Production and molecular characterisation of gentiopicroside in *Gentiana kurroo* and *Swertia chirata*

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5 Year Dual Degree Programme

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CONTENTS

CERTIFICATE

This is to certify that the work entitlied **"Production and molecular characterisation of gentiopicroside in *Gentiana kurroo* and *Swertia chirayta*"** pursued by **Payal Kotvi (123807)** in partial fulfillment for the award of degree in Masters of Biotechnology from Jaypee University Of Information and Technology, Waknaghat has been carried out under my supervision .this work has not been submitted partially or wholely to any other institution.

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ACKNOWLEDGEMENT

All praise belongs the almighty lord to whom I thank for the strength, courage and perseverance bestowed upon to me to undertake the course of the study.I hereby acknowledge with deep gratitude the cooperation and help given by all members of Jaypee University in helping with my project.With proud privilege and profound sense of gratitude, I acknowledge my indebtedness to my guide Dr. Hemant Sood , Assistant professor, Dept. of Biotechnology and Bio-informatics, for her valuable guidance,suggestions constant encouragement and cooperation.
I express my thanks to Dr. Rajinder Chauhan ,Head ,Department of Biotechnology and Bioinformatics, Jaypee University Of Information and technology
And of course Mrs. Mamta Mishra and other staff members for their constant help and support.

Payal Kotvi

Date:

LIST OF TABLES

LIST OF ABBREVATIONS

MeJA	Methyl jasmonate
SA	Salicylic acid
SNP	Sodium nitroprusside
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
ISPD	Isoprenoid synthase domain containing
MVK	Mevalonate Kinase
G10H	Geranoil 10-hydoxylase
MVA	Mevalonate pathway
MEP	Methylerythritol 4-phosphate

INTRODUCTION

CHAPTER 1

Medicinally important herbs occupy the biggest sector of the pharamceutical industry. Their ability to synthesis certain chemical compounds which fall under the category of primary and secondary metabolites, where on one aspect serve as their natural mechanism against any sought of environmental stress and on the other aspect play their role by becoming part of various medicinal formulations.

Gentiana kurroo Royle and *Swertia chirata* belonging to the family Gentianaceae are an important and highly endangered medicinal herbs used for their high medicinal properies. They are natives of North-western Himalayas and enormously found in places like Jammu & Kashmir, Himachal Pradesh, and Uttrakhand at an altitudes of 1500-3400 m. The two plant species are members of the family Gentianaceae which has about 16 genera and approximately 145 species.

Morphological characteristics

Gentiana a small perennial herb also known as Indian Gentian, Neelkanth or karu. It has leaves arising from the base which are long, thin and basally joined in pair to form a common casing. They are deep green in colour and remains throughout the life cycle. The shoots are have blooming branches which consist of cauline leave. They are restricted direct and in sets joined at base shaping a tube around blooming shoot. They are green in shading and blur its shading with development. Blooms are substantial, infundibuliform, finish, bisexual and profound violet blue from outside and whitish from inside. Sepals are five, gamosepalous, pretty much equivalent in size. Natural product are a case and dehisces longitudinally. Seeds are kept at low temperature (5ºC) subsequent to collecting to refrain from losing their feasibility. They require very much depleted soil and low temperature for development. On the opposite side Swertia otherwise called Haima, kirata Tikta and Chiravata is an erect yearly herb. *Swertia chirata* has an erect, around 2–3 ft long stem, the center segment is round, while the upper is four-calculated, with a noticeable decurrent line at each edge. The stems are two-three feet long, vigorous, spreading, and round and hollow beneath and four calculated upwards. The stem are orange darker or purplish in shading contain substantial yellowish essence. The root is straightforward, decreasing long and typically a large portion of an inch thick. The leaves are comprehensively lanceolate, fivenerved and sub-sessile. Blooms are little, stalked, green yellow, tinged with purple shading, turn and tetramerous. Organic product is a little, one celled egg shape case. The seeds are various, moment, smooth and many-calculated. It can be developed in an assortment of soils with sandy topsoil rich in carbon and humus with low temperature development (10°C).

Fig.1. Mature plants of (A) *Swertia chirata* (B) *Gentiana kurroo*

Harvesting and Seed germination

In *Gentiana kurroo* flowers starts to bloom from the third week of August till the first week of november between 15 September and 20 October. A single plant produces 20 flowers. The flowers close during night and under low light, and revive when the sun shines brightly. The first fortnight of november is the ideal time for seed harvesting. After this the capsules open, spreading the seeds. June is the ideal month for seed sowing. The seeds are ought to be stored at low temperature (below 5°C) after harvesting. Variability in temperate conditions can lead to considerable reduction in germination percentage. Seeds more than one-year old lose viability and do not germinate. Gentiana requires a pebbly soil with perfect drainage.

Swertia chirata is propagated by its seeds. Sowing is generally done during the spring when the temperature is not above 10° C and in a situation when the soil contains plenty of humus. When the seedlings have grown, they are taken out individually and planted into separate pots or containers. The young plants are replanted outdoors during the early part of summer. The plants are usually harvested for drug industry in July and September when it sets into flowering.

Use and Trade

The two plant species are traded for their pharmacologically derived interests where they serve their importance by adding their valuable metabolites to the formulations, which has been the key interest in the pharma sector. Their rhizomes and roots are used as bitter tonic, antiperiodic, expectorant, antibilious, anthelmintic, astringent, antipsychotic, anti-inflammatory, sedative, cholagogue, refrigerant, blood purifier and carminative. The roots of *Gentiana kurroo* are a source of iridoid glycosides such as gentiopicrine, gentiamarin and the alkaloid gentianin. Phytochemical screening of the plant also showed that the roots of *Gentiana kurroo* are rich in various active ingredients like flavonoids, alkaloids and terpenoids, which are responsible for its effects as analgesic, anticancer and immune-modulatory. It is traded in the market by its trade name “Trahimaan” and roots are traded in dried form. Phytochemical analysis have revealed that swertia contains many compounds that are responsible for its therapeutic properties such as alkaloids, flavonoids and glucosides. *Swertia chirata* has been proposed in the studies to contain three main metabolites swertiamarn, amarogentin and mangiferin which make it the site of interest. Chirata has also been reported to contain a yellow bitter glucosides known as gentiopicroside. It is a secoirridoid which has been present in both the plant species. It has been also reported to be used for curing skin diseases,bronchial asthma, urinary infections,

inflammations, leprosy, helminthiosis, dyspepsia, exhaustion from chronic diseases and in all cases of weakness of digestive system and loss of appetite. The anti-inflammatory properties of gentiopicroside has also contributed to its major medicinal properties.

