STUDY THE EFFECT OF SEAWEED IN *SWERTIA CHIRAYITA*, AN ENDANGERED HERB, FOR THE PRODUCTION OF SECONDARY METABOLITES

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CERTIFICATE

This is to certify that the work titled "**Study The Effect Of Seaweed In *Swertia Chirayita*, An Endangered Herb, For The Production Of Secondary Metabolites"** submitted by "**Chaynika Kapoor and Shrutkirti**" in partial fulfillment for the award of degree of **Bachelor of Technology** in **Biotechnology** of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. (Mrs.) Hemant Sood

Assistant Professor

DECLARATION

We hereby declare that the work presented in this thesis has been carried out by us under the supervision of Dr. (Mrs.) Hemant Sood, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan-173215, Himachal Pradesh, and has not been submitted for this or any degree or diploma to any other university or institute. All assistance and help received during the course of the investigation has been duly acknowledged.

Chaynika Kapoor

Shrutkirti

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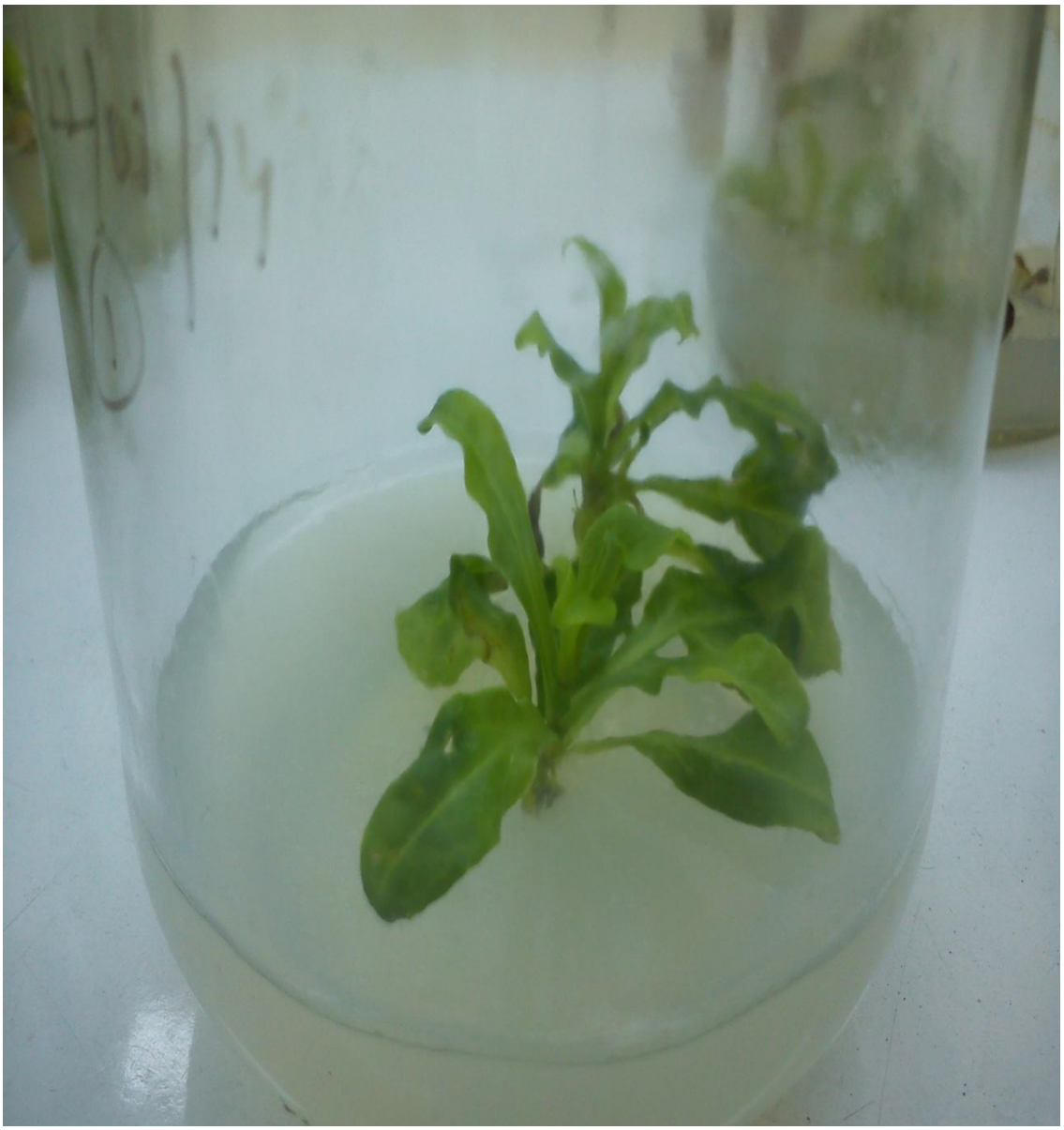
Chaynika Kapoor

Shrutkirti

CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	Meaning
°C	Degree Celsius
µM	Micro molar
GA_3	Gibberellic acid
IAA	Indole acetic acid
gm	Gram
HCl	Hydrochloric Acid
IBA	Indole -3 Butyric Acid
Kg	Kilogram
g/l	Gram per litre
NAA	Naphthalene acetic acid
KN	Kinetin
l/ lt.	Litre
m	Meters
mg	Milligram
MS media	Murashige and Skoog Media
NaOH	Sodium Hydroxide
ppm	Parts per Million
rpm	Rotations per Minute
STM	Standard Media
SW	Seaweed
GH	Growth hormones



SUMMARY

Swertia chirayita of gentianaceae family is basically found in Himalayas from Kashmir to Bhutan and Khasi hills and it is basically an upright herb. The medical importance of Swertia is also listed in various systems of medicines such as Ayurveda, Unani and Siddha. There are various species of Swertia which are found in India but among those species *Swertia chirayita* is highly famous for its medicinal values and is also used as a tonic for curing skin diseases and diabetes. Generally the plants available in market are adulterated or even substituted to close relatives of chirayita. There is a high demand of pharmaceutical products both in indigenous and world market but because of low scale commercial plantation the lack of herb continues. S. chirayita is also facing problems of loss of habitat and destructive harvesting which has severely hurt the population of the above mentioned species. Because of its high medicinal importance, the plant is recklessly collected from its natural habitat which already is in its endangered status so in vitro grown techniques is the only alternative for its large scale micropagation and for the production of important medicinal compounds. In the present study, large number of plantlets were produced which are rich in important medicinal compounds like swertiamarin, mangiferin and amerogentin. Best proliferation of shoots was obtained on medium having MS+IBA+KN+GA_3 and those in vitro grown shoots were further cultured in different type of growth hormones and seaweed extract and it has been found that metabolites are not accumulated in callus but present in shoots on best combination of 3g/l of seaweed, agar and no sucrose and no growth hormone in concentration 26.65 µg/mg swertamarin, 3.05 µg/mg mangiferin and 1.16 µg/mg amerogentin.

So, the following study is quite useful for reclaiming its natural population with quality rich shoot material and provide continuous resource for the metabolite production.

Chapter 1: INTRODUCTION

Use of medical plants and their importance in health system has increased in past few decades, which has resulted in huge demands of herbal medicines. Moreover the supply of raw materials for pharmaceutical industry is highly dependent on wild population of plants.

The field grown plants from where the herbal extracts are taken are highly vulnerable to attack from fungi, bacteria and insects which can alter medicinal content of preparation. Recent studies have shown that the demand of traditional medicines is increasing but the supply of plants is not able to meet the demand. Hence, the development of vitro systems by phytopharmaceutical industry for the production of medical plants and their extracts is the best alternative for the problem.

Isolating an active compound from plants is a highly difficult task because of their low concentration. Moreover when a raw material is scarce or chemical production is low the industry will lack methods to produce desired active compound. There is a huge interest for producing bioactive secondary metabolites of commercial importance as some active compounds are rare and found in rare plants. There has been a great interest in secondary metabolism because of evolving commercial importance and also in possibility of altering the production of bioactive metabolites. Advances in biotechnology include most notable methods of culturing plants cells and tissues. Ongoing research has mainly focused on identifying rate limiting steps in biosynthetic pathways. Medical plants which are in vitro propagated furnish a ready source of sterile, uniform and compatible plant material for biochemical characterization.

Producing industrially and medically important metabolites in cell suspension, callus, and hairy roots in various plant species (Vanishree et al. 2004; Vongpaseuth and Roberts 2007) are also included in advancements. Production of berberine from *Coptis japonica* by Mitsui Petrochemicals 2 (Morimoto et al. 1988), production of vincristine and vinblastine from *Catharanthus roseus* (Zhao et al. 2001), production of ginesinosides from *Panax ginseng* (Sivakumar et al. 2005) and production of paclitaxel from different species of *Taxus* (Tabata et al. 2004) are notable case studies wherein the tissue culture processes have been up scaled to a commercial level.

Now the focus of biotechnologists have shifted on the in vitro aspects followed by genetic engineering and they are working for the establishment of protocols which will result in maximizing outputs in the parameters of growth and also in secondary metabolite accumulation.

Swertia chirayita is an example of above said words as in vitro establishment of culture is vastly examined which has resulted in establishment of micro propagation techniques.

1.1 Swertia chirayita

Swertia chirayita has its roots belonging to family Gentianaceae which is an important herb endemic to the Himalayan region. It has a good hold in herbal industry because of its pharmacological properties like anti-diabetic, anti-malarial. *Swertia chirayita* is basically collected from the wild and very less effort have been put in developing proper agro techniques of the plant. Moreover this plant falls into endangered group but still it has high demands in pharmaceutical industry.

Swertia chirayita of gentianaceae family is basically found in Himalayas from Kashmir to Bhutan and Khasi hills and it is basically an upright herb. Raw materials Vol. X PID (CSIR) New Delhi gives detailed information on importance of genus Swertia. Swertia are also well known for their medicinal values. The medical importance of Swertia is also listed in various systems of medicines such as Ayurveda, Unani and Siddha. Among There are various species of Swertia which are found in India but among those species *Swertia chirayita* is highly famous for its medicinal values and is also used as a tonic for curing skin diseases and diabetes. Its market is huge and is increasing at a rate of 10% annually both domestic and international markets.

Generally the plants available in market are adulterated or even substituted to close relatives of chirayita. Amarogentin, swerchirin is responsible for bitterness, antihelmintic and antipyretic properties. Amarogentin is also the bitterest compound isolated till date. Medicines such as Ayush 64, Mensturyl syrup contain chirayitan extract in different concentration depending upon antifungal, antibacterial properties.

S. Chirayita is very difficult to be grown or extracted on a mass scale because of non viability of seeds or due to harvesting of plant before seed matures; hence it is deprived of natural

regeneration. Moreover it has a very low percentage of germination and it requires very delicate field handling hence the technology development of this species is very low.

Moreover there is a high demand of pharmaceutical products both in indigenous and world market but because of low scale commercial plantation the lack of herb continues. S. chirayita is also facing problems of loss of habitat and destructive harvesting which has severely hurt the population of the above mentioned species. National Medicinal Plant Board, Govt. of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria this plant is put under category of critically endangered species. National medicinal plant board of India has put S. chirayita under the tagline of highly prioritized medical plants in India. Novel techniques such as in vitro conservation and micro propagation need to be employed for production of large number of disease free plant hence the population of the species can be multiplied to meet the demands.

