Plant Material

The leaves of *Argyreia nervosa*, *Jasminum sambac*, *Passiflora foetida* and *Sapindus emarginatus* were collected from the foot hills of Tirumala, Tirupathi, Andhra Pradesh. All the plants were authenticated by Dr. Madhava Chetty, Professor, Dept of Botany, S.V. University, Tirupati. Voucher specimens were preserved in the Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Plant</th>
<th>Family</th>
<th>Parts used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Argyreia nervosa</em></td>
<td>Convolvulaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>2</td>
<td><em>Jasminum sambac</em></td>
<td>Oleaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>3</td>
<td><em>Passiflora foetida</em></td>
<td>Passifloraceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>4</td>
<td><em>Sapindus emarginatus</em></td>
<td>Sapindaceae</td>
<td>Leaves</td>
</tr>
</tbody>
</table>

Chemicals

All the chemicals used were of analytical grade and were procured from Merck or Sigma or S.D. Fine Chemicals.

Preparation of hydroalcoholic extracts

The leaf powder was defatted by petroleum ether, the marc was dried and subjected to extraction using 70% alcohol by Soxhlet exhaustive extraction process (Carter, 1987) and percentage yield of extracts is given in Table 3.
Table 3. Percentage yield of the hydro-alcoholic extract

<table>
<thead>
<tr>
<th>S. No</th>
<th>Plant</th>
<th>Yield (% W/W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Argyreia nervosa</td>
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</tr>
<tr>
<td>2</td>
<td>Jasminum sambac</td>
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</tr>
<tr>
<td>3</td>
<td>Passiflora foetida</td>
<td>2.24</td>
</tr>
<tr>
<td>4</td>
<td>Sapindus emarginatus</td>
<td>3.29</td>
</tr>
</tbody>
</table>

PHYTOCHEMICAL STUDIES

(1) Fractionation of hydroalcoholic extracts

Each extract about 30 g was suspended in distilled water (100 ml) in a superating funnel and was fractionated successively using petroleum ether (60-80°C) (3×200 ml), Chloroform (3×200 ml) and ethyl acetate (3×200 ml). The left over portion was designated as aqueous fraction (Table 4).

Table 4. Percentage yield of the fractions (%W/W)

<table>
<thead>
<tr>
<th>Extract Name</th>
<th>Pet Ether (PF)</th>
<th>Chloroform (CF)</th>
<th>Ethyl acetate (EF)</th>
<th>Aqueous (AF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANE</td>
<td>3.15</td>
<td>5.74</td>
<td>6.35</td>
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<tr>
<td>JSE</td>
<td>1.15</td>
<td>2.33</td>
<td>4.33</td>
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<tr>
<td>PFE</td>
<td>1.56</td>
<td>3.26</td>
<td>2.71</td>
<td>9.42</td>
</tr>
<tr>
<td>SEE</td>
<td>1.15</td>
<td>2.47</td>
<td>1.24</td>
<td>5.44</td>
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</tbody>
</table>
(2) Phytochemical Investigations

Preliminary phytochemical tests (Kokate et al., 2001; Khandelwal, 2000) were carried out for the hydroalcoholic extracts.

Test For Alkaloids

Five ml of the extract was added to 2ml of hydrochloric acid (HCl). To this acidic medium, 1ml of different alkaloidal reagents were added. The respective colour (or) precipitate indicated the presence of alkaloids (Trease and Evans, 1978).

Dragendroff’s test

Test solution was treated with Dragendroff’s reagent (Potassium bismuth iodide) showed reddish brown precipitate indicated the presence of alkaloids.

Mayer’s test

Test solution was treated with Mayer’s reagent (Potassium mercuric iodide) gave cream or white coloured precipitate indicated the presence of alkaloids.

Wagner’s test

Test solution was treated with Wagner reagent (Iodide in potassium iodide) gave brown precipitate indicated the presence of alkaloids.

Hagers’ test

Test solution was treated with Hager reagent (saturated picric acid solution) gave yellow precipitate indicated the presence of alkaloids.
Test for Tannins

Gelatin test

Test solution was treated with 1% solution of gelatin containing 10% sodium chloride gave white precipitate indicated the presence of tannins (Trease and Evans 1978).

Test for catechin

Test solution was treated with Conc. Hydrochloric acid warmed near a flame red coloration development indicated the presence of tannins.

Test for chlorogenic acid

The aqueous solution was exposed to air developed green colour indicated the presence of tannins.

Test for Cardiac Glycosides

Keller Killani test for Desoxy sugar

Test solution was treated with few drops of ferric chloride solution and mixed with sulphuric acid containing Ferric chloride solution forms two layers, lower layer reddish brown, upper layer bluish green indicated the presence of desoxy sugar (Trease and Evans, 2002; Kar, 2003).

