Biochemical characterization and cell immobilization of *Costus pictus* D. Don with special reference to antidiabetic property

By

T. ELIZA LINCY

THESIS

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture
Kerala Agricultural University

Centre for Plant Biotechnology and Molecular Biology
COLLEGE OF HORTICULTURE
K.A.U. P.O., THRISSUR 680 656
KERALA, INDIA

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ABSTRACT OF THE THESIS

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DECLARATION

I hereby declare that this thesis entitled "**Biochemical characterization and cell immobilization of *Costus pictus* D. Don with special reference to antidiabetic property"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara

T.ELIZALINCY
(2004-11-37)

CERTIFICATE

Certified that this thesis, entitled "**Biochemical characterization and cell immobilization of *Costus pictus* D. Don with special reference to antidiabetic property"** is a record of research work done independently by Ms. T. Eliza Lincy under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the Advisory Committee of **T. Eliza Lincy (2004-11-37)** a candidate for the degree of **Master of Science in Plant Biotechnology**, agree that this thesis entitled "**Biochemical characterization and cell immobilization of *Costus pictus*** D.Don **with special reference to antidiabetic property"** may be submitted by **T. Eliza Lincy** in partial fulfillment of the requirement for the degree.

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Dedicated to
Tom Home

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Introduction

1. INTRODUCTION

Diabetes mellitus (DM) has emerged as one of the most challenging problem of the 21st century. A metabolic disorder characterized by hyperglycemia, glycosuria, negative nitrogen balance and sometimes ketonaemia and causes a number of complications like retinopathy, neuropathy and peripheral vascular insufficiencies (Joe and Arshag, 2000). The dramatic increase in the prevalence of diabetes can be attributed to several factors. Globally diabetes has shadowed the spread of 'modern lifestyle' and can be linked to an increasingly obese and sedentary population (Moller, 2001). This chronic metabolic disorder that afflicts 150 million people now is set to rise to 300 million by 2025.

There are two major forms of diabetes. Type 1 or insulin dependant diabetes mellitus, is an autoimmune genetic disorder resulting from an absolute deficiency of insulin due to destruction of the insulin producing pancreatic β-cells. Type 2 or non insulin dependent diabetes mellitus is a multifactorial disease (Tayler *et al.*, 1994) which is characterized by insulin resistance, associated not only with hyperinsulinemia and hyperglycemia but also with atherosclerosis, hypertension and abnormal lipid profile, collectively called syndrome X. Type 2 insulin resistant diabetes accounts for 90-95% of the diagnosed cases of disease (Ramarao and Kaul, 1999). Complications are the major cause of morbidity and mortality in DM. Presently, there is no single approach to treat this disease and usually a combination therapy is adopted from different approaches.

Historical accounts reveal that as early as 700-200 B.C, DM was a well recognized disease in India and was even distinguished as two types; a genetically based disorder and other one resulting from dietary indiscretion (Oubre *et al*., 1997). In India, indigenous remedies have been used in the treatment of DM since the time of Charaka and Sushruta (6th century BC) (Grover and Vats, 2001).

Plants have always been an exemplary source of drugs and have been directly or indirectly used as medicines. The ethno botanical information reports about 800 plants that may posses anti-diabetic potential (Aguilara *et al*., 1998). Several such herbs have shown anti-diabetic activity when assessed using presently available experimental techniques (Saifi *et al*., 1971; Mukherjee *et al*., 1972; Coimbra *et al.,* 1992; Ajit Kar *et al*., 1999 and Jafri *et al*., 2000). A wide array of plant derived active principles representing numerous chemical compounds has demonstrated activity, consistent with their possible use in the treatment of NIDDM (Bailey and Day, 1989; Ivorra *et al.,* 1988 and Marles and Farnswoth, 1995). Among these are alkaloids, glycosides, galactomannan, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrate, glycopeptides, terpenoids, amino acids and inorganic ions. Even the discovery of widely used hypoglycemic drug, metformin came from the traditional approach of using *Galega officinalis*. Thus, plants are a potential source of antidiabetic drugs (and others too) but this fact has not gained enough momentum in the scientific community. The reasons may be many including the lack of belief among the practitioners of conventional medicine over alternative medicine.

Although, oral hypoglycemic agents and insulin are the main stay of treatment of diabetes and are effective in controlling hyperglycemia, they have prominent side effects and fail to significantly alter the course of diabetic complications (Rang and Dale, 1991).

Costus pictus D.Don. syn. *Costus mexicanus* (DC) belongs to the family Zingiberaceae. It is a Mexican plant brought to India during 2002-2003. The plant originated in the region from Mexico to Costa Rica. These groups of plants are called spiral ginger/stepladder plant due to its spiral appearance of the stems. Leaves eaten as antidiabetic drug so it is commonly known as ‘Insulin plant’. It is grown in gardens as an ornamental plant especially in the central parts of Kerala. Its yellow coloured flowers with orange red tips and spots are very attractive. The

leaves are fleshy with sour taste. Plant is propagated through stem cuttings and also through rhizomes.

The hypoglycemic properties of the plant has been reported both in streptozotocin and alloxan induced diabetes in animals. The toxicity study of crude extract of the plant was also well studied (Benny, 2004a; Benny *et al.*, 2004a & b)

In this context the following aspects were taken up for the present study:

1. Biochemical characterization of *Costus pictus* with other antidiabetic plants ginger (*Zingiber officinale*), gymnema (*Gymnema sylvestre)*, adhatoda (*Adhatoda vasica*), neem (*Azadirachta indica*) and tulsi (*Ocimum sanctum*) for primary and secondary metabolites.
2. Molecular characterization of *Costus pictus* with other antidiabetic plants by SDS PAGE for protein banding pattern and isozyme studies for peroxidase enzyme.
3. Cell immobilization of *Costus pictus* for the secondary metabolite production *in vitro*.

Review of Literature

2. REVIEW OF LITERATURE

Diabetes is one of the most challenging health problems in the 21st century. Currently, more than 194 million people worldwide have diabetes making it one of the most common non-communicable diseases globally. It is estimated that if adequate measures are not taken now, the number may exceed 300 million by 2025.

According to the International Diabetes Federation, in 2003 five countries with the largest number of persons with diabetes were India (35.5 million), China (23.8 million), the US (16 million), Russia (9.7 million) and Japan (6.7 million) (IDF, 2005). In India prevalence rate of diabetes is estimated to be 1 to 5% (Patel *et al*., 1986; Varma *et al*., 1986 and Rao *et al*., 1989).

It is evident from different studies that the increased oxidative stress with depleted antioxidant enzymes and vitamins are prevalent both in Type1 and Type 2 Diabetes mellitus. Hyperglycemia can increase oxidative stress and change the redox potential of glutathione. Now oxidative stress is acknowledged as a pathogenetic mechanism in the diabetic complications like diabetic retinopathy, nephropathy, and microangiopathy.

The references relevant to the topic are listed below:

2.1 DIABETES - A METABOLIC DISORDER

2.1.1. Chemical remedies

It is well known that the incidence of diabetes is high all over the world, especially in Asia. Different types of oral hypoglycemic agents such as biguanides and sulphonylurea are available along with insulin for the treatment of Diabetes mellitus, but have side effects associated with their uses. There is a growing interest in the herbal remedies because of their effectiveness, minimal side effects in clinical experience and relatively low costs (Gupta *et al*., 2005).

The worldwide epidemic of type2 diabetes (NIDDM) has been stimulating the search for new concepts and targets for the treatment of this incurable disease. Most current therapies were developed in the absence of defined molecular targets. Meanwhile, two targets, protein tyrosine phosphatase1B (PTB- 1B) and glycogen synthase kinase-3 (GSK-3), have emerged as validated targets for treating this disease. Likewise, various companies have intensively studied GSK-3, which plays a key role in the insulin-signaling pathway, as a potential target for the development of antidiabetic therapies (Gupta *et al*., 2005).

Different approaches for treatment of diabetes (Mollar, 2003; Wagman and Nuss, 2001) are insulin or insulin mimetic, enhancers of insulin release, inhibitors of hepatic glucose production (hgp), inhibitors of glucose uptake, enhancers of insulin action.

Newer oral hypoglycemic agents (OHA) and various insulin preparations have been developed in the recent past. OHA like sulphonyl urea compounds (eg. talbutamide, acetohexamide, chloropropamide, glibenclamide, glipizide and glidozide) which exert their hypoglycemic effect, mainly by increasing insulin secretion as well as potentiation of insulin action were discovered and are in use.

Another class of OHA is guanidine derivatives known as biguanides. (eg. Pheformin, metformin, and biuformin) which produce insulin like effects on several tissues and do not cause hypoglycemia in normal subjects. However, limitations of OHA in NIDDM and problems of insulin antibodies in IDDM have been recorded (Kahn and Shechter, 1990).

2.2 *Costus pictus* D.Don

2.2.1 Botany

The genus *Costus* Linn. belongs to family Costaceae, which has been separated from family Zingiberaceae on the basis of the presence of spirally arranged leaves and rhizomes being free from aromatic essential oils. More than

100 species of the genus are distributed in the tropics all over the world (Benny, 2004).

Costus pictus D. Don (Syn. *Costus mexicanus* (DC.) commonly known as Spiral ginger, Stepladder or Insulin plant is a plant originated in Mexico. In India it is grown in gardens as ornamental plant especially in Kerala. The major attraction of this plant is its stem with spiral leaves and light airy and tissue paper like flowers. Red painted stem enhances the beauty of the glossy linear leaves and strongly spiraling canes. The flowers are in a terminal cone, yellow in colour with an orange red tip and this lasts for 3 – 4 days. Usually the plant grows up to 2 –3 m and spread to1.5 – 2 m. The flowers are displayed in a dramatic form high above the leaves. While the flowers do not produce an aroma, they do make a beautiful effect sitting atop of the tall spiraling stems. Propagation is carried out through stem cuttings and also from rhizomes (Benny, 2004).

2.2.2 Cytology

Vovides and Lascurain obtained the following chromosome counts in 1995. *Costus pictus*, *C. scaber, C. pulverulentu*s all 2n = 18; *C. dirzoi* 2n = 27, 28. It is thought that a polyploid series exists in Costus.

2.2.3 Natural distribution

Commonly found in Mexico to Costa Rica in rain forests, clearings, and hill forests along watercourses and roads down to sea level, but mainly distributed between 300-1800meters in all countries.

2.2.4 Morphology

It is a perennial, tall species with red spotted stems that can be used as cut foliage. It has thin stems and narrow leaves and blooms basally in spring and terminally in summer. Height of the plant is generally 1 to 4 meters. It grows 2.1m to 2.4m in medium sun. Produces outstanding spiraling effect when grown. It needs light shade for growth. The soil should stay moist and well-drained mulch is a must.

2.2.5 *Costus pictus* D.Don leaf characteristics

Leaf in general acuminate has filiform point 2 to3 mm, leaf shape is narrowly elliptic and having a length of 10 to 25 cm, width is 2 to 6 cm. Upper leaf hairs are glabrous to sparsely strigose. Bottom leaf hairs are glabrous to densely puberulous. Base of the leaf is cunate, rounded or slightly cordate. Leaf apex is acuminate in shape.

2.2.6 Inflorescence

Costus pictus can be recognized by its reddish yellow flowers, green inflorescence, callus absent, and short ligule. Placed between *C. laevis* and *C. malortienaus*, it differs from *C. laevis* by shorter ligule, shorter petiole, smaller leaves, and smaller flowers. It differs from *C. malortienus* by leaf shape and indument. Narrow leaves with undulating margins is a characteristic feature. It also produces basal inflorescence in the spring and terminal in the summer and fall. It seems to be one of the most cold hardy and vigorous of all Costus species.

2.2.7 Medicinal properties

Costus pictus root has anodyne, antibacterial, antispasmodic, aphrodisiac, carminative, skin stimulating, stomachic, and antiasthmatic properties (Brown, 1995). It is a commonly used medicinal herb in China and is considered to be one of their 50 fundamental herbs (Duke and Ayensu, 1985).

2.2.8 *Costus pictus* and Diabetes

Costus pictus is an ornamental plant, seems to be a medicinal plant owing to its hypoglycemic properties. The raw leaves are commonly taken as a folk remedy to reduce glucose level by diabetic patients. The hypoglycemic properties of the plant has been reported both in streptozotocin and alloxan induced diabetes in animals. The toxicity study of crude extract of the plant was also well studied (Benny, 2004a; Benny *et al.*, 2004a & b)

2.2.9 Biochemicals in *Costus pictus*

Benny (2006) screened *Costus pictus* to find out the chemical constituents. It is found that the sour taste of the fresh leaves as well as the crude extract is due to the presence of oxalic acid.

She also studied the physical and chemical properties of the crude extract. Proteins were estimated by Lowry's method, Carbohydrates by Anthrones method. The fiber content was estimated by Gravimetric method. The total ash was estimated by gravimetric method and total fat by extracting with n- hexane. Calcium content was estimated by permanganometric method. The heavy metals were estimated by AAMS method. Further analysis on the chemical constituent was carried out by permanganometric titration, thin layer chromatography and by HPLC for organic acids.

Physical and chemical properties of crude extract of *Costus pictus* leaves (Benny, 2006)

Sl.NO	Parameters	Description/Results
1.	Colour and appearance	Pale brown to deep brown free flowing powder
2.	Taste	Sour, Characteristic
3.	Moisture content	4.8%
4.	pH (1% solution)	3.7
5.	Acid value	304
6.	Solubility in water (2%solution w/v)	62.5%
7.	Solubility in methanol (2%solution w/v)	44.8%
8.	Protein	49%
9.	Carbohydrate	33%
10.	Fibre	Negligible
11.	Ash	19%
12.	Fat	2.55
13.	Calcium	<1%
	Heavy metal analysis	
14.	Vanadium	10.12ppm
15.	Zinc	343.78ppm
16.	Cadmium	6.91ppm

2.3. GENUS COSTUS

2.3.1 Antidiabetic plants in genus *Costus*

The methanolic leaf extract of *Costus afer* was investigated by Anaga *et al*. (2004) for some pharmacological effects *in vivo* and *in vitro*. The extract exhibited a biphasic antihyperglycaemic activity. At 200mg/kg-body weight, it decreased the blood glucose level by 50% in streptozotocin (STZ)-induced hyperglycemia in male rats in 60 minutes post dosing. However, doses above 200-mg/kg-body weight, increased blood glucose level, potentiating the action of STZ.

Spiral ginger *(Costus speciosus)* is a rhizomatous perennial herb with pinkish white flowers in reddish bracts. It is distributed below 1500m altitudes in tropical forests throughout India. The plant is ornamental and the rhizome is a source of an anthelmintic compound and an alternative source of diosgenin (Rathore and Khanna, 1978). It is also used locally for treating diabetes.

2.3.2 Other medicinal properties of plants in genus Costus

Three polysaccharide glucans were isolated from fresh stems of *Costus spicatus* by Silva *et al*. (2003) by fractionation on Sephacryl S-300 HR and Sephadex G-25. Chemical and spectroscopic studies indicated that they have a highly branched glucan type structure composed of alpha- (1 -> 4) linked D-glucopyranose residues with (1 -> 3), (1 -> 6) branching points, and a small amount of (1 -> 6) branching to alpha- (1 -> 3) linked D-glucopyranose residues.

Shiva *et al*. (2003) studied the morphology and distribution of *Costus speciosus*, a perennial herb that yields alkaloids of medicinal importance. The details of its agro technological/cultural requirements, harvesting and storage, medicinal properties and activity were given.

The methanol extract of *Costus discolor* showed specific potent antifungal activity. All the extracts showed strong antioxidant activity comparable with or

higher than that of alpha-tocopherol. The dichloromethane extracts of *C. megalobractea*, *C. spirali*s and *Zingiber cassumunar* showed stronger activity than the methanol extracts. (Habsah *et al*., 2000)

A cDNA encoding F26G from *Costus speciosus* was introduced into a heterologous plant of *Nicotiana tabacum* via *Agrobacterium tumefaciens* using a binary vector method (Ichinose *et al*., 1999). Successful integration of the cDNA into tobacco chromosomal DNA was confirmed by PCR analysis. F26G activity was also detected in cell-free extracts of the transgenic plantlets.

A new steroidal saponin, named aferoside A, was isolated from roots of *C. afer* by Chao *et al.* (1996). Its structure was established on the basis of chemical transformations and various spectroscopic methods, mainly 2D NMR techniques (COSY, HMQC and HMBC).

Analysis of *Costus speciosus* cell extracts derived from cell suspension cultures was done (Li *et al*., 1992). The effect of temperature and pH on the separation of synthetic mixtures of nucleotides and nucleosides by reversed-phase high-performance liquid chromatography (RC-HPLC) was studied. Stem and rhizome callus contains steroidal constituents diosgenin and tigogenin (Asoklar *et al*, 1992c).

The alcoholic extract of *Costus speciosus* dried rhizomes on acid hydrolysis yields about 3.86% total sapogenins, which on further crystallization and chromatographically yields about 2.12% pure diosgenin (Gupta *et al*., 1970).

2.4 COMPARISON CROPS

2.4.1 Ginger

The juice from freshly squeezed ginger which contains gingerols has been reported to be hypoglycemic in diabetic rats (Sharma and Shukla, 1977). The diabetic state alters the micro vascular function and affects the synthesis of

prostacyclin, thromboxane and leukotrienes (Jeremy *et al*., 1983). Similarly, the gingerols have been reported to inhibit both cyclooxygenase and lipoxygenase and to diminish the production of prostaglandins and leukotrienes (Kiuchi *et al*., 1992). The chemical structures of gingerols are similar in part to those of prostaglandins (Kimura *et al*., 2005).

Ginger is a widely known spice plant, which is equally reputed for its medicinal properties. Ghayur *et al*. (2005) reported the hypotensive, endothelium-dependent and independent vasodilator and cardio-suppressant and stimulant effects of its aqueous extract, which tested positive for saponins, flavonoids, amines, alkaloids and terpenoids. Ginger juice mixed with sugar-candy and given twice daily is a good remedy for diabetes (Singh and Panda, 2005b)

Ginger rhizome contains essential oil (4%), mixture of mainly sesquiterpene hydrocarbons, based on dried ginger, ash (6.5%), proteins (12.3%) and water soluble proteins (2.3%), starch (45.25%), fat (4.5%), phospholipids trace amount sterol (0.53%), crude fibre (10.3%), cold alcoholic extract (7.3%) as oleoresin, vitamins like thiamine, riboflavin, niacin, pyridoxine and vitamin-C are also found. Reducing sugars glucose, fructose, arabinose were present in trace amount (Vermin and Prakanyi, 2005).

Indian ginger contains an aromatic volatile oil of light yellow colour containing camphene, phellandrene, zingiberine, cineole and borneol, gingerol a yellow pungent body; oleoresin contains gingerin the active principle. Two terpenoids viz., humulene monoxide humulene dioxide and humulenol along with (+) α-curcumene, farnesene, linalool, β-sesquiphellandrene, and caryophyllene epoxide were identified from essential oil. Gingerol I, II and III were isolated from rhizomes. Aspartic acid, threonine, serine, glycine, cysteine, valine, isoleucine and argentine were isolated from aerial parts and tuber. Two pigments cassumunaquinones I and II were isolated from rhizomes (CSIR, 1966a).

2.4.2 Gymnema

Gymnema sylvestre extracts have proved to be useful against both IDDM and certain types of NIDDM (Saxena and Vikram, 2004).

Gholap and Kar (2003) studied the relative efficacy of *Inula recemosa*, *Gymnema sylvestre*, *Boerhaavia diffusa* and *Ocimum sanctum* in controlling corticosteroid-induced hyperglycaemia in mice. They found that only *Inula recemosa* (root) and *Gymnema sylvestre* (leaf) extracts were effective in decreasing the serum glucose level. Moreover, the two extracts in combination were found to be more effective than the individual extracts.

In a comparative evaluation of hypoglycaemic activity of 30 Indian medicinal plants, Kar *et al.* (2003) observed that *Gymnema sylvestre* ranked third in the blood glucose lowering activity, after *Coccinia indica* and *Tragia involucrata*. Ethanolic extract of *Gymnema* leaves have shown some blood sugar-lowering effect in alloxan induced diabetic rats (KAU, 2000).

Conduritol- A is a sugar alcohol in leaves of Gymnema, which inhibits glucose absorption from intestine and hence exhibits antidiabetic ability. Miyatake *et al.* (1993) isolated conduritol-A and have standardized the procedure for its purification.

Shanmugasundaram and Panneerselvam (1981) reported that the administration of *Gymnema sylvestre* brings down the blood glucose levels in diabetic rabbits and this is mediated through increased insulin secretion. Their investigation also revealed that diabetic patients under *Gymnema* therapy showed significant change in their blood glucose and serum insulin levels indicating that administration of *Gymnema* stimulates insulin release.

The leaves contain hentriacontane, pentatricontane, α and β chlorophylls, phytin, resins, tartaric acid, formic acid, butyric acid, anthraquinones derivatives, inositil, *d*- quercitol and gymnemic acid; the leaves contain trace alkaloid known

as Gymnamagin (Chopra *et al*., 1956). Gymnemic acid is the name applied to an impure complex mixture which can be fractionated by successive extraction with petroleum ether, ether, chloroform, ethyl acetate and alcohol.

The leaves of the plant, when chewed, possess the remarkable property of paralyzing, for a few hours, the sense of taste for sweet and bitter substance; acid taste is not affected while salt taste is very slightly if at all influenced. The leaves have been sometimes used as a remedy for diabetes. It has been shown, however, that neither the leaf powder nor the alcoholic extract prepared from it has any effect on the concentration of sugar in the blood or in the urine of patients suffering from diabetes. However, they cause hypoglycaemia in experimental animals when administered orally or by injection. This effect is not due to any direct influence on the carbohydrate metabolism, but to indirect stimulation of insulin secretion by pancreas (CSIR, 1956).

2.4.3 Adhatoda

The leaves, flowers, fruits and roots are extensively used for treating cold, cough, whooping cough and chronic bronchitis and asthma, as sedative-expectorant, antispasmodic and as anthelmintic.

Several alkaloids are present in the drug and the chief principle is a quinazoline alkaloid, vasicine. The yield of the alkaloids from different samples in India ranged from 0.541 to 1.105 percent on dry basis. Yield as high as 2.18 percent on dry basis has been reported from a foreign sample of which more than half was the *l*- form and the remainder the *dl* form of alkaloids.

Vasicine alkaloid is bitter and probably occurs in nature in its *l*- form, and is racemized during the process of isolation. Vasicine forms colourless crystals, readily soluble in chloroform. The specific rotation of *l*- vasicine is strongly dependent on the nature of the solvent and the concentration of the solution. Vasicine is readily resolved through *d*-tartarate, the *l*-vasicine salt being more sparingly soluble in methanol. Recent investigations about the alkaloids in

combination (1:1) showed pronounced bronchodilatory activity *in vitro* and *in vivo* (CSIR, 1985)

Leaves and roots are having hypoglycemic property. Alcoholic extract is hypotensive, bronchodilator, respiratory stimulator, hypoglycemic and antispasmodic. A non-nitrogenous principle from leaves, feebly hypoglycemic for short duration in male rabbits has been reported (Asolkar *et al*., 1992a; Rastogi and Mehrotra, 1995)

2.4.4 Neem

Leaf extract is hypoglycemic and antihyperglycemic. It forms one of the constituents of a composition for diabetic patients. Kernal oil is also useful in diabetes. Oil and nimbidin showed hypoglycemic activity in rabbits (Asolkar *et al*., 1992b).

2.4.5 Tulsi

The plant contains volatile oil, alkaloids, glycosides, saponins and tannins. The leaves contain ascorbic acid (83mg/100g) and carotene (2.5mg/100g). The juice of the leaves possesses diaphoretic, antidiabetic, antiperiodic, stimulating and expectorant properties. The plant yields on steam distillation a volatile oil with a characteristic odour of thymol and a pungent, spicy taste. A sample of oil had ester (linalyl acetate), 2% combined alcohol, 1.5% free alcohol, 31% phenol, 38% soluble in 1.5 volume of 80% alcohol. The principal constituent is thymol (toltal phenols18-65%) *d*-limonene, α and γ-terpinene, and diterpene and terpineol are present. A decoction of leaves is used in fever and coughs. The juice of leaves is used for catarrh and as eye drops for conjunctivitis (CSIR, 1966b).

The leaves contain an essential oil, which has been studied with gas chromatography. The oil contains eugenol, carvacrol, methylchavicol, limatrol and caryophylline (Singh and Panda, 2005b)

According to Ayurveda and Siddha systems tulsi is having the following properties. Guna: laghu, ruksha. Rasa: katu, tikta. Veerya: ushna. Vipaka Katu. Dosha: kaphavatagha. Karma: swasakasahara, Parsvashulaghna, krimighna, Dipana, Ruchikara, Jwaraghna, Rasayana (Singh and Panda, 2005b).

2.5 PRIMARY METABOLITES

2.5.1 Carbohydrates

Several studies have shown that raising the carbohydrate intake doesn't adversely affect the fasting blood glucose levels, glucose tolerance or insulin requirements provided that calories are not increased. Insulin needs are more closely correlated with total calorie intake than with the carbohydrate level in the diet.

Diets high in carbohydrates and fibre improve glucose metabolism without increasing insulin secretion. They lower fasting serum and peripheral insulin concentrations in response to oral glucose administration in both diabetic and nondiabetic individuals (Srilakshmi, 2000)

2.5.2 Proteins

A diet high in protein is good for diabetes because it supplies the essential amino acids needed for tissue repair. Protein doesn't raise blood sugar during absorption as does carbohydrates and it does not supply as many calories as fat.

In patients with NIDDM, consumption of protein along with carbohydrates will lower the blood glucose concentration due to amino acid stimulation of insulin secretion seen in so many of these patients. Protein also promotes satiety and helps both types of diabetic patients to adhere to the carbohydrate allowance.

