The recent resurgence of plant based remedies resulted in the over exploitation of medicinal plants. The pharmaceutical companies usually procure materials from traders who get these from unskilled and untrained people, which result in the adulteration and substitution of the plant materials. A serious limitation encountered in the use and research of traditional medicine is the lack of standardization and quality control of raw materials forming the drug. In this era of enormous increase in demand of herbal drugs, there is a need to authenticate the exact plant material. The attributed medicinal effect will be obtained only when the original plant material is used. In the present investigation an attempt was made to prepare a standard protocol for authenticating the seven selected medicinally important plants of the family Asclepiadaceae, based on the morphological and phytochemical analysis.

The medicinal value of a plant material lies in its chemical constitution. The chemical contents of different medicinal members of the family Asclepiadaceae are compared. The investigation of amino acid profile and biologically active compounds has revealed the therapeutic potency of these medicinal plants. The chemoprofile of the plants help in the identification of the genuine crude drug. The antibacterial and antioxidant activities were carried out to understand the acceptability of their traditional use.

4.1 Phytochemistry

The chemical constitution of a plant material determines its medicinal value. These substances can impart a definite physiological
action on the human body (Hill, 1952). Phytochemistry is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, their natural distribution and their biological function (Harborne, 1998).

4.1.1 Authentication and identification

The plants under study used in different medical systems, are authenticated and the parameters undertaken, provide authentic key characters for the identification procedure. Many botanically different species are known by a single name. However, the identity of the specimens are authenticated botanically by a taxonomic expert. In this investigation, the morphological description, botanical name with the author citation and the photographs (Plate 3.1; Plate 3.2) of each species were given, which aid in easy and quick identification of the plants. These parameters will aid in identifying the plant when it is in intact form.

4.1.2 Ash content

A drug obtained in the form of plant part, dried material, dried powder or extract cannot be identified by the morphological characters. The nutritive value of the plant materials used as medicine or which form a major portion of the diet is very important. Various parameters were studied using the crushed plant material to determine the nutritive value. Determination of ash content was one such parameter (Edeoga et al., 2003). The study of ash value, the concentration of minerals and the chemoprofile of the plant help in the identification and standardisation process.

In the present study, ash values of the leaf and stem extracts give an idea of the amount of mineral content in the plants. Compared to the
stems, the leaves showed higher ash value. The total ash value varied from 7.09 ± 0.08% to 13.08 ± 0.24% in the leaves and from 4.32 ± 0.03% to 9.82 ± 0.09% in stems. The highest quantity of ash was observed in *Tylophora indica* (Burm.f.) Merr. leaf (13.08 ± 0.24%) and stem (9.82 ± 0.09%) extract and the lowest value in *Gymnema sylvestre* (Retz.) R.Br. leaf (7.09 ± 0.08%) and stem (4.32 ± 0.03%) extract. According to Indian Pharmacopoeia, the ash value of *Gymnema sylvestre* (Retz.) R.Br. is 12%. In the sample studied, the ash value of leaf was 7.09 ± 0.08% and that of stem was 4.32 ± 0.03%. If the value exceeds the standard value (12%), it is an indication of the accumulation of metallic salts, pesticides, silica and toxic elements. Hence the plant material is unsuitable in pharmaceutical industry for the preparation of Ayurvedic formulations. For all other plants, the standard ash value is not reported. Hence the ash values determined can be taken as a reference standard for future studies to determine the quality of the crude drug.

Plant accumulates a number of mineral elements essential to human nutrition, though it equally accumulates other mineral elements which are in no direct use to humans but injurious to health (Baker, 1989; Mark *et al*., 2000). The determination of ash is useful for detecting low grade products, exhausted drugs and excess of sandy or earthy matter (Biren, 2009).

The total ash content is a measure of the presence of inorganic compounds in a drug. It is used to determine the quality and purity of the crude drug (Edwin *et al*., 2006). Ash obtained after the incineration of the plant material constitutes the minerals (Belitz and Grosch, 1999). It is the general measure of quality or grade in identifying the authenticity of a food material (Kirk and Sawyer, 1997).
Mineral content is a measure of the amount of specific inorganic components present within a material. In the present investigation, evaluation of the minerals such as calcium, magnesium, sodium, potassium, zinc and iron were undertaken. All the extracts showed the presence of calcium, iron, potassium, magnesium, sodium and zinc. In the leaf extracts the concentration of calcium and potassium were high and the concentration of iron and zinc were low (Table 3.2; Fig. 3.1). The stem extracts also showed high concentration of calcium and potassium and low concentration of iron and zinc (Table 3.3; Fig. 3.2).

Sodium and potassium play a vital role in keeping a normal balance of water between the cells and fluids. Both are essential for nerve impulses to travel to the muscles including heart (Ruth and Frank, 1978). Potassium activates the glycolytic and respiratory enzymes and regulates the osmotic pressure within the cell (Belitz and Grosch, 1999). The Na/K ratio is significant, where a value of less than 1 is recommended for controlling high blood pressure (Pelletier, 1994). All the samples were rich in potassium and poor in sodium. The Na/K ratio was 0.174 in Asclepias curassavica L., 0.536 in Calotropis gigantea (L.) R.Br., 0.188 in Gymnema sylvestre (Retz.) R.Br., 0.061 in Holostemma ada-kodien Schult., 0.058 in Pergularia daemia (Forssk.) Chiov., 0.143 in Tylophora indica (Burm.f.) Merr. and 0.084 in Wattakaka volubilis (L.f.) Stapf. leaf extract. On comparison the Na/K ratio ranged from 0.058 to 0.536 in all the leaf extracts (Fig. 3.3). The Na/K ratio varied from 0.117 (Holostemma ada-kodien Schult.) to 0.309 (Wattakaka volubilis (L.f.) Stapf.) in all the stem extracts. This seemed to be ideal, and therapeutically good for hypertension. The study implied that Na/K ratios were within tolerable limit in all the samples and these could be administered internally.
Minerals cannot be made in the body and must be obtained through our diet. In many plants the mineral content contribute to its therapeutic activity (Chevallier, 2001). The high potassium content of Dandelion leaf (*Taraxacum officinale*) makes it a potent diuretic. Similarly in horse tail (*Equisetum arvense*) the high silica content makes it useful in the repair of connective tissues and so it is used in arthritis (Chevallier, 2001). These necessitated the evaluation of mineral and nutritional content of medicinal plants. Studies on the medicinal properties of plants clearly indicate the role of minerals in their therapeutic effects (Akinmoladun *et al.*, 2007). Essential minerals namely calcium, sodium, potassium and magnesium are so intertwined and interbalanced in their functions in the human body and there is danger in creating imbalances in the human body. So it is better to depend wholly on natural foods (Ruth and Frank, 1978).

Calcium is indispensable for the contraction of heart and other muscles (Kirk and Sawyer, 1997). It is also essential for clotting of blood and building of bones. Magnesium activates more enzymes in the body than any other mineral. Capillaries are destroyed if there is too little magnesium in the body. It is required for the body to manufacture protein, fat and other essentials which make up cells and intercellular materials. It also prevents the unwanted calcification of kidney and bladder.