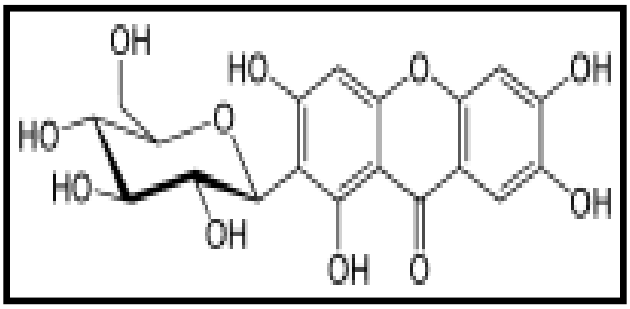
1.2 Chemical Constituents

Swertia chirayita (family Gentianaceae) extracts basically have anti diabetics and antioxidant activity, which are because of flavonoids and secoiridoids. These both are major part of functional components in methanolic extracts which are taken from aerial part of the plant. The main constituents of the plant are chiratin and ophelic acid. The aerial part of the plant contains xanthones and other whole part contains gentiamine alkaloids. It contains main chemicals mangiferin, amarogentin, amaroswerin, sweroside and swertiamarin , 1,5, 8-trihydroxy-3-methoxyxanthone, 1-hydroxyl-2, 3, 5, 7-tetramethoxyxanthone, 1-hydroxyl-3, 5, 8-trimethoxyxanthone, 1-hydroxyl-2, 3, 4, 6-tetramethoxyxanthone, 1-hydroxyl-2, 3, 4, 7-tetramethoxyxanthone, 1,8-dihydroxy-3, 5-dimethoxyxanthone, 1, 7-dihydroxy-3, 8-dimethoxyxanthone, 1, 3, 5, 8-tetrahydroxyxanthone, balanophonin, oleanolic acid , maslinic acid, and sumaresinolic acid.

MANGIFERIN:

Mangiferin is basically a xanthonoid, and this molecule is basically a natural phenol. In a study which was conducted in 2005 it showed antioxidant and antimicrobial activities (Stoilova, I.; Gargova, S.; Stoyanova, A.; Ho, L. 2005).It also showed some inhibitory effects

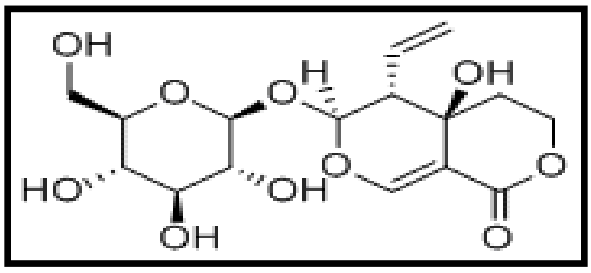
on type II 5a reductase in vitro.IN the year 2001 it showed gastroprotective and antidiabetic effects on rodents(Miura, T.; Ichiki, H.; Hashimoto, Y. et al ,2001)



MANGIFERIN

SWERTIAMARIN:

Hepatoprotective and antiedematogenic/anti-inflammatory are the pharmacological properties possessed by Swertiamarin. Swertiamarin it also has a potent lipid lowering agent which can also contribute to its cardio protective and anti atherosclerotic role(Vaidya *et al.* 2009) .It also have a very low level of toxicity and it is anti bacterial (Kumarasamay *et al.* 2003), anticholinergic. (Suparna *et al.* 1998) and antinociceptive (Jaishree *et al.* 2009).

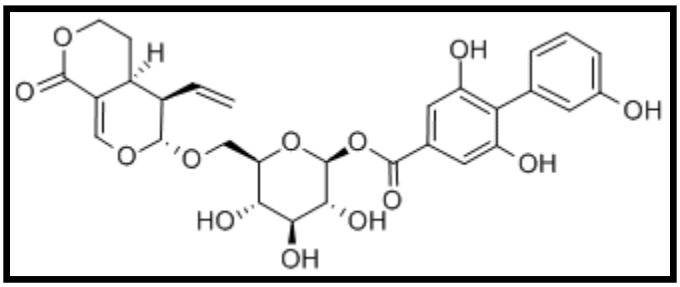


SWERTIAMARIN

AMAROGENTIN:

Amarogentin is the bitterest compound known to mankind till date. At a dilution level of 1:58,000,00 its bitterness can be tasted. It is known as topoismerase inhibitor (Ray *et al.* 1996),

chemopreventive and is reported to have anti-leishmanial (Medda *et al.* 1999) and gastroprotective properties (Niiho *et al.* 2006).



AMAROGENTIN

1.3 Rationale:

The present study was carried out with following objectives:

1. Multiplication of the *in-vitro* grown cultures under optimized conditions for cell cultures.

2. Enhanced production of metabolites using different Growth hormones, different sucrose concentrations, and different seaweed concentrations.

3. Quantification of metabolites using HPLC.

Chapter 2 - LITERATURE REVIEW

Micro propagation:

In the year 1999 Wawrosch C reported that micro propagation of the Nepalese plant *Swertia chirayita* by adventitious shoot regeneration from root explants were found most suitable method for propagation of *Swertia chirayita*. In the year 2000 Balaraju reported efficient in vitro plant regeneration for *Swertia chirayita* which is highly endangered herb of Himalayan ranges. This was developed using shoot tip explants derived from in vitro grown seedlings

An in vitro shootlet production system using Murashige and Skoog (MS) medium with various hormones such as BAP, KN and TDZ was established. BAP at 1.0 mg/l and KN, 0.1 mg/l induced highest number of multiple shoots (42.16+or-1.05) per explants. Microproliferated shoots were transferred to elongation medium amended with GA3 (0.1 mg/l) and hormone free basal medium, after which they were transferred to rooting medium. Half strength MS medium gave highest frequency of rooting (22.48+or -1.o8). Half strength medium was supplemented with NAA,0.1 mg/l after testing with different auxins.

In the year 2007 Pooja described *Swertia chirayita* as an endangered herb of the temperate Himalayas, through 4 week old seedling derived nodal explants. There was 4.5 fold multiplication on Murashige and skoog medium supplement with 4 micro M benzyl amino purine (BA) and 1.5 micro M 6-(gamma , gamma -dimethylallylamino) purine (2iP) every 4 weeks. Rooting was optimized on modified MS medium supplemented with 1 micro M naphthalene acetic acid (NAA) and 500 mg l-1 of activated charcoal (AC). There was a success rate of 94% was obtained by *in vitro* hardening in the in a greenhouse conditions.

Wang Li in the year 2009 reported ex wall in vitro. Moreover the influence of phytohormones and the medium on shoot regeneration from the leaf of *Swertia chirayita* Bunch ham.

Adulterants

There are basically 31 species of Swertia found in Nepal as reported by Joshi in the year 2008. Moreover *S. chirayita* is considered to be superior in medical properties in comparison to other species *Swertia* and hence it is traded more. Generally the species of *Swertia* are bundled and mixed with *S. chirayita* and sold substitutes such as S. nervosa, *S. angustifolia* are also traded in the non availability of S. chirayita. Chirayita is generally adulterated with *tetragonum Roxb. E. bicolor Roxb., E. pedunculatum L., Slevolgia orientalis Griesb., Swertia alata Royle., S. angustifolia Buch. -Ham., S. bimaculata Hook. f. and Thomas., S. ciliata G. Don., S. densifolia Greisb., S. elegans Wight., S. lawii Burkill., S. minor Griesb., S. paniculata Wall., S. multiflora Dalzell.* The adulterants are inferior to S. chirayita in terms of bitterness. *Andrographis paniculata* (local Indian name: Kalmegh) is also known as *chirayita* in India.

Andrographis paniculate is also used as a substitute for *S. chirayita* because of their similarities in therapeutic actions. The main phytochemicals reported in *A. paniculata* are kalmeghin, diterpes: andrographolide, andrographiside, neoandrographolide as well as panicolide, caffeic acid, chlorogenic acid and other polyphenolics (Li et al. 2007, Hossain et al. 2007). Phytochemicals found in S. chirayita are xanthones, flavanoids, iridoid and secoiridoid glycosides (Pant et al. 2000). They all are the main Phytochemicals. The pure *S. chirayita* can be differentiated from other substitutes and adulterants by its intense bitterness, brownish-purple stem (dark color), stem that is rounded at the base and terete at the upper portion, continuous yellowish, pith, green petals with dark red distinct marking and double nectaries (Joshi & Dhawan 2005)

ELICITORS :

METHYL JASMONATE (MeJA): It is avery volatile organic compound used in many diverse pathways such as seed germination, root growth, flowering, fruit ripening. Generally plant produces methyl jasmonate and jasmonic acid in its response to biotic and abiotic stresses (in particular, herbivory and wounding), which result in building damaged part of the plants. Methyl jasmonate can be used for signaling defense system of plant or it can even be spread by physical contact or even by air to produce a defensive reaction in unharmed plants. Unharmed plants

basically absorb MeJA through stomata or diffusion by cell leaf cytoplasm. Plant generally produces MeJA For its internal defense and also as a signaling compound for other plants.

MEja also helps the plants to produce different multiple types of defense chemicals namely photoalexins (antimicrobial),nicotine or proteinase inhibitors. MeJA basically activates proteinase inhibitor genes through a receptor mediated signal transduction pathway. Moreover the proteinase inhibitors interfere with the insect digestive process and helps in discouraging the insect from eating the plant again.

SALICYLIC ACID: Salicylic acid is basically a phenolic phytohormone and plays a role in growth and development, photosynthesis, transpiration, ion uptake and transport. It is also helpful in plant defense against pathogens. It is involved in the systemic acquired resistance (SAR) in which a pathogenic attack on one part of the plant induces resistance in other parts. SA is also responsible for endogenous signaling, mediating in plants defense. SA also helps in inducing some specific changes in leaf anatomy and chloroplast structure.

VANADYL(IV) SULFATE: It is a well known salt of vanadium. This is hygroscopic blue solid which is a common source of vanadium in the laboratory which indicates its high stability. Vanadyl ion can also be termed a most stable diatomic ion 1 N short is written as VO^{2+}.

YEAST EXTRACT: It is a most common name for processed yeast products made by extracting the cell contents. It basically means by removing the cell walls.

CHITOSAN: It is produced commercially by deacetylation of chitin, which is basically a structural element in exoskeleton of crustaceans, such as in crabs or shrimp and having cell walls of fungi.

Agrobacterium rhizogenes: Agrobacterium rhizogenes (updated scientific name: *Rhizobium rhizogenes*. It is basically a gram negative soil bacterium which is responsible for hairy root disease in dicotyledonous plants. A. rhizogenes basically induces formation of proliferative multi branched roots at the site of infection, hence called the hairy roots.