One gram of protein per kilogram bodyweight is adequate but more may be given and the amount of fats and carbohydrates proportionally reduced (Srilakshmi, 2000)

Chatwal (1983) reported the chemical reaction occurring while spraying ninhydrin spray reagent for proteins. With ninhydrin (indane 1,2,3 trione hydrate)

amino acids yield a coloured product. The mechanism of action is not certain. However, ninhydrin does a powerful oxidative decarboxylation of α-amino acids producing Co_2, NH_3 and an aldehyde with one less carbon atom than the parent amino acid. The reduced ninhydrin then results with the liberated ammonia, forming a blue complex, which maximally absorbs light of wavelength 570 nm. The intensity of blue colour produced under standard conditions forms the basis of an extremely useful quantitative test for α-amino acids. Amines other than α-amino acids react with ninhydrin, forming a blue colour but without evolution of Co_2 are thus indicative of a α- amino acid.

2.6 SECONDARY METABOLITES

2.6.1 Phenol

To the plant biochemist, plant phenols can be a considerable nuisance, because of their ability to complex with protein by hydrogen bonding. When plant cell constituents come together and the members are destroyed during isolation procedures, the phenols rapidly complex with protein and as a result, there is often inhibition of enzyme activity in crude plant extracts.

Olive oil is rich in oleic acid and its richness in phenolic compounds, which act as natural antioxidants and may contribute to the prevention of human disease. Individual phenolic compounds in virgin olive oil analytically separated and quantified by Pancorbo *et al*. (2005). Capillary electrophoresis might represent a good compromise between analysis time and satisfactory characterization for some classes of phenolic compounds of virgin olive oils.

Water-soluble extracts from Lamiaceae species were screened for antioxidant properties in six *in vitro* assays (Dorman *et al*., 2004). Total phenol content and qualitative-quantitative compositional analyses were also carried out. All the extracts contained Folin-Ciocalteu reagent-reactive substances, which were confirmed by the presence of polar phenolic analytes (i.e., hydroxybenzoates, hydroxycinnamates, and flavonoids).

Qimen black tea has strong inhibitory activity of canola oil oxidation. Four antioxidant compounds, theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3'-gallate (TF2B) and theaflavin digallate (TF3), were isolated from acetic acetate extract of black tea by silica gel and sephadex LH-20 column chromatography (Su *et al*., 2004). TF1, TF2A, TF2B and TF3, have stronger antioxidant activity than that of BHT (Butylated hydroxytoluene).

Lee *et al*. (2004) examined the antioxidant and anticancer activities of the petroleum ether extracts from the roots of *Platycodon grandiflorum*. Extract was fractionated and the antioxidant activities of the fractions were evaluated in terms of their inhibition of lipid peroxidation as well as their free radical scavenging activity. The antioxidant activity was closely related to the content of phenolic compounds, and the anticancer active fraction exhibited a typical UV absorbance spectrum of polyacetylene.

Ross *et al*. (2004) confirmed the hypothesis that alkylresorcinols are metabolized in humans via beta-oxidation of their alkyl chain. Alkylresorcinols, phenolic lipids present in high amounts in wholegrain wheat and rye, are of interest as potential biomarkers of the intake of these cereals. Alkylresorcinols are known to be absorbed by humans and animals, but little is known about their metabolism or resulting metabolites. Extracts were separated by thin-layer chromatography, and fractions containing alkylresorcinols and possible metabolites were identified by retention on the plate compared to standard compounds, and staining with fast blue B.

Pyrogallol, having three hydroxy groups at the adjacent positions in the benzene ring, oxidized human oxyhemoglobin to methemoglobin and reduced human methemoglobin to oxyhemoglobin. Since superoxide dismutase and catalase inhibited these reactions extensively, active oxygens such as superoxide and hydrogen peroxide were considered to be involved in the oxido-reductive reaction of human hemoglobin by pyrogallol (Miyazaki *et al*., 2004).

Coumarins and flavonoids from the point of view of chemical belonging are phenol derivatives with important pharmacological effects. Applying method of thin layer chromatography, it is detected the presence of coumarins and flavonoids substances in plant material that has been tested by Besovic and Duric (2003). Chromatograms are developed in systems cyclohexane-ethylacetate (13:7) and toluene-ether (1:1) saturated with 10% acetic acid, and visualization by observing on UV lamp (254 and 366 nm), spraying with reagents KOH (10% ethanol solution) and diphenylboryloxyethylamine (1% methanol solution).

Antioxidant drugs have significant role in the management of diabetes and as such many of the herbal drugs used in diabetes have been reported to have antioxidant activity (Sabu and Kuttan, 2003). Green tea polyphenols and methanol extract of *Phyllanthus amarus*, which were found to have antidiabetic action reduce the oxidative stress in experimental diabetes. The antioxidant activity of various fractions of non-tannin phenolics of canola hulls were analysed by Amarowicz *et al*. (2000). Fractions I and II showed the best preventive effect against the bleaching of beta-carotene.

Different extracts of *Crataegus monogyna* against H_2O_2 and HOCl, have been determined *in vitro* for antioxidant activity (Rakotoarison *et al*., 1997). All the extracts are efficient and the scavenging capacity is clearly related to the total phenol content. Among individual compounds, the flavanol-type derivatives, especially the proanthocyanidin B2, are more efficient. Thus, *in vitro* plant tissues could be an interesting source of bioactive molecules.

2.6.2 Flavoniods, Flavonols and Flavones

Flavonoids are present in plant as mixtures and it is very rare to find only a single flavonoid component in a plant tissue. In addition, there are often mixtures of different flavonoid classes. The coloured anthocyanins in flower petals are almost invariably accompanied by colourless flavones or flavonols and recent

research has established that the flavones are important co-pigments, being essential for the full expression of anthocyanin color in floral tissues. Mixtures of anthocyanins are also the rule, particular in the flowers of ornamental plants, and any one-flower tissue may contain up to ten different pigments.

The highest yield (14.4 g/kg) of naringin, the major flavonoid from the peel of *Citrus paradisi* that could be achieved by supercritical fluid extraction was obtained using supercritical carbon dioxide modified with 15% ethanol and fresh peels at 95 bar and 58.6 ^{0}C (Giannuzzo *et al*., 2003).

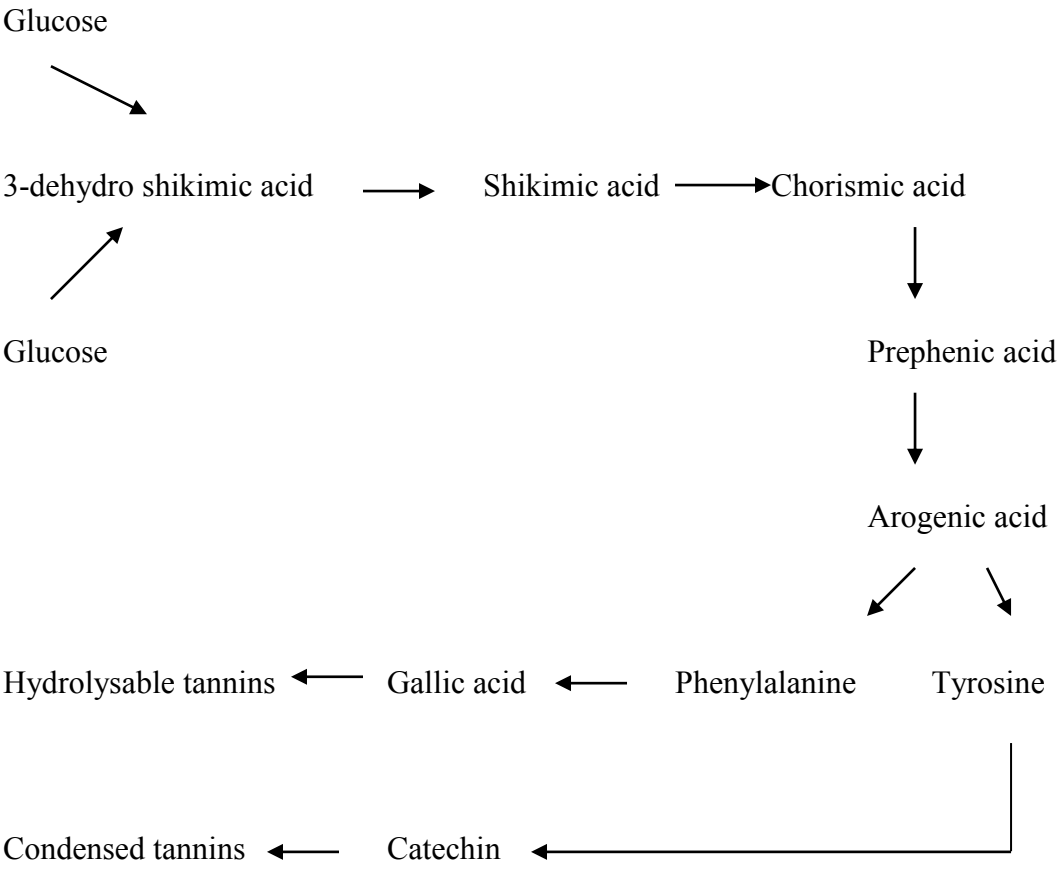
The ethyl acetate fraction obtained from the extraction of *Artemesia annua* yielded 11 flavones, four flavones glycosides and two chromone derivatives (Yang *et al*., 1995). Three new compounds were isolated were quercetin 4'-mehyl ether, 2,2-dihydroxy 6-methoxychromene and 2,2,6-trihydroxychromene.

Kaempferol (corresponding in hydroxylation pattern to the anthocyanidin pelargonidin); quercetin (cf. cyanidin) and myricetin (ef. Delphinidin), (Like the corresponding anthocyanidins can be separated clearly by simple PC).

2.6.3 Tannins

The tannins have the ability to react with protein, formatting stable water – insoluble co-polymers. Industrially, tannins are substances of plant origin, which because of their ability to cross-link with protein are capable of transforming raw animal skins into leather. In the plant cell, the tannins are located separately from the proteins and enzymes of the cytoplasm but when tissue is damaged, e.g. when animals feed, the tanning reaction may occur, making the protein less accessible to the digestive juices of the animal. Plant tissues high in tannin are in fact, largely avoided by most feeders, because of the astringent taste they impart. One of the major functions of tannins in plants is a barrier to herbivory.

2.6.3.1 Biosynthesis of Tannins (Katoh *et al*., 1989)



A TLC-densitometric method was introduced for the parallel determination of Rosmaric acid and Caffeic acid (Janicsak and Mathe, 1998). In the wavelength range 290-330 nm, it was found that the emission peak areas exhibit a maximum at 325 nm. Therefore, the densitometric evaluations of these tannins were made at 325 nm. The two compounds behaved very similarly as regards color stability. The application of TLC permits utilization of the fluorescence of both phenols, often without the need for prior purification. This fluorescence measurement makes the detection extremely sensitive. Gallic acid, the decomposed product of tannin, was in bright yellow and green color, showing a strong specificity. Lei *et al*. (1997) did TLC to detect gallic acid giving reliable indexes for quality assay of TCM preparations.

2.6.4 Terpenoids

Chemically, terpenoids are generally lipid-soluble and are located in the cytoplasm of the plant cell. Essential oils sometimes occur in special glandular cells on the leaf surface, whilst carotenoids are especially associated with chloroplasts in the leaf and with chromoplasts in the petal.

Machado *et al*. (2006) determined the presence of alkaloids and terpenes in gynoecium and fruit extracts and in fresh nectar stored in the nectar chamber using thin layer chromatography. Its main chemical components, alkaloids and terpenes, suggest that they serve a protective function and are not related to the floral nectar source or to improving nectar quality.

Astaxanthin (ASTX), a carotenoid with potent antioxidant properties, exists naturally in various plants, algae, and sea foods. Wu *et al*. (2006) investigated the in vitro ability of ASTX to protect porcine lens crystallins from oxidative damage by iron-mediated hydroxyl radicals or by calcium ion-activated protease (calpain), in addition to the possible underlying biochemical mechanisms.

The extracts *Tabernaemontana catharinensis* were fractionated and analyzed by thin-layer chromatography and gas chromatography/flame ionization detection. Pereira *et al.* (2005) determined the antioxidant activity was by the coupled reaction of beta-carotene and limonene acid. The antioxidant activity of the extracts ranged from 53% to 95%.

A new cassane furanoditerpene, 17-methylvouacapane-8 (14), -9(11)-diene (1), has been isolated from the seed kernels of *Caesalpinia crista* (Jadhav *et al*., 2003). The furanoditerpene can serve as a marker chemically to differentiate *C. crista* from the synonymous *C. bonduc*.

2.6.5 Saponins

The formation of persistent foams during plant extraction or during the concentration of plant extracts is a reliable evidence that saponins are present.

Plant secondary metabolites, such as saponins, have a considerable impact in agriculture because of their allelopathic effects. Fons *et al*., 2003 investigated the influence of saponins on rhizosphere bacteria *in vitro* and in soil conditions. The effects of gypsophila saponins on the growth kinetics of rhizosphere bacteria were studied by monitoring the absorbance of the cultures in microtiter plates.

Golba (2000) standardised the techniques for quantitative estimation of saponins from *in vitro* cultures of *Gymnema sylvestre*. He found that the solvent system chloroform: acetone: methanol (5:1:1.5) was best for eluting the saponins in to a single condensed spot.

Sinsheimer *et al.* (1970) reported that this acid is a complex mixture of nine closely related acidic glycosides which they named gymnemic acids A, B, C, D, V, W, X, Y and Z. They used techniques like thin layer chromatography (TLC), partition chromatography and column chromatography to separate these constituents.

2.6.6 Alkaloids

Adulteration of non-toxic plants by toxic ones for aristolochic acid I, tetrandrine and fangchinoline in medicinal plants detected by Koh *et al.* (2006) through HPLC-DAD chromatographic fingerprints.

Classical thin-layer chromatography (TLC) of opium alkaloids using complex eluents with strong alkaline substances to obtain a clean separation between morphinan and isoquinoline compounds was done by Pothier and Galand (2005).

The oxidation products of three Rauwolfia alkaloids having yohimbane skeleton, namely, reserpine, rescinnamine and ajmalicine have been characterised by chromatographic and spectroscopic techniques by Azeem *et al*. (2005).

El-Seedi *et al.* (2005) subjected *Lophophora williamsii*, from the collection of the Witte Museum in San Antonio, to radiocarbon dating and alkaloid analysis. Alkaloid extraction yielded approximately 2% of alkaloids.

A novel monoterpene alkaloid, named incarvillateine E, possessing three moles of incarvilline moieties, has been obtained from the aerial parts of *Incarvillea sinensis*. (Bignoniaceae). On the basis of spectroscopic evidence, the structure of incarvillateine E has been characterized by Chi *et al. (*2005).

The crude alkaloid content of transformed *Clitoria ternacia* were analysed by Malabadi and Nataraja (2003) for the identification of alkaloids using thin layer chromatography. The crude alkaloid extract was dissolved in a small volume of methanol and fractioned on a precoated silica gel plate with acetone: light petroleum ether: carbon tetra chloride: isooctane (35: 30: 20: 15) as the developing solvent system. The alkaloids in each fraction were detected after spraying with Dragendroff's reagent and identified by comparing their relative front (Rf) values.

2.6.7 Plant acids

Oxalate is an excellent ligand for metal ions, where it usually binds as a bidentate ligand forming a 5-membered MO_2C_2 ring. The affinity of divalent metal ions is sometimes reflected in their tendency to form insoluble precipitates. Thus, oxalic acid also combines with metals such as calcium, iron, sodium, magnesium, and potassium in the body to form crystals of the corresponding oxalates, which irritate the gut and kidneys. Because it binds vital nutrients such

as calcium, long-term consumption of foods high in oxalic acid can lead to nutrient deficiencies. Healthy individuals can safely consume such foods in moderation, but those with kidney disorders, gout, rheumatoid arthritis, or certain forms of chronic vulvar pain are typically advised to avoid foods high in oxalic acid or oxalates.

Conversely, calcium supplements taken along with foods high in oxalic acid can cause oxalic acid to precipitate in the gut and drastically reduce the levels of oxalate absorbed by the body. The calcium oxalate precipitate better known as kidney stones obstruct the kidney tubules (Roth and Breitenfield, 1977; Obzansky and Richardson, 1983). The root and or leaves of rhubarb and buckwheat are listed being high in oxalic acid (Harborne, 1973).

For paper chromatography, the best solvent pair is probably n – butanol acetic acid – water (BAW) and phenol – water (Harborne, 1973).

Succinic acid, an intermediate of tricarboxylic acid cycle, is produced and accumulated by anaerobic microorganisms. The long-standing interest in the production of this organic acid is because it is a key compound in producing more than 30 commercially important products. The detection of succinic acid is generally carried out by gas chromatography (GC), enzymatic assays, ion-exclusion chromatography (IEC) or by high performance liquid chromatography (HPLC) (Agarwal *et al*., 2005).

2.7 ELECTROPHORESIS

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length. i.e. the denatured polypeptides become rods of negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in

proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2- mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

The majority of proteins which have isoelectric points within the pH range 4- 7.5 and hence are weakly acidic tend to be separated best in alkaline gels in the pH range 8-9. Too high an ionic strength necessitates the use of low voltage gradients to avoid excessive heating. This leads to long electrophoresis times with risks of increased denaturation and band separation due to diffusion (Andrews, 1986).

Separation ranges of acrylamide gels of various concentrations:

Percentage	Optimum molecular weight range
3-5	Above 100 000
5-12	20 000-1 50 000
10-15	10 000- 80 000
15+	Below 15 000

2.7.1 SDS PAGE

Ishii *et al*. (2004) purified the enzyme, gamma-RA decarboxylase to homogeneity on SDS-PAGE through the steps of one ion-exchange chromatography and two kinds of hydrophobic chromatography.

Sandal (*Santalum album* L.) proteins were extracted according to modified Lowry's (Lowry *et al*., 1951) procedure and were subjected to SDS-PAGE on a mini gel system and proteins were stained using Coomassie brilliant blue R-250 and then destained with 40% methanol to remove background staining (Suma and Balasundaran, 2004).

Moncheva *et al*. (2003) employed SDS-PAGE analysis to study the seasonal qualitative and quantitative changes of phytoplankton composition at Varna Bay (Black Sea). SDS-PAGE analysis showed different protein patterns, in summertime the major protein constituents were of 14, 37, 48 and 70 k Da, while in the springtime the sizes ranged between 38 and 48 k Da.

2.8 ISOZYME ANALYSIS

Molecular markers consist of specific molecules, which show easily detectable differences among different strains of species or among different species (Singh, 1998). Genetic marker studies have the most significant impact of all currently available modern biotechnologies on crop improvement programmes and thus their integration in crop improvement and genetic conservation activities is recommended both for developed and under developed countries (FAO, 1994).

Peroxidase catalyses the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc. Peroxidase occurs in animals, higher plants and other organisms. The best studied is horse radish peroxidase (Sadasivam and Manikam, 1992).

The basis of electrophoretic analysis of isozymes (from isoenzymes) was laid down in 1957 when Hunter and Mohler discovered the isozymes. In 1959 Markert and Moler introduced the concept of isozymes, which they defined as the different molecular forms in which proteins may exist with the same enzymatic specificity (Buth, 1984). This means that different variants on the same enzymes have identical or similar functions and are present in the same individual.

2.8.1 Isozyme in relation to physiological and developmental stages of plants

Different developmental stages of somatic embryogenesis of sandal (*Santalum album* L.) was used for the isozyme analysis. Peroxidase activity was modulated during different developmental phases of embryos. In friable callus state, just before the induction of embryos, there was an increase in the activity of peroxidase (Suma and Balasundaran, 2004).

To diagnose the Fe deficiency at early stages of growth, Salama, (2000) studied the growth, active iron (Fe)+2 chlorophyll concentrations, enzyme activities such as peroxidase (POD) and isozyme analysis .

The enzymes peroxidase and polyphenol oxidase were evaluated for their involvement in the resistance mechanism of tomato plants against *Pseudomonas syringae* pv *tomato*. Four different peroxidase isozymes were found in extracts from diseased plants compared to only one in healthy plants (Bashan *et al.*, 1987).

2.8.2 Isozyme variation in relation to geographical variation

Nair (2005) did the isozyme analysis to detect the variability in *Gymnema sylvestre*. Identification of the diversity of neem (*Azadirachta indica* A. Juss) by peroxidase, esterase, aspartate amino transferase isozyme analysis were tried by Philomina and Surendran (2003).

Montarroyos *et al*., 2003 evaluated the genetic diversity among 28 accessions from the Pernambuco Agriculture Research Institute Cassava Active Germplasm Bank through isoenzymatic systems alpha, beta -Esterase (EST), Peroxidase (POX), Glutamate Oxalacetic Transaminase (GOT) and Acid Phosphatase (ACP). Based on the results obtained GOT and POX system being best suited for root tip and foliar tissue.

Five isoenzymes (peroxidase, esterase, glutamate oxaloacetate transaminase (aspartate aminotransferase, catalase, and malic dehydrogenase

(malate dehydrogenase) were analysed (Sharma and Deka, 2002) for the identification of hybrids between Cambod type and Assam type cultivars.

Fifty-three genotypes of *Pennisetum purpureum* germplasm were evaluated (Sukumar and Khan, 2001) for genetic diversity through Mahalonobis D2 analysis. The isoenzyme analysis grouped the genotypes into 2 major groups.

2.8.3. Isozyme in relation to disease detection

Investigation has been carried out (Shivakumar *et al*., 2003) on differential expression of peroxidase isoenzymes in pearl millet seedlings inoculated with downy mildew disease causing pathogen *Sclerospora graminicola*.

2.9 IN VITRO PLANT REGENERATION

Malabadi (2002) conducted a study to develop a simple and efficient protocol for the rapid propagation of spiral ginger (*Costus speciosus*). Rhizomes cultured on modified MS basal medium supplemented with 8.87 µM BA+9.29 µM kinetin (KN) +5.37 µM NAA were able to produce the maximum number of shoot buds with 10 to 11 roots. Luxuriant shoot elongation was observed when newly formed shoot buds with parental rhizome were cultured on modified MS basal medium containing 8.87 µM BA+9.29 µM KN+5.37 µM NAA further supplemented with 100 mg adenine sulphate/litre and 10% (v/v) coconut water. New rhizomes were able to produce plantlets with well-developed roots and these were successfully acclimatized and established under greenhouse conditions.

Hosoki and Sagawa (1977) developed a technique for rapid clonal propagation of ginger by tissue culture using buds from rhizomes in storage on a medium consisting of Muashige-Skoog major elements, Ring-Nitsch minor elements and vitamins, 2% sucrose, and 1-ppm 6-Benzyl amino purine. Numerous adventitious shoots with roots were produced by repeated subculture of individual plantlets on 1ppm BA medium.

2.10 CELL IMMOBILIZATION

Immobilization of biocatalysts, enzymes, microbial/ plant/animal cells and cell organelles have opened up a vast area of technological applications of these biocatalysts in process industries particularly, food, pharmaceuticals, organic chemicals and waste treatment, besides their role in analytical and therapeutic applications. In recent years, suspension cultures of plant cells have been used to produce many biochemicals, including perfumes, dyes, medicals and opiates. This technology is progressing rapidly because of its advantages in term of simplicity and potential economics compared to extraction from natural plants.

The first commercial process utilizing plant suspension culture appeared in the early 1980's, and additional processes appear to be nearing commercialization. However, plant cells show slow growth in suspension cultures and in general give very low product concentrations, which results in extremely low reactor productivity. In addition, they are very shear sensitive, making suspension culture difficult. These problems can be alleviated by cell immobilization. Immobilized cells can operate continuously without cell washout (Kilbanov, 1983)

2.10.1 Immobilization techniques

Using plant cell cultures of *Lithospermum erythrorhizon* Kim and Chang, (1993) produced shikonin by *in situ* and cell immobilization in calcium alginate beads in shake flask cultures. *In situ* product extraction and cell immobilization enhanced shikonin production and facilitated product recovery. *In situ* extraction by n-hexadecane and cell immobilization by calcium alginate gave higher specific shikonin productivities of 7.4 and 2.5 times.

Biocatalysts have been immobilized using different methods including adsorption, entrapment, covalent binding, cross-linking or a combination of two or more of these techniques. Both organic and inorganic supports have been used as carriers. Some of the characteristics of a useful support include its cost, nonhydrophilicity, porosity and surface area. The choice of the technique as well

as the support depends on the nature of the enzyme, nature of the substrate and its ultimate application in proper reactor geometry (D'Sauza, 1989; Tampion and Tampion, 1987)

Immobilization of plant cells has been developed as an alternative to suspension cultures to provide cell-to-cell contact and favorable cell differentiation to improve secondary metabolite production.

Immobilization of all high yielding cell lines for secondary metabolite production or for biotransformation is not desirable for many reasons. a) The immobilized cell should be active in uptake of nutrients for efficient bioconversion. b) The product should leach out of the cells and beads into the medium. c) For biotransformation to proceed the substrate must be taken up by the immobilized cells and converted to the products which can be released to the medium. Without the above-mentioned features the cell cultures will not be useful for immobilization (D'Sauza, 1989)

2.10.2 Callus induction

Callus is a genetic mosaic of cells owing to rapid changes in DNA during cell divisions. From these cells, selection for genetic fitness takes place and the regenerants arising in most cases will have a normal genotype almost true to the parent (Edallo *et al*., 1985).

Plant cell cultures of the Mexican species *S. chrysotrichum* were established from friable calli by Charlet *et al*. (2000). These cells produce an antifungal spirostanol saponin designated SC1. Plant cell immobilization within Ca-alginate gel beads can lead to an enhancement in secondary metabolite production.