The Ca/Mg ratio can vary from 4:1 to 1:1 (Ruth and Frank, 1978). Among the leaf samples studied, the Ca/Mg ratio was 3.39:1 in *Asclepias curassavica* L., 2.18:1 in *Calotropis gigantea* (L.) R.Br., 1.95:1 in *Gymnema sylvestre* (Retz.) R.Br., 3.02:1 in *Holostemma ada-kodien* Schult., 3.01:1 in *Pergularia daemia* (Forssk.) Chiov., 3.82:1 in *Tylophora indica* (Burm.f.) Merr. and 7.02:1 in *Wattakaka volubilis* (L.f.)
Stapf. In all the leaves, the ratio was within the ideal range, except in *Wattakaka volubilis* (L.f.) Stapf. In stem, the ratio varied from 2.65:1 (*Holostemma ada-kodien* Schult.) to 11.79:1 (*Asclepias curassavica* L.). The ratio exceeded the upper limit in *Asclepias curassavica* L. (11.79:1), *Gymnema sylvestre* (Retz.) R.Br. (4.25:1), *Tylophora indica* (Burm.f.) Merr. (6.45:1) and *Wattakaka volubilis* (L.f.) Stapf. (7.88:1) (Fig. 3.4). The plants where the ratio exceeded 4:1 cannot be used for medicinal purposes. If it has to be used as one of the ingredients in Ayurvedic formulations, it has to be supplemented with the deficient mineral to avoid the adverse side effects.

Calcium and magnesium oppose each other at the intercellular level. Thus low magnesium intake causes calcification, or high calcium storage. Magnesium is needed for proper calcium absorption. If the diet is low in magnesium but high in phosphorus and moderate in calcium, calcification occurs in both heart and kidney. If the diet is rich in phosphorus they need more magnesium for proper balancing (Ruth and Frank, 1978).

Zinc is needed for bone growth and healing of wounds. It plays an important part in the manufacture of insulin. Lack of zinc results in absence of sense of taste, poor appetite, rheumatoid arthritis etc. The amount of vitamin A available for use in the blood depends partially on the zinc status of the body (Ruth and Frank, 1978). The calcium and potassium contents were more in all the fourteen samples under study and the percentage of iron, magnesium, sodium, and zinc were low.

Minerals are used in growth and repair, and help to regulate body processes. The dietary deficiency or nonproportionate intake of minerals through food or medicine over an extended period result in a disease. Hence the mineral content and the ratio of minerals in the medicinal plants are very significant and all these are a part of standardisation.
4.1.3 Extractive values

The extractive values are useful to evaluate the chemical constituents present in the crude drug. This also helps in estimation of specific constituents soluble in a particular solvent. The soluble extractive values with solvents such as water and ethyl alcohol indicate the nature of constituents present. The amount of matter extracted from the plant, when water or ethyl alcohol is used as extracting solvent, is determined by the values of the water soluble and ethanol soluble extractives.

The water soluble extractive values of the seven leaf samples varied from 14.71% to 25.18%. The alcohol soluble extractive values of seven leaf samples ranged from 7.24% to 12.81%. In the stem samples, the water extractive values varied from 8.95% to 12.69% and alcohol extractive values ranged from 6.71% to 8.23%. From the extractive values, it was observed that *Pergularia daemia* (Forssk.) Chiov. leaf showed the highest quantity of water soluble extractives (25.18%) and *Gymnema sylvestre* (Retz.) R.Br. leaf showed the highest amount of alcohol soluble extractives (12.81%).

In the present study, it was observed that the water soluble extractive values were higher than alcohol soluble extractive values in the leaf and stem extract of all the plants. This revealed that the polar compounds were in larger quantity in the plant materials. The extractive value of the leaf was on the higher side when compared to that of the stem which indicated that the chemical constituents were more in the leaves than in stem.

4.1.4 Qualitative phytochemical analysis

The curative properties of medicinal plants are due to the presence of various complex chemical substances of different composition which occur as secondary metabolites.
Preliminary phytochemical screening revealed the presence of flavonoids, tannins, sterols, phenols, saponins and terpenoids in *Asclepias curassavica* L., *Calotropis gigantea* (L.) R.Br., *Holostemma ada-kodien* Schult. and *Wattakaka volubilis* (L.f.) Stapf. Flavonoids, tannins, sterols, phenols, saponins, terpenoids and alkaloids were observed in *Gymnema sylvestre* (Retz.) R.Br. Flavonoids, tannins, sterols, phenols, terpenoids and alkaloids were present in *Pergularia daemia* (Forssk.) Chiov. Flavonoids, sterols, phenols, saponins, terpenoids and alkaloids were observed in *Tylophora indica* (Burm.f.) Merr.

The most important of the bio-active compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds (Edeoga *et al.*, 2003). These classes (such as alkaloids, saponins, tannins, phenols and flavonoids) of compounds are known to have curative activity against several pathogens. Hence it could suggest their use traditionally for the treatment of various illnesses (Hassan *et al.*, 2004; Usman and Osuji, 2007). The present study confirms scientific validity to the use of these plants in traditional medicine.

### 4.1.5 HPTLC fingerprint profile

The chemoprofile of each plant extract provides a set of peaks with Rf values and their corresponding area percentage. Fingerprint profile of each extract is unique and specific. The different peaks and their percentage of area obtained are specific for each Rf value which indicates a chemical compound.

In the present study of the phenolic profile, among all the leaf extracts *Tylophora indica* (Burm.f.) Merr. had maximum number of peaks (seventeen), which indicated the presence of highest number of phenolic compounds. In *Wattakaka volubilis* (L.f.) Stapf. sixteen peaks
that represent sixteen different types of phenolic compounds were observed. In *Pergularia daemia* (Forssk.) Chiov. only fourteen different types of phenolic compounds were present. The lowest number of phenolic compounds (nine) was observed in *Gymnema sylvestre* (Retz.) R.Br.

The phenolic profile of the leaf extracts are presented in Fig. 3.5 (1-7), where in each graph, different peaks obtained are recorded and the corresponding Rf values, heights, percentage of heights are given. In Table 3.7, the Rf values of different extracts obtained and their major peaks are summerized. Common peaks represent common phenols. The phenolic compounds having Rf value 0.78 was present in all the leaves except *Tylophora indica* (Burm.f.) Merr. Similarly the phenolic compounds having the Rf 0.39 was present in all the plants except *Wattakaka volubilis* (L.f.) Stapf. and these were the two major peaks. The phenolic compounds with Rf value 0.35 was present in all the plants except *Gymnema sylvestre* (Retz.) R.Br. The phenolic compounds with Rf value 0.32 was present in five plants viz., *Calotropis gigantea* (L.) R.Br., *Gymnema sylvestre* (Retz.) R.Br., *Pergularia daemia* (Forssk.) Chiov., *Tylophora indica* (Burm.f.) Merr. and *Wattakaka volubilis* (L.f.) Stapf. Phenolic compounds with Rf values of 0.46 (*Calotropis gigantea* (L.) R.Br., *Pergularia daemia* (Forssk.) Chiov., *Tylophora indica* (Burm.f.) Merr. and *Wattakaka volubilis* (L.f.) Stapf.), 0.49 (*Asclepias curassavica* L., *Gymnema sylvestre* (Retz.) R.Br., *Holostemma ada-kodien* Schult. and *Pergularia daemia* (Forssk.) Chiov.), 0.57 (*Asclepias curassavica* L., *Holostemma ada-kodien* Schult., *Pergularia daemia* (Forssk.) Chiov. and *Wattakaka volubilis* (L.f.) Stapf.), 0.69 (*Asclepias curassavica* L., *Holostemma ada-kodien* Schult., *Pergularia daemia* (Forssk.) Chiov. and *Tylophora indica* (Burm.f.) Merr.) were present in the
plants. Thus a similarity in the distribution of the phenolic compounds could be seen.