Pharmacological importance

Root extracts of *Gentiana kurroo* have been used within diverse cultures since ancient times as a bitter tonic, antiperiodic, expectorant, antibilious, astringent, stomachic, anti-inflammatory, antibacterial, anthelmintic, blood purifier and carminative . It is also reported to be used for treating and curing skin diseases, bronchial asthma, urinary infections, inflammations, leprosy, helminthiosis, dyspepsia, exhaustion from chronic diseases and in all cases of weakness of digestive system and loss of appetite. Air-dried flower tops of *Gentiana kurroo* have been tested for the anti-inflammatory activity. Gentiopicroside possesses anti-inflammatory, analgesic, anticonvulsant, hypotensive, antipsychotic, sedative, diuretic, anti-malarial and anti-amoebic properties, whereas, Amaroswerin possessed gastro-protective properties. The ethanolic extract of s*wertaia.chirata* has shown hypoglycemic activity. It also contains antimicrobial activity against gram-negative and gram positive bacterias.The plant extract shows anti-leishmanial activity against Leishmania donovani in golden hamsters. A herbal drug Chirata is gathered from dried plants of Swertia species. The phytochemical analysis of the two plant species have revealed the presence of tannins, alkaloids, saponins, cardiac glycosides, terpenes, flavonoids, phenolics, and carbohydrates in the two plant species .From these studies it has been concluded that these plants can be used as an alternative in the treatment of various autoimmune diseases eg. arthritis..

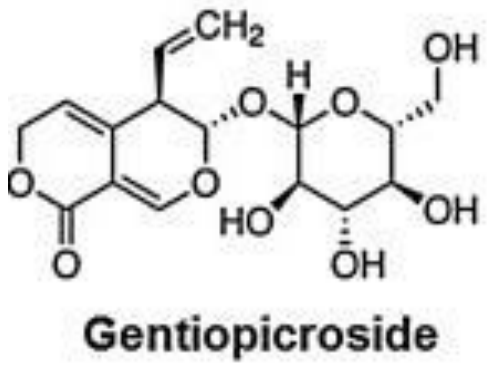

Fig .2. Chemical structure of Gentiopicroside

Major Threats

The wild population in India has declined by 80% within the span of 10 years. The current estimation depicts the decreasing trend of population to have reached upto 80% in India. Overexploitationa and utilisation of resources has lead to their extinction. Therefore, the red data book of Indian plants has listed these species as endangered and its status as critical. Major threats for this species are loss of habitat and unregulated harvesting. Habitat loss continues due to road construction and agricultural invasion. The population of the species has declined due to over grazing and industrialisation. Due to rapid deforestation change in climate has also added to the deteriotating condition.

Plants and their conservation

In order to meet the growing demand in national and international trade markets of raw plants, cultivation must be increased. In both the species seed proliferation due to seed technology offers limitations in their use due to low viability, and low germination percentages. Therefore

biotechnology offers means of improving biodiversity. Various biotechnological approaches such as micropropagation techniques has received more attention and may play a vital role in the establishment of genetically uniform plants for *Gentina* and *Swertia* industry. Several studies reported on micropropagation, somatic embryogenesis and acclimatization with the potential to produces uniform clones of *swertia. Chirata* and *Gentiana kurroo*. Synthetic seed productionis has provided a platform for micropropagation and plant germplasm conservation through cryopreservation .hence this can be utilised for mass propagation and short-term storage of genetically uniform clones require manipulation of in vitro tissue culture systems that are able to transform into complete plantlets. Micropropagation which is conducted under a controlled environment will help to prevent the current plant biodiversity conservation problems arising from over harvesting practices of wild populations and can profoundly improve the quality of bioactive secondary metabolites of the age old medicinal plant *Gentiana kurroo* and *Swertia chirata.*

IMPORTANCE OF THE PROJECT

These two plant species contain a secoiridoid glucosides gentiopicroside which has high medicinally importance. However the metabolic content of gentiopicroside varies among the species. *Gentiana kurroo* has been proposed to have gentiopicrodide content (0.2%) but their has been no research conducted on Swertia with respect to gentiopicroside. In this study attempts have been made to elicit the content of this metabolite in both the plant species and also see their vunerability with respect to elicitors under controlled parameters. Since very few efforts have been made towards understanding the molecular basis of the biosynthetic pathway of the two species, the present study provides genetic improvement stratergies to positively effect the biosynthetic machinery. To overcome this challenge 4 genes of the biosynthetic pathway (MVA & non- MEP) encoding HMGR , PMK , ISPD, G10H and CAS were taken and amplified which were seen for their expression analysis. Therefore the present study investigates about the biosynthetic pathway and their role in the production of this secondary metabolites . To conduct this study following objectives have been derived.

- To study the effect of elicitors on the plant growth and gentiopicroside content in *Gentiana kurroo* and *Swertia chirata* .
- Comparative molecular analysis of gentiopicroside in *Gentiana kurroo* and *Swertia chirayta* .

REVIEW OF LITERATURE

CHAPTER 2

Elicitors have been used to elicit the metabolite content and study their various aspects with respect to growth and development. Shimizu et.al (2008) has reported increase in the anthocyanin accumulation in roots of cultured plants which were elicited with MeJA (20-50 uM). In the sme study he has also used SA which have not been illustrated to increase any significant concentration of anthocyanin. Shoots treated with 100 uM MeJA have shown to exhibit inhibitory effects. In this study 25 uM has been used for eliciting the anthocyanin in the cultures.

