Expression analysis of key genes in shoot cultures of *Swertia chirayita* fed with different precursors *in vitro*

Thesis submitted in fulfillment of the requirements for the degree of

MASTER OF TECHNOLOGY

BY

PRATEEKSHA SINGH

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

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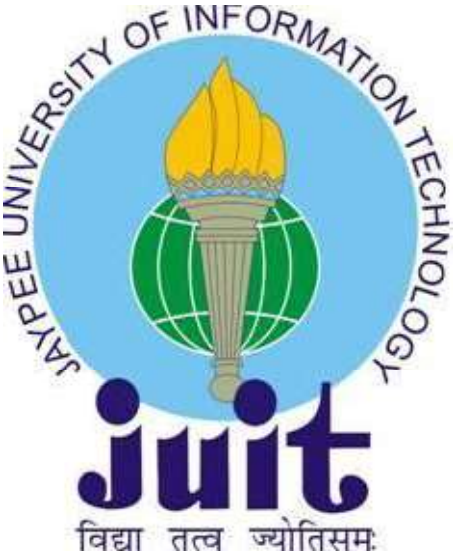

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MAY, 2017

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DECLARATION

I hereby declare that the work reported in the M-Tech thesis entitled **"Expression analysis of key genes in shoot cultures of *Swertia chirayita* fed with different precursors *in vitro*"** submitted at **Jaypee University of Information Technology, Waknaghat, India**, is an authentic record of my work carried out under the supervision of **Dr. R.S. Chauhan**. I have not submitted this work elsewhere for any other degree or diploma.

PRATEESKSHA SINGH

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, Waknaghat , India

Date :

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M-Tech. thesis entitled "**Expression analysis of key genes in shoot cultures of *Swertia chirayita* fed with different precursors *in vitro***", submitted by **PRATEEKSHA SINGH** at **Jaypee University of Information Technology, Waknaghat**, **India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

DR. R.S CHAUHAN

DEAN(BIOTECHNOLOGY)

PROFESSOR AND HEAD

DEPT. OF BIOTECHNOLGY AND BIOINFORMATICS

DATE

ACKNOWLEDGEMENT

This project in this report is an outcome of continual work over and intellectual support fromvarious sources. I would like to express my gratitude and acknowledgement for the help of allthose people who made this work possible.

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I also thank Varun,Ira,Soniyaand Vikrant for the guidance and help.Without their constant support this project might have not reached this stage of completion**.**

Prateeksha Singh

LIST OF ABBREVATIONS

% : percent

°C : degree Celsius

µg: microgram

µl: microliter

cDNA : complementary DNA

DNA: deoxyribonucleic acid

dNTP : deoxynucleotide triphosphate

HMGR : 3-hydroxy-3-methylglutaryl-CoA reductase

HMGS : 3-hydroxy-3-methylglutaryl-CoA synthase

Min : minutes

miRNA : microRNA

mL : milliliter

RNA : ribonucleic acid

RPE : ribulose 5-phosphate 3-epimerase

RPM : revolutions per minute

RT-qPCR: quantitative reverse transcription PCR

Sec: seconds

WHO : World Health Organization

w.r.t : with respect to

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ABSTRACT

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Swertia chirayita is a high value medicinal herb having therapeutic properties. The medicinal properties conferred by this herb are associated with the presence of a well-known range of secondary metabolites with pharmacological significance. These secondary metabolites include, swertiamarine,amarogentin and mangiferin. Different analysis on these compounds related key genes is already done, but there is lack of study on corresponding product which is get enhanced with the intermediate compounds or some other end product.

Different key genes which were taken in such a way for them the gene expression studies is done which give significant association with the production of these intermediate and final produced secondary metabolite. So this study identified some key genes which are having potential importance in the molecular level and in metabolic engineering of *S.chirayita*. These key genes which are used for enhancement of the secondary metabolite production and from here production of these secondary metabolite get increased

CHAPTER 1
INTRODUCTION

Process of systematic exploration and development of novel sources of commercially valuable chemical compounds, macro- and micro-organisms, genetic and biochemical resources from nature is known as “bioprospecting” or “biodiversity prospecting”.Different forms of primary and secondary metabolites which are produced by plants used as natural products. These compounds get produced because of different complex survival strategies that plants usually developed due to various stress condition.25% of current drug market are covered by mainly plant based pharmaceuticals which are derived from the natural products. The current requirement for plant-derived herbal material is ~US $ 14 billion/year, rising 15-25% annually, and likely to reach US $ 5 trillion by 2050, estimated by world health organization(WHO).

Out of the ~297,000 native floral species of the world, 10% are used as medicinal plants this is based on approximate calculation. India accounts for one amongst the seventeenth mega diversity countries of the world, it houses two major biodiversity hotspot and 7% of total biodiversity.According to the Department of Ayush, NationalMedicinal Plants Board, Ministry of Health and Family Welfare, Govt. of India, out of the total floral species of India (17,000-18000), 44% are expected to have therapeutic usage in various folk and renowned systems of medicine like Ayurveda,Homoeopathy, Unani and Siddha.Himalayan biodiversity hotspot which belongs to Himalayan region covering a geographical area of 500,000 km. Out of 8000 estimated species of different plants ~40% are endemic and approximately 1748 are known for medicinal properties.

Swertia chirayita belongs to gentian family, commonly known as ‘Chirata’. The plant is found at altitude of 1200-1300 m. *S.chirayita* is having hepatoprotective, antimalarial, antidiabetic, anti-inflammatory activities. Different phytochemicals such as flavonoids, xanthones, iridoids and seco-iridoid glycosides which are present as secondary metabolites shows various therapeutic values.

metabolites which are found in *S.chirayita*, two seco-iridoid glucosides(swertiamarin and amarogentin), and mangiferin, a xanthone C-glucoside.By taking the two biosynthetic routes of the mevalonate (MVA) andnon-mevalonate (MEP) pathways,swertiamarin and amarogentinaresynthesized by which is trailed by the seco-iridoid pathway.

Figure 1.1: A mature *Swertia chirayita* plant (https://plants.usda.gov/)

Biogenesis of sweroside, starts from geranyl diphosphate (GPP) which is a common intermediate of both these seco-iridoids. Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) together forms GPP.Cytosolic mevalonate (MVA) involving six enzymes, and the plastidic (MEP) pathway, that includes eight enzymes, forms IPP and DMAPP. After that GPP get modified to different secondary products such as loganic acid, secologanic acid and other secondary product also get formed which includesiridodio/trio and deoxyloganic acid. m-Hydroxybenzoic acid is intermediate which comes from shikimate pathway is not an intermediate of phenyalanine. Swertiamarin is produced by hydroxylation of sweroside and amarogentin is the biphenylcarboxylic acid-derivative of it. A retro biosynthetic 13C NMR study on *S.chirayita* also confirmed the synthesis of biphenylcarboxylic acid unit from m hydroxy benzoic acid. Some minor division of six enzymes, or the plastidic (MEP) pathway, that includes eight enzymes forms IPP and DMAPP. After that GPP is get modified to different secondary products such as loganic acid secologanic acid and other secondary product also get formed which includes irido dio/trio

phenylalanine-derived metabolites mightalso participate in the biosynthesis of amarogentin which is experimentally proved.