The phenolic profile of the stem extracts are presented in Fig. 3.6 (1-7), where in each graph, different peaks obtained are recorded and the corresponding Rf values, heights, percentage of heights are given. In the case of stem extracts, *Tylophora indica* (Burm.f.) Merr. and *Wattakaka volubilis* (L.f.) Stapf. showed the maximum number of peaks (ten). Eight phenolic compounds were observed in *Asclepias curassavica* L. and *Gymnema sylvestre* (Retz.) R.Br. Seven phenolic compounds were observed in *Calotropis gigantea* (L.) R.Br. and *Holostemma ada-kodien* Schult. Six phenolic compounds were observed in *Pergularia daemia* (Forssk.) Chiov. A phenol with Rf value 0.79 was present in all the plants except *Asclepias curassavica* L. and *Wattakaka volubilis* (L.f.) Stapf. Similarly the phenol with Rf value 0.33 was present in all the plants except *Asclepias curassavica* L. and *Calotropis gigantea* (L.) R.Br. The phenol with Rf value 0.40 was present in all the plants except *Asclepias curassavica* L. An HPTLC study of the phenolic compounds was not reported earlier in these plants.

From the HPTLC fingerprint profile of flavonoids in the leaf extract, the highest number (ten) of flavonoid compounds were observed in *Pergularia daemia* (Forssk.) Chiov. Nine flavonoids were seen in *Asclepias curassavica* L. Eight different types of flavonoids were observed in *Tylophora indica* (Burm.f.) Merr. and *Holostemma ada-kodien* Schult. (Table 3.9). There was similarity among the plants in the presence of similar flavonoids. The flavonoid with Rf value of 0.37 was present in *Asclepias curassavica* L., *Calotropis gigantea* (L.) R.Br., *Pergularia daemia* (Forssk.) Chiov., *Tylophora indica* (Burm.f.) Merr. and *Wattakaka volubilis* (L.f.) Stapf. The flavonoid with Rf value of 0.25
was seen in *Asclepias curassavica* L., *Gymnema sylvestre* (Retz.) R.Br., *Holostemma ada-kodien* Schult. and *Tylophora indica* (Burm.f.) Merr. The flavonoid with Rf value of 0.22 was seen in *Asclepias curassavica* L., *Holostemma ada-kodien* Schult. and *Wattakaka volubilis* (L.f.) Stapf. Similarly the flavonoid with Rf value 0.50 was seen in *Holostemma ada-kodien* Schult., *Pergularia daemia* (Forssk.) Chiov. and *Wattakaka volubilis* (L.f.) Stapf.

The flavonoid profile of the stem extracts are presented in Fig. 3.8 (1-7). In each graph, different peaks obtained are recorded and the corresponding Rf values, heights, percentage of heights are given. The Rf value in different extracts obtained, and the major peaks are summarized (Table 3.10). Common peaks represent common flavonoids. Among the stem extracts *Gymnema sylvestre* (Retz.) R.Br., *Holostemma ada-kodien* Schult. and *Tylophora indica* (Burm.f.) Merr. showed the maximum number of peaks (ten).

Chemoprofile of many of the medicinal plants have been reported earlier (Saraswathy et al., 2005; Tayade and Nagarsenker, 2007). A plant extract showing a particular HPTLC fingerprint profile will not be shown by any other plant extract (Chevallier, 2001). The different Rf values and the number of peaks given by each extract may help to identify the source plant. The chemoprofile of leaf and stem extract of all the seven plants can be used for the authentication and identification of the plant material. The unique feature of picture-like image of HPTLC coupled with digital scanning profile is more and more attractive to the herbal analysts to construct the herbal chromatographic fingerprint (Chen et al., 2006). The HPTLC fingerprint profile of the major chemical constituents along with their recorded Rf values would also provide a basis to determine the quality and purity of the drug obtained from these plants.
These fingerprint profiles can be used to find out adulteration which is very common in the raw drug industry. Adulteration of herbal formulation with undeclared synthetic drugs or by mixing analogues of prescription drugs that are created by replacing or adding functional groups to the original formulation are the recent major problems, since they may cause adverse side effects (Yean et al., 2007). HPTLC method was found to be well suited for the detection of adulterants (Shelar et al., 2011). Kanan et al. (2009) detected common synthetic adulterants in slimming herbal remedies using HPTLC.

Even though the chemical identity of each spot is not revealed, the chemoprofile can be used to identify the plants from which the extract is obtained. It is a method of chemical standardization of the drug. The fingerprint profile gives the authenticity of the drug. Many of the modern pharmacopoeias and other regulatory agencies like WHO included HPTLC as a powerful and most economical tool for true identification of plant material, especially in terms of its chemical constituents.

4.1.6 Detection of pure compounds

In the present study, important compounds such as β-sitosterol, quercetin and lupeol which exhibit an array of biological activities, were observed in the selected plants. These can be used as marker compounds. The raw material with maximum concentration of the phytochemical marker can be identified for use in any herbal formulation using HPTLC.

β-sitosterol showed a peak with Rf value 0.25 at λ.max of 206nm. In the chemoprofile of all the leaf and stem extracts a peak with Rf value 0.25 was observed thus confirming the presence of β-sitosterol in Asclepias curassavica L., Calotropis gigantea (L.) R.Br., Gymnema sylvestre (Retz.) R.Br., Holostemma ada-kodien Schult., Pergularia
daemia (Forssk.) Chiov., Tylophora indica (Burm.f.) Merr. and Wattakaka volubilis (L.f.) Stapf. (Fig. 3.14). The presence of β-sitosterol has been previously reported only in Calotropis gigantea (L.) R.Br. and Holostemma ada-kodien Schult. (Manju and Alice, 2001).

β-sitosterol inhibits proliferation of colon cancer cells with less toxicity towards normal cells in in vitro (Baskar et al., 2010). It can scavenge the nitric oxide radicals generated by DPPH and induce apoptosis of malignant cells in vitro (Muller et al., 1997). β-sitosterol has been reported to possess cytotoxic effects (Awad et al., 2007; Nguyen et al., 2005) and anti-proliferative and apoptotic potential in several cancer models (Awad and Fink, 2000).

The Rf value of quercetin is 0.81 at a λ max of 280nm. Presence of a peak with the same Rf value (0.81) was seen in the leaf and stem extracts of Holostemma ada-kodien Schult. thus confirming the occurrence of quercetin in the plant for the first time (Fig. 3.10). Quercetin has been receiving great attention in recent years, on its biological activities such as hypoglycemic (Torres-Piedra et al., 2010), anti-tumor (Chien et al., 2009; Wong and Chiu, 2010), anti-inflammatory (Rogerio et al., 2010; El-Sayed and Rizk, 2009), antiviral (Walker et al., 2009), antibacterial (Hirai et al., 2010; Ramadan and Asker, 2009), analgesic (Kumar and Goyal, 2008; Lee and Houghton, 2005) and anti-oxidant property (Chen et al., 2006; Arash, 2010).