Metabolite Analysis:

In accordance to Hostettmann K et al. plant is a rich source of novel, biologically active plant compounds. Moreover biological and chemical screening is complementary approaches for detection of new plant constituents. Biological screening later followed by activity guided fractionation has been successfully utilized at Institute de Pharmacognosie et Phytochimie, University of Lausanne, Switzerland, for the discovery of antifungal, larvicidal and molluscicidal compounds. For the screening of crude plant extract HPLC coupled to UV spectroscopy (LC/UV), mass spectrometry (LC/MS) and nuclear magnetic resonance (LC/NMR) has proved to be highly efficient. For the early recognition of mollucidial saponins in Swertia madagascariensis and Phytolacca dodecandra. The techniques of LC/MS and LC/MS/MS used with different ionisation techniques such as thermospray (TSP), continuous flow-FAB (CF-FAB) and electrospray(ES) are found to be highly effective. For the investigation of polyphenols and bitter principles in Gentianaceae species the combination of LC/UV/NMR/MS was of great value. LC/NMR Analysis of extract of African plant Swertia calycina was also produced or presented.

Use of CC and preparative TLC was done to isolate two known xanthones, decussating and gentiacaulein from aerial part of medical species. Moreover leaf segments, stem internodes and roots were excised and cultured on supplemented L Insmaaier and skoog medium. Regenerated plantlets were obtained by induction of calluses. Basically analysis of tissues yielded 3 known compound (decussatin, methyl swertianin and 1-hydroxy-3, 5, 7, 8-tetramethylxanthone) and a new compound identified as 1, 8-dihydroxy-2, 4,6-trimethoxyxanthone.

From the hairy root cultures of Swertia japonica Amarogentin, amaroswerin and four xanthones were identified by Ishimaru K in the year 1990. Inoculation of cultures with Agrobacterium rhizogenes helped in formation of hairy roots. Moreover cultures of these roots were analyzed for xanthone and bitter principles, Bellidifolin, methylbellidifolin, swertianolin and a new xanthone derivative, 8-O-primeverosylbellidifolin, were isolated. Moreover two bitter principles were identified namely amarogentin and amaroswerin.

In the year 1994 a experiment was conducted on 11 *Swertia* and related plants by HPLC to know their effective constituents. Quantative determination of 3 iridoids (swertiamarin, gentiopicroside

and sweroside) and a xanthone (swertianolin) in 11 Swertia spp., Halenia elliptica and Gentiana rigescens, mostly collected in Yunnan. C18 was used as the stationary phase, and water: ethanol:isopropanol:tetrahydrofuran (65:30:5:1) as the mobile phase.

In the year 2005 Tian Wei established a specific HPLC method for controlling the quality of total glycosides from *Swertia franchetiana.*

For the quantitative assessment of pharmaceutical extracts from S. franchetiana HPLC method was applied. Sample preparation, elucidation mode (isocratic or gradient) was optimized to obtain HPLC profile. SPD-10Avp pump, SPD-M10AVP photodiode-array detector (PAD) and SIL-IOADVP auto injector these were the constituents of HPLC system. Moreover data was processed with CLASS VP6.1 WORKSTATION.

Kromasil C18 column (250 min x 4.6 min ID, 5 micro m) with methanol and water as mobile phase were used for performing HPLC analysis. Column temperature was set at around 40^0C and rate of flow of water at 1 ml min-1. Samples and reference solution of chemical standard were injected to HPLC system but separately. Hence the results showed 16 characteristic peaks which were divided into 3 parts one peak in 0-10 min of retention time, 9 peaks containing main 1-7 peaks in 10-15 min of retention time, and 6 peaks in 15-30 min of retention time. Peak 1-7 were identified as swertiamarin (1), gentiopieroside (2), sweroside (3), isoorientin (4), swertisin (5), isoswertisin (6) and swetianolin (7) based on retention time, UV spectra and molecular weight.

Hence, it was conclude that total glycosides from S. franchetiana with a high level of specificity can be used for controlling their quantity and assure consistency.

Triterpenoids from *chirayita* plants was identified and they are commonly used in Indian medicines. At a temperature of 60 to 80 degrees under reflux air derived powered plant was extracted with petroleum. Elution of column with CHC13 oleanolic acid was crystallized from ethanol as white needles and beta Marvin was given by crystallization from ethanol of CHC13 filtrate.

DAD known as diode array detection used with (RP-HPLC), was used for detection of nine active compounds known as swertiamarin (SWM, 1), mangiferin (MA,2), gentipicroside (GE, 3), sweroside (SWO, 4), isoorientin (IS, 5), swertisin (SWS, 6), swertianolin (SWN, 7), 7-O-[alpha -L-rhamnopyranosyl-(1->2)- beta -D-xylopyranosyl]-1,8-dihydroxy-3-methoxyxanthone (RX, 8), and bellidifolin (BE, 9) used as the external standard, in Tibetan folk medicinal species *Swertia franchetiana*.

The nine samples were identified by comparing them with standard samples and then qualified by using external standard method with DAD at 254nm.Correlation coefficients of the calibration curves were found to be higher than 0.9980. RSDs stand for relative standard derivations of peak areas and the retention times for nine standards were less than 2.07% and 2.86%.

In the year 2006 Bhand developed a microanalytical HPLC technique for determining swertiamarin in Swertia species and bacoside A in bacopa monnieri.LOD (limit of detection) of swertiamarin and bacoside A are 500 pg and 4000 ng in a 2mg plant material.

Moreover the dried plant material from S cordata and S chiravita contained nearly 5.59 and 0.44% swertiamarin. B monnieri was containing around 0.12% bacoside A. The simplicity of method and accuracy of the method enabled its use for estimating marker compounds.
In the year 1988 Kulanthaivel reported isolation of new xanthone and 2-hydroxy dimethylterephthalate from Swertia petiolata. In the state of UP alpine pastures of Kumauni Himalaya provides shelter to the herb S chirayita. It is well known for its laxative properties. In the year 1987 structure of chiratanin was discovered by mandal. Chiratin is a novel dimeric xanthone. This compound is obtained from aerial parts of Swertia chirayita which is used as a liver tonic or as an antimalarial in India.

In the year of 2009 plant powder of *Swertia densiflora* was collected in different seasons by Sunita. In this the best season for harvesting *Swertia densiflora* is investigated. This powder was then extracted with methanol and chloroform. Chromatography was performed on silica gel 60 F254 HPTLC plate, with ethyl acetate:methanol:water, 7.5:1.5:1.2 (v/v), as mobile phase.

Scanning was done at 244nm in reflectance absorbance mode to achieve quantitation. The amount of swertiamarin in *Swertia densiflora* (Griscb.) Kashyap. whole plant powder was found to be 2.94 mg g-1 in the month of March, 1.59 mg g-1 in October and 1.18 mg g-1 in December. In the year of 1997 Liquid chromatography/ultra violet/mass spectrometric and liquid chromatography/nuclear magnetic resonance spectroscopic analysis of crude extracts of Gentianaceae species was done by Wolfender. HPLC stands for high performance liquid chromatography coupled with (LC/UV-DAD)ULTRAVOILET Photodiode array and mass spectrometry (LC/MS) are used highly for chemical screening of crude extracts. There are various probes which are now commercially available namely liquid chromatography (LC/NMR) and several LC/NMR probe which has given a new impulse for the coupling by upcoming of magnetic resonance spectroscopy.

So as to know the potential of this coupled technique as a compliment to LC/UV/MS for one line identification 2 characteristic Gentianaceae were analyzed. They were Swertia calycina (from Rwanda) and Gentiana ottonis (from Chile). On-line UV, MS, MS/MS 1H-NMR and 1H-1H-NMR spectra and major contituents of these plants allowed their identification directly with crude extracts. Major constituents of these plants were (flavonoids, xanthones, naphthoquinones, secoiridoids and glycosides).

The limitations of LC/NMR used with LC/UV/MS are also discussed openly in the paper.

Production of phytochemicals in Plants Cell Bioreactor

For the production of useful phytochemicals plant cell culture is a viable alternative over whole plant cultivation. There are some parameters such as cell aggregation, mixing, aeration and shear sensitivity which need to be taken into consideration for cultivating plant cell at a large scale and also for selecting suitable bioreactor. Proper cultivation strategy (Batch, Fed-batch Two-stage, etc), feeding of metabolic precursors and extraction of intracellular metabolites shall be taken into account for increased productivity in a bioreactor.

Bioreactor is basically a huge step for commercial production of secondary metabolites by plant biotechnology. For enhancing productivity of novel secondary metabolites genetic transformation can be used, especially by *Agrobacterium rhizogenes* induced hairy roots.

Culture Conditions

Media

In the year 2008 K balaraju cultured shoot tip explants were derived from in vitro grown seedling on Murashige and Skoog medium having 2% sucrose. pH level of the medium was kept at 5.8 before gelling with 0.3% phytagel. At 1.0 mg/l and KN ,0.1mg/l gave highest number multiple shoots (42.16+-1.05) per explants. Elongation medium was amended with GA3(0.1mg/l) when micro proliferated shoots were transferred to it and hormone free basal medium, after which they were transferred to rooting medium. Highest rooting frequency (22.48+-1.08) was given by half strength MS medium supplemented with NAA. Inside plant tissue culture room hardening was successfully achieved.

In the year 2007 Vibha Dhawan and Pooja Joshi identified rapid protocol for micro propagation through auxiliary multiplication from 4 week old seedling. They obtained a 4.5 fold multiplication every 4 weeks. It was obtained on Murashige and Skoog (MS) medium supplemented with 4 μM benzyl amino purine (BA) and 1.5 μM 6-(γ,γ-dimethylallylamino) purine (2iP). Moreover a success rate of 94% was achieved in vitro hardening in growth room and ex vitro hardening in greenhouse condition. This study was very important for mass multiplication of elite genotypes of critically endangered species and can be used for multiplying large quality planting.

Temperature

In the year 2011 B K Pradhan studied seed germination which was collected from Six microhabitats at 20^0C ,25^0C and 30^0C under a 14/10 h light/dark photoperiod and in continuous darkness. It was indicated by two way ANOVA that temperature and microhabitat does affect

germination rate, seed germination and germination recovery. Seeds that were collected from under the canopy had a high germination rate in comparison to tweeds collected from the open at 20°C, 25°C, and 30°C (14/10 h light/dark photoperiod.

Initially there was negligible germination in continuous darkness but after transfer to 14/10 h light/dark photoperiod, the seeds which were collected from under the canopy recovered at 20°C and 25°C and these showed the highest germination percentage as compared to seeds that were collected from under the tree base, stump base and grassy slope. Highest GR percentage was recorded from seeds recovered from under the canopy at 30°C. Among the microhabitats tested, variation in GR rate was significant ($p < 0.05$)

Terpenoid Elicitation

- **Biotic**

Xanthone production in *Hypericum perforatum* (HP) suspension cultures in response to elicitation by *Agrobacterium tumefaciens* co-cultivation was studied by G.Franklin et al (2008). RNA blot analyses of HP cells co-cultivated with *A. tumefaciens* showed a rapid up-regulation of genes encoding important enzymes of the general phenylpropanoid pathway (PAL, phenylalanine ammonia lyase and 4CL, 4-coumarate:CoA ligase) and xanthone biosynthesis (BPS, benzophenone synthase). Analyses of HPLC chromatograms of methanolic extracts of control and elicited cells (HP cells that were co-cultivated for 24 h with *A. tumefaciens*) revealed a 12-fold increase in total xanthone concentration and also the emergence of many xanthones after elicitation. Methanolic extract of elicited cells exhibited significantly higher antioxidant and antimicrobial competence than the equivalent extract of control HP cells indicating that these properties have been significantly increased in HP cells after elicitation. Four major *de novo* synthesized xanthones were identified as 1,3,6,7-tetrahydroxy-8-prenyl xanthone, 1,3,6,7-tetrahydroxy-2-prenyl xanthone, 1,3,7-trihydroxy-6-methoxy-8-prenyl xanthone and paxanthone. Antioxidant and antimicrobial characterization of these *de novo* xanthones revealed that xanthones play dual function in plant cells during biotic stress: (1) as antioxidants to protect the cells from oxidative damage and (2) as phytoalexins to impair the pathogen growth.