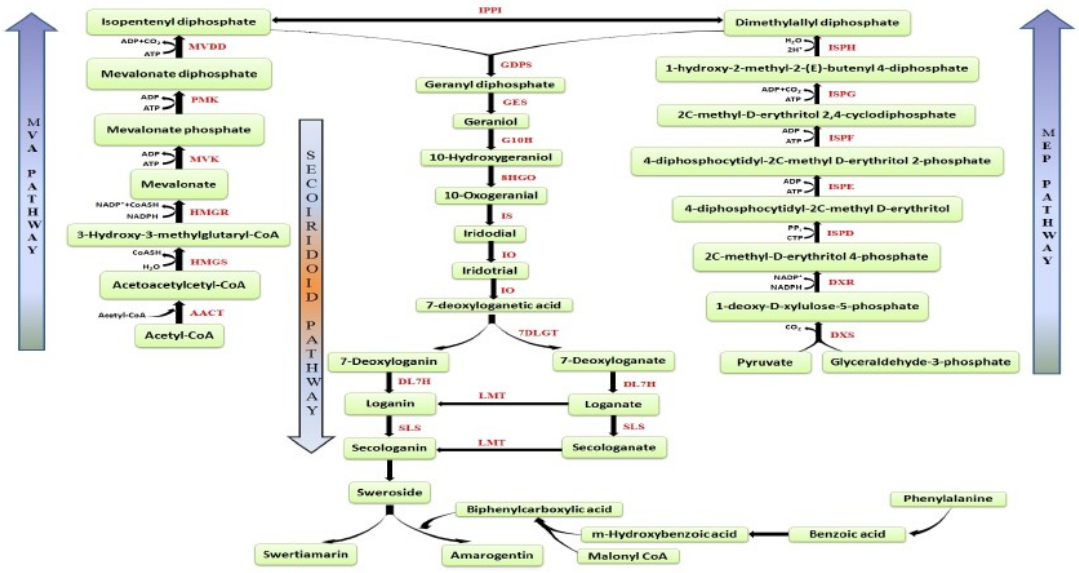


Figure 1.2:Biosynthetic pathway of swertiamarine and amarogentin (Padhan et al. 2015)

After all these complete picture of all pathways and their related biosynthesis at molecular level of all these compound is still lacking. There is no related and particular information are availablein the expression and biosynthetic level of these compounds such as swertiamarine,amarogentin and mangiferin.Other species of Swertia for which different studies have been taken as pathway correlation and their significant and related products.

Expression profiling of these pathway genes with respect to the different metabolite levels would give a clue to the relative contribution of each gene on the biosynthetic machinery of these major metabolites and all these pathway get co related with each other. Synthesis of primary and secondary metabolite get affected with different intrinsic and extrinsic factors such as humidity, mode of nutrition, intensity of light, temperature, carbon sources and other nutrition which are used for affecting the pathways. Photoperiod also affect the synthesis of primary and secondary pathways, such as plant is photo hetrotrophic or it is photoautotrophic. Murashige and skoog medium is mainly use for *S.chirayita* while the plants which are whichare grown in the green-house used soil mixture and amount of photosynthesis provided such as humidity, mode of nutrition, intensity of light, temperature, carbon sources and other nutrition which are used for affecting the pathways. Photoperiod also affect the synthesis of to

these plants. Different method of nutrition are used which are as photohetrotrophic nutrition and photoautotrophic nutrition. All these should help in affective towards different pathways, gene supplementation and product which are formed. Mode of nutrition, photoperiod also influenced the different autotrophic and heterotrophic mode of nutrition.

Plants traits which are related with biomass yield are also influenced by biochemical,molecular and physiological process.The physiobiological characters such as leaf size,stem diameter,fruit size,flower size,leaf sensescence also effect the biomass yield. Different pathways such as glycolysis,TCA,pentose phosphate pathway, photosynthesis implicates biosynthesis,degradation and inter conversion of different compounds.Different metabolic lead product are mainly depends on metabolic products which are formed such as intermediate compounds e.g., shikimic acid,pentose-5-phosphate and glycosides,erythrose-4-phosphate.Efficiencies and states of all secondary metabolites depends on state of these intermediates compounds which help for formation of secondary metabolites and other associated products,which provide precursor for products.In contrast phytochemicals which are related to these compounds,in such a way that production of these are related and effect the final products which are formed.

From past few decades all gene functions there regulations exploration is associated with how the production of secondary metabolites occurs.Small RNA(sRNA),their role in defense mechanism,regulation of genes andformation of gene products are also identified from different secondary metabolite products.siRNA is of two types:miRNA and siRNA which are micro RNA and short interfering RNA.These are important for different methods such as silencing,formation of different types compounds there regulation and further association with protein can also be identified and used by these type of RNA.

New strategies which enhanced different development and improvement which are involved in the crop resistance, biomass improvement and yield of desired chemicals.Understanding at the deeper level for various regulatory mechanism is related with different pre-requisite methods.For their functional cues different forms of culture and environmental conditions are offered.

Observing different pharmacological importance of *S. chirayita*, and obscure knowledge on the biosynthesis pathways and the regulators thereof, and the lesser known molecular processes channeling towards secondary metabolite levels, the current research work was carried out with the following objectives:

Objective1: To investigate relative contribution of different pathway modules of Swertiamarin, Amarogentin and Mangiferin biosynthesis in *Swertia chirayita.*

Objective 2: To validate the biosynthetic routes of Swertiamarin, Amarogentin and Mangiferin along with the associated most critical steps involved in their biosynthesis.

CHAPTER 2

REVIEW OF LITERATURE

Because of modern drug resistance and toxicity over different products natural products are becoming important part of human health. Plant drugs which are traditionally used by different developing countries are nowadays again play an important role in health sector. Approximately 80% of the countries are relies on the plant drug which is estimated by WHO. Mainly 25% of the plants are used as medicinal plants and from there its pharmacological importance are also get increased as different prototypes are formed with these plants .Plants produced a series of different compounds which are structurally found in different plants and other associated pathways and compounds. Primary metabolites which are produced by different precursors with the help of primary metabolic pathways while secondary metabolites are produce in such a way so that primary metabolites are used as precursor for them. Secondary metabolites are of different types such as alkaloids,terpenoids, natural phenols and polyketides. Secondary metabolites which are produced are having medicinal important properties which are used for different cure and protection from diseases.

Out of the ~297,000 native floral species of the world, 10% are used as medicinal plants this is based on approximate calculation. India accounts for one amongst the seventeenth mega diversity countries of the world, it houses two major biodiversity hotspot and 7% of total biodiversity.Out of the total floral species of India (17,000-18000), 44% are expected tohave therapeutic usage in various folk and renowned systems of medicine like Ayurveda,Homoeopathy, Unani and Siddha.

2.1Swertia:

Genus of the Gentiana family which includes different annual and perennial herbs,contains the plant Swertia. Approximately 170 species of Swertia has been reported out of which 40 had been identified in India.Out of these 40 species, 32 are found in Himalayan regions and remaining are found in North-West Himalayas.A number of herbal medicines and medicinal potential values are identified for Swertia and its different species. Prominent trade significance and pharmacological values are important for different species of Swertia. Other species are used as substitute for different species of different medicinal important properties

and this helps with the occurrence of different chemical important and secondary important plants.

2.2 *Swertia chirayita*:

Swertia chirayita is mainly found in temperate regions of Himalayas. It is found in the shady and slopy region of Himalayas which is found from Kashmir to Bhutan and other Khasia range. It is found at the elevation of 1200-4000 m.

This herb contain very important position in Indian medicinal system and it is used as an antidiarroetic,bloodpurifier,digestive,carminative,antihelmintic,expectorant,febrifuge,antiperodic and laxative agent. Plant is having usefulness in flatulence, skin disease, bronchial asthma, malarial fever.It is also used in dying industry for coloring of cotton cloth as yellow in color and because of its bitterness property ii is used in alcohol industry. It is used as stimulant of central nervous system(CNS). Formulations like Ayush-64 (The Central Council for Research in Ayurvedic Sciences,India, Unijules Life Sciences Ltd.,Vhca Ayurveda), Diabecon (Himalaya HerbalHealthcare), Citazil (Unexo Laboratories Pvt. Ltd.), Lekoril (Unexo Laboratories Pvt.Ltd.), Lekorin (Unexo Laboratories Pvt. Ltd.), Livnol (Unexo Laboratories Pvt. Ltd.),Malarin (Unexo Laboratories Pvt. Ltd.), Mahasudarshanchurna (Zandu PharmaceuticalWorks Ltd.), Melicon V ointment (Cadila Pharmaceuticals Ltd.) etc.

2.3Systematics:

The plant is a member of the family Gentianaceae. A detailed account of the taxonomic classification of the plant is given in Table 2.1.

