Introduction

*N*ature has bestowed on us a very rich botanical wealth and number of diverse types of plants which grow wild in different parts of the country. The compounds of medicinal value from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries. Hence these compounds give formulation to various medicines. Therefore standardizatition of these compounds from plant materials is the need of the day. Hence the modern methods describing the identification and quantification of active constituents of medicinal potency in the plant species may be useful for their proper standardization of herbs and their further formulation. The WHO has emphasized the need to ensure the quality of medicinal plant products using modern controlled techniques and applying suitable standards (Sharma *et al*., 2010). Authentication of medicinal plants at genetic and chemical and medicinal level is a critical step in the use of these for both research purposes and commercial preparations.

Preliminary biochemistry of medicinal plants may be useful in the detection of their bioactive principles and subsequently may lead to drug discovery and development. Further, these tests facilitate their quantitative estimation and qualitative separation of pharmacologically active chemical compounds. Although, some studies on *Tinospora cordifolia* have been done by many researchers to obtain purified plant chemicals, very few screening programmes have been initiated on crude extracts of this plant.

It has also been widely observed and accepted that the medicinal value of plants lies in the bioactive phytochemicals present in the plant. Thus, chapter deals with the biochemical analysis of *Tinospora cordifolia* plants parts *in vivo* and *in vitro.* The investigations were carried out on the following parameters:

6.1 Preliminary biochemical studies and primary metabolites *in vivo* and *in vitro*

6.2 Qualitative and Quantitative determination of secondary metabolites *in vivo* and *in vitro*

6.1 PRELIMINARY PHYTOCHEMICAL STUDIES AND PRIMARY METABOLITES *IN VIVO* AND *IN VITRO*

Preliminary biochemical studies are helpful in finding chemical constituents in the plant material that may well lead to their quantitative estimation. Biochemical studies of the plants are required for standardization, which helps in understanding the significance of phytoconstituents in terms of their observed activities. Different parameters of preliminary biochemical studies i.e. ash value, extractive value, qualitative analysis and presence of different organic constituents are important for the evaluation and biochemical investigation of the identified plant metabolites.

Biochemical investigations of *Tinosora* species have been carried out earlier by various workers. *T. cordifolia* has been found to be a rich source of various biochemical compounds like flavonoids, alkaloids, triterpenoids, steroids, glycosides, anthocyanins etc.

(Shrivastava, 1953; Tripathi *et al.* 1973; Joshi *et al*., 1974; Chattopadhyaya and Pakra.shi, 1975; Laxmi *et al*. 1976; Agarwal and Mishra, 1979; Khurdiya and Anand, 1981; Ali *et al.,* 1982; Morton, 1987; Parveen *et al.* 2012). However *Tinospora* species still needs further biochemical investigation to elaborate and utilize their medicinal and pharmaceutical values to the fullest.

6.1A PRELIMINARY PHYTOCHEMICAL STUDIES

Preliminary biochemical studies of *Tinospora cordifolia* plant parts (*in vivo* and *in vitro*) included the following tests:

6.1A(a) Determination of ash content

6.1A(b) Determination of extractive values

6.1A(c) Qualitative analysis of phytochemicals.

6.1A(a) DETERMINATION OF ASH CONTENT

There is a relationship between the element content of the plant and its nutritional status. It is clear that mineral nutrition is important to maintain good health and because of that determination of As, Ca, Fe, Mg, Na, K, Zn, Ni, Co etc. have been added to Ayurvedic Pharmacopoeia of India (The Ayurvedic Pharmacopoeia of India, 1999). Ash usually represents the inorganic part of the plant. The amount and composition of ash remaining after combustion of plant material varies considerably according to the part of the plant, age, treatment etc. The objective of ashing plant material is to remove all traces of organic matter, which might otherwise interfere in analytical determination.

When the powdered drug is ignited at 700^0C a white coloured ash is obtained. It is total ash that contains inorganic salts in oxide form. Water soluble ash contains inorganic salts which are soluble in water.

MATERIAL AND METHODS

Ash content in the *in vivo* and *in vitro* plant parts of *Tinospora cordifolia* was calculated by the methods given below:

(i) Determination of total ash

Five grams of the ground plant material was taken in a silica crucible previously ignited and weighed. The ground plant material was then spread in a fine and even layer on the bottom of the crucible. It was then incinerated in a muffle furnace by gradually increasing the heat not exceeding dull red heat until free from carbon and then cooled and weighed. The residue was collected on an ash less filter paper which was then incinerated. The ash percentage was calculated with reference to the air dried material.

$$\text{Ash percentage} = \frac{W2-W1}{W} \times 100$$

Where, w1= weight of crucible (g)

w2= weight of crucible with the ash (g)

w = weight of dried sample (g)

(ii) Determination of Water Soluble Ash

100 mg of ash was boiled for five minutes with 10 ml of distilled water. The insoluble matter was collected in a silica crucible or on an ash less filter paper. It was washed with hot water and then ignited to constant weight at low temperatures. The weight of the insoluble matter was subtracted from the weight of the ash. The percentage of water soluble ash was calculated with reference to the amount of ash taken.

$$\text{Water soluble ash (\%)} = \frac{\text{Weight of acid insoluble ash}}{\text{Weight of sample taken for ashing in total}} \times 100$$

(iii) Determination of Acid Insoluble Ash

The total ash was boiled for five minutes with 25 ml of 10% HCl. The insoluble ash was collected in a silica crucible or on an ashless filter paper. It was washed with hot water and then ignited and weighed. The weight of the insoluble matter was subtracted from the weight of ash. The difference in weight represents the acid insoluble ash. The percentage of acid insoluble ash was calculated with reference to the amount of ash taken.

$$\text{Acid insoluble ash (\%)} = \frac{\text{Weight of water soluble ash}}{\text{Weight of sample taken for ashing in total}} \times 100$$

OBSERVATIONS AND RESULTS

The powdered plant parts of *Tinospora cordifolia* found to contain an average of total ash value 12.58%, 11.48% and 8.34% in callus, leaf and stem powders respectively. Out of which 6.46%, 5.96% and 6.06% respectively were water soluble ash and 3.29%, 2.13% and 1.87% respectively of ash content were acid insoluble ash. (Table- 7.1).

Total ash value indicated the presence of inorganic constituents. Thus among these three plant parts (*In vivo* and *in vitro*) in *Tinospora cordifolia* callus has maximum inorganic constituents whereas stem has least. Among *in vivo* and *in vitro* parts, *in vitro* part (calli) has maximum inorganics in all three plant parts.

6.1A(b) DETERMINATION OF EXTRACTIVE VALUES

Medicinal principles are present in different parts of the plant like root, stem, bark, heartwood, leaf, flower, fruit or plant exudates. These medicinal principles are separated by different processes; the most common being extraction.

The present study on *Tinospora* species was aimed to know the extractive values of *in vivo* and *in vitro* plant parts of *Tinospora cordifolia* plant powders with different solvents; that isolation and quantification of chemical substances found in plant species can be easily carried out with the solvents so of higher extractive values and solvents with lower extractive value may be avoided.

MATERIAL AND METHODS

Estimation of extractive value was done according to Regina (2003). The different plant materials eg. *in vivo* (stem and leaf) and *in vitro* (9weeks old callus) plant parts of *Tinospora cordifolia* were taken for investigation. The materials were kept on blotting paperand left for air drying and then powdered. Then, a known quantity (5gms) of plant part powders were taken separately. Extraction was made in soxhlet apparatus with different solvents *i.e.* petroleum ether, ethyl acetate, benzene, methyl

alcohol, chloroform, ethyl alcohol and water. The extracts were filtered in the flasks of known weight and the solvents were evaporated, accurate weights of extracts were taken. The extractive percentage (%) was calculated with reference to air dried plant powders.

$$\text{Extractive Value (\%)} = \frac{\text{Weight of extract}}{\text{Weight of dried sample taken}} \times 100$$

OBSERVATION AND RESULTS

It is observed from the Table-7.2, that the extractive values were found maximum with methanol for all tested plants and plant parts of *Tinospora cordifolia*. Extractive values with ethanol, ethyl acetate and dist. water were also considerable.

6.1A(c) QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

The traditional medicine involves the use of different plant extracts or their bioactive constituents. Secondary metabolites are responsible for medicinal activity of plants. Hence in the present study phytochemical screening of *in vivo* and *in vitro* elements of *Tinospora cordifolia* were carried out. For qualitative phytochemical analysis, these plant parts were extracted in different solvents like ethanol, methanol, ethyl acetate, hexane, petroleum ether, chloroform and distilled water. This was aimed to confirm the presence of various phytochemicals like alkaloids, tannins, glycosides, flavonoids, steroids, terpenoids, phenols, coumarins, and resins in different extracts.

MATERIAL AND METHODS

The *in vivo* plant materials (stem, leaf and root) *Tinospora cordifolia* were collected and gently washed to remove all the soil particles. The mature calli (9weeks old) of test plants were taken as *in vitro* material. The separated plant parts were allowed to dry at room temperature. The dried plant materials were ground to powder form and used for the experiments. The prepared by the method described by Savithramma *et al*. (2011). 5gm of all plant samples (leaf, stem and callus) were weighed and were soaked in 25ml of ethanol, methanol, hexane, chloroform, petroleum ether, ethyl acetate and distilled water in the ratio 1:5 (weight: volume). These were allowed to stand at ambient room temperature for 48 hours. All the extracts were centrifuged at 5000rpm for 15mins. The supernatants were collected and tested for the presence of various metabolites. For this purpose, conventional procedures illustrated by Chitravadivu *et al.* (2009), Savithramma *et al.* (2011) and Waliullah *et al.* (2011) were followed.

QUALITATIVE ANALYSIS

1. Alkaloids

0.2g of each of the fractions was warmed with 2% H_2SO_4 for two minutes. The reaction mixtures were filtered and few drops of Dragendroff's reagent (0.8 gm Bismuth nitrate pentahydrate dissolved in 40 ml dist. water added with 10ml glacial acetic acid and mixed with 8.0gm Potassium iodide in 20ml dist. water) was added to each filtrate. Orange red precipitate indicates the presence of alkaloids moiety.

2. Tannins

A small quantity of each extract was mixed with water and heated on water bath and filtered. A few drops of ferric chloride were added to each of the filtrates. The dark green solution indicated the presence of tannins.

3. Anthraquinones

0.5 g of each extract was boiled with10% HCl for few minutes on water bath. The reaction mixtures were filtered and allowed to cool. Equal volume of HCl was added to each filtrate. Few drops of 10% ammonia was added to each mixture and heated. Rose pink colour formation indicated the presence of anthraquinones.

4. Glycosides

Each extract was hydrolyzed with HCl and neutralized with NaOH solution. A few drops of Fehling's solution A and B were added to each mixture. Formation of red precipitate indicated the presence of glycosides.

5. Saponins

0.2 g of each extract was shaken with 5ml of distilled water and heated to boiling. Frothing (appearance of creamy miss of small bubbles) showed the presence of saponins.

6. Flavonoids

0.2 g of each extract was dissolved in diluted NaOH and few drops of HCl were added. A yellow solution that turns colourless indicated the presence of flavonoids.

7. Steriods/ Terpenoids

1 ml of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turned red and sulphuric acid layer showed yellowish green fluorescence. That indicated the presence of steroids.

8. Phenols

To 2ml of test solution, alcohol and then few drops of neutral ferric chloride solution were added. Reddish brown coloured appearance indicated the presence of phenols.

9. Coumarins

3 ml of 10% NaOH was added to 2 ml of aqueous extract, formation of yellow colour indicated the presence of coumarins.

10. Resins

To the 0.2 gm of each extract added 10ml of glacial acetic acid then heated and cooled. A drop of concentrated H_2SO_4 was added. Purplish red colour showed the presence of resins.

OBSERVATION AND RESULTS

According to the observations of various chemical reactions done for the purpose of screening of *Tinospora cordifolia* plant parts for various metabolites are tabulated in Tables-7.3. From the results it was observed that this plant contain majority of tested metabolites. For the purpose of screening methanolic, ethanolic and ethyl acetate extracts of plants were found better suited for maximum metabolites viz. alkaloids, tannins, glycosides, flavonoids, steroids, terpenoids, phenols, coumarins, and resins. While chloroform fractions of plant parts were found to contain tannins, saponins, steroids, terpenoids, phenols and resins. Extracts in sterile distilled water revealed the presence of tannins, anthraquinones, flavonoids and phenols. Hexane and petroleum ether solvents were found least suitable for extraction purpose. *In vivo* parts of *Tinospora cordifolia* was found to be least in metabolites than callus. *Tinospora cordifolia* was found to contain most of the screened metabolites in different solvents while alkaloids, steroids and flavanoids were abundant in plant extracts.

Overall, qualitative phytochemical analysis of *in vivo* and *in vitro* plant parts of *Tinospora cordifolia* in different solvents showed that the medicinal value of these plants can be explained on the basis of presence of diverse bioactive secondary metabolites.

