the highest inhibition of lipid peroxidation. Lipid peroxidation inhibitory activity mainly depends on the solubility and hydrophobicity of the compounds present in the respective extracts (Son and Lewis, 2002). The highest lipid peroxidation inhibition of ethanol extract may be due to the presence of high amounts of polyphenols and flavonoids. Flavonoids are known for their antioxidant effect on lipid peroxidation as a result of scavenging of hydroxyl radicals at the stage of initiation and termination of peroxyl radicals (Hussain et al., 1987). They may donate hydrogen atoms forming a flavonoid radical and flavonoid radical in turn are able to react with free radicals there by terminating the radical chain reaction (Baderschneider and Winterhalter, 2001). It is reported that lipid peroxidation can be inhibited by flavonoids, possibly through their activity as strong oxygen scavengers and singlet oxygen quenchers (Baumann et al., 1980; Bergman et al., 2003). Earlier study by Prasad et al. (2005) has shown the lipid peroxidation inhibitory activity of a flavonoid isolated from *Ipomea aquatica* leaf. In all the three plant extracts, there was a gradual decrease in the inhibition of lipid peroxidation even with the increase in concentration of the extract which may be due to the degradation or peroxidation of the source (Bendich et al., 1986).
Superoxide scavenging activity

The superoxide radical scavenging ability of *A. paniculata* leaf extracts in different solvents is depicted in Figure 2.10. There was a scavenging of 51.06% of superoxide anions with ethanol extract at a concentration of 0.9 mg/ml (IC$_{50}$ value). Eventhough other solvent extracts showed superoxide scavenging, IC$_{50}$ values could not be achieved with the concentration used in this assay.

**Fig. 2.10 : Superoxide scavenging activity of *Andrographis paniculata* leaf extracts**

![Graph showing superoxide scavenging activity of *Andrographis paniculata* leaf extracts](image)

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)

The superoxide radical generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. Figure 2.11 represents the superoxide quenching ability of *T. cordifolia* leaf extracts. The superoxide scavenging ability was generally low with all the solvent and aqueous extracts. As with ethanol extract, even at 0.7 mg/ml concentration the percentage radical scavenging was 40.13%. Per cent radical scavenging abilities of other extracts were lower than the ethanol extract.

**Fig. 2.11 : Superoxide scavenging activity of *Tinospora cordifolia* leaf extracts**

![Graph showing superoxide scavenging activity of *Tinospora cordifolia* leaf extracts](image)

Values are means of three independent replicates
Mean values with different superscripts are significantly different from each other as indicated by Turkey’s HSD (P ≤ 0.05)
The decrease in absorbance at 560 nm with *T. foenum-graecum* extracts and the reference compound quercetin indicates their abilities to quench superoxide radicals in the reaction mixture. As shown in Figure 2.12, the quenching ability was generally low with all the solvent extracts. Ethanol extract showed the highest scavenging of 33.3% at 0.9 mg/ml. Aqueous extract recorded the least scavenging activity when compared to all other extracts. Quercetin was found more potent having an IC\textsubscript{50} value 0.155 mg/ml.

**Fig. 2.12 : Superoxide scavenging activity of Trigonella foenum-graecum leaf extracts**

Superoxide radicals are generated during the normal physiological process, mainly in mitochondria. Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded can lead to production of extremely reactive hydroxyl radicals (Wolff and Dean, 1987; Jiang *et al.*, 1990). Although, superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen both of which contribute to oxidative stress (Dahl and Richardson, 1978; Halliwell and Gutteridge, 1990; Meyer and Isaksen, 1995; Pietta, 2000). Therefore superoxide radical scavenging by antioxidants has physiological implications.
From the present study it was found that the superoxide scavenging activity of *A. paniculata, T. cordifolia* and *T. foenum-graecum*, leaf extracts was generally low with all the solvent extracts. But there was a positive correlation with the polyphenol and flavonoid contents and superoxide scavenging activity of the extract (Policegoudra *et al.*, 2007). This shows that the antioxidant activity of the extract was due to the presence of polyphenols and flavonoids in it. Robak and Gryglewski (1988) reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions.

From the results it appeared that the superoxide scavenging activity of the plant extract is negligible compared to the standard quercetin. It was shown that IC$_{50}$ value could be reached only with ethanol leaf extract of *A. paniculata*. As with other solvent extracts of *T. cordifolia* and *T. foenum-graecum*, IC$_{50}$ values could not be reached. Even though the IC$_{50}$ values could not be reached, there was a positive correlation with flavonoid content and superoxide radical scavenging activity of the extract. The result supports the earlier study by Mathew and Kuttan (1997) on *T. cordifolia*, which showed that the IC$_{50}$ value for superoxide scavenging could be as high as 6 mg/ml.