Simlilar studies have been conducted by **Fang et.al (1998)** but using varied concentration as compared to the previous study. He has reported 0.5 uM MeJA alone to produce 2-3 fold increase in anthocynin production.

The effect of SA acid has been reported by **Vicente and Plasencia (2011). In their study** they have shown SA to exhibit inbitory effects on mitochondrial electron transport where SA (20-500 uM) has contributed to inhibited ATP synthesis in the elicited tobacco cultures.

Loreti et.al (2008) have indicated in their studies that GA, JA, and ABA may interact with sucrose to form a complex web of signalling pathway that co-ordinate anthocyanin accumulation. JA has enhanced the PAP1 and PAP2 availability and increased entire biosynthetic pathway.

Obinata et.al(2003) have reported increase in the activity of phenyalanine ammonia-lyase and chalcone-flavonone isomerise in grape cell which contribute towards procyanidin and anthocyanin by treatment with SA (0.1mM) by 3 fold.

The *in vitro* grown shoots were exposed to different concentrations of the abiotic elicitors such as chitosan(50 and 100mg/L), methyl jasmonate (25 , 50 , 75 µM), salicylic acid (10mM , 20mM ,30mM), vanady lsulphate (10mM , 20mM ,30mM), biotic elicitors such as Agrobacterium rhizogenes(10, 20µL)and yeast extract (0.2mg/L) and growth hormones to study the metabolite variation in the plantlets. Observations were recorded and the sampling was done every 10days for a month and the plantlets were stored at -80^{0}C for HPLC. HPLC analysis was done for evaluating the metabolite content. **Kumar et.al (2013)** have reported increase in the quantity of swertiamarin significantly (9.33 µg/mg) in the shoots cultured on media supplemented with IBA (2mg/L) and GA3 (3mg/L) in just 20 days. This lead to increase in the amount by 1.5 fold for swertiamarin and 2 fold for amarogentin when compared to the control. The greatest levels of mangiferin (2.99µg/mg) and amarogentin (1.03 µg/mg) were observed in shoots cultured which were supplemented with a IBA (2mg/L) and KN (2mg/L) combination with incubation of 30 days. Mangiferin was estimated to be 1.5-fold higher than the control and 1.94-fold higher for amarogentin.The lowest content of swertiamarin was in shoots grown on IBA (2mg/l)+GA3(3mg/l)+KN(2mg/l).

Seaweeds are macroscopic multi-cellular marine algae that commonly populate the coastal regions of the world's oceans. According to **Holdt and Kraan (2011) & Choknacka et. al (2012)** seaweeds serve as a reservoir of biologically active compounds These active compounds contain polysaccharides, proteins, polyunsaturated fatty acids (PUFAs), pigments, polyphenols,

minerals and plant growth hormones**.** Seaweed and seaweed derivative products have been used in various field of crop production and improvement where immense potential of the vitamins ,minerals and plant growth enhancing compounds present in the seaweeds are utilized (**Khan et. al , 2009).**

Crouch et. al , (1990); Rathore et.al , (2008); Yeong et. al , (2013) These high-valued products make possible the use of micropropagation techniques that may be more costly than conventional mariculture.With the advent of time , seaweed extracts have gained popularity due to their potential in the field of agriculture where they have shown to improve mineral absorption, reducing the use of fertilizers, and enhancing the plant growth.

Commercial seaweed preparations often exhibit enhanced growth and development. According to the studies the amount of mineral nutrients present in SWC is too small to explain the observed growth responses, hence some other factors must be involved .The presence of hormone such as cytokinins , auxins gibberellins, ABA, ethylene have been confirmed by gas chromatography/ mass spectroscopy nuclear magnetic spectroscopy. The authors **Crouch and Staden (1993)** has utilized the potential of high pressure liquid chromatography (HPLC) to identify cytokinins such as trans zeatin , dihydrozeatin and iso- pentenyladenine.The sudy on the commercial seaweed extract of from brown alga *Ecklonia maxima* , exhibited root promoting activity. Analysis of the active biological compounds promoting rooting via HPLC concluded for the presence of IAA. The rice microdrop bioassay tested and confirmed the presence of gibberellins activity in seaweed. However the presence of ethylene in seaweed still remains to be demonstrated. **Neha et.al** have used seaweed extract to carry out elicitation in *picrorihza kurroo.* Filtered sterilized seaweed extract (2%) showed high growth and development.

To understand the biosynthetic pathway *aconitum heterophyllum* **malhotra et.al** have taken 15 genes to check expression of MVA/MEP pathway in roots and shoots which differ in artisine content. Genes like DXPS, ISPD, HDS,HMGR,HMGS,MVK were to check expresson analysis. Overall 8 genes showed decrease in the artisine content when they were checked for expression in roots which gave high (0.37%) and shoots gave low (0.14%) artisine content. Similar studies were carried out by **padhan et.al** where he carried out expression analysis of 24 genes of mangiferin and amarogentin and 15 genes of swertiamarin to understand the biogenesis pathaway in *swertia chirata*. He has checked the expression profile of these genes in green house roots ,green house leaves, green house flowers, tissue cultured leaves and tissue cultured roots. PMK, ISPD and IS showed elevated levels i.e 57–104 fold increase in roots, and EPSPS of mangiferin biosynthesis showed an about 117 fold increase in transcripts in leaf tissues of the greenhouse grown plants.

MATERIALS AND METHODS

CHAPTER 3

Collection of plant material

Swertia chirayita and *Gentiana kurroo* plants were acquired from Himalayan forest Research Institute Mehli, Shimla, Himachal Pradesh . Green house grown plants of *Swertia chirayita* and *Gentiana kurroo* were raised and maintained in Jaypee University of Information technology, Waknaghat, Himachal Pradesh, India. The invitro plantlets of *Swertia chirata* and *Gentiana kurroo* which were maintained in the culture room (25°C ± 2) with light intensity (5,800 W m–2)and relative humidity (≈ 75%) with photoperiod of (16 h day/8 h night).Tissue samples from the invitro grown plants were taken and further used for expression studies.