Table 2.1:Classification of *Swertia chirayita*

CLASSIFICATION	DETAILS
Taxonomy ID	137887
Super kingdom	Eukaryota

Kingdom	Viridiplantae
Phylum	Streptophyta
Class	Magnoliopsida
Sub-class	asterids
Order	Gentianales
Family	Gentianaceae
Tribe	Gentianeae
Genus	*Swertia*
Species	*Chirayita*

2.4 Biological description of plant: **2.4Biological description of plant**:

2.4.1: Macroscopic:

It is described as annual herb mainly by different scientists,but some based on its properties and products known as biennial and perennial herbs.Plant grows upto 0.6 to 1.5 m.Main stem appears in the month of March/April and leaves which are also grow and shows senescence in the month of same year till November. The stem of this plant is 6mm in diameter in 1 mm in length.It is yellowish brown to purplish in the color and distinguished pith is also found which is dark green in color.The plant is having two types of leaves one which produce during the reproductive phase is known as cauline phase and other which is present at vegetatively is radical leaves.The radical leaves are a huge rosette, light green-purplish dark green in color, subsessile, broadly lanceolate and 5-7 nerved, 24 -29 cm in length and 5.5-6.4 cm in width, lanceolate with multicostate and pinnately reticulate(convergent) venation. Radical leaves show decrease in their number as they grow in stem,they are completely absent as they reach till floral bud. Lower lamina can be distinguished as when they grown up at the start are of green in color then they get changed into the purple in color. Branching is found in different patterns and these help to form different aestivation system.Flowers are leafy and large having panicles with yellow color ,granular depression is found near the base of each corolla base.

Thecalyx is quadrilobed,quardriplex,twisted,gamosepalous,regular,valvateaestivation.Stamens are present as alternated form and with corolla. Filaments are equal in size and equal in length with the stamens.Seeds are minute, dark brown in color and secondary,tertiary roots get

developed with this.Gynocium is having bicarpellary, superior, syncarpous, ovoid elliptical sections which contains small size ovules which are partially arranged. Stigma is very short and contain slender shape style. Seeds which are important and they help for getting primary and secondary type of roots.

2.4.2: Microscopic:

ROOT:Like other plants Swertia is having parts which are like cork, cortex, phloem, xylem etc.2-4 layers of cork can be seen. Secondary cortex is made up of 6-12 layers.Secondary phloem is made up of thin sieves, phloem parenchyma and companion cells. Secondary xylem contains xylem fibers, vessel elements,tracheids. Centre is less spongy and hollow in most of cases while made up of woods.Fiber contains pits and these are generally mucilage in structure.

STEM: Transverse section shows single layer of epidermis.In younger stem the epidermis layer is covered with cuticle while in older it remains as such. Endodermis is distinctly visible and it covered with thin layer.it is followed by pericycle. Xylem vessel is made up of thin layer of vessels. Some vessels which are present are less in number while tracheids are present in abundance. Vessels contains simple pits while these can be differentiate from tracheids.

LEAF:Transverse section of leaf shows very little differentiation in comparison with upper layer and lower layer is found. Thickness of upper layer is more than the lower layer.The cells become less elongated.Spongy cells which are found must be associated with other cells which are connected to the palisade cell tissues.These tissues must be associated with the particular set of transverse type of cells.

2.5 Medicinal properties of *Swertia chirayita*:

S.chirayita is used as medicine form the ancient system as the herbal drug and advance medicine form.Several studies are done for its medicinal properties.Some of them are as follow:

2.5.1Anti diabetic:

Anti-diabetic property which is because of the presence of swerchirin,which is one of the active constituent found and present as the hexane compound fraction. Different studies have been involved for *S.chirayita* where a significant drop in sugar level was observed after oral

administration for 7 hours.This occurs because of compound hexane which is extracted from plant. Bellidiofin and swerchirin are active compounds which used to show hypoglycemic effect, this effect was completely dose dependent. Different studies has been done for glycemic effect of *S.chirayita* in which rat models are taken for which were fed,fasted and glucose loaded.

2.5.2Anti-carcinogenic:

Bellidifolin, swertiamarine and their other isoforms are having mutagenic activities, mainly found in methanol extracts.These mutagenic activities which are found in such a way that. Methanol extraction is used for different carcinogenic activities.Different detoxification experiments and enzymes which are involve in the anti carcinogenic activities are observed.

2.5.3Anti-malarial:

In-vitro malarial testing was done on *Plasmodium falciparum* in which inhibit the growth of these malarial agents.Ayush-64 and Malarian are used as anti-malarial drug. *Plasmodium bergheiis* is also tested for the effect of this anti-malarial activities. Different syrups such as Ayush,malarin is also used for anti malarial syrup.

2.5.4Hepatoprotective:

Hepatoprotective activity was because of sweroside against lipopolysaccharides.Carbon tetra chloride was used for induced liver toxicity. Sweroiside is having hepatoprotective activity against lipopolysaccharides. It was also observed against methanol extracts.Mainly this was induced against carbon tetra chloride toxicity.

2.5.5Anti-inflammatory:

Diethoxy compound was observed for anti-inflammatory activity.It was tested over rats .Carrageenin induced and formalin induced is used for this effect. The peadal edema which was reduced with this, effectively was found 50% and 57%.Dose dependent tumour necrosis factor which was cytokinin induction is used as inflammation reactant which is associated with particular reactions. Tumor necrosis factor - α (TNF - α), interleukin - 1β (IL - β), interferon - γ (IFN - γ) and interleukin - 10 (IL - 10), modulation of tumor necrosis were effective because of the presence of amarogentin, mangiferin.*S.chirayita* plant was taken as model for these inflammatory effect.

2.5.6Antimicrobial:

Gram positive and gram neagative bacteria are having significant effect against *S.chirayita*. Antimicrobial activity was observed with methanol and aqueous extracts was evaluated for bacteria and fungi. Better antimicrobial activity was observed in methanol extracts. These antimicrobial effect was maximum shown in *E.coli*, *Baccilus cereus*.

. **2.5.7As hair tonic**:

Swertiamarine is used as hair tonic without mixing of any other ingredients.It is very effective hair tonic which is observed presence of swertiamarine in *S.chirayita*.

2.5.8Action on CNS:

Swertiamarine is used as depressing and stimulating effect. CNS stimulating effect mangiferin activity which is responsible for activation of CNS.

2.5.9Gastroprotective activity:

S.chirayita is used for decreasing gastric mucosal intensity by methacin and necrotizing agents. Pre-treatment will cause protection in gastric mucosa with the help of exhaustion and establishment of sulphur compounds.

2.5.10Analgesic and Antipyretic activity:

These properties which are present due to sweritamarine and amarogentin produced compounds whichstimulatesthese activity. Acetic acid induction was completely stopped with the help of ethanol extracts which were found effective with effectiveness of *S.chirayita*.

2.5.11Antileishmenial activity:

Swertiamarine and amarogentin are used for *Leshmeniadonovani* which is used to effect the binary complex formation which effect the human body.

2.6 Molecular biology of *S.chirayita*:

Different analysis has been done for the molecular understanding of *S.chirayita*. The plant is reported as diploid with gametic chromosome number 13.Different cytotypes has been reported with different chromosome number such as 24 and 22.It is also considered as allotetraploids. Different cytotypes which are reported are as n=10,24,36,38.Dysploidy is define as dysploidy. Several karyo morphological and meiotic analyses have been performed in *S.chirayita* to understand the plant at the molecular level. The plant has been reported to be a diploid (2n = 26) plant, with gametic chromosome number n = 13.

However, different cytotypes of *S. chirayita* with 2n = 20 and 2n = 24 [210] have also been reported. Raina et al.describe *S. chirayita* to be a genomic allotetraploid (2n =26). However, different cytotypes of *S. chirayita* with 2n = 20 and 2n = 24 [210] have alsobeen reported. Raina et al.describe *S. chirayita* to be a genomic allotetraploid (2n =26). They argue the secondary origin of Chirata, as it exhibits the base number of 13 (x =13), because plants showing base number more than 12 are of secondary origin as reported earlier. Also, the genus Swertia had been reported with cytotypes 2n = 14,16, 18, 20, 21, 24, 26, 28, 38, 42, 52 and 60, and with 7, 9 and 12 base numbers .Based on these reports they logically hypothesize that *S. chirayita* has come up either because of a cross between two progenitors with x = 7 and x = 6, or due to dysploidy(ascending or descending) in cytotypes with 2n = 12 or 14, that is followed by chromosome doubling.

2.7 Micropropagation study on *S.chirayita*:

Micropropagation is done to regenerate and potential to conserve valuable plant genetic sources.Different growth elicitors and hormonal effect can be observed on the plant.Transformed root culture has been observed and amount of amarogentin and swertiamarin get increased. Production of viable seeds is supplemented with MS media.Multiple shoot regeneration is done with shoot tip explants when grown with MS media.Regeneration characterization and development of different shoot culture has been used for different seeds. Developed plantlet are being transplanted into the soil and fertile seed plant get developed.Shoot generation is also read on this plant where effect of different growth hormones and there other phytochromes.MS supplementation is best with the combination of BAP which provides more shoot regeneration. Axillary shoot multiplication is needed for growth of bud culture.All these sources which are related with the production and

supplementation of different type of hormones and phytochemicals can be observed in the plant.

