3.1 Introduction

The plants selected for the present study are *Delonix regia*, *Lallemantia royleana*, *Phyllanthus maderaspatensis*, *Plantago ovata*, *Rosa indica* and *Solanum nigrum*. The crude extracts are screened for their anti-oxidant activities.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Scientific name of Plant</th>
<th>Family</th>
<th>Common Name</th>
<th>Part used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Delonix regia</em></td>
<td>Fabaceae</td>
<td>Gulmohor</td>
<td>Leaves and Petals</td>
</tr>
<tr>
<td>2.</td>
<td><em>Lallemantia royleana</em></td>
<td>Lamiaceae</td>
<td>Tukhm-e-balanga</td>
<td>Seeds</td>
</tr>
<tr>
<td>3.</td>
<td><em>Phyllanthus maderaspatensis</em></td>
<td>Euphorbiaceae</td>
<td>Kanocha</td>
<td>Seeds</td>
</tr>
<tr>
<td>4.</td>
<td><em>Plantago ovata</em></td>
<td>Plantagiaece</td>
<td>Isabghol</td>
<td>Seeds</td>
</tr>
<tr>
<td>5.</td>
<td><em>Rosa indica</em></td>
<td>Rosaceae</td>
<td>Gulab-e-surkh</td>
<td>Petals</td>
</tr>
<tr>
<td>6.</td>
<td><em>Solanum nigrum</em></td>
<td>Solanaceae</td>
<td>Makoi</td>
<td>Berries, Leaves and Flowers</td>
</tr>
</tbody>
</table>

*Table No. 3.1:* Ethno-botanical information of some medicinal plant species selected for the study.
3.2 Collection of Plant Material

The plants parts were collected from in and around the campus of Integral University, Lucknow. Some of the plant parts were also purchased from Unani practitioners. The plant samples were taxonomically identified from the Herbarium Department at NBRI, Lucknow, India. Each part was separated, cleaned, tap washed followed by washing with distilled water and then air dried. The dried plant materials were then stored in air-tight containers until used.

3.3 Preparation of the Plant Extract

The plant extracts were prepared by the method of Alade and Irobi (1993). Briefly, 30 g of powdered plant materials were soaked in 100 ml of methanol for 72 h in dark. The mixture was stirred every 24 h using a sterile glass rod. At the end of the extraction the extract was passed through Whatman filter paper No. 1 (Whatman, UK). The alcoholic filtrate obtained was concentrated in vacuo at 40°C in a rotavapour and stored at 4°C until further use.

3.3.1 Preparation of Crude Extracts in Methanol Using Soxhlet Apparatus

The finely ground dry plant parts were weighed 30 g and kept in a muslin cloth. The muslin cloth containing dry extract were placed in a chamber of the soxhlet apparatus (Figure 3.1). The extraction solvent was added in the flask and heated and its vapours condense in condenser. The condensed solvent containing the plant extract was collected in another flask, for almost 72 hours or till all the extract has been extracted from the plant (Handa et al., 2008). The methanol was used for crude extract preparation. All the extracts were air dried in petri plates and the extracts were weighed and kept in eppendroff at -20°C until used.

3.4 Determination of Extraction Yield of Plant Extract (% yield)

The yield (% w/w) from all dried extracts was calculated by the formula given below.

\[
\text{Yield} \, (\%) = \frac{W_2 - W_1}{W_0} \times 100
\]

Where, \(W_2\) is the weight of the extract and the container, \(W_1\) weight of the container alone and \(W_0\) the weight of the plant powder (Anokwuru et al., 2011).
3.5 Phyto-chemical Analysis of Plant

The plant extracts were qualitatively analysed for the presence of the phyto-chemicals. The phyto-chemicals were estimated qualitatively using a number of assays as mention below. (Tiwari, et. al., 2011; Mir, et. al., 2013; Trease and Evan, 1983; Kokate, et. al., 1997 and Hegde and Joshi, 2010).

3.5.1 Qualitative Analysis

3.5.1.1 Test for the presence of Alkaloids (Wagner’s test)

Wagner’s reagent is prepared by dissolving 2g of iodine and 6g of potassium iodide in 100ml of water. The plant extract solution was prepared by dissolving 500 mg of dried plant extract in 500 ml of methanol for 20 minutes, on a water bath. The extract was then filtered and allowed to cool. 2ml of plant extract was taken and treated with few drops of Wagner’s reagent. A reddish brown coloured precipitate indicates the presence of alkaloids.
3.5.1.2 Test for the Presence of Anthraquinone (Borntrager’s test)

Approximately 0.5 g of the plant extracts was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of chloroform was added to the filtrate. Few drops of 10% ammonia were added to the mixture and heated again. Formation of rose-pink color indicates the presence of anthraquinones.