2.10.3 Suspension cultures

Analysis of *Costus speciosus* cell extracts derived from cell suspension cultures were studied by Li *et al*. 2003.

For the production of diosgenin, the seedling calli of *Costus speciosus* maintained for 18 months as static cultures were transferred to RT liquid medium supplemented with 0.1ppm of 2, 4-dichlorophenoxy acetic acid and grown as suspension culture for 6-8 months by frequent sub culturing for 4-6 weeks (Rathore and Khanna, 1978) The cultures of *C. speciosus* showed root formation, so callus was transferred to RT liquid medium supplemented with 1ppm of 2, 4-D, which resulted in undifferentiated cells.

2.10.4 Immobilization techniques

2.10.4.1 Using calcium alginate

The cells were immobilized in the calcium alginate gel bead coated with a cell-free gel film. Iizuka *et al*. (2005) then performed the batch cultures with the addition of various volumes of coconut oil. The total scopoletin production increased about 16 times larger than that in the suspension culture without solvent.

Micrometer sized calcium alginate beads referred to as biobeads encapsulated plasmid DNA molecules carrying a reporter gene. In order to evaluate the efficiency of the biobeads in mediating genetic transfection, protoplasts isolated from cultured tobacco cells were transfected with biobeads containing a plasmid that carries the modified green fluorescent protein gene CaMV 35s GFP.

The first report of plant cell immobilization was by Brodelius and coworkers. They entrapped viable cells of *Catharanthus roseus, Morinda citrifolia* and *Digitalis lanata* in Calcium alginate. Plant cells cannot be entrapped by adsorptive techniques such as attachment to glass or plastic as used for animal or microbial cells (Lindsey and Yeoman, 1985).

2.10.5 Configuration of reactors for immobilized cell cultures

Configuration for a typical packed bed reactor consists of a column with polymeric beads. The nutrient medium is circulated through the beads and is recycled back to the reservoir. This can be operated in continuous mode by providing the feed and effluent stream to the nutrient. For bioreactors limited by external mass transfer rate, the airlift and fluidized bed reactors may be a better choice, since they provide better hydro dynamical conditions around support particles and therefore improve the external mass transfer limited bioconversion rate (Venkataraman and Ravishankar, 1990).

2.10.5.1 Flask cultures

The immobilization capacities in a 3-day flask incubation of coffee cells were 0.38, 0.32 and 0.26 g immobilized cells/g sponge for core + peripheral, core and peripheral parts respectively. In a bubble column, a high immobilization capacity of 0.42 g immobilized cells/g sponge for coffee cells after 3 days of incubation was obtained using the core + peripheral parts (Liu, 1998).

2.10.6 Immobilized plant cells for the metabolite production

The Phytochemicals of cell culture origin find applications in pharmaceuticals, chemical and food industries. Atleast 50 compounds of economic value have been produced in plant cell culture with the yield comparable to intact plants. The most promising ones are shikonin (for skin ailments) berberine (for intestinal infections), vincristine and vinblastine (anticancer), digoxin (heart ailments), safranal, capsaicin (food additives), pyrethrins (biopesticides), anthocyanine (colour) and haumatin (sweetner) (Ravi shankar *et al*., 1990).

The immobilized plant cells have been used for basic studies on biochemistry and for the production of valuable phytochemicals. They have been applied to studies on biotransformation, biosynthesis and permeability of products to the exterior of the cells (D'Sauza, 1989). A rare anthocyanin in mutated carrot

cells was obtained with high stability of pigments at 2-5 pH for 6 months (Vunsh *et al.,* 1986)

Lindsey and Yeoman (1985) have done extensive work on the production of capsaicin. They reported several hundred-fold increases in capsaicin production in immobilized *Capsicum frutescens* cells. They found that the immobilization process channels the precursor (phenylalanine) to capsaicin (alkaloid) production rather than using the same for cell growth.

A three-fold increase in β- carotenes and lycopene content was obtained in mutant carrot cells treated with N-methyl-nitrosoguanidine. An increase in serpentine and ajamalicine (2% dry weight) was reported by treating Catharanthus cell cultures with gamma rays (Nishi *et al*., 1974).

2.10.7 Enhancing the metabolite production

2.10.7.1 Hormones

Growth regulators play an important role in growth and differentiation of cells and also influence secondary metabolite synthesis.

The production of tobacco alkaloids by callus, suspension and immobilized cells of *Nicotiana spp*. was investigated (Cho, 1992). In undifferentiated callus cultures nicotine production was stimulated by addition of low concentrations of auxin, but suppressed by high levels. Kinetin had a similar effect on nicotine production. This was frequently associated with the differentiation of buds and shoots.

Lower levels of both 2, 4-D and Kinetin favoured diosgenin production in *Costus speciosus* (Jain *et al*., 1984). Tobacco cells have been shown to be sensitive to 2, 4-D for nicotine production. GA is reported to increase berberine production. Presence of auxin 2, 4-D and NAA increase scopoletin and scopolin production in tobacco tissue cultures (Okasaki *et al*., 1982). Ethephon induces a range of secondary metabolites. Hence hormones are important constituents of medium for metabolite synthesis.

MATERIALS AND METHODS

3.MATERIALS AND METHODS

The study entitled, "Biochemical characterization and cell immobilization of *Costus pictus* D. Don with special reference to antidiabetic property" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, from January 2004 to November 2006. The experimental materials and the methodologies of the study are given below.

3.1. MATERIALS

3.1.1 Plant materials

The antidiabetic plants such as *Costus pictus*, ginger, chakkarakolli, adalodakam, neem and tulsi were maintained in an area of 5 cent at CPBMB field. Leaf samples were analyzed for biochemical characterization and callus cultures were used for cell immobilization. Fresh leaf samples were collected from the field for analysis.

3.1.2 Chemical, glasswares and laboratory wares

The chemicals of good quality (AR/GR grade) from Sigma, USA, Merck India Ltd and Sisco Research Laboratories, British Drug House (BDH) were used for the study. The glassware were purchased from Borosil India Ltd. BioRad PAGE Electrophoresis apparatus was used for biochemical characterization of proteins and peroxidase enzyme.

3.1.3 Laboratory equipments

The equipment item available at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and the Biochemistry Laboratory, College of Horticulture were used for the present study. The equipments used are listed in Appendix I

3.2 BIOCHEMICAL CHARACTERIZATION

Qualitative tests, thin layer chromatography and paper chromatography were adopted for the analysis of primary and secondary metabolites. Polyacrylamide gel electrophoresis was carried out for the protein and isozyme analysis.

3.3 Primary Metabolites

3.3.1 Carbohydrates

Procedures given by Sadasivam and Manikam (1992) was followed.
Composition of the reagents is given in Appendix II (a)

3.3.1.1 Molisch's test

Two drops of Molisch's reagent (5% 1-naphthol in alcohol) was added to two ml of leaf extract. Mixed well. The test tube was inclined and 1ml of concentrated sulphuric acid was added to the inside of the test tube.

3.3.1.2 Iodine Test

Few drops of iodine solution were added to 1ml of the leaf extract.

3.3.1.3 Fehling's Test

To 1 ml of Fehling's solution A, 1ml of Fehling's B solution was mixed and a few drops of leaf extract was added and boiled for few minutes.

3.3.1.4 Benedict's test

To 2 ml of Benedict’s reagent, five drops of the leaf extract was added and boiled in a water bath and the solution was cooled.

3.3.1.5 Seliwanoff's Test

To 2 ml of Seliwanoff's reagent two drops of leaf extract was added and heated the mixture to just boiling.

3.3.1.6 Bial's Test

To 5ml of Bial's reagent 2ml of the extract was added and warmed gently. When bubbles rose to the surface the tube was cooled under the tap.

3.3.2 Proteins

Procedures were referred from Sadasivam and Manikam, 1992.

3.3.2.1 Xanthoproteic Reaction

One ml of concentrated HNO_3 was added to five ml of the leaf extract and boiled. After cooling 40% of NaOH was added.

3.3.2.2 Glyoxylic Reaction for Tryptophan

Two ml of the leaf extract was added to 2ml of glacial acetic acid in a test tube. Then 2 ml of concentrated sulphuric acid was added carefully down the sides of the test tubes.

3.3.2.3 Modified Million's Test

One ml of the leaf extract was added to 1ml of 10% mercuric sulphate in 10% sulphuric acid and boiled for half a minute.

3.3.3 Amino acids

3.3.3.1 Thin Layer Chromatography

Materials:

1. Glass plate (20 x 20 cm or 20 x 10cm)
2. Glass tank with lid.
3. Spreader
4. Developing solvents
5. Absorbent silica gel G.
6. Sample
7. Standards
8. Spraying agents
9. TLC sprayer.

3.3.3.1.1Preparation of plates

Procedure:

1. Dry glass plates of 20 x 20 cm were taken.
2. Prepared slurry of the silica gel G in water in the ratio 1:2 (w/v) prepared.
3. The applicator and glass plates were cleaned using acetone dipped in cotton and kept in flat surface.

4.The slurry was stirred thoroughly for 1-2 min and poured into applicator positioned on the head of the glass plate.

5.The slurry was coated over the plates at a thickness of 0.25nm by moving the applicator at a uniform speed from one end to the other.

6.The plates were allowed to dry at room temperature for 15-30 min.

7.Finally the plates were heated in oven at 100 -120 ^{o} C for 1-2 h to remove moisture and to activate the absorbent on the plate.

Readymade plates of Merck India Ltd were also used.

3.3.3.2 TLC for amino acids

The leaf samples of 1gm were taken and macerated using 75% ethanol and filtered and applied in the chromatogram. In order to prepare amino acid standards, 0.05g was dissolved in specific solvents. Tryptophan was dissolved in NaOH and Tyrosine and Phenyl alanine dissolved in HCl. Other amino acids were dissolved in distilled water. Solvent system BAW 4:1:1 was used for the separation of proteins and amino acids. The TLC plates were sprayed using 0.1% ninhydrin in acetone and dried. The Rf values were calculated by,

$$\text{Rf value} = \frac{\text{Distance moved by the sample}}{\text{Distance moved by the solvent front}} \times 100$$

3.4 SECONDARY METABOLITES

3.4.1 Phenols and phenolic acids

3.4.1.1 Sample Preparation

Fresh samples of both tender and mature leaves were collected for phenol analysis. 1g-leaf sample was weighed accurately and extracted in different solvents for the analyzing phenolic compounds.

3.4.1.2 Selection of appropriate solvent for extraction

Leaf extracts in distilled water, ethanol and methanol solvents were used for chromatography. Better separation was obtained when the samples were acid hydrolyzed using HCl and extracted with diethyether.

Acid- hydrolysis was carried out with 2M HCl for 0.5h. 1g of leaf sample was macerated in pestle and mortar using 25 ml of 2M HCl. The resultant solutions were cooled and filtered using filter paper. The filtrate was taken in the separating funnel for ether extraction with diethyl ether. Twenty five ml of diethyl ether was added to the separating funnel having filtrate. On shaking and keeping on the stand for 15 minutes separation into two layers was achieved. The top ether layer was collected and evaporated to dryness over a water bath.

3.4.1.3 Preparation of Standards

The phenol standards of 0.1gm orcinol, resorcinol, pyrogallol, phloroglucinal, catechol, pyrocatechol, hydroquinone and phenolic acids such as vanillic, salicylic acids were taken in 1 ml of 2M HCl.

3.4.1.4 Application of sample on TLC Plates

Both pre-coated silica gel 60 F_{254} of Merck India Ltd and plates prepared in our laboratory (0.25mm thickness) were used. Sample application was done at a distance above 2cm above from lower side of the plate. Each sample was spotted at a distance of 1.5cm distance. 5µl of sample was applied using micropipette and/or capillary tube. Sample was spotted in minimum area by using hair drier.

3.4.1.5 Developing system of TLC Plates

The running solvent systems Acetic acid (HOAC)-Chloroform ($CHCl_3$) 1:9 and Ethyl acetate (EtOAC) – Benzene (C_6H_6) 9:11 were tried. Varying the proportion of different solvents was also tried. For the betterment of band separation the Ethyl acetate - Benzene 1:1 gave good separation (Harborne, 1973).

Procedure:

The solvent system was taken in the glass chromatography chamber to a depth of 0.5cm and allowed to saturate with the solvent by keeping for 30 minutes. The spotted TLC plate was placed in the chamber in a slanting position and closed with a lid having hole on one side. The chromatogram was developed to a distance of 10 cm. Then the plates were removed and air-dried and viewed in UV chamber and or spray reagent was applied to detect the spots.

The phenols and phenolic acids on TLC plates absorb the short UV and were detected in a wavelength 253 nm as dark absorbing spots on plates of silica gel containing fluorescent indicator.

3.4.1.6 Preparation of spray reagent

Folin- Ciocalteu reagent was the best to detect phenols. Folin- Ciocalteu reagent -water 1:1 was sprayed using the sprayer as fine spray from bottom to top in an ascending manner. Phenols present in plates appeared as blue spots.

3.4.2 Flavonoids

3.4.2.1 Preparation of sample

Fresh leaf tissues were extracted 5-10 min with boiling 95% ethanol. Filtered extract was concentrated on a watch glass and dried under exhaust fan. During evaporation chlorophyll and other impurities deposited on the watch glass and the aqueous concentrates was applied to the plate by capillary tube (Harborne, 1973).

3.4.2.2 Chromatographic methods

i) Paper chromatography

Two Dimensional paper chromatography was tried using Whatmann No: 3 paper in BAW, 5% aqueous acetic acid. One-dimensional PC was using BAW solvent system 4:1:5 and forestal (acetic acid conc. HCl –water 30:3:10) (Harborne, 1973).

ii) Thin layer chromatography

Ready made silica gel 60 F_{254} was used. Only the aqueous layer was taken for spotting in case of 95% ethanol extract. Three to five µl of sample was spotted and dried by using hair drier.

Chromatography chamber preparation:

The solvent was freshly prepared, mixed and poured in to the chamber to obtain 0.5cm layer at the bottom. Then the chamber was allowed for saturation of running solvent for 30 min. Rest of the steps were same as mentioned early 3.4.1.5. The plate was air-dried followed by oven drying.

3.4.2.3 Colour properties of flavonoids in visible & UV light

Visible colour	Colour in UV	Indication.
Orange Red Mauve	Dull orange, red or mauve Fluorescent yellow Cerise or Pink	Anthocyanidin 3-glycosides Anthocyanidin 3,5 diglycosides .
Bright yellow	Dark brown or Black Bright yellow or yellow-green	6- hydroxylated flavonols & flavones, some chalcone glycosides Aurones
Very pale yellow	Dark brown	Most flavonol glycosides
None	Dark mauve Faint blue Dark mauve	Most isoflavones flavanonols 5-desoxyisoflavones&7,8 dihydroxy flavonones Flavanones flavanonol 7–glycosides.

3.4.3 Flavonols and Flavones

Flavonols are very widely distributed is plants, both as co-pigments to anthocyanins in petals and also in leaves of higher plants (Harborne, 1973).

3.4.3.1 Sample Preparation

One gram leaf sample was ground in pestle and mortar using 25ml of 2M HCl heated at 100^{o}C for 30 min. Cooled and filtered the sample solution and then extracted twice with ethyl acetate. Ethyl acetate layer was taken to dryness and dissolved in small volume of ethanol for chromatography.

3.4.3.2 Selection of Solvent

Forestal: (acetic acid- HCl- water) 30:3:10

BAW: 4:1:5

Procedure:

1. Whatman No. 3 filter paper was taken and cut into 10 x 10 cm size.
2. Using capillary tubes the ethanol extract was taken and spotted on the filter paper.
3. The paper was dried using hair drier.
4. Then the spotted paper was placed in BAW and Forestal solvent systems.
5. Running was done in ascending manner and the paper was dried is chromatography oven at 110^{o}C for 2 min. Then the paper was viewed under UV light 253nm & 366nm.

3.4.4 Tannins

Tannins occur widely in vascular plants, their occurrence in the angiosperms being particularly associated with woody tissues.

There are two types of tannins, which are distributed unevenly throughout the plant kingdom. They are condensed tannins and hydrolysable tannins.

3.4.4.1 Condensed tannins

Proanthocyanidins were detected in green plant tissues by immersion in boiling 2M HCl for 0.5 h. The production of red colour in the extract of amyl / butyl alcohol showed the presence of proanthocyanidins (Harborne, 1973).

3.4.4.2 Hydrolysable tannins

Fresh leaf sample on acid hydrolysis using 2M HCl was used for extraction with ethyl acetate. Ethyl acetate layer (upper layer) was collected and evaporated to dryness. This sample was used for detection of gallic acid and/or ellagic acid (Harborne,1973).

3.4.4.3 Thin layer chromatography

The dried leaf sample was extracted with 100% methanol and concentrated to a minimum quantity and applied the methanol extract using capillary tube. The samples were spotted at distance of 1cm in a minimum area by blowing hot air from hair drier.

3.4.4.4 Paper chromatography

Fresh leaf was extracted with aqueous acetone (70%) to avoid the hydrolysis of ester linkages and obtain the native tannins. This extract was applied in Whatmann No: 3 filter paper and followed the standard procedure of chromatography.

3.4.4.5 Solvent System

The paper chromatography chamber was saturated with BAW/Forestal Solvent systems prior to running. 3cm width Whatmann No.3 paper strip was spotted with sample as mentioned early and kept the paper straight touching the lower end above the spot. Running was carried out up to ¾th of the paper. Air-drying followed with oven drying of the paper for 15 min was done for further steps (Harborne, 1973).

3.4.4.6 Visual observation of tannins

TLC plates were viewed under UV light 253nm and 366nm.

3.4.5 Terpenoids

3.4.5.1 Sample extraction

Chemically terpenoids are lipid soluble and located in the cytoplasm of plant cell. They are normally extracted from plant tissues with light petroleum ether or chloroform and can be separated by chromatography on silica gel/ alumina using the same solvent system mentioned above (Harborne, 1973).

Fresh leaf was taken in mortar and pestle and extracted with aqueous solvent acidified with 2M HCl. Partition chromatography was adopted to transfer terpenoids from the crude extract. The top layer (ether layer) was collected, evaporated to dryness and dissolved in small quantity of ether for further analysis (Harborne, 1973).

3.4.5.2 Selection of appropriate solvent system

Three solvent systems, Benzene – Chloroform (1:1) Hexane-Chloroform (3:2) and Benzene-ethyl acetate (9:11) also were tried. For $AgNo_3$ impregnated plates, methylenedichloride - ethyl acetate - n-propanol (45:45:4.5:4.5) was used. For paraffin coated plates 70% methanol was used as solvent (Harborne, 1973).

3.4.5.3 Plate preparation

1) Terpene alcohols were best separated on paraffin impregnated plates in 70% methanol. So 5% paraffin in petroleum was prepared and the activated silica gel plates were first immersed in 5% paraffin for 1 min and then allowed to dry before use.
2) To separate terpenes according to the number of double bonds, silver nitrate impregnated silica gel plate was used. TLC on silica gel plates spread as a slurry with 2.5% aqueous $AgNo_3$.

Five µl of sample was spotted along with the markers, essential oils like geranium oil, ginger oil, vetiver oil that contain terpenoids.

3.4.5.4 Preparation of spray reagent

TLC plates were sprayed with (1) 0.2% aqueous $KMNo_4$, (2) 5% antimony chlorides in chloroform (3) Vanillin-H_2SO_4. Vanillin H_2So_4 was prepared freshly by adding 8ml ethanol to 0.5g vanillin and the solution was mixed with 2ml H_2So_4. Spraying was done as mentioned early. The plates were heated at 100-105^oC after spraying until colour developed fully.

3.4.6 Saponins

The running solvent system developed by Golba (2000) to elute saponins from *Gymnema* was adopted. The saponins were eluted into a single condensed spot using chloroform: acetone: methanol (5:1:1.5) as the solvent system. The saponin spot was obtained at an Rf value of 0.5. An initial run in a pure solvent was also included so as to elute the non-saponins to the solvent front without affecting the saponins. Three different solvents, hexane (100 per cent), chloroform (100 per cent) and ethyl acetate (100 per cent) were tried.

3.4.7 Carotenoids

3.4.7.1 Sample Preparation

Fresh leaves of 1 g was ground in cold acetone (2 ml) saturated with $MgCO_3$ and light petroleum ether (b.p 60-80^oC) (4ml). The clear extract was taken in a capillary tube and spotted in activated silica gel plate (Harborne, 1973).

Procedure:

Clean and dry chromatography chamber was saturated with solvent system of benzene-petroleum-ethanol-water (10:10:2:1) and made it ready for running.

The plates were placed inside the chamber for running following the standard procedure mentioned earlier. After ¾th running, the plates were taken for air-drying.

The spots were visible in normal light. β-Carotene moves first followed by xanthophylls, chlorophyll a and b.

3.4.8 Plant acids

Plant acids were easily recognized by their taste in solution and by the low pH of crude aqueous plant extracts. Though the *Costus pictus* leaves are sour in taste, they were subjected to plant acid analysis by TLC technique.

3.4.8.1 Sample extraction

Initially one gram sample was collected from each plant and since this was not sufficient for analysis. Twenty gram was taken for further analysis.

Twenty gram fresh leaf sample was taken for extraction. Clear extract was spotted directly and another lot of 20 g was extracted with hot 75% ethanol (25 - 30ml) and clear filtrate was spotted for chromatography. Ethanol extract was acidified with 2M HCl and chromatography was done as mentioned earlier. The ethanol extract with 2M HCl gave good results.

Finally 20g leaf was taken and macerated with 25 ml of 75% hot ethanol. Clear extract was acidified with 2M HCl using 1ml for the hydrolysis and release of organic acids (Harborne, 1973).

3.4.8.2 Standard preparation

Tartaric acid and citric acid of plant origin and tricarboxylic acid and oxalic acid of non-plant origin were taken in 75% ethanol as standard for chromatography.

3.4.8.3 Solvent system

Benzene –methanol –acetic acid (79:14:7)

3.4.8.4 Preparation of spray reagent

100 ml of 1:4 mixture of water methanol and 0.3g of Bromocresol green were mixed together and added 8 drops of 30% NaOH for spray reagent.

3.4.8.5 Detection of spots

After development of the TLC plates, air-drying was done for removing the solvent and sprayed with 0.3% bromocresol green solution. The carboxylic acids stain as yellow – green on blue background was obtained.

3.4.9 Alkaloids

3.4.9.1 Mayer's test

3.4.9.1.1 Preparation of Mayer's reagent

Solution A was prepared by dissolving 0.36g of mercuric chloride in 60ml distilled water. By dissolving 5g potassium iodide in 10ml distilled water, solution

B was prepared. Both these solutions were mixed thoroughly and made up to 100ml to make Mayer's reagent solution.

3.4.9.1.2 Procedure

Dried sample of 0.5g was taken and extracted with 5ml chloroform. This extract was filtered through whatmann no: 1 filter paper to get clear solution. From this solution, 2 ml aliquot was taken and added, 5 ml mayer's reagent was added and it was shaken gently.

3.4.9.2 Wagner's test

3.4.9.2.1 Preparation of Wagner's reagent

Wagner's reagent was prepared by dissolving 2g of potassium iodide in to 5ml distilled water. Then this solution was added to 100ml 0.1N iodine solution.

3.4.9.2.2 Procedure

The procedure for this test was as same as the Mayer's test.

3.4.9.3 Dragendorff test

3.4.9.3.1 Preparation of Dragendorff reagent

Solution A and solution B was prepared by adding 8g bismuth sub nitrate in 20 ml conc. nitric acid and by dissolving 27.2g potassium iodide in 50 ml of distilled water respectively. These two solutions were mixed well and allowed to stand for sometime. Then the precipitate of potassium nitrate was obtained. From that, the supernatant was taken and made up to 100 ml.

3.4.9.3.2 Procedure

The steps for this experiment were also as same as above tests.

3.5 ELECTROPHORESIS STUDIES

3.5.1 Protein extraction

3.5.1.1 Phosphate buffer

A: 0.2M solution of monobasic sodium phosphate (27.8g in 1000 ml)

B: 0.2M solution of dibasic sodium phosphate (53.65g in 1000 ml)

39ml of A solution and 61ml of B solution was taken and pH adjusted to 7.0 and diluted to a total of 200 ml.

3.5.1.2 Tris buffer

A: 0.2M solution of Tris (hydroxymethyl) amino methane (2.42g in 100ml)

B: 0.2 M HCl 100ml

50 ml of A and 44.2 ml of B was diluted to a total volume of 200 ml.

3.5.1.3 Tris buffer + ingredients

The Tris extraction buffer was prepared as explained above and the ingredients like citric acid 2.5µM, ascorbic acid 6µM, cystein-HCl 6µM and sucrose 0.5M was added and the pH was made up to 7.2 and the volume was made upto 200ml.

The fresh leaf samples of 0.5g were taken, macerated using 1ml of above said buffer in a pre-chilled pestle and mortar in ice-cold condition. The extract was transferred into the eppendrof tube and centrifuged at 12,000 rpm at 4^0C for 15 min. The supernatant was used for the protein or enzyme electrophoresis.

3.5.2 Sodium dodecyl sulphate - Poly acryl amide gel electrophoresis

Electrophoresis is the migration of charged molecules in solution in response to an electric field.

3.5.2.1 Polyacrylamide

It is easy to handle and to make at higher concentrations, is used to separate most proteins and small oligonucleotides that require a small gel pore size for retardation. 7.5%, 10%, 12.5% and 15% gels were tried for the separation of proteins (Andrews, 1986).

3.5.2.2 Buffer system

Chloride is a good choice as it has a high mobility over a wide range and in most cases has no damaging effect on sample constituents such as proteins.