Conventional and *Agrobacterium rhizogenes*-transformed root cultures were studied by Michael Keil et al (2000) with respect to growth and amarogentin content following cultivation in various

growth media. The fastest growth rate was observed using Nitsch medium. The best amarogentin content was obtained after cultivation in root culture (RC) medium for which the slowest growth rate was noticed. Addition of sucrose at 6 % and 9% (w/v), respectively, also resulted in better growth rates and increased total but unaltered relative amarogentin content compared to 3 % (w/v) sucrose. No change in amarogentin content was observed upon addition of elicitors, putative precursors of amarogentin biosynthesis, and plant growth hormones with the exception of salicylic acid and chitosan: at 100 mM salicylic acid a reduction and at 25 mg/L chitosan an increase of amarogentin were observed at significant levels. The cultivation of *S. chirayita* roots in a 2-L stirred-tank bioreactor was successful only with a stainless-steel mesh fitted inside the culture vessel for immobilization of the roots. A 15-fold enhancement of amarogentin content in the medium was achieved by a root permeabilisation treatment using Tween 20 at 1.3 % (v/v) final concentration in the bioreactor.

- **Abiotic**

Swertia chirayita Buch ham is a medicinal plant having high medical values. This plant comes under endangered species. By NaCl elicitation was done to understand in vitro induction of marker secondary metabolites. On a 1 month old static culture shoots 50 and 100 mM of NaCl concentrations were applied. Plants were checked out for anti oxidative enzymatic system and production of secondary metabolites. Increase was noticed in secondary metabolite at 50Mm NaCl without cellular damage. There was a increase in secondary metabolites metabolic content after treatment with NaCl which falls back to its normal value after seven days. There was concurrent induction of scavenging enzymes during this period. Moreover biochemical relationship between anti oxidative enzymes and production of secondary metabolites has further been discussed in light of physiological requirements.

L. Beerhues and U.Berger identified that Cell-suspension cultures of *Centaurium erythraea* and *Centaurium littorale* (Gentianaceae) respond to methyl jasmonate and yeast extract with a differential accumulation of xanthones. Methyl jasmonate induced the formation of 1-hydroxy-3,5,6,7-tetramethoxyxanthone, the amount of which increased in both cell cultures around 10 h after addition. A substantial increase in the activity of phenylalanine ammonia-lyase

(PAL) was not observed. When challenged with yeast extract the cell cultures accumulated l,5-dihydroxy-3-methoxyxanthone. This appeared rapidly after addition of yeast extract in *C. erythraea* but its amount in *C. littorale* increased only after a lag phase of 25 h. While PAL activity in *C. erythraea* was strongly suppressed a fourfold increase in its activity was found in *C. littorale*. Both elicited xanthones accumulated intracellularly.

- **SEAWEED**

Recent studies have shown that seaweeds are very beneficial to the growth and development of plants. They not only provide various nutrients for the growth but also help plants in defending themselves against variety of stresses. They are eco-friendly, non-toxic, non-polluting, environmentally benign and safe for the health of humans. They also have following benefits:

- Seaweeds are important marine renewable resources.
- They are used as food, feed, fodder, fertilizer, agar, alginate, carrageenan and source of various fine chemicals.
- Seaweeds extracts are marketed as liquid fertilizers and biostimulants since they contain many growth regulators such as auxins , gibberellins, betaines, macronutrients such as Ca, K, P, and micronutrients like Fe, Cu, Zn, B, Mn, Co, and Mo, necessary for the development and growth of plants.
- Seaweeds and seaweed extracts also enhance soil health by improving moisture holding capacity and by promoting the growth of beneficial soil microbes.
- Seaweed extracts are known to enhance the growth of vegetables, fruits, and other crops.
- Seaweed extracts when applied to seeds, added to the soil or sprayed on crops at vegetative and flowering stages, can stimulate seed germination, growth of various crops.
- Plants treated with seaweed extract have been reported to show increased nutrient uptake, better plant growth, and deep root development by improving lateral root formation and increased total volume of the root system.

- Seaweed extract also enhances plant defense against pest and diseases increase salt tolerance, drought tolerance, and heat tolerance.

Gaurav Kumar & Dinabandhu Sahoo in 2011 has shown the effect of seaweed liquid extract on growth and yield of *Triticum aestivum* var. Pusa Gold. Their experiment went as following:

The effect of seaweed liquid extract (SLE) of Sargassum wightii on germination, growth and yield of *Triticum aestivum* var. Pusa Gold was studied. Different concentrations of SLE (5, 10, 20, 30, 40 and 50%) were prepared by diluting this extract with distilled water. The seaweed extract was stored at 4°C for further applications. Seeds with uniform shape, size, color, and weight were selected for soaking in seaweed extract. One hundred seeds in ten replicates each were soaked for each concentration of seaweed liquid extract for 24 h. Control seeds were soaked in distilled water for 24 h. After an incubation period of 24 h at room temperature (24±2°C),seeds were placed on Petri plates containing filter paper. Seed containing Petri plates were placed at temperature 21°C,light intensity of approximately 100 μmol photonsm−2 s−1 for 12 h in a germinator. The filter paper was kept moist by regular addition of distilled water for control seeds and SLE for treatment seeds. Parameters such as seed germination, root length, shoot length, and number of lateral roots was regularly monitored every fifth day, till 25 days of growth. The soaked seeds were also transferred to the field and were regularly monitored with an interval of 5 days, for number of branches, number of kernels, kernel length, number of seeds per kernel, and dry weight of seeds. The experiment was terminated after a period of 4 months.

The effect of the 20% SLE concentration gave an 11% increase in seed germination over control. An increase in several growth parameters such as root length, number of lateral roots, shoot length and number of branches were also found to be highest for the 20% SLE treatment, and declining at higher concentrations. Root length was highest for the 20% treatment. Application of 20% SLE showed a 63.38% increase in the number of lateral root as compared to control. A 6.7% increase in shoot length over control was measured 25 days after germination and a 46.15% increase in the number of branches plant−1 for the same 20% treatment. The number of kernels plant−1 and kernel length were observed to be highest that was 54.16% and 18.75%, respectively, increased over control at 20% and lowest at the 100% SLE treatment. Treatment of 20% also gave a 13.69% increase in number of seeds kernel−1 and 22.86% increase in dry weight of seeds over control.

This study showed that the application of *S. wightii* seaweed liquid extract increases seed germination percentage. Seed treatment with 20% seaweed extract gave highest percentage of seed germination while 100% concentration gave least. The increased germination percentage at low concentrations may be due to the presence of some growth regulators, micro- and macronutrients. The effects of the application of seaweed fertilizers in improving total fruit production may be related to the effect of cytokinins. Finally, the application of low SLE concentrations may increase plant growth and deliver substantial economic and environmental benefits to farmers. Thus, the present study demonstrated that seaweed liquid extract could serve as an alternative biofertilizer as they are eco-friendly, cheaper and can overcome the ill effect of chemical fertilizers along with pesticides.

In an another study, by Jayaraj Jayaraman & Jeff Norrie & Zamir K. Punja (2011), it was found that commercial extract from the brown seaweed *Ascophyllum nodosum* reduces fungal diseases in greenhouse cucumber. *Ascophyllum nodosum* is a temperate seaweed found in the Atlantic and Arctic seas and has been widely studied for its properties, which include plant growth promotion and use in animal feed. In order to evaluate efficacy of seaweed extract on greenhouse cucumber, they utilized a commercial formulation of seaweed extract, "Stimplex™", which is water-soluble and contains a plethora of compounds, including carbohydrate molecules mostly in the form of oligosaccharides, including oligogalacturonides, and some polysaccharides.

They examined different methods and dosages of application, integration of fungicide, and their effects on four commonly occurring fungal pathogens including *Alternaria cucumerinum*, *Didymella applanata*, *Fusarium oxysporum*, and *Botrytis cinerea.*

Greenhouse cucumber plants were treated with Stimplex™ either as spray or root drench or using both methods, and treated plants showed generally lower infection levels of different pathogens. Two concentrations (0.5% and 1%) of Stimplex™ were tested, of which, 0.5% was found to be more effective than 1%. Further, 1% spray showed some moderate degree of phytotoxicity on leaves. Disease incidence levels were the lowest in plants sprayed and root drenched (T3) with 0.5% Simplex™. The following were the percentile disease reduction levels observed for various pathogens inoculated under the above treatment: Alternaria, 70% Didymella, 47%: and Botrytis, 46%, Fusarium root rot incidence levels were less in plants either sprayed or sprayed and drenched with 0.5% Stimplex™.

The percent disease reduction was between 85% and 88% at the above treatments. However, the best disease control effect was noticed when 0.5% Stimplex™ was integrated with one fungicide application (chlorothalonil, 2g L^{-1}). The disease reduction was at the level of 75% for Alternaria, 60% for Didymella, and 55% for Botrytis, respectively. Whereas in the case of Fusarium-inoculated plants, spraying with 0.5/1% Stimplex™ along with fungicide drench or fungicide drench alone provided complete reduction of disease. In Fusarium-inoculated plants, the plant biomass (shoot and root) levels were higher in Stimplex™ 0.5% sprayed/sprayed and drenched plants than control, fungicide, and remainder of the treatments

Stimplex™ application resulted in a significant reduction in disease incidence of all the pathogens tested. The disease control effect was greater for Alternaria and Fusarium infection, followed by Didymella and Botrytis. Combined spray and root drenching with Stimplex™ was more effective than either spray or root drenching alone. The alternation of one fungicide application, alternated with Stimplex™ application, was highly effective and found to be the best treatment in reducing the disease ratings. Plants treated with Stimplex™ showed enhanced activities of various defense related enzymes including chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, and lipoxygenase. Altered transcript levels of various defense genes, were observed in treated plants. Cucumber plants treated with Stimplex™ also accumulated higher level of phenolics compared to water controls. These results suggest that seaweed extracts enhance disease resistance in cucumber probably through induction of defense genes or enzymes.

Wajahatullah khan (2009) summarized the use of seaweed extracts as Biostimulants in the plant growth and development in his review article that says seaweed products exhibit growth-stimulating activities, and the use of seaweed formulations as biostimulants in crop production is well established. Biostimulants are defined as ‘‘materials, other than fertilizers, that promote plant growth when applied in small quantities’’ and are also referred to as ‘‘metabolic enhancers’’ (Zhang and Schmidt 1997). Seaweed components such as macro- and microelement nutrients, amino acids, vitamins, cytokinins, auxins, and abscisic acid (ABA)-like growth substances affect cellular metabolism in treated plants leading to enhanced growth and crop yield (Crouch and others 1992; Crouch and van Staden 1993a; Reitz and Trumble 1996; Durand and others 2003; Stirk and others 2003; Or dog and others 2004). Seaweed extracts are bioactive at low concentrations (diluted as 1:1000 or more) (Crouch and van Staden 1993a). Although many of the various chemical components of seaweed extracts and their modes of action remain

unknown, it is plausible that these components exhibit synergistic activity (Fornes and others 2002; Vernieri and others 2005).