6.1A CONCLUSIONS

From the overall observations and results on preliminary phytochemical studies in *Tinospora cordifolia* following important points were concluded:

- *Tinospora cordifolia* was found to be rich of inorganic elements in analysis of their ash values as the average ash values ranges from 7.34±0.26% to 12.58±0.10%. Among *in vivo* and *in vitro* parts, *in vitro* part (calli) has rich in inorganics.
- The extractive values were found maximum with methanol in most of the plant parts. Extractive values with ethanol, ethyl acetate and dist. water were also considerable. But the extractive contents with hexane, chloroform and petroleum ether were poor for *Tinospora cordifolia*.
- All the tested metabolites were found to be present in different parts of *Tinospora cordifolia*. *In vivo* parts of *Tinospora cordifolia* found to be lesser in metabolites than *in vitro*.

6.1B QUANTIFICATION OF PRIMARY METABOLITES

Medicinal plants form the backbone of Traditional System of medicine in India. Pharmacological studies have acknowledged the value of medicinal plants as potential source of bioactive compounds (Prusti et al., 2008). Phytochemicals from medicinal plants serve as lead compounds in drug discovery and design. Medicinal plants are rich source of novel drugs that forms the ingredients in Traditional System of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs. This emerging field is oriented towards the characterisation of small metabolites that act as substrates, products, ligands and signalling entities in cells. Biological system are under constant challenges from the environment. Adaptation to environmental stimuli is reflected in alterations in the genome, the transcription of

genes, the expression level and post-translational modifications of proteins and in the primary and secondary metabolism. Deciphering of the plant metabolome is one of the most difficult analytical tasks in functional genomic research (Stobiecki and Kachlicki, 2005). The term metabolomics stands out from any other organic compound analysis in scale and in chemical diversity, i.e., all metabolites are aimed to be described, both primary and secondary metabolites, present in an organism or biological system. In plants, the characterization of endogenous primary and secondary metabolites is of interest for the quality and improvement of crops, as well in the study of e.g. physiology, ecology and development phenomena in plant biochemistry. (Bino et al., 2004;). Primary metabolic routes produce primary metabolites, which are present almost everywhere in nature and are essential for all life forms. These compounds include the common carbohydrates, fats, proteins and nucleic acids that are needed to create and maintain life. Apart from fats, the compounds are polymeric and usually chemically large molecules. Typically they are involved in the energy regulation of organisms and with growth and development of tissues; in short, they are the building blocks of organisms. Through the ages, human have relied on nature for their basic needs. Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population (Talreja, 2011). Primary metabolite analysis is necessary for knowing the nutritional potential of plants and then also from the precursors for the synthesis of secondary metabolites (Tatsuta and Hosokawa, 2006; Vijayvergia and Kumar, 2007). Primary plant metabolites are simple molecules or polymers of simple molecules synthesized by plants, generally do not possess therapeutic as such but essential for the life of plants and contain

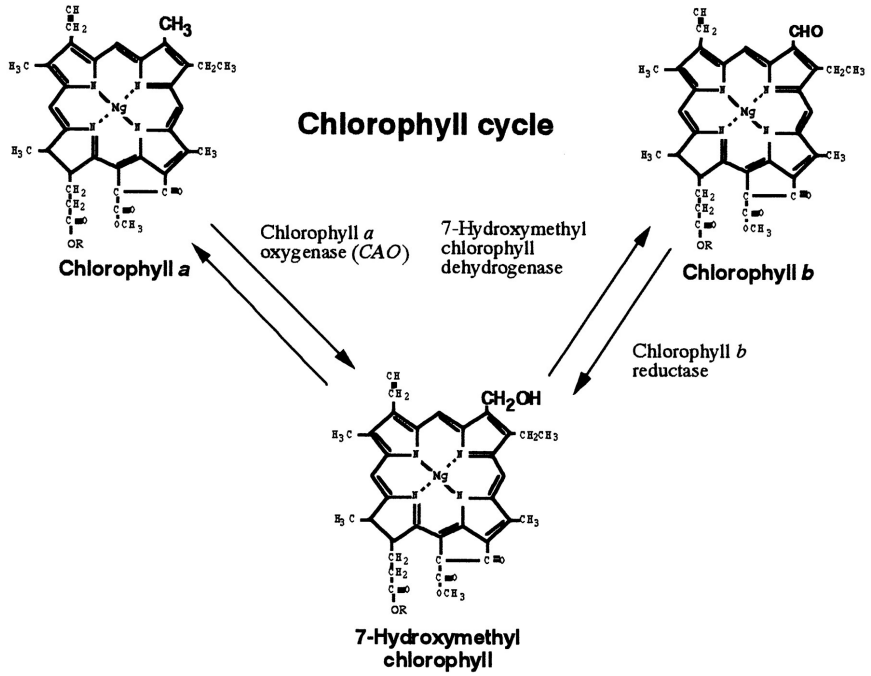
high-energy bonds. These are used up for the biosynthesis of secondary metabolites (Harada and Fukusaki, 2009).

Keeping in view the importance of these primary metabolites, during the studies various potent primary metabolites viz., chlorophyll, carbohydrates (sugar, starch), total protein, ascorbic acid and total phenol have been investigated from in vivo root, stem, leaf and *in vitro* callus in *Tinospora cordifolia*.

CHOLOROPHYLL

INTRODUCTION

The Chlorophyll is one of the essential components for photosynthesis in plants. They occur in chloroplast as green pigments in all the photosynthetic tissues. Chemically, each chlorophyll molecule contains a porphyrin nucleus with a chelated magnesium atom at the centre and a long chain hydrocarbon attached through a carboxylic group. The different types of chlorophyll like chlorophyll a, b and carotenoids are present in a whole pigment extract of green plant tissues.



PRINCIPLE:

Chlorophyll is extracted and the absorption at 663 nm and 645 nm are read using spectrophotometer. Using the absorption coefficient the amount of chlorophyll is calculated by Arnon's method, 1949, using the formula:

$$\text{Chlorophyll a} = \frac{(12.3 \times \text{O.D. at } 663 - 0.86 \times \text{O.D. at } 645) \times V}{1000 \times W}$$

$$\text{Chlorophyll b} = \frac{(19.3 \times \text{O.D. at } 645 - 3.6 \times \text{O.D. at } 663) \times V}{1000 \times W}$$

Total chlorophyll= a+b

Where,

V= Final volume of chlorophyll extract in 80% acetone

W= Fresh weight of tissue extracted

MATERIAL:

Dilute analytical grade acetone to 80% prechilled acetone.

METHOD:

(i) Extraction: 1 gm of plant material was crushed in chilled 80% acetone and extracted thrice by centrifugation at 5000rpm for 5 minutes. All the supernatants were pooled and the volume of the extract was made upto 4 ml.

(ii)Estimation: Absorbance of this solution was read at 645 and 663 nm against 80% acetone used as blank. Results presented were average of three replicates.

\RESULT:

Different amount of chlorophyll a content was observed in various plant parts used in the plant, which are summarized in the following manner:

Leaf (5.37 gm/gdw) > Stem (5.27 gm/gdw) > Callus (1.28 gm/gdw) > Root (0.00 gm/gdw) (Table 6.4).

Different amount of chlorophyll b content was observed in various plant parts used in *Tinospora cordifolia*, which are summarized in the following manner:

Leaf (4.67 gm/gdw) > Stem (4.23 gm/gdw) > Callus (1.18 gm/gdw) > Root (0.00 gm/gdw) (Table 7.4).

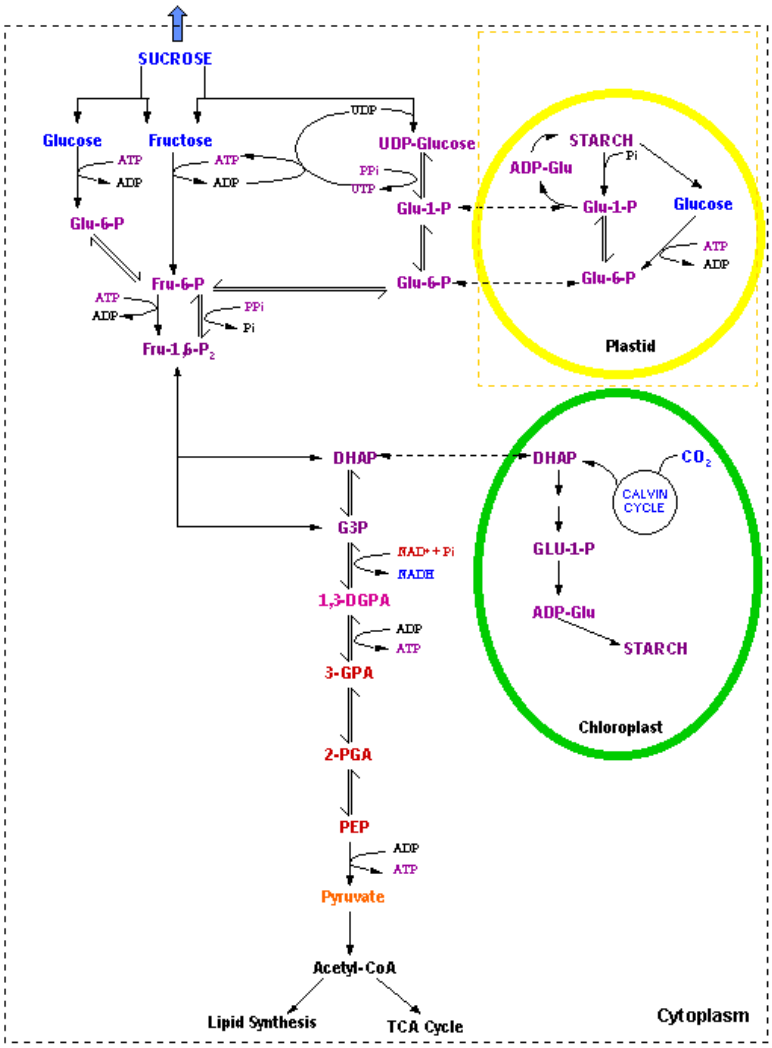
CARBOHYDRATES

INTRODUCTION

Carbohydrates are widely prevalent in the plant kingdom, comprising the mono-, di-,oligo-, and polysaccharides. The common monosaccharides are glucose, fructose, galactose, ribose etc. The disaccharides *i.e*. the combination of two monosaccharides include sucrose, lactose and maltose etc. Starch and cellulose are polysaccharides consisting of many monosaccharide residues. Cellulose is the most abundant organic compound on this planet, since it forms the part of the cell wall in plants.

Some carbohydrates are digestible by humans and therefore provide an important source of energy, whereas others are indigestible and therefore do not provide energy. Indigestible carbohydrates form part of a group of substances known as dietary fiber, which also includes lignin. Consumption of significant quantities of dietary fiber has been shown to be beneficial for human nutrition, help to reduce the risk of certain types of cancer, coronary heart disease, diabetes and constipation. Being an important source of energy and dietary fiber, carbohydrates also contribute to the sweetness, appearance and texture characteristics of many foods. Plants take about one quarter of the carbon dioxide found in the atmosphere and convert it into simple sugars (carbohydrates) through

photosynthesis. It is important to determine the type and quantity of carbohydrates in foods for health purpose .



PRINCIPLES

Carbohydrates are estimated by Anthrone's method (Yemm and Willis, 1954). They are hydrolysed in to simple sugars by dilution in acidic medium. Glucoses dehydrated to hydroxyl methyl furfural. This

compound with anthrone forms a green coloured product with absorption maximum at 630 nm.

MATERIAL AND METHODS

SUGAR

A. MATERIAL

1. Anthrone reagent: Dissolve 200 mg anthrone in 100ml ice cold 95% H_2SO_4 (freshly prepared).
2. 80% ethanol
3. 2,3 N-HCl
4. Standard glucose

STOCK SOLUTION:

10gm glucose was dissolved in 10 ml of distilled water.

Working Standard

Dilution Series were made from 0.05 to 0.1mg/l

1.0.1mg/ml= 1.0 ml of stock standard

2.0.075mg/ml= 0.75ml of stock standard +0.25 ml of distilled water

3.0.05mg/ml= 0.5ml of stock standard +0.5 ml of distilled water

4.0.025mg/ml= 0.25ml of stock standard +0.75 ml of distilled water

5.0.05mg/ml= 0.05ml of stock standard +0.95 ml of distilled water

PROCEDURE

1. METHOD OF EXTRACTION

Plant Sample (1 mg)

↓

Macerated in 80% ethanol (10ml)

↓

Centrifuged at 3000 rpm for 10-15 min

↓

Supernatant was saved

2. ESTIMATION

a. Take 1.0ml of aliquot and add 2.0 ml of anthrone reagent.

b. Heat the mixture in water bath for 12 minutes and then cool.

c. O.D was recorded at 630 nm.

d. Reference curve was prepared by using glucose.

e. Three replicates were taken and carbohydrate contents were expressed as mg/ml of tissues.