3.5.2.3 pH

. Low ionic strengths permit high rates of migration, while high ionic strength gives slower rates but in practice sharper zones of separation than low ionic strength buffers (Maurer, 1971)

3.5.2.4 Use of SDS

The figure of about 1.4 g SDS per gram of protein is often quoted as a typical value (Andrews, 1986). This means that the number of SDS molecules bound is of the order of half the number of amino acid residues in the polypeptide chain.

3.5.2.5 Current and voltage

The current was turned on to 10-15mA until the samples travel through the stacking gel. Then the run was continued at 30mA until the bromophenol blue reaches the bottom of the gel (about 3h). The gel also run at high current (60-70mA) for short period (1hr) with proper cooling. Heating causes variations in both the current and voltage. In order to minimize these fluctuations it is usual to carry out electrophoresis with power supplies, which can be regulated to provide an output at constant voltage or current (Andrews, 1986).

Composition of the reagents is given in Appendix II (b)

Procedure

1. The glass plates were thoroughly cleaned and dried and assembled properly. The assembly held together with clips in upright position.

2. Sufficient volume of separating gel mixture was prepared (15ml for a chamber prepared by mixing the following:

	For 15%gel	for 10%gel
Stock acrylamide soltion	10ml	6.65ml
Tris-HCl	4ml	4ml
Water	5.7ml	9.05ml

Ammonium per sulphate solution	0.1ml	0.1ml
10% SDS	0.2ml	0.2ml
TEMED	10µl	10µl

3. The gel solution was mixed gently and carefully, poured in the chamber between the glass plates. Distilled water was layered on the top of the gel and allowed to set for 30min.

4. Staking gel (4%) was prepared by mixing the following solutions (total volume 10ml)

Stock acrylamide solution	0.675ml
Tris-HCl (pH 6.8)	0.5ml
Water	3.75ml
Ammonium persulphate solution (5%)	25µl
10%SDS	0.1ml
TEMED	10µl

Water was removed from the top of the gel and washed with a little stacking gel solution. Stacking gel solution was poured and the comb was placed in the stacking gel solution and allowed to set for 60min.

5. After the stacking gel has polymerized, the comb was removed and without distorting the shapes of the well. The gel in between the glass plates was carefully installed in electrophoresis apparatus after removing the clips. The apparatus was filled with tank buffer and avoided the formation of air bubbles at the bottom of the gel. Cathode was connected and the current was turned on. The tank buffer and the plates were kept cool by placing the apparatus at 15^0C.

6. The samples were prepared as mentioned in the protein extraction procedure. The centrifuged supernatant (45µl) was taken and mixed with sample buffer (5µl) and the sample solution was heated in boiling water for 2-3 min to ensure complete interaction between protein and SDS.

7. The sample solutions were cooled and 45µl was taken in a pipette and carefully injected into a sample well.

8. The current was turned on to 10-15mA until the samples travel through the stacking gel. Then the run was continued at 30mA until the bromophenol blue reaches the bottom of the gel (about 3 h). The gel also runs at high current (60-70mA) for short period (1 h) and kept cool by placing the apparatus at 15^0C.

9. After the run is complete, the gel was carefully removed from plates and immersed in Coomassie brilliant blue R 250 solution for atleast 3h or overnight with uniform shaking and Silver staining were also tried.

10. The gel was transferred to a suitable container with atleast 200-300 ml destaining solution and shaken gently and continuously. Dye that was not bound to protein was thus removed. The destainer was changed frequently, particularly during initial periods, until the background of the gel was colourless. The protein fractioned into band were seen coloured blue. As the proteins of minute quantities were stained faintly, destaining process was stopped at appropriate stage to visualize as many bands as possible.

11. The gel was photographed and documented in gel doc machine.

3.5.2.6 Silver staining

Staining was done either with coomassive brilliant blue 250 or amido black10B dye helps in visualing the fractioned polypeptides on gels. The dye can be detected a band containing as little as 0.1µg of polypeptide. On many occasions, the available protein for electrophoresis is so small or some proteins occur in minute amounts the detection becomes extremely difficult with these dyes. Under such circumstances a higher sensitive detection system is required. Silver staining is a very useful method in this regard with about 100 fold greater sensitivity over dye staining (Sadasivam and Manikam, 1992).

Materials

a) Washing solution

1ml of formaldehyde (analytical grade, 37%), 40ml of methanol and 60ml of distilled water were mixed and used.

b) Sodium thiosulfate: Dissolved 200mg in a litre of water

c) Silver nitrate solution (0.1%)

d) Developer: Sodium carbonate 3g(w/v) dissolved in 80ml water. 1ml of the above sodium thiosulfate solution was added to 1ml formaldehyde and finally volume made up to 100ml.

e) Stopper: 5%citric acid or 5% acetic acid solution.

Procedure

1. After electrophoresis, the gel was transferred to a clean container and washed in the washing solution with slow shaking for 10 min.
2. The wash solution was discarded and the gel rinsed with plenty of water for 2 min.
3. The gel was soaked in sodium thiosulfate solution for1-2 min.
4. The gel was washed with water twice, each time 1-2 min. The wash water was drained.
5. The gel was soaked in silver nitrate solution for 10 min with gentle shaking.
6. The gel was washed in water as in step 4.
7. Developer was poured to the plastic container and shaked slowly, gently. The proteins reduce silver nitrate to silver and the yellow to dark brown colour band was appeared.
8. When sufficient intensity of bands developed adding either citric acid or acetic acid solution stopped the reaction.
9. The protein-banding pattern was recorded by photography.

3.5.3 Isozyme analysis (Peroxidase enzyme)

The isozyme markers have also been extensively used in identifying provenance, characterizing phenotypes and developing a biochemical marker for oil-bearing capacity (Anagadi *et al*., 1998).

Isozymes (or isoenzymes) are powerful tool for gene variability within and between populations of plants and animals, yet nowadays-new molecular technique based on DNA are used.

Composition of the reagents for peroxidase enzyme is given in Appendix II (c)

Procedure

1. The glass plates were thoroughly cleaned and dried and assembled properly. The assembly was hold together with clips in upright position.

2. A sufficient volume of separating gel mixture was prepared (15ml for a chamber prepared by mixing the following

	For 15%gel	for 10%gel
Stock acrylamide solution	10ml	6.65ml
Tris-HCl	4ml	4ml
Water	5.7ml	9.05ml
Ammonium per sulphate solution	0.1ml	0.1ml
TEMED	10µl	10µl

3. The gel solution was mixed gently and carefully, poured in the chamber between the glass plates. Distilled water was layered on the top of the gel and allowed to set for 30 min.

4. Staking gel was (4%) prepared by mixing the following solutions (total volume 10ml)

Stock acrylamide solution	0.675ml
Tris-HCl (pH6.8)	0.5ml
Water	3.75ml
Ammonium persulphate solution (5%)	25µl
TEMED	10µl

The water was removed from the top of the gel and washed with a little stacking gel solution. Stacking gel solution was poured and the comb was placed in the stacking gel solution and allowed to set for 60 min.

5. After the stacking gel has polymerized, the comb was removed without distorting the shapes of the well. The gel, after removing the clips, was carefully was installed in electrophoresis apparatus. The apparatus was filled with electrode buffer; the formation of air bubbles at the bottom of the gel was avoided. Cathode was connected and the current was turned on. The electrode buffer and the plates were kept cool by placing the apparatus inside the refrigerator.

6. The samples were prepared as mentioned in the protein extraction procedure. The centrifuged supernatant (45µl) was taken and mixed with sample buffer (5µl) and carefully injected into a sample well.

7. The current was turned on to 10-15mA until the samples travel through the stacking gel. The stacking gel helps concentration of samples. Then the run was continued at 30mA until the bromophenol blue reaches the bottom of the gel (about 3h). However, the gel may be run at high current (60-70mA) for short period (1h) with proper cooling.

8. After the completion of run, the gel was carefully removed from between plates and immersed in staining solution for atleast 3h or overnight with uniform shaking.

9. The enzymes absorb the benzidine and bright blue colour bands appeared. The bands were destained by immersing the gels in large volume of 0.67% sodium hydroxide or 7% acetic acid solution for 10 min.

10. The gel was photographed and documented in gel doc machine.

3.6 CELL IMMOBILIZATION

3.6.1 Requirements for immobilization

3.6.1.1 Preparation of tissue culture medium

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of plant tissue culture media. Stock solutions (Appendix II (d)) of major and minor nutrients were prepared and stored in pre-cleaned glass bottles in refrigerated conditions. Stock 3 was stored in amber coloured bottle.A clean steel vessel, rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel. For preparing media of full strength 20 ml was pipetted from 50X stocks and 10ml from 100X stocks. A small volume of distilled water was added to it and later on, sucrose 30g and inositol 0.1g were added and dissolved in it. The pH of the medium was adjusted to 5.7 using 0.1N NaOH. Adding distilled water made up the desired volume to one litre.

For solid medium, agar was added at 0.75 per cent (w/v) concentration, after adjusting the pH. The melted agar was poured into culture vessels and was plugged with absorbent cotton. For solid media, test tubes (15 cm x 2.5 cm) were used whereas for liquid media conical flasks or Erlenmeyer flasks (100, 250 and 500ml) were used as culture vessels. Fifteen ml medium was poured in each test tube, 30 ml medium in 100 ml conical flask, 120 ml in 250 ml conical flask and 250 ml in 500 ml conical flask. Vessels containing media were sterilized in an autoclave at 121ºC in 15psi for 20min. The medium was allowed to cool to room temperature and stored in culture room until used.

3.6.1.2 Antibiotics

The stock solutions of antibiotics were prepared fresh under sterile conditions. Aliquots were taken from them and were added to the sterilized media. The solid media was first melted, cooled to 40 ºC. Then 250µl of antibiotic

cefotaxime was added in 250 ml of melted media in conical flask and poured in test tubes in laminar airflow cabinet.

3.6.1.3 Culture room

The cultures were incubated at 26 ± 2 ºC in air-conditioned culture room with 16 h light photoperiod (1000 lux) from fluorescent tubes for plant regeneration. Dark condition was provided by black cotton cloth fixed in culture racks.

3.6.1.4 Collection and preparation of explants

The plants for taking explants were maintained in CPBMB, COH, Vellanikkara. The explants viz., leaf for callus induction and bud for plant regeneration was taken. They were washed in tap water to remove dust and kept in detergent solution for 10 minutes followed by washing in distilled water to remove detergent solution. This was followed by treatment with Bavistin 0.1% solution for 30 minutes. Then the explants washed thoroughly and treated with 0.1% streptocyclin 1h. The sample was then thoroughly washed with distilled water and dried on blotting paper. The explants were then wiped with 50% ethanol.

Surface sterilization of explants was carried out under aseptic condition in laminar airflow cabinet. The explants were sterilized with mercuric chloride ($HgCl_2$) 0.1% followed by washing thoroughly in sterile water. Finally, the explants were dried carefully transferred them on filter paper pieces in sterile Petri dish. The leaf pieces were trimmed on the four sides and reduced to 0.5 cm^2.

3.6.2 Plant regeneration

The *Costus pictus* rhizome buds were kept first in medium ½ MS + 3ppm BA to obtain *in vitro* leaves for the callus induction. Then transferred to *Costus speciosus* multiplication medium, ½ MS+ 8.87µM BA +9.29 µMKN +5.37 µM NAA (½ MS+ 2ppmBA+ 2ppmKN+ 1ppmNAA).

3.6.3 Callus induction

Leaf bits of *Costus pictus* were inoculated in ½ MS + 1ppm 2,4-D+ 0.5ppm BA, ½ MS+ 1ppm BA+ 3ppm NAA medium callus was induced. The callus was sub cultured in ½ MS + 0.5ppm 2,4-D and ½ MS+ 0.25ppm 2,4-D supplemented with 3% sucrose at 4 weeks interval.

3.6.4 Suspension culture

The callus was taken out from the media in laminar flow chamber and transferred to liquid media ½ MS + 0.5ppm 2,4-D. The mouth of bottle was flamed well to prevent the microorganism entry. Suspension cultures were obtained by agitating the callus cells in ½ MS liquid medium + 0.5ppm 2,4-D at 90 rpm on rotary shaker.

3.6.5 Immobilized cell culture

Materials/ Chemicals required

Sodium alginate	3%
Calcium chloride	1.35g in 150ml water
Capillary tubes	3 mm diameter
Erlenmeyer flask	150 ml capacity

In laminar air flow chamber, freely suspended cells were mixed with 3% sodium alginate and extruded into Ca Cl_2 (0.08M) by using micropipette tip having diameter 3mm. The beads thus formed were allowed for 30min in $CaCl_2$ to stabilize the beads. The beads were later washed thrice with sterile distilled water before transferring to the nutrient medium. The beads were grown in MS liquid medium kept in rotary shaker at 30^{o}C under light intensity of 2000 lux and harvested periodically.

3.6.6 Estimation of secondary metabolites

The secondary metabolites produced inside the beads will be leached out to the medium. The secondary metabolites were recovered by extracting the medium and cells into ethyl acetate using separating funnel. The medium

containing immobilized cells and MS liquid medium (control) were extracted using ethyl acetate. Top layer was collected and secondary metabolite analysis was done by thin layer chromatography. The plates were developed using Ethyl acetate (EtOAC) – Benzene (C_6H_6) 1:1 solvent system for phenols, Benzene – Ethyl acetate 19:1 for terpenoids and Benzene- Methanol- Acetic acid (76:14:7) for plant acids. The developed chromatographic plates were dried in hot air. The dried plates were sprayed with respective spray reagents. ½ MS liquid media was also extracted using ethyl acetate and taken as a control.

Results

4. RESULTS

The study on "Biochemical characterization and cell immobilization of Costus pictus D. Don with special reference to antidiabetic property" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 2004- 2006. The results of the experiments are described in this chapter.

4.1 BIOCHEMICAL CHARACTERIZATION

4.1.1 Primary Metabolites

4.1.1.1 Carbohydrates

The results obtained for carbohydrate qualitative tests are presented in the Table 1.

a) Molisch's test

Appearance of a violet ring at the junction was observed in the mixture of reagent and extracts of the entire plant sample. Slight variation in the intensity of colour was observed in the ring formed. Costus pictus, ginger, gymnema, adhatoda, neem and tulsi showed almost same intense violet ring and neem had a dark violet ring with H_2SO_4.

b) Iodine test

The appearance of deep blue colour with almost same intensity was obtained in all leaf samples except in neem and tulsi in which light blue colour was recorded.

c) Fehling's test

Formation of yellow or brownish - red precipitate was observed in all leaf samples. Costus pictus, ginger, gymnema and tulsi gave medium red, adhatoda light red and neem deep red precipitate indicated the presence of sugars.

d) Benedict's test

Formation of red, yellow or green colour precipitate was observed in the extracts of all the plant samples with varying colour intensities. In Costus pictus

Table1. The colour intensities observed in qualitative tests for carbohydrates in selected plants

Selected plants	Molisch's test	Iodine test	Fehling's test	Benedict's test	Seliwanoff's test
Costus	Violet ring	Blue	Medium red	Red	Light red
Ginger	Violet ring	Blue	Medium red	Red	Medium red
Gymnema	Violet ring	Blue	Medium red	Red	Light red
Adhatoda	Violet ring	Blue	Light red	Green	Medium red
Neem	Dark violet ring	Light blue	Deep red	Brick red	Dark red
Tulsi	Violet ring	Light blue	Medium red	Red	Dark red

Table 2. The colour intensities observed in xanthoproteic reaction for proteins and amino acids in selected plants with different reagents

Selected plants	HNO_3	NaOH
Costus	Light yellow	Light orange
Ginger	Light yellow	Light orange
Gymnema	Dark yellow	Dark orange
Adhatoda	Dark yellow	Medium orange
Neem	Dark yellow	Dark orange
Tulsi	Dark yellow	Dark orange

ginger, gymnema and tulsi gave red precipitate. Adhatoda showed green colour. Brick red precipitate was observed in neem, indicated the presence of sugars.

e) Seliwanoff's test

Light red colour was observed in Costus pictus and gymnema. Ginger and adhatoda gave medium red colour. Neem and tulsi gave dark red colour.

4.1.1.2 Proteins and amino acids

a) Xanthoproteic reaction

On adding acid, yellow colour was observed in the leaf samples. When NaOH was added deep orange colour was observed. All the leaf samples showed the yellow and orange colour change. Variation in the colour intensities are mentioned in Table 2.

b) Glyoxylic reaction for tryptophan

Violet ring was formed at the junction of all sample extract- reagent mixture with varying colour intensities. The colour intensities observed are mentioned in Table 3.

c) Modified Million's test

Costus pictus sample gave yellow precipitate with $HgSO_4$ and pinkish red colour with $NaNO_3$ where as ginger gave yellow precipitate$HgSO_4$ and pinkish red with $NaNO_3$. Gymnema gave yellow precipitate with $HgSO_4$ and dark red with $NaNO_3$. Adhatoda gave yellow precipitate with $HgSO_4$ and dark red with $NaNO_3$. Neem gave yellow precipitate with $HgSO_4$ and medium red with $NaNO_3$. Tulsi gave yellow precipitate with $HgSO_4$ and medium red with $NaNO_3$. The colour intensities observed in selected plant samples with different reagents are presented in the Table 4.

4.1.1.3 Amino acids

The TLC of twenty amino acids as 'reference' was carried out. Details are given in Table 5. Chromatography of protein and amino acids of the samples were

Table 3. The colour intensities observed in glyoxylic reaction for proteins and amino acids in selected plants

Costus	*Light violet ring*
Ginger	*Light violet ring*
Gymnema	*Light violet ring*
Adhatoda	*Light violet ring*
Neem	*Prominent dark violet ring*
Tulsi	*Light violet ring*

Table 4. The colour intensities observed in modified millions test for proteins and amino acids in selected plants with different reagents

Selected plants	$HgSO_4$	$NaNO_3$
Costus	*Yellow precipitate*	*Pinkish red*
Ginger	*Yellow precipitate*	*Pinkish red*
Gymnema	*Yellow precipitate*	*Dark red*
Adhatoda	*Yellow precipitate*	*Dark red*
Neem	*Yellow precipitate*	*Medium red*
Tulsi	*Yellow precipitate*	*Medium red*

Table 5. Rf values (x100)and colour of spots developed on TLC for amino acid standards

Sl.No	Amino acids	Rf values	Colour of the spot
1	Glycine	22.3	Violet pink
2	Alanine	25.8	Pink
3	Serine	21.1	Violet pink
4	Cystein	14.7	Pink
5	Threonine	25.8	Pinkish violet
6	Valine	47.0	Pink
7	Leucine	62.9	Pink
8	Isoleucine	61.1	Pink
9	Metheonine	55.2	Pink
10	Aspartic acid	23.5	Violet
11	Asparagine	22.3	Brown
12	Glutamic acid	28.2	Pinkish violet
13	Glutamine	24.7	Pinkish violet
14	Arginine	17.6	Violet
15	Lysine	20.0	Violet
16	Proline	28.2	Yellow
17	Phenyl alanine	69.4	Greyish violet
18	Tyrosine	57.6	Greyish violet
19	Tryptophan	52.9	Greyish violet
20	Histidine	18.8	Greyish violet

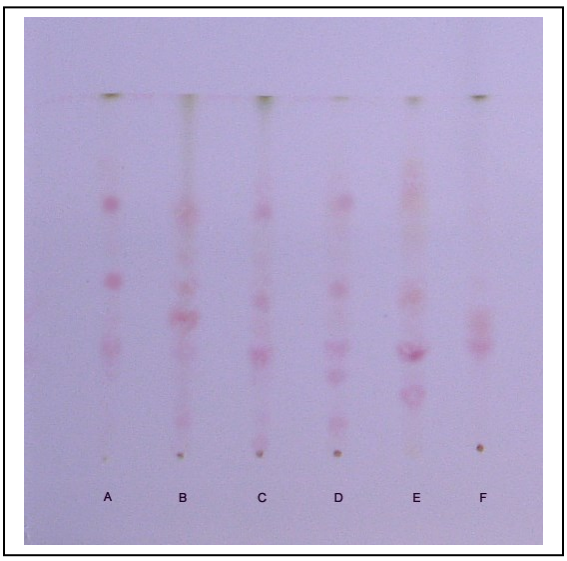
Table 6. Rf values(x100) and colour of the amino acid spots developed on the TLC plate for selected plants

Costus		Ginger		Gymnema		Adhatoda		Neem		Tulsi	
Rf	Clr	Rf	Clr	Rf	Clr	Rf	Clr	Rf	Clr	Rf	Clr
								81.2	pink		
72.5	pink										
		68.7	pink	68.7	pink	68.7	pink	68.7	pink		
								62.5	pink		
50	pink									50	pink
		47.5	pink	47.5	pink	47.5	pink				
								43.7	pink		
		37.5	pink							37.5	pink
31.2	pink			31.2	pink	31.2	pink			31.2	pink
						6.25	pink				

Table 7. Efficiency of different solvents for phenol extraction

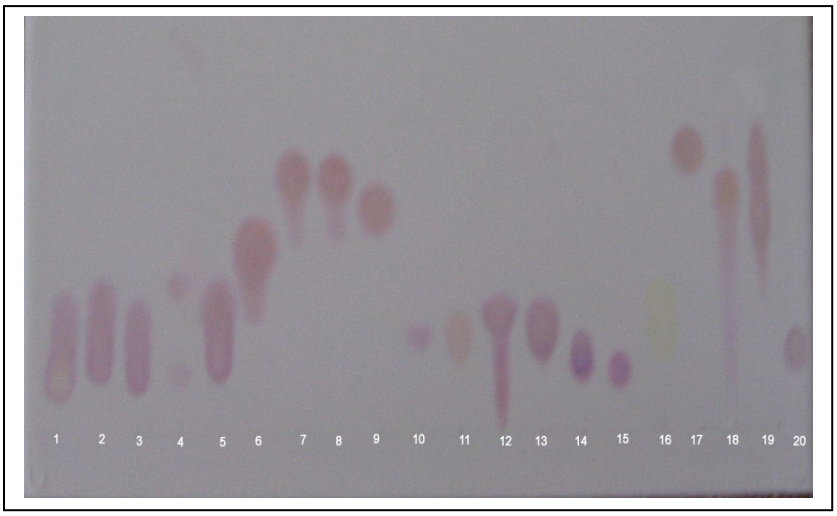
Sl.No	*Extracting solvent*	*Efficiency*
1	*Water*	*Poor*
2	*Ethanol*	*Average*
3	*Methanol*	*Average*
4	*Sample treated with acid- ether*	*Good*

a) Selected plants



A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

b) Standards



1) Glycine
2) Alanine
3) Serine
4) Cystein
5) Threonine
6) Valine
7) Leucine
8) Isoleucine
9) Metheonine
10) Aspartic acid
11) Asparagine
12) Glutamic acid
13) Glutamine
14) Arginine
15) Lysine
16) Proline
17) Phenyl alanine
18) Tyrosine
19) Tryptophan
20) Histidine

Plate 1. Amino acid spots of selected plants detected through ninhydrin spray on TLC

also carried out separately. In the chromatogram, the protein and amino acid spots appeared as pink. α- amino acids produced pink colour; proline, hydroxy proline and amines produced yellow colour and free amide group produce brown colour with ninhydrin. Butanol-acetic acid –water (4:1:5) was used as a solvent to separate the amino acids. The 100Rf values of the standard were between 14.7 and 69.4 for standards. The Rf value of the samples were different from the standard values, indicated the presence of varying quality of the different plant amino acids in the selected samples. Three to four pink spots with different Rf values were observed for each sample. The 100Rf values ranged between 6.25 to 81.2 (Table 6 and Plate1).

4.1.2 SECONDARY METABOLITES

4.1.2.1 Phenolics

4.1.2.1.1 Sample extraction

The extraction of sample was carried out using water, ethanol and methanol. Efficiency of different solvents for phenol extraction is mentioned in Table 7. All the solvents expressed streaking in the TLC plate. So, acid hydrolysis was adopted for extraction. So acid hydrolysis of leaf tissues was carried out, followed by ether extraction gave good separation of phenol.

4.1.2.1.2 Selection medium (immovable)

Paper chromatography with Whatmann No.1, Whatmann No.3, TLC plates of silica gel G and TLC aluminium sheets of silica gel 60 F_{254} were tried. Among the above media silica gel G and TLC aluminium sheets of silica gel 60 F_{254}. Whatmann No.1 was not giving clear bands where as Whatmann No.3 showed better performance in PC. At the same time aluminium sheets of silica gel 60 F_{254} plates performed well in the spot formation.