2.8Genetic diversity:

Genetic diversity minimizes the frequency of unfavorable traits.It is related with the survival and the adaptability of any organism.It is also necessary to differentiate between intra and inter populations.Different biochemical,genetic andmolecular markers are used to identify these traits.Molecular markers are used to identify in comparison to other markers such as these do not get affected with environmental changes.DNA based markers are used for proper identification and authentication is also very easy as the lack of
polymorphism.There are different techniques which are involved such as PCR can be also used for these identification of diversity.

2.9Molecular basis of biosynthesis of major secondary metabolites:

S. chirayita possesses a wide range of phytochemicals belonging to different classes. Swertiamarin, amarogentin and mangiferin are some of the major secondary metabolites of the plant as reported earlier . Swertiamarin and amarogentin are secoiridoid glucosides (terpenes), whereas mangiferin is a xanthone C-glucoside. Earlier studies demonstrated that various structural and regulatory genes control the biosynthesis and accumulation of terpenoids at the molecular level in different plant species.

Very few efforts have been gone in the direction of understanding the molecular basis of biosynthesis of these secondary metabolites in *S. chirayita*, that include identification of metabolic pathways, and the genes/enzymes thereof. Till date, no breakthrough has been achieved in this area, and the complete knowledge of molecular basis of their biosynthesis is still elusive. Some of the modern approaches taken towards elucidation of biosynthesis pathway(s) and the missing links involve, correlating the expression profiles of genes (or enzymes) with the accumulating level(s) of the chemical constituents, including their intermediates .However, the emergence of advanced technologies such as transcriptome sequencing by using advance form of sequencing which is next generation sequencing (NGS) technology has enabled us in understanding the particular and important change in the different metabolic processes ,differential transcriptomics, evolutionary genomics, and gene discovery . Swertiamarin and amarogentin are biosynthesized through a combined route involving the mevalonate (MVA), non-mevalonate (MEP), and the secoiridoid pathway.

There is a clear picture of the biosynthesis pathway till the formation of secologanin, a secoiridoid. C-1 position of the pyran ring gets glycosylated (O-linked glycosilation) in secoiridoids. Geraniol (a monoterpenoid alcohol) is considered to be the precursor in the biosynthesis pathway of secoiridoid glycosides, as indicated by several studies.

CHAPTER 3
MATERIALS AND METHODS

3.1Plant material and tissue collection

Plants of *S.chirayita* were raised and maintained in the green house of *S.chirayita* Waknaghat, Himachal Pradesh, India (1500 m altitude; 31°01' N, 77°04' E), in controlled green house chambers [light (intensity 1200-4800 W m–2),temperature (25°C), relative humidity (approximately 75%) and photo time with particular intensity(14 h day/10 h night).These plants were raised with MS media supplemented salts, supplemented with different growth hormones [Kinetin (2.2 mg/L), Indole-3-butyric acid (2.5 mg/L) and gibberellic acid (3.2 mg/L)], sucrose (35 g/L), agar-agar (8.9 g/L), pH 5.9-6.7, and maintained at the chambers [light (intensity 6,800 W m_{-2}), temperature (25°C), relative humidity (75%) and photoperiod (20 h day/ 10h night)] at the tissue culture facility of the university.These plants are 2 years old and in-vitro plants were used for expression analysis of *MVA,MEP* pathway. Different collected samples of roots and shoots were collected for primary genes related with secondary pathways .For transcriptome analysis plants which are taken such as 1.5 years from green house and 1.5 from *in-vitro* cultivation.

Precursor feeding was done with the help of different combinations such as these combinations are used in the different combinations. In this different combinations are used for all three secondary metabolite compounds.

Amarogentin (AM) - Benzoic acid (BA), Benzoic acid (BA) + Geraniol (GE),

Swertiamarin (SW) - Pyruvate (PYR) + Geraniol (GE)

Mangiferin (MN) - p-Coumaric acid (pCA)

All three (AM+SW+MN) - BA+GE+Cinnamic Acid(CA)+PEP

Concentration of the fed samples which was used 2.5mg/100mL.These samples are used in such a way that collection of these different samples were done accordingly such as after 10,20,30days samples were collected and from there sampling was done accordingly for different reactions. All these samples were analyzed then for their quantification, RNA isolation and further gene expression analysis by using RT-PCR and their expression profiling.

3.2 Quantification of swertiamarine,amarogentin and mangiferin:

Determination of different levels of three secondary metabolites mangiferin, swertiamarine, amarogentin were done by using high performance liquid chromatography on Waters HPLC system (Waters Corporation, USA) equipped with Waters 525 HPLC pumps, Waters 727 autosampler, Waters 2996 photodiode array detector .

Homogenization was done with the help of mortar and pestle(pre chilled) using liquid nitrogen .The powdered samples were kept in methanol 80%,vortex and sonicated at room temperature for 10 minutes. Centrifugation of the samples was done for 10 minutes at 10000rpm.Supernatant was filtered with 0.22µm filter and diluted to 80% methanol and quantification with the help of HPLC was done in the following way:

- Different days grown tissue amples were taken.
- Homogenization was done by using mortar and pestle and fine powder mixture get formed.
- Sonication was for 10 minutes by using 80% methanol.
- After sonication,centrifugation was done at 10000 rpm for 15 minutes.
- Filteration was done by using membrane filter.
- Further quantification was done with HPLC.

For different fed samples which are taken quantification was done and from there control and different precursor feeding samples get compared to each other .All these samples which are taken when get compared to the control then there was significant increment in the different precursors which are fed over. For this different combinations which are taken and from there comparison significantly shows that several time folds increase. Those samples are chosen which shows significant increment in comparison to control. These are having increment in comparison to fold and from there all these can be isolated and there precursor feeding was checked and compare with the control. All these are related with the several fold increment and this increment help in the comparison.

3.3 Total RNA isolation and preparation of cDNA:

Isolation of a large amount of RNA from the aforementioned tissues of *S. chirayita* was done by using Reagent method. (Ambion Inc., USA).Trizol method is used for RNA isolation process.Method for RNA isolation is in the following way:

Tissue samples which were already fed samples and analysed with the help of HPLC was taken. These samples were weighed, approximately 100mg of tissue samples were taken. Crushing was done with the help of liquid nitrogen. Then these samples were kept in the different eppendorfs.800µL of trizol was added,which required 10 minutes of incubation. Centrifugation was needed for 12000rpm.After collection of supernatant chloroform was added.Again incubation for 5 minutes and centrifugation at 12000 rpm is needed. Collection of supernatant was done and addition of isopropyl alcohol. Finally washing get started with DEPC water, then dried at room temperature.

Different samples which were taken and Qualitative and quantitative estimation of the isolated RNA was done by spectrophotometric (A260 and A280) measurements (NanoDrop, Thermo Scientific, USA), whereas integrity was assessed on agarose gel stained with 1% (w/v) ethidium bromide.

cDNA was synthesized from total RNA by using the Verso cDNA synthesis kit,in which first strand of cDNA get synthesized.Priming was done by the anchored oligo(dT) primers and removal of contaminating genomic DNA was done using Verso RT Enhancer supplied with the kit. For miRNA expression studies cDNA was prepared using NCode™ miRNA First-Strand cDNA Synthesis (Thermo Fisher Scientific, USA) by selective poly-A tailing of miRNA and subsequent cDNA synthesis from poly-A miRNAs.

For 1X cDNA verso DNA needs different concentration in the following way:

Table3.1: cDNA synthesis

COMPONENETS	VOLUME(µL)
5x cDNA buffer	4
dNTP mixture	2
Oligo dT	1
RT enhancer	1
VERSO enzyme mix	1
Water	10

Template of prepared RNA should be added as 1µL.

