APPENDICES

APPENDIX I

ESTIMATION OF TOTAL FLAVONOIDS
(Nabavi et al., 2008a and Ebrahimzaded et al., 2009a)

Principle
Aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids.

Reagents
1. Methanol
2. Aluminium chloride
3. Potassium acetate

Procedure
Plant extracts (0.5 ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M Potassium acetate, and 2.8 ml of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam UV/Visible spectrophotometer. Total flavonoid contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions of different concentrations (12.5 to 100 mg/ml) in methanol.

APPENDIX II

DPPH SCAVENGING ASSAY
(Mensor et al., 2001)

Principle
Reduction of stable nitrogen–centered free radical DPPH by the antioxidants of plant extract via the process of hydrogen or electron donation, causes the color changes from violet to yellow. The color change depends on their ability to scavenge DPPH which measured at 518 nm.
Results and Discussion

Compatibility of Pongamia pinnata biofuel / diesel blends with few industrial metals

Reagents
1. Methanol
2. 1mM DPPH (Diphenyl-2-picryl hydrazyl radical) in Methanol

Procedure
3ml of in Methanolic solution of 1mM DPPH (3ml) was added to 100 µl of PBS containing 10-100µg of the flavonoid fraction. DPPH solution with methanol was used as a positive control and methanol acted as a control. When DPPH reacts with antioxidant in the sample, it was reduced and the color changed from deep violet to light yellow. This was measured at 518nm. The percentage scavenging activity was calculated by the following formula:

\[
\text{Scavenging activity (Per cent)} = \frac{A_{518}[\text{control}] - A_{518}[\text{sample}]}{A_{518}[\text{control}]} \times 100
\]

APPENDIX III
NITRIC OXIDE SCAVENGING ASSAY
(Green et al., 1982)

Principle
Scavenging of nitric oxide is based the principle that aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce the chromophore nitrite ion, which is measured colorimetrically at 546 nm.

Reagents
1. Phosphate buffered saline (7.2 pH)
2. Sodium nitroprusside (100mM)
3. Griess reagent (1 per cent sulfanilamide, 2 per cent H₃PO₄, 0.01 per cent Naphthyl ethylenediamine dihydrochloride)

Procedure
Three ml of reaction mixture containing sodium nitroprusside in PBS and extract was incubated at 25°C for 150 minutes. Controls were kept without test compound in an identical manner. After incubation, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546nm.
The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.

\[
Scavenging \; activity \; (\text{Per cent}) = \frac{A[\text{control}] - A[\text{sample}]}{A[\text{control}]} \times 100
\]

APPENDIX IV

HYDROGEN PEROXIDE SCAVENGING ASSAY
(Ruch et al., 1989)

The ability of the plant extracts to scavenge H$_2$O$_2$ was attributed to their phenolics which can donate electrons to H$_2$O$_2$ and neutralizing it to water and oxygen.

Reagents
1. Phosphate buffer (pH7.2)
2. H$_2$O$_2$ (4mM)

Procedure
A solution of H$_2$O$_2$ (4mM) was prepared in phosphate buffer (pH 7.2). H$_2$O$_2$ concentration was determined spectrophotometrically from its absorption at 230nm was determined after 10 minutes against a blank solution containing phosphate buffer without H$_2$O$_2$.

The scavenging activity of H$_2$O$_2$ by plant extract and the standard compounds was calculated using the formula

\[
Scavenging \; H_2O_2 \; (\text{Per cent}) = \frac{A_0 - A_1}{A_0} \times 100
\]

APPENDIX V

CYTOTOXICITY ASSAY
(Salomi and Panikkar, 1989)

Principle
The viable cells exclude the dye and remained unstained, while non-viable cells take up the dye and are stained blue.
Reagents

1. 0.2M - PBS pH 7.2
   KCl – 40 mg
   KH₂PO₄ – 20 mg
   Na₂HPO₄·2H₂O – 57.5 mg
   NaCl – 900 mg
   Distilled water – 100 ml

2. Trypan blue – 0.1 per cent in PBS

Procedure

The Ehrlich Lymphoma Ascite cells were propagated in the peritoneal cavity of mice were taken and washed with saline thrice by centrifuging at low speed. 0.1 ml of PBS containing 1×10⁶ cells was used for the in vitro assay.