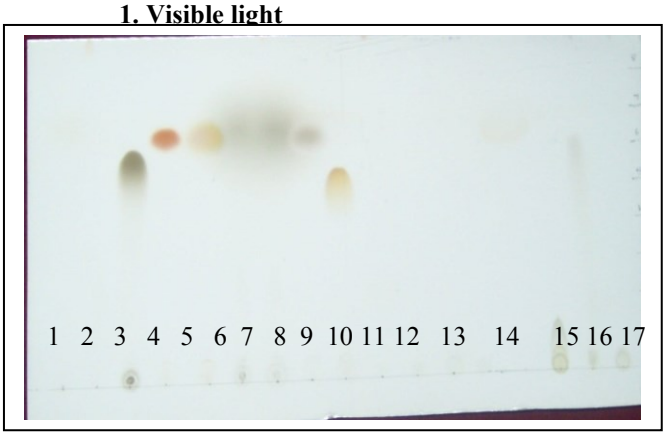
4.1.2.1.3 Selection of running solvent

For TLC acetic acid: chloroform of varying proportions was tried. The spot development was not good in all the proportions of acetic acid and

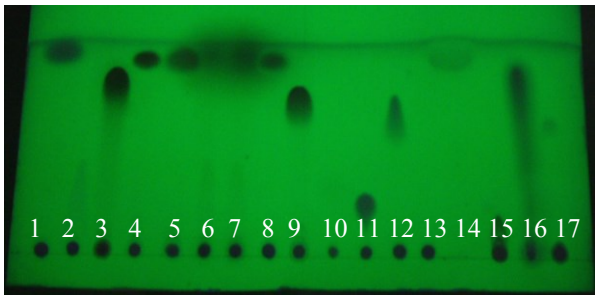
Table 8. Rf values(x100) and colour of the standard phenolic compounds developed on the TLC plate observed under 253 & 366 nm

		Colour (visible light)	253nm	366nm
A	**Simple phenols**			
1	Orcinol	Invisible	87	
2	Resorcinol	Invisible	90	
3	Catechol	Dark brown	92	
4	Pyrocatechol	Red	92	
5	Hydroquinone	Dark pink	86	
6	Pyrogallol	Brown	92	
B	**Phenolic acids**			
7	Vanillic acid	Light brown	91.2	F.G
8	Salicylic acid		41.2	
C	**Phenyl propanoids**			
9	3,4 Dimethyl phenol	Invisible	92	
10	Tannin	Dark brown	17	
D	**Quinones**			
11	8-Hydroxy quinoline	Invisible	84	Fluorescent yellowish green
12	Ferron	Invisible	7.0	Fluorescent Green
E	**Other phenols**			
13	Cinnamic acid	Invisible	68.7	
14	Caffic acid	Invisible	25	
15	Guaicol	Invisible	93.7	
16	Phenol	Invisible	100	

1. Visible light



2. UV light 253 nm

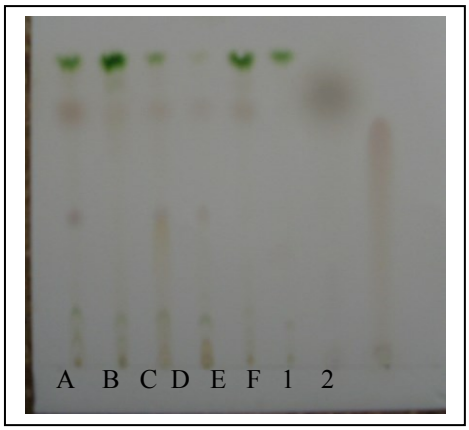


3. UV light 366 nm

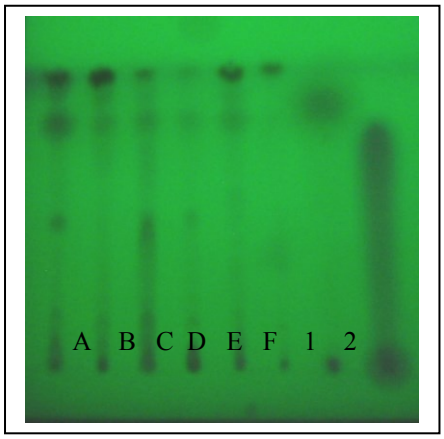
7) Vanillic acid
8) Salicylic acid
9) Pyrogallol
10) Orcinol
11) Resorcinol
12) Catechol
13) Pyrocatechol
14) Hydroquinone
15) Phloroglucinol
16) Phenol
11) Caffic acid
1) Cinnamic acid
2) 3,4 Dimethyl phenol
3) Guaicol
4) Tannin
5) 8-Hydroxy quinoline
6) Ferron

Plate 2a. Phenol spots of standards observed on TLC

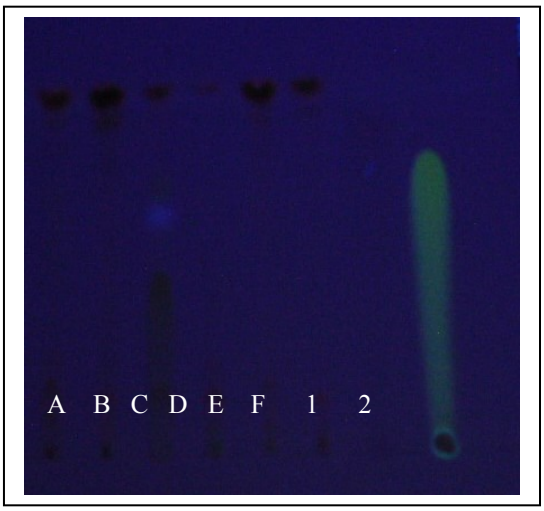
1. Visible light



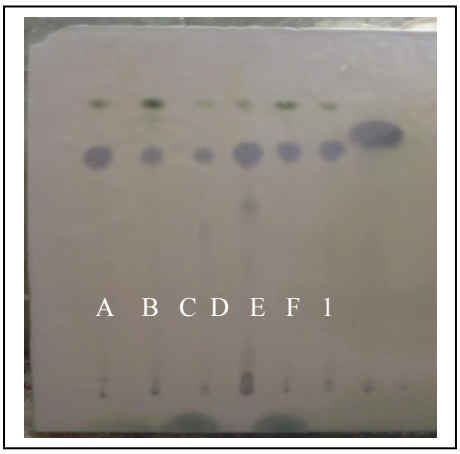
2. UV light 253 nm



3. UV light 366 nm



4. Folin-Ciocalteu spray reagent



A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

1)Pyrocatechol, 2) 8-hydroxy quinoline

Plate 2b. Phenol spots of selected plants observed on TLC

Table 9. Rf values(x100) of phenolic compounds developed on TLC plate for selected plant samples observed under 253 nm

Costus	Ginger	Gymnema	Adhatoda	Neem	Tulsi
83.7	83.7	83.7	83.7	83.7	83.7
				64.2	
57.1		57.1	57.1		
		53.5			
21.4		21.4			
14.2	14.2	14.2	14.2		14.2
	7.1		7.1	7.1	

Table 10. Performance of solvent systems for flavonoids

Sl.No	Type of chromatography		Solvent system	Resolution
1.	PC	Two dimensional PC	BAW(4:1:5), 5% aqueous acetic acid	Poor
		One dimensional PC	BAW(4:1:5), Forestal (acetic acid- HCl- water) (30:3:10)	Medium
2.	TLC	One dimensional TLC	BAW (4:1:5)	Good

Table11. Rf values(x100) of flavonoids developed on TLC plate for selected plant samples observed under 253 nm

Costus	Ginger	Gymnema	Adhatoda	Neem	Tulsi
				88.2	88.2
	82.3				
	76.4		76.4		
				71.5	
70.0	70.0				
			64.7	64.7	64.7
	58.8	58.8			58.8
		47.0	47.0		
					35.2
			29.4		
	23.5	23.5			23.5

chloroform (1:9) system was tried. So, a different solvent system of ethyl acetate: benzene (9:11) was tried. Good spots were observed in 1:1 of ethyl acetate: benzene.

4.1.2.1.4 Identification of phenolic compounds

The samples were spotted in silica gel plates containing fluorescent indicator. The plates were viewed under UV viewer at 253 nm and 366 nm. The phenols appeared as dark absorbing spots at 253 nm and fluorescent spots at 366 nm. The Rf values of synthetic phenolic compounds were also observed under 253 & 366 nm are mentioned in the Table 8 and depicted in Plate 2a. The Rf values of the synthetic compounds are different from the actual leaf samples (Table 9 and Plate 2b), indicated the presence of different phenol compounds in the samples.

In ginger, fluorescent violet spot was observed with an 100Rf value of 14.2 at 366nm where as neem showed 7.1 at 366nm, indicated the difference in the nature and properties of the phenolic compounds in the natural secondary products (Plate 2b).

Phenolic compounds were identified as blue spots when the TLC plates were sprayed with folin- ciocalteu reagent in all samples. Fuming with ammonia vapour intensified the spots. The phenol spot recorded at 100Rf value of 83 with folin-ciocalteu reagent was recorded in all the plant samples (Plate 2b). Gymnema expressed maximum phenolic compounds (5 nos) where as tulsi showed the least (2 nos). Costus pictus also showed the presence of 4 phenolic compounds.

4.1.2.2 Flavonoids

4.1.2.2.1 Sample extraction

The plant samples were extracted using ethanol.

4.1.2.2.2 Selection of method and running solvent

Table12. Rf values(x100) and colour of the flavonoids developed on the TLC plate for selected plant samples observed under 366 nm

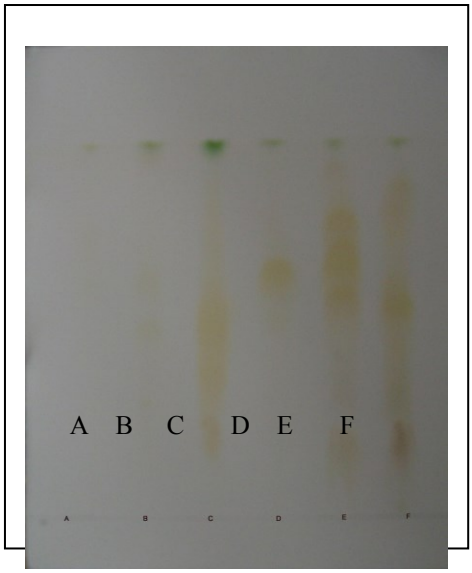
Costus	Ginger	Gymnema	Adhatoda	Neem	Tulsi
100(F.R)	100(F.R)	100(F.R)	100(F.R)	100(F.R)	100(F.R)
90(F.Y)					
			76.4(F.Y)		
	35.2(F.V)	35.2(F.V)			
		23.5(F.G)			
				17.6(F.G)	

F.R- fluorescent red; F.Y- florescent yellow; F.V- fluorescent violet; F.G- florescent green.

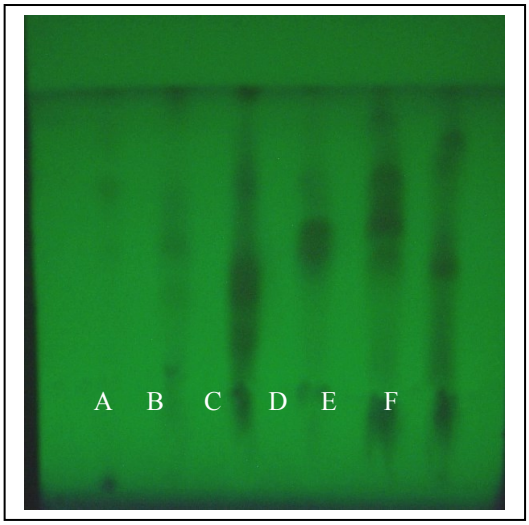
Table 13. Rf values(x100) of flavones developed on the TLC plate for selected plant samples observed under 253 nm

Costus	Ginger	Gymnema	Adhatoda	Neem	Tulsi
	91.6				
		58.8	58.8	58.8	58.8
25.0					

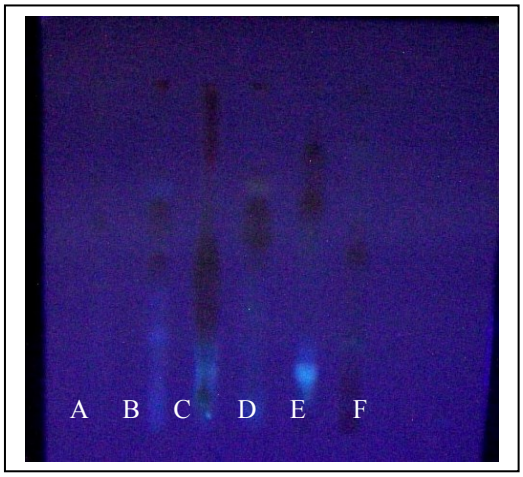
1. Visible light



2. UV light 253 nm



3. UV light 366 nm

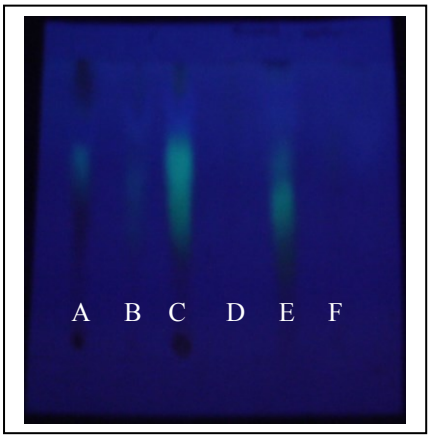


A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

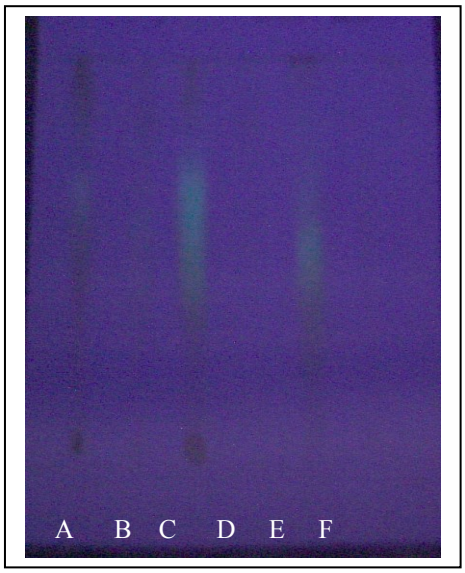
Plate 3. Flavonoid spots of selected plants observed on TLC

1. Visible light

2. UV light 253 nm



3. UV light 366 nm



A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

Plate 4. Flavonol spots of selected plants observed on PC

Two methods such as paper chromatography and TLC were carried in which TLC was found to be the best for the present analysis. Both one dimensional and two dimensional paper chromatography were not found successful in this study. The resolution of selected solvent systems is mentioned in Table 10. Among the solvent systems BAW (4:1:5) gave good result.

4.1.2.2.3 Identification of flavonoids

Flavanoids appeared as dark spots at 253 nm and as fluorescent spots at 366 nm. At 253 nm, only a single spot was observed in Costus pictus with an 100Rf value of 70. All the other plant samples developed three to four spots at 253 nm indicating the presence of different flavonoids. The 100Rf values ranges between 23.5 to 88.2. At 366 nm two to three fluorescent spots were observed in all plant samples. The fluorescent red spot was recorded at solvent front in all the plant samples. The details of Rf values of spots at 253nm and 366nm are presented in tables 11 and 12 and plate 3.

4.1.2.3 Flavones

4.1.2.3.1 Selection of running solvent

The solvent systems such as Forestal (acetic acid- HCl- water) 30:3:10 and BAW: 4:1:5 were used for Paper chromatography. The best separation was obtained in BAW 4:1:5 but streaking of bands were also observed. In 253nm all the plants exhibit only one spot in PC. Costus pictus showed only one spot with an (100Rf- 100) 25.0. The Rf values of the plant sample are presented in the Table13 and Plate 4.

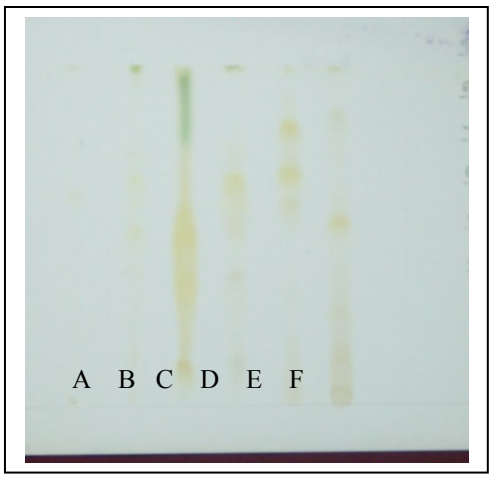
4.1.2.4 Tannins

4.1.2.4.1 Sample extraction

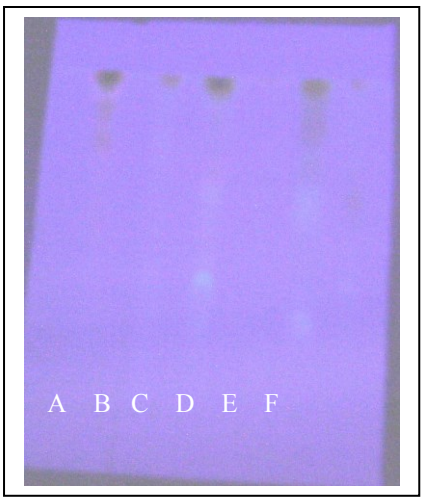
Isoamyl alcohol and N-butanol were tried for the extraction of condensed tannin. Both of them gave red which indicated the presence of proanthocyanidins in neem and tulsi leaf samples. The leaf samples of other selected plants were not expressed red colour. Only one method of extraction as mentioned in the methods and material was carried out for the extraction of condensed tannin.

TLC

1. Visible light



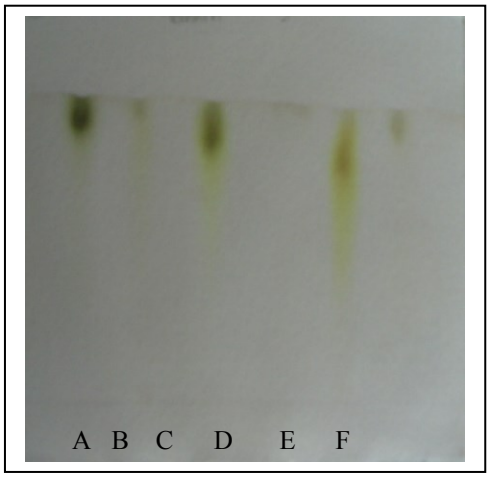
2. UV light 253 nm



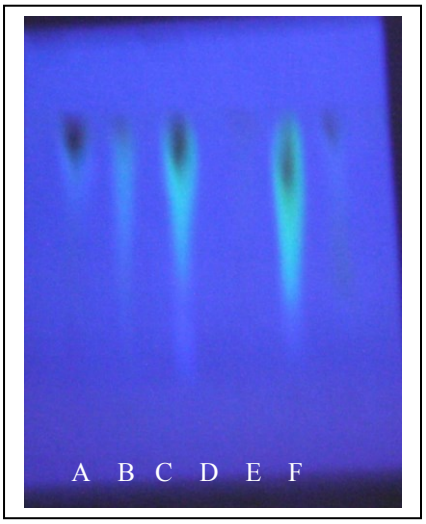
3. UV light 366 nm

PC

1. Visible light



2. UV light 253 nm



A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

Plate 5. Tannin spots of selected plants and standards observed on TLC and PC

Table14. Rf values(x100) of hydrolysable tannin developed on TLC plate for selected plant samples observed under 253 nm

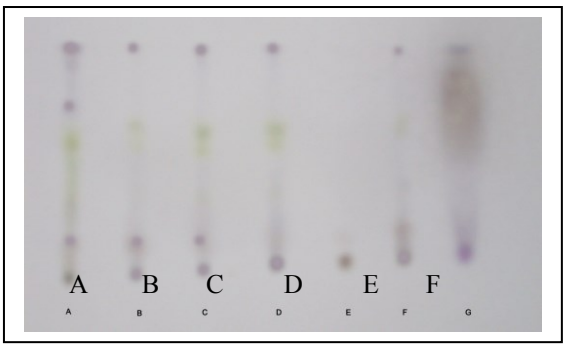
Costus	Ginger	Gymnema	Adhatoda	Neem	Tulsi
	89.3				
86.6		86.6			
				85.3	
					84
80					
73.3					
		66.6		66.6	66.6
		40			
			26.6	26.6	
	20		20		20
13.3					

Table 15. Rf values(x100) of terpenoids developed on the TLC plate for selected plant samples sprayed using Vanillin-H_2SO_4

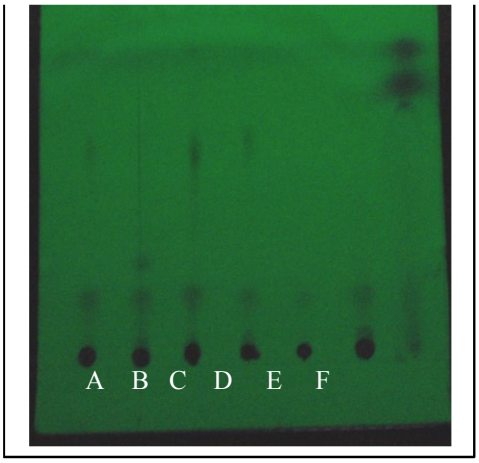
Costus	Ginger	Gymnema	Adhatoda	Neem	Tulsi
		90		90	-
			85		-
71.4	71.4				-

a) Selected plants

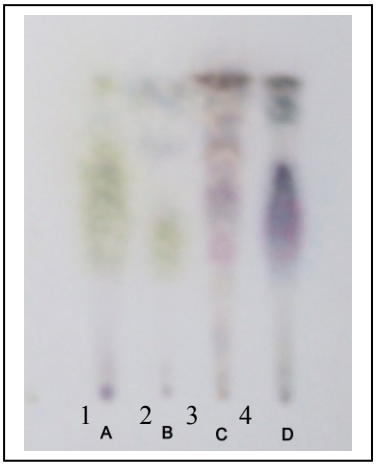
1. Visible light



2. UV light 253 nm



b) Standards



1) Geraniiol
2) Citronella oil
3) Ginger pil
4) Vettiver oil

Plate 6. Terpenoid spots of selected plants and standards observed on TLC

1. Visible light

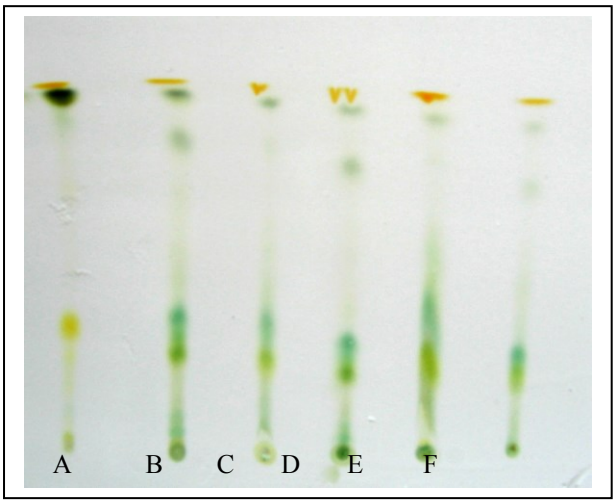


Plate 7. Carotenoid spots of selected plants observed on TLC

A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

4.1.2.4.2 Selection of running solvent for hydrolysable tannin

The solvent systems Forestal (acetic acid- HCl- water) 30:3:10 for PC and BAW 4:1:5 were tried for TLC. In PC streaking of spots were observed (Table 14 and Plate 5). The best separation was obtained using BAW: 4:1:5 in TLC. The Rf values were in between 13.3 to 89.3. Costus pictus showed four bands.

At 366nm, Costus pictus had a fluorescent yellow spot with the 100Rf value of 80. Ginger also has shown fluorescent violet spot in 366 nm with an 100Rf value 68.

4.1.2.5 Terpenoids

Sample was extracted as mentioned in methods and material (3.4.5.1)

4.1.2.5.1 Selection of running solvent

Three solvent systems, benzene – chloroform (1:1) and hexane-chloroform (3:2) benzene- ethyl acetate (9:11) were tried initially. Best separation was observed in benzene – chloroform (1:1) (table 15 and plate 6a).

For $AgNO_3$ impregnated plates, methylenedichloride - ethyl acetate - n-propanol (45:45:4.5:4.5) was used. For paraffin coated plates 70% methanol was used as solvent (Harborne, 1973). Both of them were not found successful in spot development.

4.1.2.5.2 Selection of spray reagent

Different spray reagent such as 0.2% aqueous $KMnO_4$, 5% antimony chloride in chloroform and Vanillin-H_2SO_4 were tried. Vanillin-H_2SO_4 spray regent recorded best result all the samples. Terpenoid spots at 253nm showed dark colour. Vanillin-H_2SO_4 spray regent showed pink / violet colour with terpenoids disappeared within minutes after spraying. The 100Rf values of the samples were between 71.4 to 90.0. In standard geraniol, citronella oil and ginger oil and vetiver oil were showing a good number of terpenoid spots in TLC plate. The Rf values of the same are presented in Table 16 and Plate 6b. All the plant samples showed

Table 16. Rf values(x100) of terpenoid standards developed on TLC plate sprayed with Vanillin-H_2SO_4

Standards	Rf value
Geraniol	16.6
Citronella oil	76.9
Ginger oil	93.2, 71.5, 46
Vetiver oil	84.6, 69.2

Table 17. Solvent systems tried for separation of carotenoids

Sl.No	Solvent system	Separation
1.	Water	Poor
2.	Methanol	Better
3.	Saturated $MgCO_3$ in cold acetone (2ml) and petroleum ether (4ml)	Good

only one spot of different Rf values. Costus pictus and ginger showed spots of same 100Rf (71.4). Similarly gymnema and neem also expressed spots of same 100Rf (90.0)

4.1.2.6 Saponins

Gymnema and neem showed stable foam formation during the gentle shaking of extract in methanol indicated the presence of saponins. The running solvent system adopted by Golba (2000) to elute saponins from gymnema was used in this study. The saponin was eluted as a single spot in chloroform: acetone: methanol (5:1:1.5) for gymnema and neem. They showed pink spots when sprayed with Vanillin- H_2SO_4 with the 100Rf value of 90 and 20 respectively. Saponin was not detected in other plant samples including Costus pictus.

4.1.2.7 Carotenoids

4.1.2.7.1 Sample extraction

The sample extraction was carried out using water, methanol and a solvent system of saturated Mg CO_3 in cold acetone (2ml) and petroleum ether (4ml). The best separation of carotenoids was recorded in saturated $MgCO_3$ in cold acetone (2ml) and partition chromatography was carried out with petroleum ether (4ml) (Table 17).