Baljet test

Test solution was treated with sodium picrate, yellow to orange color development indicated the presence of glycosides.

Bromine Water test

Test solution was treated with bromine water, yellow precipitate indicated the presence of glycosides.

Raymond test
Test solution was treated with dinitrobenzene in hot methanolic alkali, violet colour indicated the presence of glycosides.

Legal test

Test solution was treated with pyridine and was made alkaline by adding sodium nitroprusside solution pink colour indicated the presence of glycosides.

Test for Anthraquinone Glycosides

Borntrager test

The powder drug was boiled in 5 ml of 10% sulphuric acid for 5 minutes, filtered while hot the filtrate was cooled and was shaken gently with equal volumes of benzene. Benzene layer was separated and then treated with solution of ammonia (10%) allowed to separate, ammonical layer acquired pink colour indicated the presence of anthraquinones.

Modified Borntrager’s test

C-glycosides of anthraquinone require more drastic conditions for hydrolysis. Hydrolysis of the drug was carried out with 5 ml of dilute hydrochloric acid and 5 ml of 5% solution of ferrichloride, hydrochloric extract was used for to perform the Borntrager’s test.

Test for Cyanogenic Glycosides

Grignard’s test

The test solution was taken in flask stoppered with strips of sodium picrate. Care was exercised not to touch the inner side of the test tube with paper strips. The content was warmed for half an hr. The strip of paper turned to red colour indicated the presence of cyanogenic glycosides.

Ferriferrocynaide test
Test solution was treated with 5 ml alcoholic potassium hydroxide solution and was allowed to stand for 5 minutes, then transferred to an aqueous solution containing ferrous sulphate and ferric chloride and was warmed over a water bath for 10 minutes. The appearance of Prussian blue colour indicated the presence of hydrogen cyanide.

Precipitation of mercury from mercury nitrate

Test solution was treated with mercury nitrate solution, reduction of mercury by hydrogen cyanide indicated the presence of hydrogen cyanide.

Cuprocynaide test

Test solution was made to contact with the paper saturated with guaic resin in absolute alcohol and was moistened with copper sulphate solution turned to distinct stain indicated the presence of hydrogen cyanide.

Test for Coumarin Glycosides

With ammonia

Test solution was placed over a paper impregnated with ammonia developed fluorescence indicated the presence of coumarins (Kokate, 1999).

With alkali solution

Test solution was extracted with alcohol and made alkaline showed blue or green fluorescence indicated the presence of coumarins.
Material and Methods

Test For Iridoidal Glycosides

*Trim–Hill test*

The test solution was treated with 5 ml of 1% aqueous HCl for 3-6 hours. About 0.1 ml was decanted into another test tube containing Trim Hill reagent (10 ml of acetic acid, 1ml of 0.2 % Copper sulphate, and 0.5 ml Conc. Hydrochloric acid) and it was heated over a flame, production of blue colour indicated the presence of iridoids (Harbourne, 2007).

Test For Flavonoids

*Shinoda test*

1 g of the powder was extracted with 10 ml of ethanol (95%) for 15 minutes on a boiling water bath and filtered. To the filtrate a small piece of magnesium ribbon and 3 drops of hydrochloric acid was added, formation of red colour indicated the presence of flavonoids.

*Fluorescence test*

1 g of powder was extracted with 15 ml methanol for 2 min on a boiling water bath and was filtered while hot and evaporated to dryness. To the residue 0.3 ml boric acid solution (3%v/v) and 1 ml oxalic acid solution (10%w/v) were added. The mixture was evaporated to dryness and the residue was dissolved in 10 ml of ether. The ethereal layer showing greenish fluorescence under Ultra Violet light indicated the presence of flavonoids.
Test For Saponins

Froth test

0.1 g powder was shaken vigorously with 5 ml of water in a test tube for 30 minutes and was left undisturbed for 20 minutes. Persistent froth indicated the presence of saponins.

Test for Steroidal saponins

The extract was hydrolysed with dilute sulphuric acid and extracted with chloroform. The chloroform layer was tested for sterols.

Test for Triterpenoidal saponins

The extract was hydrolysed with dilute sulphuric acid and extracted with chloroform. The chloroform layer was tested for triterpenoids.

Test For Sterols

Libbermann - Burchadt test

1 g powder was moistened with 1 ml of acetic anhydride on a clean tile and 2 drops of sulphuric acid was added. The powder was mixed well and the colour gained was observed. The formation of green blue colour indicated the presence of sterols.

Salkowski reaction

2 ml of extract was treated with chloroform and 2 ml of conc. Sulphuric acid and was shaken well with chloroform, red precipitate indicated the presence of sterols.
Test For Carbohydrates

Reduction of Fehling’s solution

Test solution was taken in test tube and heated, an equal volume of Fehiling’s solution A and B were added and heated over a water bath, formation of brick red precipitate indicated the presence of reducing sugar.