Various concentrations (10 to 100 µg/0.1ml of PBS) of flavonoid fraction containing 1×10⁶ ELA tumor cells were incubated at 37°C for three hours. At the end of the incubation period 0.1 ml of trypan blue was added and layered the cells on the haemocytometer for counting. The dead cells were blue in color and counted to calculate the percentage of dead cells (per cent cytotoxicity).

\[
\text{Per cent Cytotoxicity} = \frac{\text{Dead cell count}}{\text{Dead cell count} + \text{Viable cell count}} \times 100
\]

APPENDIX VI

ESTIMATION OF ASPARTATE AMINO TRANSAMINASE (AST)
(Reitman and Frankel, 1957)

Principle

Serum glutamine oxaloacetic transaminase catalyses the reversible transfer of an amino group from aspartate to α-keto glutarate forming glutamate and oxaloacetate. SGOT catalyses the following reaction:

\[
L - \text{Aspartate} + \alpha - \text{Keto glutarate} \rightarrow \text{Oxaloacetate} + L - \text{Glutamate}
\]

\[
\text{Oxaloacetate} + 2,4 \text{ DNPH} \rightarrow 2,4 \text{ dinitrophe nyl hydrazine (Brown Colored)}
\]
Reagents

1. Tris buffer, pH 7.5 - 100mmol/l
2. L-aspartate - 500mmol/l
3. 2-oxoglutarate - 15mmol/l
4. 2, 4 dinitrophenyl hydrazine reagent
5. Working sodium hydroxide (4N)

Procedure

0.5ml of buffered substrate was incubated at 37°C for 3 minutes and 0.1 ml of serum was added, mixed well and incubated at 37°C for 30 minutes. Then 0.5ml of 2, 4 -dinitrophenyl hydrazine (DNPH) reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5ml of 4N working sodium hydroxide was added, and kept at room temperature for 10 minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505 nm. Activity of SGOT was expressed as U/L.

APPENDIX VII

ESTIMATION OF ALANINE AMINO TRANSAMINASE (ALT)
(Reitman and Frankel, 1957)

SGPT catalyses the reversible transfer of amino group from L-alanine to alpha ketoglutarate with the formation of pyruvate and glutamate. The pyruvate so formed is allowed to react with 2-4 dinitrophenylhydrazine (DNPH) to produce 2, 4- dinitrophenyl hydrazine derivative, which is measured photometrically.

\[ \alpha - \text{Keto glutarate} + L - \text{Alanine} \xrightarrow{SGPT(pH 7.4)} L - \text{Glutamate} + \text{Pyruvate} \]

\[ \text{Pyruvate} + 2,4 \text{ DNPH} \xrightarrow{\text{Alkaline Medium}} 2,4 \text{ dinitrophenyl hydrazine(Brown Colored)} \]

Reagents

1. Tris buffer, pH 7.5 - 100mmol/l
2. L-alanine - 500mmol/l
3. 2-oxoglutarate - 15mmol/l
4. 2, 4 dinitrophenyl hydrazine reagent
5. Working sodium hydroxide (4N)
**Procedure**

0.5ml of buffered substrate was incubated at 37°C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37°C for 60 minutes. Then 0.5ml of DNPH reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5ml of 4N working sodium hydroxide was added and kept at room temperature for 10 minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505 nm. Activity of SGPT was expressed as U/L.

**APPENDIX VIII**

**ESTIMATION OF ALKALINE PHOSPHATASE**

*(King, 1965)*

**Principle**

Alkaline phosphatase was an enzyme which catalyses the splitting of phosphoric acid from certain monophosphoric esters. In this method disodium phenyl phosphate was hydrolyzed with the liberation of phenol and formation of sodium phosphate. The amount of phenol formed was estimated in a spectrophotometer at 650nm.

**Reagents**

1. **Disodium phenyl phosphate (0.01M)**  
   Dissolved 1.09g of disodium phenyl phosphate in water and made up to 500ml.  
   Boiled, cooled and added little chloroform and kept in refrigerator (Solution A).

2. **Sodium carbonate-sodium bicarbonate buffer (0.1M)**  
   Dissolved 3.18g of anhydrous sodium carbonate and 1.68g of sodium bicarbonate in water and made up to 500ml (Solution B).

3. **Buffered substrate for use**  
   Equal volume of solution A and solution B was mixed which has pH of 10.

4. **Tricholoro acetic acid (20%)**

5. **Acid molybdate reagent**  
   5g of ammonium molybdate dissolved in 5N sulphuric acid.
6. 1, 2, 4 – ANSA

0.25% of 1,2,4 – ANSA was prepared by adding 0.5g of dry powder ANSA to 190 ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite stoppered the bottle and shaken until it dissolved.