After preparation of cDNA, one more reaction cDNA check was also done,which is checked with the help of NanoDrop. Amount of produced cDNA is very less so amplification of the sample is needed. Reaction should be prepared in this way:

Table3.2: cDNA check reaction

Components	Volume for 1X reaction(µL)
5X cDNA	4
dNTP mixture	2
Oligo dT	1
RT enhancer	1
Verso enzyme	1
RNA	1
Water	10

After preparation of this cDNA amplification should be done by using PCR amplification reaction, which should be in following way,

Table 3.3:PCR amplification reaction

COMPONENTS	VOLUME FOR 1X REACTION
PCR buffer	1.25
dNTPs	0.25
Forward primer	0.5
Reverse primer	0.5
Template	1
Taq	0.125
Water	8.875

Amplification get checked with 1% agarose gel. Then further amplified products are taken for the RT PCR. For this reaction was made diluted upto 100ng.For RT PCR reaction should be prepared in this way:

Table 3.4: RT-PCR reaction

COMPONENTS	VOLUME(μL)
SYBR green	6.25
Forward primer	0.5
Reverse primer	0.5
Template	1
MilliQ water	4.25

For this reaction different primers which were taken *LMT, IS, GES, DL7H*.For these genes expression analysis was checked.

Primers which are used for RT-PCR reaction having following properties:

GENES	LEFT PRIMER SEQUENCE	RIGHT PRIMER SEQUENCES	ANNEALING TEMPRATURE	AMPLICON SIZE (bp)
LMT	ATGAAAGGTGGTGATGACTC	AATGCGGAAAGGTTTGATTG	48	156
IS	GGCTGAGTTAATGAAGGATA	CAAGAACCCTAATTCCTTAC	57.4	164
GES	GAGTGTTCTTCCTTACCTA	GACTAAGGCCATATATGATC	50	151
DL7H	TGAATTCTGCTGCATCATC	TGACCATGATATTGCTCGAG	53	180

CHAPTER 4

RESULTS AND DISCUSSION

4.1Tissues grown as different fed concentrated samples

Different plant cultures were grown in such a way that its feeding for different precursor were done. It was taken as 2.5mg/mL. These precursors were use to fed different samples which are used as tissues for further reactions. These tissues were monitor on different days,such as collection was done with the comparison of control and different combinations which were used for different grown conditions. Samples were collected at different days such as 10,20 and 30 days. Comparison with control sample was done and from there different grown conditions are observed. Growth of different cultures are found is such a way:

RESULTS

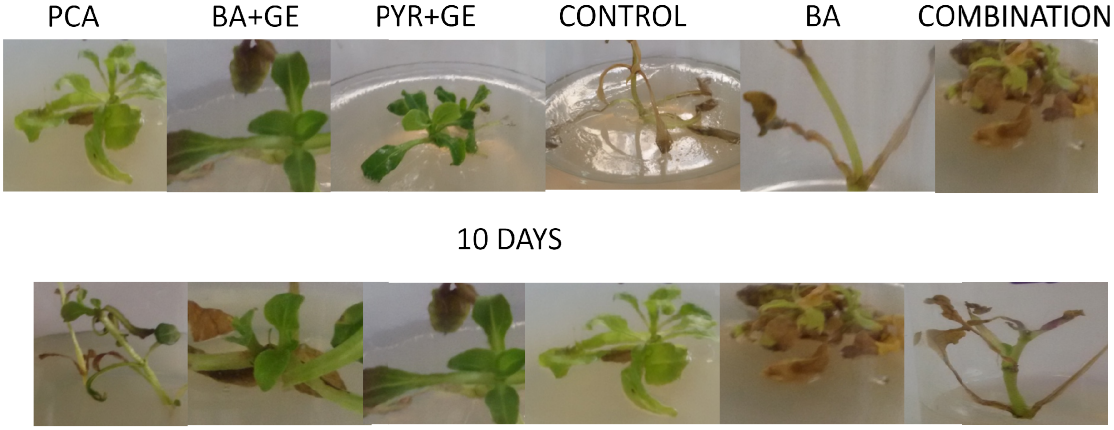


Figure 4.1: Fed plants of *Swertia chirayita*

Here we can find growth of different plants with the different fed cultures and from there comparison at different days was easy to done. These fed samples were taken and used as source for further determination of different types of reaction such as HPLC and for gene expression which is related with RNA isolation, cDNA preparation and futher RT-PCR reactions.

4.2 Differential concentrations of Swertiamarine,amarogentin and mangiferin by HPLC: HPLC analysis revealed variable biogenesis of major secondary metabolites (swertiamarin, mangiferin and amarogentin) in the studied tissues of *S.chirayita*.From the HPLC chromatogram of the above secondary metabolites is given in concentration are as:

Table 4.1: HPLC quantification for different precursors

Control				PCA				BA+GE			
	SW	AM	MN		SW	AM	MN		SW	AM	MN
0	0.11	0.06	0.39	0	0	0	0	0	0	0	0
10	1.36	0.05	0.33	10	2.93	0.06	0.61	10	0.67	0.05	0.04
20	1.52	0.06	0.12	20	4.73	0.14	1.73	20	2.65	0.1	1.06
30	1.72	0.08	0.53	30	1.63	0.08	0.81	30	3.01	0.12	2.37
40	1.27	0.06	0.73	40	0.32	0.06	0.67	40	1.9	0.11	2.23

PYR+GE				BA				BA+CA+GE+PEP			
	SW	AM	MN		SW	AM	MN		SW	AM	MN
0	0	0	0	0	0	0	0	0	0	0	0
10	0.3	0.09	0.48	10	0.65	0.05	0.13	10	0.07	0.01	0.32
20	0.48	0.06	0.7	20	4.81	0.16	2.25	20	0.12	0.03	0.53
30	1.6	0.07	1.49	30	0.83	0.05	0.36	30	0.25	0.03	0.39
40	0.87	0.07	0.62	40	2.56	0.15	1.73	40	0.32	0.12	0.56

ONLY 30 DAYS	CONTROL	PCA	BA+GE	PYR+GE	BA	BA+CA+GE+PEP
SW	1.72	1.63	3.01	1.9	0.83	0.25
AM	0.08	0.08	0.12	0.07	0.05	0.03
MN	0.53	0.81	2.37	1.49	0.36	0.39

From these given observations different concentration of these secondary metabolites are observed in different compounds such as p-coumaric acid(pCA), Benzoic acid (BA), Benzoic acid, Geraniol (GE), Pyruvate (PYR), Geraniol (GE),Cinnamic Acid(CA),Phosphoenol pyruvate(PEP).These compounds were found as a great concentration increment with comparison to the control and there are different combinations which are found in all three secondary metabolites.These three are as follow:

Amarogentin (AM) - Benzoic acid (BA), Benzoic acid (BA) + Geraniol (GE)

Swertiamarin (SW) - Pyruvate (PYR) + Geraniol (GE)

Mangiferin (MN) - p-Coumaric acid (pCA)

All three (AM+SW+MN) - BA+GE+Cinnamic Acid(CA)+PEP

The concentration of precursors used was 2.5mg/d .These findings of different concentration was observed and comparison was done with the help of control and then further values which are taken can be observed as several folds increment.

Major biogenesis of swertiamarine,amarogentin and mangiferin is revealed by HPLC analysis. This chromatogram is associated with the levels of these compounds in different fed samples.In the hplc analysis it was obtained that there is difference of 0.01%-0.1%,0.01%-0.2% between these three compounds.

From HPLC analysis as the quantification was done from which it was observed that there is increment in the different compound concentration and these are several folds increments. Such as in Swertiamarine increment into found several folds which is approximately two to three times, than comaparison towards amarogentin is found as increment is five to six folds as in different compounds, in case of mangiferin it was increase as two to four folds.