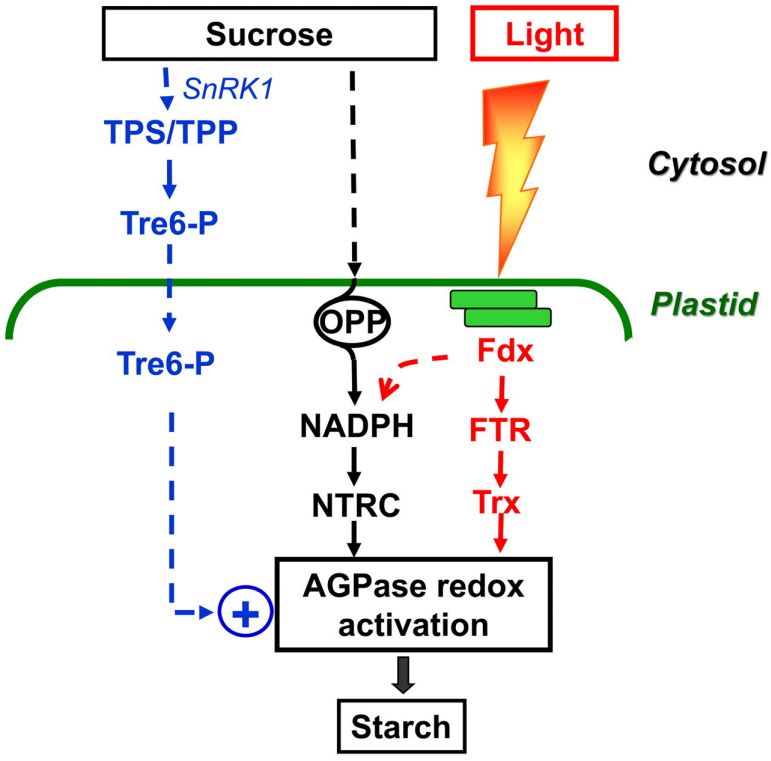
RESULTS:

Different amounts of sugar were observed in various plant parts used in this plant, which are summarized in the following manner:

Callus (7.96 gm/gdw) > Leaf (6.17 gm/gdw) > Stem (4.87 gm/gdw) > Root (0.67 gm/gdw) (Table 7.4).

STARCH

Starch is biodegradable and renewable in nature. Starch is the major carbon reserve in most plants. It is a mixture of amylose and amylopectin and is deposited as granules inside plastids (chloroplasts in leaf, amyloplasts in non-photosynthetic tissues). The initial attack on starch granules in leaf and non-photosynthetic tissues is by α--amylase and a debranching enzyme.



They are increasingly being considered as an eco-friendly alternative to the use of synthetic additives in many other products, including plastics, detergents, pharmaceutical tablets, pesticides, cosmetics and even oil-drilling fluids (Garth *et al.,* 1998).

Supernatant (1ml)

↓

Supernatant in 5 ml of 52% perchloric acid + 65 ml of distilled water

↓

Shake (5 min.)

↓

Centrifuged (2500 rpm)

↓

Repeat it thrice

↓

Supernatant of the sample was pooled and the volume was raised to 100 ml with distilled water

↓

1 ml of aliquot was separated

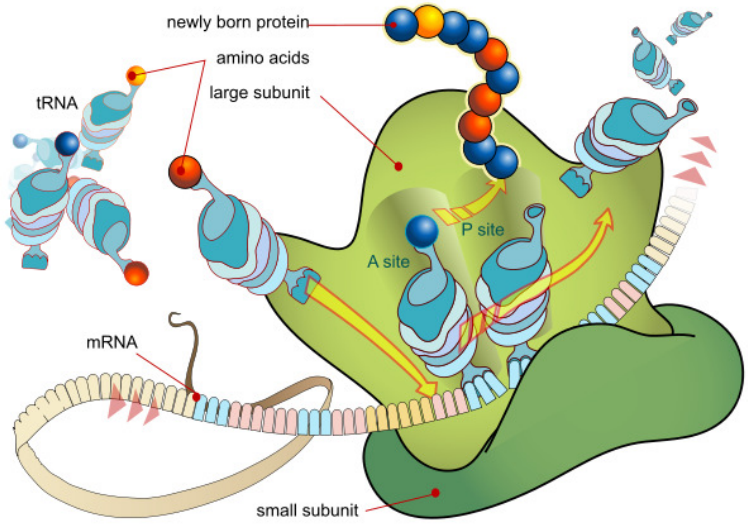
RESULTS:

Varying amount of starch contents was observed in various plant parts used in *Tinospora cordifolia*, which given in following way:

Callus (10.37 gm/gdw) > Stem (9.10 gm/gdw) > Leaf (8.44 gm/gdw) > Root (8.36 gm/gdw) (Table 7.4).

PROTEINS:

Proteins are the primary components of living being. The presence of higher protein level in the plants towards the possible increase in food value as reported (Thomsen *et al.,* 1991). Protein is an important part of life and nutrition, it is the substance that composes a large portion of body's structure. Proteins are made up of amino acids arranged in different combinations. Next to water, protein is the most abundant substance in the human body. It is a part of all body cells and is a vital building block for growth, maintenance and repair of the body tissues.



PRINCIPLE:

It has been estimated by Lowry *et al.,* (1951). The blue colour developed by the reduction of the phosphomolydicphoshpho tungstic components in the Folin- Ciocalteau reagent by the amino acid, trysoine and tryptophan in the protein.

MATERIAL AND METHODS:

A. Chemicals

1. Reagent A: 2.0% sodium carbonate in 0.1N sodium hydroxide.

2. Reagents B: 0.5% copper sulphate ($CuSO_4.5H_20$) in 1% potassium sodium tartarate.

3. Reagent C: 50 % of reagent A + 1.0 ml of reagent B.

4. Reagent D: Folin- Ciocalteau reagent

1. Protein Solution: Protein standard solution was made by dissolving 50 mg BSA (bovine serum albumin) in distilled water, final volume was made up to 50 ml.

2. Working Standard: 10 ml of stock solution was diluted by adding 40 ml of distilled water. Concentration range 10 µg -200 µg protein was prepared from this by making a series range from 0.1-1.0 ml of solution.

B. Procedure

2. Method of Extraction:

Plant Sample (1mg)

↓

Macerated in 80% ethanol (10 ml)

↓

Centrifuged at 3000-4000 rpm for 10-15 min

↓

Supernatant discarded and residue was pooled

↓

Residue was suspended in 5% perchloric acid

↓

Recentrifuged at 3000 rpm for 10 -15 min.

↓

Residue was pooled and supernatant discarded

↓

Residue was resuspended in mixture of ethanol,

ether and chloroform (2:1:1)

↓

Residue was again centrifuged and washed with 1 N cold TCA (Trichloro acetic acid)

↓

Recentrifued

↓

Again supernatant was discarded and pellet was

dissolved in 1 N NaOH

↓

Made up the solution and incubated for 1 hour at room temperature.

3. ESTIMATION

a. Added 5 ml of reagent C in 0.1 ml of residue.

b. Then added 0.5ml of reagent D and incubated at room temperature for 30 min.

c. Blue colour developed and its OD was measured at 660nm.

d. Standard curve was prepared with BSA (Bovine serum albumin) and three replicates were taken for each estimation.

e. Protein contents were expressed in terms of µg/gm fresh weight.

RESULTS:

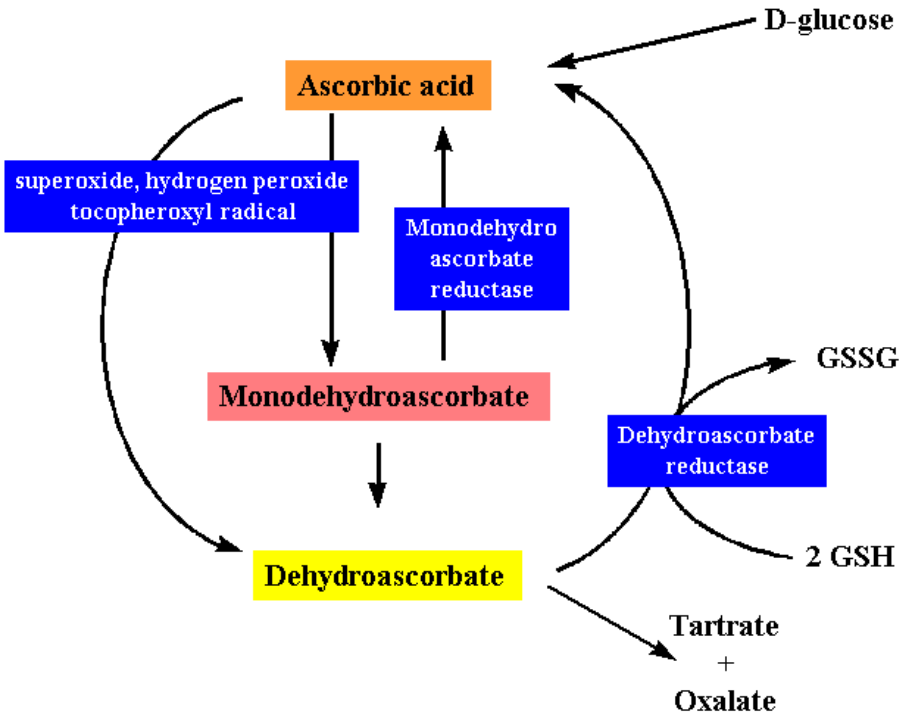
Different amount of proteins was observed in various plant parts used in this plant, summarized in the following order:

Stem (88.57 µg/mg) > Leaf (76.06 µg/mg) > Callus (67.09 µg/mg) > Root (59.33 µg/mg) (Table 7.4).

ASCORBIC ACID

INTRODUCTION

Ascorbic acid is also known as "Vitamin C" is an antiscorbutic. Usually, it is present in all fresh vegetables and fruits. It is water soluble and heat liable vitamin. It promotes seed germination, growth and flowering in plants. Besides, it also stimulates amylase, protease and RNA activity in various crops. It is found in actively metabolizing cells. Ascorbic acid (vitamin C) is a familiar molecule because of its dietary significance, most aspects of its metabolism and some aspects of its functions in plants are very poorly understood (Sadasivam and Manickam, 1996).



PRINCIPLE

Norit reagent is used in this method, which oxidizes ascorbic acid in the presence of Trichloro acetic acid.

MATERIAL AND METHODS

A. REAGENTS

a. 2% 2,4-dinitrophenyl hydrazine: 2gm of compound dissolved in 100 ml of 9N H_2SO_4.

b. Norit reagent: 2% activated charcoal (Norit) dissolved in 100 ml of 6% TCA. It was filtered through Whatman filter paper No.42.

c. 10% Thiourea: 10 gm of thiourea dissolved in 100 ml of 90% alcohol.

d. Standard ascorbic acid solution : 50 mg of ascorbic acid was dissolved in 50 ml of distilled water. A concentration range from 10µg-100µg of ascorbic acid by undertaking a volume series from 0.1-1.0 ml of the standard solution was prepared.

B. Procedure

a. Method of Extraction

Plant sample (500mg)

↓

Macerated in Norit reagent (10 ml)

↓

Centrifuged at 1000 rpm for 10-15min

↓

Supernatant was pooled

b. ESTIMATION

1. 1.0 ml of 2-4 dinitrophenyl hydrazine was added to 4.0 ml of the prepared solution.

2. At the same time, one drop of thiourea was added to it and the reaction mixture was boiled for 15 min at 100ºC.

3. This was followed by dipping these tubes in ice bath for cooling.

4. In this reaction mixture, 5.0 ml of 85% sulphuric acid was added and subjected to an incubation period of 30 minutes.

5. Different concentrations of ascorbic acid (ranging from 10 μg -100 μg) were used to prepare the standard curve.

6. Three replicates were taken and content of ascorbic acid was expressed in terms of μg/mg fresh weight of the tissue.

RESULTS:

Ascorbic acid concentration was different in various plant parts of *Tinospora cordifolia* are narrated in the following manner:

Callus (49.11 gm/gdw) > Leaf (43.19 gm/gdw) > Root (42.08 gm/gdw) > Stem (38.9 gm/gdw) (Table 7.4).

TOTAL PHENOL

INTRODUCTION

Phenols are very stable products in plant organisms. Generally, they are characterized by a benzene ring having one hydroxyl group (-OH) at its ortho position. They can be converted into lignin, which is the main phenolic polymer in plants (Kefeli *et al.,* 2003). Grains and seeds containing high amount of polyphenols are resistant to bird attack. It includes an array of compounds like tannins, flavonoids etc. Phenolic compounds occur in a variety of simple and complex forms. They are oxidized by oxidative enzymes such as peroxidases and phenol oxidizes

PRINCIPLE

Phenolic contents were measured by the method of Mahadevan (1975).Phenols react with phosphomolybdic acid in Folin Ciocalteau reagent in alkaline medium and produce blue coloured complexes (Molybdenum blue).

MATERIAL AND METHODS

a. Reagents

1. 80% ethanol
2. Folin Ciocalteau reagent
3. 20% sodium carbonate (Na_2CO_3)
4. Standard phenol solution.

STOCK STANDARD: 50 mg Catechol dissolved in 50 ml of distilled water.

WORKING STANDARD: 10 ml of stock standard was diluted to 100 ml for preparing of working standard. A series of volume from 0.1-1.0 ml of this standard gives a concentration range from 10µg-100 µg and proceeded as that of the sample.

b. PROCEDURE

1. METHOD OF EXTRACTION

Plant sample (500mg)

↓

Homogenized in 80% ethanol (10 ml)

↓

Centrifuged at 1000-3000 rpm for 20 min

↓

Supernatant was saved and residue was re-extracted with 80% ethanol

↓

Centrifuged

↓

Supernatant was pooled

2. ESTIMATION

a. Pipette out 2.0 ml of aliquot in to a test tube.

b. It was made 3.0 ml with distilled water.

c. Added 0.5 ml of Folin- Ciocalteau reagent.

d. After 3 minutes, 2.0 ml of 20 % Na_2CO_3 solution was added.

e. Mixed thoroughly and the tubes were placed in boiling water for exactly one minute. After cooling OD or absorbance at 650 nm against blank was taken.

f. Standard curve was prepared by using different concentrations of stock standard.

g. Three replicates were taken and total phenols were expressed in terms of μg/mg fresh weight.