Chapter 3- MATERIALS & METHODS

The present experiments of growth and elicitation of *Swertia chiravita* for increasing metabolite content and replacement of existing MS media with the less expensive seaweed media were carried out in the Department of Biotechnology, Jaypee University of Information Technology, Waknaghat, during 2013-2014.

The Experiments and methodology followed to carry out the above-mentioned experiments have been described under the following headlines:

3.1 Establishment of plants under *in vitro* conditions

3.1.1 Procurement of plant material

3.1.2 Cleaning of glassware

3.1.3 Preparation of media (Solid + Liquid)

3.1.4 Multiplication of shoot cultures

3.2 Elicitation Responses in the plants

3.2.1 Seaweed as a bioelicitor

3.3 Analysis

3.3.1 Observation of growth and development of plant

3.3.2 Sampling

3.3.3 Estimation of Swertiamarin , Mangiferin and Amarogentin by HPLC analysis

3.1.1 Procurement of plant material

1. *In vitro* grown plants of *Swertia chirayita* were available to us.
2. They were further multiplied and sub-cultured on optimized media and culture conditions under temperature 25˚C.
3. Shoot cultures were maintained.

3.1.2 Cleaning of Glassware

1. All the glassware to be used was washed with a dilute solution of labolin with the test tube brush and then rinsed thoroughly with tap water. Next it was wiped with 70% ethanol.
2. The glassware with contaminated cultures was first autoclaved for 15 mins at 121 lbs. and the molten media was disposed off. The glassware was then cleaned as mentioned above before reusage.

3.1.3 Preparation of media

1. Modifications of MS media supplemented with different concentrations and combinations of GA_3, IBA and KN were prepared (Murashige and Skoog, 1962).
2. The pH of the media was adjusted to be between 5.6 - 5.7 using 0.1N HCl and 0.1N NaOH, sucrose 3% (w/v) was added and finally agar-agar 0.9% (w/v) was added as a gelling agent.
3. The media was autoclaved at 121^oC and 15 lb/in^2 pressure for 15-20 minutes.

3.1.4 Multiplication of shoot cultures

1. For the purpose of shoot multiplication, we tried different combinations and concentrations of KN, IBA and GA_3.
2. The cultures were incubated 25˚C in plant tissue culture chamber with 70% relative humidity under photoperiod provided by cool fluorescent light.
3. Plants were checked after every 2 days for shoot formation and any contamination.

The cultures were sub cultured after every 4 weeks on shoot proliferation media for 2 months so as to obtain good growth and multiplication.

3.2 Elicitation Responses in the plants

3.2.1 Seaweed as a bioelicitor

Recent studies have shown that seaweed extracts protect plants against a number of biotic and abiotic stresses and offers potential for field application (Wajahatullah khan and others, 2009). The benefits of seaweeds as sources of organic matter and fertilizer nutrients have led to their use as soil conditioners for centuries (Blunden and Gordon 1986; Metting and others 1988; Temple and Bomke 1988). Some 15 million metric tonnes of seaweed products are produced annually (FAO 2006), a considerable portion of which is used for nutrient supplements and as biostimulants or biofertilizers to increase plant growth and yield. A number of commercial seaweed extract products are available for use in agriculture and horticulture. Other benefits include seed germination, improved crop performance and yield, resistance to biotic and abiotic stress, enhanced postharvest shelf-life of perishable products and they are also environmentally benign and safe for the health of humans.

Preparation of media containing seaweed with following composition:

1. MS+ SW+ Agar+ Sucrose- GH (I:K::1:3)
2. MS+ SW+ Agar+ Sucrose+ GH (I:K::1:3)
3. MS(liq)+ Sucrose
4. SW(liq)+ Sucrose
5. MS(liq)- Agar
6. SW (2g/l) + Agar - Sucrose – GH (I:K::1:3)
7. SW (2g/l)+ Agar +Sucrose + GH (I:K::1:3)
8. MS + Agar – Sucrose – GH (I:K::1:3)
9. SW + GH (I:K::1:3)
10. MS + GH (I:K::1:3)
11. SW (3g/l) + Agar - Sucrose – GH (I:K::1:3)

12. SW (1g/l) + Agar - Sucrose – GH (I:K::1:3)
13. MS + IBA (1) + Kn (3) + GA_3 (1) [control]

3.3 Analysis

3.3.1 Growth analysis of plant

The plants of *Swertia chirayita* were regularly monitored every 10 days to analyze the growth of the plant. Parameters such as number of leaves and shoot length were noted arising from the mother plants.

3.3.2 Sampling

The plants of *Swertia chirayita* were regularly sampled every 10 days, their shoot lengths and numbers of leaves were counted and recorded in a tabular form. The sampled plants were covered in aluminium foil, correctly labeled and stored at -20^0C.

3.3.3 Estimation of Swertiamarin , Mangiferin and Amarogentin by HPLC analysis

The accumulation status of metabolites was determined in plants by subjecting samples from different experiments to chemical analysis.

The quantification of Swertiamarin, Mangiferin and Amarogentin was carried by reverse phase High Performance Liquid Chromatography (HPLC Waters 515) through C18 (5µ) 4.6 x 250mm Waters Symmetry Column using PDA detector (Waters 2996). The plant material was ground separately in liquid nitrogen and suspended in 80% methanol. The sample mixture was sonicated for 0 mins. Following day, the samples were centrifuged at 10,000 rpm for 10-15 min. and the supernatant was filtered through 0.22µ filter. The filtrate was diluted 10x and 20x and injected into above mentioned column. Two solvent systems were used for running the test samples i.e. Solvent **A** (0.05% trifluoroacetic acid) and Solvent **B** (1:1methanol/ acetonitrile mixture). Solvent **A** and **B** were used in the ratio of 70:30 (v/v). The column was eluted in the gradient mode with a flow rate of 1.0 ml/min. The metabolites were detected at absorbance of nm

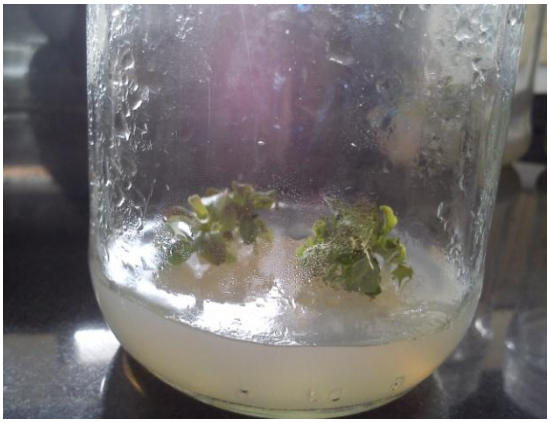
wavelength in a cycle time of 40 min at 300C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from ChromaDex Inc. The quantification was recorded for each sample and the data were interpreted in terms of amount of Swertiamarin , Mangiferin and Amarogentin present.

Chapter 4- EXPERIMENTAL RESULTS & DISCUSSION

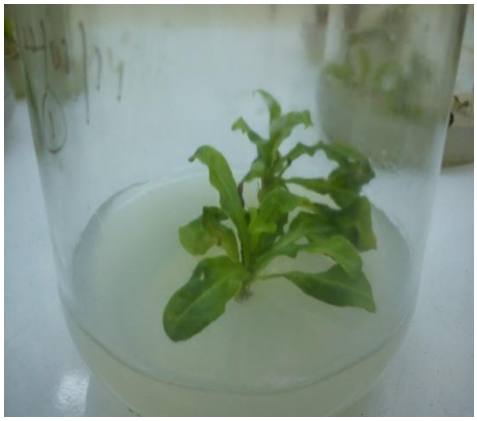
The plants were sub-cultured regularly and sampled after every 10 days. Their growth was monitored and recorded in the form of two parameters of shoot length and number of leaves.

A

B



C



D

Fig. 1: in vitro multiplication of *Swertia chirayita* from day 1 to day 40.

A: shows the plantlet on day 1 of culturing, B: shows the growth after 20 days, C: shows the growth after 30 days and D: shows the growth after 40 days.

Table 1: Composition of media for multiplication:

Media	**observation**
MS + Sucrose + IBA(1mg/l) + Kn(3mg/l) + GA_3(1mg/l)	Best growth
MS + Sucrose + IBA(1mg/l) + Kn(3mg/l)	Normal growth
MS - Sucrose + IBA(1mg/l) + Kn(3mg/l)	-

Table 2: Effect of different SW media compositions on *in vitro* shoot multiplication and growth in *Swertia chiravita*

MEDIA	**DAYS**	**SHOOT LENGTH**	**NO. OF LEAVES**	**REMARKS**
1. MS+ SW+ Agar+ Sucrose- GH (I:K::1:3)	10 20 30	5.1 5.6 4.5	9 9 20	No rooting
2. MS+ SW+ Agar+ Sucrose+ GH (I:K::1:3)	10 20 30	6.1 7.5 4.5	13 18.5 19	No rooting
3. MS(liq)+ Sucrose	10 20 30	- 5 -	- 12.5 -	No rooting
4. SW(liq)+ Sucrose	10 20 30	6.5 Dead 4.25	7 - 12	No rooting
5. MS(liq)- Agar	10 20 30	1.5 - Dead	4 - -	No rooting
6. SW (2g/l) + Agar - Sucrose – GH (I:K::1:3)	10 20 30	6 6.25 6.25	12 12 14	No rooting

7. SW (2g/l)+ Agar +Sucrose + GH (I:K::1:3)	10 20 30	6 6.25 7	9 13 13	Rooting obs
8. MS + Agar – Sucrose – GH (I:K::1:3)	10 20	4.75 5	7 12	No rooting
9. SW + GH (I:K::1:3)	10 20	7 7.5	9 13	No rooting
10. MS + GH (I:K::1:3)	10 20	5.5 5	17 20	No rooting
11. SW (3g/l) + Agar - Sucrose – GH (I:K::1:3)	10 20	5.25 6.5	9 12	No rooting
12. SW (1g/l) + Agar - Sucrose – GH (I:K::1:3)	10 20	6.5 6.5	11 12	Rooting obs Rooting obs
13. MS + IBA (1) + Kn (3) + GA_3 (1) [control]	10 20	5.75 5.5	10 16	No rooting

The plants cultured in media containing seaweed and no sucrose and no growth hormone showed good growth parameters. Some of these plants even showed rooting (fig.2).

