In vitro multiplication and regeneration of medicinally important and critically threatened herb *Swertia chirayita*.

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CERTIFICATE

This is to certify that the work titled "***In vitro* multiplication and regeneration of medicinally important and critically threatened herb *Swertia chirayita***" submitted by "**Ms ARTI JASWAL, Ms DEEPANJALI SODHA and Ms SANDEEP KAUR**" in partial fulfillment for the award of degree of **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.

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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.

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SUMMARY

Despite a descent hold in the herbal industry, *Swertia chirayita* is still collected from the wild; it is sparsely cultivated and negligible efforts have gone into developing proper agrotechniques of the plant. Little research has been done to identify the existing diversity among different populations of *S. chirayita*. As plant is critically endangered and have high requirement in the pharmaceutical industry so its availability with authencity is a prerequisitive, i.e. in the present research, we explore all the means for carrying out its micro propagation along with callus induction and regeneration so that along with the development of clones of this herb, we have developed the variants of *Swertia*. Furthermore MS + KN (3mg/l) + GA3(2mg/l) + IBA (1mg/l) is identified as best media for shoot multiplication in *Swertia chirayita* and for root induction the best recorded media is MS+KN(2mg/l)+GA3(2mg/l)+IAA(2mg/l) and rooting occurred in 9-10 days of culturing with 80% of shoots forming roots. Leaf discs from field grown plants were used to establish callus cultures. MS medium supplemented with 2, 4-D (3 mg/l) + IBA (3 mg/l) + sucrose 3% (w/v) +agar-agar (0.8%) was found to be the best for callus induction and GA3 (2mg/l) + KN (2mg/l) + IBA (2mg/l) + sucrose 3% (w/v) +agar-agar (0.8%) was the best for shoot regeneration from callus . Also, liquid cultures were best reported on MS + KN (2mg/l) + GA3(2mg/l) + IAA (2mg/l) . As S*wertia chirayita* grow at low temperature regions, so some of the cultures were also kept at 15°C. In this way we developed micro propagation technique for successful growth of *Swertia chirayita* and *in vitro* grown plants were also successfully transferred to field for hardening. *Swertia chirayita* is a medicinally important plant with a range of secondary metabolites present in it. We have established the culture conditions for the *in vitro* production of plantlets and it would act as an authentic source for the production of metabolites, so that the burden on natural habitat could be relieved and its medicinal benefits should be exploited for the beneficiary of human beings.

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LIST OF ABBREVIATIONS

Abbreviation	*Meaning*
°C	DegreeCelsius
GA3	Gibberellic acid
IAA	Indole acetic acid
Gm	Gram
IBA	Indole -3 Butyric Acid
Kg	Kilogram
NAA	Naphthalene acetic acid
KN	Kinetin
1 / lt.	Litre
mg	Milligram
MS media	Murashige and Skoog Media

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CHAPTER 1 –INTRODUCTION

1.1 ***Swertia chirayita*** of the Gentianaceae family is a slender, upright herb found in the Himalayas from Kashmir to Bhutan and Khasi Hills. *Swertia* species are known for their medicinal value.The Wealth of India, Raw materials Vol X PID (CSIR) New Delhi provide in detail full accountof distribution economic importance and uses of genus *Swertia*. Its medicinal usage is reported in Indian pharmaceutical codex, the British and the American pharmacopoeias and in different traditional systems of medicines such as the Ayurveda, Unani and Siddha. Among the different species of *Swertia* reported in India, *Swertia chirayita* is considered the most important for its medicinal properties. The plant is used as a bitter tonic in treatment of fever and for curing various skin diseases. *S. chirayita* has an established domestic (Indian) and international market, which is increasing at a rate of 10% annually. The plant available in the market many a times is adulterated and substituted by close relatives of chirayita. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin (most bitter compound isolated till date), swerchirin, swertiamarin and other active principles of the herb. Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon V ointment contain chirayita extract in different amounts for its antipyretic, hypoglycemic, antifungal and antibacterial properties. Despite a descent hold in the herbal industry, the plant is still collected from the wild; it is sparsely cultivated and negligible efforts have gone into developing proper agro-techniques of the plant. Little research has been done to identify the existing diversity among different populations of *S. chirayita*. The lacunae in the related research field raise concerns regarding the vulnerability of the species, emphasizing the need for research. Presently, supply depends on wild sources that have been depleted by over harvesting and progressive habitat clearance. It would, therefore, be helpful to use a tissue culture procedure for large scale propagation and conservation. Beside the strategy evolved should maintain quality and homogeneity of herbs.

The widespread use of *S. chirayita* in traditional medicine reflects its pharmacological importance. However, existing populations of *S.chirayita* are diminishing. Hence according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, *S. chirayita* has been categorized as critically endangered. This leads to a need for conservation of the plant. *S. chirayita* has been prioritized by the National Medicinal Plant Board(Government of India) for conservation and cultivation in Uttaranchal, emphasizing the need to Develop agro-technology packages. The novel technique of in vitro conservation and micropropagation can help in conservation and production of a large number of disease-free, true-totype plants. Swertia chirayita enjoys a good domestic and international market. About 45% of chirayita in the Himalayan region is collected from Nepal.Tissue culture is useful for multiplying and conserving the species, which are difficult to regenerate by conventional methods and save them from extinction. Genetic transformation may provide increased and efficient system for in-vitro production of secondary metabolites and for engineering the metabolic pathways for enhancement of active component. But so far this has been confined only to *Swertia chirayita* ; therefore it still needs lots of attention for various other species of this genus exploited for pharmaceutical purposes.

1.2 CHARACTERISTICS OF *SWERTIA CHIRAYITA*

1.2.1 Taxonomy

Swertia comprises 100 species (Airy Shaw, 1973) of which 27 species are reported from Nepal (Hara *et.al*, 1982). Among them *Swertia chirayita* is considered superior in quality.

Family – Gentianaceae

Local Name - Chiraito, Chiraita, Tite, Tikta

English name – Chiretta

Chirayita is an erect, biennial herb, 50-125 cm tall. Stem is robust, branching, cylindrical below, 4- angled upwards, containing large pith. Leaves are broadly lanceolate, 5-nerved, and sub sessile. Capsules are egg-shaped, seeds are minute, smooth, and many angled. It grows up to about 1.5 meters in height. It has leaves in opposite pair about 10 cms long, without stalks, pointed at the tip. The plant has numerous flowers, pale green in color, tinged with purple, with long white or pink hairs and minute sharp pointed fruits. Fruit is a small, one-celled capsule with a transparent yellowish pericarp. It dehisces from above, septicidally into two valves. Seeds are numerous, minute, many-sided and angular. Floral characteristics such as colorful corolla and presence of nectaries support crosspollination in the species. Generally, bees (Apoidae, Hymenoptera) are the pollinators of *S. chirayita.* The whole plant, collected in its flowering stage and dried, constitutes the drug. The trade name *chirayata* is based on the local name of the plant. It has long been used by the ayurvedic physicians as a bitter tonic.

