2.1 Plant materials

Seven species of the family Asclepiadaceae were selected for the present study. They are

1) *Asclepias curassavica* L.
2) *Calotropis gigantea* (L.) R.Br.
3) *Gymnema sylvestre* (Retz.) R.Br.
4) *Holostemma ada-kodien* Schult.
5) *Pergularia daemia* (Forssk.) Chiov.
6) *Tylophora indica* (Burm.f.) Merr.
7) *Wattakaka volubilis* (L.f.) Stapf.

2.2 Chemicals

All the chemicals used in the analytical methods and reagent preparation were of analytical grade with maximum available purity supplied by Hi-media (Bombay), E. Merck (Bombay). 1,1-diphenyl-2-picryl hydrazyl (DPPH), β sitosterol, quercetin and lupeol were procured from Sigma Chemical Company, USA. Vanillin, Folin-Ciocalteau reagent, aluminium backed HPTLC plates coated with 0.2 mm layers of silica gel 60 F$_{254}$ plates and ascorbic acid were from E-Merck Ltd., Mumbai, India. Culture media, Gentamycin disc (10mcg/disc) were from Hi-media Laboratories Ltd., Mumbai, India. Bacterial cultures were procured from MTCC Chandigarh.
2.3 Collection and authentication of plant materials

All the plant materials (samples) for the present investigation were procured from the plants cultivated in the garden from Ernakulam and Thrissur district as well as from the natural habitats of Palakkad district. The samples for quantitative investigation were collected at different stages of growth (young and mature), in different seasons (rainy and dry) and from the plants grown wild and cultivated in the garden. The collected plant materials were identified on the basis of taxonomical characters with the help of *Flora of the Presidency of Madras* (Gamble, 1956), *Flora of British India* (Hooker, 1883-1885), *Flora of Tamilnadu Carnatic* (Matthew, 1983), *Flowering plants of Thrissur Forests* (Sasidharan and Sivarajan, 1996) and authenticated by the direct comparison with herbarium specimens available at the herbarium of Botanical Survey of India, Coimbatore (MH). For the pollinial study, fresh flowers in the form of inflorescence were collected between 8am and 11 am and pollinia were lifted out from freshly opened flowers collected at random.

2.4 Preparation of herbarium

The seven plants selected for the study were made into herbarium specimens for future reference. The herbarium was made according to the method suggested by Jain and Rao (1977). The herbarium sheets of all the plants under study were deposited at BSI (MH), at Fr. Gabriel Herbarium, Ayurvedic Research Centre, Amala Cancer Hospital, Thrissur, Kerala and the Botany Research Laboratory, St. Teresa’s College, Ernakulam, Kerala. The identity of the entire voucher specimens were authenticated with the help of identified specimens kept at BSI (MH). The authenticated specimen numbers of each species are as follows:
1) *Asclepias curassavica* L. 29072
2) *Calotropis gigantea* (L.) R.Br. 32705
3) *Gymnema sylvestre* (Retz.) R.Br. 44900
4) *Holostemma ada-kodien* Schult. 63241
5) *Pergularia daemia* (Forssk.) Chiov. 33191
6) *Tylophora indica* (Burm.f.) Merr. 72078
7) *Wattakaka volubilis* (L.f.) Stapf. 45150

### 2.5 Phytochemical studies

The plants under study were evaluated by its phytochemical profile. The parameters included in the study were determination of ash value, analysis of minerals in the ash, ethanol soluble extractive value, water-soluble extractive value, preliminary phytochemical screening, TLC studies, HPTLC studies, detection of β sitosterol, quercetin and lupeol, estimation of total flavonoids and phenols and its variation based on season, age and habitat and estimation of amino acids.

#### 2.5.1 Sample preparation

The leaves and stems of the plant materials were collected separately and washed with tap water to remove any foreign material and dried in the shade. After optimum drying, the leaves and stems were powdered separately using sample disc type grinder (SWAN TECH. (German) S.J. 500). The leaf and stem powder of each plant species were stored in well-closed containers.