A

B

Fig 2: A: plant showing the roots grown on medium containing SW (2g/l) + Agar +Sucrose + GH

B: plant showing the roots grown on medium containing SW (1g/l) + Agar - Sucrose – GH

Day 0: Culturing

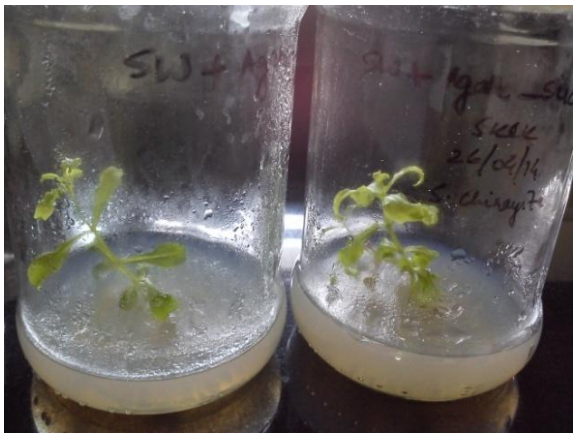
Day 10: First sampling

SW (2g/l) + Agar - Sucrose – GH

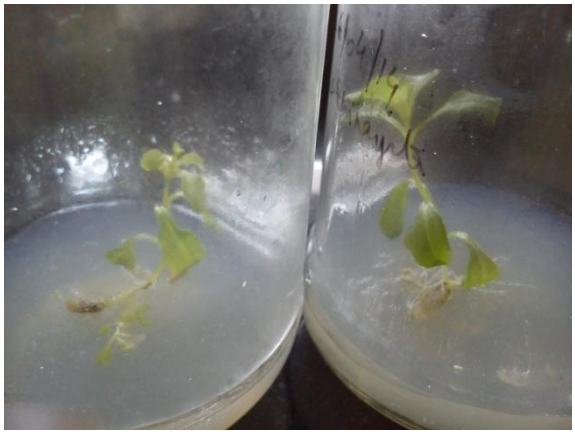
SW (1g/l) + Agar - Sucrose – GH

Control plant

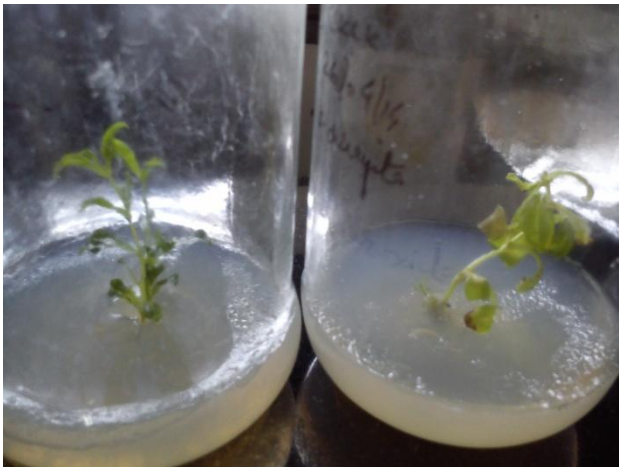
Day 20: Second sampling



SW (2g/l) + Agar - Sucrose – GH



SW (1g/l) + Agar - Sucrose – GH



Control plant

HPLC ANALYSIS

Table 3: standard amount of metabolites

Standards	Area	Weight	µg/mL
Swertiamarin	177329	1.07	21.4
Mangiferin	862793	1.11	22.2
Amarogentin	315110	0.93	18.6

Table 4: percentage of metabolites in test cultures

	Sample	Area			% present		
Swertia chiravita	**Weight**	Swertiamarin	Mangiferin	Amarogentin	Swertiamarin	Mangiferin	Amarogentin
6^{10}	105.10	141866	529900	23227	0.81	0.65	0.07
6^{20}	104.34	100459	190544	23225	0.58	0.23	0.07
7^{10}	85.46	174756	87501	15484	1.23	0.13	0.05
7^{20}	106.40	245256	186502	17487	1.39	0.23	0.05
8^{10}	104.50	100447	116520	11796	0.58	0.14	0.03
8^{20}	100.10	186739	317464	22092	1.13	0.41	0.07
11^{10}	105.40	220208	334961	18466	1.26	0.41	0.05
11^{20}	105.70	466862	250379	41542	2.67	0.30	0.12
12^{10}	101.11	181444	143728	23911	1.08	0.18	0.07
12^{20}	106.00	269417	173002	27946	1.53	0.21	0.08
13^{10}	102.70	184835	130463	14396	1.09	0.16	0.04
13^{20}	100.20	164917	123610	13527	0.99	0.16	0.04
14	101.80	575353	733561	57548	3.41	0.93	0.17

Table 5: amount of metabolites in test cultures

Swertia chirayita	Swertiamarin (**µg/mg**)	Mangiferin (**µg/mg**)	Amarogentin (**µg/mg**)
6^{10}	8.14	6.49	0.65
6^{20}	5.81	2.35	0.66
7^{10}	12.34	1.32	0.53
7^{20}	13.91	2.26	0.49
8^{10}	5.80	1.43	0.33
8^{20}	11.26	4.08	0.65
11^{10}	**12.61**	4.09	0.52
11^{20}	**26.65**	**3.05**	**1.16**
12^{10}	10.83	1.83	0.70
12^{20}	15.34	2.10	0.78
13^{10}	**10.86**	1.63	0.41
13^{20}	**9.93**	**1.59**	**0.40**
14(explant)	34.10	9.27	1.67

Chromatograms

Fig. 3: chromatogram of standard

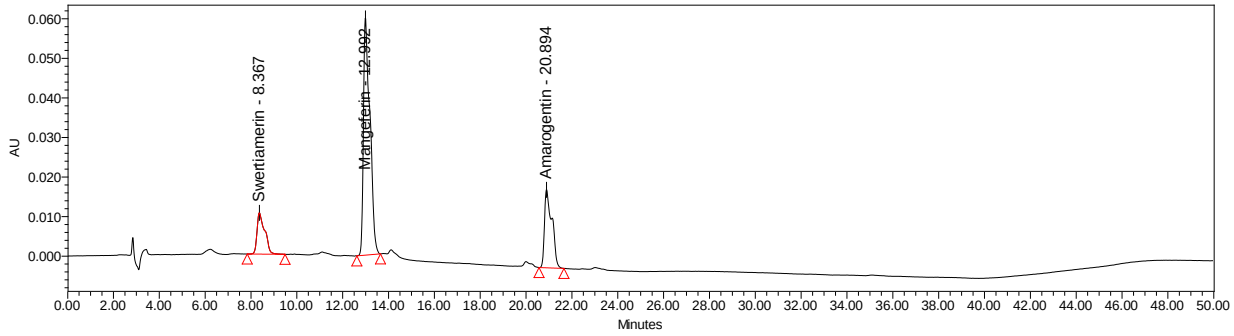


Fig. 4: chromatogram of explant used to subculture

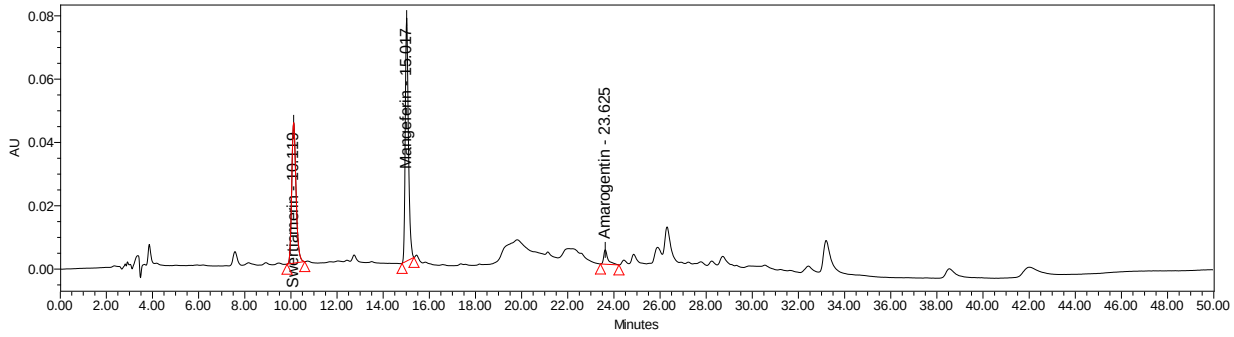


Fig. 5: chromatogram of culture sampled after 10 days grown on the media SW (2g/l) + Agar - Sucrose - GH

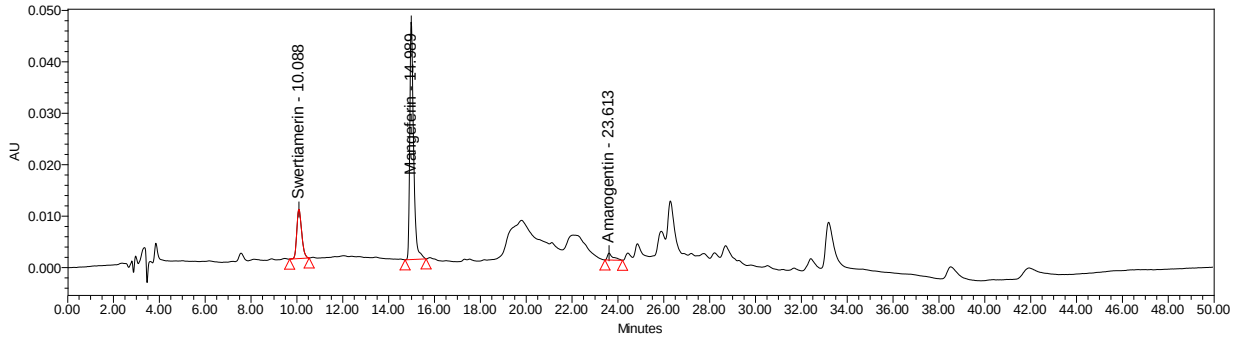


Fig. 6: chromatogram of culture sampled after 20 days grown on the media SW (2g/l) + Agar - Sucrose - GH

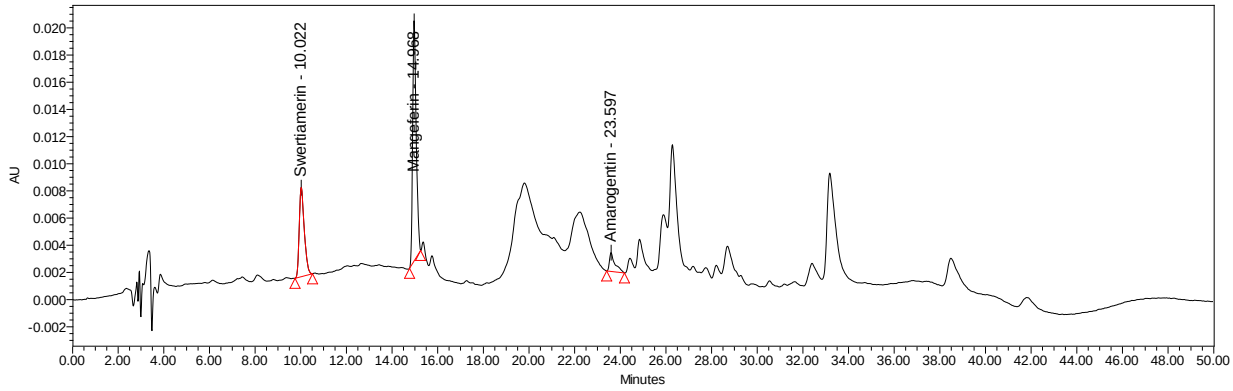


Fig. 7: chromatogram of culture sampled after 10 days grown on the media SW (2g/l) + Agar + Sucrose + GH