7. Stock Phosphate solution

Dissolved 2.194g of pure potassium dihydrogen phosphate in water and made up to 500ml. Add few drops of chloroform (1mg/1ml of phosphate).

8. Working standard: Diluted 2 ml of stock standard to 500ml.

Procedure

Pipetted out 6 ml of buffered substrate in test tube and placed it in water bath at 37°C for few minutes. Added 0.3ml of serum mixed well and incubated for 15 minutes. At the same time control and blank was also kept. For blank 0.3ml of water was added to 6ml buffered substrate. For control 0.3ml of serum was added to 6ml of distilled water. After that added 1.2ml of 20% TCA and shaken well. 5ml of filtrate was taken in separate test tubes. To blank and control added 0.8ml of acid molybdate followed by 0.2ml of ANSA. Mixed well and allowed it to stand for 10 minutes at 37°C and the color developed was read at 650nm.

Pipetted out 1.0 to 4.0ml of standard solution and made up to 5ml with distilled water. 0.8ml of acid molybdate was added followed by 0.2ml of ANSA. Standards were also read at 650nm. Alkaline phosphatase activity in serum was expressed as 1U/L. The activity in tissue homogenate was expressed as µmole of phenol liberated/min/mg protein.

APPENDIX IX
ESTIMATION OF CATALASE
(Luck, 1974)

Principle

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of H₂O₂ by catalase, the absorption decreases with time. The enzyme activity can be calculated from this decrease.
Reagents

1. 0.067M Phosphate buffer of pH 7.0
   Dissolved 3.522g of KH$_2$PO$_4$ and 1.218g of Na$_2$HPO$_4$.H$_2$O in distilled water and made up the volume to 1 litre.

2. H$_2$O$_2$ – Phosphate buffer
   Diluted 0.16 ml of H$_2$O$_2$ (10% w/v) to 100ml with phosphate buffer. Freshly prepared and used. The absorbance of the solution should be about 0.5 at 240nm with a 1cm light path.

Procedure

Pipetted out 3.0 ml of H$_2$O$_2$ Phosphate buffer into the experimental cuvette and mixed in 0.01 – 0.04ml sample with a glass rod flattened at one end. Noted the time $\Delta t$ required for a decrease in absorbance from 0.45 to 0.4 at 240nm. This value was used for the calculations. If $\Delta t$ was greater than 60 seconds, then the measurements was repeated with more concentrated solution of the sample. Calculated the activity and expressed as nmoles H$_2$O$_2$ decomposed/min/mg protein.

APPENDIX X
ESTIMATION OF SUPEROXIDE DISMUTASE
(Misra and Fridovich, 1972)

Principle
Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of NBT reduction, the extent of which can be assayed spectrophotometrically.

Reagents
1. 50mM potassium phosphate buffer, pH 7.8
2. 45µM Methionine
3. 5.3µM Riboflavin
4. 84µM Nitro Blue Tetrazolium (NBT)
5. 20mM Potassium cyanide

Procedure
The incubation medium contained a final volume of 3 ml, 50 mM potassium phosphate buffer (pH 7.8), 45µ M methionine, 5.3 µM riboflavin, 84 µM NBT and 20mM
Potassium cyanide. The tubes were placed in an aluminium foil lined box maintained at 25°C and equipped with 15 W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600 nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of enzyme. Calculated the activity and expressed in unit / mg / protein. One unit of the enzyme activity was defined as the amount of enzyme giving 50 per cent inhibition of the reduction of NBT.

**APPENDIX XI**

**ESTIMATION OF GLUTATHIONE PEROXIDASE**

(Rotruck *et al.*, 1973)

**Principle**

A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of reduced glutathione (GSH) for a specified time period. Glutathione peroxidase converts GSH to oxidized glutathione. Then the remaining GSH was measured at 421 nm.

\[
2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O
\]

**Reagents**

1. 0.4 M Tris buffer, pH 7.0
2. 10 mM Sodium azide solution
3. 10 per cent Trichloroacetic acid
4. 0.4 mM EDTA
5. 20 mM Hydrogen peroxide solution
6. 2 mM Glutathione solution

**Procedure**

To 2ml of Tris buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.5ml of tissue homogenate were added. 0.2ml of glutathione and 0.1ml of hydrogen peroxide were also added. The contents were mixed well and incubated at 37°C for 10 minutes along with a tube containing the reagents except tissue homogenate. After 10 minutes, the reaction was arrested by the addition of 0.5ml of 10 per cent TCA, centrifuged and the supernatant was assayed for glutathione by the method of Moron *et al.*, 1979 as in Appendix – XIV

The activities were expressed as nmoles of GSH oxidized/min/ mg protein.
APPENDIX XII

ESTIMATION OF VITAMIN A
(Bayfield and Cole, 1980)

Principle
The color produced by vitamin A acetate or palmitate with TCA is proportional to its concentration, which is measured at 620nm in a spectrophotometer.