Molisch test

Test solution was treated with alpha napthol and Conc. Sulphuric acid formation of purple precipitate formation at the junction of two liquids indicated the presence of sugars.

Test for pentose

Test solution was heated in a test tube with an equal volume of hydrochloric acid containing little phlororoglucinol, red color production indicated the presence of pentose sugars.

Test for ketoses

Test solution was warmed with equal volume of hydrochloric acid and a crystal of resorcinol over a water bath, formation of rose colour indicated the presence of ketose sugar.

Test For Phenolic Compounds

To the extract few drops 5% alcoholic. Ferric chloride solution was added. Bluish black (or) dark green colour formation indicated the presence of tannins and phenolic compounds.
Test for Proteins and Amino Acids

Million’s Test

To the various plant extracts the Million’s reagent was added and heated on water bath, red colour formation indicated presence of proteins.

Biuret Test

To few ml of extract, equal volume of 10% sodium hydroxide and 0.5% copper sulphate solution was added drop by drop and heated. Purple blue colour indicated presence of proteins.

Ninhydrin Test

To 5 ml of test extract 0.5ml of 0.1% Ninhydrin was added and heated for 1-2 min, appearance of purple colour showed the presence of amino acids.

Test For Lignin

The extract was treated with alcoholic solution of phloroglucinol and allowed to dry and Conc. Hydrochloric acid was added, pink colour formation indicated the presence of lignin.

Test for Fixed Oils and Volatile Oils

A small quantity of extract was pressed separately between two filter papers. Oil stains on the paper indicated the presence of fixed oil, evaporation of oil at room temperature indicated the presence of volatile oil.
Detection of DNA

The fresh plant material reacts with non-ionic detergent and forms complex with total cellular nucleic acid (form a wide array of plant genera and tissue types) was precipitated by adding 0.6 volumes of isopropanol. The precipitate was eluted by electrophoresis and viewed under Ultra Violet light (366 nm) fluorescence indicated the presence of DNA (Dellaporte et al., 1983).

(3) Thin Layer Chromatography (TLC)

TLC was used for the detection of phytochemicals in extracts and for monitoring the progress of column chromatography and for testing the homogeneity of the isolates (Stahl, 1969; Wagner et al., 1984).

Preparation of Chromatographic plates

The chromatographic plates of size 15 × 8 × 0.4 cm were cleaned thoroughly and dried in hot air oven at 105°C. A uniform suspension of silica gel G was prepared by dispersing one part of adsorbent in 2.5 parts of distilled water using a glass mortar and a pestle. The suspension was applied to plates as films, care was taken to eliminate air bubbles in the slurry. The plates were allowed to dry at room temperature and then activated at 105°C for one hour in a hot air oven. The plates were taken out and cooled to room temperature before use. The following solvent systems were used as mobile phases.

- Chloroform : methanol 80 : 20 solvent system (lipids)

- Chloroform : glacial acetic acid : methanol : water 64 : 32 : 12 : 8 (for saponins)

- Ethyl acetate : ethanol : water 100 : 13.5 : 10 (for analysis of polar compounds (glycosides), alkaloids, anthroquinone glycosides, bitters, flavonoids and saponins).
Material and Methods

- Ethyl acetate : formic acid : glacial acetic acid: water 100 : 11 : 11 : 26 (for flavonoids)

- Ethyl acetate : ethanol : water 7 : 2 : 1 (Deoxy sugars)

Detection of compounds

The extracts and isolated compounds were observed under Ultra Violet light and exposed to ammonia vapors / Iodine vapors.

Spray Reagents

- 5% alcoholic neutral ferric chloride solution (phenols)

- 10% ethanolic sulfuric acid (sugars)

- Detection of steroids / triterpenoids, flavonoids and their glycosides were carried out by using Vanillin – sulfuric acid reagent.

  Solution I: 5% ethanolic solution of sulfuric acid

  Solution II : 1% ethanolic solution of vanillin

  The plate was sprayed vigorously with solution I so as to wet it, followed by solution II and then heated for 5-10 minutes at 110°C under observation. Steroids / triterpenoids and their glycosides give blue, blue-violet or pink spots. The yellow colour of the flavonoids and their glycosides gets intensified.
Material and Methods

(4) HPTLC

The hydroalcoholic extract of AN, JS, PF and SE was subjected to HPTLC studies with the following specifications and its Rf values, percentage and area were determined.

Instrument used: HPTLC (Shimadzu)
Stationary phase: HPTLC precoated silica plate (Merck, Mumbai)
Software: Wincats: 1.4.3

Preparation of sample: extract was dissolved in alcohol
Sample application: Linomat 5.
Migration distance: 7 cm
Detection: @254nm, @366nm in Densiometry TLC Scanner III,
Spraying agent: vanillin –sulphuric acid and was heated at 105° for 5 minutes.