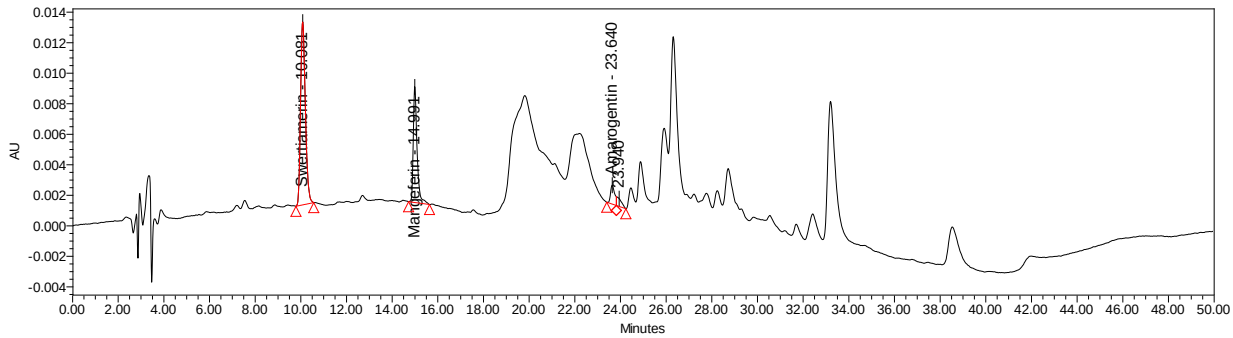


Fig. 8: chromatogram of culture sampled after 20 days grown on the media SW (2g/l) + Agar + Sucrose + GH

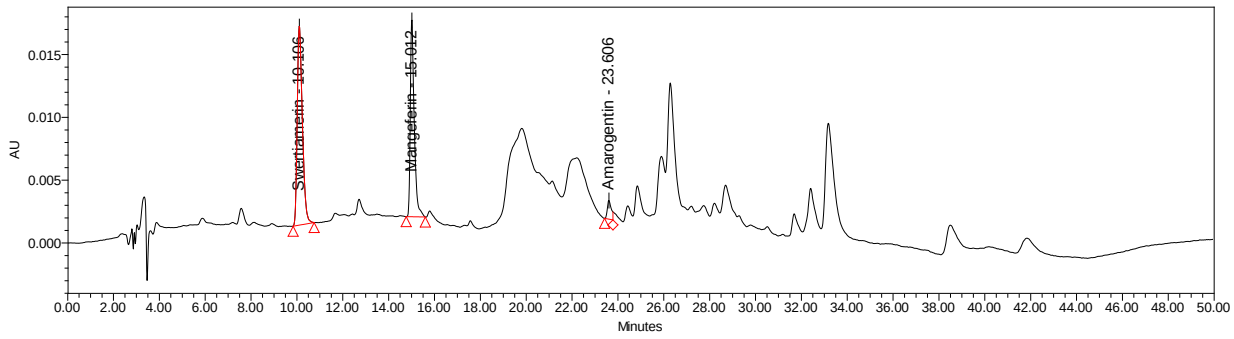
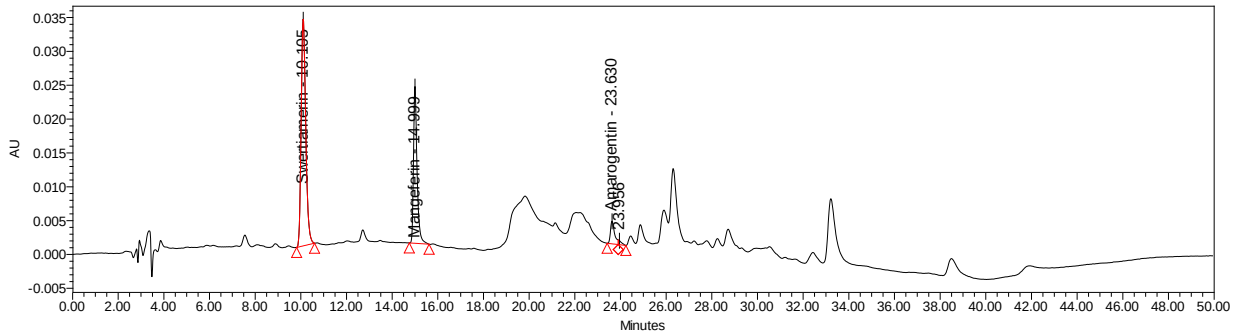


Fig. 9: chromatogram of culture sampled after 20 days grown on the media SW (3g/l) + Agar - Sucrose - GH



Many studies have shown that seaweeds are very beneficial to the growth and development of plants. They not only provide various nutrients for the growth but also help plants in defending themselves against variety of stresses. They are eco-friendly, non-toxic, non-polluting, environmentally benign and safe for the health of humans. Our experiment was based on the utilization of seaweed in plant culture media for increasing the metabolite production. The growth parameters were best in the solid media containing 2g/l of seaweed. Several cultures grown on media containing seaweed even showed rooting. This is so because they contain many

growth regulators such as auxins , gibberellins, betaines, macronutrients such as Ca, K, P, and micronutrients like Fe, Cu, Zn, B, Mn, Co, and Mo, necessary for the development and growth of plants. HPLC analysis showed maximum increase in media containing 3g/l of seaweed which was somewhere near to the explants metabolite content. However, the metabolite was significantly higher in seaweed cultured plants as compared to control plants grown on MS media containing IBA, Kn and GA_3. The control plant showed 10.86 µg/mg of swertiamarin content and it showed an increase in the content with an amount of 12.34 µg/mg in the plant grown on the media containing 2 g/l of seaweed, sucrose and growth hormones IBA and kinetin(table 2). It was comparable in the media containing 2g/l of seaweed, agar and no sucrose and no growth hormone. The same was the case with mangiferin. The control showed the content of 1.63 µg/mg and the plant grown on the media containing 2g/l of seaweed and no sucrose and no growth hormones showed the content of 6.49 µg/mg. In the case of amerogentin the control showed the content of 0.41 µg/mg and the plants grown on the media containing 2g/l of seaweed showed the content of 0.65µg/mg without sucrose and growth hormones. The plants grown on the media containing 3g/l and 1g/l of seaweed also showed significant increase in metabolite content when compared with control plants (table 2). The best media which showed highest increase in metabolite content contained 3g/l of seaweed, agar and no sucrose and no growth hormone.

Chapter 5- CONCLUSION

Due to its endangered status, it is difficult to meet the global demand of metabolites through natural population. So, the in vitro grown plants can serve this demand efficiently. In vitro propagation is the only alternative for the large scale production of authentic raw material. So, we have optimized the technology where the large scale production is carried out in 3g/l of seaweed, agar and no sucrose and no growth hormone.

As the in vitro grown shoot contain the metabolite content of 9.93 µg/mg, in vitro grown shoot was further tested in different combinations of seaweed as an elicitor. Out of all the test media the media containing 3g/l of seaweed, agar and no sucrose and no growth hormone gives the best accumulation of all three metabolites: swertiamarin (26.65 µg/mg), mangiferin (3.05 µg/mg) and amerogentin (1.16 µg/mg).Thus, we have applied successful techniques for its micro propagation with the use of various seaweed concentrations giving significantly higher metabolite content.

The best media contained no sucrose and no growth hormone which implies that seaweed also helped in decreasing the cost of media. So this complete process can be used as an alternative to conserve this medicinally important herb by growing it in large number in lab on the seaweed media. These could then act as a source of pure and collectible metabolites and provide regular support to pharmaceuticals. This will help in establishing agro techniques for promoting its cultivation and hence help in the conservation of the plant. This novel technique can help in conservation and production of a large number of disease-free, true-to-type and high metabolite containing plants. *S. Chiravita* enjoys a good domestic and international market. S chiravita extracts are used in a number of medicinal formulations and it is required in large amounts by the pharmaceuticals companies. So if this technique is translated to field for practical purposes at a large scale, this will reduce the burden on the wild for its collection. Beside the strategy evolved would maintain quality and quantity of metabolites in the plant.

APPENDIX

Media components (Inorganic)

Solutions	Components	Amount (gm/lt.)
STOCK A [10X]	KNO_3	19.00
	$MgSO_4.7H_2O$	3.70
	KH_2PO_4	1.70
STOCK B [20X]	NH_4NO_3	33.00
STOCK C [100X]	$CaCl_2.2H_2O$	44.00
STOCK D [100X]	Na_2 EDTA	3.726
	$FeSO_4.7H_2O$	3.785
STOCK E [100X]	KI	0.083
STOCK F [100X]	H_3BO_3	0.62
	$CoCl_2.6H_2O$	0.0025
	$ZnSO_4.7H_2O$	0.86
	$CuSO_4.5H_2O$	0.0025
	$MnSO_4.4H_2O$	2.23
	$Na_2MoO_4.2H_2O$	0.025
STOCK G [100X]	m – Inositol	10.00
	Glycine	0.20
STOCK H [100X]	Pyridoxin HCl	0.05
	Nicotinic Acid	0.05
	Thiamine HCl	0.01

REFERNCES:

Kirtikar, K. R. and Basu, B. D. (eds), *Indian Medicinal Plants*, Allahabad, 1984, vol. III, pp. 1664–1666.

Clarke, C. B., In *The Flora of British India* (ed. Hooker, J. D.), L. Reeve and Co, London, 1885, vol. IV, p. 124.

Bentley, R. and Trimen, H., *Medicinal Plants* (eds Bentley, R. and Trimen, H.), J and A Churchill, London, 1880, p. 183.

Anon, In *The Wealth of India: Raw Materials*, Publication and Information Directorate, CSIR, New Delhi, 1982, vol. X, pp. 78–81.

Raina, R., Johri, A. K. and Srivastava, L. J., Seed germination studies in *Swertia chiravita* L. *Seed Res.*, 1994, **22**, 62–63.

Basnet, D. B., Evolving nursery practices and method of cultivation of high value medicinal plant *Swertia chirayita* Ham. *Environ. Ecol.*, 2001, **19**, 935–938.