#### 2.5.2 Determination of physico-chemical constants

##### 2.5.2.1 Ash value

5gms of the powdered material were accurately weighed in a silica crucible, which was previously heated, cooled and weighed. The
powdered drug was spread as an even layer at the bottom of the crucible. It was heated in a muffle furnace (KEMI, KMF-4) at 450ºC for four hours by ensuring that no evolution of smoke was observed. The crucible was then cooled and weighed. This process was repeated till the weight became constant and consequently the ash became white or grey coloured. The percentage of the total ash was calculated with reference to the air-dried material. The values were the average of three determinations. The ash value of leaf and stem powder of all the seven plant species were calculated (Anonymous, 2001).

2.5.2.2 Analysis of minerals in the ash

After determining the total ash value, the ashes of all the seven plant species were analyzed for the presence of mineral elements. The ash of each sample was digested with 5 ml HNO₃ and 2 ml of HClO₄ and was made up to 50 ml using HPLC grade distilled water. The filtered sample was analyzed for sodium, potassium, calcium, iron, magnesium and zinc using inductively coupled plasma atomic emission spectrometer (ICP-AES of Thermo electron corporation, IRIS INTREPID).

2.5.2.3 Extractive value

The extracts obtained by exhausting the crude drug were the indication of approximate measures of certain chemical constituents.

a) Ethanol soluble extractive value

5 gms of air-dried coarsely powdered drug was macerated with 100 ml of 95% ethanol in a closed flask for 24 hours, with frequent shaking during the first 6 hours and then allowed to stand for 18 hours. Thereafter filtered rapidly, taking precaution against loss of ethanol, evaporated the filtrate to dryness in a tarred flat-bottomed shallow dish, dried at 105ºC and weighed. The percentage of ethanol soluble extractive value of the leaf and
stem samples were calculated with reference to the air-dried material (Anonymous, 2001). The values were the average of three determinations.

b) Water-soluble extractive value

Macerated 5 gms of air-dried coarsely powdered drug with 100 ml distilled water in a closed flask for 24 hours, shaking frequently during first 6 hours and then allowed to stand for 18 hours. Thereafter it is filtered rapidly, taking precaution against loss of water, evaporated the filtrate to dryness in a tarred flat-bottomed shallow dish, dried at 105°C and weighed. Calculated the percentage of water soluble extractive value of the leaf and stem samples, with reference to the air-dried material (Anonymous, 2001). The values were the average of three determinations.

2.5.3 Qualitative phytochemical screening

The different qualitative chemical tests were performed for establishing profiles of given extracts for their nature of chemical composition. The leaf and stem extracts of the seven plants were subjected to the following chemical tests for the identification of various phytoconstituents (Kokate, 1999; Wagner and Bladt, 1996; Evans, 2002).

Tests for alkaloids

The Dragendorff’s reagent was added to the methanolic extract. Formation of reddish brown precipitate confirmed the presence of the alkaloids. Formation of reddish brown precipitate with Wagner’s reagent also confirmed the presence of alkaloids. The alkaloids formed cream coloured precipitate with Mayor’s reagent.

Tests for flavonoids

The addition of Mg turnings and con.HCl drop wise to the methanolic extract (Shinoda test) gave pink or crimson red colour. On addition of zinc
hydrochloride, red colour development confirmed the presence of flavonoid. Intense yellow colour formation on addition of NaOH to the methanolic extract and its disappearance on addition of drops of dilute acid (Alkaline reagent test) also confirmed the presence of flavonoids. All the extracts showed the presence of flavonoids.

**Tests for phenolic compounds**

To the methanolic extract, lead acetate and ferric chloride were added. A blue black precipitate with lead acetate and green brown with ferric chloride was obtained for all the extracts, which confirmed the presence of phenolic compounds.

**Test for sterols**

The extracts of leaf and stem were prepared in methanol. Libermann’s Burchard reagent was added to the extract. Formation of green colour indicated the presence of sterols.

**Test for triterpenoids**

On addition of few drops of con.H$_2$SO$_4$ to the methanolic extract, formation of yellow colour confirmed the presence of triterpenoids.

**Test for saponins**

When powdered drug was shaken well with water, formation of stable froth or foam confirmed the presence of saponins.

**Test for amino acids**

The extract when boiled with few drops of 5% solution of Ninhydrin, a violet colour appeared in all the extracts. This confirmed the presence of amino acids in all the samples.
Test for tannins

Appearance of blue, black or green colour during the addition of few drops of ferric chloride reagent to the water extract of the sample confirmed the presence of tannins.