RESULTS:

Amount of phenol contents varied with the type of explant used as summarized in the following manner:

Stem (77.56 gm/gdw) > Callus (61.34 gm/gdw) > Leaf (53.16 gm/gdw) > Root (44.21 gm/gdw) (Table 7.4).

Digestion of plant parts of *Tinospora cordifolia* by Pests :

The inducing factors produced by the pests largely affect the primary and secondary metabolism of the plants, resulting in the enhanced production of certain metabolites, e.g. amino acids, sugars and total phenols.

Due to the eating of plant parts by the pests, the quantity of primary metabolites lowers down. In present study we also studied the fluctuation in the amount of primary metabolites.

Results showed that the amount (Table 7.5) of proteins, carbohydrates and phenols increased due to infection, which was caused by pests or in other words plants produced P.R. proteins (Pathogen Resistance) as well as also increased the level of carbohydrates and phenols to prevent itself from pest attack, Whereas other primary

metabolites concentration decreased due infestation and digestion by pests.

DISCUSSION:

Plants are the sources of many bioactive compounds containing many primary metabolites like, carbohydrates (starch, sugar), proteins, phenols, ascorbic acid etc., are useful for flavoring, fragrances, insecticides, sweeteners and natural dyes (Kaufman *et al.,* 1999). During the present research work, root, stem, leaf and calli of the plant were evaluated quantitatively for the analysis of chlorophyll, sugars, starch, protein, ascorbic acid and phenols. Keeping in view the importance of these primary metabolites the present studies for biochemical evaluation of primary metabolites from different parts of *Tinospora cordifolia* was undertaken. In the present investigation, it was observed that maximum amount of Chlorophyll a and chlorophyll b and is found in leaf and minimum in root of *Tinospora cordifolia* . In consonance to this Rainha *et al*. (2011) also found best result in the anatomical part of Hypericum foliosum. In contrats to this Arts *et al*., (2001) and Havsteen, 2002 found maximum chlorophyll result in callus of *Triticum aestivum* and other medicinally important plants.

During the present investigation, it was observed that maximum sugar is found in callus and minimum in root of the plant. In consonance to this Singh *et al*., (2010 and 2011) also showed maximum amount of sugar in *in vitro* derived callus of *Commiphora wightii* and *Andrographis paniculata*, respectively. Furthermore, root part of *Alangium salviifolium* also reported to have appreciable amount of sugar

(Tanver et al., 2010), which is also in favour of our studies However, in contrast to our findings Leaf and flower of *N. indicum* showed higher soluble sugar as compared to other plant parts investigated by Vijayvergia and Kumar, 2007.

Starch is biodegradable and renewable in nature. They are increasingly being considered as an eco-friendly alternative to the use of synthetic additives. During the present research studies, callus of the plant showed higher amount of starch whereas, stem contain moderate whreas root contain lower amount of starch content. This is in agreement with the observations made by Sharma *et al*. (2010) for starch content in *Semecarpus anacardium* (L). On contrary to this, fruits of *Momordica charantica* (Ullah *et al*., 2011) contain maximum amount of starch.

Numerous polysaccharides purified from Chinese medicinal herbs are bioactive and possesses immunomodulating, anti-tumour and antibacterial activities (Wong *et al*., 1994). Proteins are the primary components of living beings. The presence of higher protein level in the plant points towards their possible increase food value or that a protein base bioactive compound could also be isolated in future (Rishi and Sarin, 2009). During the present studies, stem contain maximum amount of protein followed by leaf, callus and root in the experimental plant species. Similar result, showing maximum protein content in the stem part was also observed in *Commiphora wightii*. (Singh *et a*l., 2011). However on contrary to this, the total amount of protein was found to be higher in seeds, Leaf and pods of *Leucaena leucocephala* (Alabi and Alausa 2006), *Clitoria ternatea* (L.), *Guazuma ulmifolia* (Lam.) & *Madhuca indica* (Gmel.) (Shekhawat and Vijayvergia, 2010), *Maytenus*

emarginata and *Pongamia pinnata* (Sagwan *et al.,* 2010) and *Moringa oleifera* (Talreja, 2011), respectively.

The biochemistry and medicines are intimately related to health depend on harmonious balance of biochemical reaction occurring in the body, disease reflects abnormalities in biomolecules in biochemical reactions (Robert, 2006). Phenols have immunomodulating, anti-tumour, antibacterial activities. In the present studies, different plant parts and *in vitro* callus of the plant evaluated for total phenol content. It was observed that callus contains maximum amount of phenol as compared to other plant parts. Similarly, Rajore (2002) and Singh *et al*. (2011) also examined the presence of appreciable amount of phenol in *in vitro* callus culture of *Jatropha curcas*, *Cordia gharaf* and *Withania Somnifera*, respectively. However, in contrast to this, stem contain higher level of phenol in N. indicum and has antibacterial and anti-inflammatory activities (Wong *et a*l., 1994; Vijayvergia and Kumar, 2007).

Ascorbic acid (vitamin C) is a familiar molecule because of its dietary significance, it is not only an important antioxidant, but it also appears to linked during flowering time, developmental senescence, programmed cell death and responses to pathogens through a complex signal transduction network. During the present research investigation, ascorbic acid was found maximum in callus and minimum in stem of the plant species. Similar results showing higher amount of ascorbic acid content in callus was also reported by Sharma (2005) in *Withania somnifera* (L.) and *Vitex negundo* (L,) respectively. However, in oppugnance to this, total levels of ascorbic acid were found to be maximum in leaf and pods of *Azadirachta indica* (Shekawat, 2002),

Amaranthus hybridus (Akubugwo *et al*., 2007), *Pavetta indica* (Linn.) (Prasad and Bisht, 2011) and *Moringa oleifera* (Talreja, 2011), respectively. Primary metabolite analysis is necessary for knowing the nutritional potential of plants and their role as precursors for the synthesis of secondary metabolites

6.3 QUALITATIVE AND QUANTITATIVE DETERMINATION OF SECONDARY METABOLITES

INTRODUCTION

Since the early days of mankind, plants with secondary metabolites have been used by humans to treat infections, health disorders and illness (Wyk and Wink, 2004). Many higher plants are major sources of useful secondary metabolites which are used in pharmaceutical, agrochemical, flavor and aroma industries. Secondary metabolites are chemicals produced by plants for which no role has yet been found in growth, photosynthesis, reproduction, or other "primary" functions. These chemicals are extremely diverse; many thousands have been identified in several major classes. Each plant family, genus and species produces a characteristic mix of these chemicals, and they can sometimes be used as taxonomic characters in classifying plants. Humans use some of these compounds as medicines, flavorings, or recreational drugs.

Plants because of the presence of various secondary metabolites have been used in the preparation of traditional medicine for a long time and most of these folk medicines were prepared from locally grown wild plants. Knowledge about the uses of plants was compiled by trial and error and passed down from one generation to another verbally. Now a

days, world markets are turning to plants as the sources of ingredients in healthcare products. Consumers are also more preferred to use plants as producers of secondary metabolites (Holm and Hiltunen, 2002). These plant secondary metabolites were found to be the sources of various phytochemicals that could be used directly or as intermediates for the production of pharmaceuticals, as additives in cosmetic, food or drink supplements (Ramlan and Mohamad, 2000). In recent years, there has been a resurgence of interest in the discovery of new compounds from plants with the aim of finding novel treatment against a variety of illnesses. Many medicinal plants that reported to have the potential for medicinal purpose were investigated for useful active compounds. For example *Artemisia annua* L, a medicinal plant traditionally used by the Chinese for fevers and malaria, had resulted to the isolation of artemisinin (qinghao) (Christen and Veuthey, 2001; Charles and Simon, 1990). Garlic (*Allium satrum* L), a medicinal plant since ancient times, was found to have anti-bacterial and anti-fungal activity with the discovery of active compound called allin. It had also been proven to have cholesterol-lowering and anti-hypertensive properties (Khory, 1984).

Moreover, it has also been noted that, plant secondary products are often produced only in small quantities in most of the plant species. It is not always feasible to isolate secondary compounds from intact plants. Besides, plants are endangered by a combination of factors such as over-collecting, unsustainable agriculture practices, urbanization, pollution and climate change, no proper regulation on management and conservation. Therefore, Biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives to production of desirable

medicinal compounds from plants (Rao and Ravishankar, 2002). On a global scale, medicinal plants are mainly used as crude drugs and extracts. Hence, Plant cell and tissue culture techniques can be use as an important tool to maintain sustainability supply of plant materials for producing bioactive compounds continuously under artificially controlled conditions (Thorpe, 2006; Mohd, 2000). Thus, the search for new plant-derived chemicals should be a priority in current and future efforts towards sustainable conservation and rational utilization of biodiversity (Philipson, 1990).

For this reason, the present investigation has been lead down to isolate, and estimate the various secondary metabolite present in *Tinospora cordifolia* using various techniques like NMR and IR spectra.

(A) IDENTIFICATION, ISOLATION AND ESTIMATION OF FLAVONOIDS *IN VIVO* AND *IN VITRO*

Flavonoids are a series of related water soluble polyphenolic glycosides, having in common a basic structural unit- the C_{15} skeleton of flavone derived from two separate biosynthetic pathways- acetate pathway and shikimnic acid pathway. Flavonoids occur almost universally in higher plants but are uncommon in cryptogams (non seeded plants). They are not synthesized by animals; the flavones found in animal kingdom (in certain moths and butterflies) are of dietary origin. Flavonoid compounds are highly characteristic to plants; many of them

are easily recognizable as the pigments in flowers and fruits while some occur in all parts of plants.

MATERIAL AND METHOD

Plant parts (root, stem and leaf) of *Tinospora cordifolia* used in present investigation were collected from the Botanical Garden of University of Rajasthan, Jaipur . Plant parts were separated, dried and powdered for extraction of their flavonoid contents. Three replicates were used for each plant part and mean values were taken. Unorganized cultures of *Tinospora cordifolia* raised and maintained, were harvested at maximum growth age of Nine weeks. These tissue samples were separately dried, powdered and used for extraction of flavonoids.

EXTRACTION PROCESS

Each of the dried and powdered test samples were separately soxhlet extracted by Subramanian and Nagarajan, (1969) method in 80% methanol (100 ml/gdw) on a water bath for 24 hrs. The methanolic extracts thus obtained were filtered and concentrated *in vacuo*. Each of the extracts was concentrated and re-extracted in petroleum ether 40-60°C (Fraction- I), ethyl ether (Fraction- II) and ethyl acetate (Fraction-III) in succession. Fraction-I was discarded in each case due to its fatty components, whereas, Fraction-II and III were analyzed for free and bound flavonoids respectively. Fraction-II was stored until analysis but the Fraction-III which contained flavonoid glycosides, was acid hydrolysed by refluxing with 7% H_2SO_4 (10 ml/g) for 2 hrs. The mixture was filtered and the filtrate was extracted with ethyl acetate in separating funnel. The ethyl acetate layer (upper layer) was washed throughly with

distilled water till neutrality and concentrated *in vacuo*. Both II and III fractions were reconstituted in ethanol before chromatographic analysis.

CHROMATOGRAPHIC ANALYSIS

QUALITATIVE ANALYSIS

(i) Thin layer chromatography (TLC)

Thin glass plates (20×20 cm) coated (wet thickness, 0.2- 0.3 mm) with Silica gel 'G' (30 gm / 60 ml) were air dried at room temperature. The dried plates were activated at 100° C for 30 min in an oven, cooled at room temperature and used to examine free and bound aglycones of each sample obtained as above. Each of the extracts was applied 1 cm above the edge of the chromatographic plates alongwith the reference flavonoids used as markers and developed in an air-tight chromatographic chamber which was already saturated with 200 ml of the solvent (Benzene : Acetic acid : Water; 125 : 72 : 3; Wong and Francis, 1968).

Several other solvent system such as n-butanol : acetic acid : water (4:1:5; upper phase), ethyl acetate saturated with water , acetic acid-water (6 : 4 ; 85 : 15), t- butanol : acetic acid : water (3 : 1 : 1; TBA) and Forestal system (acetic acid : conc. HCL : water (10 : 3 : 30) were also tried but benzene : acetic acid : water (125 : 72 : 3 ; solvent I) gave better separation.

Each of the developed chromatograms was examined under UV light (254 nm) alone and in the presence of ammonia fumes (100 ml wide –mouthed bottle containing conc. Ammonia was held in close contact with each spot for 5 – 10 sec.