1.2.2 Habitat and Range

The plant is a native of temperate Himalayas, found at an altitude of 1200–3000 m (4000 to 10,000 ft), from Kashmir to Bhutan, and in the Khasi hills at 1200–1500 m (4000 to 5000 ft). It can be grown in sub-temperate regions between 1500 and 2100 m altitudes. The plant can be grown in a variety of soils with sandy loam rich in carbon and humus. It is also found in open ground and recently slash-and-burnt forests.

1.2.3 Ecology

Chiraito is a biennial herb, which shows a rosette form in the first year whereas two years old plant has elongated stem with yellow flower. Distribution of Chiraito is not uniform; it depends upon the altitude and slope. It prefers to grow on north facing slopes. It grows in south facing slope between 1500m and 3000m. While on the north facing slope, it descends below 1500m. In general, 2000m altitude is most preferable range (Bhattarai, 1996). Chiraito prefers to grow in acidic soil condition with pH of 4.7 to 5.5 (Bhattarai & Shrestha, 1996). Chiraito is found being mixed with other species. The most common associate are: Bhuin Kaphal (*Fragaria indica*), Bukephool (*Anaphilis triplinervis*), Chari Amilo (*Oxalis corniculata).*

1.2.4 Regeneration

The plant is harvested for the drug industry when it sets into flowering in July–September. The natural regeneration of plant takes place by seeds, when the seeds become biologically mature having high potentiality of viability during November (Bhattarai, 1996). The viability of seeds is very low if seeds are collected before November. The seeds stored in bad condition have no

viability at all. The viability decreases after next October. If seeds are collected after November and cleaned properly, the percentage of germination is reported to be up to 90% (Bhattarai, 1991). To start a Chiraito Nursery in November it is possible collect Chiraito seeds from the forest. The seeds collected should be sown within a year of collection. Before February, the soil is too cold to sow the seeds. It is better to sow between February and April, into moist, fertile nursery beds. The seeds are covered with a thin layer of soil (depth twice the size of the seeds). Mulching is necessary for better germination. Frequent water spraying is done to maintain the moisture content of soil. When the seeds start to germinate, the mulching materials should be removed. After the seedlings attained the height of 6-8 cm, then they are ready for transplantation in field. Chiraito should be harvested after three years of growth when the plant is well developed, after the plant has flowered and produced fruit. If harvesting is done after the seeds mature, then the plant can naturally regenerate. Harvesting Chiraito without considering the age of the plant and seed maturity reduces regeneration significantly.

Only a few scattered reports in the literature suggest germination studies and nursery practices of *S. chirata*. Low germination percentage and viability of the seeds, long gestation periods and delicate field-handling are some of the factors which discourage commercial cultivation of the plant.

1.2.5 Secondary Metabolites

Extracts from *Swertia chirata* (family Gentianaceae) have ant diabetics and antioxidant activity, largely attributed to the flavonoids and secoiridoids, which are a major class of functional components in methanolic extracts from aerial part of plants.

The whole plant contains gentiamine alkaloids and the aerial part contains xanthones. The main chemical constituents of this plant are ophelic acid and chiratin. The plant also contains resins, tannin, gum, carbonates, phosphates and 4 to 6 per cent ash.

A number of workers have shown that the drug contains bitter glycosidal components, chiratin and amarogentin, swerchirin, phytosterol, also a number of acids and phenolic compounds. The bioactive constituents include the xanthone and secoiridoid glycosides consisting of mangiferin, amarogentin, amaroswerin, sweroside and swertiamarin. *Swertia chirata* Buch-Ham (Fam. Gentianaceae) is widely used in India to treat fever and malaria. Xanthone derivatives like mangostin, isomangostin and mangostin triacetate are known to possess significant anti-inflammatory activities. The total xanthones of Swertia species produce significant CNS stimulant action. Reports also suggest that, several varieties of xanthones show potent anti-platelet activity, anti-cancer, anti-fungal and antimalarial effects. The plant has been reported to possess hypoglycemic activity, anti-inflammatory activity, hepatoprotective activity , wound healing activity as well as antibacterial activity on selected microbial strain. Various parts of this plant, including the root, stem, flower and leaves are recommended for the treatment of fever in combination and separately. The root juice is given for the relief of fever in whole part of India. *Swertia chirata* is proved key ingredient of Mahasudarshan churna, an Ayurvedic formulation which are widely used in India.

1.3 Medicinal Uses of *Swertia chirayita*

The herb *Swertia chirayita* possesses digestive, hepatic (conditions pertaining to the liver) and tonic properties. In fact, this bitter herb promotes digestion, particularly of fats, and aid in regulating blood sugar levels. At the same time, the herb is an effective medication for leishmaniasis - a parasitic disease usually found in tropical regions. Chiretta is especially beneficial for certain health conditions, including diabetes and nausea.

Here is a brief discussion regarding the use of this herb to treat these precise health problems.

- Additional animal studies with chiretta have discovered that this herb is more effectual in regulating blood sugar levels compared to the regular anti-diabetic drug Orinase(Tolbutamide).

- The astringent flavor of chiretta sets of an impulsive response that promotes the production of saliva and gastric enzymes. This reflex reaction owing to the use of the herb not only stops nausea (queasiness), but also helps to cure indigestion, bloating and hiccups. In addition, chiretta also encourages the secretion of bile that promotes digestion as well as improves appetite.

- *Swertia chiretta* has an attractive chemistry that is to a great extent akin to gentian (Gentianalutea), a widely used healing tonic for the digestive system. The plant also encloses xanthones that are supposedly effectual against malaria and tuberculosis.

In addition, chiretta also contains amarogentin - a glycoside that perhaps fortifies the liver against toxicity caused by carbon tetrachloride. As discussed earlier, this herb also has a valuable impact on the liver, encourages the flow of bile and heals constipation. It is also beneficial for curing dyspepsia.

- Chirayita is a valuable bitter tonic. It is laxative and an appetizer. It also corrects the disordered process of nutrition and restores the normal function of the system.

Chirayita is an effective drug for reducing fevers. It is especially beneficial in the treatment of malarial fevers. It is also effective in hysteria and convulsion.