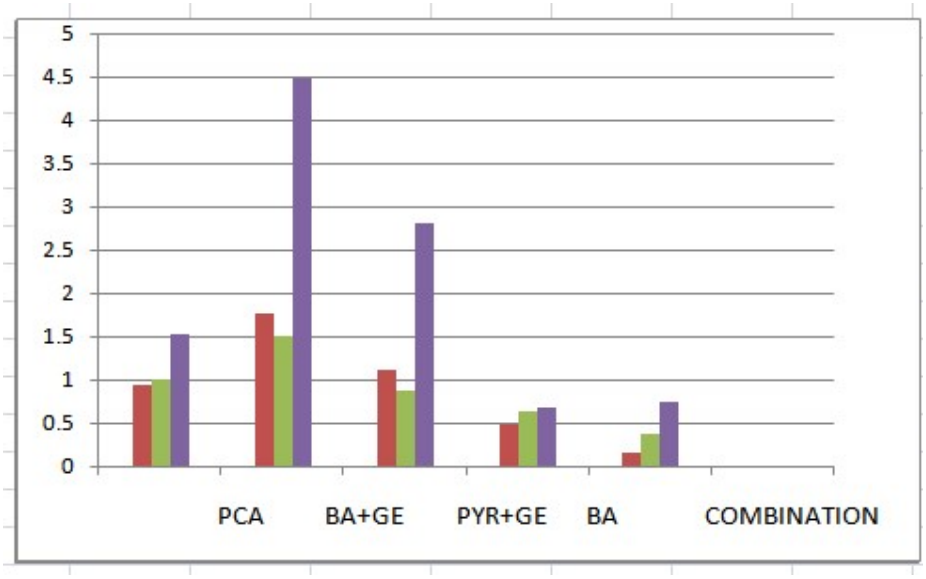


Figure4.2: Fold accumulation of amarogentin, swertiamarine and mangiferin in different fed samples

Here different fed samples are taken in which comparison was done on the basis of different compounds and combinations which are needed. All these combinations which are taken show significant increase in different samples of SW,AM,MN. To show effect of different combinations which are taken in such a way that combination of benzoic acid and gereniol shows significant increment in the mangiferin compound this may be because of increment in the intermediate compounds or because of the end product which is formed. Comparatively increment in the amarogentin, mangiferin and swertiamarin is found in the combinations of benzoic acid and geraniol many folds increase in the case swertiamarine. In each and every fed samples mangiferin was showing significant increment in the combination of amarogentin

,swertiamarine, andmangiferin. These graphs show significant increment which is 3-folds,4-folds increment in the different concentration of these secondary metabolites.

4.1 RNA isolation:

RNA isolation was done with the different samples and different compounds which were observed in comparison to the control such as two bands were there out of which one was rRNA and other was of mRNA. All 6 different samples which were taken in at a particular sequences. Nanodrop reading is also taken for samples which were used as the quantification of the different compounds.

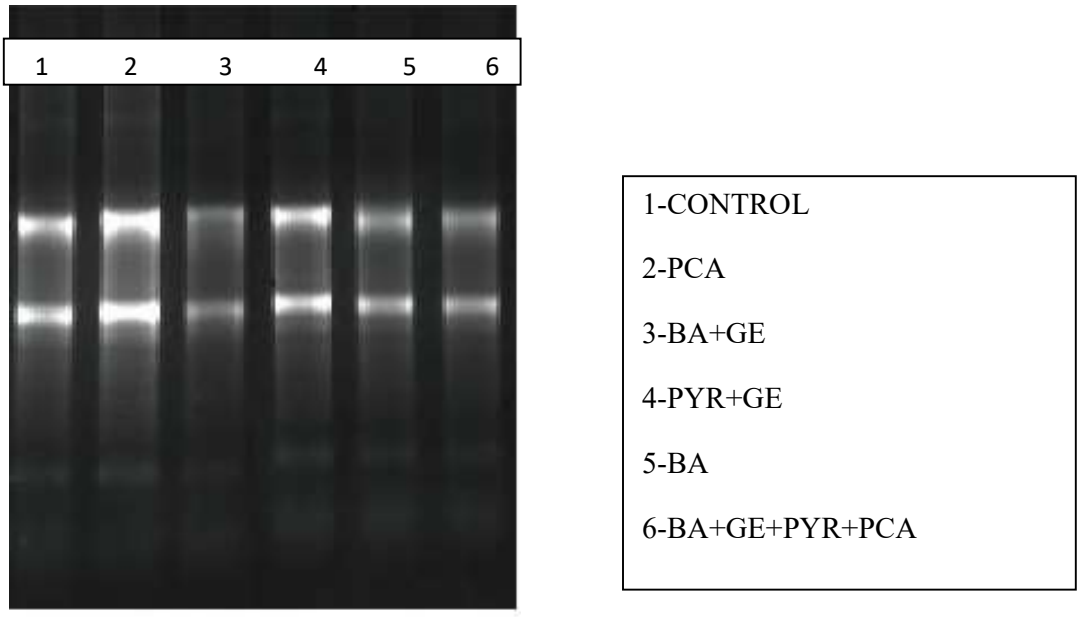


Figure 4.2:RNA Bands

Isolation of RNA in the plant samples which are taken such as Swertiamarine,amarogentin and mangiferin are taken. Samples which are taken have shown bands of 16s rRNA and 23s r RNA. These bands show isolation of RNA and with these confirmation of plant samples also has been done. These are found as the no other contamination is there which shows confirmation of the presence of these plant gene samples.

4.2 RNA quantification:

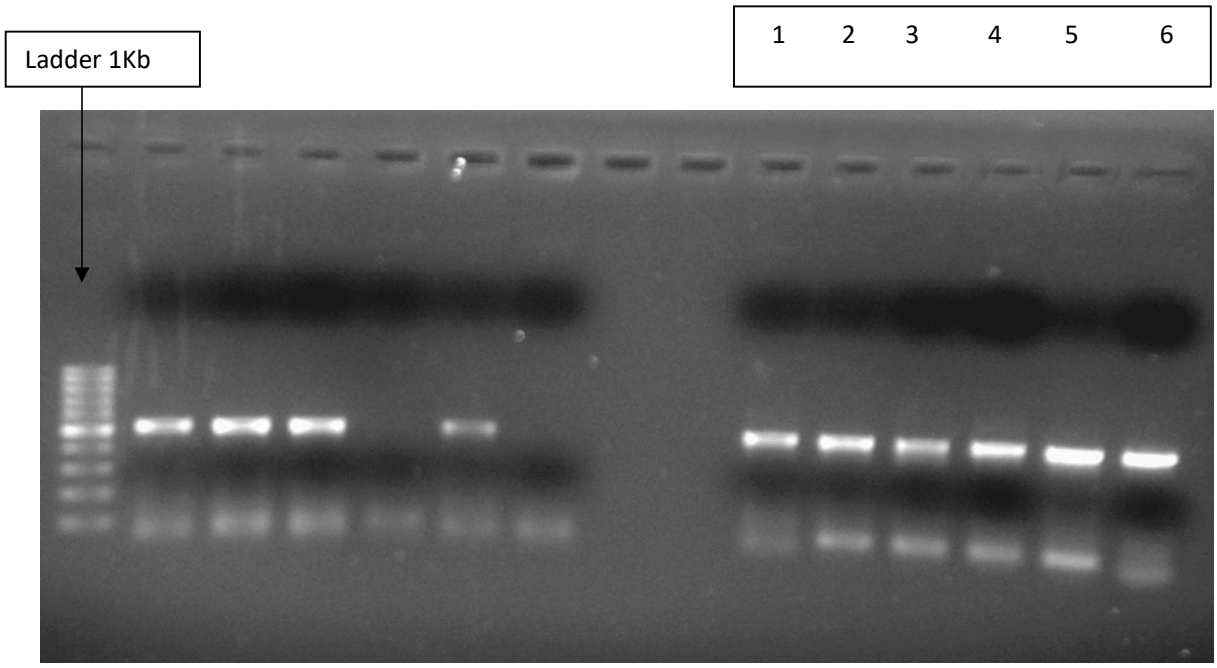
RNA quantification was done with the help of nanodrop where the value was determined in nanogram.After isolation of RNA further process which is related with gene expression get started. In which cDNA preparation, quantification is associated with this.

Table 4.2: Concentration of RNA

SAMPLES	RNA CONCENTARTION(ng/µg)
CONTROL	2370
PCA	1550
BA+GE	1480
PYR+GE	782
BA	598
BA+GE+CA+PEP	681

RNA quantification is done with nanodrop in which concentration of RNA is found maximum in p-coumaric acid and least in the Benzoic acid samples.

4.3:cDNA isolation:



1-CONTROL

2-PCA

3-BA+GE

4-PYR+GE

5-BA

6-BA+GE+PYR+PCA

Figure 4.3:cDNA bands

Isolation from RNA and formation of complementary DNA is mainly done for expression of gene, initially the first step is formation of complementary DNA where it is associated with concentration of The bands appeared on the gel corresponding to the DNA ladder.DNA ladder confirms the formation of cDNA that contain tubulin gene sequence, and hence it has expressed.