In *Tinospora cordifolia* the extract (free and bound flavonoids) of root resolved into two spots (Rf 0.87, yellow green ; Rf 0.78, bright yellow green), stem resolved into three fluorescent spots (Rf 0.86, yellow green ; Rf 0.79, bright yellow green ; Rf 0.83, yellow), leaf dipicted three spots (Rf 0.86, yellow green ; Rf 0.78, yellow green ; Rf 0.82, yellow), and the extract of *Tinospora cordifolia* callus also showed three spots (Rf 0.85, yellowish brown green ; Rf 0.80, yellow) (Plate: 7.1).

Some of the developed chromatograms were exposed to I_2 vapours and the yellowish brown spots were observed against a white background. Later, I_2 was evaporated by continuous heating and a few plates were than sprayed with 5% ethanolic $FeCl_3$ as also with 1% alcoholic $AlCl_3$ separately and heated in an oven at 100° C for 5 min. Kaempferol, Quercetin and Kaempferol-7-O-glucoside in *Tinospora cordifolia* gave positive reactions to the spraying reagent used.

(ii) Preparative Thin Layer Chromatography (PTLC)

Preparative TLC was performed on silica gel 'G' (45 gm/ 80 ml) coated plates (wet thickness 0.4 – 0.5 mm), activated, cooled and separate plates were used for each of the diethyl ether (Fr.II) and ethyl acetate (Fr. III) fractions. These plates were then developed in an organic solvent (Benzene: Acetic acid: Water; 125: 72: 3), and visualized under UV light (254 nm). The fluorescent spots coinciding with the reference Kaempferol, Quercetin and Kaempferol-7-O-glucoside in *Tinospora cordifolia* extracts were marked, scrapped and eluted with ethyl acetate.

Each of the eluates was dried over anhydrous Na_2SO_4, reconstituted in chloroform and crystallized using methanol.

(iii) Identification of flavonoids

Each of the isolated compounds was subject for its mp, UV, IR and NMR spectral studies along with the authentic samples. Later, on the basis of the colour reactions, TLC behaviour and the spectral data, the isolated compounds were identified by comparing with that of the standards (Marby *et al*., 1970; Harborne *et al.*, 1975).

QUANTITATIVE ANALYSIS

The identified Kaempferol (K), Quercetin (Q), Kaempferol-7-O-glucoside (Kg) were quantitatively estimated adopting the spectrophotometric methods of Mabry *et al*., (1970), Kariyone *et al*., (1953) and Naghski *et al*., (1950) , respectively. Further the computation of their regression curves was done.

Stock solutions (1 mg / ml) of Kaempferol, Quercetin, Kaempferol-7-O-glucoside, were prepared in methanol, out of which different concentrations (20 µg to 60 µg) were separately spotted on TLC plates (Silica gel 'G'). These were developed in the solvent used for qualitative purposes as above, air dried and visualized under the UV spectrophotometer, the Aliquot was scrapped and dissolved in seprate test tubes, 5 ml of spectroscopic methanol was added, shaken vigorously,

centrifuged and the supernatant was raised to 10 ml by methanol, to which 3 ml of 0.1 M $AlCl_3$ solution was added, shaken vigorously and kept at room temperature for 20 min. The optical density of each of the samples was taken on a spectrophotometer set at 426 nm for K and Kg, and 440 nm for Q, and 296 nm for against the blank (10 ml of spectroscopic methanol + 3 ml of 0.1 M $AlCl_3$) and the average of five replicates of each was calculated. A regression curve for each of the authentic compounds Kaempferol, Quercetin, Kaempferol-7-O-glucoside, was plotted in between the various concentrations and their respective optical densities which following the Beer's law.

Correspondingly, each of the free and bound fractions (II and III), of *Tinospora cordifolia* were dissolved in 1 ml of methanol, spotted (0.5 ml) on TLC alongwith the reference markers as described above. The fluorescent spots coinciding with those of the reference compounds were marked, scrapped, eluted and processed as mentioned above. The optical densities were recorded and the flavonoid contents in each case was calculated (mg/gdw) from the computed standard calibration curves. Three such replicates were run and their average value was recorded.

OBSERVATION AND RESULT

In the present investigation, flavonoids profile has been studied *in vivo* and *in vitro* from *Tinospora cordifolia*, where Kaempferol, Quercetin and Kaempferol-7-O-glucoside from different plant parts and callus of *Tinospora cordifolia* have been evaluated for their

chromatographic, spectroscopic and colour reactions data have been presented in the Table- 7.6.

Presence of two flavonoids kaempferol (Rf 0.86, mp 312-313°C, UV max 253sh, 266sh, 294sh, 368sh), quercetin (Rf 0.79, mp 309-311°C, UV max 255sh, 269sh, 301sh, 374 sh), have been identified and confirmed in all plant parts and unorganized cultures of *Tinospora cordifolia,* whereas kaempferol-7-O-glucoside (Rf 0.83, mp 317-329°C, UV max 235sh, 240sh, 259sh, 374sh, 424sh) have been identified and confirmed in only stem, leaf and callus of *Tinospora cordifolia.* The characteristic IR spectral peaks were coinciding with those of their respective standard reference compounds of Kaempferol, Quercetin and Kaempferol-7-O-glucoside in particular samples.

Estimation of flavonoids- ***In vivo of Tinospora cordifolia***

Among all the plant parts of *Tinospora cordifolia.*(root, stem and leaf) collected leaf showed minimum amount of total flavonoids (0.54 mg/ gdw) and maximum was calculated in stem (0.69 mg/gdw). Increasing amount of total flavonoid content was observed in order as: leaf (0.54 mg/gdw) < root (0.59 mg/gdw) < stem (0.69 mg/gdw).

Maximum concentration of kaempferol was found in root (0.35 mg/gdw) and minimum was found in leaf (0.26 mg/gdw). Increasing amount of kaempferol concentration was observed in order as leaf (0.26 mg/gdw) < stem (0.28 mg/ gdw) < root (0.35 mg/gdw). Maximum concentration of quercetin was found in stem (0.34 mg/gdw) and minimum in leaf (0.19 mg/ gdw). Increasing amount of quercetin concentration was observed in order as leaf (0.19 mg/gdw) < root (0.24

mg/ gdw) < stem (0.31 mg/gdw). Whereas kaempferol-7-O-glucoside concentration was recorded only in stem (0.10 mg/ gdw).

Estimation of flavonoids- ***In vitro of Tinospora cordifolia***

Nine weeks old callus (maximum GI) was analyzed for identification and estimation of flavonoids in *Tinospora cordifolia.*

Presence of three flavonoids kaempferol, quercetin, kaempferol-7-O-glucoside in *Tinospora cordifolia* was also confirmed in their tissue cultures (calli). The amount of individual flavonoid as well as total flavonoid content in callus was higher than *in vivo* samples (root, stem and leaf). Occurrence of above these three flavonoids (kaempferol, quercetin, kaempferol-7-O-glucoside) in callus, the maximum concentration was found of kaempferol (0.45 mg/gdw) and the lowest concentration was found of kaempferol-7-O-glucoside (0.12 mg/gdw). Increasing amount of flavonoid (K, Q, Kg) content in callus was observed in order as: Kg (0.12 mg/gdw) < Q (0.35 mg/gdw) < K (0.45 mg/gdw).

DISCUSSION

Phytochemistry is the study of phytochemicals produced in plants, describing the isolation, purification, identification and structure of the large number of secondary metabolic compounds found in plants. Plants are capable of synthesizing an overwhelming variety of small organic molecules, called secondary metabolites, usually with very complex and unique carbon skeleton structures and many of them of high interest to the pharmaceutical and chemical industry. These plant produced secondary compounds have immense importance in commercial and

industrial aspects representing a large market value; about 25% of today's pharmaceuticals containing at least one active ingredient of plant origin (Rischer *et al*., 2006).

Thus, these secondary metabolites are also of interest because of their use as dyes, fibers, oils, waxes, flavouring agents, drugs, antibiotics, insecticides and herbicides (Croteau *et al*., 2000; Dewick 2002). The medicinal value of various plant based drugs can be attributed to these secondary metabolites *viz*; alkalods, glucosides, flavonoids, terpenoids, resins, volatile oils, gums, tannins etc. Though, secondary metabolites are not directly essential for the growth of the plants. Yet, they play pivotal roles such as, pollen attractants, chemical adaptors to environment stress, and defensive, protective or offensive chemicals against microorganism, insects, higher herbivores, predators and even against other higher plants (Gupta *et al*., 1992; Alfermann and Peterson, 1995; Sharma *et al*., 1996).

The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's Market place for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. However, it is not only commercial significance that drives the research initiatives. The deliberate stimulation of defined chemical products within carefully regulated *in vitro* cultures provides an excellent forum for in-depth investigation of biochemical and metabolic pathways, under highly controlled micro environmental

regimes. . Plant *in vitro* cultures can generate valuable natural products. Plants and cell cultures are the resources for flavours, fragrances, bio based fuels and plastics, enzyme preservatives, cosmetics, natural pigments and important bioactive compounds.

Hence**,** Plant tissue culture research has become a thrust area during the last decade due to the renewed emphasis it has received in the production of secondary metabolites in plants. The production of flavors, sweeteners, natural colorants as well as pharmaceuticals has been achieved with the help of tissue culture. Present study deals with the isolation, quantification and identification of flavonoids and sterols from different plant parts and unorganized tissue culture of *Tinospora cordifolia.*

Tinospora cordifolia is a prosperous source of flavonoids, many workers have observed flavonoids in different parts. A review report on flavonoids of *Tinospora crispa* has been reported (Umi Kalsom *et al*], 1995) and recently Yen Chin Koay, 2013 has been reported luteolin 4'-methyl ether 7-glucoside, genkwanin 7-glucoside, luteolin 4'-methyl ether.

In the present investigation, flavonoids profile has been studied *in vivo* and *in vitro* of *Tinospora cordifolia*, where kaempferol, quercetin, and kaempferol-7-*O*-glycoside from different plant parts of *Tinospora cordifolia* have been evaluated their chromatographic, spectroscopic and color reactions data, have been presented in the Table-7.6.

The eluted compounds from TLC were pooled together according to their TLC behaviour and isolate them with the solvents and evaporated

yielding three flavonoids kaempferol, quercetin, and kaempferol-7-*O*-glycoside. The spectral analyses of the active , (a) kaempferol (b) quercetin and (c) kaempferol-7-*O*-glucoside from the different plant parts of selected plant species are shown below: -

(a) Kaempferol : brownish needles on crystallization (mp 312°-313°C)

UV light absorption MeOH: 253 sh, 266 sh, 294 sh, 322 sh, 368 sh

IR : νcm^{-1}/ max KBr: 3420 (O–H), 2830 (C-H), 2240 (C=C), 1700 (C=O), 1600, 1610 (C≡C), 1560, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010, 815

^{1}HNMR (300MHz, $CDCl_3$): 2.35(H_1), 7.01(H_2), 7.18 (H_3), 6.29 (H_4), 6.37 (H_5), 2.35 (H_6), 5.39 (H_7), 5.36 (H_8), 7.18 (H_9), 7.01 (H_{10})

^{13}C NMR(300MHz, $CDCl_3$): 1.36 (C_1), 129.8 (C_2), 126.8 (C_3), 131.9 (C_4), 147.4 (C_5), 154.2 (C_6), 114.6 (C_7), 137.5 (C_8), 124.0 (C_9), 136.0 (C_{10}), 121.1 (C_{11}), 149.4 (C_{12}), 106.9 (C_{13}), 131.9 (C_{14}), 126.1 (C_{15})

(b) Quercetin : yellowish needles on crystallization (mp 309°-311°C)

UV light absorption MeOH: 255 sh, 269 sh, 301 sh, 374 sh,

IR: νcm^{-1}/ max KBr: 3420, 3380(O–H), 2800 (C-H), 2100 (C=C), 1680 (C=O), 1610 (C≡C), 1560, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010

^{1}HNMR (300MHz, $CDCl_3$): 2.45, (H_1), 2.55 (H_2), 6.79 (H_3), 6.98 (H_4), 6.49 (H_5), 2.33 (H_6), 6.38 (H_7), 2.36 (H_8), 5.37 (H_9), 1.4 (H_{10})

^{13}C NMR (300MHz, $CDCl_3$): 137.3 (C_1), 137.9 (C_2), 14.2 (C_3), 127.0

(C_4), 126.1 (C_5), 133.8 (C_6), 142.4 (C_7), 158.2 (C_8), 114.6(C_9), 134.5 (C_{10}), 123.0 (C_{11}), 138.0 (C_{12}), 121.1 (C_{13}), 149.4 (C_{14}), 108.9 (C_{15}), 127.8

(C)Kaempferol-7-*O*-glucoside:brownish needles on crystallization (mp 317°-329°C).

UV light absorption MeOH: 235 sh, 240 sh, 259 sh, 374 sh, 424 sh

IR: νcm^{-1}/ max KBr: 3600 (glycoside), 3420 (O–H), 2210 (C≡C), 1700 (C=O), 1600 (C=C), 1610, 1560, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010, 815.