Reagents
All reagents were prepared freshly. Exposure of samples and reagents to light was avoided.
1. Saturated TCA in chloroform
   15g clear TCA crystals were dissolved in 25ml of alcohol-free chloroform, stored in dark.
2. Standard vitamin A
   1.5mg vitamin A palmitate was dissolved in 10ml of chloroform.

Procedure
Aliquots of the standard were pipetted out into a series of clean, dry test tubes in the concentration range of 0-7.5 µg. The volumes in all the test tubes were made up to 0.1ml with chloroform. From a fast delivery pipette, added 2 ml of TCA reagent rapidly, mixing with the contents of the tube. The absorbance was recorded immediately at 620nm in a spectrophotometer. The procedure was repeated for the sample tubes. Constructed a standard graph and read off the concentration in the samples. The results were expressed as µg/gram wet tissue.

APPENDIX XIII

ESTIMATION OF VITAMIN E
(Rosenberg, 1992)

Principle
Tocopherols can be estimated using Emmerie-Engel reaction which is based on the reduction of ferrous ions by tocopherols, which then forms a red color with 2, 2’ dipyridyl. Tocopherols and carotenes are first extracted with xylene and the extinction read at 460nm to measure carotenes. Correlation is made for these after adding ferric chloride and reading at 520nm.
Reagents

1. Absolute alcohol
2. Xylene
3. 2, 2’ dipyridyl – 1.2g in 1 litre of n-propanol
4. Ferric chloride solution
   1.2g of FeCl₃.6H₂O in one litre of ethanol and stored in a brown bottle
5. Standard solution of D, L-α–tocopherol (10mg/L in absolute alcohol)
   91mg of α- tocopherol is equivalent to 100mg of tocopherol acetate.

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank) pipetted out 1.5 ml of each tissue extract, 1.5ml of the standard and 1.5ml of water respectively. To the test and blank added 1.5ml of ethanol and to the standard added 1.5ml of water. Added 1.5ml of Xylene to all the tubes, stoppered, mixed well and centrifuged.

Transferred 1.0ml of xylene layer into another stoppered tube and care should be taken not to include any ethanol or protein. Added 1.0ml of 2, 2’ dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml of the mixtures into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then, in turn with the blank, added 0.33ml of ferric chloride solution. Mixed well and after exactly 15 minutes read test and standard against the blank at 520nm. The amount of Vitamin E can be calculated using the formula,

\[
\text{Amount of tocopherol} = \frac{\text{Reading of test at 520nm} - \text{Reading of test at 460nm}}{\text{Reading of standard at 520nm}} 
\times 0.29
\]

The results were expressed as µg per gram wet tissue.

APPENDIX XIV

ESTIMATION OF REDUCED GLUTATHIONE
(Moron et al., 1979)

Principle

Reduced glutathione (GSH) is measured Edman’s reaction with DTNB (5, 5’-dithio-2-nitro benzoic acid) to give a compound that absorbs at 412 nm.

\[
2\text{GSH} + H_2O_2 \xrightarrow{GPx} \text{GSSG} + 2H_2O
\]
Results and Discussion

Compatibility of Pongamia pinnata biofuel / diesel blends with few industrial metals

Reagents
1. 0.2M sodium phosphate buffer (pH 8.0)
2. 0.6 mM DTNB in 0.2 M phosphate buffer
3. 5 per cent TCA
4. 25 per cent TCA
5. Standard GSH solution (M.W. 307.33)
   Dissolved 10 mg of GSH in 100ml of 5 per cent TCA

Procedure

The tissues (1g each) were homogenized in 5 per cent TCA. 125 μl of 25% TCA was added to give 20 per cent homogenate. The precipitated protein was centrifuged down at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1ml of the supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0). 0.2 ml of freshly prepared DTNB solution (0.6mM in 0.2M phosphate buffer pH 8.0) was added to the tubes and the intensity of the yellow color formed was read at 412nm in a spectrophotometer after 10 minutes.