2.5.4 Chromatographic analysis

Chromatography is an important analytical tool used for the separation and identification of different classes of natural products and authentication of plant materials. TLC studies of the plant materials were carried out in order to conclude the best solvent system to carry on HPTLC studies.

2.5.4.1 Thin layer chromatography (TLC)

Among the many chromatographic methods available, TLC is widely used for the rapid analysis of drugs and drug preparations. The time required for the determination of the constituents of a drug by TLC is very short. TLC provides a chromatographic fingerprint profile of the phytochemical preparation and the drug constituents (Wagner and Bladt, 1996; Harborne, 1973).

Preparation of extracts for the detection of phenols

Test solutions of the leaves and stems of seven plants under study were prepared separately. 2gms of powder of each sample were dissolved in 15 ml of methanol and extracted for 15 minutes under reflux. The supernatant was filtered and the filtrate was used as the test solution (Wagner and Bladt, 1996).

Preparation of extracts for the detection of sterols

Test solutions of the leaves and stems of seven plants under study were prepared separately. 2gms of powder of each sample were dissolved
in 15 ml of methanol and extracted for 15 minutes under reflux. The supernatant was filtered and the filtrate was used as the test solution (Wagner and Bladt, 1996).

**Preparation of extracts for the detection of flavonoids**

2 gms of powder of each sample was dissolved in 15 ml of 80% ethanol and extracted for 15 minutes under reflux. The supernatant was filtered and the filtrate was used as the test solution.

**Preparation of extracts for the detection of terpenoids**

2 gms of powder of each sample was dissolved in 15 ml of methanol and extracted for 15 minutes under reflux. The supernatant was filtered and the filtrate was used as the test solution.

**Identification and standardization of a suitable solvent system for TLC analysis**

The solvent systems were selected based on the chemical constituents identified in the qualitative phytochemical analysis. Pure solvents or mixture of solvents were used as mobile phase.

**Application of extract**

10 mcl of the extract was applied on TLC plates. Pre-coated silica gel plates (Merck 60F254) were used for TLC studies. The extract was sucked within capillary tube and spotted on the TLC plate 2 cm above its bottom end. The plates were developed in different solvent systems separately. After developing, the plate was air dried and observed under UV light and visible light. In each analysis, better solvent system was selected after spraying with Folin’s - ciocalteu (phenols), Vanillin sulfuric acid (saponins), Liebermann - Burchard reagent (sterol) and NP/PEG reagent (flavonoids). The selection of solvent system for mobile phase was important in TLC, because the same solvent system can be
used in HPTLC. The better solvent system, which gave good separation and more number of spots, was selected for each extract and they are as follows:

- Phenol: Toluene: Ethyl acetate: Formic acid (5: 3: 0.4)
- Sterol: Hexane: Diethyl ether: Acetic acid (8: 2: 0.1)
- Flavonoids: Butanol: Ethyl acetate: Acetic acid: Formic acid (3: 1: 1: 0.4)
- Terpenoids: Toluene: Ethyl acetate: Formic acid (5: 3: 0.4)

2.5.4.2 High performance thin layer chromatography (HPTLC)

HPTLC (CAMAG, Switzerland) is a sophisticated and automated technique for qualitative and quantitative analysis of chemical constituents in plants and herbal drugs. After confirming the presence of phenols, sterols, terpenoids, and flavonoids by TLC analysis, a good solvent system was selected with the help of a good detecting reagent. The best solvent system which gave good separation and maximum number of spots in TLC analysis was selected as the mobile phase for HPTLC analysis.

The extracts of leaves and stems of all the plants under study were applied to HPTLC plates of size 20 x 10 cm that were pre-coated with Silica gel (Merck 60F254) for the detection of phenols, sterols, flavonoids and terpenoids. The extracts were applied as bands with a CAMAG automatic TLC III (Linomat). The separations were performed in CAMAG twin trough chambers previously saturated by the solvents for 30 minutes at room temperature. The solvent systems selected as the better mobile phase for TLC were then used as the mobile phase. The quantity of samples applied were 10 µl each. The samples were applied as a band of 10 mm and 8 mm apart from each other. 20 cm x 10 cm plates
were used. The plates were developed upto a distance of 8 cm, and then the plates were taken out of the chamber and dried in the air. The air dried plates were observed under UV light at 254 nm.