The herb is an excellent drug for strengthening the stomach and promoting its action. It is used in the treatment of dyspepsia and diarrhea.

1.3.1 Medicinal formulations based on *Swertia chirayita*

1. Diabecon (GlucoCare)
2. Ayush-64
3. Mensturyl syrup
4. Melicon V ointment
5. Anigest

1.3.2 Biological activities attributed to *Swertia chirayita*

1. Hypnotic
2. Antihelmintic
3. Stomachic
4. Anticholinergic
5. Anticonvulsant
6. Antiinflammatory
7. Antimalarial
8. Antipyretic
9. Bitter
10. Hypoglycemic/antidiabetic
11. CNS depressant
12. Hepatoprotective
13. Cardio stimulant

OBJECTIVES:

- To carry out large –scale in vitro multiplication of plantlets and their transfer to soil.
- Optimization of media for callus induction from different explants and shoot regeneration.
- In vitro root induction of micropropagated shoots.
- Transfer to greenhouse and hardening of *in vitro* grown plants.
- Determining concentration of pharmacologically important secondary metabolites produced in the plant under different *in vitro* conditions using HPLC.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Importance:

Leena et al. (2003) published a paper stating that medicinal plants are the most important source of life saving drugs for the majority of the world's population. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. In-vitro regeneration holds tremendous potential for the production of high-quality plant-based medicine. Cryopreservation is long-term conservation method in liquid nitrogen and provides an opportunity for conservation of endangered medicinal plants. In-vitro production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants. Bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology. Genetic transformation may be a powerful tool for enhancing the productivity of novel secondary metabolites; especially by *Agrobacterium rhizogenes* induced hairy roots. This article discusses the applications of biotechnology for regeneration and genetic transformation for enhancement of secondary metabolite production in-vitro from medicinal plants.
In the paper titled Wild useful plant genetic resources of Uttaranchal Himalayas, India (2004) Anjula et al. wrote about some wild useful species of flowering plants from Uttaranchal Himalayas, India, including the edible types, fodder-yielding species, medicinal types, fibre yielding species, wood species used for miscellaneous purposes and dye-yielding types. Some species such as *Aisandra butyracea [Diploknema butyracea], Nardostachys jatamansi, Picrorhiza kurroa, Podophyllum hexandrum* and *Swertia chirata* are exploited at the commercial scale from wild sources and thus demand special attention by the conservationists. Clinical evaluation of *Swertia chirata* for the treatment of P. vivax malaria was done by Panda-A-K et al. in 2004 on malaria patients in Kolkata, West Bengal, India to investigate the use of S.chirata for curing malaria caused by Plasmodium vivax.

Eighteen P. vivax-positive patients were administered with tablets containing air-dried powder of *S. chirata* (500 mg) at 50 mg/kg per day, in 2 divided doses for 5 days. Twelve out of 18 cases (66%) were parasite-free on day 6, while the rest showed reduction in parasite number. Febrile episode was not observed in 13 treated cases and no early or late treatment failure cases was observed. Rajesh-C et. al. in 2006 gave a list of Ayurdevic medicinal plants used to treat diabetes, highlighting the use and mode of action of *Gymnema sylvestre, Azadirachta indica, Momordica charantia, Ocimum sanctum [O. tenuiflorum], Pterocarpus marsupium, Swertia chirata* and *Syzygium cumini* against the disease. Notes are also presented on the symptoms, as well as the use of metals such as zinc and lead in the treatment of the disease.
The effect of Catliv, a herbal liver tonic from extracts of *Swertia chirata, Eclipta alba, Fumaria vaillentii, Picorrhiza kurrora, Andrographis paniculata, and Phyllanthus niruri,* on induced hepatopathy in cows was investigated by Pradhan et al. in 2001. It was concluded that Catliv is an effective liver tonic for treatment of hepatopathy in calves. Comparative efficacy of Ayush-64 vs. chloroquine in vivax malaria was done by Neena-V et. al. in 2000.

A phase II prospective comparative randomized clinical trial was conducted in India in patients with Plasmodium vivax malaria.Ayush-64 is a combination of extracts of *Alstonia scholaris* bark, *Picrorhiza kurroa* rhizome, *Swertia chirata* whole plant and *Caesalpinia crista* seed pulp. Patients received an oral dose of either 1 g Ayush-64, three times a day for 5-7 days or a total dose of 1500 mg chloroquine over 3 days. Peripheral smears were examined every day for 3 days or until they were negative and then weekly up to 28 days. At day 28, only 23 of 47 patients in the Ayush-64 group and all 41 in the chloroquine group were cured ($P<0.05$). In the 23 patients in the Ayush group, parasite clearance time was longer than that of chloroquine (3.16 vs. 1.5days). Both regimens were generally well tolerated. Blood sugar lowering activity of *Swertia chirata* (Buch-Ham) extract was reported by Mukherjee-B et al. in 1987. According to the report this species is used widely in Indian medicine as a tonic, stomachic, febrifuge and anthelmintic. A 90% ethanol extract of the plants lowered the blood sugar level in fed, fasted or glucose loaded rats.

The extract not only enhanced but also prolonged the sugar-lowering effect of tolbutamide when it was administered orally. Suparna-M et al. in 1998 reported that Iridoids, a widely distributed class of natural products have shown encouraging biological activities including anticancer, hepatoprotective, immunostimulant and antileishmanial. Recent reports on the promising biological activities of iridoids isolated from plant sources are reviewed. Particular reference is made to kutkin and picroliv isolated from Picrorrhiza kurrooa [Picrorhiza kurroa], arbortristosides from seeds of Nyctanthus arbortristis [Nyctanthes arbor-tristis], swertiamarin from *Swertia japonica*, amarogentin from *S. chirata* and *S. japonica*, aucubin from Aucuba japonica, scandoside methylester from Hedyotis colymbosa [H. corymbosa] and 4 iridoids from Scrophylaria [Scrophularia] koelzii. Amarogentin, a naturally occurring secoiridoid glycoside and a newly recognized inhibitor of topoisomerase I from *Leishmania donovani.* In a study conducted by Sutapa-R,et.al.in 1996 a methanol extract of *Swertia chirata* (Gentianaceae) inhibited catalytic activity of topoisomerase I from L. donovani. After fractionation the extract yielded 3 secoiridoid glycosides: amarogentin, amaroswerin and sweroside. Amarogentin is a potent inhibitor of type I DNA topoisomerase from L. donovani and exerts its effect by interaction with the enzyme, preventing binary complex formation. Trigonella foenum-graecum (methi) and *Swertia chirayita* (chirayita) are employed as traditional Ayurvedic herbal medicines. In this study, Bajpai et al. have investigated the blood sugar lowering activities of the 95% ethanol extracts of methi (seeds) and chirayita (whole plant) in normal fasted, fed and glucose loaded male albino rats using oral route of administration. Significant blood sugar lowering activity was found at a single dose (250 mg/kg body weight) in fasted and fed models. Both extracts also suppressed the peak value significantly and improved the glucose tolerance in rats.