(5) Standardization of extracts

Determination of the total phenols

The total phenolic content of the extracts were determined with the Folin-Ciocalteau assay. An aliquot (1 ml) of extracts or a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l) was added to a 25 ml volumetric flask, containing 9 ml of distilled deionised water (H2O). A reagent blank using double distilled H2O was also prepared. One milliliter of the Folin-Ciocalteu’s phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na2CO3 solution was added to the mixture. The solution was diluted to 25 ml with H2O and mixed. After incubation for 90 min at room temperature, the absorbance against the prepared reagent blank was determined at 750 nm with an UV-VIS Spectrophotometer. The
data for the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per 100 grams dry mass (mg GAE/100g). All samples were analysed in triplicate (Marinova et al., 2005).

**Determination of the total flavonoids**

The total flavonoid content was measured with an aluminum chloride colorimetric assay. An aliquot (1 ml) of extracts or a standard solution of (+)-Quercetin (20, 40, 60, 80 and 100 mg/l) was added to a 10 ml volumetric flask, containing 4 ml of distilled deionized water (H₂O). To the flask was added 0.3 ml 5 % sodium nitrate. After 5 min, 0.3 ml of 10 % Aluminum Chloride was added. At the sixth minute, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with H₂O. The solution was mixed well and the absorbance was measured against a prepared reagent blank at 510 nm with an UV-VIS Spectrophotometer. The data of the total flavonoid content was expressed as milligrams of (+) Quercetin equivalents (QE) per 100 g dry mass (mg QE/100 g dw). All samples were analyzed in triplicates (Marinova et al., 2005).

**Determination of total tannins**

The total tannin content in the two extracts was determined by modified method of Polshelettiwar et al., (2007). The sample (0.1 ml) was mixed with 0.5 ml of Folin-Denis reagent followed by 1 ml of sodium carbonate (0.5% w/v) solution and distilled water (up to 5 ml). The absorbance was measured at 755 nm within 30 min of the reaction against the blank. The total tannin in the extract was expressed as the equivalents of tannic acid (g TAE/g extract).

(6) **Isolation of phytochemicals**

*Column chromatography*
The hydroalcoholic extract of AN, JS, PF and SE were subjected to column chromatography using silica gel 60-120 or 100-200 mesh as adsorbent (Column chromatography grade, SD Fine Chemicals) by gradient dilution. The extract was packed over the surface of the silica gel and the solvents were made to run according to increasing order of polarity (Remington, 2005).

**Column chromatography of hydroalcoholic extract of AN**

Preliminary qualitative chemical investigations and TLC studies on hydroalcoholic extract of AN revealed the presence of alkaloids / tannins / phenolic compounds / flavonoids / sterols / sugars / lignin / deoxy ribonucleic acid. Therefore the hydroalcoholic extract of AN was subjected to column chromatography using silica gel 60-120 mesh as adsorbent by gradient dilution to isolate the phytoconstituents.

The bottom of the column (45 cm length, 12 cm diameter) was packed with adsorbent cotton, above which silica gel (60 g) was filled as a slurry in ethyl acetate. Care was taken to prevent entrapment of air bubbles into the column. About 3 g of the hydroalcoholic extract of AN was dissolved in 25 ml of alcohol, dispersed uniformly in 20 g of silica gel and loaded over the filled column. The top portion of the column was covered with a piece of Whatman No.1 filter paper, above which mobile phase level was maintained. The column was prepared in ethyl acetate and left overnight. Next morning, the column contents were eluted with gradient elution starting with pet ether : toluene followed by chloroform, ethyl acetate, methanol and water (90:10, 70:30, 50:50, 30:70 and 10:90). Each time, 100 ml of elute was collected. Elution of different components was monitored by TLC on silica gel-G to know the nature of compounds in the fractions and homogeneity of the compounds. Chloroform:Ethyl acetate (80:20) and ethyl acetate : Methanol (20:80) elutes produced a single spot on TLC with silica gel – G. The fractions were combined and concentrated under
Material and Methods

Institute of Pharmaceutical Technology, SPMVV, Tirupati

reduced pressure, yielded a white substance, which was recrystallized from acetone. It was found to be homogenous by TLC studies and was designated as AN - 1 and AN - 2. The isolated compound was subjected to physical, chemical and spectral studies.

Column chromatography of hydroalcoholic extract of JS

Preliminary qualitative chemical investigations and TLC studies on hydroalcoholic extract of JS revealed the presence of tannins / phenolic compounds / flavonoids / sterols / triterpenoids / sugars / coumarins / deoxy ribonucleic acid. Therefore the hydroalcoholic extract of JS was subjected to column chromatography using silica gel 60-120 mesh as adsorbent by gradient dilution to isolate the phytoconstituents.