Densitometric scanning was done at λ max of 230nm for the detection of lupeol where it showed maximum absorption and a peak with Rf value 0.80. Lupeol was found to be present in leaf and stem extracts of Calotropis gigantea (L.) R.Br., Gymnema sylvestre (Retz.) R.Br, Holostemma ada-kodien Schult., Pergularia daemia (Forssk.) Chiov.,
Tylophora indica (Burm.f.) Merr. and Wattakaka volubilis (L.f.) Stapf. (Fig. 3.18). Its occurrence was previously reported only in Holostemma ada-kodien Schult. (Manju and Alice, 2001). Lupeol inhibits growth of highly metastatic tumors of human melanoma origin at a dose that has no toxic effect on normal human melanocytes (Saleem et al., 2008). It exhibits biological activities like anti-inflammatory and anti-arthritic activities (Wal et al., 2011) which support the traditional use of Calotropis gigantea (L.) R.Br. and Wattakaka volubilis (L.f.) Stapf. for arthritis and to reduce inflammation.

The overlapping chromatograms show the super imposition of the compounds present in the extracts. This super lay of chromatograms of seven plants very clearly indicates the presence of similar compounds in the members of the family Asclepiadaceae. The phytochemical results, which are being reported for the first time, can be useful in the preparation of the herbal monograph.

4.1.7 Amino acid profile

In the present investigation, the presence of amino acids which contribute to the anti diabetic and neurostimulating effect were observed in the selected plants.

4.1.7.1 Amino acids with antidiabetic effect

Cluster analysis of the seven plants using amino acid profile grouped the four plants viz., Gymnema sylvestre (Retz.) R.Br., Holostemma ada-kodien Schult., Pergularia daemia (Forssk.) Chiov. and Wattakaka volubilis (L.f.) Stapf. under one cluster. These plants are traditionally used in the treatment of diabetes among the seven plants selected. The anti hyperglycemic effect of these plant extracts has been confirmed (Virdi et al., 2003; Kar et al., 2003; Wahi et al., 2002).
Evidences suggest that amino acids may be potentially important in the prevention of diabetes and diabetes associated complications. Individual amino acids especially the ones bestowed with anti-oxidant property like N-acetyl cysteine and taurine seem to have beneficial effects by their ability to reduce intracellular oxidative stress generation and glycol-oxidation. Other amino acids like glycine and lysine are good for the prevention of glycation. Nutritional intervention with taurine, phenyl alanine or branched chain amino acids can improve insulin sensitivity and post-prandial glucose disposal (Anuradha, 2009).

The amino acids lysine, arginine, alanine, aspartic acid and glutamic acid showed varying degrees of antiglycating effect (Sulochana et al., 2001). The insulin response can be substantially increased by the co-ingestion of amino acid, protein mixture containing free leucine, phenyl alanine and an ample source of essential amino acids (Van Loon et al., 2003). Amino acids are shown to inhibit the binding of glucose with proteins, the first step in the pathway of glycation cascade, by competitive inhibition thereby offering protection (Manduteanu et al., 1992). Nakaya et al. (2001) observed that glycine improves insulin sensitivity in an experimental model of type 2 diabetes. In another study glycine diminished hyperglycemia, hypercholesterolemia and glycated hemoglobin concentrations in diabetic rats (Alvarado-Vasquez et al., 2003).

A derivative of isoleucine present in fenugreek (Trigonella foenum graecum) seeds display insulinotropic effect both in vivo and in vitro (Broca et al., 1999). It appears that this has pancreatic and extra pancreatic effects and holds great promise of a novel class of anti diabetic agent (Broca et al., 2004). Various in vitro studies using incubated beta-cells of the pancreas have described strong insulinotropic effects of
arginine, leucine and phenyl alanine (Malaisse et al., 1989; Sener and Malaisse, 1981; Schwanstecher et al., 1998).

In the present study, amino acids isoleucine, alanine, aspartic acid, glutamic acid, glycine, leucine and phenyl alanine which support the prevention of diabetes and diabetic complications were observed in Gymnema sylvestre (Retz.) R.Br. The plant stimulates the release of insulin in vitro by increased membrane permeability (Persaud et al., 1999). Gymnema sylvestre (Retz.) R.Br. leaves contain triterpene saponin, gymnemic acid. Gymnemic acid molecules fill the receptor location in the absorptive external layers of the intestine thereby preventing the sugar molecule absorption by the intestine, which results in low blood sugar (Sahu et al., 1996). This compound has also been found to increase fecal excretion of cholesterol (Persaud et al., 1999) thereby decreasing the storage of triglycerides in the adipose tissue.

The oral administration of the leaf extract of Holostemma ada-kodien Schult. has showed significant reduction of blood glucose level in alloxan induced diabetic rats from the fourth day of the study (Janapathi et al., 2009). In the investigation of the amino acid profile the presence of alanine, aspartic acid, glutamic acid, glycine, leucine, isoleucine and phenyl alanine were observed in this plant. This adds further support to the use of this plant for the prevention of diabetes.

Ethanol extract of Wattakaka volubilis (L.f.) Stapf. possesses significant antidiabetic, antihyperlipidaemic and antioxidant effects in alloxan induced diabetic rats (Maruthupandian et al., 2010). Aerial parts of the plant Pergularia daemia (Forssk.) Chiov. reported to have the pharmacological activities like hepatoprotective (Sureshkumar and Mishra, 2006) and anti-diabetic property (Wahi et al., 2002). In the present study it was observed that both these plants contain alanine,
aspartic acid, glutamic acid, glycine, leucine, isoleucine and phenyl alanine which support the prevention of diabetes.

Several authors have reported that flavonoids, sterols, terpenoids and phenolic acids are known to be bioactive antidiabetic principles (Oliver-Bever, 1986; Rhemann and Zaman, 1989). There are evidences to prove that flavonoids and tannins have antidiabetic activity (Iwu, 1980). Flavonoids are known to regenerate the damaged beta cells in Alloxan diabetic rats (Chakravarthy et al., 1980). Phenolics are found to be effective antihyperglycemic agents (Manickam et al., 1997). Some flavonoids and polyphenols, as well as sugar derivatives have showed significant antidiabetic potential (Jial et al., 2003).

The phytochemical analysis has shown the presence of potent phytochemicals like flavonoids, terpenoids, tannins, sterols, phenols and saponins in the above plants. It denotes that the antidiabetic effect of Gymnema sylvestre (Retz.) R.Br., Holostemma ada-kodien Schult., Pergularia daemia (Forssk.) Chiov. and Wattakaka volubilis (L.f.) Stapf. may be due to the presence of more than one antihyperglycemic principle and their synergistic effects.

Deficiency of one or more amino acids have been observed in diabetes and the beneficial effects of amino acids in some studies are positively correlated with the increase in plasma levels of these amino acids. The inclusion of individual amino acids or amino acid mixture, perhaps as a combinational therapy with conventional treatment protocols can be of therapeutic interest (Anuradha, 2009). The results of amino acid analysis provide scientific validity to the traditional use of Gymnema sylvestre (Retz.) R.Br., Holostemma ada-kodien Schult., Pergularia
daemia (Forssk.) Chiov. and Wattakaka volubilis (L.f.) Stapf. in the treatment of diabetes.

Amino acid availability is of considerable importance, since amino acid deficiency will be an important factor in the etiology of some diabetic complications (Franconi et al., 2004; Rosenlund, 1993). It is known that the antidiabetic drugs currently available produce undesirable effects after long term consumption. The synthetic hypoglycemic agents used in clinical practices have serious side effects like hematological effects, coma, disturbs the functions of liver and kidney. In addition they are not suitable for use during pregnancy (Larmer, 1985). When compared with synthetic drugs, drugs derived from plants are frequently considered to be less toxic and with fewer side effects (Moming, 1987). Future research on the use of plant extracts as supplements, to improve insulin sensitivity and post-prandial glucose disposal is worthwhile. Such dietary supplements can lower patient’s dependency on the conventional glucose lowering medication.