3.5.1.3 Test for the presence of Flavonoids

The crude powder of dried plant extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution and a yellow coloration confirmed the presence of flavonoids.

3.5.1.4 Test for the presence of Phlobatannins

An aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid (HCl) to observe the deposition of red precipitate for the presence of flavanoids.

3.5.1.5 Test for the presence of Glycosides (Fehling’s test)

The crude plant extract powder of 0.5 g was dissolved in 5 ml of methanol. 2ml of this mixture was taken and to it 10 ml of 50% HCl was added. The mixture was heated in a boiling water bath for 30 min. Then to the mixture add 5 ml of Fehling’s solution and the mixture was again boiled for 5 min to observe a brick red precipitate as an indication for the presence of glycosides.

3.5.1.6 Test for the presence of Saponins (Frothing test)

About 0.2 g of the dried plant extract was dissolved with 5ml of distilled water and then heated to boil. Frothing (appearance of creamy layer of small bubbles) showed the presence of saponins.
3.5.1.7 Test for the presence of Steroids (Salkowski test)

1ml of the plant extract was taken and to it few drops of concentrated sulphuric acid (H₂SO₄) was added. The presence of red coloration indicates the presence of steroids.

3.5.1.8 Test for the presence of Tannins (Ferric chloride test)

The presence of tannins was tested by taking 0.5 g of the crude plant extract powder and dissolve in 10 ml of distilled water. The extract was filtered and ferric chloride reagent was added to the filtrate, a blue-black precipitate was taken as an indicator for the presence of tannin.

3.5.1.9 Test for the presence of Terpenoids (Salkowski test)

The presence of terpenoids was tested in 0.2 g of the extract of the plant sample and mixed with 2 ml of chloroform and concentrated sulphuric acid (H₂SO₄) (3 ml) was added carefully to form a layer. A reddish brown coloration of the interface was formed to indicate positive results for the presence of terpenoids.

3.5.2. Quantitative Analysis

3.5.2.1 Estimation of Total Phenols by Gallic Acid Assay

The phenolic compounds were determined by the method of Folin ciocalteau (Singleton and Rossi, 1965). The stock solution of 1mg/ml gallic acid was prepared in dimethyl sulfoxide. From the stock different concentration of gallic acid taken (400 µl, 320 µl, 160 µl, 80 µl, 40 µl, 20 µl and 10 µl) to make a final volume of 400 µl. To the solution of gallic acid 1.6 ml sodium carbonate (Na₂CO₃). The sodium carbonate solution was prepared by taken 7.5 g of Na₂CO₃ dissolved 100 ml of distilled water (7.5%). After the addition of sodium carbonate solution, add 2 ml of Folin ciocalteau (0.2 N) was added. Similarly, the plant crude extract was prepared at the concentration of 50 µl and 100 µl for controls. All the samples were incubated at 37° C in dark for 1 h and the absorbance was
taken at 765 nm in a UV-VIS spectrophotometer (Chemito). All determinations were carried out in triplicate and the results were expressed in mean g/gallic acid equivalent (GAE)/g of the extract. A blank reagent was prepared using distilled water instead of the plant extract. The amount of phenolic compound in the extract were determined from the standard curve produced with varying concentrations of gallic acid used as a standard. The results were calculated according to calibration curves for gallic acid. Values were expressed as means (n=3) ± standard deviations (SD).

3.5.2.2 Estimation of Total Tannins by Tannic Acid Assay

The total tannins were estimated by the Folin-Denis reagent method. The air dried plant extract powder of 2 g was refluxed with 75 ml of double distilled water for 30 min for complete extraction of tannins. The mixture was allowed to cool and filtered using Watmann filter paper no. 1. The filtrate was centrifuged at 2000 rpm for 20 min. The supernatant was collected in 100 ml volumetric flask and the volume was made up to 100 ml by double distilled water. The blank used in the protocol was a solution of tannic acid.

3.6 Determination of Anti-oxidant Activity

There is evidence that indigenous anti-oxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural anti-oxidants contained in spices, herbs and medicinal plants. The plants were screened for anti-oxidant study.