The bottom of the column (45 cm length, 12 cm diameter) was packed with adsorbent cotton, above which silica gel (60 g) was filled as a slurry in ethyl acetate. Care was taken to prevent entrapment of air bubbles into the column. About 3 g of the hydro alcoholic extract of JS was dissolved in 25 ml of alcohol, dispersed uniformly in 20 g of silica gel and loaded over the filled column. The top portion of the column was covered with a piece of Whatman No.1 filter paper, above which mobile phase level was maintained. The column was prepared in ethyl acetate and left overnight. Next morning, the column contents were eluted with gradient elution starting with pet ether:toluene followed by chloroform, ethyl acetate, methanol and water (90:10, 70:30, 50:50, 30:70 and 10:90). Each time, 100 ml of elute was collected. Elution of different components was monitored by TLC on silica gel-G to know the nature of compounds in the fractions and homogeneity of the compounds. Chloroform :Ethyl acetate (80:20) and ethyl acetate : Methanol (20:80) elutes produced a single spot on TLC with silica gel–G respectively. The fractions were combined and concentrated under reduced pressure, yielded a white substance, which was recrystallized from acetone. It was found to be homogenous by TLC studies and was designated as JS - 1 and JS - 2. The isolated
compound was subjected to physical, chemical and spectral studies.

**Column chromatography of hydroalcoholic extract of PF**

Preliminary qualitative chemical investigations and TLC studies on hydroalcoholic extract of PF revealed the presence of alkaloids / tannins / phenolic compounds / glycosides / flavonoids / sterols / sugars / lignins / deoxy ribonucleic acid. Therefore the hydroalcoholic extract of PF was subjected to column chromatography using silica gel 60-120 mesh as adsorbent by gradient dilution to isolate the phytoconstituents.

The bottom of the column (45 cm length, 12 cm diameter) was packed with adsorbent cotton, above which silica gel (60 g) was filled as a slurry in ethyl acetate. Care was taken to prevent entrapment of air bubbles into the column. About 3 g of the hydro alcoholic extract of PF was dissolved in 25 ml of alcohol, dispersed uniformly in 20 g of silica gel and loaded over the filled column. The top portion of the column was covered with a piece of Whatman No.1 filter paper, above which mobile phase level was maintained. The column was prepared in ethyl acetate and left overnight. Next morning, the column contents were eluted with gradient elution starting with pet ether:toluene followed by chloroform, ethyl acetate, methanol and water (90:10, 70:30, 50:50, 30:70 and 10:90). Each time, 100 ml of elute was collected. Elution of different components was monitored by TLC on silica gel–G to know the nature of compounds in the fractions and homogeneity of the compounds. Chloroform (100) and ethyl acetate : Methanol (20:80) elutes produced a single spot on TLC with silica gel – G respectively. The fractions were combined and concentrated under reduced pressure, yielded a white substance, which was recrystallized from acetone. It was found to be homogenous by TLC studies and was designated as PF - 1 and PF - 2. The isolated compound was subjected to physical, chemical and spectral studies.

**Column chromatography of hydroalcoholic extract of SE**
Preliminary qualitative chemical investigations and TLC studies on hydroalcoholic extract of SE revealed the presence of tannins / phenolic compounds glycosides / sterols / triterpenoids / saponins / sugars / lignins / deoxy ribonucleic acid. Therefore the hydroalcoholic extract of SE was subjected to column chromatography using silica gel 60-120 mesh as adsorbent by gradient dilution to isolate the phytoconstituents.

The bottom of the column (45 cm length, 12 cm diameter) was packed with adsorbent cotton, above which silica gel (60 g) was filled as a slurry in ethyl acetate. Care was taken to prevent entrapment of air bubbles into the column. About 3 g of the hydro alcoholic extract of SE was dissolved in 25 ml of alcohol, dispersed uniformly in 20 g of silica gel and loaded over the filled column. The top portion of the column was covered with a piece of Whatman No.1 filter paper, above which mobile phase level was maintained. The column was prepared in ethyl acetate and left overnight. Next morning, the column contents were eluted with gradient elution starting with pet ether : toluene followed by chloroform, ethyl acetate, methanol and water (90:10, 70:30, 50:50, 30:70 and 10:90). Each time, 100 ml of elute was collected. Elution of different components was monitored by TLC on silica gel-G to know the nature of compounds in the fractions and homogeneity of the compounds. Toluene : Chloroform (20:80) and ethyl acetate : Methanol (50:50) elutes produced a single spot on TLC with silica gel – G respectively. The fractions were combined and concentrated under reduced pressure, yielded a white substance, which was recrystallized from acetone. It was found to be homogenous by TLC studies and was designated as SE - 1 and SE - 2. The isolated compound was subjected to physical, chemical and spectral studies.