Optimization of culture condition for elicitation in shoot cultures of *Gentiana kurroo* and *Swertia chirata*

Invitro grown shoots for the two species are taken and cultured on MS media supplemented with growth hormones and elicitors. Shoot explants of Swertia were cultured on MS media containing IBA(1mg/l)+KN(3mg/l) where on the other hand shoot explants of Gentiana were established on the MS media containing IBA(1mg/l)+KN(3mg/l) with 15 gms of sucrose and 8 grams of agar. Both the cultures were supplemented with elicitators viz. Methyl jasmonate

,salicylic acid, SNP, and seaweed.(Table 2) The elicitators were filter sterilised using 0.22 um sterile filter.The cultured were kept for optimisation under 25± 2ºC light conditions for 30 days.

Table .1. Concentration of different plant growth elicitors.

S.NO	Elicitors	Concentration
1	MeJa	50, 75,100 uM
2	SNP	25,50,75 uM
3	Salicylic acid	25,50 uM
4	Seaweed	2%

Data collection

The elicitated and control shoots were collected at an interval of 15 and 30 days of their culturing. The data was collected for number of shoots formation shoot biomass and gentiopicroside production by carrying out HPLC analysis.

Quantification of gentiopicroside by HPLC

The plant materials (100 mg) of the two species supplemented with elicitors were grounded using motor pestle with liquid nitrogen and thereafter were suspended in 80% methanol for overnight incubation. The samples were then centrifuged at 10,000 rpm for 15 min. Supernatent was then filtered through 0.22 um filter. The desired separation was obtained by implying a gradient method where mobile phase A was composed of 0.1% TFA in water and mobile phase

B was a mixture of acetonitrile: water in the ratio of 70:30. The linear gradient at a flow rate of 1.0ml/min was start with 15% B; 20% B in next 5 min, 70% B in next 25 min, hold for 5 min; 15% B in next 5 and equilibrated for 5 min at 240 nm UV wavelength. The phytochemicals were separated and recognized based on their retention time (RT) and UV-spectra assessment.

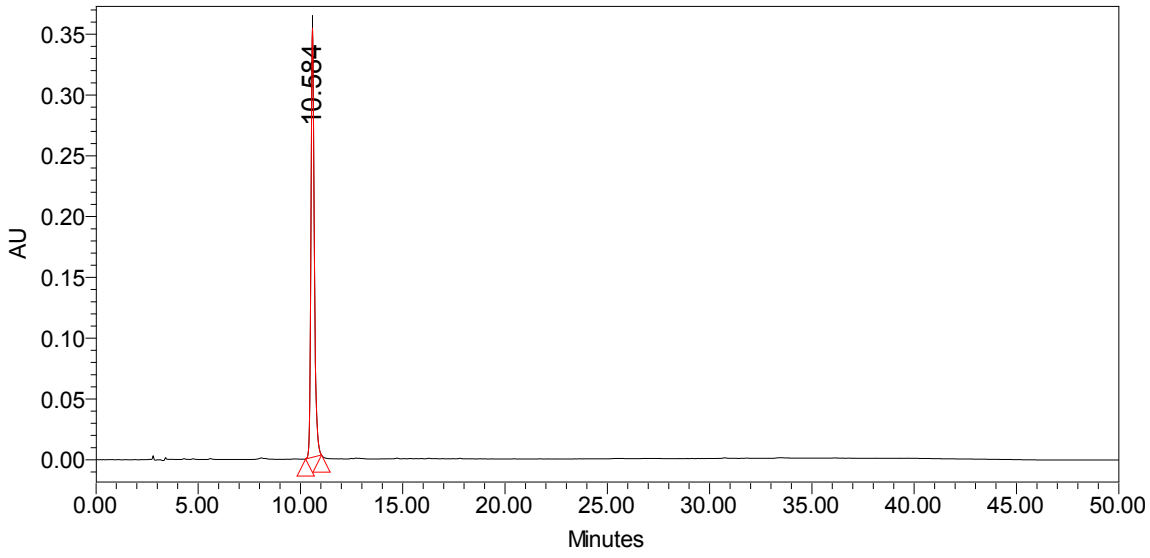

Fig.3. Cromatogram of gentiopicroside standard.

RNA isolation and preparation of cDNA

RNA was isolated from the elicited tissues of S. chirayita and Gentiana kurroo using Trizol Reagent (Ambion Inc., USA). The isolated RNA was quantified using spectrophotometric (A260 and A280) measurements (NanoDrop, Thermo Scientific, USA). Further integrity of the RNA was assessed on agarose gel stained with 1% (w/v) ethidium bromide. 100ug of RNA was taken

to prepare cDNA using verso cDNA kit using verso oligo dT and verso RT enhancer which was visualised after running the samples on 1% ethidium bromide stained gel.

Identification of secondary metabolic pathway genes in *Swertia chirata* and *Gentiana kurroo.*

The metabolic route for synthesis of gentiopicroside i.e a seco-irridoid involves the MVA, MEP, secoiridoid and phenylpropanoid route. Six different genes from the MVA and MEP pathway were finalised based on the previous conducted studies. The primers of the selected genes were procured from Padhan et. al. 2015.These primers were used for the gene expression analysis in samples of *Gentiana kurroo* and *Swertia chirata* elicited plants.

RESULTS

CHAPTER 3

The shoot explants of *Gentiana kurroo* and *Swertia chirayta* cultured on MS media supplemented with plant growth elicitors (Table 1) under 25 ± 1°C were observed in the interval of 15 days The 0 day sample were also collected. After 30 days final sampling was conducted and fresh weight of the samples of the two species was recorded.(Table 2).The data was collected to carry out futher studies on morphological and gentiopicroside content.(Table 3)

Table 2. Fresh weight of *Gentiana kurroo* and *Swertia chirata* after 15 and 30 days.