The antiinflammatory effect of a xanthone derivative (1,5-dihydroxy-3,8-dimethoxy xanthone) obtained from S. chirata (SC-I) was investigated in acute, sub-acute and chronic experimental models using male albino rats by Shivaji-B et.al. in 2000. Aerial parts of S. chirata (collected from India) were extracted with organic solvent and purified by chromatographic procedure.

Anthelmintic activity of roots of *Swertia chirata* was studied by Manjunath-K-P et. al. in 2006. Ethanolic crude extract of S. *chirata* root (from Meghalaya, India) was fractionated with seven different solvents (petroleum ether, solvent ether, ethyl acetate, chloroform, benzene, butanone and butanol) and the fractions were evaluated for anthelmintic activity on earthworm Pheretima postuma. Butanol and ethyl acetate fractions exhibited significant anthelmintic activity. According to Khan-M-Y medicinal plants are the most important source of life saving drugs for the majority of the world's population. Plant secondary metabolites are economically important as drugs, fragrances, pigments, food additives and pesticides. The biotechnological tools are important to select, multiply, improve and analyze medicinal plants. In-vitro production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants and bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology. Genetic transformation is a powerful tool for enhancing the productivity of novel secondary metabolites; especially by *Agrobacterium tumefacians*. Combinatorial biosynthesis is another approach in the generation of novel natural products and for the production of rare and expensive natural products. DNA profiling techniques like DNA microarrays serve as suitable high throughput tools for the simultaneous analysis of multiple genes and analysis of gene expression that becomes necessary for providing clues about regulatory mechanism, biochemical pathways and broader cellular functions.

2.2 Micro propagation:

It was reported by Wawrosch-C et al.(1999) that micro propagation of the threatened Nepalese medicinal plant *Swertia chirata* Buch.-Ham. by adventitious shoot regeneration from root explants was found to be the most suitable method for the propagation of *Swertia chirata.* In another study done in 2000 Balaraju et al. reported an efficient*in vitro* plant regeneration protocol for *Swertia chirata* Buch.-Ham. ex Wall (Gentianaceae), a critically endangered Himalayan medicinal herb.

This was developed using shoot tip explants derived from in vitro grown seedlings. Media with 2% sucrose and various types of hormones markedly influenced invitro propagation of S. chirata. An in vitro shootlet production system using Murashige and Skoog (MS) medium with various hormones such as KN and IBA was established. KN at 2 mg/l ,GA3 at 2mg/l and IBA at 2mg/l induced highest number of multiple shoots. The highest frequency of rooting was obtained in half-strength MS medium supplemented with 2,4D and IAA in ratio of 3:3 mg/l after testing with different auxins at various concentrations within 4 weeks of transfer to the rooting medium. Pooja joshi described the micro propagation of *Swertia chirayita*, an endangered medicinal herb of the temperate Himalayas, through axillary multiplication from 4-week-old seedling-derived nodal explants. A 4.5-fold multiplication every 4 week on Murashige and Skoog(MS) medium supplemented with 4 micro M benzyl amino purine (BA) and 1.5 micro M 6-(gamma , gamma - dimethylallylamino) purine (2iP). 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). A success rate of 94% was obtained by *in vitro* hardening in the growth-room and by ex vitro hardening in greenhouse conditions. Influence of phytohormones and medium on the shoot regeneration from leaf of *Swertia chirata* Buch.-Ham. Ex wall. *in vitro* was reported by Wang-Li et al. in 2009.

2.3 Callus Induction:

Bisht-S et. al.in 2008 gave a protocol for callus induction and in-vitro plant regeneration through organogenesis for *Swertia angustifolia* Buch-Ham. (Gentianaceae). Callus was induced on Murashige and Skoog basal medium supplemented with cytokinin (Kn or BA) and auxin (2, 4-D/IBA/NAA) from leaf, petiole and stem explants of this medicinal plant.

It was also reported that higher concentrations of Kn and 2, 4-D (2.5-3.0 mg l-1) exhibited best callus and higher concentration of another cytokinin BA (1.5-2.0 mg l-1) produced best calli when combined with 2.0 mg l-1 of IBA in both leaf and petiole explants.

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2.4 Hardening:

Hardening was successfully attained under controlled conditions inside the plant tissue culture room but with low survival rate by Balaraju-K et al. in the year 2000.

2.5 Metabolite Analysis:

According to Hostettmann-K et al.plants provide a rich source of novel, biologically active compounds. Biological and chemical screenings are complementary approaches for the rapid detection and isolation of interesting new plant constituents. Biological screening followed by activity-guided fractionation has been used successfully at the Institute de Pharmacognosie et Phytochimie, University of Lausanne, Switzerland, for the discovery of antifungal, larvicidal and molluscicidal compounds. 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 chemical screening of crude plant extracts. In particular LC/MS and LC/MS/MS used with different ionisation techniques such as thermospray (TSP), continuous flow-FAB (CF-FAB) and electrospray (ES) have proved to be very efficient for the early recognition of molluscicidal saponins in Swartzia madagascariensis and Phytolacca dodecandra. The combination of LC/UV/NMR/MS was of great value for the investigation of polyphenols and bitter principles in Gentianaceae species. Among other examples, the LC/NMR analysis of the antifungal crude extract of the African plant *Swertia calycina* is presented.Two known xanthones, decussatin and gentiacaulein were isolated from the aerial parts of this medicinal species using CC and preparative TLC by Fukamiya et al. Apical meristems, leaf segments, stem internodes and roots were excised and cultured on supplemented Linsmaier &Skoog medium.

Calluses were induced and regenerated plantlets were eventually obtained. Analysis of cultured tissues yielded 3 known compounds (decussatin, methyl swertianin and 1-hydroxy-3, 5, 7, 8-tetramethylxanthone) and a new compound identified as 1, 8-dihydroxy-2, 4, 6-trimethoxy xanthone. The last named xanthone was methylated to give one known and 2 new derivatives.