4.1.7.2 Amino acids with neurostimulating effect

The deficiency of amino acids specially glutamic acid and aspartic acid lead to decreased brain function, including number of abnormalities such as impaired memory function, intelligent quotient, mental performance, abnormal behavioral pattern, autism and lack of concentration (Sourkes, 1968; James, 1980; Leon, 1986). Glutamic and aspartic acids are required in higher concentration to keep the brain in proper functioning order (Ansell and Richter, 1954; Sourkes, 1968). Glutamic acid is also known as “Brain Fuel” (Leon, 1986). It is the only amino acid which can easily pass the blood brain barrier and can stimulate the cell in the central nervous system by increased membrane permeability (Streit et al., 1988). Furthermore, these two amino acids act
as donor and precursor for the synthesis of other amino acids which are required for the proper functioning and improving the mental abilities of the brain. Glutamine is a major excitatory neurotransmitter in the brain and spinal cord (Goodman and Gillman, 1991). Aspargine take part in the metabolic control of the brain and nervous system (Chatterjee, 1977; James, 1980). Glutamic and aspartic acids act on the excitatory receptors in the hippocampus and thereby enhance the discharge of neutral pathways. These receptors seem to be involved in memory and learning ability of the brain (Olten and Wenk, 1988; Raigorodsky and Urca, 1990).

In the present study, aspartic acid was found to be present in all the seven plants in comparatively larger quantity and its quantity was exceptionally high viz., Asclepias curassavica L., Calotropis gigantea (L.) R.Br. and Tylophora indica (Burm.f.) Merr. These plants can be used as a good source of aspartic acid. Glutamic acid was present in Gymnema sylvestre (Retz.) R.Br., Holostemma ada-kodien Schult., Pergularia daemia (Forssk.) Chiov. and Wattakaka volubilis (L.f.) Stapf.

4.1.7.3 Essential and non essential amino acids

In the present investigation, among the eight essential amino acids, threonine, isoleucine, leucine, phenyl alanine and tryptophan were observed to be present in all the seven plants viz., Asclepias curassavica L., Calotropis gigantea (L.) R.Br., Gymnema sylvestre (Retz) R.Br., Holostemma ada-kodien Schult., Pergularia daemia (Forssk.) Chiov., Tylophora indica (Burm.f.) Merr. and Wattakaka volubilis (L.f.) Stapf. (Table 3.11). The highest percentage of essential amino acids was observed in Gymnema sylvestre (Retz) R.Br. followed by Calotropis gigantea (L.) R.Br. and Pergularia daemia (Forssk.) Chiov. (Fig. 3.19). The highest percentage of non-essential amino acids was observed in
Calotropis gigantea (L.) R.Br. followed by Asclepias curassavica L. and Gymnema sylvestre (Retz) R.Br. (Fig. 3.20).

Threonine helps to maintain proper protein balance in the body. It is important for the formation of collagen, elastin and tooth enamel. It aids liver and lipotropic function when combined with aspartic acid and methionine; prevents the buildup of fat in the liver and assists metabolism and assimilation. Isoleucine is needed for hemoglobin formation; stabilizes and regulates blood sugar and energy levels and is valuable to athletes, as it aids in the healing and repair of muscle tissue (McCarty and Mark, 1985). Leucine is responsible for regulating the blood sugar concentrations, growth and repair of muscles, hormone production, wound healing and energy production. Its deficiency causes dizziness, headaches, fatigue, depression, confusion, irritability and hypoglycemia in infants. Phenylalanine is used by the brain to produce nor-epinephrine that transmits signals between nerve cells in the brain. It promotes alertness and vitality; elevates mood; decreases pain, aids memory and learning; treat arthritis, depression, menstrual cramps, migraines and obesity. Tryptophan is a natural relaxant; reduces anxiety, depression and stabilizes mood. It helps in the treatment of migraine; aids in weight control by reducing appetite; enhances the release of growth hormones and control hyperactivity in children. The highest percentage of non-essential amino acid was observed in Calotropis gigantea (L.) R.Br. followed by Asclepias curassavica L. and Gymnema sylvestre (Retz) R.Br. (Fig. 3.20).

The essential amino acids are not synthesized in the body and must be supplemented through diet. Since all the plants under study are rich in five essential amino acids, all of them can be recommended for use as a supplement source of these essential amino acids. Leon (1986) reported that glutamic and aspartic acid are the only non-essential amino acids,
which under certain conditions become contingency nutrient and essential. They are used in higher concentration to stimulate and stabilize the brain activities. In the present study, presence of larger quantity of aspartic acid was observed in plants viz., *Asclepias curassavica* L., *Calotropis gigantea* (L.) R.Br. and *Tylophora indica* (Burm.f.) Merr. Glutamic acid was present in larger quantity in *Gymnema sylvestre* (Retz) R.Br. and *Pergularia daemia* (Forssk.) Chiov. These plants can be used as a good source of these amino acids.

In the estimation of amino acids, the content of aspartic acid, serine, glutamic acid, alanine and valine showed significant variation among the plants (Table 3.11; Table 3.47). The estimated value of threonine, proline, glycine, cysteine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine and tryptophan did not show much difference among the plants in the family (Table 3.11; Table 3.47).

4.1.8 Significance of phytoconstituent studies

In the present study irrespective of the season, habitat and age of the plant, the highest polyphenolic and flavonoid content was recorded in *Pergularia daemia* (Forssk.) Chiov. followed by *Tylophora indica* (Burm.f.) Merr. The lowest polyphenol and flavonoid content was found in *Calotropis gigantea* (L.) R.Br.

Medicinal plants contain many antioxidants such as vitamins, carotenoids, flavonoids, polyphenols, saponins, enzymes and minerals (Ray and Hussan, 2002). There are evidences to prove that flavonoids and tannins have antidiabetic activity (Iwu, 1980 and 1983). Flavonoid compounds appear to play vital role in defence against pathogens and predators (Brenda, 1998). Flavonoids do possess antioxidant activity due to the presence of phenolic ring in the moiety (Mikamo et al., 2000). The
phenolics, particularly polyphenols exhibit a wide variety of beneficial biological activities in mammals, including antiviral, antibacterial, immunostimulant, anti allergic, anti hypertensive, hypocholesterolemic, hepatoprotective, anti-inflammatory and anti carcinogenic (Svobodova et.al., 2003). Natural antioxidants possess anti-viral, anti-inflammatory, anti-cancer, antimutagenic, anti-tumour, and hepatoprotective properties.