(7) Spectroscopy

UV-Spectra

The UV spectra and absorption maxima ($\lambda_{\text{max}}$) for the isolated compounds were
recorded using Systronics UV-Visible Spectrophotometer.

**IR Spectra**

IR spectra for the pure isolates were recorded in KBr pellet using Perkin Elmer Spectrum one FT-IR Spectrophotometer.

**$^1$H NMR-Spectra**

$^1$H NMR Spectra were recorded using $^1$H NMR AV 400 MHz high resolution multinuclear FT-NMR Spectrometer (Bruker).

**$^{13}$C NMR-Spectra**

$^{13}$C NMR Spectra were recorded using $^{13}$C NMR AVIII 500 MHz high resolution multinuclear FT-NMR Spectrometer (Bruker).
**Material and Methods**

**Mass Spectra**

Mass spectra were recorded using HP 1100 MSD series Spectrometer for Electro Spray Ionization Mass Spectra (ESIMS).

IR, $^1$H NMR, $^{13}$C NMR, Mass spectra were recorded at *Indian Institute of Technology, Chennai.*

**PHARMACOLOGICAL STUDIES**

(1) **In-vitro antioxidant studies**

**DPPH radical scavenging assay**

DPPH scavenging activity was measured with spectrophotometer method (Sreejayan and Rao, 1996). To the extract solution of concentration ranging from 40 microgram to 200 microgram 4 ml of DPPH was added and was made upto 5ml with ethanol, incubated for 30 minutes at room temperature. The absorbance was measured at 517nm against blank. The percentage of inhibition of DPPH was calculated as follows. Ascorbic acid was used as standard and the scavenging effect of DPPH was expressed in terms of ascorbic acid equivalents and its results are presented in Table 15.

\[
\text{\% inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

**Scavenging activity of hydrogen peroxide**

The scavenging effect of hydrogen peroxide was determined as per Ruch, 1989. 1 ml of extract solution was treated with 0.6 ml of hyrogen peroxide for 10 minutes, the absorbance was read at 230 nm against blank. Ascorbic acid was used as standard and the scavenging effect of hydrogen peroxide was expressed in terms of ascorbic acid equivalents.
Material and Methods

and its results are presented in Table 16.

\[
\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

% inhibition

**Scavenging activity of nitric oxide**

Nitric oxide was generated from sodium nitroprusside and its scavenging effect was determined as per Green et al., 1982; Morocci et al., 1994. Different concentration of extract solution in phosphate buffer was incubated with sodium nitroprusside for 5 hours at 25 °C. Control experiments were performed with equal amount of buffer instead of extract solution. After 5 hours of incubation, 0.5ml of supernatant liquid was removed and 0.5ml of Griess reagent was added. The absorbance of the chromphore formed during diazotization with sulphanilamide and its subsequent coupling was read at 546nm. Ascorbic acid was used as standard and the nitric oxide scavenging was expressed in terms of ascorbic acid equivalents and its results are presented in Table 17.

**Total antioxidant capacity**

The total antioxidant capacity was determined by Spectrophotometric method of Prieto et al., 1999. Extract test solution of concentration ranging from 40 to 200 µg was taken in eppendorf tube and 1ml of reagent containing 0.6mM sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybadate were added. The tubes were incubated at 95°C for 90 minutes and were cooled to room temperature, the absorbance was read at 695 nm. Ascorbic acid was used as standard and the total antioxidant capacity was expressed in terms of ascorbic acid equivalents and its results are presented in Table 18.

**Assay of Reducing Power**

The reducing power assay was determined by Spectrophotometric method of
Oyazizu, 1986. The extract solution of various concentrations ranging from 40 to 200 microgram was treated with 2.5 mL of 0.2 M phosphate buffer (pH 6.6), 2.5 ml of 1% potassium ferricyanide incubated at 50°C for 20 minutes cooled to which 2.5 ml of trichloroacetic acid (TCA) was added and centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution was removed and 2.5 ml of methanol and 0.5 ml of (0.1% ferric chloride) solutions were added, the absorbance of the resulting solution was read at 700 nm. Ascorbic acid was used as standard and the reducing power was expressed in terms of ascorbic acid equivalents and its results are presented in Table 19.

(2) In-Vivo antidepressant studies

Animals

Adult healthy Swiss Albino mice of either sex (20-30g) were used. The animals were acclimatized for the laboratory conditions for a period of ten days i.e. room temperature (27±3°C), relative humidity (65±10%), and 12h light/dark cycle. All animals were fed with rodent-pellet diet and water was allowed ad libitum under strict hygienic conditions.

Acute toxicity study

The acute toxicity study was conducted as per the OECD guidelines 423 (OECD, 2001) where the limit test dose of 2000 mg/kg was used (Lipnick et al., 1995; Kulkarni, 1993). Observations were made and recorded systemically 1, 2, 4 and 24 h after dose administration for skin changes, morbidity, aggressiveness, sensitivity of the sound and pain, as well as respiratory movement.