Amarogentin, amaroswerin and four xanthones from hairy root cultures of Swertia japonica were identified by Ishimaru-K et.al.in 1990. Formation of hairy roots was induced by inoculation of cultures with Agrobacterium rhizogenes; cultures of these roots were analyzed for xanthone and bitter principles, Bellidifolin, methylbellidifolin, swertianolin and a new xanthone derivative, 8-O-primeverosylbellidifolin, were isolated. Two bitter principles, amarogentin and amaroswerin, were also identified. Determination of effective constituents in 11 Swertia and related plants by HPLC was done by Gao et al. in 1994.They described the quantitative 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. micro -Bondapak C18 was used as the stationary phase, and water: ethanol: isopropanol: tetrahydrofuran (65:30:5:1) as the mobile phase. Tian-Wei, et .al. in 2005 established a sensitive and specific HPLC method for controlling the quality of total glycosides from *Swertia franchetiana.*
HPLC method was applied for qualitative and quantitative assessment of the pharmaceutical extracts from S. franchetiana. Sample preparation, HPLC column, mobile phase, elution mode (isocratic or gradient) and gradient programme were optimized to obtain HPLC profile. The HPLC system consisted of a SPD-10Avp pump, SPD-M10AVP photodiode-array detector (PAD) and SIL-IOADVP auto injector . Data were processed with the CLASS-VP6.1 Workstation . HPLC analysis was performed on a Kromasil C< sub>18</ sub> column (250 min x 4.6 min ID, 5 micro m) with methanol and water as mobile phase. The column temperature was set at 40 degrees C and the flow-rate was 1 ml min-1. The reference solution of chemical standards and sample were injected into HPLC system, separately.

Results : The HPLC chromatographic fingerprinting of the total glycosides showed 16 characteristic peaks which were partitioned 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. Comparison of the HPLC profiles of the total glycosides, the extracts prepared using another process and the plant indicated that they were closely related to each other. Conclusion: The HPLC profiles and quantitative assessment of the total glycosides from S.franchetiana with high specificity can be used to control their quality and assure lot-to-lot consistency.

Sharma et al. isolated and identified triterpenoids from chirayata plants (*Swertia chirata* Buch.-Ham.) commonly used in Indian medicine. The air dried powdered whole plant was extracted with petroleum ether at 60-80 deg under reflux. The concentrated extract was chromatographed over silica gel and eluted with petrolium ether, benzene and chloroform. Petrolium ether and benzene elutes on evaporation did not give any solid. After elution of the column with CHCl3, oleanolic acid was crystallized from ethanol as white needles and evaporation and crystallization from ethanol of the CHCl3 filtrate yielded beta-amyrin. Identity of these compounds was confirmed by TLC and m.m.p. with authentic samples. Li-YuLin, et .al. reported a sensitive and specific reversed-phase high performance liquid chromatography (RP-HPLC) method with diode array detection (DAD) for the quantitative determination of the nine active components, namely, 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*. Based on the baseline chromatographic separation of most components from the methanolic extract of Swertia franchetiana on a reversed-phase Eclipse XDB-C8 column with water-acetonitrile-formic acid as mobile phase, the nine components were identified by comparison with standard samples and qualified by using the external standard method with DAD at 254 nm.

The correlation coefficients of all the calibration curves were found to be higher than 0.9980. The relative standard deviations (RSDs) of the peak areas and retention times for the nine standards were less than 2.07% and 2.86%, respectively.Micro-LC determination of swertiamarin in Swertia species and bacoside-A in Bacopa monnieri.

Bhand et al. in 2006 developed a micro analytical HPLC technique for determination of swertiamarin in Swertia species and bacoside-A in Bacopa monnieri. Small quantities (>=2 mg) of powdered plant material are required for analysis. The limits of detection (LOD) of swertiamarin and bacoside-A are 500 pg and 4,000 ng, respectively, in 2 mg plant material. Dried plant material from S. cordata and S. chirata contained 5.59 and 0.44% swertiamarin, respectively, and B. monnieri contained 0.12% bacoside-A. The simplicity, accuracy, and reproducibility of the method enable its use for estimation of marker compounds. Isolation of a new xanthone and 2-hydroxy dimethyl terephthalate from *Swertia petiolata* in 1988 was reported by Kulanthaivel et al.. S. petiolata, a subspecies of the Ayurvedic herb S. chirata, is found in the alpine pastures of Kumaun Himalaya, Uttar Pradesh; it is known for its laxative and antimalarial properties. Structures for the 2 title compounds are assigned.

Structure of chiratanin, a novel dimeric xanthone was described by Mandal et al. in 1987.the compound was isolated from the aerial parts of *Swertia chirata* which is used locally in India as an antimalarial and liver tonic. Chemical constituents of the Gentianaceae. V: Tetraoxygenated xanthones of *Swertia chirata* Buch.-Ham.were described by Ghosal et al. in 1973. S. chirata yielded 9 tetraoxygenated xanthones, also heterosides, triterpenes and monoterpene alkaloids. The medicinal properties of S. chirata are attributed to the xanthones.

Liquid chromatography/ultra violet/mass spectrometric and liquid chromatography/nuclear magnetic resonance spectroscopic analysis of crude extracts of Gentianaceae species was done by Wolfender et al. Quantification of Swertiamarin from whole plant powder of *Swertia densiflora* (Griscb.)Kashyap.collected in different seasons was done by Sunita et al. (2009.)

In this paper, the comparative quantification of Swertiamarin from *Swertia densiflora* (Griscb.) Kashyap in different periods, in order to identify the best period for harvest is investigated. The plant powder was first extracted with methanol and then in chloroform. The final residue was reconstituted in methanol and used for quantitation. 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. Quantitation was achieved by densitometric scanning at 244 nm (lambda max) in reflectance-absorbance mode. The response to swertiamarin was linear function of concentration over the range 30 to 100 micro L mL-1 in the extract of *Swertia densiflora* (Griscb.)Kashyap. 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.
Liquid chromatography/ultra violet/mass spectrometric and liquid chromatography/nuclear magnetic resonance spectroscopic analysis of crude extracts of Gentianaceae species was done by Wolfender et al.*in* 1997. According to the paper High performance liquid chromatography (HPLC) coupled with ultraviolet photodiode array (LC/UV-DAD) and mass spectrometry (LC/MS) detection has been used routinely for the chemical screening of crude extracts of Gentianaceae species. The recent progress realised in the field of nuclear magnetic resonance spectroscopy (NMR) has given a new impulse to the coupling of this detection method with liquid chromatography (LC/NMR) and several LC/NMR probes are now commercially available. In order to evaluate the potential of this coupled technique as a complement to LC/UV/MS for the on-line identification of plant metabolites, 2 characteristic Gentianaceae species were analysed, namely Swertia calycina (from Rwanda) and Gentiana ottonis (from Chile). On-line UV, MS, MS/MS 1H-NMR and 1H-1H-NMR spectra of the major constituents of these 2 plants (flavonoids, xanthones, naphthoquinones, secoiridoids and glycosides) allowed their identification directly within the crude extracts. The potential and the limitations of LC/NMR used together with LC/UV/MS for the on-line analysis of plant metabolites are also discussed in the paper.