The carotenoids were of varying colours such as green, blue green, yellow green and yellow in ascending order in TLC plate which are considered to be chorophyll b, chorophyll a, xanthophyll and carotene respectively. The degree of absorbability of carotene, xanthophyll, chlorophyll a & b are different and it is in the ascending order. The 100Rf values of chlorophyll b were in between 22.2 to 33.3. The 100Rf values of chlorophyll were in between 38.8 to 44.4. The 100Rf values of xanthophyll ranges in between 61.1 to 64.4. The 100Rf value of carotene is 100. The Rf values are mentioned in the Table 18 and depicted on Plate 7. The yellow spot representing carotene at the solvent front was common for all plant samples where as the spots of chlorophyll a, chlorophyll b and xanthophyll showed slight variation in Rf values of all leaf samples of selected plants.

Table 18. The Rf values(x100) and colour of the carotenoids developed on the TLC plate for selected plant samples observed under visible light

	Chlorophyll b (green)	Chlorophyll a (blue green)	Xanthophyll (yellow green)	Carotene (yellow)
Costus	27.7	44.4	64.4	100
Ginger	33.3	42.2	61.1	100
Gymnema	27.7	42.2	61.1	100
Adhatoda	27.7	42.2	61.1	100
Neem	22.2	38.8	61.1	100
Tulsi	27.7	44.4	-	100

Table 19. Efficiency of different solvents for plant acid extraction

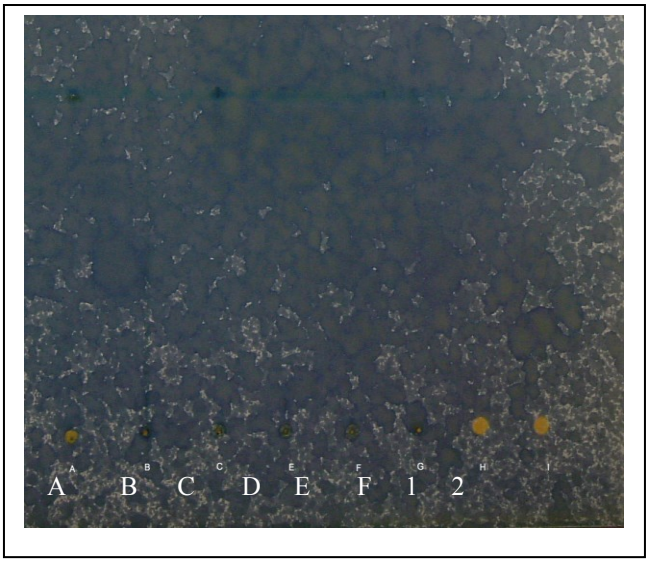
Sl.No	Solvent	Separation
1.	Plant extract without solvent	Bad
2.	75% ethanol	Poor
3.	0.1N HCl	Moderate
4.	2M HCl	Best

Table 20. Rf values(x100) of plant acid standards

Standards	Rf value
Tricarboxylic acid	80
Oxalic acid	3.1
Tartaric acid	3.1
Citric acid	3.1
Sample (Costus)	3.1

Table 21. Colour intensities of qualitative tests for alkaloids in selected plants

Plants	Mayers test	Wagners test	Dragendroffs test
Costus	Light white precipitate	Dense brown flocculent	Orange red precipitate
Ginger	Light white precipitate	Dense brown flocculent	Orange precipitate
Gymnema	White precipitate	Light brown flocculent	Orange red precipitate
Adhatoda	White precipitate	Light brown flocculent	Orange red precipitate
Neem	White precipitate	Dense brown flocculent	Orange red precipitate
Tulsi	Light white precipitate	Light brown flocculent	Orange red precipitate



A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

1) Oxalic acid
2) Citric acid
3) Tricarboxylic acid 4)Tartaric acid

Plate 8. Plant acids spots of selected plants and standards detected through bromocresol green spray on TLC

4.1.2.8 Plant acids

4.1.2.8.1 Sample extraction

Twenty gram plant sample was taken for plant acid analysis and efficiency of solvent systems for plant acid extraction was studied (Table 19). The sample extracted with 2M HCl gave the best results by way getting yellow spot in blue back ground in TLC plate.

Only Costus pictus gave spots similar to that of the standard oxalic acid, with an 100Rf value of 3.1. No other spot was observed in any other plant samples. The Rf values are given in the Table 20 and depicted in Plate 8.

4.1.2.9 Alkaloids

The colour intensities are presented in the Table 21.

a) Mayer's test

White precipitates with varying colour intensities were observed for the leaf samples. Light white precipitate was observed for the leaf sample of Costus pictus.

b) Wagner's test

Brown flocculent was formed with varying colour intensities. In the case of Costus pictus there was dense brown flocculent in the qualitative test.

c) Dragendroff's test

Orange red precipitate was observed with same colour intensities.

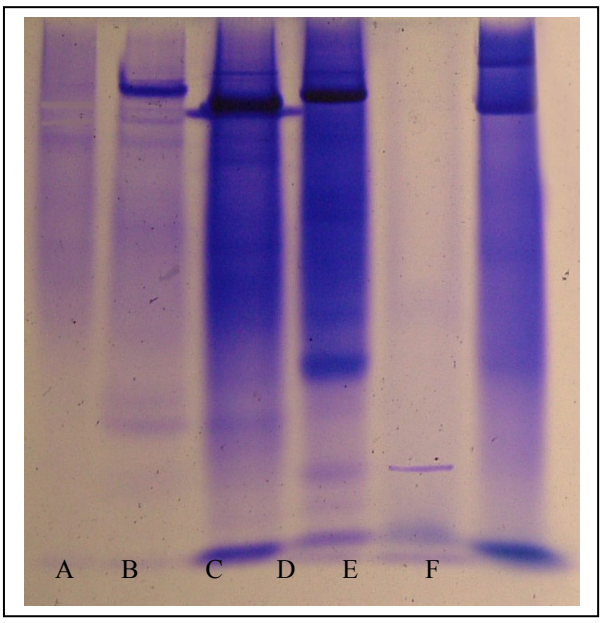
Presence of alkaloid was present a common factor for all leaf samples.

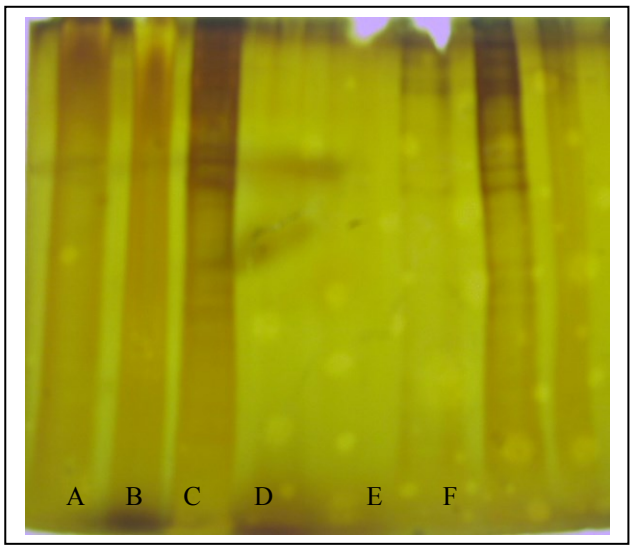
4.2 ELECTROPHORESIS STUDIES

4.2.1 SDS Polyacrylamide gel electrophoresis

4.2.1.1 Standardization of protein extraction

For the protein extraction three buffer systems viz, phosphate buffer, Tris buffer, Tris+ ingredients as suggested by Harborne (1973) were tried. Tris+ ingredients buffer was found to be the best for protein extraction which had maximum number of bands.

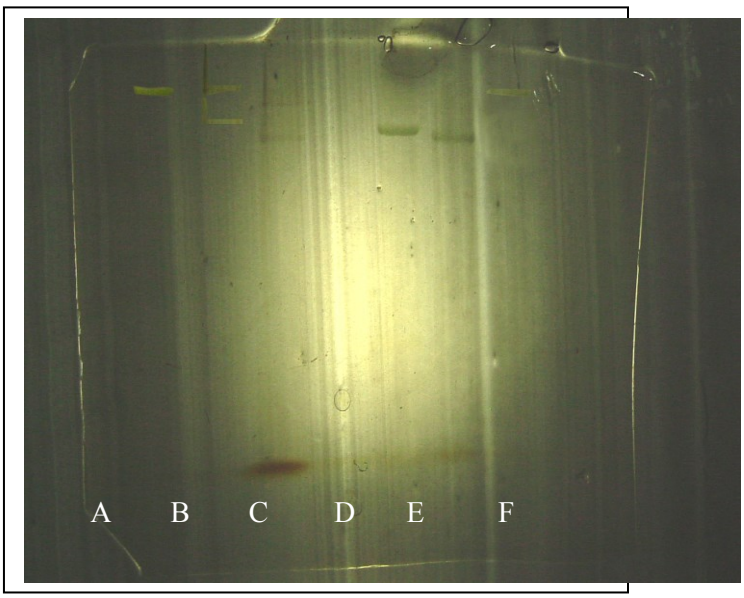




a) Coomassie brilliant blue **b) Silver staining**

A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

Plate 9. Protein banding pattern in selected plants



a) Benzidine

A- Costus, B- Ginger, C- Chakkarakolli, D- Adalodakam, E- Neem, F- Tulsi

Plate 10. Peroxidase banding pattern in selected plants

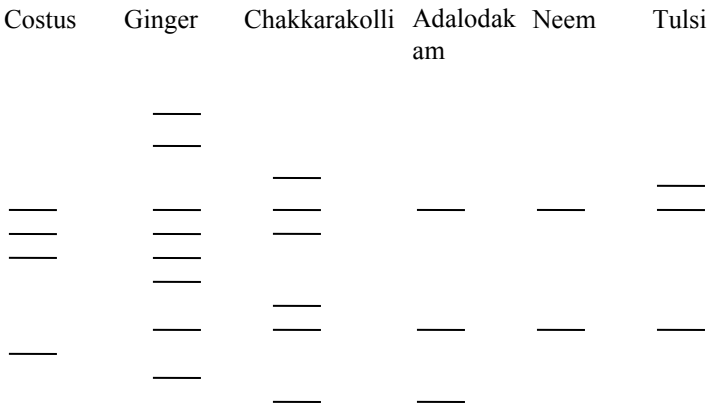


Figure 1. Protein banding pattern in selected plants

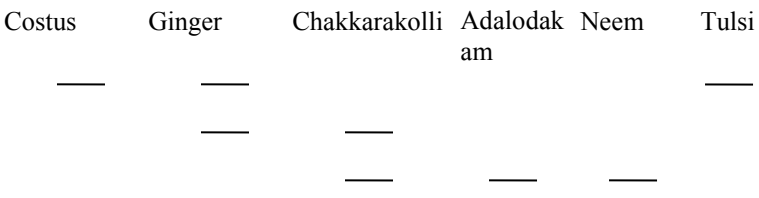


Figure 2. Peroxidase banding pattern in selected plants

4.2.1.2 Properties of gel

Acrylamide stock solutions of two different concentrations viz, acrylamide 30%+ bisacrylamide 0.8% and acrylamide 30% + bisacrylamide 0.1% were tried. The first one was found best for all leaf samples. The concentration of gels 7.5%, 10%, 12.5% and 15% were tried in which 10% gel gave good separation of proteins in SDS Page for all leaf samples.

4.2.1.3 Current and voltage

The current was turned on to 10-15mA until the samples travel through the stacking gel. The stacking gel helps to concentrate the sample protein and there by obtaining clear bands. Then the run was continued at 30mA until the bromophenol blue reaches ¾ of the gel (about 3h). High current of 60-70mA for short period of 1h with proper cooling was also tried which was not successful in the separation process. The resolution of bands was better at low current system.

4.2.1.4 Staining

Coomassie brilliant blue R 250 was effective than silver staining for good resolution. The protein-banding pattern is shown in Figure 1. Costus pictus showed four thin three bands of which were similar to ginger. Actually there were 10 bands in ginger. Gymnema showed six protein bands, adhatoda three protein bands, neem two protein bands and tulsi four protein bands respectively (Plate 9).

4.2.2 Isozyme Analysis

4.2.2.1 Peroxidase enzyme

Phosphate buffer was used for enzyme extraction. Extraction was carried out in ice-cold pestle and mortar. Cold conditions prevented the denaturing of enzymes during extraction. For isozyme analysis non-denaturing gels were used for enzyme separation. 7.5% and 10% gels gave good results. The banding patterns of isozymes (peroxidase) in leaf samples are given in the Figure 2 and Plate 10. Costus pictus showed only one peroxidase band. Ginger and tulsi also

showed the same band in pattern. Ginger and gymnema showed two bands in which one band was common for both samples. Costus pictus, adhatoda, neem and tulsi showed only one band of different mobility.

4.3 CELL IMMOBILIZATION

Immobilization studies were carried out in Costus pictus as the initial step for exploiting in the future.

4.3.1 Plant regeneration

Since in vitro studies on Costus pictus plant are not reported, regeneration media reported for ginger (½ MS + 3ppmBA) and Costus speciosus (½MS + 2ppmBA+ 2ppmKN+ 1ppm NAA) were tried for Costus pictus plant regeneration. Rhizome bud was taken as the explant. Medium for Costus speciosus was found the best for rapid regeneration from rhizome (Plate 11). Effect of media for rate of plant regeneration from rhizome buds are presented in Table 22.

4.3.2 Callus induction

For the callus induction leaf sample was taken as the explant. Media tried were ½ MS+ 1ppm BA+ 3ppm NAA, ½ MS + 1ppm 2,4-D + 0.5ppm BA, ½ MS+ 0.25 ppm 2,4-D and ½ MS + 0.5 ppm 2,4-D. Creamy white callus was obtained in ½ MS+ 1ppm BA+ 3ppm NAA which was considered to be the best among the media tried.(Table 23 and Plate 12).

4.3.3 Suspension Culture

Suspension cultures were obtained by agitating the callus cells in ½ MS liquid medium + 0.5ppm 2, 4-D at 90 rpm on rotary shaker for one full week to separate single cells (Plate 12).

4.3.4 Immobilization of cells

The alginate beads formed by mixing the cell aggregates with sodium alginate in $CaCl_2$ solution (.08 M) for 30 minutes was effective for

Table 22. Effect of media for plant regeneration from rhizome buds

Media composition	Performance
½ MS + 3ppmBA	Slow growth
½MS + 2ppmBA+ 2ppmKN+ 1ppm NAA	Fast growth

Table 23. Effect of media in callus appearance

Media composition	Callus appearance
½ MS+ 1ppm BA+ 3ppm NAA	Creamy white glossy callus
½ MS + 1ppm 2,4-D + 0.5ppm BA	Brown callus from sides of the leaf bits
½ MS + 0.5 ppm 2,4-D	Brown callus
½ MS+ 0.25ppm 2,4-D	Brown callus

Table 24. Rf values(x100) of secondary metabolites produced through cell immobilization

Secondary Metabolites	Rf values
Phenols	44.4, 77.7, 11.1
Terpenoids	16.6
Plant acids	3.1

immobilization of callus. The beads were later washed thrice with sterile distilled water, to remove excess $CaCl_2$ from beads before transferring to the nutrient medium. The cultures were grown in MS liquid medium (Plate12) kept in rotary shaker 90 rpm at 30^{o}C under a light intensity of 2000 lux and the media was harvested for the analysis of secondary metabolite.

4.3.3.1 Qualiative analysis of secondary metabolites

The liquid medium containing immobilized cells and liquid medium without immobilized cells (control) were extracted with ethyl acetate and spotted in silica gel plates to observe the presence of secondary metabolites (Plate 12). The Rf values are presented in the Table 24.

Thin Layer Chromatography

a) Phenols

Ethyl acetate: Benzene (1:1) solvent system was used to separate phenols. Three spots were observed in liquid medium containing immobilized cells and there was no similar spot in the control. These spots gave blue colour with folin-ciocalteu reagent.

b) Terpenoids

Benzene – chloroform (1:1) solvent system was used to develop the chromatography plates. Two terpenoid spots were observed in liquid medium containing immobilized cells and in control one light pink spot of round shape was observed when the plates were sprayed with Vanillin -H_2SO_4. The spot in the control may be due to the contamination or chemical reaction of the spray reagent with the medium component.

c) Plant acids

Benzene –methanol –acetic acid (79:14:7) solvent system was used to develop the chromatography plates. Light yellow spot was observed in liquid medium containing immobilized cells when sprayed with bromocresol green. No spot was observed in control.

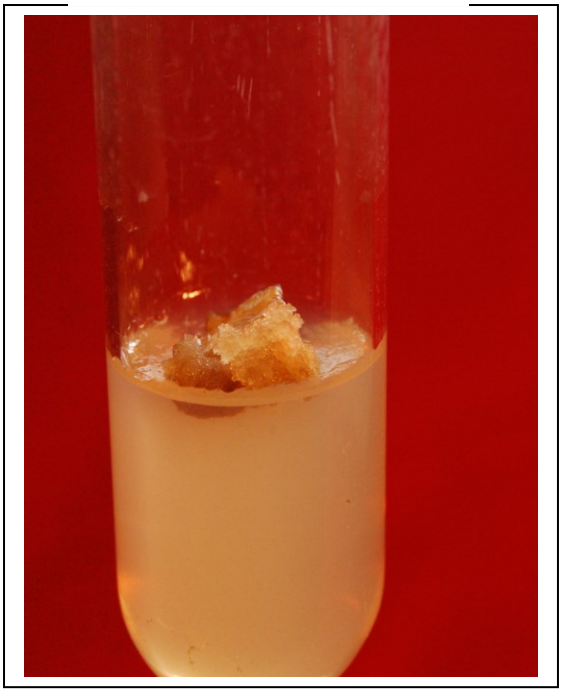
Plate. 11 Plant regeneration from rhizome bud

Plate.12 Steps in cell immobilization

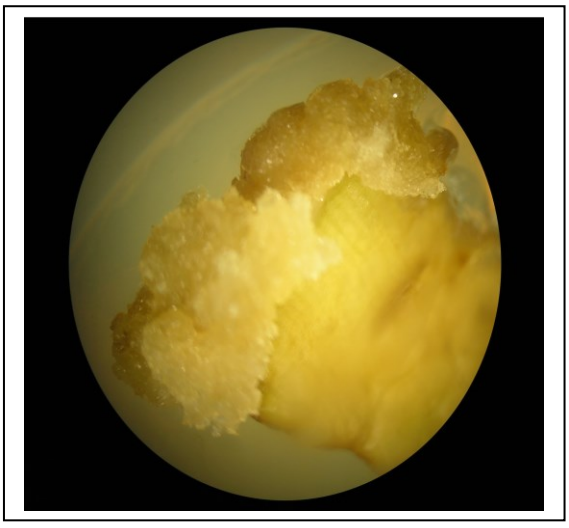
A. Callus induction

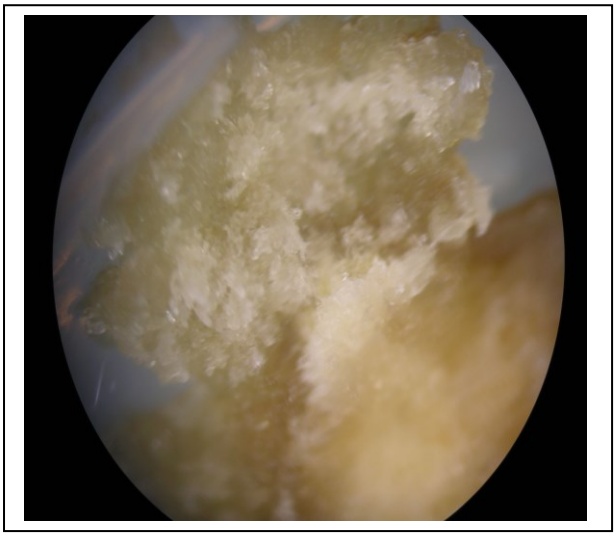
Explant kept on media for callus induction

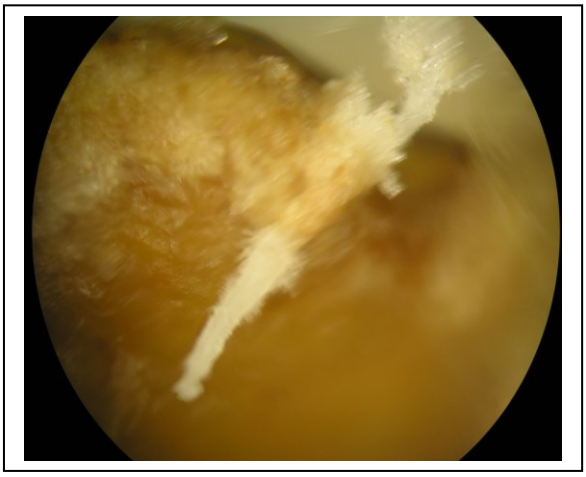
Callus formation in media



Microscopic view of the callus a & b







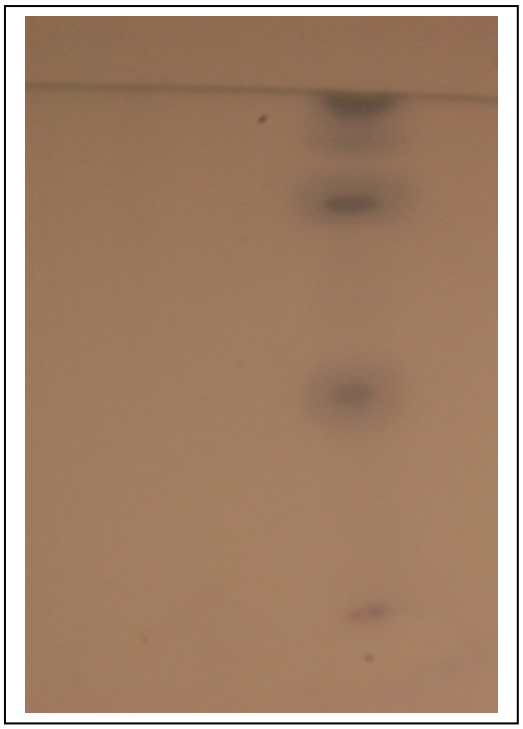
Glossy callus in media

B. Suspension culture

C. Immobilized cells using sodium alginate

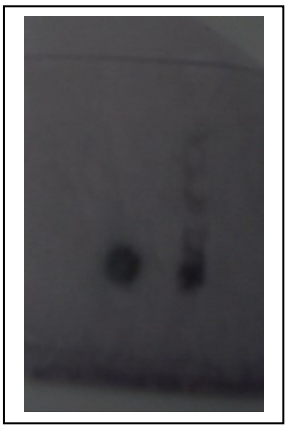
D. Secondary metabolite production

Phenol



Plant acids

Terpenoids



DISCUSSION

5. DISCUSSION

The study entitled “Biochemical characterization and cell immobilization of *Costus pictus* with special reference to antidiabetic property” was taken up with the objective of screening of *Costus pictus* for biochemical constituents and standardization of cell immobilization technique.

5.1. QUALITATIVE EVALUATION

5.1.1 Primary metabolites

5.1.1.1 Carbohydrates

Amount and type of carbohydrate intake affects our blood glucose levels. Carbohydrates have the most immediate effect on blood glucose levels, since carbohydrates are broken down into glucose early during digestion. It is important to eat the suggested amount of carbohydrate at each meal, along with some protein and fat. Certain carbohydrates are attached to other biomolecules which may be a medicine or a precursor for the production of useful metabolites for normalizing the metabolism.

a) Molisch's test

The colour of this test was due to the reaction of alpha-naphthol with furfural and /or its derivatives formed by the dehydration of sugars by concentrated sulphuric acid. If oligosaccharides or polysaccharides are present, they are first hydrolyzed to constituent monosaccharides that are then dehydrated. Pentoses produce furfural and hexoses yield 5-hydroxy methyl furfural. The present study indicated the presence of sugars in all the leaf samples. *Costus pictus*, ginger, gymnema, adhatoda and tulsi have shown almost the same intense violet ring and neem has shown dark violet ring with H_2SO_4. Neem contains high concentration of carbohydrates than all other plants compared.

b) Iodine test

Iodine forms coordination complex between the helically coiled polysaccharides chain and will be centrally located within the helix due to adsorption. All the leaf samples indicated the presence of starch (Sadasivam and Manikam, 1992).The iodine colour obtained with the polysaccharides depends upon the length of the branched or unbranched chain available for the complex formation. Amylose, a linear component of starch gives deep blue colour, Amylopectin; a branched chain component of starch gives purple colour. Glycogen gives reddish brown colour, where as dextrin, partially hydrolyzed starch gives colour ranging from brown to red colour depending upon the size of the molecules. But cellulose and inulin have no colour with iodine. *Costus pictus*, ginger, gymnema and adhatoda gave blue colour in the present study. So presence of amylose was confirmed. Neem and tulsi showed light blue colour indicating the presence of very low quantity of polysaccharides.

c) Fehling's test

The blue alkaline cupric hydroxide present in fehling's solution, when heated in the presence of reducing sugars, get reduced to yellow or red cuprous oxide and it get precipitated (Sadasivam and Manikam, 1992). Hence, formation of the red precipitate in the study indicated the presence of reducing sugars in all plant samples. Neem has shown deep red colour. So the presence of high concentration of sugars in neem was confirmed. Adhatoda has shown light red colour, so the presence of low concentration of reducing sugars was indicated.

d) Benedict's test

Reducing sugars (all mono and disaccharides), because of having potentially free aldehyde or keto group, reduce cupric hydroxide in alkaline solution to red coloured cuprous oxide (Sadasivam and Manikam, 1992). In *Costus pictus*, ginger, gymnema and tulsi gave red precipitate. Adhatoda showed

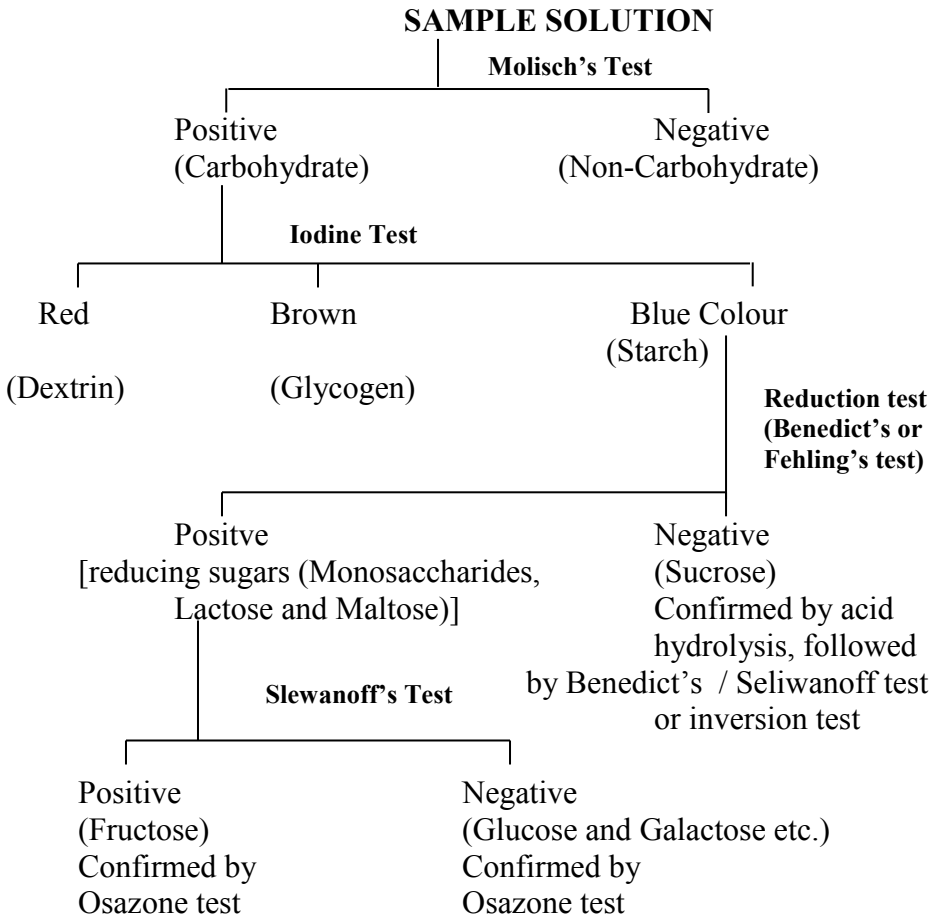
green colour. Brick red precipitate was observed in neem, indicating the presence of reducing sugars in high concentration.

e) Seliwanoff's test

In concentrated HCl, ketoses undergo dehydration to yield furfural derivatives more rapidly than do aldoses. These derivatives form complexes with resorcinol to yield deep red colour. It is a time bound colour reaction specific for ketoses.

