CHAPTER VIII
ANTIOXIDANT POTENTIAL STUDY

8.1: Methodology

There are many methods that employed in the estimation of antioxidant potential of a given sample based on scavenging activity or antioxidant capacity. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity is one of the widely used assay method which was first described by Blois (1958) and later on slightly modified by various researchers; Another method that widely employed in the estimation of antioxidant potential is ABTS radical scavenging method that was developed by Rice-Evans and Miller (1994) and then modified by Re et al., in 1999; these methods rely on the total phenolic contents based on Folin-Ciocalteu method was developed reducing power. Total phenolic contents based on Folin-Ciocalteu method was developed reducing power. Total phenolic contents based on Folin-Ciocalteu method was developed reducing power. Total phenolic contents based on Folin-Ciocalteu method was developed reducing power.

Chemicals and Solvents: The chemicals 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Gallic acid, ferric chloride, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Sigma-Aldrich (Munich, Germany). Merck’s Folin-Ciocalteu (Merck (Mumbai, India)) other reagents and solvents used were analytical grade (RANKEM New Delhi, India).

Preparation of crude extract

*Solanum spirale* (leaf, berry and root), *Pouzolzia bennettiana* (shoot), *Allium hookeri* (whole plant), *Solanum kurzii* (berry), *Zanthoxylum rhetsa* (shoot), *Clerodendrum colebrookianum* (shoot) and *Phoebe cooperiana* (fruit) were collected from various locations from study site (from Pasighat market and villages of East Siang District of Arunachal Pradesh). And wash in tap water to remove soil and dirt then wash with distilled water. Shoots were shed dried and berry and roots were dried in an oven at below 40 degree Celsius till constant weight was achieved. Dried samples were grinded in laboratory mill and kept in air tight container for further use. 100g each powder were soaked in 500 ml methanol for 48 hours and filtered through Whatman paper No.41. The residue was extracted twice with 500ml of methanol each. The total filtrate was concentrated by rotatory evaporator at 45°C under reduced pressure.
8.1.1: Determination of Antioxidant Activity using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Method

The antioxidant activity was determined according to the method of Aoshima et al., (2004). Briefly, to 100 µl of sample extract, or standard, 2.9 mL of DPPH reagent (0.1mM in methanol) was added and vortexed vigorously. The reaction mixture was stored in the dark for 30 minute at room temperature and decolouration of DPPH was measured against a blank at 517 nm using an ultraviolet-visible (UV-Vis) spectrophotometer (Lamba-25, Perkin Elmer, Cambridge, UK). Linear calibration curves were produced with \( R^2 = 0.9994 \) (Fig: 8.1) and result was calculated as trolox equivalent per gram dry sample. The inhibition % was calculated using the formula:

\[
\text{Inhibition}\% = \frac{A\ (\text{control}) - A\ (\text{test sample})}{A\ (\text{control})} \times 100
\]

**Figure 8.1: Trolox concentration vs absorbance for DPPH standard curve.**
8.1.2: ABTS Free Radical Scavenging Assay

The ABTS radical cation scavenging activity was performed according to Re et al., (1999) with slight modifications. The ABTS solution (7mM) was reacted with potassium persulfate (2.45mM) solution and kept overnight in dark to yield a dark green-colored solution containing ABTS radical cation. Prior to use in the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.700± 0.02 at 743nm using UV-Vis spectrophotometer with the temperature set at 30 °C. Free radical scavenging activity was assayed by mixing 100μL of test sample with 2.9ml of an ABTS working standard in a microcuvette. The decrease in absorbance was measured at exactly 1 minute after mixing the solution and then at 1 minute intervals up to 6 minutes when final absorbance was recorded. Linear calibration curves were produced with $R^2 = 0.9988$ (Fig: 8.2) for evaluation of antioxidant activity in ABTS and result was calculated as trolox equivalent per gram dry sample. The inhibition % was calculated using the formula:

$$\text{Inhibition} = \frac{A \text{ (Control)} - A \text{ (test sample)}}{A \text{ (control)}} \times 100$$