Forward 5'GGAATGGATCTGACGGCAAG3'-20 bases

Reverse 3'GGTCAGGAGTTGCAAAGCG5'-19 bases

4.4 Nanodrop reading for cDNA concentration

Table 4.3: cDNA concentration

SAMPLES	cDNA CONCENTRATION(ng/µg)
CONTROL	994
PCA	1040
BA+GE	1103
PYR+GE	934
BA	1190
BA+GE+CE+PEP	863

Nanodrop reading which is taken for cDNA concentration which is found maximum in benzoic acid and minimum in combination of different compounds.

4.5 Expression analysis:

Expression analysis of key genes in in-vitro cultures precursor fed shoots at the molecular level, tissue specific variation six samples for different fed samples were taken for which four genes are teken. It was observed that genes of swertiamarine, amarogentin and mangiferin are highly expressed and were analyzed in five tissues of S.*chirayita* using RT-qPCR technique. It was observed that most of the genes of the swertiamarin/amarogentin biogenetic pathways were active and highly expressed in green house grown *S. chirayita* when compared with the in vitro cultured plants. Sixteen genes of swertiamarin/amarogentin biosynthesis pathway, coding for GES, IS,7DLGT, DL7H and LMT showed higher expression levels (≥ 3folds).GES showed 24 folds higher expression, IS showed 105 folds increment, GES showed 24 folds,DL7H shows 273 folds increment. Aside from leaves, gene expression profiles of mangiferin biogenetic pathway did not exhibit specific behaviour in connection withmangiferin levels in all the tissues. This finding was in tune with an earlier study, where the expression status of genes of phenylpropanoid pathway did not exhibit considerable correlation with the levels of shikonin . It can be inferred from the observation that these highly expressed pathway genes are associated with the biogenesis of other phytochemicals. The significance of PAL gene of mangiferin biosynthesis pathway has been demonstrated in

developing shoot and root tissues of Populus tremuloides Michx. , where expression levels of the gene varied relative to the contents of condensed tannins (CT) and lignin.

EPSPS, a key gene identified in our study has been reported to modulate the aromatic amino acid biosynthesis pathway and act as glyphosate target. In flowers, the mangiferin biosynthetic pathway genes did not show specific behaviour in relation to mangiferin levels. This may advocate the connection of a transport mechanism in which the secondary metabolites are biosynthesized outside the floral tissue and later on gets transported to the sink-tissue. A similar mechanism of transport of nicotine by Nt-JAT1, a (MATE)-type transporter in Nicotiana tabacum from roots to the central vacuoles of leaves, and glucosinolates transport from the maternal tissue to seeds of Arabidopsis thaliana, through the NRT/PTR transporters, have been reported.

All these studies sturdily sustain the findings of our report and advocate the suitability of these genes in order to be considered as key targets for regulating various metabolic traits of utmost importance. The candidate genes with significantly higher expression profiles relative to the metabolites levels can be modulated to increase the levels of desired secondary metabolites. As an illustration, overexpression of DXS, HMGR and prenyl transferases have been demonstrated to enhance the contents of terpenoids in various crop plants [286]. Identification and modulation of various transcription factors (TFs) associated with the biosynthetic pathway(s) is another.

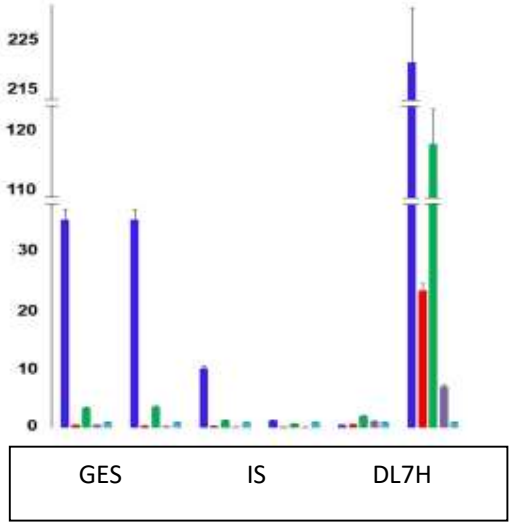


Figure4.4: Fold increment expression in different genes

CHAPTER-5

CONCLUSION

Understanding of the complete biosynthetic pathway is a prerequisite in devising different secondary metabolites pathway in different plants which are fed with perticular precursors. The current research was carried out keeping in view the medicinal importance of *S. chirayita*, the obscure knowledge on the molecular basis of biosynthesis of major secondary metabolites like swertiamarin, amarogentin and mangiferin, and their regulation.These metabolites get increased with different precursor feeding compounds and from here different key genes which are responsible for these metabolite production get identified and proved with the help of different increment in the folds and expression level of all those genes.These genes which are identified and associated with the particular metabolites. This study involves different key genes which are present and are related with the production of secondary metabolites which are needed for the enhancement of the products which are associated with these genes.

These genes show significant increment in the amount of the particular products which are get beneficial as this plant is having medicinal importance and key genes enhancement which is needed is the most significant increase in the metabolite production.

CHAPTER-6

REFERENCES

[1] World Health Organization, "Essential medicines and health products information portal," World Health Organization, Geneva. Retrieved Jun, vol. 14, p. 2014, 2014.

[2] M. Rao, M. Palada, and B. Becker, "Medicinal and aromatic plants in agroforestry systems," in New Vistas in Agroforestry, ed: Springer, pp. 107-122, 2004.

[3] C. P. Kala, P. P. Dhyani, and B. S. Sajwan, "Developing the medicinal plants sector in northern India: challenges and opportunities," Journal of Ethnobiology and Ethnomedicine, vol. 2, p. 1, 2006.

[4] S. Z. Hasan, V. Misra, S. Singh, G. Arora, S. Sharma, and S. Sharma, "Current status of herbal drugs and their future perspectives," in Biological Forum-An International J, pp. 12-1, 2009.

[5] A. Sharma, "Global medicinal plants demand may touch $5 trillion by 2050," Indian Express, vol. 29, 2004.

[6] K. Joshi, P. Chavan, D. Warude, and B. Patwardhan, "Molecular markers in herbal drug technology," Current Science, vol. 87, pp. 159-165, 2004.

[7] U. Schippmann, D. J. Leaman, and A. Cunningham, "Impact of cultivation and gathering of medicinal plants on biodiversity: global trends and issues,"Biodiversity and the ecosystem approach in agriculture, forestry and fisheries,2002.

[8] J. Maheswari, "Patenting Indian medicinal plants and products," Indian Journal of Science and Technology, vol. 4, pp. 298-301, 2011.

[9] S. Samant, U. Dhar, and L. Palni, "Medicinal Plants of Indian Himalaya: Diversity," Distribution and Potential Values,(GB Pant Institute of Himalayan Environment & Development. Gyanodaya Prakashan), 1998

[10] P. Joshi and V. Dhawan, "Swertia chirayita - an overview," Current Science, vol.89, pp. 635-640, 2005.

[11] E. M. Williamson, Major herbs of Ayurveda: Churchill Livingstone, 2002.

[12] G. P. Bhat and N. Surolia, "In vitro antimalarial activity of extracts of three plants used in the traditional medicine of India," The American journal of tropical medicine and hygiene, vol. 65, pp. 304-308, 2001.

[13] K. D. Alam, M. S. Ali, S. Mahjabeen, M. R. Hassan, M. F. Rahman, and R. Chowdhury, "Potential hypoglycemic effect of Swertia chirata - an Indian subcontinent herb with important medicinal value," Pharmacologyonline, vol. 2, pp. 642-647, 2011

.

[14] R. Arya, S. Sharma, and S. Singh, "Antidiabetic effect of whole plant extract and fractions of Swertia chirayita Buch.-Ham," Planta Medica, vol. 77, p. P_138,2011.

[15] R. Nagalekshmi, A. Menon, D. K. Chandrasekharan, and C. K. K. Nair, "Hepatoprotective activity of Andrographis paniculata and Swertia chirayita," Food and Chemical Toxicology, vol. 49, pp. 3367-3373, 2011.

[16] V. K. Verma, K. K. Sarwa, A. Kumar, and M. K. Zaman, "Comparison of hepatoprotective activity of Swertia chirayita and Andrographis paniculata plant of North–East India against CCl4 induced hepatotoxic rats," Journal of Pharmacy Research, vol. 7, pp. 647-653, 2013.