CHAPTER 3: MATERIALS AND METHODS

3.1 Collection of plant material

Swertia chirayita plants were procured from Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni (Solan).Plants were potted in a pot and kept in the greenhouse of the department for their further growth and development.

3.2 Cleaning of Glassware

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. The glassware with contaminated cultures was first autoclaved for 15 minutes at 121 lbs. and the molten media was disposed off. The glassware was then cleaned as mentioned above before reusage.

3.3 Preparation of media

Modifications of MS media supplemented with different concentrations and combinations of IAA, IBA and KN were prepared (Murashige and Skoog, 1962). The pH of the media was adjusted to be between 5.5 - 5.7 using 0.1N HCl and 0.1N NaOH, sucrose 3% (w/v) was added and finally agar-agar 0.8% (w/v) was added as a gelling agent. The media was autoclaved at 121^{o}C and 15 lb/in$_2$ pressure for 15-20 minutes. The cultures were incubated at 25±1^{o}C in plant tissue culture chamber with 70% relative humidity under 16 hr photoperiod provided by cool fluorescent light. Data was collected on days to multiple shoot formation, per cent shoot apices with multiple shoots, and number of shoots per explants. The data were analyzed for test of significance. The cultures were sub cultured after every 4 weeks on shoot proliferation media for 2 months so as to obtain good growth and multiplication.

3.4 Surface sterilization of explants

Shoot apecies of field grown plants were washed with sterile water to remove dirt and debris and surface sterilized in 0.5% Bavistin and 0.1% Mercuric Chloride followed by 4-5 washings in sterile water. Surface sterilized seeds were kept in autoclavable petri plates having filter papers in culture room at 25oC by maintaining optimum moisture(60-70%). Data will be collected for % survival of sterilized explants.

3.5 Multiplication of shoot cultures

For the purpose of shoot multiplication, we tried 10 different combinations and concentrations of KN, IBA and GA3. One set of cultures was incubated at 16/8hr light/dark cycle at 25±1^0 C in plant tissue culture chamber. The other set of cultures was incubated at low temperature 15±20 C with the same light and dark cycle. Data were collected on days to multiple shoot formation, number of shoots per explants and per cent shoot apices with multiple shoots. The data were analyzed for test of significance. The cultures were sub cultured after every 4 weeks on shoot proliferation media for approximately 2 months, so as to obtain good growth, and highly proliferated shoots.

3.6 Induction of roots in shoots

Individual shoots were excised from the parent cultures and transferred onto MS media supplemented with different concentrations and combinations of IBA,IAA for root induction. The cultures were incubated under the same culture conditions as mentioned above. Data for days to root initiation, number of roots/shoot and per cent rooting were recorded and analyzed for test of significance.

3.7 Callus induction from different explants

Explants such as leaf discs and roots were taken from *in vitro* grown plantlets of *S chirayita* and cultured on MS medium supplemented with different concentrations and combinations of 2,4-D, IAA, NAA and IBA with sucrose 3% (w/v). The pH of the media was adjusted to 5.7 using 0.1N HCl and 0.1N NaOH and finally agar-agar 0.8% (w/v) was added as a gelling agent. The media were autoclaved at 121^{o}C and 15 lb/in$_2$ pressure for 15-20 minutes. The explants were excised aseptically and cultured on above mentioned media. The cultures were incubated at 16hr light /8hr dark cycle at 25±2^{o}C in plant tissue culture chamber. Data were recorded on days to initiation of callus formation, per cent explants forming calli, days to compete callus formation, callus morphology, and calli forming excessive roots. The data were statistically analyzed for test of significance. The cultures were sub-cultured after every 15- 20 days on callus induction media prior to regeneration.

3.7 Regenration from calius cultures

After the explants were completely transformed into callus mass,the calli or parts thereof were transferred onto regeneration media consisting of MS salts supplemented with different concentrations and combinations of IBA and KN for differentiating into shoots. Calli of cream color originating from different explants were transferred onto regeneration media and incubated in the plant tissue culture chamber maintained at 16hr light/8hr dark photoperiod. The data were recorded on days to shoot primordial initiation, per cent calli forming shoot primordial, number of primordial/callus and number of shoots/callus. The regeneration frequency was determined by counting the number of calli forming shoot primordial and plantlets. The data were statistically analyzed for test of significance.

3.8 Hardening and acclimatization of plantlets

Once the initial formation of roots could be seen, the rooted shoots were gently removed from the culture vessels, washed under running tap water, treated with 0.5% Bavistin and transferred to pots containing different combination and concentrations of sand, soil, vermiculite, perlite and coco peat in the glasshouse conditions for acclimatization and hardening.

3.8.1 Vermiculite

Vermiculite is a micaceous mineral that is expanded in a furnace, forming a lightweight aggregate. Handled gently, vermiculite provides plenty of air space in a mix. Handled roughly, vermiculite compacts and loses its ability to hold air. Vermiculite holds water and fertilizer in the potting mix. It also contains calcium and magnesium and has a near-neutral pH.

3.8.2 Perlite

Perlite is an amorphous volcanic glass that has relatively high water content, typically formed by the hydration of obsidian. In horticulture perlite can be used as a soil amendment or alone as a medium for hydroponics or for starting cuttings. It is sterile and pH-neutral. When added to a soil mix, perlite can increase air space and improve water drainage. Its pieces create tiny air tunnels that allow water and air to flow freely to the roots. Perlite will hold from three to four times its weight in water, yet will not become soggy. It helps prevent water loss and soil compaction.

3.8.3 Coco peat

Coco peat, also known as coir pith, coir fiber pith, coir dust, or simply coir, is made from coconut husks, which are byproducts of other industries that use coconuts. Raw coconuts are washed, heat-treated, screened and graded before being processed into coco peat products of various granularity and denseness, which are then used for horticultural and agricultural applications and as industrial absorbent. It is used as a soil additive. It holds water well, re-wets well from dry, holds around 1000 times more air than soil and is mixed with sand, compost and fertilizer to make good quality potting soil. Once the plants are transferred, they are kept covered with jars and poly bags for 10 – 15 days for preservation purposes and after that are exposed by uncovering for a period of 10 – 15 mins every day for the first few days during early mornings or late evenings. The exposure time is gradually increased over time.