**Scanning of the HPTLC plates**

The plates were scanned under UV with the help of HPTLC scanner. The scanning data was subjected to integration through the WIN CATS Software. The fingerprint developed by this was used for the detection of phytoconstituents present in the leaf and stem extracts of the seven plants. The Rf, height and area of each spot was recorded in the chromatogram. Rf value is the ratio of the distance moved by the solute to the distance moved by the solvent front.

For the detection of the presence of pure compounds (lupeol, β-sitosterol and quercetin) in the extracts, along with the leaf and stem extracts, standard pure compounds were applied to the HPTLC plates. The separations were performed using the solvent systems selected as the best mobile phase using TLC. The plates were developed and scanned under UV with the help of HPTLC scanner. The presence of the pure compound in the extract was confirmed by comparing the Rf value of the pure compound with that of the extract at the particular λ max of the pure compound.

**2.5.5 Estimation of total polyphenols**

50 gms of powdered drug (leaf) were extracted 3 times with 100 ml methanol. The filtrate was evaporated to dryness and the residue was dissolved in 50ml of distilled water. The aqueous solution was extracted with 250 ml of petroleum ether and discarded the ether layer. The aqueous layer was extracted with 25 ml chloroform and discarded the chloroform layer. The aqueous layer was extracted with 25 ml of diethyl ether and discarded the diethyl ether solution. The aqueous layer was then
extracted with 25 ml of ethyl acetate 3 times. The ethyl acetate layer was collected after passing through sodium sulphate and evaporated to dryness. The residue was dried at 105°C and weighed as polyphenols. The total polyphenol content was estimated. The seasonal (dry and rainy), age wise (mature and young) and habitat wise (wild and cultivated) variation of the content in the leaves were estimated. The values were the average of twelve determinations.

2.5.6 Estimation of total flavonoids

Total flavonoid content was estimated according to Rajpal (2002). Refluxed 3 gms leaf powder with 50 ml of methanol for 30 minutes and filtered. The above process was repeated twice. The methanol was evaporated from the filtrate and the residue was shaken with 25 ml hot water. The above aqueous extract was shaken with 25 ml ethyl acetate. The above solution was transferred to a separating funnel and collected the ethyl acetate layer in a previously weighed flask \( w_1 \). The above process was repeated with 20 ml, 15 ml hot water and ethyl acetate. The same process was continued till the ethyl acetate layer turned colourless, distilled off the ethyl acetate and evaporated to dryness and weighed the final residue \( w_2 \). The total flavonoid content was estimated.

\[
\text{Percentage of flavonoids} = \frac{w_2 - w_1}{\text{weight of the sample}} \times 100
\]

The seasonal, age wise and habitat wise variation of the flavonoid content in the leaves were estimated. The values were the average of twelve determinations.

2.5.7 Estimation of amino acids by HPLC

Amino acids of the samples were estimated using the specially designed HPLC amino acid analyser (Model Schimadzu, LC – 10 AS Japan).
The samples for analysis of amino acids other than Tryptophan were prepared by acid hydrolysis. 100mg plant sample was hydrolysed using 10 ml 6N HCl at 110°C for 24 hours in heat sealed evacuated tubes.

The acid hydrolysed samples were filtered through a Whatmann No. 42 filter paper after breaking open the tubes. The filtrate was flash evaporated to remove HCl and made up to a definite volume in 0.05 M HCl using sodium citrate caprylic acid buffer of pH 2.2 (8 ml sodium perchloric acid and 50 ml n-caprylic acid). The buffered samples were again filtered using 0.45 µM membrane filter and 20 µl of the filtrate of each sample was injected through the sample loop at 60°C.

The HPLC column was packed with a strongly acidic cation exchange resin made up of styrene divinyl benzene copolymer with sulfinic group. The column used was ISC – 07/S/1504/Na with a length of 19 cm and diameter of 5 mm. The guard column prevented the entry of impurities into the column. The amino acids were separated inside the column. The post column derivatisation of the components was carried out with O-phthalaldehyde and hypochlorite of individual amino acids at the reaction coil. These components were detected by spectrofluorometer.