A standard curve of GSH was prepared using concentrations ranging from 2 to 10 nanomoles of GSH in per cent TCA. The results were expressed as mean GSH in nmoles/gram of wet tissue.

APPENDIX XV

ESTIMATION OF TISSUE THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS)
(Nichans and Samuelson, 1963)

Principle

The pink chromogen formed by the reaction of 2-thiobarbituric acid with breakdown products of lipid peroxidation malondialdehyde and other thiobarbituric acid reactive substances (TBARS) in acidic solution was read at 535 nm.

Reagents
1. Trichloro acetic acid (TCA) – 15%
2. Hydrochloric acid (HCl) – 0.25N
3. Thiobarbituric acid (TBA) – 0.38% in hot distilled water
4. TCA-TBA-HCl reagent-solution: 1,2 and 3 were mixed freshly in the ratio of 1:1:1.
5. Stock standard –(MDA bis diethyl acetate) or (1,1,3,3-tetra methoxy propane) was made upto 100ml with double distilled water.

Procedure

The tissue homogenate was prepared in Tris-HCl buffer (pH 7.5) 1.0 ml of the tissue homogenate was treated with 2.0ml of TBA-TCA-HCl reagent and mixed thoroughly. The mixture was kept in boiling water bath for 15 minutes. After cooling, the tubes were centrifuged for 10 minutes and the supernatant was taken for measurement. The absorbance of chromophore was read at 535 nm against the reagent blank. The amount of TBARS was calculated using the extinction co-efficient $1.56 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$. The results were expressed as mM per 100 mg tissue.

APPENDIX XVI
HISTOLOGICAL TECHNIQUE
(Culling, 1974)

At the end of the treatment period, the different organs of the normal extract, extract plus the ELA tumor cells and ELA tumor cells treated mice were removed and histological studies were carried out to reveal the anticarcinogenic effect of the protein fractions of the selected medicinal plants.

The following steps were followed in the histological techniques.

1. Autopsy bits are preserved in 10 per cent formalin solution for minimum 1 hour.
2. Dehydration of biopsy bits were done by 3 changes of acetone (each 500ml).
3. Cleaned the bits from acetone by 3 changes of xylene (each 500ml) for about 3 hours.
4. Incubated the processed tissue bits in paraffin wax - 2 changes for 3-4 hours in an incubator at 58 – 60°C.
5. Embedded the tissue in paraffin wax after incubation in melted paraffin.
6. The sections were cut from autopsy bit embedded in wax (Sections are 1-3 μm thick autopsy bit).
7. Sections were taken on the glass slide
8. Sections on glass slide were cleaned from wax by immersing in xylene.
Sections were histochemically reacted with haemotoxylin and eosin staining to evaluate the morphology and cellular composition.

APPENDIX XVII

Characterization of Flavonoid constituents

(Geissman, 1962)

<table>
<thead>
<tr>
<th>Flavonoid type</th>
<th>Aq. OH</th>
<th>Con. H$_2$SO$_4$</th>
<th>Mg-HCl (Shinoda Test)</th>
<th>Na/Hg and HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chalones</td>
<td>Orange to red</td>
<td>Orange, red or magenta</td>
<td>None</td>
<td>Very pale yellow</td>
</tr>
<tr>
<td>Dihydrochalcones</td>
<td>Colorless to pale yellow</td>
<td>Colorless to pale yellow</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Aurones</td>
<td>Red to purple</td>
<td>Red to magenta</td>
<td>None</td>
<td>Very pale yellow</td>
</tr>
<tr>
<td>Flavonones</td>
<td>Yellow to Orange in the cold; deep red or purple on heating red</td>
<td>Orange to crimson</td>
<td>Red, Magenta, Violet, blue</td>
<td>Red</td>
</tr>
<tr>
<td>Flavones</td>
<td>Yellow</td>
<td>Intense yellow to fluorescent orange</td>
<td>Yellow to red</td>
<td>Red</td>
</tr>
<tr>
<td>Flavonols</td>
<td>Yellow to orange</td>
<td>Intense yellow to fluorescent orange</td>
<td>Yellow to magenta</td>
<td>Yellow to pale red</td>
</tr>
<tr>
<td>Flavanols</td>
<td>Very pale yellow, quickly changing to brown</td>
<td>Reddish yellow</td>
<td>Red to magenta</td>
<td>Brownish yellow</td>
</tr>
<tr>
<td>Leucoanthocyanins</td>
<td>Yellow</td>
<td>Crimson</td>
<td>Pink with HCl, deepening with Mg</td>
<td>Very pale pink</td>
</tr>
<tr>
<td>Anthocyanidins and anthocyanins</td>
<td>Blue to violet</td>
<td>Yellowish orange</td>
<td>Red fades to pale pink</td>
<td>Yellowish orange</td>
</tr>
<tr>
<td>Catechins</td>
<td>Yellow changing to red and brown</td>
<td>Red</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Pale pink or pink</td>
</tr>
<tr>
<td>Isoflavonones</td>
<td>Yellow</td>
<td>Yellow</td>
<td>None</td>
<td>Red</td>
</tr>
</tbody>
</table>
APPENDIX - XVIII
HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY.
(Khushboo et al, 2009)