Quantitative estimation of polyphenols and flavonoids revealed the presence of high quantity of flavonoids and phenols in the wild plants when compared to the cultivated plants. In the wild species of Asclepia curassavica L. (P<0.01), Calotropis gigantea (L.) R.Br. (P<0.05) and Pergularia daemia (Forssk.) Chiov. (P<0.05) the polyphenol content was high when compared to the cultivated species. In Gymnema sylvestre (Retz.) R.Br., Holostemma ada-kodien Schult., Tylophora indica (Burm.f.) Merr. and Wattakaka volubilis (L.f.) Stapf. (P>0.05) the difference observed was not so significant. The flavonoid content in the wild plants were distinctly higher than in the cultivated plants. It is worth mentioning that the increase in the total flavonoid content in the wild plants were significant in Asclepia curassavica L. (P < 0.01), Calotropis gigantea (L.) R.Br. (P<0.01), Gymnema sylvestre (Retz.) R.Br. (P<0.01) and Wattakaka volubilis (L.f.) Stapf. (P<0.01). In Pergularia daemia (Forssk.) Chiov and Tylophora indica (Burm.f.) Merr. the increase was not so significant.

It is interesting to note that higher accumulation of tested compounds were observed in wild plants. Plants growing wild were subjected to many biotic and abiotic stress factors. These plants evolve various defence mechanisms to resist the damage caused by these stress factors (Carrasco et al., 2001). Phenolic compounds have been considered to be involved in the chemical defence mechanism of plants against plant pathogens,
herbivores, soil salinity and water scarcity (Close and Mc Arthur, 2002). Thus the phytoconstituents showed comparative increase in wild plants than cultivated ones.

Due to increased importance gained by Ayurvedic and other Indian system of medicines, medicinal plants are on the verge of becoming are or in endangered condition. Conservative measures should be adopted for the preservation of medicinally important wild plants in their natural habitat. Harvesters should be made aware of the fact that harvesting should be done only after the dispersal of seeds.

On quantification of phenol and flavonoids during dry and rainy season, it was found that there was notable increase in the content during dry season. The polyphenol content showed significant difference (P<0.01) in plants (*Asclepias curassavica* L., *Calotropis gigantea* (L.) R.Br., *Gymnema sylvestre* (Retz.) R.Br., *Holostemma ada-kodien* Schult., *Pergularia daemia* (Forssk.) Chiov., *Tylophora indica* (Burm.f.) Merr. and *Wattakaka volubilis* (L.f.) Stapf.) collected during dry season when compared to that of rainy season. The total flavonoid content also showed marked increase (P<0.01) in plants (*Asclepias curassavica* L., *Calotropis gigantea* (L.) R.Br., *Gymnema sylvestre* (Retz.) R.Br., *Holostemma ada-kodien* Schult., *Pergularia daemia* (Forssk.) Chiov. and *Wattakaka volubilis* (L.f.) Stapf.) collected during dry season. The difference in the content was highly significant.

It is reported that cool temperatures and irrigation have been shown to increase accumulation of isoflavones in seeds of *Glycine max* (Bennett *et al*., 2004). Phenolic acid, anthocyanin and flavonol contents and antioxidant activity in fruit juice of *Fragaria ananassa* are significantly influenced by different growing temperature combination (Wang and Zheng, 2001). A positive correlation between flavonoid content and the
duration of exposure to sunshine in the leaves of *Angelica keiskei* have been demonstrated (Lee *et al*., 2003). The effects of UV-A and UV-B irradiance in *Lactuca sativa* (red lettuce) reduce the plant growth, and a negative correlation is observed on accumulation of total anthocyanins, phenolics, and flavonoids in leaves and ultraviolet radiation (Tsormpatsidis *et al*., 2008). Thus the environmental factors influence the content of phytoconstituents.

In the phenolic content obtained from mature and young plants significant increase was observed in the mature plants of *Calotropis gigantea* (L.) R.Br. and *Wattakaka volubilis* (L.f.) Stapf. (P<0.01). But the difference was not significant in *Asclepias curassavica* L., *Gymnema sylvestre* (Retz.) R.Br., *Holostemma ada-kodien* Schult., *Pergularia daemia* (Forssk.) Chiov. and *Tylophora indica* (Burm.f.) Merr. There was no significant change in the flavonoid content in mature plants of *Pergularia daemia* (Forssk.) Chiov., *Tylophora indica* (Burm.f.) Merr. and *Wattakaka volubilis* (L.f.) Stapf. when compared to the young plant. In the flavonoid content a major difference at 1% level of significance was observed in the mature plants of *Calotropis gigantea* (L.) R.Br. and *Holostemma ada-kodien* Schult.

In short, leaves from wild plants collected during dry season showed higher polyphenol and flavonoid content. A significant variation was not observed in the phytochemical content between the young and mature plants. Hence during the preparation of an Ayurvedic formulation, the season of harvest, the habitat and the age of the plant also determine its quality.

In the case of plants used for medicinal purposes, all these factors must be considered, besides the post harvesting managements (Caicedo *et al*., 2007). Although the biosynthesis of secondary metabolites is
controlled by genetic factors, it is affected by environmental influences and developmental stages. The present investigation revealed that collection of the plants for therapeutic use should be done on scientific basis. Thus, variations in total phenolic and flavonoid contents observed in the present study can be explained by qualitative and quantitative changes of environmental circumstances. It is difficult to determine which environmental factor is mainly responsible for the observed variations because the changes are small and irregular. Moreover, the plants collected have been growing in natural conditions, so it is not easy to separate effects of individual factors from multifactorial influence of the environment.

4.2 Biological activity

Biological activity or pharmacological activity describes the beneficial or adverse effects of a drug on living matter. Activity is generally dosage-dependent.

4.2.1 Antibacterial activity

The rise of antibiotic resistant micro organisms is one of the severe problems in health care systems of the world. Therefore, it is essential to find new compounds that have antimicrobial properties and it is worthwhile to screen plant species which have the above properties to synthesize new drugs (Nascimento et al., 2000). Plants with their complex chemical storehouse of biodynamic compounds, serve as plant defense mechanisms against invasion by microorganisms and insects. It can provide valuable sources of natural antibacterial agents (Abel and Busia, 2005; Roosita et al., 2008). The active principles isolated from plants appear to be one of the important alternatives, when compared to many sub-standard orthodox synthetic medicines, because of their less or no side effects and better bioavailability (Scazzocchio et al., 2001).
In the present study the zones of inhibition obtained against pathogenic microbe *Staphylococcus aureus* by *Asclepias curassavica* L. (21 ± 2.01mm), *Calotropis gigantea* (L.) R.Br. (19 ± 1.18mm), *Gymnema sylvestre* (Retz.) R.Br. (17 ± 1.32mm), *Holostemma ada-kodien* Schult. (16 ± 1.31mm), *Pergularia daemia* (Forssk.) Chiov. (21 ± 1.62mm) and *Tylophora indica* (Burm.f.) Merr. (17 ± 1.41mm) proved the bactericidal activity of methanolic extracts. The methanolic extract of *Holostemma ada-kodien* Schult. showed a zone of inhibition of 23 ± 1.81mm diameter against *Salmonella typhymurium*. Ethanolic extract of *Calotropis gigantea* (L.) R.Br. (Table 3.25), *Holostemma ada-kodien* Schult. (Table 3.27) and *Tylophora indica* (Burm.f.) Merr. (Table 3.29) showed considerable antibacterial activity. The most resistant organism in the present study was found to be *Klebsiella pneumoniae* - MTCC 3384 and *Escherichia coli* - MTCC 727. Bactericidal activity of different extracts against different bacterial strains were evident from the diameter of the inhibitory zone (Table 3.24; Table 3.25; Table 3.26; Table 3.27; Table 3.28; Table 3.29; Table 3.30).