		Swertia (fresh weight in gm)		**Gentiana (fresh weight in gm)**	
S.NO	Sample	After 15 days	After 30 days	After 15 days	After 30 days
1	Control	0.2493	0.2675	0.2451	0.2565
2	MeJA	0.5744	0.3009	0.2317	0.3870
3	SNP	0.1664	0.1568	0.2649	0.4784
4	Seaweed	0.2791	0.3197	0.3902	0.6971
5	Salicylic acid	0.2860	0.3920	0.3343	0.3215

Table 3. Effect of elicitors on growth and development in *Gentiana kurroo* and *Swertia chirata*

		Swertia Tissue browning		Gentiana Tissue browning	
S.NO	Sample	After 15 days	After 30 days	After 15 days	After 30 days
1	Control	-	-	-	-
2	MeJA	+	++	++	+++
3	SNP	-	-	-	-
4	Seaweed	-	+	-	++
5	Salicylic acid	-	-	-	-

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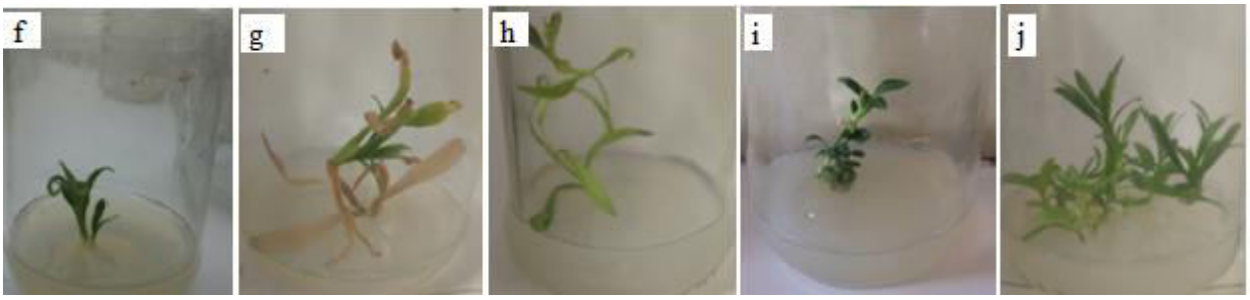

Fig.4.1.Effect of elicitors on growth and development *Gentiana kurroo* after 15 days (f) Control (g) MeJa (h) SNP (i) Seaweed (j) Salicylic acid

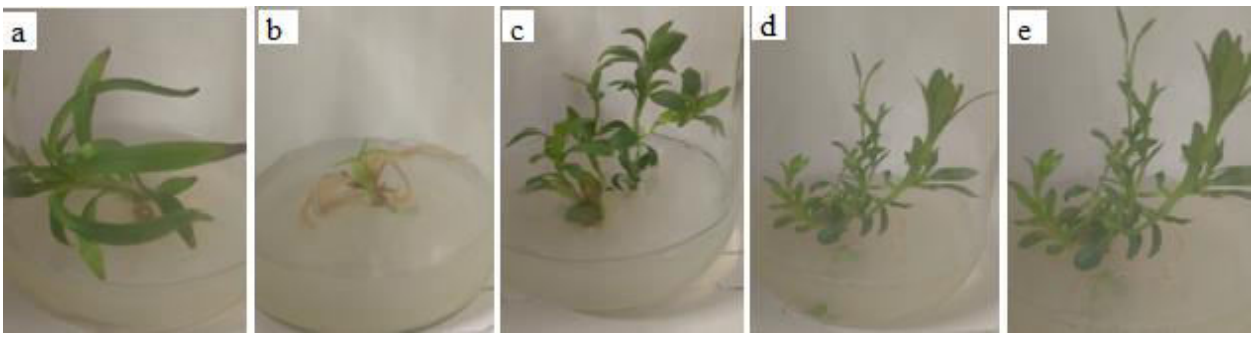

Fig.4.2. Effect of elicitors on growth and development *Gentiana kurroo* after 30 days (a) Control (b) MeJA (c) SNP (d) Seaweed (e) Salicylic acid

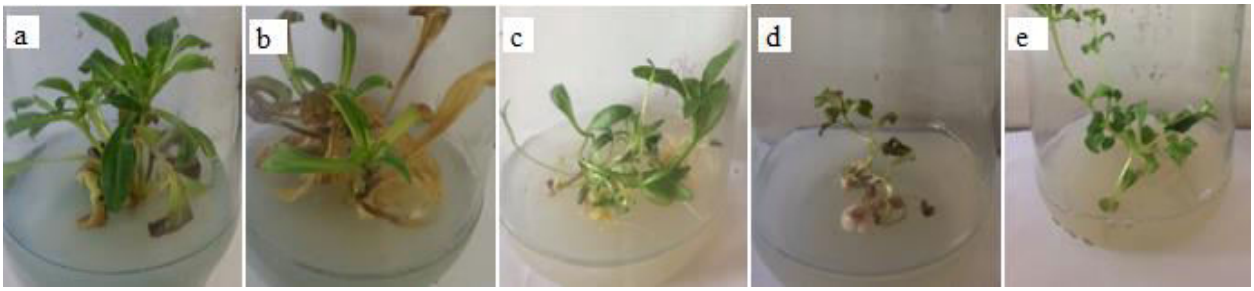

Fig.4.3. Effect of elicitors on growth and development in *Swertia chirata* after 15 days (a) Control (b) MeJa (c) SNP (d) Salicylic acid (e) Seaweed

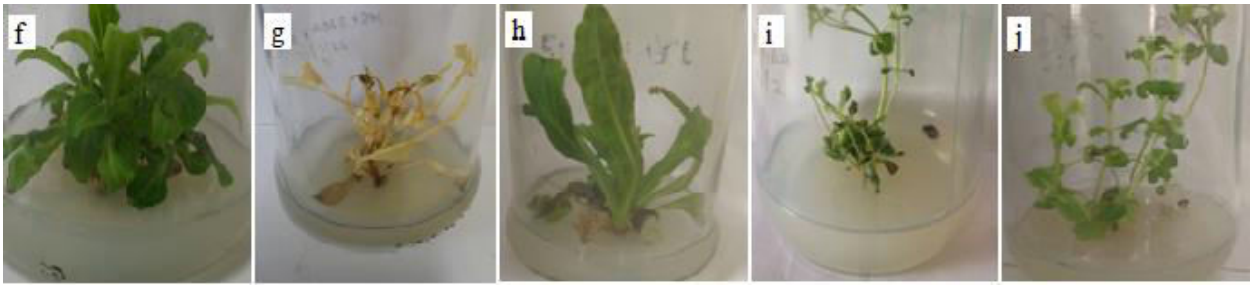

Fig.4.4. Effect of elicitors on growth and development in *Swertia chirata* after 30 days (f) Control (g) MeJa (h) SNP (i) Salicylic acid (j) Seaweed