^{1}H NMR(300MHz, $CDCl_3$): 5.1 (H_1), 6.68 (H_2), 7.64 (H_3), 6.04 (H_4), 6.03 (H_5), 5.20 (H_6), 6.81 (H_7), 7.16 (H_8), 6.70 (H_9), 5.91 (H_{10}), 3.91 (H_{11}), 2.37 (H_{12}), 3.40 (H_{13}), 2.48 (H_{14}), 3.76 (H_{15}), 2.41 (H_{16}), 3.49 (H_{17}), 2.31 (H_{18})

^{13}C NMR(300MHz, $CDCl_3$): 70.6 (C_1), 75.3 (C_2), 78.8 (C_3), 92.4 (C_4), 154.8 (C_5), 154.2 (C_6), 114.6 (C_7), 137.5 (C_8), 124.0 (C_9), 136.0 (C_{10}), 121.1 (C_{11}), 149.4 (C_{12}), 97.5 (C_{13}), 123.1 (C_{14}), 129.0 (C_{15})

^{13}C NMR (300MHz, $CDCl_3$): 71.8 (C_1), 81.1 (C_2), 66.4 (C_3), 28.0 (C_4), 156.3 (C_5), 95.2 (C_6), 156.6 (C_7), 94.0 (C_8), 155.5 (C_9), 99.2 (C_{10}), 130.7 (C_{11}).

Presence of three flavonoids kaempferol, quercetin, kaempferol-7-O-glucoside in *Tinospora cordifolia* was observed maximum in callus as compared to *in vivo* samples like root, stem and leaf . The increasing

order of different flavonoid contents in different plant parts of *Tinospora cordifolia* are as follows:

Kaempferol: leaf (0.26) < stem (0.28) < < root (0.35) < callus (0.45) mg/gdw

Quercetin: leaf (0.19) < root (0.24) < stem (0.31) < callus (0.35) mg/gdw

Kaempferol-7-*O*-glucoside: leaf (0.09) < stem (0.1) < callus (0.35) mg/gdw (Plate 7.6 ; A).

Similarly, Singh and Sharma (2009) have reported the presence of kaempferol, quercetin and Kaempferol-7-*O*-glucoside in different plant parts of *C. nodosa*, *C. renigera* and *C. pumila.* In consonance to this, Meena and Patani, (2008) have quantified quercetin from *in vivo* (leaf, stem, fruit and root) and *in vitro* (callus developed from nodal segments) plant parts of *Citrullus colocynthis (Linn.)* Schrad. and evaluated that amount of quercetin was lower in callus tissues developed from nodal segments. Besides this, quercetin has also been quantified from *in vivo* and *in vitro* plant parts of different sps. i.e. *Scutellaria baicalensis* (Yamamoto *et al*., 1986), *Acacia catechu* (Jain *et al*., 2007) and *Ricinus communis* L. (Khafagi, 2007) etc.

In contrast to this, Arya *et al*. (2008) have studied the quercetin content in callus tissue of *Pluchea lanceolata* Oliver & Hienr. They revealed that maximum amount of quercetin was present in 6 week old callus tissues (0.23 mg/gdw) followed by 8 week callus tissue (0.20 mg/gdw), while 4 and 2 week callus tissue showed 0.17 mg/gdw and 0.12 mg/gdw quercetin, respectively.

Quercetin has also been reported in tissue cultures of *Carotalaria juncea* (Jain and Khanna, 1974); *Datura sps*. (Jain *et al*., 1975); *Emblica officinalis* (Khanna *et al.*, 1982); *Trigonella foenum-graecum* (Aminuddin *et al.*,1977). Presence of quercetin and kaempferol has been also reported from tissue culture of *Lycopersicon esculentum, Agave cantala, A. wightii, Cheiranthus cheiri* (Khanna *et al*., 1980), *Peganum harmala* (Harsh, 1982), *Lycium barbarum* (Grover, 1985). Presence of catechin has been reported in tissue culture of *Rheum ribes* (Farzami and Ghorbant, 2005).

Flavonoids have been reported from *in vivo* and *in vitro* tissue cultures of a number of plant species *viz. Scutellaria baicalensis* (Zhou *et al*., 1997), *Glycyrrhiza glabra* (Asada *et al*., 1998), *Polygonum hydropiper* (Nagao *et al*., 1999), *Vaccinium myrtillus* (Hohtola *et al*., 2005), *Momordica charantia* (Agarwal and Kamal, 2007) and *Crataegus sinaica* (Maharik *et al*., 2009).

Present study confirms the presence of three flavonoids Kaempferol (K), Quercetin (Q), Kaempferol-7-O-glucoside (Kg) *in vivo* and *in vitro* in *Tinospora cordifolia*. Presence of higher concentration of flavonoids in callus, in comparison to plant parts is a landmark in the field of tissue culture. Hence, it can be concluded that large scale production of flavonoids can be achieved by tissue culture for need of society.

IDENTIFICATION, ISOLATION AND ESTIMATION OF ALKALOIDS- *IN VIVO* AND *IN VITRO*

Materials and Methods

Material and Reagents

Berberine chloride dehydrate was purchased from Sigma (St. Louis, MO, USA). All organic solvents used were analytical reagent grade.

The authenthetic sample of *Tinospora cordifolia* from University of Rajasthan was collected. The dried stem were chopped into small pieces and dried in a hot air oven (50ºC) for 1 hour. Sample was ground and passed through a sieve with mesh number 20. The powdered samples are pharmacognostically identified (macroscopic, microscopic and TLC characteristics) by comparing with the characters of the authentic sample. Comparision of the yield of crude extract as well as the berberine content in each sample was analyzed statistically using one-way ANOVA and significant differences were set at $p<0.05$.

Apparatus and Equipment

A Camag TLC system consisting of TLC Scanner III, application device Linomat IV, twin trough plate development chamber, CATS 4.0 software (Camag, Muttenz, Switzerland) was used. Chromatography was performed on silica gel GF_{254} plates (20 cm x 10 cm, 0.2 mm thickness, E. Merck, Germany) with a 100µl Camag syringe. The samples were streaked as narraow bands of 6 mm in length, 10 mm from the lower edge

using a nitrogen aspirator. Development of the plates was carried out with 9 hours for solvent saturation of the tank at ambient temperature. A solvent system consisting of butanol: glacial acetic acid : water (14:3:4) was used. Total volume of solvent mixture was 30 ml and the migration distance was 80 mm. Chromatograms were evaluated by peak area after scanning in absorbance mode at 415 nm with a scanning speed of 20 mm/s and slit dimension of 5 mm x 0.45 mm.

Preperation of the Extracts

The powdered plant material (100 g) was macerated with 80% ethanol (500 ml) for 160 hours, shaken 80 hours at a speed of 200 rpm and allowed to stand 80 hours. The extract was filtered through a whatman filter paper No. 1. The marc was re- extracted for 48 hours, shaken 24 hours and allowed to stand 24 hours (300 ml x 9). The combined extract was concentrated using a rotary evaporator and evaporated to dryness on a water- bath. The dried residue was cooled in a desicator for 30 minutes, then accurately weighed. The extraction was performed in triplicate.

Preparation of Standard and sample solutions

A stock solution of standard berberine was prepared by dissolving 4.8 mg berberine chloride dihydrate (equivalent to 3.96 mg of berberine) in 10 ml methanol. The solution was then diluted with methanol to obtain the working standard solution at a concentration of 120 µg/ ml.

TLC Fingerprints

The solution of each extract was prepared to obtain the

concentration of 10 mg/ml in 80% ethanol. The extract (3 µl) and the standard (0.48 mg/ml, 5 µl) were spotted on a TLC plate. The plate was developed in solvent system consisting of ethyl acetate : butanol : formic acid : water (50:30:12:10). The developing was 80 mm. After removing from the chamber, the plate was dried in air in a fume hood for 30 minutes, and examined under UV light (254 and 366 nm). The plate was sprayed with anisaldehyde-sulphuric acid reagent and heat at 110ºc for 10 minutes.

Determination of Berberine content

A volume of sample solution (5 µl) was applied in triplicate on a TLC plate and analyzed by the proposed method. The calibration curve was determined by using the standard solution of 120 µg/ml in methanol. Two to ten microlitre volumes of the standard solution were applied on the plate corresponding to concentrations of 240-1,200 ng/spot.

Observation and Results

Thin layer chromatographic fingerprints, showed berberine with Rf value 0.65. TLC showed green-yellow fluorescent spot of berberine as a major chemical constituent. Berberine and other alkaloid's appeared as brown-orange spots after spraying with Dragendroff;s reagent.

Leaf (2.27) < Root (3.79) < Bark (4.12) < Stem (5.78) mg/gdw

Estimation of alkaloid- *In vitro*

Nine weeks old callus (maximum GI) was analyzed for identification and estimation of phytosterols in *Tinospora cordifolia.*

Occurrence of Beberine in *Tinospora cordifolia* was also confirmed in their tissue cultures (calli). The amount of alkaloid content in callus was higher than *in vivo* samples (root, stem and leaf). Occurrence of Berberine in callus, the maximum concentration was found of callus (5.89mg/gdw) was found in maximum amount.

Leaf (2.27) < Root (3.79) < Bark (4.12) < Stem (5.78) < Callus (5.89) mg/gdw (Plate 7.6 ; B).

The results of biochemical studies of the both plant species show that *in vitro* plant cell cultures have potential for commercial production of secondary metabolites. The introduction of newer techniques of molecular biology, so as to produce transgenic cultures and to effect the expression and regulation of biosynthetic pathways, is also likely to be a significant step towards making cell cultures more generally applicable to the commercial production of secondary metabolites.

IDENTIFICATION, ISOLATION AND ESTIMATION OF PHYTOSTEROLS- *IN VIVO* AND *IN VITRO*

Phytosterols (also called plant sterols) are a group of steroid alcohols, phytochemicals naturally occurring in plants. Phytosterols occur naturally in small quantities in vegetable oils, especially sea buckthorn oil (1640 mg/100g oil), corn oil (968 mg/100g), and soybean oil (327 mg/100g oil) (Thomas *et al*., 2006; Pennington & Douglas, 2005). These are the derivatives of cyclopentano-perhydroxy-phenanthrene or sterane. Steroids containing hydroxyl groups are often referred to as

sterols. Most abundantly found phytosterols are β-sitosterol (29 C), stigmasterol (29 C) and campesterol (28 C). Lanosterol (cryptosterol 30 C) an intermediate in biosynthesis of cholesterol, has been reported from yeast. Stigmasterol is abundantly found in soybean and wheat germ oil, while spinasterol can be obtained from spinach and cabbage. Phytosterols have been studied for their biological effects by Farquar, 1996; Johns,1996; Sharma *et al*., 1996; Wang and lee, 1998; Heber *et al*., 1999; Visioli and Galli, 2001 etc. They are white powders with mild, characteristic odor, insoluble in water and soluble in alcohols. They have applications in medicine and cosmetics and as a food additive taken to lower cholesterol.

MATERIAL AND METHOD

Plant parts (root, stem and leaf) of *Tinospora cordifolia* used in present investigation were collected from the Botanical Garden of University of Rajasthan. Plant parts were separated, dried, powdered and analyzed for phytosterols estimation. Three replicates were used for each plant part and mean values were taken. Unorganized cultures of *Tinospora cordifolia* raised and maintained were harvested at their maximum growth. These tissues were dried at 100°C for 15 min to inactivate enzymes followed by 60°C till constant weight was achieved. Samples obtained from tissue culture were then separately dried, powdered and used for their phytosterol content.

EXTRACTION PROCESS

Each of the dried and powdered test samples were defatted in a soxhlet apparatus in petroleum ether (60-80°C) for 24 hrs on a water bath. The residual defatted tissues were dried and then refluxed with 15% (v/v) HCl in 70 % ethanol for 4 hrs (Tomita *et al*., 1970). Each of the hydrolysates was extracted with ethyl acetate repeatedly; the ethyl acetate layers were pooled, neutralized by washings with distilled water and taken over anhydrous Na_2SO_4 to remove any trace water. Later, these were dried in vacuo, reconstituted in chloroform, filtered and analysed chromatographically.

CHROMATOGRAPHIC ANALYSIS

QUALITATIVE ANALYSIS

(i) Thin layer chromatography (TLC)

TLC plates coated with Silica gel 'G' (0.2-0.3 mm) were dried, activated and used for the separation of steroids. Each of the extracts dissolved in chloroform was applied on these plates along with the reference steroids as markers and developed in an organic solvent mixture of hexane : acetone (8 : 2; Fazli and Hardman,1968). The developed plates were air-dried , sprayed with 50% H_2So_4 (Bennett and Heftmann, 1962) or anisaldehyde reagent (prepared by mixing 0.5 ml anisaldehyde, 1 ml H_2SO_4 and 50 ml glacial acetic acid) separately and heated to 100°C for few min until characteristic colors developed. Later, such chromatograms were visualized under UV light (366 nm). The time

required for the initial appearance of the color reaction, the initial color in day light, and after heating for 10 min and the color in UV light were also noted.

Some other solvent system (benzene: ethyl acetate, 85: 15) Heble *et al*., 1968; (acetone : benzene, 1: 2) Khanna and Jain 1973; (hexane ; ethyl acetate, 3: 1) Kaul and Staba, 1968 were also tried but hexane : acetone (80:20,v/v) solvent mixture gave excellent results, in the present investigation.