The results indicated that the different extracts had variation in inhibitory potencies. It was due to the differences in the phytoconstituents of the extracts. The methanolic and ethanolic extracts had higher antimicrobial activity than the water and hydro alcoholic extracts. In the present analysis it was observed that the plant extracts have considerable bactericidal activity against gram positive (*Staphylococcus aureus* - MTCC 3160 and *Bacillus subtilis* - MTCC 3053) and mild activity against gram negative (*Klebsiella pneumoniae* - MTCC 3384, *Salmonella typhymurium* - MTCC 98 and *Escherichia coli* - MTCC 727) bacteria.

Tona *et al.* (1999) and Rhoda (2001) have reported that the alcoholic extract of *Phyllanthus amarus* showed significant antimicrobial activity against multi-drug resistant, clinically isolated microorganisms. The organic
extracts provided a more powerful antimicrobial activity, as compared to the aqueous extracts (Cowan, 1999). Similar results showing that the alcoholic extract had the best antimicrobial activity were also reported by Jana and Shekhawat (2010) in Anethum graveolens and Preethi et al. (2010) in Leucas aspera and Holarrhena antidysenterica.

Flavonoids are becoming the subject of anti-infective research and many groups have isolated and identified the structures of flavonoids possessing antimicrobial and cytotoxic activities (Cragg et al., 1997). Flavonoids, triterpenoids and other compounds of phenolic nature are classified as active antimicrobial compounds.

In the present investigation flavonoids extracted from the plants were showing comparatively higher bactericidal property. The results agreed with other reports that flavonoid compounds appeared to play vital role in defence against pathogens (Brenda, 1998). The extracts preserved its antibacterial activity even after the boiling temperature during extraction. From the determination of MIC value, the flavonoids of Asclepias curassavica L. and Tylophora indica (Burm.f.) Merr. were found to possess more bactericidal activity on Staphylococcus aureus when compared to the other plants. The flavonoids of Wattakaka volubilis (L.f.) Stapf. was proved to have the least bactericidal activity (Table 3.31). These findings could form the basis of further studies to isolate active flavonoid compounds, elucidate them against wider range of bacterial strains with the goal to find new therapeutic principles.

4.2.2 Antioxidant activity

As oxidative stress is an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied. Antioxidants are widely used as ingredients in the dietary supplements in order to maintain
health and to prevent diseases such as cancer and coronary heart disease (Bielakovic et al., 2007).

In the present investigation, the highest DPPH free radical scavenging activity was found in *Pergularia daemia* (Forssk.) Chiov. followed by *Holostemma ada-kodien* Schult., *Asclepias curassavica* L., *Wattakaka volubilis* (L.f.) Stapf., *Tylophora indica* (Burm.f.) Merr., *Gymnema sylvestre* (Retz.) R.Br. and the lowest activity was found in *Calotropis gigantea* (L.) R.Br. (Table 3.39; Fig. 3.34). DPPH assay method used is quick, reliable and reproducible and is widely used to test the ability of compounds as free radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts (Mosquera et al., 2007).

Medicinal plants contain many antioxidants such as vitamins, carotenoids, flavonoids, polyphenols, saponins, enzymes and minerals (Ray and Hussan, 2002). Positive role of antioxidant is established in many of the disease states like liver toxicity (Naparro et al., 1993), diabetes (Sabu and Kuttan, 2002), cancer (Kaur et al., 2002), neurodegenerative and several cardiovascular diseases (Svobodova et al., 2003; Tiwari, 2004). The antioxidant capacity of phenolic compounds is based on their ability to scavenge free radicals, chelate pro-oxidant metal-ions and to inhibit some enzymes. A direct relationship has been reported between the levels of phenolic compounds and antioxidant potential of plants (Robards et al., 1999). It has been reported that the antioxidant activity of many compounds of botanical origin is proportional to the phenolic content (Rice-Evans et al., 1997), suggesting a causative relationship between total phenolic content and antioxidant activity (Veglioglu et al., 1998). Cai et al. (2004) show a linear correlation between antioxidant activity and
total phenolic content ($R^2>0.95$) in the 112 traditional Chinese medicinal plants associated with anticancer properties.

4.2.2.1 Statistical analysis

From the analysis of the result obtained, it was observed that the plant which had the highest polyphenolic content was found to possess the best antioxidant activity and the one which had the lowest polyphenolic content was found to possess the least antioxidant activity. This is in accordance with the earlier reports (Zhu et al., 2004). There was direct correlation between polyphenol content and antioxidant activity (correlation coefficient $R^2 = 0.6557$). The $P$ value obtained was 0.027 which is statistically significant at 5% level. Hence it was inferred that the phenolic compounds have a significant antioxidant activity and proved the positive association between these two.

From the present investigation it was observed that a positive correlation occur between the flavonoid content in the plant and the antioxidant activity. The strong correlation coefficient ($R^2$) value 0.8337 suggests that flavonoid compounds present in these plants contribute well to the antioxidant capacity. The results agreed with the earlier reports that medicinal plants contain many antioxidants such as vitamins, carotenoids, flavonoids, polyphenols, saponins, enzymes and minerals (Ray and Hussan, 2002).

The results obtained in the present study indicated that the leaves of these medicinal plants have great importance as therapeutic agents in preventing oxidative stress related degenerative diseases. These plants represent promising sources of natural antioxidants. Antioxidants and natural remedies have two common roles in the health management, i.e., preventive and curative. Natural drugs enhance the body’s defence
capacity against various causative factors and improves the metabolic processes in the body. In recent years much attention has been devoted to natural antioxidants and their association with health benefits (Ali et al., 2008). However, conformation of its in vivo activity should be carried out. Such antioxidants could replace synthetic toxic antioxidants. Thus, the present study has provided scientific validity for the ethno pharmacological uses of these plants in the treatment and prevention of various diseases and disorders.

4.3 Cluster analysis

Cluster analysis puts objects into clusters by minimizing variability within clusters and maximizing variability between clusters. Several phytochemicals have been proved as excellent tools for establishing taxonomic positions of controversial taxa (Pandey, 2011). Several pioneering botanists like Abbott (1886) and Eykman (1888) have employed chemical data in plant classification.

Cluster analysis using the parameter amino acid quality and quantity revealed that the seven plants under study fall distinctly into two clusters (Fig. 3.37). Holostemma ada-kodien Schult., Wattakaka volubilis (L.f.) Stapf., Pergularia daemia (Forssk.) Chiov., and Gymnema sylvestre (Retz.) R.Br. in one cluster. These four plants are traditionally used for the treatment of diabetes among these seven medicinal plants. Asclepias curassavica L., Calotropis gigantea (L.) R.Br., and Tylophora indica (Burm.f.) Merr. form the second cluster. The similarity in amino acid content of Tylophora indica (Burm.f.) Merr. with that of Asclepias curassavica L. and Calotropis gigantea (L.) R.Br. supports the modified classification proposed by Swarupanandan et al. (1996); but the amino acid profile of Holostemma ada-kodien Schult. is different.
Cluster analysis, using the phenol profile, have grouped the seven plants into another three different groups. *Asclepias curassavica* L. and *Wattakaka volubilis* (L.f.) Stapf. were closely related and formed first cluster. *Calotropis gigantea* (L.) R.Br. and *Tylophora indica* (Burm.f.) Merr. formed the second cluster. *Gymnema sylvestre* (Retz.) R.Br. *Holostemma ada-kodien* Schult. and *Pergularia daemia* (Forssk.) Chiov. formed the third cluster (Fig. 3.38).