The amino acids were eluted step-wise starting from acidic amino acids, then neutral and finally alkaline amino acids. The chromatogram was recorded by a data processor. Total nitrogen of the sample was estimated by Kjeldahl method and the amount of amino acid was expressed in gm amino acid per 16gN.

Tryptophan was estimated separately by colorimetric method after alkali hydrolysis. 200 mg sample was hydrolysed with 10 ml of 5% NaOH at 110°C for 24 hours in a sealed tube filled with pure nitrogen.
The hydrolysate was neutralized with 6N HCl. It was then filtered through Whatmann No. 1 filter paper and the filtrate used for estimation.

Estimation of tryptophan

0.1 ml of 2.5% sucrose and 0.1 ml of 0.6% thioglycolic acid were successively added to test tubes containing 4 ml of 50% sulphuric acid and kept for 5 minutes in a water bath at 45-50°C and cooled, 0.1 ml of standard tryptophan containing 10 µg/ml was then added and mixed. The volume was made up to 5 ml with 0.1 N HCl and allowed to stand for five minutes for the development of colour. The absorbance at 500 nm wavelength was measured after 5 minutes using spectrophotometer. The commercial sample of tryptophan was used as standard. Amino acid estimation was done in all the leaf samples.

2.6 Biological activities
2.6.1 Antibacterial activity

Antibacterial activity of the all the leaf extracts were done by agar well diffusion method (Perez et al., 1990). Two gram positive bacteria Staphylococcus aureus (MTCC 3160) and Bacillus subtilis (MTCC 3053) and three gram negative bacteria Klebsiella pneumoniae (MTCC 3384), Salmonella typhymurium (MTCC 98) and Escherichia coli (MTCC 727) were used as the test organisms. The extract was prepared by dissolving 2 grams of leaf powder in 20 ml of distilled water and extracted for 20 minutes under reflux. The supernatant was filtered and the filtrate was used. Similarly 50% hydro alcoholic, ethanolic and methanolic extracts were prepared. The plates were prepared by using Muller Hinton agar (MH agar) (Hi Media). 18 hours old cultures of test organisms in Nutrient Broth (Hi Media) were used as inoculum. To that, sterile swabs were dipped and pressed at the side walls of the tube in order to remove the excess inoculum and swabbed it evenly on the MH agar plate. Then the
wells were made in the agar plate by using sterile cork borer with suitable diameter. To this 150 µl each of the extract was added aseptically using micropipettes and sterile tips. Then Gentamycin disc (10mcg /disc) was placed in the plate aseptically from a sufficient distance from the well containing the leaf extract using sterile forceps. Gentamycin disc (10mcg /disc) was used as the positive control and pure water, methanol, ethanol and 50% hydro alcohol were used as negative control. After keeping it in the rest position for two hours for the proper diffusion of the extract, the plates were incubated at 37°C for 24 hours and the zone of inhibition was measured in mm. The same procedure was repeated to check the antimicrobial activity of the flavonoids (100 mg/ ml of methanol) isolated from the leaves of the seven plants under study. Pure methanol was used as negative control. Minimum inhibitory concentration (MIC) of flavonoid against Staphylococcus aureus (MTCC 3160) was also assessed by agar well diffusion method.

2.6.2 Antioxidant studies

The 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay method (Brand-Williams et al., 1995) was employed to measure the free radical scavenging activity. The assay method is based on the reduction of a methanolic solution of the coloured free radical DPPH by free radical scavenger. The decrease in absorbance of DPPH at its absorbance maximum of 516nm is proportional to the concentration of free radical scavenger added to the DPPH reagent solution. The activity was expressed as IC_{50} (i.e. the concentration of the test solution required to give 50% decrease in absorbance compared to that of a control solution).

The DPPH reagent was prepared in methanol (2 mg/ml). Ascorbic acid was used as the standard. To a set of clean and dry test tubes containing 3 ml of methanol, 50µl of DPPH reagent was added and
mixed thoroughly. The initial absorbance of each test tube was measured at 516 nm. To these test tubes different dilutions of leaf extract was added in increasing concentration. The solution was mixed, allowed to stand for 20 minutes in the dark at room temperature and the final absorbance was measured at 516 nm. The percentage reduction in absorbance was calculated from the initial and final absorbance at each level. The concentration of test solution required for producing 50% reduction in absorbance (IC$_{50}$) was calculated by plotting the linear regression calibration curve of log of concentration of test solution on X-axis against percentage reduction in absorbance on Y-axis. Ascorbic acid pure was used as a standard.