Molisch's test, Iodine test, Fehling's test, Benedict's test and Seliwanoff's test confirmed the presence of carbohydrates in *Costus pictus* and in all selected plants with varying concentrations. Depending on carbohydrate concentration, colour varied from light to dark as mentioned in Table1. Carbohydrate tests indicated the presence of monosaccharides of ketohexoses origin in all the selected plants including *Costus pictus*. There was an indication of low quantity of keto sugar production in *Costus pictus*. Plants like adhatoda expressed light red precipitate for Fehling's test where as *Costus pictus* showed medium red precipitate. *Costus pictus* was dissimilar to neem in all carbohydrate tests with respect to colour intensity.

The keto sugar present in *Costus pictus* may be either some of biomolecules having carbohydrate moiety or some glucosamine or glycosyl compounds. It is evident from the screening flow chart given below.



Result of the protein test as well as the sugar test is supporting the presence of glycosylate biomolecule of diverse origin in *Costus pictus*.

5.1.1.2 Proteins and amino acids

Hypoglycemic peptide, Polypeptide-p, has been isolated from fruit, seeds, and tissue of bitter gourd by Khanna and Jain (1981). Amino acid analysis indicates a minimum molecular weight of approximately 11,000 (166 residues). Polypeptide-p is a very effective hypoglycemic agent when administered subcutaneously to gerbils, langurs and humans. With this background the analysis of selected plants with special reference to *Costus pictus* was carried out.

a) Xanthoproteic reaction

The yellow color was due to the nitro derivatives of the aromatic amino acids like phenylalanine, tyrosine and tryptophan present in the side chain of

protein and free amino acids in the plant extract. The sodium salts of nitro derivatives are orange in color. So the presence of protein having aromatic amino acids and/ or free aromatic amino acids in all leaf samples indicated a positive trend of xanthoprotein in the plants.

b) Glyoxylic reaction for tryptophan

The indole group of tryptophan reacts with the Glyoxylic acid released by the action of concentrated H_2SO_4 on acetic acid to give a purple color. So the presence of tryptophan was confirmed in all the selected plants. The indole group in the plant samples in the present study is an indication of the production of organic compounds of pharmaceutical importance.

c) Modified Million's test

The yellow precipitate formed in all the sample extracts was due to the precipitation of selected protein. Mercury combined with tyrosine of the protein and or free amino acids. Yellow precipitate with $HgSO_4$ and red colour with $NaNO_3$ in varying intensities were observed in all leaf samples. The red colour was due to the reaction of the precipitate with the nitrous acid.

Xanthoproteic reaction, Glyoxylic reaction for tryptophan and Modified Million's test confirmed the presence of proteins having aromatic amino acids in *Costus pictus* and in all selected plants. The colour intensity varied from light to dark colour based on the protein concentration as mentioned in Table 2, 3 and 4. In *Costus pictus* presence of low concentration of protein, the presence of aromatic amino acids and free amino acids including tryptophan was confirmed.

The analysis of free amino acids and/or the amino acids of side chain of plant protein were made by TLC methods. In all the selected plants, the amino acid spots appeared as pink in colour. The intensive blue/ pink product is generally characteristics of those amino acids that are having α- amino group (Sadasivam and Manikam, 1992). Most of the plants expressed three spots in TLC, except adhatoda and neem in which one more additional amino acid of

different Rf value was observed. 100Rf value of 31.2 was recorded for the four plants whereas a 100Rf value of 72.5 was recorded for *Costus pictus* only. The identity of selected plants can be observed from the variation of Rf values of amino acid. The Rf value of free aromatic amino acids of standards varied with the amino acids of plant origin in TLC. Nature, properties and sequence of amino acids of plant origin might have influenced the movement and colour development in TLC plate. Presence of aromatic amino acids in proteins of related plants were ascertained by the three protein tests and variation in spot pattern in TLC revealed the flexibility of protein presence and functioning in biological system. Considering the presence of hexoketoses, free amino acids and /or soluble proteins, the possibility of amyloproteins in selected plants especially in gymnema and *Costus pictus* cannot be ruled out.

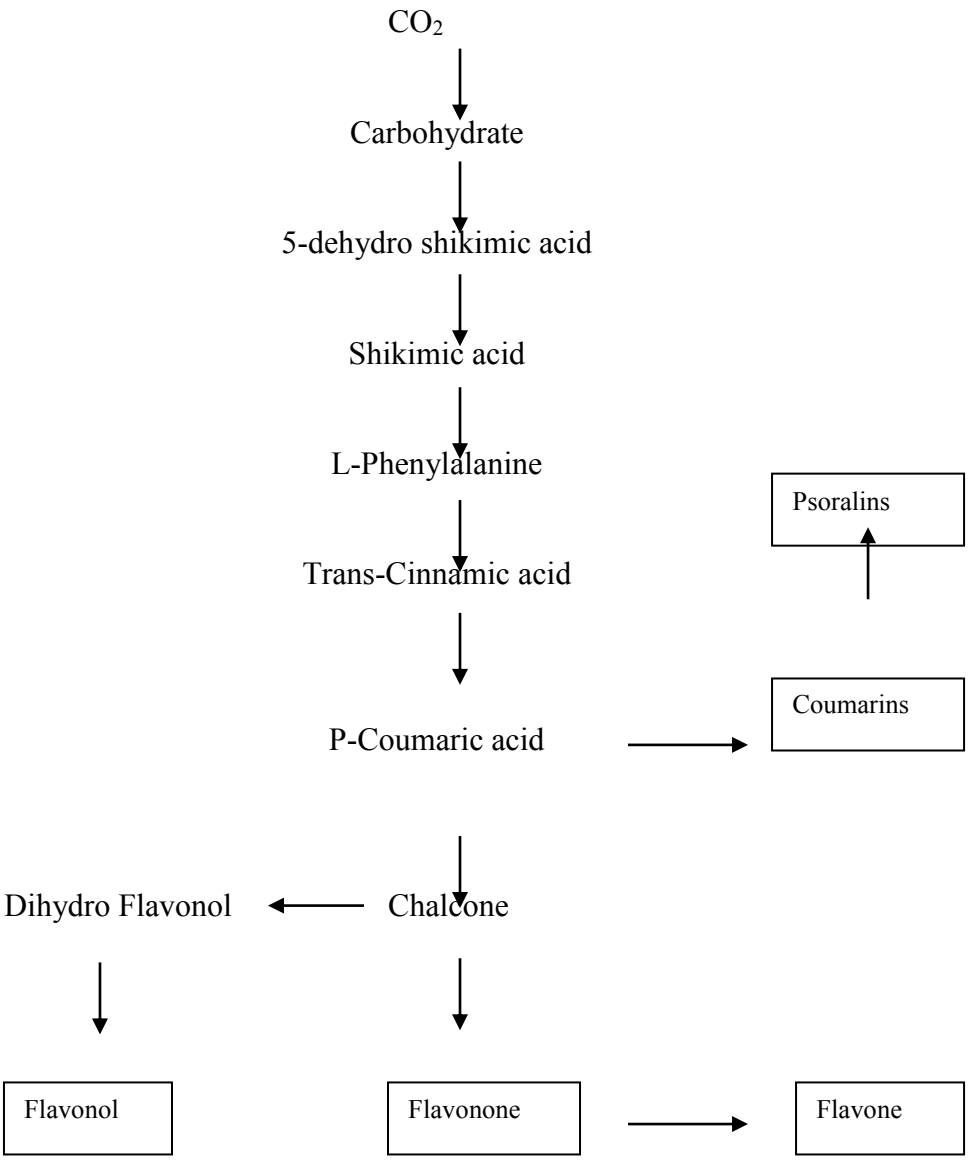
5.1.2 Secondary metabolites

5.1.2.1 Phenols

Based on the observations it can be very well presumed that the *Costus pictus* has a specific metabolic pathway. Plant phenolics are biosynthesized by different routes. The presently accepted biochemical pathway through tyrosine and phenylalanine results from the studies of *E.Coli* biochemical mutants can be adopted for *Costus pictus* and other selected plants.

In general, shikimic acid pathway participates in the biosynthesis of most plant phenolics. It converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the aromatic amino acids.

An outline of the biosynthesis of plant phenolics showing the involvement of shikimic acid pathway is also indicated in the scheme given below.



Phenols react with phosphomolybdic acid in Folin- Ciocalteu reagent in alkaline medium and produce blue coloured complex (molybdenum blue). Phenols with catechol or hydroquinone nuclei appear as blue spots immediately after spraying. Other phenols showed blue- gray spots when the plate was fumed with ammonia vapour.

The best separation of phenolic spots occurred in all plant samples in the present study. The modified solvent system ethyl acetate: benzene (1:1) was found to be best for all the plants studied and is in conformity with the finding of Stahl (1969) that ethyl acetate: benzene (9:11) as the best solvent system. Best

separation of phenolic spots occurred on acid hydrolysis (Harborne, 1973). The plates were viewed in UV range 253nm and 366nm. The phenols appeared as dark absorbing spots in 253nm and fluorescent spots in 366nm. Four compounds were isolated in TLC from *Costus pictus* where as gymnema expressed five spots in which four of them were similar to *Costus pictus* (Plate 2). This indicates the similarity of *Costus pictus* with gymnema in phenolics production.

Preventive effect of the phenol antioxidants in treatment of patients suffering from diabetes mellitus with vascular complications was indicated by Bobyreva (1997) on alloxan diabetes model. In occurrence with this finding the phenolics present in *Costus pictus* and gymnema may be of value in diabetes treatment.

5.1.2.2 Flavonoids

Ethanol was used as the solvent for the extraction of flavonoids from leaf samples. One dimensional paper chromatography and TLC were found good for obtaining clear spots. The running solvent system Forestal (acetic acid- HCl-water) 30:3:10 for PC and BAW 4:1:5 for TLC gave clear spots. The best separation of spots observed in TLC plates was developed in BAW 4:1:5 solvent system. The fresh leaf tissues were extracted 5-10 min with boiling 95% ethanol to release the flavonoids (Harborne, 1973).

There was only one spot (100Rf value 70) at 253nm in Costus pictus. Same was the result at 366nm but the 100Rf value was 90.The Rf values of other crops were different at 253nm except the fluorescent red spot (100Rf value 100). The pigment aurone (Harborne, 1973) recorded an 100Rf value of 100 at the solvent front and this was common to all plants (Plate 3).

Flavonoids, as antioxidants, are reported to prevent the progressive impairment of pancreatic β-cell function due to oxidative stress and may thus reduce the occurrence of type 2 diabetes. Hence the presence of flavonoids in all the plants studied substantiates their use as antidiabetic plants and gymnema is rated to have highest value with respect to number of flavonoid compounds. Song

et al., 2005 have indicated the association of dietary flavonol and flavone intake with type 2 diabetes, and biomarkers of insulin resistance and systemic inflammation.

5.1.2.3 Flavones

Felgines *et al*. (2000) have compared kinetics of absorption of naringenin a predominant flavanone in grapefruit and its glycosides in rats. They have reported that flavanones are efficiently absorbed after feeding to rats and that their bioavailability is related to their glycosidic moiety.

Citrus peel extract containing polymethoxylated flavones (PMFs) is reported to have beneficial effects on cholesterol-levels. Biomarkers of insulin resistance, diabetes and obesity were observed at a significant decrease after supplementation with PMF. Hence the presence of flavones in all the plants studied may be substantiating their use as antidiabetic plants but the relative merit of the flavones observed has to be studied further.

5.1.2.4 Tannins

The red colour production observed in Neem and tulsi leaf samples in qualitative tests may be due to the presence of proanthocyanidins. In the TLC for hydrolysable tannins, fluorescent violet spot was observed in ginger. Violet colour of ellagic acid appears on the plates of forestal solvent system for myricetin and quercetin. Galloyl esters usually appear as violet fluorescing spot (Harborne, 1973). *Costus pictus* expressed four spots of tannin, of which spot at 86.6 was similar to that of gymnema (Plate 5). The presence of the maximum of four hydrolysable tannins in *Costus pictus* indicates its usefulness in medicine.

5.1.2.5 Terpenoids

Acid hydrolysis followed by ether extraction was the best method for separating terpenoids through TLC. Clear separation was observed in benzene – chloroform (1:1) solvent system. Terpenoids spots recorded dark absorbing spot

under UV light; where as vanillin spray expressed pink colour spots, which disappeared within minutes after spraying. TLC of *Costus pictus* and ginger expressed same compound at 100Rf value 71.4. Gymnema, adhatoda and neem had different spots and there was no indication of terpenoids in tulsi (Plate 6). Eventhough the Rf values differed each other, the colour test indicated the common origin or derivatives of same compound in all the plants except tulsi.

Sato *et al*. (2002) isolated the triterpene acid compound dehydrotrametenolic acid from dried sclerotia of *Poria cocos* (Polyporaceae). These terpenoids and thiazolidine type of antidiabetic agents such as Ciglitazone, although structurally unrelated, share many biological activities: both induce adipose conversion, activate peroxisome proliferator-activated receptor gamma (PPAR gamma) *in vitro*, and reduce hyperglycemia in animal models of NIDDM. Dehydrotrametenolic acid is a promising candidate for a new type of insulin-sensitizing drug. This finding is very important for the development of insulin sensitizers that are not of the thiazolidine type. The presence of terpenoids in *Costus pictus* and other selected plants may be substantiating their use as antidiabetic plants in the light of the above finding.

5.1.2.6 Carotenoids

Saturated $MgCO_3$ in cold acetone (2ml) and petroleum ether (4ml) showed best separation of carotenoids in leaf samples. The solvent system of benzene-petroleum-ethanol-water (10:10:2:1) gave good separation of carotenoids like carotene, xanthophylls, chlorophyll a & b. The carotenoids observed under visible light in different coloured spots such as green, blue green, yellow green and yellow in ascending order. *Costus pictus* expressed same Rf values for chlorophyll a and b as that of tulsi. At the same time the 100Rf value of xanthophylls was at 64.4, which was not similar to any of the plants (Plate 7). In general carotenoids in *Costus pictus* expressed a different system, which may contribute in the efficiency of photosynthetic system.

Saponins appeared as pink spot with Vanillin H_2SO_4 in gymnema and neem. All other plants including *Costus pictus* did not have saponins, which points to the fact that *Costus pictus* is a plant of acidic nature.

5.1.2.7 Plant acids

Carboxylic acids stain yellow- green on a blue background (Harborne, 1973). Only *Costus pictus* gave spot (100Rf value 3.1) similar to that of oxalic acid, tartaric acid and citric acid (Plate 8). The high concentration of oxalic acid in *Costus pictus* was already reported by Benny, 2006. So the presence of oxalic acid in *Costus pictus* was confirmed in support of the acidic taste of the leaves. In *Costus pictus*, there was no report of oxalic acid poison or oxalate formation in biological system, the mechanism and efficiency of oxalate oxidase in the plant has to be studied.

5.1.2.8 Alkaloids

The alkaloid tests explained by Chatwal (1983) were followed in the present study. The presence of alkaloid in *Costus pictus* and selected plants were ascertained by testing with reagents like Mayer's reagent, Dragendorff's reagent and Wagner's reagent. An insoluble precipitate with varying colour was formed for each reagent. These insoluble precipitates had characteristic colours and are used for the detection and quantification of alkaloids. All tests showed positive result for the presence of alkaloid in all the plant samples. Among the above three tests Wagner's test gave dense brown flocculent precipitate in *Costus pictus*. This may be an indication of the presence of distinct amino acid precursors for the alkaloid production.

Tecoma stans is a plant traditionally used in Mexico for the control of diabetes. Tecomine was shown to be one of the compounds responsible for the hypoglycemic action. Tecomine was unable to modify glycemia; the only effect seen being a decrease in plasma cholesterol levels. On the contrary, when tested (Costantino *et al*., 2003) *in vitro* on glucose uptake in white adipocytes, the

compound showed a marked effect. Hence, the alkaloids present in the plant samples, especially *Costus pictus* are worthy of investigation as antidiabetic drugs.

5.2 QUALITATIVE EVALUATION OF *COSTUS PICTUS* WITH RELATED PLANTS

5.2.1 Comparison of ginger with *Costus pictus*

Sharma and Shukla (1977) reported that the juice from freshly squeezed ginger contains gingerols that is hypoglycemic in diabetic rats. In ayurvedic preparations like decoction- vidangadi kashayam, churanam- mehamakshika churanam, capsules- megamuthgara rasam, khrutham- bruhadadi madyam arishtam- paribhatharishtam, devadharvyarishtam, levangasavam dried ginger is utilized (Vaidyan, 1988).

Costus pictus and ginger showed almost same intense violet ring with H_2SO_4 in Molish's test and light blue colour in Iodine test. So presence of amylose was confirmed. Fehling's test confirmed the presence of reducing sugars in both the plants by red coloured precipitate with same intensity. In Benedict's test, red precipitate was observed in both the plants indicating reducing sugar. In Seliwanoff's test light red colour was observed in *Costus pictus* where as ginger gave medium red colour. The above results indicated the presence of monosaccharides of ketohexoses origin. They were found in all selected plants including *Costus pictus* with less colour intensity (light red). All tests of carbohydrates except Seliwanoff's test gave same trend for both ginger and *Costus pictus*. From the results it can very well assume that low quantity of rare keto sugars is present in *Costus pictus* (Table 1).

In protein analysis *Costus pictus* and ginger gave light yellow colour with HNO_3 and light orange with NaOH in Xanthoproteic test. In Glyoxylic reaction for tryptophan, *Costus pictus* and ginger gave light violet ring and confirming the

presence of tryptophan. In Modified Millions test, *Costus pictus* and ginger gave yellow precipitate with $HgSO_4$ and pinkish red colour with $NaNO_3$. Presence of three spots in ginger and *Costus pictus* with some difference in Rf values of amino acids. Amino acids test indicated the presence of free amino acids in *Costus pictus* and ginger. Presence of aromatic amino acids in ginger and *Costus pictus* were confirmed by three protein tests and amino acid pattern in TLC.

Variation in the spot pattern of TLC of ginger and *Costus pictus* revealed the flexibility of protein functioning in both plants and the production of secondary metabolites.

Ginger and *Costus pictus* exhibited the phenol spots of same 100Rf value of 83 with Folin-ciocalteu reagent. As these two plants belong to the same family, these two compounds can be considered as one of the factors governing the common characteristics of the family. For flavonoids one spot was similar to ginger with the 100Rf value of 70 at 253nm. Fluorescent red band in solvent front was also similar at 366nm. In tannins and plant acids, there were no similar bands in both the plants. The terpenoid spot with the 100Rf value 71.4 were present in both plants. Carotene was present in both plants in solvent front. In Wagner's test for alkaloids, *Costus pictus* and ginger gave dark brown flocculent colour.

In general the presence of various metabolites present in ginger and *Costus pictus* varied in Rf values and colour expression. So the mechanism of metabolism might have basic difference in both plants.

5.2.2 Comparison of gymnema with *Costus pictus*

Gymnema sylvestre grows in South-East Asia. Its therapeutic role in relation to diabetes mellitus, rheumatic arthritis and gout was well known for a long time**.** However, gymnema is best known for its benefits in diabetes**.** The hypoglycemic activity of gymnema has been reported by several workers (Srivastava *et al*., 1988, Persaud *et al*., 1999 and Siddhiqui *et al*., 2000). There are a number of earlier reports on the quantitative analysis of saponins, the major secondary metabolites produced by gymnema. Different techniques based on HPLC (Yokota *et al*., 1994), TLC (Golba, 2000) and HPTLC (Puratchimani and Jha, 2004) were employed for this purpose. Puratchimani and Jha (2004) had quantified saponins separated on precoated silica gel plates using densitometric scanning.

Yoshikawa *et al.* (1997) had observed that the leaves of Gymnema contained dammarane type saponins which stained pink and violet, and oleanane type saponins which stained blue and violet on TLC plates sprayed with 30 per cent H_2SO_4. Golba (2000) had used vanillin (3 per cent) - H_2SO_4 (5 per cent) to detect saponin extracted from *in vit*ro cultures of gymnema.

Costus pictus and gymnema showed almost same violet ring with H_2SO_4 in Molish's test. *Costus pictus* and gymnema gave blue colour in Iodine test. So the presence of amylose was confirmed. Fehling's test confirmed the presence of reducing sugars in both the plants by red precipitate with same intensity. In Benedict's test, red precipitate was observed in *Costus pictus* and gymnema. In Seliwanoff's test *Costus pictus* and gymnema gave medium red colour. The carbohydrate tests indicated the presence of monosaccharides of ketoses origin in same quantity in both gymnema and *Costus pictus*. All carbohydrate tests are similar for both plants. It may be an indication of the presence of different rare ketoses and or glycoproteins in both plants.

Xanthoprotein test for *Costus pictus* gave light yellow with HNO_3 and light orange with NaOH but gymnema gave dark yellow with HNO_3 and dark orange with NaOH. In Glyoxylic reaction for tryptophan, *Costus pictus* and

gymnema gave light violet ring. So presence of tryptophan was confirmed in both the plants. In Modified Million's test, *Costus pictus* gave yellow precipitate with $HgSO_4$ and pinkish red colour with $NaNO_3$ but gymnema gave yellow precipitate with $HgSO_4$ and dark red precipitate with $NaNO_3$. Presence of α- amino acids were confirmed by getting pinkish violet spots in *Costus pictus*. The 100Rf value 31.2 amino acid spot in gymnema was similar to *Costus pictus*.

Gymnema and *Costus pictus* had closely related three spots of amino acids in TLC. Protein tests and TLC for free amino acids and carbohydrate tests were indicating the similarity of the functioning of biomolecules in gymnema and *Costus pictus*. Gymnema is an accepted antidiabetic plant in which Conduritol-A is a sugar alcohol. More than 70% of all proteins are glycoxylated in plants. The presence of monosaccharides of ketoses origin in same quantity in gymnema and *Costus pictus* may be supporting their medicinal property.

When comparing gymnema with *Costus pictus*, the phenol spots exhibited the same 100Rf value of 83 with Folin-ciocalteu reagent. The 100Rf values of 83.7, 57.1, 21.4 and 14.2 were observed similar when the spots were viewed under UV light 253 nm and 366 nm. For flavonoids and flavones no similarity with gymnema was observed in TLC. In tannins, one spot with the 100Rf value of 86.6 in gymnema is similar to *Costus pictus*. No similarity was observed in terpenoids also. Carotene and chlorophyll b exhibited the same Rf values in both plants. All tests for alkaloids were positive. In Wagner's test for alkaloids, *Costus pictus* gave dark brown flocculent but gymnema gave medium brown flocculent.

Similarity of carbohydrate, protein, phenols and tannin between gymnema and *Costus pictus* may be exploited for their medicinal use. Presence of all secondary products at varying degree and low primary products like ketosugars may be an indication of the medicinal use of *Costus pictus*.

5.2.3 Comparison of adhatoda with *Costus pictus*

Leaves and roots of adhatoda are reported to have hypoglycemic property (Singh and Panda, 2005a). A non-nitrogenous principle from leaves was reported to be feebly hypoglycemic of short duration in male rabbits (Asolkar *et al*, 1992 a; Rastogi and Mehrotra, 1995). It is utilized in ayurvedic preparations like, decoction- musthathi kashyam, mahalothrathi kashayam, kashayam kanji, khrutham, lehyam- ekanayakara rasayanam, arishtam- devadharvyarishtam (Vaidyan, 1988).