3.6.1 Superoxide Scavenging Activity of Plant Extracts by Alkaline DMSO Method

The superoxide anion and the products formed by the reduction of superoxide anion have been implicated as causes of mutation in bacteria (Weitzman and Stosse, 1981 and Levin et. al., 1982) and eukaryotic cells (Cunningham and Lokesh, 1983 and Cunningham et. al., 1984), chromosome aberrations (Sofuni and Ishidate, 1984), and transformation in mammalian cells (Troll and Wiesner, 1985 and Cerutti, 1985).
3.6.1.1 Chemicals and Reagents

The dimethyl sulfoxide was purchased from Merck Co. (Germany), Mumbai. Nitro-blue tetrazolium was purchased from SD Fine Chemicals. All the other reagents used were of analytical grade and double distilled water was used for all the experiments.

3.6.1.2 Preparation of standard solution

The required quantity of standard (BHT) was dissolved in methanol to give the concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.81, 3.90 and 1.95 μg/ml.

3.6.1.3 Preparation of Test Sample

To the 25 mg of methanolic extract was dissolved in 25ml of dimethyl sulfoxide to give stock solution of 1 mg/ml. Dilution were done with same dimethyl sulfoxide to give different concentrations of μg/ml.

3.6.1.4 Preparation of Reagents

Alkaline DMSO: 1 ml alkaline DMSO was prepared by adding, 5 mM of NaOH in 0.1 ml of water and 0.9 ml of dimethyl sulfoxide.

NBT: 25 mg of nitro-blue tetrazolium was dissolved in 25 ml of Dimethyl sulfoxide to give concentration of 1 mg/ml.

3.6.1.5 Protocol for Estimation of Superoxide Scavenging Activity

To the reaction mixture containing 0.1 ml of NBT (1 mg/ml solution in DMSO) and 0.3 ml of the plant extract in DMSO, 1 ml of alkaline DMSO (1 ml DMSO containing, 5 mM of NaOH in 0.1 ml water) was added to give a final volume of 1.4 ml and the absorbance was measured at 560 nm. Different concentration of plant extracts ((1000, 500, 250,125, 62.5, 31.25, 15.625, 7.813, 3.906 and 1.953 μg/ml) were added to a hydrogen peroxide solution (0.6 ml, 40 mM). 300μl of plain DMSO, 0.1 ml NBT solution and 1 ml alkaline DMSO was then mixed and absorbance was taken at 560 nm and this was taken as control reading. Alkaline DMSO radical
scavenging assay were determined by the method of Kunchandy and Rao (1990) with slight modification (Sanja et. al., 2009 and Vaijanathappa et. al., 2008). The plant extract were prepared at different concentration (1000 µg/ml to 1.95 µg/ml). The reaction mixture prepared of 0.1 ml of nitro blue tetrazolium (1 mg/ml in DMSO) and 1 ml of alkaline DMSO (1 ml of DMSO containing sodium hydroxide 5 mM in 0.1 ml of water). To the reaction mixture 0.3 ml of the crude extract prepared in DMSO was added. To give a final volume of 1.4 ml and the absorbance was measured at 560 nm spectrophotometrically. The percentage of super oxide radical scavenging by the plant extracts and a standard compound was calculated as follows:

\[
\text{Percentage super oxide} = \frac{\text{Test absorbance} - \text{Control absorbance} \times 100}{\text{scavenging activity} \times \text{Test absorbance}}
\]

3.6.2 Determination of Hydrogen Peroxide (H₂O₂) Scavenging Activity

3.6.2.1 Chemicals and Reagents

Hydrogen peroxide was purchased from Merck Co. (Germany), Mumbai. Di-sodium hypo phosphate was purchased from SD Fine Chemicals.

3.6.2.2 Preparation of Standard Solution

The required quantity of standard (BHT) was dissolved in methanol to give the concentration of 1000, 500, 250, 125, 62.50, 31.25, 15.625, 7.81, 3.90 and 1.95 µg/ml.

3.6.2.3 Preparation of Reagents

H₂O₂ Solution: 20 mM of H₂O₂ in 1.0 l of PBS.