HPTLC is a valuable tool for the investigation of herbal products with respect to
different aspects of their quality. The advantage of HPTLC over other techniques is that
large number of samples can be simultaneously analyzed using small volume of mobile
phase unlike HPLC, thus lowering analysis time and cost per analysis.

Extraction of plant material

Air dried (35 – 50°C) leaves (1.0 g) of C. dactylon and T. catappa were
extracted with solvents (3 _ 25 mL each) of varying polarity viz. chloroform, ethyl
acetate, methanol and ethanol, in order to optimize the extraction method for the most
efficient extraction of andrographolide and its derivatives. Extracts were concentrated
under vacuum, redissolved in methanol, filtered, re-concentrated and finally made up to
5 mL volume with methanol prior to HPTLC analysis.

HPTLC Instrumentation

Chromatography was performed on pre-activated (at 110°C) silica gel 60
F254 HPTLC plates (10 - 10 cm; 0.25 mm layer thickness). Samples and standard
compounds were applied to the layer as 8 mm wide bands, positioned 10 mm from the
bottom of the plate, using an automated TLC applicator Linomat IV (Camag, Muttenx,
Switzerland) with nitrogen flow providing delivery from the syringe at a speed of
10s/mL. These critical parameters were maintained for all analyses performed.

The mobile phase components were ethyl acetate, methanol, and water
(10:1.65:1.35). 1% ethanolic aluminium chloride were used as the spray reagent over
the plate and heated at 110 ºC for 5 minutes in hot air oven. UV detection at 366 nm.

APPENDIX XIX
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
(Vesna et al., 2004)

HPLC analysis

The described HPLC procedure could be useful for the qualitative and
quantitative analysis of flavonoids in plant materials.

HPLC was performed on a liquid chromatograph HP1090 (Hewlett-Packard)
with diode-array detector (DAD). Hypersil MOS column (200 × 2.1 mm) with a 5 µm
particle size was used at the flow rate of 0.2 ml/min. The mobile phase was methanol with water 50:50. The injected volume was 10 µl of 1% ethyl acetate and methanolic solution of extracts and standards. The spectra were acquired in the range 190 - 800 nm in both UV and visible. All injections were performed in duplicate. The identification of compounds was performed by comparison with retention times and mass spectra of pure standards (Vesna et al., 2004).

APPENDIX XX
FOURIER TRANSFORM-INFRARED SPECTROSCOPY
(Mohd Nasir et al., 2006)

IR spectroscopy in mainly in biochemical research for intermediate-sized molecules such as drugs, metabolic intermediates and substrates. It is an ideal and rapid method for measuring certain contaminants in foodstuffs.

Principle
IR is very much helpful in examining the peak variation of amino groups and carboxylic groups. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample, which corresponds to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present.

Procedure
Infrared spectroscopy of Shimadzu Corporation of model IR prestige 21 was used. A drop of each extract was applied on a sodium chloride cell to obtain a thin layer. The cell was mounted on the IR and scanned through the IR region.

APPENDIX XXI
$^{13}$C- NUCLEAR MAGNETIC RESONANCE SPECTROMETRY
(El-Sayed et al., 2011)

The use of NMR in the determination of molecular structure has been a major growth point in Biochemistry. Developments in instrumentation and accumulation of basic data have allowed this technique to be applied to determine macro molecular structure and interactions.
Instrumentation

Melting points (uncorrected) were determined on a D. Electrothermal 9100 (U.K.). Mass spectra were measured using Shimadzu QP-2010 Plus, 70 ev. 1H NMR (300 MHz) and 13C NMR (75 MHz) were measured on Bruker- VX-500 NMR instrument. The NMR spectra were recorded in MeOH and chemical shifts were given in δ (ppm) relative to TMS as internal standard. IR spectra were measured on Jasco FT/IR-460 plus, Japan Infrared Spectrophotometer.