Experimental protocol for antidepressant activity

Mice were randomly divided into 10 groups with six animals in each group. Group I received only vehicle (1% tween 80 solution p.o, daily) and served as control; group II received standard antidepressant drug- Imipramine (25mg/kg p.o, daily); group III and group
IV received AN 250mg/kg and 500mg/kg (p.o, daily) in 1% tween 80 respectively, group V and VI received JS 250 and 500mg/kg in 1% tween 80 respectively, p.o, daily, group VII and VIII received PF 250 and 500mg/kg in 1% tween 80 respectively, p.o. daily, group IX and X received SE 250 and 500mg/kg in 1% tween 80 respectively, p.o. daily. At the end of experimental period (7 days of treatment) the animals were fasted overnight and sacrificed by cervical dislocation. The brains were excised immediately and the brain tissue was homogenized and used for further analysis.

Behavioral parameters

Test for locomotor activity

The locomotor activity was measured using Actophotometer. It consists of cage which has 30 X 30 X 30 cm, and at the bottom six lights and photocells were placed in the outer periphery of the bottom in such a way that a single mouse blocks only one beam. Photocell is activated when the rays of light falls in photocells, the beam of light is interrupted as and when animal crosses the light beam, the number of interruptions were recorded for a period of 5 minutes (Goyal, 2005).
**Tail suspension test**

The total duration of immobility by tail suspension was measured according to the method of Steru et al., 1985. Mice both acoustically and visually isolated and suspended 50cm above the floor by adhesive tape placed approximately 1cm from the tip of the tail, immobility time was recorded during a 15minutes test for animals of all groups.

**Forced swim test**

FST is the most widely used pharmacological in vivo model for assessment of antidepressant activity. In this model, mice were forced to swim in condition from which they cannot escape and rapidly become immobile, floating in an upright position and making only small movements to keep their heads above water. The development of immobility reflects the cessation of persistent escape directed behavior or learned helplessness, and a decrease in the duration of immobility, is interpreted as an antidepressant like effect. Mice were placed individually in a glass cylinders (height: 21 cm, diameter: 14.5 cm) containing 15 cm of water at 23 ± 1°C. First 2 min were allowed for acclimatization and the duration of climbing, swimming and immobility during 4 min were recorded (Porsolt et al., 1977a).

**Biochemical estimations**

**Neurotransmitter estimations**

It has been described that the antidepressant effects of drugs in the FST are related to changes in monoaminergic neurotransmitters (Miura et al., 1999). To address some of the mechanisms involved in antidepressant activity of selected extracts the neurotransmitter estimations were done.

Mice were sacrificed after the treatment period (7 days) by decapitation and brains were rapidly removed. The brains were placed in 800µl of ice-cold 0.1M perchloric acid. Individual brain samples were homogenized and centrifuged at 20000 x g at 4°C and stored in
a dark freezer at -70°C until further analysis. The pellets were dissolved in 10mM NaOH solution for protein determination using the Bradford protein assay (Bradford, 1976).

The samples were filtered through a 0.45-µm filter and 10 µl was injected in an HPLC system. The HPLC eluent system consisted of aqueous component of citrate buffer and acetonitrile (95:5, v/v) as organic phase, with an aqueous component of 12.16 mM citric acid, 11.60 mM (NH₄)₂HPO₄, 2.34 mM sodium octylsulfonate, 3.32 mM DBAP and 1.1 mM disodium EDTA. The pH of the eluent was adjusted to 3.71 with NaOH 2N after acetonitrile addition and filtered through a 0.45-µm filter; the eluent was used at a flow rate of 1ml/min (pressure was approximately 13.1 MPa) (Alburges et al., 1993).

**Conditions for HPLC**

The Wakosil C-18 (stainless steel column of 250mm x 4.6mm internal diameter, was packed with porous silicon spheres of 5µm diameter, 100Å° pore diameter- I15C-18rs-100a, 5µm, 4.6 x 250mm) was used at 27±0.2°C. The chromatographic system consists of Jasco PU - 2085 plus pump, Jasco AS – 2057 auto sampler, and a mellinium 32 software system. The detection system consisted of Antec Leyden model II electrochemical detector with an Antelec Leyden model sensel ISAAC flow- cell assembly (range 1nA, filter 0.005Hz, E_{ox}= 0.60V, basal±0.001V, I_c =2.72nA).
Material and Methods