Phytochemical analysis

HPLC analysis of the elicited plants of *Gentiana kurroo* and *Swertia chirata* have revealed the effect of the elicitors on the content of the secondary metabolite gentiopicroside. Gentiopicroside content have varied in range from 0.01-0.34 % of fresh weight in the two above mentioned species. The highest level of gentiopicroside has been accumulated in the invitro grown shoot cultures of *Gentiana kurroo* which was elicited by seaweed i.e 0.34% of fresh weight within 15

days of incubation. Whereas no elevation has been noticed when compared with the standards. Plants elicited by SA have also accumulated 0.20 % of fresh weight in 30 days. Elevated levels of gentiopicroside have also been seen to accumulate in elicited plants *geniana kurroo* with SNP. A considerable decrease has been witnessed in *gentiana kurooo* elicited with seaweed and seaweed elicited cultures within 30 days of incubation. The decrease metabolite content might be due to feedback inhibition or rechannaleization of metabolic flux of their biosynthetic pathways. Whereas SA acid have shown to increase the metabolite content within 15 days of incubation.

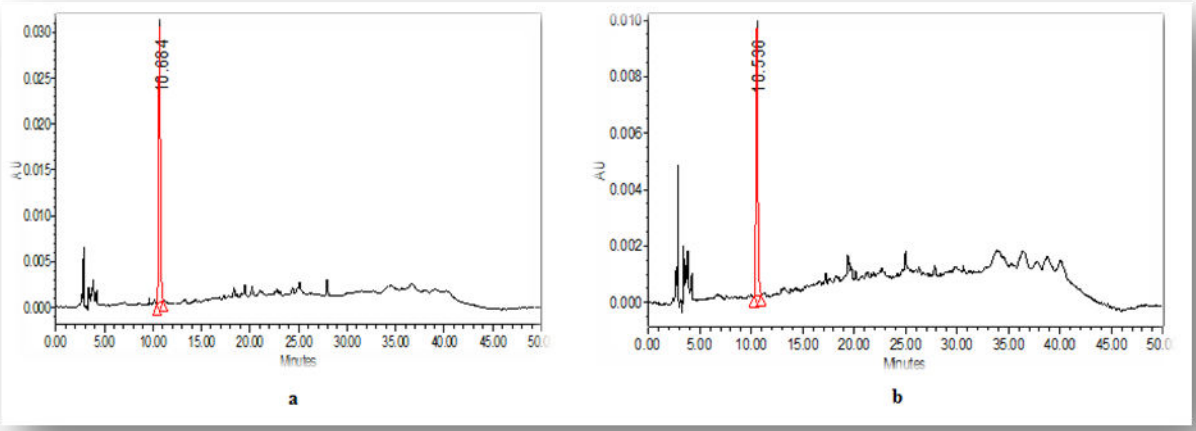

Fig 5: HPLC analysis of seaweed elicited shoots of *Gentiana kurroo* plant elicitor (a) After 15 days (b) After 30 days

On the other hand, invitro grown elicited plants of *Swertia chirata* have accumulted a constant range of gentiopicroside i.e 0.1% of fresh weight. No elevation has been found in any of the elicited plants of *Swertia chirata.*

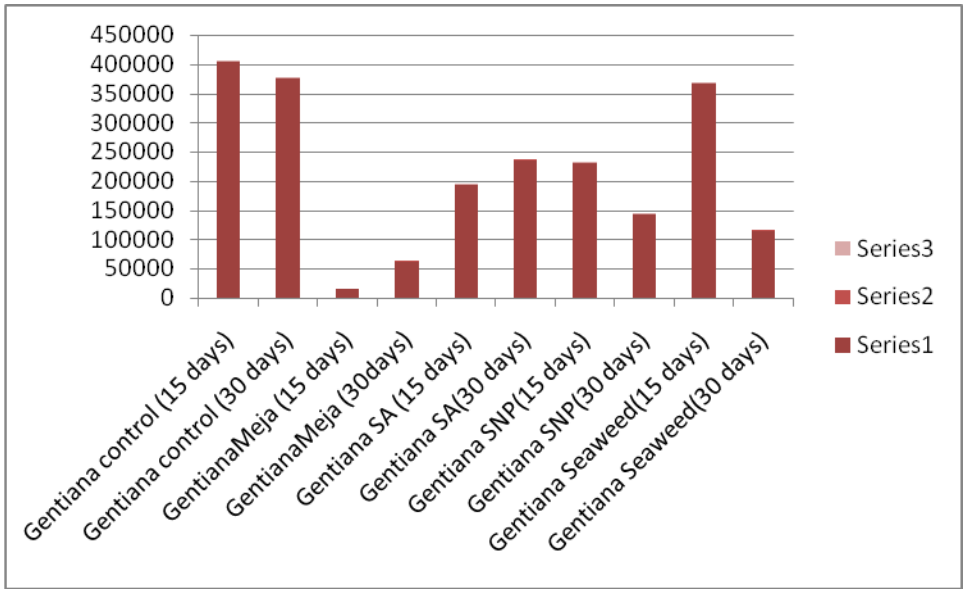

Fig.6. Quantification of gentiopicroside in Gentiana kurroo

RNA isolation

RNA of the quantified samples were extracted using TRizol method. Quantity,concentration and purity of the RNA was estimated by spectrophotometric measurements at A260 and A280 nm (Table.5.). The samples were further visualised on the ethidium bromide gel**.**

Table 4. Spectrophometric measurement of the RNA samples at A260 andA280.