Adhatoda and *Costus pictus*, had almost same intense violet ring with H_2SO_4 in Molish test. *Costus pictus* and adhatoda gave blue colour in Iodine test. So the presence of low concentration of amylose was confirmed. Fehling's Test confirmed the presence of reducing sugars in *Costus pictus* by giving medium red colour and adhatoda by giving light red precipitate. In Benedict's test, red precipitate was observed in *Costus pictus* while adhatoda showed green colour. Reducing sugar was indicated in lesser quantity in adhatoda when compared to *Costus pictus*. In Seliwanoff's test light red colour was observed in case of *Costus pictus* and adhatoda. Monosaccharide of ketose origin was recorded for both adhatoda and *Costus pictus* with variation in quality and quantity. This may be an indication of variation in expression of monosaccharide in both plants.

In protein analysis, *Costus pictus* gave light yellow colour with HNO_3 and light orange with NaOH but adhatoda gave dark yellow colour with HNO_3 and medium orange with NaOH. In Glyoxylic reaction for tryptophan, *Costus pictus* and adhatoda gave light violet ring. So the presence of tryptophan was confirmed at varying rate. In Modified Million's test, *Costus pictus* gave yellow precipitate with $HgSO_4$ and pinkish red colour with $NaNO_3$ but adhatoda gave yellow precipitate with $HgSO_4$ and dark red precipitate with $NaNO_3$. Adhatoda expressed four spots in TLC for amino acid but the Rf values of three spots were dissimilar to *Costus pictus*. The expression of a spot of 100Rf

value 31.2 in adhatoda was similar to that of *Costus pictus*. Free amino acids and/or glycoxylate protein might have contributed the different medicinal properties of adhatoda. Intensity of various tests for protein and carbohydrate in adhatoda and *Costus pictus* expressed dissimilarity at different level of qualitative test and concentration.

The phenol spots exhibited the same 100Rf value of 83 with Folin-ciocalteu reagent for *Costus pictus* and adhatoda. The 100Rf values of 83.7, 57.1 and 14.2 were observed similar for the two plants when the spots were viewed under UV light of 253 nm. For flavanoids, flavones, tannins, terpenoids there was no similarity in spots between adhatoda and *Costus pictus.* Carotene and chlorophyll b exhibited same Rf values in both the plants. All alkaloid tests were positive and the Wagner's test gave dark brown flocculent colour in *Costus pictus* where as adhatoda gave medium brown flocculent colour.

The above qualitative tests revealed the dissimilarity in metabolite production of leaf sample of adhatoda and *Costus pictus*.

5.2.4 Comparison of neem with *Costus pictus*

Neem oil and nimbidin have been reported to have hypoglycemic activity (Asolkar *et al*, 1992b). In many ayurvedic preparations for diabetes, neem bark, leaf, root, fruit and flower are used to prepare decoction. In musthathi kashayam, mahalothrathi kashayam preparations for diabetes neem bark is utilized. Neem leaves are utilized for the diabetic capsule preparations (Vaidyan, 1988).

Neem and *Costus pictus* expressed violet ring with H_2SO_4 in Molish's test. Neem showed dark violet ring whereas *Costus pictus* gave violet ring and not the dark –violet ring. *Costus pictus* gave blue colour and neem gave light blue colour in Iodine test. So the presence of low concentration of amylose was confirmed in neem. Fehling's Test confirmed the presence of reducing sugars in both the plants. Medium red precipitate for *Costus pictus* and deep red precipitate for neem were observed. In Benedict's test, red precipitate was observed in *Costus pictus* and brick red precipitate was observed in neem. In Seliwanoff's test, neem gave dark red colour and light red colour in *Costus pictus* leaves. Even though the taste of

neem leaf appeared to be bitter, the presences of carbohydrates were more in number with high intensity of colour expression except in Iodine test. The presence of phenolic compounds in neem might have also contributed the intensity of colour in carbohydrates because of the complex metabolic pathways involved in the neem plants. The light blue colour for Iodine test indicated the presence of low amylopectin and amylase where as the *Costus pictus* expressed standard blue colour for Iodine test, which indicated the presence of substantial quantity of amylose. *Costus pictus* was dissimilar to neem in all carbohydrate tests with respect to quantity and qualitaty.

In protein analysis, *Costus pictus* gave light yellow with HNO_3 and light orange with NaOH but neem gave dark yellow with HNO_3 and dark orange with NaOH in Xanthoproteic test. In Glyoxylic reaction for tryptophan, *Costus pictus* gave light violet ring but neem gave dark violet ring. So presence of tryptophan was confirmed in neem as in neem. In Modified Millions test *Costus pictus* gave yellow precipitate with $HgSO_4$ and pinkish red colour with $NaNO_3$ but neem showed yellow precipitate with $HgSO_4$ and medium red with $NaNO_3$.

Rf values of spots in neem were not similar to that of *Costus pictus* in amino acid analysis. Neem expressed four spots of 100Rf values 81.2, 68.7, 62.5 and 43.7. *Costus pictus* showed comparatively low quantity of carbohydrates and proteins and/or free amino acids. Presence of aromatic amino acids in the protein was confirmed by these tests in neem. The protein flexibility of both neem and *Costus pictus* can be very well observed from the analytical data of carbohydrate test and protein analysis.

Phenol spots of *Costus pictus* and neem exhibited the same 100Rf value of 83 with Folin-ciocalteu reagent and similar when the spots were viewed under UV light 253 nm. For flavonoids, flavones, tannins and terpenoids there was no spot similarity with neem. Carotene was present in both the plants. Alkaloid tests were positive for both the plants. Even though a few tests were positive in the quality tests of both neem and *Costus pictus*, the dissimilarity was dominating in majority of the test for metabolites.

5.2.5 Comparison of tulsi with *Costus pictus*

The leaves of tulsi contain essential oils with eugenol, carvacrol, methylchavicol, limatrol and caryophylline as chemical constituents (Singh and Panda, 2005b).The presence of essential oil in *Costus pictus* has not been reported earlier.

Tulsi and *Costus pictus*, showed almost same intense violet ring with H_2SO_4 in Molish test. *Costus pictus* gave blue colour and tulsi gave light blue colour in Iodine test. So the presence of low concentration of amylose was confirmed in tulsi. Fehling's test confirmed the presence of reducing sugars in both the plants by red precipitate with same intensity. In Benedict's test, red precipitate was observed indicating reducing sugar in both the plants. In Seliwanoff's test light red colour was observed in case of *Costus pictus* and dark red colour in tulsi. The dark red colour of tulsi sample for Seliwanoff's test indicated the presence of high quantity of hexoketoses.

In protein analysis, *Costus pictus* gave light yellow colour with HNO_3 and light orange with NaOH during the reaction but tulsi gave dark yellow colour with HNO_3 dark orange colour with NaOH in Xanthoproteic test. In Glyoxylic reaction for tryptophan, *Costus pictus* and tulsi gave light violet ring. So tryptophan presence was confirmed in tulsi as in *Costus pictus*. In Modified Millions test *Costus pictus* gave yellow precipitate with $HgSO_4$ and pinkish red colour with $NaNO_3$ but tulsi gave yellow precipitate with $HgSO_4$ and medium red with $NaNO_3$. Tulsi expressed two spots similar to *Costus pictus* with a third spot similar to that of ginger. High quantity of hexoketoses in tulsi might have contributed glycosylated protein and medicinal properties of tulsi now in *Costus pictus* also.

There were three spots of phenolics present in tulsi, of which *Costus pictus* and tulsi exhibited the same 100Rf value of 83 with Folin -ciocalteu reagent. The 100Rf values of 83.7and 14.2 were observed similar for both the plants under UV light 253 nm. There was a third spot of 100Rf value 64.2 at 366nm in tulsi only. For flavonoids, flavones, tannins and terpenoids no spot similarity was observed

for *Costus pictus* and tulsi. Carotene, chlorophyll a and b were present in both the plants with same Rf value.

Alkaloid tests were positive for both the plants. In Wagners test for alkaloids, *Costus pictus* gave dark brown flocculent colour but tulsi gave medium brown flocculent colour. The 100Rf value of 50 and 31.2 for amino acids in TLC were similar to *Costus pictus*

5.3 ELECTROPHORESIS STUDIES ON SELECTED ANTIDIABETIC PLANTS WITH SPECIAL REFERENCE TO *COSTUS PICTUS*.

5.3.1 Standardization of protein extraction

Most of the proteins separated electrophoretically were soluble enzymes. Their extraction from cell resulted breaking of the cell wall or membrane and releasing the enzyme/ protein into appropriate buffer solution like mild Tris-HCl buffer. In some cases reducing agents are also required to prevent the oxidation or inhibition of many of the enzymes and they were included in the extraction buffer (May, 1994). In the present study, Tris-HCl, Tris–HCl + ingredients and Phosphate buffers were tried. The Tris-HCl + ingredients buffer was giving clear banding pattern with maximum number of bands. Tris-HCl in the extraction buffer might have maintained the osmotic stability and citric acid, ascorbic acid and cystein-HCl protected the proteins from denaturation.

5.3.2 Poly acrylamide gel electrophoresis

The separation of bands was very sharp and also this gave stiff gels, which enabled easy handling of gel. The 10% gel gave good separation of bands. In the gel preparations 10% SDS was added to denature the proteins. Andrews (1986) already reported the optimum current for PAGE. Initial run with 15mA and in separating gel 30mA current was found good.

Silver staining and coomassie brilliant blue staining were compared for selecting the most suitable stain. In coomassie brilliant blue staining, the proteins absorb the coomassie brilliant blue whereas in silver staining the amino acids particularly aromatic amino acids in the protein reduce silver nitrate and form

complexes with metallic silver of yellowish brown-to-brown colour. The destaining of the dark background was found difficult in silver staining.

While comparing *Costus pictus* with other plants with respect to protein-banding pattern, *Costus pictus* exhibited one band similar to adhatoda. Ginger expressed 3 bands. Next to ginger, gymnema, neem and tulsi showed similar 2 bands corresponding to it.

In this study the extraction buffer played a major role in the protein banding pattern of the leaf samples of plants which were having acidic, neutral and/or basic nature. The efficiency of the protein banding can be obtained only by considering the properties of each plant separately. The present study also supported the above statement by showing maximum bands in Tris+ ingredients buffer of *Costus pictus* leaf. Purpose of including this work was to get an idea of the protein banding at molecular level. It can be taken up as separate project for screening at molecular level.

5.4 ISOZYME STUDIES ON SELECTED PLANTS WITH SPECIAL REFERENCE TO *COSTUS PICTUS*.

The antioxidant effect of an aqueous extract of *Phaseolus vulgaris* pods, an indigenous plant used in Ayurvedic medicine in India, was studied in rats with streptozotocin-induced diabetes by Venkateswaran and Pari (2002). Oral administration resulted in a significant reduction in thiobarbituric acid reactive substances and hydroperoxides. The extract also causes a significant increase in reduced glutathione, superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase in the liver and kidneys of rats with streptozotocin-induced diabetes. These results clearly revealed the antioxidant property of the extract. The effect of the extract at 200 mg/kg body weight was more effective than glibenclamide.

While comparing isozyme banding pattern, *Costus pictus,* ginger and tulsi showed similar peroxidase-banding pattern in the zymogram. Ginger had one

additional band also. The presence of oxalic acid and oxalate oxidase enzyme in *Costus pictus*, might have produced H_2O_2. The released H_2O_2 in *Costus pictus* may contribute the performance of peroxidase enzyme and expressed difference in banding pattern from other plant samples.

Costus pictus is a plant surviving in marshy land. It may have the ability to protect the plant from rot diseases and survival in waterlogged area due to the balanced mechanism of hydrogen peroxide production and peroxidase activity. Hydrogen peroxide production may be due to the availability of oxalic acid and oxalate oxidase enzyme. The hydrogen peroxide from the oxalic acid may become the substrate for the peroxidase enzyme in *Costus pictus* . The above mechanism and environmental factors might have influenced the growth and rooting pattern of *Costus pictus*.

5.5 IMMOBILIZATION STUDIES ON *COSTUS PICTUS*

The major advantage of plant cell immobilization is that it provides high cell concentration per unit volume of reactor, better cell to cell contact, and more favourable conditions for cell differentiation, resulting in higher yields of secondary metabolites (D'Sauza, 1989).

High productivity has also been attributed to rudimentary differentiation achieved by immobilization process. Immobilization of plant cells i.e. physically restraining the cells on a fixed support can be seen as an intermediate stage between homogenous suspension culture and the highly structured tissue matrix of the whole plant (Lindsey and Yeoman, 1985)

5.5.1 Plant Regeneration

Regeneration was obtained from nodal buds of *Costus pictus* in MS supplemented with 2ppm BA and 2ppm KN and 1ppm NAA as mentioned by Malabadi (2002).

5.5.2 Callus Induction

Costus pictus belongs to the family Zingiberaceae and this plant lacks *in vitro* studies. So the media tried in Zingiberaceous crops were exploited. ½ MS+ 1ppm BA+ 3ppm NAA was found the best because it gave creamy white glossy callus. But it took more time from callus initiation to callus formation. In ½ MS + 1ppm 2,4-D + 0.5ppm BA leaf bits showed rapid callusing from the sides. The colour of the callus was brown and easy callus formation was observed. Since phenolics interference was there in the callus by expressing brown colour, the viability of callus for long time is doubtful

5.5.4 Suspension culture

Suspension cultures were obtained by agitating the callus cells in ½ MS liquid medium + 0.5ppm 2,4-D at 90 rpm on rotary shaker for one week for the separation of single cells. To obtain friable callus 2,4-D was added in the liquid media.

5.5.3 Immobilization of cells

The alginate beads after immobilization were kept in ½ MS liquid media for the secondary metabolite production and kept in rotary shaker 90 rpm at 30^0C under light intensity of 2000 lux and the media harvested 15^{th} day for secondary metabolite analysis.

5.5.3.1 Secondary metabolite production

Fresh ½ MS liquid media was also extracted using ethyl acetate and taken as a control, because media also contains many chemicals.

The TLC of samples from immobilization gave positive result for phenols, terpenoids, plant acids and amino acids. Thus, the secondary metabolites produced by the immobilized plant cells were observed to be leached out into the media.

Based on the analysis it is evident that phenolics production in *Costus pictus* is there even in the immobilized cells of callus. This observation is an

indication of the presence of phenolics-the inevitable evil in all types of cell are present in the callus cells of Costus *pictus.* Considering the medicinal use of selected phenolics and production of desired phenol by immobilization and other techniques in the preliminary work carried out in *Costus pictus* revealed that the phenolics observed were different from the leaf samples. This is a promising observation which can be exploited for the production of desired phenolics from callus of *Costus pictus.*

So the immobilization improved technological properties of biocatalysts, converting from water soluble to water insoluble molecules and thereby permitting their use in conventional chemical reactors can be adopted from the cells of *Costus pictus*.

In conclusion it can very well be presumed that the *Costus pictus* plant provide a cheap, efficient and safe system for the production of secondary metabolites for human therapeutics and plant disease control.

Summary

6. SUMMARY

India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants for human health care purposes. Nearly 80 per cent of the population of developing countries like ours relies on traditional medicines for their primary health care needs (Kamboj, 2000). Under such a situation comes the importance of the medicinal plant *Costus pictus* D.Don. a folk remedy for blood sugar control. The present study aims to identify the biochemical constituents in *Costus pictus* and compare with selected antidiabetic plants. The salient findings of the research are summarized below:

- The biochemical characterisation of *Costus pictus* and selected plants to identify its primary and secondary metabolites was carried out by qualitative tests and also by TLC. Qualitative tests were conducted for primary metabolites such as carbohydrates and proteins. TLC for amino acids of proteins/free amino acid present in selected plants was carried out.
- Carbohydrate test indicated the presence of monosaccharides of ketohexose origin in selected plants. *Costus pictus* expressed low quantity of ketohexoses. It was dissimilar to Neem in all carbohydrate tests with respect to colour intensity. The presence of glycosylated biomolecules of diverse origin may be available in *Costus pictus*.
- Qualitative tests revealed that *Costus pictus* has low concentration of protein with aromatic amino acids and / or free amino acids, including Tryptophan. The pink spot in TLC confirmed the presence of 2- amino acids in all selected plants. Variation in spot pattern in TLC revealed the flexibility of protein functioning in biological system. Considering the presence of hexoketoses, free amino acids and / or soluble protein; amyloproteins in Gymnema and *Costus pictus* cannot be ruled out.
- Gymnema and *Costus pictus* recorded four similar compounds of phenolic origin. This similarity might have contributed by enzymes of glycoprotein

origin. The presence of hydrolysable tannins in *Costus pictus* and Gymnema also attracts the medicinal use of plants as antidiabetic plants.

- Secondary metabolites such as phenols, tannin, flavonoids, quinones, terpenoids, saponins, carotenoids, alkaloids, and plant acid were also expressed in the selected plants.
- For the cell immobilization study, Callus induction and proliferation from leaf bits obtained in ½ MS + 1ppm 2, 4-D+ 0.5ppm BA, ½ MS+ 1ppm BA+ 3ppm NAA medium.½ MS + 3ppm BA was found good for regeneration from rhizome buds. Suspension cultures were obtained by agitating the callus cells in ½ MS liquid medium + 0.5ppm 2,4-D at 90 rpm on rotary shaker. Immobilized cells as alginate beads in ½ MS liquid media showed the presence of phenolics, terpenoids, plant acids and amino acids in qualitative tests. Immobilization technique can be exploited for the production of secondary metabolites from *Costus pictus* plant.

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∗ Originals not seen

Appendices

APPENDIX I

Laboratory equipments used for the study

Spectrophotometer	Spectronic Genesys – 5, Spectronic Instrument, USA
Refrigerated centrifuge	Kubota, Japan
Water purification system	Millipore, Germany
Vertical electrophoresis system	Mini-Protein system, Biorad, USA
Ice flaking machine	Ice matics
Gel documentation system	1. Biorad 2. Alpha Imager

APPENDIX II

a) Reagents for carbohydrate qualitative tests

Iodine Solution: A few crystals of iodine solution to 2% potassium iodide solution was added till the color becomes deep yellow.
Fehling's Reagent A and Fehling's Reagent B
Benedict's Reagent: 173g of sodium citrate and 100g sodium carbonate dissolved in about 800ml of water and the solution was heated to dissolve the salts.
Seliwanoff's Reagent: Dissolve 0.05g of resorcinol dissolved in 100ml diluted (1:2) hydrochloric acid.
Bial's Reagent: 1.5g orcinol was dissolved in 500 ml of concentrated HCl and 20 drops of 10% ferric chloride was added.

b) Reagents for SDS PAGE

a) Stock Acrylamide solution
Acrylamide 30% 30g
Bisacrylamide 0.8% 0.8g
Water to 100ml

b) Separating gel buffer
1.875M Tris-HCl 22.7g pH8.8
Water to 100ml

c) Staking gel buffer
0.6M Tris-HCl 7.26g pH6.8
Water to 100ml

d) Polymerizing agents
a) Ammonium persulphate 5% 0.5g/10ml, prepared freshly before use
b) TEMED fresh from the refrigerator

e) Electrode buffer

0.05M Tris	3g	
0.192M Glycine	7.2g	pH 8.2-8.4
0.1% SDS	0.5g	No adjustment required
Water to	500ml	

f) Sample buffer (5x concentration)

Tris-HCl buffer pH 6.8	5ml
SDS	0.5g
Sucrose	5g
Mercaptoethanol	0.25ml
Bromophenol blue	1ml
(0.5%W/V solution in water)	
Water to	10ml

Stored frozen small aliquots. Diluted to 1x concentration and used.

g) SDS	10% solution	stored at room temperature

h) Protein stain solution

Coomassie brilliant blue R250	0.1g
Methanol	40ml
Acetic acid	10ml
Water	50ml

First dissolve the dye in methanol and proceed. Use fresh preparation every time.

i) Destainer

Methanol	40ml
Acetic acid	10ml
Water	50ml

c) Reagents for isozyme analysis

All reagents without adding SDS were used.

Staining solution	
Benzidine	1.04g
Acetic acid	9ml
Hydrogen	
Peroxide (3%)	50ml
Water	40ml

d) Composition of MS plant tissue culture media

Constituent	Murashige and Skoog (1962) – MS (mg l^{-1})	Gamborg's (1968) – B_5 (mg l^{-1})
KCl	-	-
$MgSO_4.7H_2O$	370	250
$NaH_2PO_4.H_2O$	-	150
$CaCl_2.2H_2O$	440	150
KNO_3	1900	2500
$CaCl_2$	-	-
Na_2SO_4	-	-
NH_4NO_3	1650	-
KH_2PO_4	170	-
$Ca(NO_3)_2.4H_2O$	-	-
$(NH_4)_2SO_4$	-	134
$FeSO_4.7H_2O$	27.8	-
$MnSO_4.4H_2O$	22.3	-
$MnSO_4.H_2O$	-	10
KI	0.83	0.75
$CoCl_2.6H_2O$	0.025	0.025
$Ti(SO_4)_3$	-	-
$ZnSO_4.7H_2O$	8.6	2
$CuSO_4.5H_2O$	0.025	0.025
H_3BO_3	6.2	3
$Na_2MoO_4.2H_2O$	0.25	0.25
$Fe_2(SO4)_3$	-	-
EDTA disodium salt	37.3	-
EDTA-Na ferric salt	-	43
m-inositol	100	100
Thiamine	0.1	1.0
Pyridoxine	0.5	1.0
Nicotinic acid	0.5	1.0
Glycine	2	-
Cysteine	-	10
Sucrose	30,000	20,000

Dedicated to my loving family

Biochemical characterization and cell immobilization of *Costus pictus* D. Don with special reference to antidiabetic property

By

T. ELIZA LINCY

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture
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Centre for Plant Biotechnology and Molecular Biology
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ABSTRACT

Kerala, a state known for its indigenous knowledge and traditional healing practices, is endowed with thousands of medicinal plants. More than 1200 species of plants are being used in the indigenous system of medicine in the state. The use of synthetic chemicals in modern medicine has been causing several side effects. Hence, more and more scientific and commercial activities are now directed towards plant-based medicines.

The present study was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2004-2006. The objective of the study was the biochemical characterization and cell immobilization of *Costus pictus* with special reference to antidiabetic property.The hypoglycemic properties of the plant has been reported both in streptozotocin and alloxan induced diabetes in animals. The toxicity study of crude extract of the plant was also studied (Benny, 2004a; Benny *et al.*, 2004a & b) and reported a positive sign of non-toxicity. Based on the above facts the biochemical characterisation and cell immobilization of *Costus pictus* was carried out and this was compared with antidiabetic plants like ginger, gymnema, adhatoda, neem and tulsi.

Molisch's test, Iodine test, fehling's test, benedict's test and seliwanoff's test confirmed the presence of carbohydrates in *Costus pictus* and in all selected plants with varying intensities. Carbohydrate tests indicated the presence of monosaccharides of ketohexoses origin in all selected plants including *Costus pictus*. xanthoproteic reaction, glyoxylic reaction for tryptophan and modified million's test confirmed the presence of proteins having aromatic amino acids in *Costus pictus* and in all selected crops. TLC also confirmed the presence of α-amino acids by giving pink colour with ninhydrin.

The best separation of phenolic spots occurred on acid hydrolysis. The phenols appeared as dark absorbing spots in 253 nm and fluorescent spots in 366 nm. Phenol spots appeared as blue with folins reagent. Four compounds were isolated in TLC from *Costus pictus*. Ethanol was used as the solvent for the

extraction of flavonoids from leaf samples. There was only one spot (Rf value 70) at 253nm in *Costus pictus*. The red colour production observed in neem and tulsi leaf samples confirmed the presence of condensed tannins. The presence of four hydrolysable tannin in *Costus pictus* was confirmed.

The TLC for terpenoids, *Costus pictus* and ginger expressed same compound at Rfx100 value 71.4. Gymnema, adhatoda and neem had different spots. In TLC the carotenoids expresed different coloured spots such as green-chlorophyll b, blue green-chlorophyll a, yellow green-xanthphyll and yellow-β-carotene in ascending order under visible light. TLC for plant acids, *Costus pictus* only gave spot only similar to that of oxalic acid. Mayer's test, dragendorff's test and wagner's test showed positive result for alkaloid in all the plants studied.

All the above antidiabetic plants for comparison were subjected to electrophoresis. Protein extraction was carried out using Tris-HCl, Tris-HCl + ingredients and Phosphate buffers. The Tris-HCl +ingredients buffer was found to be more appropriate on account of discrete and distinct banding pattern. Protein-banding pattern in SDS denatured gel conditions and isozyme (peroxidase) banding in non denaturing gels were also studied. The 10% gel gave good separation of bands. The bands were clear in coomassive brilliant blue stain than in the silver staining. *Costus pictus* exhibited four bands. Isozyme analysis was carried out using peroxidase enzyme system. *Costus pictus* exhibited four bands in which three are similar to that of ginger.

The cells of *Costus pictus* were immobilized using sodium alginate. Immobilized cells kept for secondary metabolite production in ½ MS liquid media showed the presence of phenolics, terpenoids different Rf values and plant acids, indicating the utility of the technique of producing secondary metabolites from *Costus pictus in vitro*. Considering the presence of similar hexoketoses, free amino acids and / or soluble protein and phenolic compounds in gymnema and *Costus pictus* cannot be ruled out for the medicinal use of *Costus pictus* as an antidiabetic plant. Detailed study on *Costus pictus* with respect to protein and oxalic acid are needed to obtain more information of the antidiabetic property and toxicity to biological system.