2.7 Study of pollinial morphology

Fresh flowers in the form of inflorescence were collected between 8 am and 11 am and pollinia were lifted out from freshly opened flowers. Twelve pollinia were collected randomly and pollen morphological features were studied by both LM and SEM observations. For scanning electron microscope studies the specimens were mounted on specimen stubs, coated with gold. Then viewed, scanned and photographed.

2.8 Data analysis

2.8.1 t Test

The differences in the content of phytochemical constituents at different conditions were compared using t test.

The t test assesses the statistical difference between the means of two groups.

\[
t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{n_1 s_1^2 + n_2 s_2^2}{n_1 + n_2 - 2} \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}}
\]
where $\bar{x}_1$ and $\bar{x}_2$ denote the average of the content in 1) matured plant and young plant 2) dry season and rainy season 3) wild plant and cultivated plant respectively for polyphenol content.

$n_1$ and $n_2$ are respective sample size and $s_1$ and $s_2$ are respective sample standard deviation.

Similarly the $t$ value was calculated for flavonoid content at different conditions.

Null Hypothesis: $H_0 : \mu_1 = \mu_2$ against alternative hypothesis $H_1 : \mu_1 > \mu_2$

$H_0$: The mean quantity of the polyphenol in the mature and young plant (dry season and rainy season; wild and cultivated plant) is the same.

$H_1$: The mean quantity of polyphenol in the mature plant is higher than that of young plant; in dry season it is higher than rainy season; in wild plant it is higher than in cultivated plant.

$n_1 + n_2 - 2$ denote degrees of freedom. If computed $t$ statistic value is higher than critical value drawn from table of $t$ distribution for the specific degrees of freedom, we reject the null hypothesis $H_0$. Evidently the alternative hypothesis is accepted. The standard levels of significance 5% and 1% are considered in appropriate cases. For convenience, P value is calculated. If P value is less than 0.05 (0.01) we reject the Null hypothesis $H_0$ at 5% (1%) level of significance. Similarly $t$ test was conducted to check whether there is significant age-wise, season-wise and habitat-wise variation in the flavonoid content.

### 2.8.2 Correlation

Correlation coefficient is the extent of relationship between two variables. In order to study the relationship between the polyphenol content and antioxidant activity and between flavonoid content and
antioxidant activity, correlation coefficient was computed applying Karl Pearson’s Coefficient of Correlation \((r)\). Correlation coefficient is a pure number which lies between -1 and +1, denoted by \(r\). If the value of \(r\) is nearer to 0, there will be less positive correlation and if its value is nearer to 1, there will be high positive correlation.

\[
r = \frac{\sum_{i=1}^{N} (x_i - \bar{x})(y_i - \bar{y})}{N \sigma_x \sigma_y}
\]

\(x_i, y_i = \text{Variables}\)

\(\bar{x}, \bar{y} = \text{Averages}\)

\(N = \text{No. of pairs}\)

\(\sigma_x, \sigma_y = \text{Standard deviation of } x, y\)

### 2.8.3 Cluster analysis

Clustering is the grouping of similar objects in a sample or a population. Cluster analysis develops tools and methods to build natural subgroups or clusters of individuals, from a data with multivariate measurements on a large number of individuals or objects. The aim of the cluster analysis is to construct groups with homogenous properties, out of heterogeneous large samples. There are essentially two types of clustering methods: hierarchical algorithms and partitioning algorithms. The hierarchical algorithms can be divided into agglomerative and splitting procedures. The first type of hierarchical clustering starts from the finest partition possible (each observation forms a cluster) and grouping them together. (Johnson and Wichern, 1996; Anderson, 1984).

A graphical representation of the sequence of clustering (grouping) is called dendrogram. It displays the sequence of clustering and the distances between the clusters. The vertical axis displays the indices of
the points, whereas the horizontal axis gives the distance between the clusters. Large distances indicate the clustering of heterogeneous groups. (Johnson and Wichern, 1996; Anderson, 1984).