SAMPLE	CONCENTRATION OF RNA (260/280) (ng/ul)
CONTROL SWERTIA	589
CONTROL GENTIANA	651
SWERTIA SNP	756
GENTIANA SNP	482
SWERTIA SEAWEED	2373
GENTIANA SEAWEED	1553

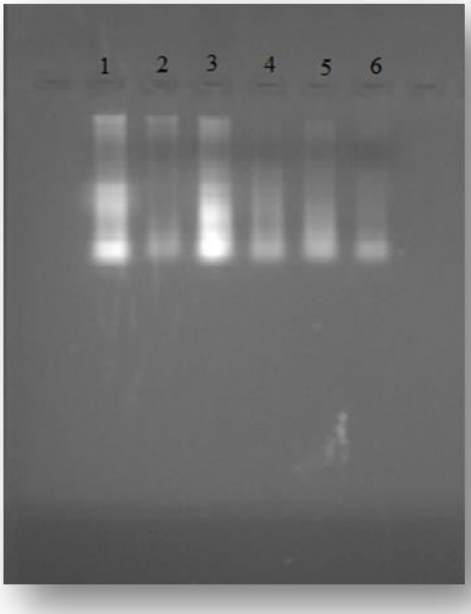

Fig.7. RNA isolation (1) Control Swertia (2) Control Gentiana (3)Swertia seaweed(4) Gentiana seaweed (5) Swertia SNP (6) Gentiana SNP .

cDNA synthesis

100ug of RNA was taken to prepare cDNA using verso cDNA kit using verso oligo dT and verso RT enhancer. A band of 525 bp was visualised after running the samples on 1.2% ethidium bromide stained gel (Fig.7.).Quantity, purity and concentration of the RNA was estimated by spectrophotometric measurements at A260 and A280 nm (Table.3).

Table .5. Spectrophometric measurement of the CDNA samples at A260 and A280.

SAMPLES	CONCENTRATION OF cDNA (260/280) (ng/ul)
CONTROL SWERTIA	994
CONTROL GENTIANA	1043
SWERTIA SNP	1003
GENTIANA SNP	934
SWERTIA SEAWEED	1903
GENTIANA SEAWEED	863

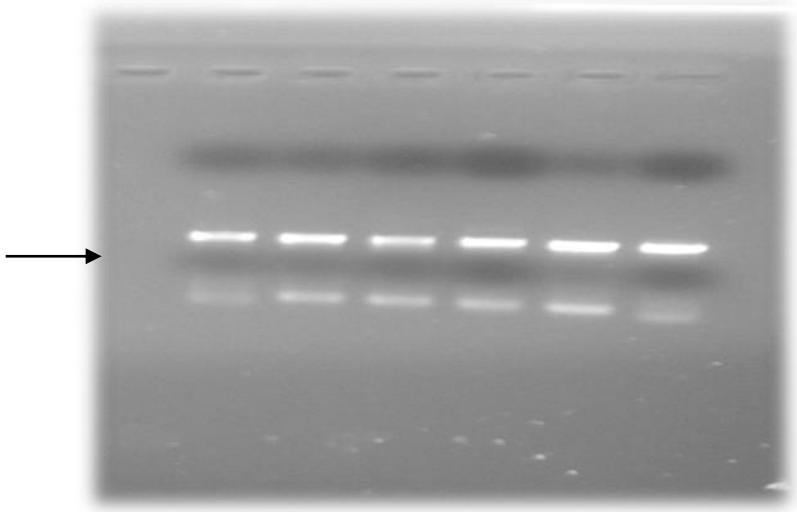

Fig.8. cDNA synthesis (1) Control Swertia (2)Control Gentiana (3)Swertia seaweed(4) Gentiana seaweed (5) Swertia SNP (6) Gentiana SNP .

Identification of pathway genes for *Gentiana kurroo* and *Swertia chirata*

To understand the biogenesis of the gentiopicroside production a total of 4 genes were selected based on their role in mangiferin, amarogentin and swertiamarin biosynthesis in *Swertia chirayita* as described in Padhan et al. 2015. The primers of the selected genes were procured from Padhan et al. 2015.These primers were used for the gene amplification in samples of elicited plants of *Gentiana kurroo* and *Swertia chirata.*

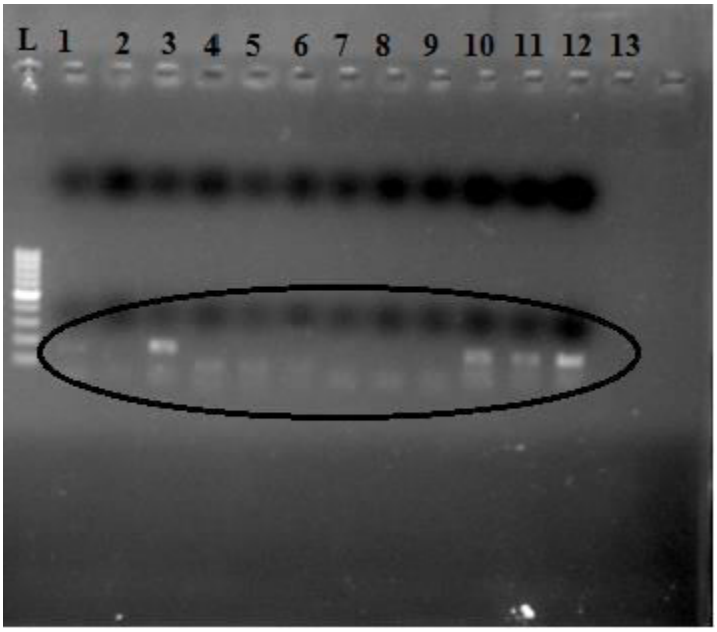

Fig.9. Gene amplification in *Gentiana kurroo* (a)L –Ladder (b) 1-3 HMGR (c) 4-6 MVK (d) 7-9 ISPD (d)10-12 G10H