Wolfender,-J-L; Rodriguez,-S; Hostettmann,-K; Hiller, W Liquid chromatography/ultra violet/mass spectrometric and liquid chromatography/nuclear magnetic resonance spectroscopic analysis of crude extracts of Gentianaceae species. Phytochemical-Analysis. 1997; 8(3): 97-104

Demizu-S; Ohshima-Y; Hiraga-Y; Takahashi-K J.Chromatogr.; High-performance liquid chromatographic analysis of Swertia herb, (1986) 360, 1, 307-11

Jayaraj Jayaraman & Jeff Norrie & Zamir K. Punja, Commercial extract from the brown seaweed *Ascophyllum nodosum* reduces fungal diseases in greenhouse cucumber, Journal of Applied Phycology (2011), Volume 23, Issue 3, pp 353-361

Sakamoto-I; Morimoto-K; Tanaka-O; Inouye-H , Application of high performance liquid chromatography and field desorption mass spectroscopy to seperative analysis of bitter secoiridoid glucosides of Swertia Herba,Chem.Pharm.Bull.; (1983) 31, 1, 25-30

Tian-Wei; Chen-ZhaoHui; Zhai-Jing; Chen-LiRen; Li-YongMin , HPLC fingerprinting of total glycosides of *Swertia franchetiana*. Acta-Pharmaceutica-Sinica. 2005; 40(5): 447-452

Shekarchi,-M; Hajimehdipoor,-H; Khanavi,-M; Adib,-N; Bozorgi,-M; Akbari-Adergani,-B A validated method for analysis of Swerchirin in *Swertia longifolia* Boiss. by high performance liquid chromatography,Pharmacognosy-Magazine. 2010; 6(21): 13-18

Negi,-J-S; Pramod-Singh; Rawat,-M-S-M; Pant,-G-J Qualitative and quantitative determination of major xanthones in *Swertia speciosa* by high performance liquid chromatography, Medicinal-Plants. 2010; 2(1): 45-50

Xu-KangPing; Shen-Jian; Li-FuShuang; Liu-JianFeng; Liu-GuoRu; Tan-JianBing; Tan-GuiShan, Determination of six active components in three species of genus Swertia by HPLC multiwavelength with detection. China-Journal-of-Chinese-Materia-Medica. 2009; 34(11): 1384-1389

Khan,-M-Y; Saleh-Aliabbas; Vimal-Kumar; Shalini-Rajkumar, Recent advances in medicinal plant biotechnology. Indian-Journal-of-Biotechnology. 2009; 8(1): 9-22

Tian-LuanYuan; Chen-JiaChun; Huang-FengJiao; Fang-JinBo, Simultaneous determination of four active components in Swertia by RP-HPLC.Chinese-Journal-of-Natural-Medicines. 2008; 6(6): 444-449

Wang-ZhiGang; Ma-ChaoMei; Tang-ShuHan; Xiao-Huai; Kakiuchi,-N; Kida,-H; Hattori,-M Qualitative and quantitative analysis of Swertia herbs by high performance liquid chromatography-diode array detector-mass spectrometry (HPLC-DAD-MS), Chemical-and-Pharmaceutical-Bulletin. 2008; 56(4): 485-490

Xia-CongLong; Liu-GuangMing; Zhang-Hao. Determination of four effective constituents in*Swertia binchuanensis* by HPLC, Journal-of-China-Pharmaceutical-University-. 2007; 38(1): 43-46

Li-YuLin; Wang-LingYun; Wang-XiaoYan; Suo-YouRui; Chen-GuiChen; Hu-FengZu; Du-YuZhi; You-JinMao Separation and determination of the major active components in Tibetan folk medicinal species *Swertia franchetiana* by HPLC with DAD, Journal-of-Liquid-Chromatography-and-Related-Technologies. 2007; 30(9/12): 1687-1696
Philadelphia, USA: Taylor & Francis.

Bhandari,-P; Kumar,-N; Gupta,-A-P; Singh,-B; Kaul,-V-K Micro-LC determination of swertiamarin in Swertia species and bacoside-A in Bacopa monnieri Chromatographia-. 2006; 64(9/10): 599-602

Maninder-Karan; Karan-Vasisht; Handa,-S-S , Morphological and chromatographic comparison of certain Indian species of Swertia. Journal-of-Medicinal-and-Aromatic-Plant-Sciences. 1997; 19(4): 955-963

Gao,-G-Y; Li,-M; Feng,-Y-X; Tan,-P Determination of effective constituents in 11 Swertia and related plants by HPLC. Acta-Pharmaceutica-Sinica. 1994; 29(12): 910-914

Sunita-Shailajan; Abhishek,-J-S , Quantification of Swertiamarin from whole plant powder of *Swertia densiflora* (Griscb.) Kashyap. collected in different seasons, Asian-Journal-of-Chemistry. 2009; 21(6): 4351-4356

Hostettmann,-K; Wolfender,-J-L The search for biologically active secondary metabolites. Pesticide-Science. 1997; 51(4): 471-482

Keil-M; Haertle-B; Guillaume-A; Psiorz-M , Production of amarogentin in root cultures of *Swertia chirata*. Planta-Med.; (2000) 66, 5, 452-57

Wawrosch-C; Maskay-N; +Kopp-B , : Micropropagation of the threatened Nepalese medicinal plant *Swertia chirata* Buch.-Ham. ex Wall. Plant-Cell-Rep.; (1999) 18, 12, 997-1001

Sharma-P-V, Triterpenoids of *Swertia chirata*, Indian-J.Pharm.Sci.; (1983) 45, 5, 222

Arvind-Bhatt; Rawal,-R-S; Uppeandra-Dhar , Ecological features of a critically rare medicinal plant, *Swertia chirayita*, in Himalaya.

Abhishek-Kumar; Verma,-S-P, Efficacy of Rumec powder in the treatment of acidotic goats.
Shi-GaoFeng; Lu-RunHua; Yang-YunShang; Pan-HongCheng; Guan-YouWei; Han-ChunFang , Studies on chemical constituents of essential oil from *Swertia chirayita*. 2004

Bhupender-Dutt; Sharma,-Y-P , Screening of chirata plant, *Swertia chirayita*, from Himachal Pradesh for bitterness variability(2003).

Wang-ChangZeng; Maier,-U-H; Eisenreich,-W; Adam,-P; Obersteiner,-I; Keil,-M; Bacher,-A; Zenk,-M-H , Unexpected biosynthetic precursors of amarogentin - a retrobiosynthetic 13C NMR study (2001).

Basnet,-D-B , Evolving nursery practices and method of cultivation of high value medicinal plant *Swertia chirata* Ham (2001).

Gaurav Kumar & Dinabandhu Sahoo, Effect of seaweed liquid extract on growth and yield of *Triticum aestivum* var. Pusa Gold, J Appl Phycol (2011) 23:251–255

Keil,-M; Hartle,-B; Guillaume,-A; Psiorz,-M Production of amarogentin in root cultures of *Swertia chirata*. (2000)

Shivaji-Banerjee;Sur,-T-K;Suvra-Mandal;Das,-P-C;Sridhar-Sikdar, Assessment of the anti-inflammatory effects of *Swertia chirata* in acute and chronic experimental models in male albino

rats. (2000)

Rai,-L-K; Pankaj-Prasad; Sharma,-E Conservation threats to some important medicinal plants of the Sikkim Himalaya.(2000)

Balaraju,-K; Agastian,-P; Ignacimuthu,-S Micropropagation of *Swertia chirata* Buch.-Hams. ex Wall.: a critically endangered medicinal herb(2009)

Wang-Li; An-LiZhe; Hu-YanPing; Wei-LiXin; Li-Yi , Influence of phytohormones and medium on the shoot regeneration from leaf of *Swertia chirata* Buch.-Ham. Ex wall. *in vitro* (2009).

Alka-Shiva, Methods of sustainable harvesting and value addition for economic uplift and biodiversity conservation series 35. Important medicinal plant species: *Gloriosa superba, Gymnema sylvestre, Hedychium coronarium, Nardostachys jatamansi & Swertia chirayita*. (2007)

Madhuri,-S; Govind-Pandey , Some dietary agricultural plants with anticancer properties. (2008)

Chaudhuri,-R-K; Amita-Pal; Jha,-T-B Production of genetically uniform plants from nodal explants of *Swertia chirata* Buch.-Ham. ex Wall. - an endangered medicinal herb. (2007)

Bhandari,-P; Kumar,-N; Gupta,-A-P; Singh,-B; Kaul,-V-K Micro-LC determination of swertiamarin in Swertia species and bacoside-A in *Bacopa monnieri*(2006)

Manjunath,-K-P; Shivakumar,-H; Prakash,-T; Patil,-K-S; Veeranagouda,-A; Jayakumarswamy,-B-H-M; Venkatesh,-J-S; Rao,-R-N Anthelmintic activity of roots of *Swertia chirata*. (2006) Record 34 of 65 - Horticultural Science Database

Arvind-Bhatt; Rawal,-R-S; Uppeandra-Dhar Ecological features of a critically rare medicinal plant, *Swertia chirayita,* in Himalaya. (2006)

Hatjimanoli,-M; Favre-Bonvin,-J; Kaouadji,-M; Mariotte,-A-M Monohydroxy- and 2,5-dihydroxy terephthalic acids, two unusual phenolics isolated from Centaurium erythraea and identified in other Gentianaceae members.(1988)

Sekar,-B-C; Mukherjee,-B; Chakravarti,-R-B; Mukherjee,-S-K Effect of different fractions of Swertia chirayita on the blood sugar level of albino rats. (1987)

Mukherjee,-B; Mukkerjee,-S-K Blood sugar lowering activity of *Swertia chirata* (Buch-Ham) extract.(1987)

Ghosal,-S; Sharma,-P-V; Chaudhuri,-R-K; Bhattacharya,-S-K Chemical constituents of the Gentianaceae. V: Tetraoxygenated xanthones of *Swertia chirata* Buch.-Ham.(1973)

Wawrosch,-C; Maskay,-N; Kopp,-B Micropropagation of the threatened Nepalese medicinal plant *Swertia chirata* Buch.-Ham. ex Wall.(1999)

Karan,-M; Vasisht,-K; Handa,-S-S , Antihepatotoxic activity of *Swertia chirata* on paracetamol and galactosamine induced hepatotoxicity in rats.(1999)

Karan,-M; Vasisht,-K; Handa,-S-S , Antihepatotoxic activity of *Swertia chirata* on carbon tetrachloride induced hepatotoxicity in rat.(1999)

Maninder-Karan; Karan-Vasisht; Handa,-S-S Morphological and chromatographic comparison of certain Indian species of Swertia (1997)

Mukherjee,-S; Sur,-A; Maiti,-B-R Hepatoprotective effect of *Swertia chirata* on rat. (1997)

Raina,-R; Johri,-A-K; Srivastava,-L-J Seed germination studies in Swertia chirata L (1994)

Pandey,-R-K; Shrivastava,-J-L : Indigenous wild medicinal plants in the forest of Baiga Chak of Mandla District. (1994)

Girach,-R-D; Aminuddin-; Khan,-S-A Andrographis paniculata - a possible substitute for Swertia chirata in southeastern India (1994)
Shrestha,-J-N; Joshi,-S-D Tissue culture technique for medicinally important herbs - Orchis incarnata and *Swertia chirata*. (1992)

Mandal,-S; Das,-P-C; Joshi,-P-C; Chatterjee,-A; Islam,-C-N; Dutta,-M-K; Patra,-B-B Antiinflammatory action of *Swertia chirata* (1992)

Chakravarty,-A-K; Sibabrata-Mukhopadhyay; Binayak-Das Swertane triterpenoids from *Swertia chirata. (1991)*

Wajahatullah khan,usha P. Rayirath, sowmyalakshmi subramanian , mundaya N. Jithesh , prasanth rayorath, D. Mark hodges, alan T. Critchley, james S. Craigie, jeff norrie, balakrishan prithiviraj, Seaweed extracts as biostimulants of plant growth and development, J plant growth regulators (2009) 28:386–399.

Sobia tabassum, sidra mahmood, javeria hanif, maryam hina, bushra uzair, An overview of medicinal importance of *Swertia chirayita,* International journal of applied science and technology (2012) vol. 2 no.1

Prawez alam, mohammad ali, rahul singh, faiyaz shakeel, Estimation of swertiamarin in enicostemma littorale and marketed formulations using HPLC-UV method, volume 44, issue 2, pp 95-102.