In *Tinospora cordifolia* three spots were observed in the extract of root (Rf 0.94. 0.86, 0.33), stem (Rf 0.95, 0.86, 0.30) and leaf (Rf 0.92, 0.84, 0.28). and in callus (Rf 0.92, 0.87, 0.84, 0.30) (Plate: 6.8, Fig. A). All the spots were sprayed with 50% H_2So_4 and coincided to the authentic markers, (1) β-amyrin (Rf 0.98, brown- dull red); (2) Lanosterol (Rf 0.92, dark brown), (3) β-sitosterol (Rf 0.84, dull red), (5) Campesterol (Rf 0.29, grayish dull brown).

(ii) Preparative Thin Layer Chromatography (PTLC)

PTLC was performed to isolate the above identified co-chromatographically from the extracts of the selected plant parts of the both plants, PTLC was performed on silica gel 'G' coated plates (0.4 – 0.5mm) along with the reference markers. These plates were developed in hexane: acetone (80:20), air dried and exposed to I_2 vapors where upon the spots were marked. Later, I_2 was evaporated by heating in an oven and one column in each plate was sprayed with 50%H_2SO_4 (Hiai *et al*., 1976) to relocate the spots corresponding to the markers. The spots were scrapped separately eluted with chloroform and the process was repeated

until sufficient crystallizable (methanol-acetone) amount of each of the substances was obtained (Kaul and Staba, 1968). Each was retested by Co-TLC, revealing their homogenous nature and subjected for further identification.

Identification of steroids

Melting points of each of the isolated compounds were determined using a Toshniwal Melting point apparatus. NMR and IR spectra of each of the isolated compounds were also taken. A comparison of TLC behaviour, physical data and spectral properties were made and found to be in accordance with those reported for the authentic samples (Heilbron and Bunbury, 1953).

QUANTITATIVE ANALYSIS

Quantitative estimation of β-amyrin, β-sitosterol, lanosterol, campsterol and stigmasterol in each of the test samples (root , stem, leaf and callus of *Tinospora cordifolia*) cultures of the plants was estimated spectrophotometrically using the method of Das and Banerjee (1980).

Stock solutions (1 mg/ml) of β-amyrin (β-am), β-sitosterol (β-st), lanosterol (Lan), campsterol (camp) and stigmasterol (stig) were separately prepared in chloroform. From the stock solutions, different concentrations (0.01 to 0.09 mg/ml) of each was spotted on TLC (Silica gel 'G'; hexane : acetone, 80 : 20) and the developed chromatograms after air drying were exposed to I_2 vapors. The positive spots were marked on these plates and heated to 100°C for 15 min to remove any

iodine. The spots were marked, scrapped, eluted with 5 ml of chloroform, centrifuged and the supernatants were transferred to separate test tubes which were later evaporated to dryness. To each of these, 3 ml of glacial acetic acid was added and shaken on a vortex mixture for 1 min at room temperature and then immersed in crushed ice. To these, 2 ml of freshly prepared chromogenic reagent (0.5 ml of 0.5 % anhydrous $FeCl_3$ in glacial acetic acid and 100 ml of 36 N H_2SO_4; (Klyne, 1965) was added dropwise at 0°C and mixed thoroughly.

All the reaction mixtures were incubated at 40°C for 30 min and their optical density was measured in spectrophotometer set at 540 nm against a blank (3 ml of glacial acetic acid + 2 ml of the chromogenic reagent). In each case, the average of five replicates of each concentration was plotted against their respective concentrations to compute regression curves, which followed Beer's law.

The crude extract obtained from various plant parts and culture samples were separately dissolved in chloroform and spotted with reference β-amyrin , β-sitosterol, lanosterol , campesterol and stigmasterol on silica gel 'G' coated and activated glass plates and developed in a solvent system of hexane : acetone (80 : 20). These spots coinciding with those of the authentic samples of (β-am), (β-st), (Lan), (camp) and (stig) were marked in all the samples. Each of the spots were eluted and extracted as described earlier. Elutes were dried taken up in 5 ml of chloroform and processed further as mentioned above. The concentration of (β-am), (β-st), (Lan), (camp) and (stig) present in various tissue samples was calculated (in mg/gdw) by comparing the optical density of the experimental sample with the regression curves of β-

amyrin , β-sitosterol, lanosterol , campesterol and stigmasterol separately. Three such replicates were examined in each case and their mean values were determined.

OBSERVATION AND RESULT

In the present investigation, steroids profile has been studied *in vivo* and *in vitro* of the *Tinospora cordifolia*, where β-sitosterol, lanosterol , campesterol, β-amyrin and stigmasterol from different plant parts and callus of *Tinospora cordifolia* have been evaluated their chromatographic, spectroscopic and colour reactions data have been presented in the Table- 7.7.

β-amyrin (Rf 0.98, mp 198°C, UV max 240sh, 258sh, 289sh, 340sh), lanosterol (Rf 0.92, mp 131-133°C, UV max 228sh, 245sh, 322sh, 540sh), β-sitosterol (Rf 0.98, mp 135-137°C, UV max 206sh, 268sh, 356sh, 540sh) and campesterol (Rf 0.29, mp 137-138°C, UV max 210sh, 267sh, 316sh, 540sh) have been identified and confirmed in plant parts and unorganized cultures of both *Tinospora cordifolia* but the prescence and quantity are vary in different plant parts. Occurrence of stigmasterol (Rf 0.88, mp 143-144°C , UV max 235sh, 270sh, 345sh, 540sh) have been identified and confirmed only in callus and stem part of *Tinospora cordifolia*.

The characteristic IR spectral peaks were coinciding with those of their respective standard reference compounds of β-amyrin , β-sitosterol, lanosterol , campesterol and stigmasterol in particular samples.

Estimation of phytosterols- ***In vivo of Tinospora cordifolia***

Between all the plant parts of *Tinospora cordifolia* (root, stem and leaf) collected leaf showed minimum amount of total steroids (1.15 mg/ gdw) and maximum was calculated in stem (6.18 mg/gdw). Increasing amount of total steroid content was observed in order as leaf (1.15 mg/gdw) < root (3.46 mg/gdw) < stem (6.18 mg/gdw).

Maximum concentration of β-sitosterol was found in leaf (0.52 mg/gdw) and minimum was found in root (0.39 mg/gdw). Increasing amount of β-sitosterol concentration was observed in order as root (0.39 mg/gdw) < stem (0.41 mg/gdw) < leaf (0.52 mg/gdw).

Maximum concentration of lanosterol was found in leaf (0.25 mg/gdw) and minimum in stem (0.09 mg/ gdw). Increasing amount of lanosterol concentration was observed in order as stem (0.09 mg/gdw) < root (0.11 mg/ gdw) < leaf (0.25 mg/gdw).

Maximum concentration of campesterol was found in leaf (0.23 mg/gdw) and minimum in stem (0.05 mg/ gdw). Increasing amount of campesterol concentration was observed in order as stem (0.05 mg/gdw) < root (0.18 mg/gdw) < leaf (0.23 mg/gdw).

Whereas, stigmasterol concentration was recorded only in stem (2.81 mg/ gdw).

Estimation of phytosterols- ***In vitro of Tinospora cordifolia***

Nine weeks old callus (maximum GI) was analyzed for identification and estimation of phytosterols in *Tinospora cordifolia.*

Occurrence of five steroids β-sitosterol, β-amyrin , lanosterol , campesterol and stigmasterol in *Tinospora cordifolia* was also confirmed in their tissue cultures (calli). The amount of individual phytosterol as well as total steroid content in callus was higher than *in vivo* samples (root, stem and leaf). Occurrence of above these five steroids in callus, the maximum concentration was found of callus β-sitosterol (0.98 mg/gdw) was found in maximum amount and β-amyrin (0.15 mg/gdw) in minimum amount. Increasing amount of steroids (β-st, lan, camp, stig, β-am) content in callus was observed in order as: β-am (0.15 mg/gdw) < stig (0.18 mg/gdw) < lan (0.21 mg/gdw) < camp (0.27 mg/gdw) < β-st (0.98 mg/gdw).

DISCUSSION

Although, a number of plant species have been investigated for the phytosterols by a number of workers but not much attention has been paid on the genus *Tinospora* to investigate systematically. Other plants were also investigated for the phytosterols *viz.* β-sitosterol has been isolated from leaf and stem of *M. buchananii* (Dinarello, 1996), aerial parts of *M. forsskaoliana* (Kuteny *et al*., 1981), and bark of *M. laevis* (El Deeb *et al*., 2003) whereas β-sitosterol glycoside has been isolated from stem of *M. diversifolia* (Nozaki *et al*., 1986). However, β-amyrin has been isolated from stem of *M. diversifolia* (Cespedes *et al*., 2001), aerial parts of *M. forsskaoliana* (El deeb *et al*., 2003), leaf of *M. salicifolia* (Miranda *et al*., 2006), wood and stem of *M. nemerosa* (Sheng-Ding *et al*., 1984), root bark of *M. horrida* (Gonzalz *et al*., 1987), leaf of *M. arbutifolia* and *M. heterophylla* (Orabi *et al*., 2001) and aerial parts of *M. undata* (Muhammed *et al*., 2000). Hence, in the present study attempts have

been made to investigate the phytosterols, qualitatively and quantitatively, in the *Tinospora cordifolia*.

The TLC were isolated and eluted with benzene-ethyl acetate (1: 3), these TLC were pooled, concentrated and crystallized. Five phytosterol compounds were identified on the basis chromatography, color reaction (Table- 7.7), and spectral analysis.

(a) β-sitosterol (b) β-amryin (c) stigmasterol (d) lanosterol (e) campasterol

(a) β -sitosterol : brownish crystallization (mp 135-137°C)

UV light absorption MeOH: 206 sh, 268 sh, 356sh, 540sh

IR : νcm^{-1}/ max KBr: 3400 (O–H), 2700(C-H), 2210 (C≡C), 1700 (C=O), 1640 (C=C), 1610, 1570, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010, 815

^{1}HNMR (300MHz, $CDCl_3$): 0.98 (H_1), 1.38 (H_2), 1.35 (H_3), 1.36 (H_4), 1.11 (H_5), 1.82 (H_6), 1.21 (H_7), 1.35 (H_8), 1.45 (H_9), 1.16 (H_{10}), 1.64 (H_{11}), 1.47 (H_{12}), 1.26 (H_{13}), 1.15 (H_{14}), 1.75 (H_{15}), 1.27 (H_{16}), 1.27 (H_{17}), 1.96 (H_{18}), 1.24 (H_{19}), 1.66 (H_{20}), 1.06 (H_{21}), 1.21 (H_{22}), 1.46 (H_{23}), 0.86 (H_{24}), 0.86 (H_{25}), 1.26 (H_{26}), 1.29 (H_{27}), 1.29 (H_{28}), 1.51 (H_{29}), 1.47 (H_{30})

^{13}C NMR (300MHz, $CDCl_3$): 14.5(C_1), 21.7 (C_2), 34.3 (C_3), 42.9 (C_4), 30.4(C_5), 20.2(C_6), 20.6 (C_7), 30.8 (C_8), 35.7 (C_9), 29.7 (C_{10}), 18.7 (C_{11}), 48.8 (C_{12}), 20.3 (C_{13}), 20.4 (C_{14}), 40.1 (C_{15}), 27.5(C_{16}), 295.35 (C_{17}), 36.9 (C_{18}), 36.8 (C_{19}), 32.1 (C_{20}), 26.9 (C_{21}), 36.2 (C_{22}), 364.81 (C_{23}), 269.86 (C_{24}), 25.2 (C_{25}), 257.26 (C_{26}), 34.5 (C_{27}), 19.3 (C_{28}), 23.6(C_{29})

(b) β-amryin : White crystalline powder (mp 198°C)

UV light absorption MeOH: 240 sh, 258 sh, 289sh, 340sh

IR : νcm^{-1}/ max KBr: 3397, 2932 (OH), 1645, 1465 (CH), 1379 (C=O) 1029, 900, 5.12, 3.23, 1.13 (aromatic), 0.99, 0.97,0.94, 0.87, 0.83, 0.79 (CH3).

^{1}H NMR (300MHz, $CDCl_3$): 1.76 (H_1), 0.91 (H_2), 1.42(H_3), 0.91 (H_4), 1.42 (H_5), 1.95 (H_6), 1.03 (H_7), 1.81 (H_8), 1.42 (H_9), 1.95 (H_{10}), 1.35 (H_{11}), 1.13 (H_{12}), 1.35 (H_{13}), 1.81 (H_{14}), 1.81 (H_{15}), 1.26 (H_{16}), 1.28 (H_{17}), 0.68 (H_{18}), 1.03 (H_{19}), 1.09 (H_{20}), 1.03 (H_{21}), 0.91 (H_{22}), 1.03 (H_{23}), 0.77 (H_{24}), 1.01 (H_{25}), 1.23 (H_{26}), 0.91 (H_{27}), 1.64 (H_{28}), 0.77 (H_{29}), 0.77 (H_{30})

^{13}C NMR (300MHz, CDCl3): 38.14 (C-1), 27.51(C-2), 79.12 (C-3), 38.88 (C-4), 55.27 (C- 5), 18.44 (C-6), 33.03 (C-7), 38.82 (C-8), 47.81 (C-9), 37.00 (C-10), 23.48 (C-11),121.82 (C-12), 145.28 (C-13), 42.18 (C-14), 26.12 (C-15), 2737 (C-16), 32.04 (C-17), 47.22 (C-18), 46.93 (C-19), 31.34 (C-20), 34.83 (C-21), 37.26 (C- 22), 28.22 (C-23), 15.47 (C-24), 15.72 (C-25), 16.97 (C-26), 25.26 (C-27),28.44 (C-28), 33.44 (C-29), 23.48 (C-30).