![Graph showing trolox concentration vs absorbance for ABTS standard curve.](image)

Fig 8.2: Trolox concentration vs absorbance for ABTS standard curve.
8.1.3: Determination of Total Phenolic Content

Total phenolic content was determined by the Folin-Ciocalteu method described by Singleton and Rossi (1965). Briefly, to 900μL of distilled water and 1mL of the Folin-Ciocalteu reagent 100μL of filtered extract was added. After 5 minutes, 2mL of saturated sodium carbonate (75g.L-1) and 2 mL water was added. Absorbance of the resulting blue-colored solution was measured at 765nm using UV-Vis spectrophotometer after incubation at 30°C for 1.5 h with intermittent shaking. Quantification measurement was performed based on a standard calibration curve of 20, 40, 60, 80 and 100mg/100mL of Gallic acid in 80% methanol. Total phenolic content was expressed as Gallic acid equivalent (GAE) in the dry sample. Linear calibration curves were produced with $R^2=0.9989$ (Fig 8.3).

![Graph showing Gallic acid standard curve for TPC.](image)

Fig.8.3: Gallic acid standard curve for TPC.
8.1.4: Determination of Total Flavonoid Content

Total flavonoid content was determined by using the colorimetric method of Sahreen and Khan (2010) with slight modification. 50mg of sample was dissolved in 10 ml of 80% aqueous methanol and filtered through Whatman filter paper No.42 (125mm). In a 10mL test tube, 0.3ml of extract, 3.4 mL of 30% methanol, 0.15 mL of 0.5M sodium nitrite, and 0.15 mL of 0.3 M aluminium chloride hexahydrate were added and mixed. After 5 minutes, 1mL of 1M sodium hydroxide was added. The absorbance of the mixture was measured at 510 nm using UV-Vis spectrophotometer (Lambda-25, Perkin Elmer Cambridge, UK) and values were express as rutin equivalent antioxidant capacity. Linear calibration curves were produced with $R^2=0.9994$ (8.4).

![Graph]

**Fig. 8.4: Rutin standard curve for TFC.**

**Statistical Analysis:** All the assays were carried out in triplicate and the experimental results obtained were expressed as mean±SD.
8.2: Results

The antioxidant potential studied on the selected species for the thesis is given below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>TPC (mg GAE/g)</th>
<th>TFC (µMRE/g)</th>
<th>ABTS (µM/g)</th>
<th>DPPH (µM/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zanthoxylum rhetsa shoot</td>
<td>117.95±3.22</td>
<td>120.14±2.31</td>
<td>167.69±3.21</td>
<td>851.42±3.44</td>
</tr>
<tr>
<td>Solanum kurzii</td>
<td>14.60±1.12</td>
<td>89.00±2.11</td>
<td>30.70±1.34</td>
<td>257.74±2.31</td>
</tr>
<tr>
<td>mature berry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phoebe cooperiana</td>
<td>33.93±1.14</td>
<td>941.5±5.31</td>
<td>268.114±4.13</td>
<td>405.76±2.24</td>
</tr>
<tr>
<td>mature fruit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allium hookeri (whole plant)</td>
<td>10.51±2.53</td>
<td>40.31±3.01</td>
<td>76.03±2.36</td>
<td>93±1.16</td>
</tr>
<tr>
<td>Solanum spirale (shoot)</td>
<td>18.64±2.12</td>
<td>5.29±1.01</td>
<td>29.85±1.25</td>
<td>45.4±0.37</td>
</tr>
<tr>
<td>Solanum spirale (mature berry)</td>
<td>11.72±1.42</td>
<td>50.52±1.03</td>
<td>38.27±1.33</td>
<td>52±0.92</td>
</tr>
<tr>
<td>Pousolzia bennettiana (whole plant)</td>
<td>4.80±2.11</td>
<td>120±1.25</td>
<td>111.69±1.03</td>
<td>278.48±1.01</td>
</tr>
<tr>
<td>Clerodendrum colebrookianum (shoot)</td>
<td>11.84±2.32</td>
<td>10.88±1.12</td>
<td>24.84±1.27</td>
<td>86.24±1.28</td>
</tr>
</tbody>
</table>