Flavonoids are useful secondary metabolites in assessing the relationship among closely related species or in studies of infra specific variations. They are also occasionally useful in assessing phylogenetic relationships at higher levels (Bate-Smith, 1968; Gornall *et al.*, 1979; Harborne and Turner, 1984). So the flavonoid profile was used to demarcate these seven taxa. Analysis of the flavonoid profiles solved the taxonomic controversy among the five plant taxa of *Solanum nigrum* Complex (Ayesha *et al.*, 2009). The percentage variation of the flavonoids observed in the seven taxa by HPTLC analysis was used to group them statistically. It was represented in the dendrogram (Fig. 3.39). *Gymnema sylvestre* (Retz.) R.Br. and *Tylophora indica* (Burm.f.) Merr. formed a closely related cluster with a high similarity index showing much similarity in their flavonoid profiles. *Calotropis gigantea* (L.) R.Br., *Wattakaka volubilis* (L.f.) Stapf. and *Asclepias curassavica* L. form another cluster. *Holostemma ada-kodien* Schult. and *Pergularia daemia* (Forssk.) Chiov. formed the third cluster. This grouping was different from that of the amino acid clustering.

In the present investigation, it was noteworthy that, the results obtained by cluster analysis in the three distributions were not uniform. The closest groups formed by these cluster analysis revealed that in
one distribution, two particular species stand close but in other distributions two others stand close. This was seen in all the distributions. The clusters developed were not tallying with any of the already existing classifications (Brown, 1810; Bentham and Hooker, 1876; Hooker, 1883-1885; Swarupanandan et al., 1996). Hence it can be inferred that these types of studies would be more useful in understanding the affinities among the tribes, rather than between sub tribes and genera. For conclusive results more genetically controlled parameters should be included.

4.4 Morphological diversification of pollinia

Results obtained from the cluster analysis using amino acid, flavonoid and phenol profile were not uniform. Hence a genetically controlled, stable floral character of the family was studied to find the similarity among the plants.

Members of the family Asclepiadaceae are unique due to the association of pollen grains that form a sac- like definite structure called pollinia (singular : pollinium). They are the product of only one anther, but are transferred during pollination as a single unit. Pollinia of different genera and species vary in morphological respects (Galil and Zeroni, 1969; Schill and Jakel, 1978).

Pollinial morphology is taxonomically significant like the pollen morphology of other Angiosperms. Pollen aperture characters are more conservative and phylogenetically significant (Erdtman, 1952; Nair, 1970; Walker, 1974; Walker and Doyle, 1975). Nature of corpusculum, caudicle, position and size of pollen sac are the palynological features of pollinia (Sajith and Sreedevi, 2005). Pollinia have a pair of pollinial sac connected to a central corpusculum through a pair of caudicles.
Pollinial sacs are thin and flat. Their size, shape and colour differ according to the genera. In the present study the largest pollinial sacs were seen in *Calotropis gigantea* (L.) R.Br. (2.976 ± 0.06 mm) and the smallest in *Gymnema sylvestre* (Retz.) R.Br. (0.069 ± 0.01 mm). The shape of the pollinial sacs are oval in *Asclepias curassavica* L., *Calotropis gigantea* (L.) R.Br., *Pergularia daemia* (Forssk.) Chiov. and *Wattakaka volubilis* (L.f.) Stapf., globular in *Gymnema sylvestre* (Retz.) R.Br. and *Tylophora indica* (Burm.f.) Merr. and elongated in *Holostemma adakodien* Schult.

The pollinium orientation has been used as a diagnostic character for systematic studies in the family Asclepiadaceae. The classic division of Asclepiadaceae is based on the orientation of pollinium relative to the translator (Swarupanandan et al., 1996). In *Calotropis gigantea* (L.) R.Br., *Asclepias curassavica* L., *Holostemma adakodien* Schult. and *Pergularia daemia* (Forssk.) Chiov. pollinia are pendulously oriented and erect orientation was found in *Gymnema sylvestre* (Retz.) R.Br. and *Wattakaka volubilis* (L.f.) Stapf. It was horizontally oriented in *Tylophora indica* (Burm.f.) Merr. (Table 3.50).

Position of attachment of translator or caudicle to the pollinia is also an important diagnostic feature to evaluate the morphological diversification of pollinia of different genera of Asclepiadaceae. The translator attachment is either apical as in *Asclepias curassavica* L. (Plate 3.10), *Calotropis gigantea* (L.) R.Br. (Plate 3.11), *Holostemma adakodien* Schult. (Plate 3.13), *Pergularia daemia* (Forssk.) Chiov. (Plate 3.14) and *Tylophora indica* (Burm.f.) Merr. (Plate 3.15) or basal as in *Gymnema sylvestre* (Retz.) R.Br. (Plate 3.12) and *Wattakaka volubilis* (L.f.) Stapf. (Plate 3.16). From the present SEM studies surface sculpturing of pollinial sac seemed to vary in different
Irregular concavities were found in *Asclepias curassavica* L. (Plate 3.10), *Calotropis gigantea* (L.) R.Br. (Plate 3.11), *Holostemma ada-kodien* Schult. (Plate 3.13) and *Wattakaka volubilis* (L.f.) Stapf. (Plate 3.16) while surface was convoluted in *Gymnema sylvestre* (Retz.) R.Br. (Plate 3.12), *Pergularia daemia* (Forssk.) Chiov. (Plate 3.14) and *Tylophora indica* (Burm.f.) Merr. (Plate 3.15). Nature of corpusculum and caudicle also varied in different species under study (Table 3.50).

In the present investigation of quantitative characters of pollinia, the size of the pollinial sac, corpusculum and caudicle varied in different species. Largest pollinial sac and corpusculum were observed in *Calotropis gigantea* (L.) R.Br. and smallest in *Gymnema sylvestre* (Retz.) R.Br. respectively. Large caudicle was observed in *Asclepias curassavica* L. and small in *Gymnema sylvestre* (Retz.) R.Br.

The size and shape of pollinial sacs, colour of pollinia, nature of corpusculum, orientation of pollinia, structure of caudicle or translator etc. are important features for the analysis of phylogenetic study (Sanjith and Amal, 2011). Based on the pollinial characters, the species diversification was established in this study. These studies concluded that the pollinia of different genera vary in morphology. So utilizing the pollinial morphology and evidences from the present work, a key was provided below for taxa under study.
Key to the species studied based on qualitative characters of pollinia

Pollinia  pendulous
   Caudicle attachment to pollinia - apical
      Surface sculpturing of pollinial sac-irregular concavities
         Corpusculum simple, triangular
            Shape of pollinial sac - oval  ...............Asclepias curassavica
         Corpusculum complex, elongated
            Shape of pollinial sac - oval  ...............Calotropis gigantea
            Shape of pollinial sac - elongated  ............Holostemma ada-kodien
   Surface sculpturing of pollinial sac- convoluted
      Corpusculum simple, triangular
         Shape of pollinial sac - oval  ...............Pergularia daemia
         Shape of pollinial sac - globular  ...............Tylophora indica

Pollinia erect
   Caudicle attachment to pollinia - basal
      Corpusculum complex, elongated
         Surface sculpturing of pollinial sac - irregular concavities
            Shape of pollinial sac - oval  ...............Wattakaka volubilis
         Surface sculpturing of pollinial sac- convoluted, reticulate
            Shape of pollinial sac - globular  ...............Gymnema sylvestre