[17] P. Saha, S. Mandal, A. Das, P. C. Das, and S. Das, "Evaluation of the anticarcinogenic activity of Swertia chirata Buch. Ham, an Indian medicinal plant, on DMBA‐induced mouse skin carcinogenesis model," Phytotherapy Research, vol. 18, pp. 373-378, 2004.

[18] S. Banerjee, T. K. Sur, S. Mandal, P. C. Das, and S. Sikdar, "Assessment of the anti-inflammatory effects of Swertia chirata in acute and chronic experimental models in male albino rats," Indian Journal of Pharmacology, vol. 32, pp. 21-24,2000.

[19] S. C. Das, S. Bhadra, S. Roy, S. K. Saha, M. S. Islam, and S. C. Bachar, "Analgesic and anti-inflammatory activities of ethanolic root extract of Swertia chirata (Gentianaceae)," Jordan Journal of Biological Sciences, vol. 5, pp. 31-36,2012.

[20] P. Roy, F. I. Abdulsalam, D. Pandey, A. Bhattacharjee, N. R. Eruvaram, and T. Malik, "Evaluation of antioxidant, antibacterial, and antidiabetic potential of two traditional medicinal plants of India: Swertia cordata and Swertia chirayita," Pharmacognosy research, vol. 7, p. S57, 2015.

[21] A. Laxmi, S. Siddhartha, and M. Archana, "Antimicrobial screening of methanol and aqueous extracts of Swertia Chirata," International Journal of Pharmacy & Pharmaceutical Sciences, vol. 3, pp. 142-6, 2011.

[22] L. L. Nyein, "Analysis of different fractions of Swertia chirata against gram positive and gram negative bacteria," The Open Conference Proceedings Journal, vol. 4, pp. 29-32, 2013.

[23] K. Patil, S. Dhande, and V. Kadam, "Therapeutic Swertia chirata - An Overview,"Research Journal of Pharmacognosy and Phytochemistry, vol. 5, pp. 199-207, 2013.

[24] H. Badola and M. Pal, "Endangered medicinal plant species in Himachal Pradesh," Current Science, vol. 83, pp. 797–798, 2002.

[25] J. A. Bhat, M. Kumar, A. Negi, and N. Todaria, "Informants' consensus on ethnomedicinal plants in Kedarnath Wildlife Sanctuary of Indian Himalayas," Journal of Medicinal Plants Research, vol. 7, pp. 148-154, 2013.

[26] S. IUCN, "The IUCN Red List of Threatened Species, 1994-2007 version," ed: Switzerland, 2008.

[27] CITES. (2014). Available: http://www.bsienvis.nic.in/Database/bsi_3949.aspx (Last accessed on 06.03.2017)

[28] S. Phoboo, M. D. S. Pinto, A. C. L. Barbosa, D. Sarkar, P. C. Bhowmik, P. K. Jha, et al., "Phenolic‐linked biochemical rationale for the anti‐diabetic properties of Swertia chirayita (Roxb. ex Flem.) Karst," Phytotherapy Research, vol. 27, pp. 227-235, 2013.

[29] S. S. Mahmoud and R. B. Croteau, "Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase," Proceedings of the National Academy of Sciences, vol. 98, pp. 8915-8920, 2001.

[30] D. Hampel, A. Mosandl, and M. Wüst, "Biosynthesis of mono- and sesquiterpenes in strawberry fruits and foliage: 2H labeling studies," Journal of agricultural and food chemistry, vol. 54, pp. 1473-1478, 2006
.

[31] K. Miettinen, L. Dong, N. Navrot, T. Schneider, V. Burlat, J. Pollier, et al., "The seco-iridoid pathway from Catharanthus roseus," Nature communications, vol. 5, 2014.

[32] C.-Z. Wang, Ulrich H. Maier, W. Eisenreich, P. Adam, I. Obersteiner, M. Keil, et al., "Unexpected Biosynthetic Precursors of Amarogentin − A Retrobiosynthetic 13C NMR Study," European Journal of Organic Chemistry, vol. 2001, pp. 1459-1465, 2001.

[33] H. Kuwajima, N. Hayashi, K. Takaishi, K. Inoue, Y. Takeda, and H. Inouye, "Studies on monoterpene glucosides and related compounds. LXV. Biosynthesis of the biphenylcarboxylic acid moiety of amarogentin and amaroswerin," Yakugaku Zasshi= Journal of the Pharmaceutical Society of Japan, vol. 110, pp. 484-489, 1990.

[34] M. Fujita and T. Inoue, "Biosynthesis of mangiferin in Anemarrhena asphodeloides: intact incorporation of C6-C3 precursor into xanthone," Tetrahedron Letters, vol. 18, pp. 4503-4506, 1977/01/01 1977.

[35] J. Wang, Y. Liu, Y. Cai, F. Zhang, G. Xia, and F. Xiang, "Cloning and functional analysis of geraniol 10-hydroxylase, a cytochrome P450 from Swertia mussotii Franch," Bioscience, Biotechnology, and Biochemistry, vol. 74, pp. 1583-1590, 2010.

[36] J. Wang, C. Zhao, C. Liu, G. Xia, and F. Xiang, "Introgression of Swertia mussotii gene into Bupleurum scorzonerifolium via somatic hybridization," BMC Plant Biology, vol. 11, p. 1, 2011.

[37] K. Mosaleeyanon, S. Zobayed, F. Afreen, and T. Kozai, "Relationships between net photosynthetic rate and secondary metabolite contents in St. John's wort," Plant Science, vol. 169, pp. 523-531, 2005.

[38] P. V. Fine, Z. J. Miller, I. Mesones, S. Irazuzta, H. M. Appel, M. H. H. Stevens, et al., "The growth–defense trade ‐ off and habitat specialization by plants in Amazonian forests," Ecology, vol. 87, 2006.

[39] T. Vogt, "Phenylpropanoid biosynthesis," Molecular plant, vol. 3, pp. 2-20, 2010.

[40] J. J. Casal and M. J. Yanovsky, "Regulation of gene expression by light," International Journal of Developmental Biology, vol. 49, pp. 501-511, 2004.

[41] T. Kawoosa, H. Singh, A. Kumar, S. K. Sharma, K. Devi, S. Dutt, et al., "Light and temperature regulated terpene biosynthesis: hepatoprotective monoterpene picrosideaccumulation in Picrorhiza kurrooa," Functional & Integrative Genomics, vol. 10, pp. 393-404, 2010.

[42] M. T. S. Paulilo, F. S. Lapa, and M. d. B. Falkenberg, "Effect of light intensity and growth substratum on plant development and production of secondary metabolites in Cordia curassavica (Jacq.) Roem. & Schult," Revista Árvore, vol. 34, pp. 417-423, 2010.

[43] B. P. Singh, Biofuel crops: production, physiology and genetics: CABI, 2013.

[44] J. Zhang, E. L. Wrage, R. Vankova, J. Malbeck, and M. M. Neff, "Over-expression of SOB5 suggests the involvement of a novel plant protein in cytokinin-mediated development," The Plant Journal, vol. 46, pp. 834-848, 2006.

[45] J. Parkash, T. Vaidya, S. Kirti, and S. Dutt, "Translation initiation factor 5A in Picrorhiza is up-regulated during leaf senescence and in response to abscisic acid," Gene, vol. 542, pp. 1-7, 2014.

[46] P. M. Dewick, "Secondary metabolism: the building blocks and construction mechanisms," Medicinal Natural Products: A Biosynthetic Approach, Second Edition, pp. 7-34, 2001.

[47] Y. Huang, J.-H. Cheng, F.-N. Luo, H. Pan, X.-J. Sun, L.-Y. Diao, et al., "Genome-wide identification and characterization of microRNA genes and their targets in large yellow croaker (Larimichthys crocea)," Gene, vol. 576, pp. 261- 267, 2016.

[48] H. Hwang and J. Mendell, "MicroRNAs in cell proliferation, cell death, and tumorigenesis," British Journal of Cancer, vol. 94, pp. 776-780, 2006.

[49] P. Xu, M. Guo, and B. A. Hay, "MicroRNAs and the regulation of cell death," TRENDS in Genetics, vol. 20, pp. 617-624, 2004.

[50] H. Hamzeiy, J. Allmer, and M. Yousef, "Computational methods for microRNA target prediction," miRNomics: MicroRNA Biology and Computational Analysis, pp. 207-221, 2014.