3.9 Estimation of important metabolites

The *in vitro* biosynthesis and accumulation status of medicinally important secondary Metabolites of *Swertia chirayita* was determined in shoot and callus cultures of *S. chirayita* by subjecting fresh samples from different experiments to chemical analysis. The shoots including leaf material and roots were harvested separately while the leafy material was green, actively growing,and non-senescent stage. The quantification of metabolites was carried by reverse phase High Performance Liquid Chromatography. Shoots and callus were ground separately in liquid nitrogen and suspended in 80% methanol. The sample mixture was vortexed and kept overnight at room temperature. 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 isocratic mode with a flow rate of 1.0 ml/min. The metabolites were detected at absorbance of 254 nm wavelength in a cycle time of 30 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.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Establishment of cultures for multiple shoot formation:

The surface sterilized shoot apices were cultured on MS media containing IBA (0 - 2mg/l), GA3 (0 – 2 mg/l) and KN (1 - 3 mg/l) in different combinations . Out of the various different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN (2mg/l) + IBA(2mg/l) + GA3 (2mg/l) + sucrose 3% (w/v) was found to be the best with 95 % of shoot apices proliferating into multiple shoots within 8-9 days of inoculation (**Table 4.1**).

The effect of various media combinations on shoot multiplication and shoot development (after 4 weeks) were tested. (**Fig 4.1**) The excised shoots developed in primary cultures were cultured on media to test the effect of a range of concentration of various plant growth regulators, kinetin, gibberellic acid, supplemented to modified MS basal medium to find the best or a satisfactory treatment for shoot multiplication. Subcultures were done after every 4 weeks on the same media formulation. Since Swertia grows at high altitudes where temperatures are low, we thought that incubating*in vitro* cultures at low temperature might help to provide better growth . The shoot apices were also cultured and incubated at low temperature (15±2oC) with the same photoperiod in order to see whether better shoot growth can be obtained. The shoot apices proliferated into multiple shoots within 5-6 days of culture with no significant differences with respect to number of shoots per explant as observed at 25±20 °C but shoot growth and leaf sixe was definitely better at 25°C (**Table4.1**).

Table 4.1: Different concentrations and combinations of growth hormones supplemented in MS medium tried for in vitro shoot formation in *S. chirayita.*

KN	GA3	IBA	Shoots/ Explant	Shoot Length (cm)	No. of roots per explant
0	0	0	NA	NA	NA
0	1	0	4	6.0	1
0	1	2	5	7.0	5
1	1	0	9	6.7	7
2	2	1	17	8.1	4
2	1	2	22	7.2	9
2	2	2	29	7.5	7
3	2	2	20	6.3	9
3	0	1	11	7.0	9

Figure 4.1: Fully grown Shoots

4.2 Establishment of cultures for multiple shoot formation on liquid media

The surface sterilized shoot apices were also cultured on liquid MS media. Same media combination which were tested for solid cultures were used, MS medium containing KN (2mg/l) + IBA(2mg/l) + GA3 (2mg/l) + sucrose 3% (w/v) + no agar , was found to be the best with 80% of shoot apices proliferating into multiple shoots within 4-5 days of inoculation. On an average 22 shoots were obtained from a single shoot.The effect of various media combinations on shoot multiplication and shoot development (after 4 weeks) were tested. **(Fig 4.2).**

The excised shoots developed in primary cultures were cultured on media to test the effect of a range of concentration of various plant growth regulators, kinetin, gibberellic acid, supplemented to modified MS basal medium to find the best or a satisfactory treatment for shoot multiplication. Subcultures were done after every 4 weeks on the same media formulation.

Table 4.2: Different concentrations and combinations of growth hormones supplemented in MS liquid medium tried for in vitro shoot formation in *S. chirayota.*

KN	GA3	IBA	Shoots/ Explant	Shoot Length (cm)	No. of roots per explant
0	0	0	NA	NA	NA
0	1	0	5	6.1	2
0	1	2	6	7.2	4
1	1	0	8	6.3	6
2	2	1	16	7.1	5
2	1	2	20	7.3	8
2	2	2	30	8.5	8
3	2	2	19	5.3	10

Figure 4.2: Fully grown plants in Liquid media

4.3 Establishment of Callus cultures

Callus cultures were initiated from different explants such as leaf discs and nodal segments of *S. Chirayita* on MS salts supplemented with different concentrations of 2,4-D (2-3 mg/l) , IAA (1- 3mg/l).Callus formation initiated in around 82% of explants within 8 weeks at the cut surfaces in all test media combinations. Overall, MS medium supplemented with 2, 4-D (3mg/l) + IBA (3 mg/l) + sucrose 3% (w/v) +agar-agar (0.8%) was found to be the best for callus induction. (**Table 4.3**) The calli derived from nodal and leaf segments turned browner with the passage of time and most of the calli developed into shoots even on the callus induction media after sub culturing. Callus cultures derived from different explants were sub-cultured for 6-8 weeks so as to proliferate the calli on suitable callus induction media. During sub culturing, some of the calli turned brown and eventually their proliferation stopped. Those calli were not carried further for regeneration. The effect of various media combinations on callus culture (after 4 weeks) were tested. **(Fig 4.3)**.

2,4-dichlorophenoxyacetic acid (2,4-D)	indole-3-acetic acid (IAA)	Callus growth
3	2	Less growth
2	1	Medium growth
3	3	Maximum growth