Phosphate Buffer Saline: 5.751 gm of di-sodium hypo phosphate, 1.482 gm of sodium di-hypo phosphate and 9 gm of sodium chloride in one liter of double distilled water.
3.6.2.4 Protocol for Estimation of H$_2$O$_2$ Scavenging Activity

The protocol used for hydrogen peroxide assay was the procedure done by Ruch et. al., (1989) method with minor modification. A solution of hydrogen peroxide (20 mmol/ L) was prepared in phosphate buffer (pH 7.4). The test samples were prepared at different concentrations ranging from 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906 and 1.953 μg/ml and the test sample was added to the hydrogen peroxide solution (20 mmol/ L, 2 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of samples was calculated using the following formula:

\[
\text{Percent scavenged [H}_2\text{O}_2] = \left[\frac{(A0 - AI)}{AI}\right] \times 100
\]

Where A0 was the absorbance of the control,

And AI was the absorbance of the sample.

3.6.3. Determination of Nitric Oxide Free Radical Scavenging Activity

3.6.3.1 Chemicals and Reagents

Chemicals which were required to perform nitric oxide test are sodium nitroprusside and Griess reagent which were purchased from Merck Co. (Germany).

3.6.3.2 Preparation of Standard Solution

The required quantity of standard (BHT) was dissolved in methanol to give the concentration of 1000, 500, 250, 125, 62.50, 31.25, 15.625, 7.81, 3.90 and 1.95 μg/ml.

3.6.3.3 Preparation of Test Sample

Stock solutions of samples were prepared by dissolving 10 mg of dried methanolic extract in 10 ml of methanol to give concentration of 1 mg/ml.
3.6.3.4 Protocol for Estimation of Nitric Oxide Scavenging Activity

The plant extracts (1000 µg/ml – 1.953 µg/ml) 1 ml was mixed with 1 ml phosphate buffer saline and 4 ml (10 mM) sodium nitroprusside and was kept for incubation at room temperature at 25°C for 150 min. After incubation, 0.5 ml of reaction mixture and 1 ml sulphanilic acid reagent (0.33% in 20% glacial acetic acid) were added and incubated for 5 min at room temperature (for diazotization reaction). Then 1 ml N-(1-naphthyl) ethylenediamine dihydrochloride was added and kept in diffused light for 30 min and absorbance was measured at 540 nm (Badami et. al., 2005). Butylated hydroxy toluene (BHT) was used as control.

Scavenging activity (%) = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100

3.6.4. DPPH Free Radical Scavenging Activity

3.6.4.1 Chemicals and Reagents

Chemicals which were required to perform DPPH test were DPPH and methanol which were purchased from Merck Co. (Germany).

3.6.4.2 Preparation of Standard Solution

The required quantity of standard (BHT) was dissolved in methanol to give the concentration of 1000, 500, 250, 125, 62.50, 31.25, 15.625, 7.81, 3.90 and 1.95 µg/ml.

3.6.4.3 Preparation of Test Sample

Stock solutions of samples were prepared by dissolving dried methanolic extract in methanol to give the concentration of 1000, 500, 250, 125, 62.50, 31.25, 15.625, 7.81, 3.90 and 1.95 µg/ml.

3.6.4.4 Preparation of DPPH Solution

The solution of 4.3 mg of DPPH was dissolved in 3.3 ml methanol and it was protected from light by covering the test tubes with aluminium foil.
3.6.4.5 Protocol for Estimation of DPPH Scavenging Activity

DPPH radical scavenging activities of all the fractions were determined by the method of Blois (1958) with some modification. The crude plant extracts were prepared at different concentrations (1000 µg/ml to 1.95 µg/ml) in methanol, initially 10 µl of plant extracts was mixed with 200 µl of 100 mM DPPH (dissolved in methanol). The reaction mixtures were incubated for 30 min at 37° C under dark condition. Butylated hydroxy tolene was used as control, while methanol was used as blank. The DPPH radical scavenging activity was determined by measuring the absorbance at 490 nm spectrophotometrically (Ara and Nur, 2009). The percentage inhibitions were calculated by the formula given

\[
\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100
\]

3.6.5 Determination of Total Anti-oxidant Capacity by Phosphomolybdenum Method

3.6.5.1 Chemicals and Reagents

Sulphuric acid was purchased from Merck Co. (Germany), Mumbai. Di-sodium hypo phosphate and ammonium molybdate was purchased from SD Fine Chemicals.

3.6.5.2 Preparation of Reagents

Reagent Solution was prepared by adding 0.6 M of sulphuric acid, 28 mM of sodium phosphate with 4 mM of ammonium molybdate.