Estimation of Monoamine oxidase A and B

Brain tissue was homogenized in ten volumes of cold sodium phosphate buffer (200mM, pH 7.4) containing 320mM sucrose, at 4°C for 30 seconds, using a Teflon glass homogenizer. The homogenate was centrifuged at 600g for 10min at 4°C to remove nuclei and debris. The mitochondrial fraction was obtained by further centrifugation at 1500g for 120min at 4°C and resuspended in buffer. The protein concentrate was determined the MAO assay mixture contained 500μl of mitochondrial protein in the phosphate buffer. 300μl of 4mM serotonin and norepinephrine were added as substrate for MAO A and B. Mixtures were incubated at 37°C for 20min, followed by the addition of HCl (600μl, 1M). Reaction products were extracted into 4ml butyl acetate or cyclohexane respectively. The organic phases were collected and absorbance was measured at 280nm for MAO A or 242nm for MAO B respectively (Zheng and Liu, 2002; Zhou et al., 2006).

Antioxidant studies

Brain tissue samples were homogenized in 50 mM phosphate buffer (pH-7.0) containing 0.1 mM of EDTA to give 5% w/v homogenate. The homogenates were centrifuged at 10000 rpm for 10 min at 0°C in cold centrifuge; the resulting supernatant was used for further studies.

Lipid peroxidation estimation

MDA level was measured according to the method of Ohkawa et al., 1979 at room temperature. 200 μl of supernatant was added to 50 μl of 8.1% sodium dodecylsulphate, vortexed and incubated for ten min at room temperature. 375 μl of thiobarbituric acid (0.6%) was added and placed in a boiling water bath for 60 min and then the sample was allowed to cool to room temperature. A mixture of 1.25ml of butanol:pyridine(1.5:1) was added, vortexed and centrifuged at 1000rpm for 5min. the colored layer (500μl) was measured at
532nm on a (ELICO, 171) Spectrophotometer. The values were expressed in nmoles of MDA formed for mg protein/hr/min (Ohkowa et al., 1979).

**Reduced glutathione assay**

Reduced glutathione was measured according to the method of Ellman, 1959 at room temperature. 0.75 ml of supernatant was mixed with 0.75 ml of 4% sulfosalicylic acid then centrifuged at 1200 rpm for 5min at 4 °C, from this 0.5 ml of supernatant was taken and added to 4.5 ml of 0.01 M DTNB and absorbance was measured at 412 nm using a (ELICO,171) UV-Visible Spectrophotometer (Ellman, 1959).

**Determination of Vitamin C**

The level of vitamin C was determined by the method of Oayama et al., 1994. To 0.5ml of brain homogenate, 0.5 ml of water and 1 ml of trichloroacetic acid were added, mixed thoroughly and centrifuged. To 1 ml of supernatant 0.2 ml of DTC reagent was added and incubated at 37°C for 3 hrs. Then 1.5 ml of sulphuric acid was added mixed well and the solutions were allowed to stand at room temperature for another 30 min. the color developed was read at 520nm in a (ELICO,171) UV-Visible spectrophotometer. The values were expressed as µg/mg protein (Oayama, 1994).

**TOXICOLOGICAL STUDIES**

**Sub-acute toxicity studies**

Rats were randomly divided into 9 groups with six animals in each group. The plant extracts at the dose of 250 and 500 mg/kg body weight was administered orally to 9 groups of five rats respectively at every 24 h for 28 days and control received vehicle at the same volume. Group I received only vehicle (1% tween 80 solution p.o, daily) and served as control; group II and group III received AN 250mg/kg and 500mg/kg (p.o, daily) in 1% tween 80 respectively, group IV and V received JS 250 and 500mg/kg in 1% tween 80
respectively, p.o, daily, group VI and VII received PF 250 and 500mg/kg in 1% tween 80 respectively, p.o. daily, group VIII and IX received SE 250 and 500mg/kg in 1% tween 80 respectively, p.o, daily. The toxic manifestations such as body weight, mortality, food and water intake was monitored. After 28 days all surviving animals were fasted overnight and anaesthetized with ether. The heparinized blood samples were collected for determining hematological indices like Haemoglobin (Hb), Red Blood Corpuscles (RBC), White Blood Corpuscles (WBC), Packed Cell Volume (PCV); liver function parameters like (AST), (ALP), (ALT); Kidney function parameters like urea, blood urea nitrogen (BUN), creatinine; and the metabolic indices like low density lipoproteins (LDL), high density lipoproteins (HDL), very low density lipoproteins (VLDL), triglycerides (TG) and Glucose. Haematological parameters were determined by cell counter and biochemical functional tests were performed using commercial kits. Animals were sacrificed after blood collection and the internal organs were removed and weighed to determine the relative organ weights and observed for gross lesions (Mounnissamy et al., 2010).

**Statistical analysis**

All data were expressed as mean ± SEM. Differences in mean values between groups were analyzed by one – way analysis of variance (ANOVA) followed by Dunnett’s test in order to detect inter-group differences. A value of p<0.05 was considered as statistically significant.