Table 8.1: TPC, TFC, ABTS and DPPH value.
8.3: Discussion

All forms of aerobic life are constantly subjected to oxidative pressure from reactive oxygen species (ROS), produced during the biochemical utilization of O₂ (Guetens et al., 2001) and under stress, our bodies produce more reactive oxygen species (Krishnaiah et al., 2011). When excess is ROS produced in body it perturb the redox balance and produce a variety of changes though lipid peroxidation and protein and nucleic acid damage eventually be responsible for cancer, cardiovascular diseases, ageing, and neurodegenerative disorders (Cho & Kleeberger, 2007; Kinnula & Crapo, 2004; Hyun, Hernandez, Mattson, & de Cabo, 2006). The adverse effects of oxidative stress on human health have become a serious issue and a lack of antioxidants, which can quench the reactive free radicals, facilitates the development of degenerative diseases (Shahidi et al., 1992). One solution to this problem is to supplement the diet with antioxidant compounds that are contained in natural plant sources (Knekt et al., 1996). These natural plant antioxidants can therefore serve as a type of preventive medicine.

Based on epidemiological studies, flavonoid-rich diet is correlated with the increased longevity and decreased incidence of cardiovascular diseases in populations despite high intake of fat by various researchers (Burr, 1995), (de Lange, 2007), (Rosenkranz et al., 2002). The antioxidant capacities of many flavonoids are much stronger than those of vitamins C and E (Prior and Cao, 2000). In the present studies total phenolic and flavonoids is taken as the base study to correlate antioxidant potential of the selected medicinal food plants by using free radical scavenging methods of DPPH and ABTS in which Gallic acid, Trolox and Rutin were used as standards.

In this study it was recorded that S. spirale leaf contain 18.64 mg Gallic acid equivalent per gram total phenolic, 5.29μMRE/g total flavonoid content with scavenging activity of 29.85μM/g in ABTS method and 45.4 μM/g scavenging in DPPH method. Comparatively, the phenolic content is more than flavonoid content which may be attributed for considerable free radical scavenging activity in ABTS and DPPH study.

S. spirale berry is recorded to contain 11.72 mg total phenolic content calculated in Gallic acid equivalent per gram, 50.52μM per gram total flavonoid content calculated in equivalent to Rutin with scavenging capacity of 38.27μM/g in ABTS method and scavenging capacity of 52 μM per gram in DPPH method. The considerable antioxidant potential of this berry may be attributed to the flavonoid and phenolic compounds, further,
correlation in the total content of flavonoid and DDPH scavenging activities is observed in the *S. spirale* berry.

According to earlier workers, Boruah and Handique (2014) have also reported total phenolic, total flavonoid and DPPH scavenging activity from methanol extract of *S. spirale* but they have not categorically mentioned the parts taken in their study like root, berry or shoot. However, they have reported 64.14 DPPH activities without mentioning unit of study, 45.95 mg/g Gallic acid equivalent TPC and 31.95 mg/g TFC equivalent to Catechin. However their DPPH recorded value come close to our DPPH activity value of *S. spirale* berry which was recorded as of 52 µM per gram in DPPH calculated to Gallic acid standard.

Keawsa-ard *et al.*, (2012) have also reported total phenolic content of the unripe berry from *S. spirale* and recorded 32.90 mg/g equivalent to Gallic acid standard total phenolic from methanol extract, in our study, total phenolic content of ripe fruit was recorded as 11.72 mg/g equivalent to Gallic acid standard which is almost half in the total phenolic content as reported by Keawsa-ard *et al.*, (2012). The difference in result may be due to the ecotype as they took study material from Phayao Province, Thailand or may be due to stage of the collection of fruit as they used unripe berry while in this study we used ripe berry.