(c) Stigmasterol : Reddish brown crystallization (mp 143-144°C)

UV light absorption MeOH: 235 sh, 270 sh, 345 sh, 540sh

IR : νcm^{-1}/ max KBr: 3480 (OH), 2950 (C-H), 1600 (C=O)1365, 1570, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010, 815

^{1}H NMR (300MHz, $CDCl_3$): 1.04 (H_1), 1.92 (H_2), 1.05(H_3), 1.54 (H_4), 1.16 (H_5), 1.15 (H_6), 1.35 (H_7), 1.54 (H_8), 1.76 (H_9), 1.48 (H_{10}), 1.23 (H_{11}), 1.14 (H_{12}), 1.45 (H_{13}), 1.25 (H_{14}), 1.29 (H_{15}), 1.16 (H_{16}), 1.21 (H_{17}), 1.28 (H_{18}), 1.66 (H_{19}), 1.06 (H_{20}), 1.29 (H_{21}), 0.86 (H_{22}), 0.86 (H_{23}), 1.29 (H_{24}), 1.29 (H_{25}), 1.29 (H_{26}), 1.51 (H_{27}), 1.47 (H_{28})

^{13}C NMR (300MHz, $CDCl_3$): 19.3 (C_1), 33.6 (C_2), 19.7 (C_3), 39.6 (C_4), 73.5 (C_5), 32.6 (C_6), 33.8 (C_7), 29.9 (C_8), 18.4 (C_9), 48.3 (C_{10}), 20.7 (C_{11}), 27.8 (C_{12}), 40.6 (C_{13}), 27.3 (C_{14}), 32.27 (C_{15}), 22.2 (C_{16}), 34.4 (C_{17}), 32.7 (C_{18}), 29.9 (C_{19}), 27.0 (C_{20}), 33.8 (C_{21}), 462.4 (C_{22}), 267.88 (C_{23}), 25.7 (C_{24}), 27.74 (C_{25}), 24.5 (C_{26}), 19.3 (C_{27}), 23.4 (C_{28})

(d) Lanosterol : Colourless crystallization (mp 131-133°C)

UV light absorption MeOH: 228 sh, 245 sh, 322 sh, 540sh

IR: νcm^{-1}/ max KBr: 3420 (OH), 2915 (C-H), 2220 (–C≡C–), 1615 (C=C), 1440 (C=O), 1365, 1570, 1510, 1450, 1400 (aromatic), 1385, 1310, 1270, 1180, 1010.

^{1}H NMR (300MHz, $CDCl_3$): 1.11 (H_1), 0.96 (H_2), 1.02 (H_3), 0.87 (H_4), 1.22 (H_5), 1.25 (H_6), 1.03 (H_7), 1.67 (H_8), 1.49 (H_9), 1.24 (H_{10}), 1.17(H_{11}), 1.48 (H_{12}), 1.28 (H_{13}), 1.25 (H_{14}), 1.16 (H_{15}), 1.24 (H_{16}), 1.24 (H_{17}), 1.66 (H_{18}), 1.06 (H_{19}), 1.16 (H_{20}), 1.06 (H_{21}), 2.1 (H_{22}), 0.86 (H_{23}), 1.29 (H_{24}), 1.24 (H_{25}), 1.25 (H_{26}), 1.16 (H_{27}), 1.43 (H_{28}), 1.46 (H_{29})

^{13}C NMR (300MHz, $CDCl_3$): 22.5 (C_1), 6.24 (C_2), 22.6 (C_3), 39.5 (C_4), 24.3 (C_5), 35.7 (C_6), 30.8 (C_7), 18.9 (C_8), 43.9 (C_9), 33.8 (C_{10}), 15.0 (C_{11}), 27.4 (C_{12}), 22.8 (C_{13}), 22.3 (C_{14}), 31.9 (C_{15}), 22.5 (C_{16}), 33.0 (C_{17}), 27.1 (C_{18}), 31.8 (C_{19}), 30.8 (C_{20}), 15.1 (C_{21}), 46.6 (C_{22}), 24.6 (C_{23}), 25.8 (C_{24}),

29.79 (C_{25}), 50.0 (C_{26}), 11.5 (C_{27}), 29.1 (C_{28}), 19.0 (C_{29})

(e) Campesterol : Colourless powder (mp 137-138°C)

UV light absorption MeOH: 210 sh, 267 sh, 316 sh, 540sh

IR: νcm^{-1}/ max KBr: 3380 (OH), 2920 (C-H), 2270 (C≡C), 1760 (C=O), 1610 (C=O), 1560, 1520, 1450, 1430 (aromatic), 1395, 1310, 1270, 1180, 1010, 815

^{1}HNMR(300MHz, $CDCl_3$): 1.05 (H_1), 1.87 (H_2), 1.15 (H_3), 0.87 (H_4), 0.84 (H_5), 1.08 (H_6), 1.68 (H_7), 1.17 (H_8), 1.44 (H_9), 1.65 (H_{10}), 1.17 (H_{11}), 1.27 (H_{12}), 1.28 (H_{13}), 1.69 (H_{14}), 1.04 (H_{15}), 1.25 (H_{16}), 0.88(H_{17}), 0.88 (H_{18}), 1.27 (H_{19}), 1.25 (H_{20}), 1.69 (H_{21}), 1.54 (H_{22}), 1.46 (H_{23}), 1.48 (H_{24}), 1.25 (H_{25}), 1.27 (H_{26}), 1.01 (H_{27})

^{13}C NMR (300MHz, $CDCl_3$): 17.8 (C_1), 38.9 (C_2), 17.5 (C_3), 42.3 (C_4), 21.3 (C_5), 25.8 (C_6), 31.9 (C_7), 30.9 (C_8), 18.8 (C_9), 48.2 (C_{10}), 20.7 (C_{11}), 44.4 (C_{12}), 40.1 (C_{13}), 27.4 (C_{14}), 25.20 (C_{15}), 22.5 (C_{16}), 34.2 (C_{17}), 32.4 (C_{18}), 29.8 (C_{19}), 27.8 (C_{20}), 38.3 (C_{21}), 42.6 (C_{22}), 27.7 (C_{23}), 25.9 (C_{24}), 29.74 (C_{25}), 34.7 (C_{26}), 19.4 (C_{27}), 23.8 (C_{28}), 30.6 (C_{29}), 9.8 (C_{30})

During the phytochemical investigation, occurrence of five phytosterol β-sitosterol, β-amryin, stigmasterol, campesterol and lanosterol in *Tinospora cordifolia* were also confirmed maximum in their unorganized callus culture as compared to *in vivo* samples like root, stem and leaf. The increasing order of different flavonoid contents in different plant parts of *Tinospora cordifolia* are as follows:

β-sitosterol: root (1.69) < stem (4.19) < leaf (4.52) < callus (4.98) mg/gdw

β-amryin: leaf (0.08) < callus (0.15) mg/gdw

Stigmasterol: stem (0.13) < callus (0.18) mg/gdw

Lanosterol: stem (0.05) < root (0.10) < leaf (0.18) < callus (0.21) mg/gdw

Campesterol: stem (0.13) < root (0.15) < leaf (0.18) < callus (0.27) mg/gdw. (Plate 7.6 ; C)

In the present study, β-sitosterol was isolated from root, leaf of the plant and the same was reported in many other species including *Tephrosia strigosa* and *Heliotropium indicum*, *Ajuga macrosperma* aerial part of *Brillantaisia palisatii, Elaphoglossum spathulatum, Parahancornia amapa, Conyza bonariensis, Lilium longiflorum* and *Tulipa gesneriana*, Zhongguo zhongyao, *Atractylodes chinenese, Eulophia epidendraea* (Sreenivasulu and Sarma, 1996; Pandey *et al*., 1996; Dinda *et al*.,1997; Carvalhoa *et al*.,2001, Kong *et al*.,2001; Berrondo *et al*.,2003; Socolsky *et al*.,2003; Endoh *et al*.,1981; Maridass and Ramesh, 2010).

Beta-sitosterol (*β*-sitosterol) and its glucoside (*β*-sitosterolglucoside) are the most abundant sterols found in plants. In common with other phytosterols they are not endogenously synthesised in the human body and are derived exclusively from the diet (Ling *et al*.,1995). Although they differ from cholesterol by only an extra ethyl group in the side chain, they show profound biological effects in a number of experimental animal models. These include, inter alia, reduction of carcinogen-induced colon cancer, anti-inflammatory (Yamamoto *et al*., 1991), and anticomplement activity (Yamada *et al*.,

1987). Bouic *et al*., (1996), reported that the *β*-sitosterol and *β* -sitosterol glucoside stimulate the proliferation of human peripheral blood lymphocytes and they can be used as an immunomodulatory agents. Donald *et al*., (1997) reported that the *β*-sitosterol and *β*-sitosterolglucoside were used in the treatment of pulmonary tuberculosis.

In the present work, the compound *β*-amyrin was also isolated from the b plant which was also found in several other plants i.e. *Tephrosia strigosa* and *Heliotropium indicum*, *Brillantaisia palisatii*, *Lychnophora pinaster*, *Luxemburgia nobilis*, *Chiococca braquiata, Parahancornia amapa, Atractylodes chinenese*, *Atractylodes chinenese* (Sreenivasulu and Sarma, 1996; Pandey *et al*., 1996, Carvalhoa *et al*.,2001; Ding *et al*.,2000; Berrondo *et al*.,2003, Silveira *et al*.,2005; Oliveira *et al*.,2002; Lopesa *et al*.,2004).

Quercetin, a phytochemical belonging to the flavonoids, has antioxidant activity, inhibition of protein kinases (Davies *et al*., 2000) and DNA topoisomerases (Constantinou *et al*., 1995) regulate gene expression (Moon *et al*., 2003) and also modulate gene expression related to oxidative stress and in the antioxidant defence system (Moskaug *et al*.,2004). According to Van Wiel *et al*., (2001) and Tsanova- Savova and Ribarora (2002), the most common flavonoids in grape wine were flavonols (quercetin, kaempferol, and myricetin). Betes- Saura *et al*., (1996) detected quercetin, kaempferol in leaf and exocarps of grape *Vitis labruscana* cv. Kyoho and, *Vitis vinifera* L. fruits. *Lilium auratum, L. henryi, L.martagon, L. myrciphyllum* and *L. willmottiae. L. henryi, L. martagon, L. myrciphyllum, L. willmottiae, L. leichtlinii*.Apigenin and luteolin which were structurally elucidated from 13C-NMR, where

spectral data reported earlier (Loo *et al*., 1986; Wagner, 1976). These compounds were isolated from the root of *Glossostemon bruguieri, Conyza bonariensis* (Meselhy, 2003; Kong *et al*., 2001).

Luteolin was also isolated from the fruit of *Terminalia chebula (*Klika *et al*., 2004). So far anti-venom compounds isolated from plants include *β*-sitosterol, *β*-sitosterolglucoside, *β* - amyrin, kampferol and quercetin (Martz ,1992 ;Houghton *et al*.,1993; Abubakar *et al*.,2000; Reyes-Chilpa *et al*.,1994).

Similarly, Singh and sharma (2009) has been reported the presence of β-sitosterol, stigmasterol, campesterol and lanosterol in different plant parts of *C. nodosa*, *C. renigera* and *C. pumila.*

Sterols have been reported from *in vivo* and *in vitro* tissue cultures of a number of plant species such as β-sitosterol, stigmasterol, campesterol and lanosterol from seeds of *Nicotiana tabacum* (Benveniste *et al*., 1966, 1969), Campesterol, β-sitosterol, and stigmasterol from above plant parts of *Corchorus olitorius* (Trang and Stohs, 1975), *Digitalis lanata* (Helmbold *et al*.,1978), *Dioscorea deltoidea* (Stohs *et al*., 1975 b), *D. totoro* (Tomita *et al*., 1970), *Withania somnifera* (Yu *et al*., 1974) and *Yucca glauca* (Stohs *et al*., 1975 a) , β-sitosterol and stigmasterol from *D. metel* (Nag, 1976), *E. officinalis* (Taparia, 1975), *Seasamum indicum* (Khanna and Jain, 1973), *Solanum elaegnifolium*, *S. nigrum* (Khanna *et al*., 1978), *M. charantia* (Khanna and Mohan, 1973) and *Tylophora indica* (Benjamin and Mulchandani, 1973), whereas only β-sitosterol in tissue cultures of *Apocynum cannabinum* (Lee *et al*., 1972), *D. metel* (Nag, 1976), *Helianthus annus* (Sharma , 1975), *Morus alba*

(Kulkarni *et al*., 1970), *S. aviculare* (Gaur, 1978), *S. xanthocarpum* (Heble *et al*.,1968), *Ageratum conyzoides* (Kamboj and Saluja, 2011).

Present study showed that phytosterols which are considered as precursors of growth and reproductive hormones are present in sufficient amount in all plant parts as well as tissue cultures of *Tinospora cordifolia.* Depending upon the needs amount of sterols can be further increased in tissue culture by supplementing with many PGR's with the growth medium with various precursors of sterols.