DISCUSSION

CHAPTER 4

Gentiana kurroo and *Swertia chirata* have been explored for their medicinally important aspects contributing in the pharmaceutical sector. These species have been studied at their molecular and genetic levels to understand their biogenesis pathways concerning production of secondary metabolites. Attempts have been made to elicit these secondary metabolites by using elicitors like MeJA ,SA, SNP and seaweed. In the past studies different concentrations of MeJA (20-50 uM) have shown to enhance the levels of anthocyanin in the roots of *gynuria bicolour* [1]. Similiar studies on the use of MeJA (0.5 uM) as an elicitor has also shown to enhance the anthocyanin content by 2-3 fold *vacinium phallaea* over its control [2]. To the contrary, in our present study MeJA (75 uM) has shown to exhibit tissue browning followed by inhibitory effects on the gentiopicroside production in both plant species genus. SA has also been a potential elicitors to study the variability of the compounds with its different concentrations. In a study conducted earlier SA has not been seen to contribute towards the increase in the anthocyanin content [1]. SA acid (20 uM- 500 uM) has shown to induce inhibitory effects on the ATP synthesis in tobacco cultures [3].SA in concentration of 50 ppm has shown to enhance seed germination in the groundnut cultivars [4]. With respect to our present study SA (50 uM) acid has not shown any conciderable increase in the gentiopicroside content in *gentiana kurroo* with respect to the control. The quantification analysis of the effects of SA on *swertia chirata* to enhance gentiopicroside content has been reported for the first time. Earlier the plant has only

been utilised for its potential secondary metabolites as swertiamarin amarogentin amd mangiferin. SA acid has also contributed towards enhancing the content of procyanidin and anthocyanin in cultured grape cells. In the same study positive effects have also been observed towards enhancing the enzyme activity of phenylalanine ammonium lyase and chalconeflavanone isomerase which contribute towards photochemical production [5]. Studies have also been conducted with combinations of plant elicitors such as MeJA and SA contributing towards taxol production [6]. Similar results have also been seen ehers MeJA and SA has strongly increased anthraquinone accumulation in transgenic and non-transgenic [7]. In a study conducted SA (50 uM) has enhanced the production of hypericin (7.98-fold) and pseudohypericin (13.58-fold) in *Hypericum hirsutum* [8]. Effect of seaweed has also been seen to enhance the metabolite content. Seaweed (2%) has shown 3.23, 1.55, 2.42, 2.52 and 2.41 folds enhancement in the various tissues of the plant species *picrorihza kurroo*. Studies have also indicated to stimulate P-I production by 2.60, 2.01, and 1.35-fold by using SNP and seaweed [9].Similarly in our present study seaweed has contributed towards the maximum gentiopicroside concentration (0.31%) in *gentiana kurroo* but no additive enhancement with respect to control. Further more to the study their has been a constant effect of the gentiopicroside content (0.1%) in *swertia chirata.*

Another aspect of the present study was molecular characterisation of the two plant genus for gentiopicroside production in swertia chirata and gentiana kurroo was carried out where padhan et.al has identified 16 genes in sweria chirata for the production of marker compounds. Genes (HMGR , MVK , ISPD , CAS, G10H) from the conserved pathways were selected in the present study for gentiopicroside production and characterization [10]. Further amplification of these genes were carried out on *gentiana kurroo and swertia chirata* elicited cultures to

understand its biogenesis of gentiopicroside production. Amplification of HMGR gene was observed in seaweed elicited shoot culture at 0.31% concentration in *gentiana kurroo*. Whereas there was no HMGR amplification visualized for *swertia chirata*. Respectively the other 4 gene were amplified on *gentiana kurroo* and *swertia chirata* elicited shoot cultures. Amplification of G10H was seen with respect to SNP, seaweed, and SA elicited shoot cultures in *gentiana kurroo*.

So, this study infers that the selected conserved pathway genes contributing for gentiopicroside production in *gentiana kurroo* showed no amplification for HMGR, MVK ,ISPD and G10H in elicited shoots of swertia chirata at 0.1%.

CONCLUSION AND FUTURE PROSPECTS

CHAPTER 5

As we already know that the potential metabolite gentiopicroside which is anormously present in *gentiana kurroo* has high medicinal importance. Therefore in the present study attempts have been made to explore and elicit the role of gentiopicroside in *swertia chirata* along with *gentiana kurroo* by using various elicitors. The effect of these elicitors were observed by performing phytochemical analysis by HPLC where we found the seaweed elicited shoot of gentiana kurroo gave 0.31% of gentipicroside as compared to 0.1% in sweria chirata. wheras other elicitors didnot make any significant improvement in both genus. This study also throws light on the aspects of molecular characterisation where common potential genes of MVA and non-MVA pathway such as MVK ,HMGR , ISPD and G10H were taken and amplified in the above mentioned to genus. The present study gives the idea about the role of potential genes in gentiopicroside pathway which has not been explored till now. So the present work can be further extended by carrying out expression analysis for the above set gene in both genus under different invitro grown conditions. So we would conclude the present findings can be used as a lead for the exploration of gentiopicroside production in related genus.

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ANNEXURE

STOCKS	CHEMICALS	ORIGINAL STRENGTH(mg/l)	STOCK(g/l)	FINAL VOLUME
A-10X	KNO_3	1900	19	
	$MgSO_4.7H_20$	370	3.7	100 ml/l
	KH_2PO_4	170	1.70	
B-20X	NH_4NO_3	1650	33	150 ml/l
C-100X	$CaCl_2.2H_2O$	440	44	10 ml/l
D-100X	Na $_2$EDTA	37.26	3.72	10 ml/l
	$FeSO_4.7H_2O$	27.85	2.28	
E-100X	KI	0.83	0.083	100 ml/l
F-100X	H_3BO_3	6.2	0.62	
	$CoCl_2.6H_2O$	0.025	0.0025	
	$ZnSO_4.7H_2O$	8.6	0.86	10 ml/l
	$CuSO_4.5H_2O$	0.025	0.0025	
	$MnSO_4.4H_2O$	22.3	2.23	
	$Na_2MbO_4.2H_2O$	0.25	0.025	

G-100X	m-INOSITOL	100	10	10 ml/l
	GLYCINE	2	0.2	
H-100X	PYRIDOXINE-HCL	0.5	0.05	
	NICOTINE ACID	0.5	0.05	10 ml/l
	THIAMINE	0.1	0.01	

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