*Pouzolzia bennettiana* shoot contain low phenolic content of 4.80 mg/g calculated in Gallic acid equivalent, high flavonoid content of 120 µM/g calculated in Rutin equivalent, while ABTS methods gave scavenging result of 111.69 µM/g calculated in Trolox equivalent and DPPH scavenging method gave a value of encouraging 278.48 µM/g calculated in Trolox equivalent. The encouraging antioxidant potential of this folk medicinal food plant may be due to considerable TFC in this plant.
Allium hookeri contain 10.51 mg/g of total phenolic content calculated equivalent to Gallic acid, 40.31 μM/g total flavonoid content calculated to Rutin equivalent with scavenging activity of 76.03 μM/g in ABTS method and 93.1 μM/g in DPPH free radical scavenging method. The considerable antioxidant potential activity may be due to the presence of considerable amount of flavonoid and phenolic compounds in this plant. Singh and Singh (2014) have also reported antioxidant potential of A. hookeri root and recorded 40.60 polyphenol contents from A. hookeri root in mg/ml equivalent to Gallic acid standard and in our study we recorded 10.51 mg/g of total phenolic content equivalent to Gallic acid standard; the mentioned study has been studied in different unit hence, no much significance could be drawn out from the reference.

Zanthoxylum rhetsa contain 117.95 mg GAE/g TPC, 120.14 μMRE/g TFC and recorded 167.69 μM/g scavenging in ABTS method and 851.42 μM/g in DPPH scavenging method. It is recorded to contain high phenolic and flavonoid content with high potential scavenging capacity, direct correlation of the phenolic and flavonoid contents is recorded with the antioxidant potential in Z. rhetsa. The high scavenging potential in ABTS and DPPH may be attributed to the high phenolic and flavonoid content as antioxidant of a plant is largely contributed by presence of phenolic compounds and flavonoids (Williams, 2004). Highest antioxidant potential was recorded in this medicinal food plant. The recorded data shows that this folk medicinal food plant is extremely fit to be included in nutraceutical food.

Solanum kurzii berry was recorded to contain 14.60 mg GAE/g TPC, 89 μMRE/g TFC with scavenging activity of 30 μM/g in ABTS and 257 μM/g in DPPH method. The high activity in DPPH method may be due to presence of considerable presence of total flavonoids.
*Clerodendrum colebrookianum* contain 11.84mg GAE/g TPC, 10.88μMRE/g TFC with scavenging activity of 24.84μM/g in ABTS free radical scavenging method and 86.24μM/g in DPPH method. Though *C. colebrookianum* is recorded with considerable antioxidant potential activity but in compare to other studied species low phenolic, flavonoid, scavenging activity in DPPH as well as in ABTS was recorded in *C. colebrookianum*.

*Phoebe cooperiana* contain 33.93 mg GAE/g TPC, 941μMRE/g TFC, free radical scavenging activity of 268.11μM/g in ABTS method and 407.76μM/g in DPPH scavenging method. Relation between total flavonoid and scavenging activity is observed in its fruit. The high content of flavonoid and considerable antioxidant potential in this fruit is recorded. Direct correlation of the flavonoid contents is recorded with the antioxidant potential in its fruit. The high scavenging potential in ABTS and BPPH may be attributed to the considerable phenolic and high flavonoid content as antioxidant of a plant is largely contributed by presence of phenolic compounds and flavonoids (Williams, 2004)). The recorded data shows that this folk medicinal food plant is extremely fit to be included in nutraceutical food. This nutraceutical folk food plant may be useful in the development of herbal drugs in near future. Fruit and vegetables are major sources of dietary antioxidant (Namiki, 1999).