3.6.5.3 Protocol for Determination of Total Anti-oxidant Capacity

Total anti-oxidant capacities of the plant extracts and butylated hydroxy tolene (BHT) were determined by the method of phosphomolybdenum (Prieto 1999). An aliquot (0.1 ml) of plant extracts were combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°
C for 90 min. After incubation the samples were cooled to 25° C, the absorbance was measured at 695 nm against blank. The blank contained 1 ml of reagent solution without the sample. The total anti-oxidant activity was expressed as the absorbance value at 695 nm. The high absorbance value of the sample indicates its strong anti-oxidant activity. The total anti-oxidant capacity may be attributed to their chemical composition and phenolic acid content (Jayaprakasha et. al., 2003 and Prasad et. al., 2009).

3.7 Identification of Compounds by Thin Layer Chromatography and Characterization by GC-MS

The compounds were identified by thin layer chromatography and compounds were separated by high performance chromatography. The characterization of crude extract was done by GC-MS.

3.7.1 Identification of Compounds by Thin Layer Chromatography

Thin layer chromatography (TLC) is a sophisticated method and a type of planar chromatography used in the present study to identify the components in the plant extract such as alkaloids, phenols, flavonoids etc. The separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase, (driven by capillary action) travel along the stationary phase. During the movement the compound with higher affinity to stationary phase travels slowly while others travel faster. All the silica gel glass plates contained a reference spot along with the plant extract. The gallic acid and tannic acids were used as reference. The silica gel glass plates were developed in an iodine chamber in the presence of iodine fumes. The formation of yellow to purplish pink colour spots was observed. The \( R_f \) of the unknown compound is compared with \( R_f \) of the known compound (gallic acid and tannic acid). \( R_f \) is the retention factor, indicates the movement of compound on the silica gel plates.

TLC was carried out using Silica gel plates. The plate is spotted with small amount of test samples and standard samples 0.5 cm from bottom of TLC plate. The spotted plate is immersed in the solvent system (methanol and
chloroform in a ratio of 9:1). The system is kept 5 min to equilibrate the atmosphere in TLC chamber and let solvent front run up the plate. When the plate has run far enough (solvent front approx 1 - 0.5 cm from top of plate) remove plate from TLC chamber. The plate is left to dry and then the $R_f$ value is calculated for each sample and the results compared with the standard.

$R_f$ value is the distance travelled by a given component divided by the distance travelled by the solvent front. For a given system at a known temperature, it is a characteristic of the component and can be used to identify components. The $R_f$ values of the extract and standard were observed, calculated and compared. It was found that the chromatogram had been showing bands at the similar distances as that of the band of Tannic acid and Gallic acid and their $R_f$ values calculated were similar.

$$R_f = \frac{\text{Distance travelled by component}}{\text{Distance travelled by solvent}}$$

### 3.7.2 Characterization of Crude Extract by Gas chromatogram and mass spectrometer (GC-MS)

The methanolic crude extracts were characterized by GC-MS technique. The analysis was done by Mr. Ajai Kumar at Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi. The instrument used for analysis was GCMS-QP2010 Plus. Analytical conditions for Gas chromatogram and mass spectrometer are given below.

**GCMS-QP2010 Plus (Shimadzu)**

<table>
<thead>
<tr>
<th>Column</th>
<th>Rts- 5 MF (30 m x 0.25 mm internal diameter x 0.25 µm film thickness)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Electron ionization (EI)</td>
</tr>
<tr>
<td>Source temperature</td>
<td>230° C</td>
</tr>
<tr>
<td>Scan time</td>
<td>0-60 min</td>
</tr>
<tr>
<td>Transfer line temperature</td>
<td>280° C</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Split mode 10:0</td>
</tr>
<tr>
<td>Injection temperature</td>
<td>250° C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>1 µl</td>
</tr>
</tbody>
</table>
Materials and Methods

Carrier gas  Helium; Flow rate: 1.5 ml/min
Oven temperature  80°C - 310°C

3.7.2.1 Identification of Components

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technique (NIST05) and WILEY8 spectral database having more patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST05 and WILEY8 library. The Name, Molecular weight, Molecular formula were obtained.

3.8 Statistical Analysis

All results are expressed as mean ± standard deviation (SD). The mean, standard deviation and percentage inhibition values were calculated in MS excel. Linear regression analysis was used to calculate the efficient concentration (IC\textsubscript{50}) values. The results were compared using one-way analysis of variances (ANOVA) and considered significantly different where probability values were found to be equal to or less than 0.05. All ANOVA tests were performed using Graph Pad Prism (Graph Pad Software, Inc., San Diego, USA).
References:


Materials and Methods