Newly cultured callus

Callus after one week

Callus after 3 weeks

Fully Grown Callus

Figure 4.3.1: Fully grown callus

Figure 4.3.2: Well developed plant from callus

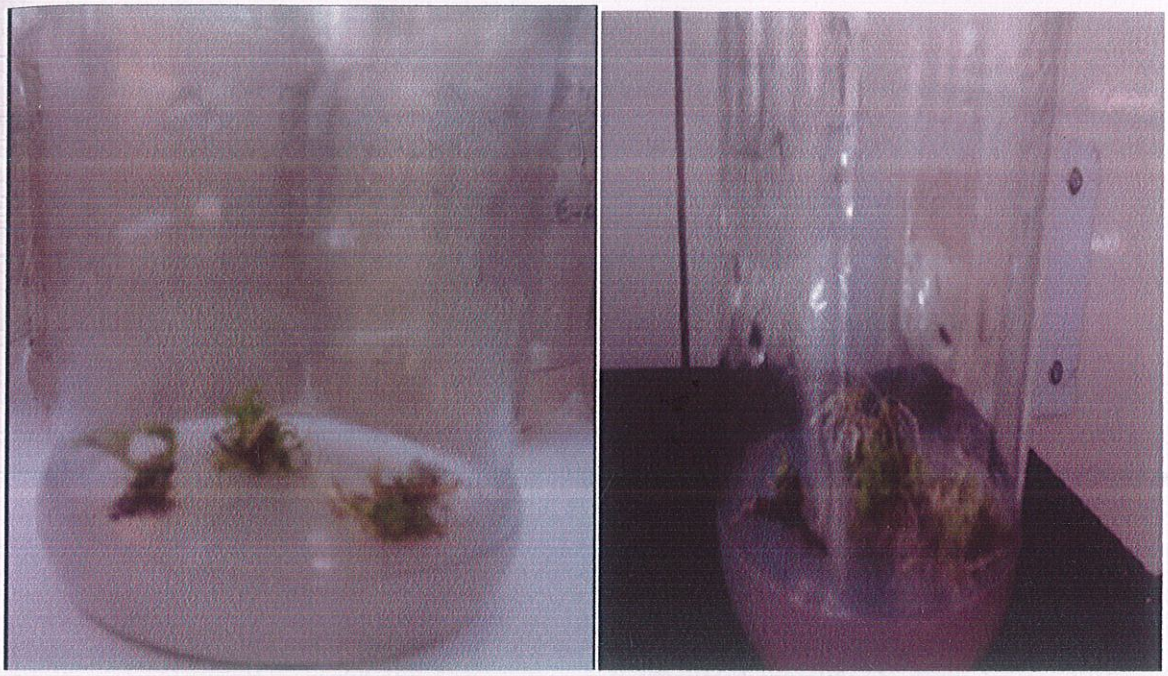


Figure 4.3.3: Callus having roots

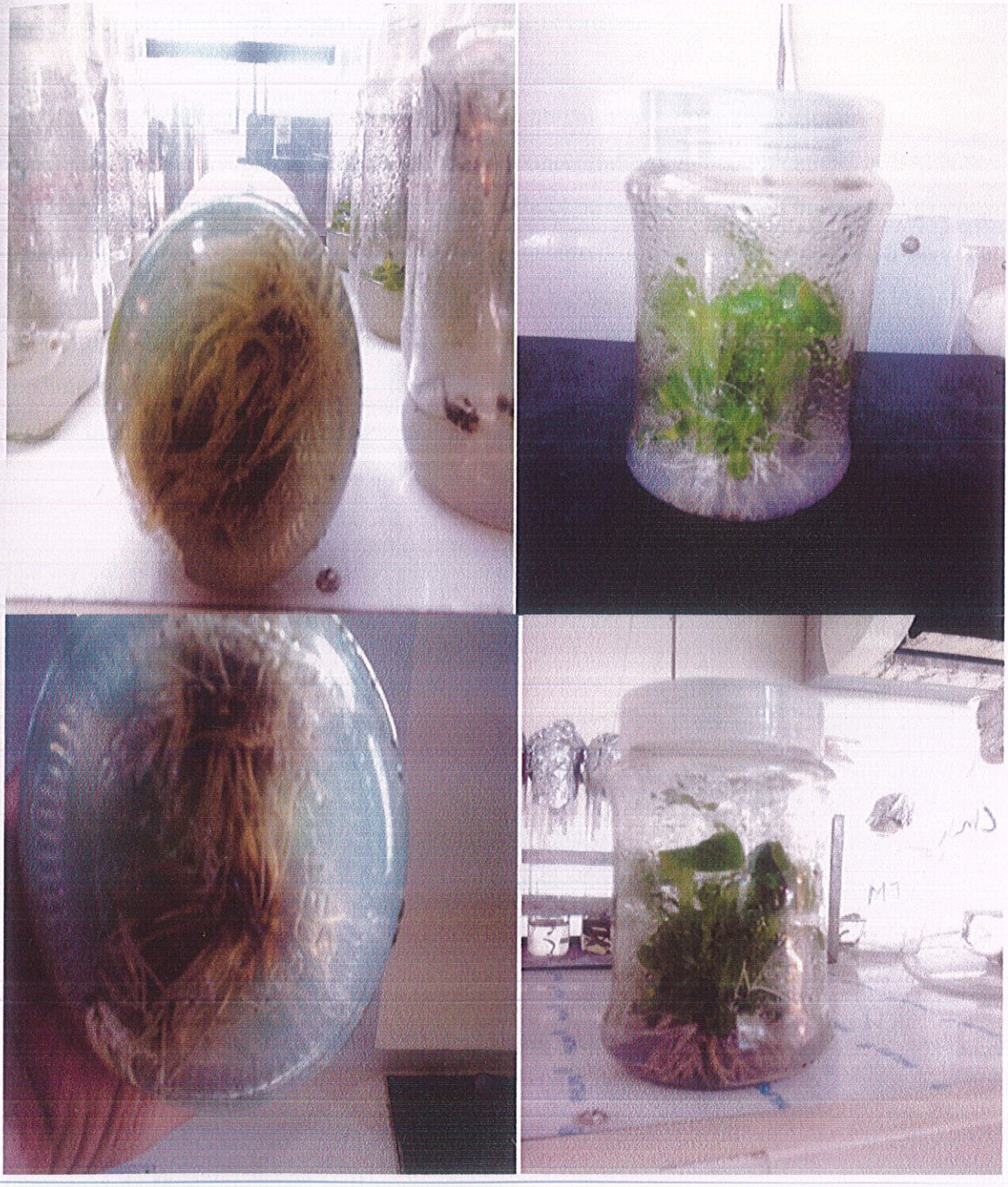


Figure 4.3.4: *In*-vitro root formation in *S. chirayota*

4.4 Maintenance and regeneration of callus cultures

Proliferating callus cultures or parts thereof were sub-cultured onto regeneration media containing MS salts supplemented with different concentrations and combinations of BA, KN and IBA. Media containing BA and KN induced shoot primordia formation in the form of green nodular structures. No shoot primordia formation occurred in callus cultures derived from nodal segments on any of the test regeneration media. MS medium supplemented with 2, 4-D (3mg/l) + IAA (3mg/l) + sucrose 3% (w/v) +agar-agar (0.8%) to be the best for regeneration. The same medium combination was found most suitable for obtaining maximum primordia/callus and the highest number of shoots/callus from calli of both the explants.

Figure 4.4: Regenerated callus

4.5 Induction of roots *in vitro* grown shoots

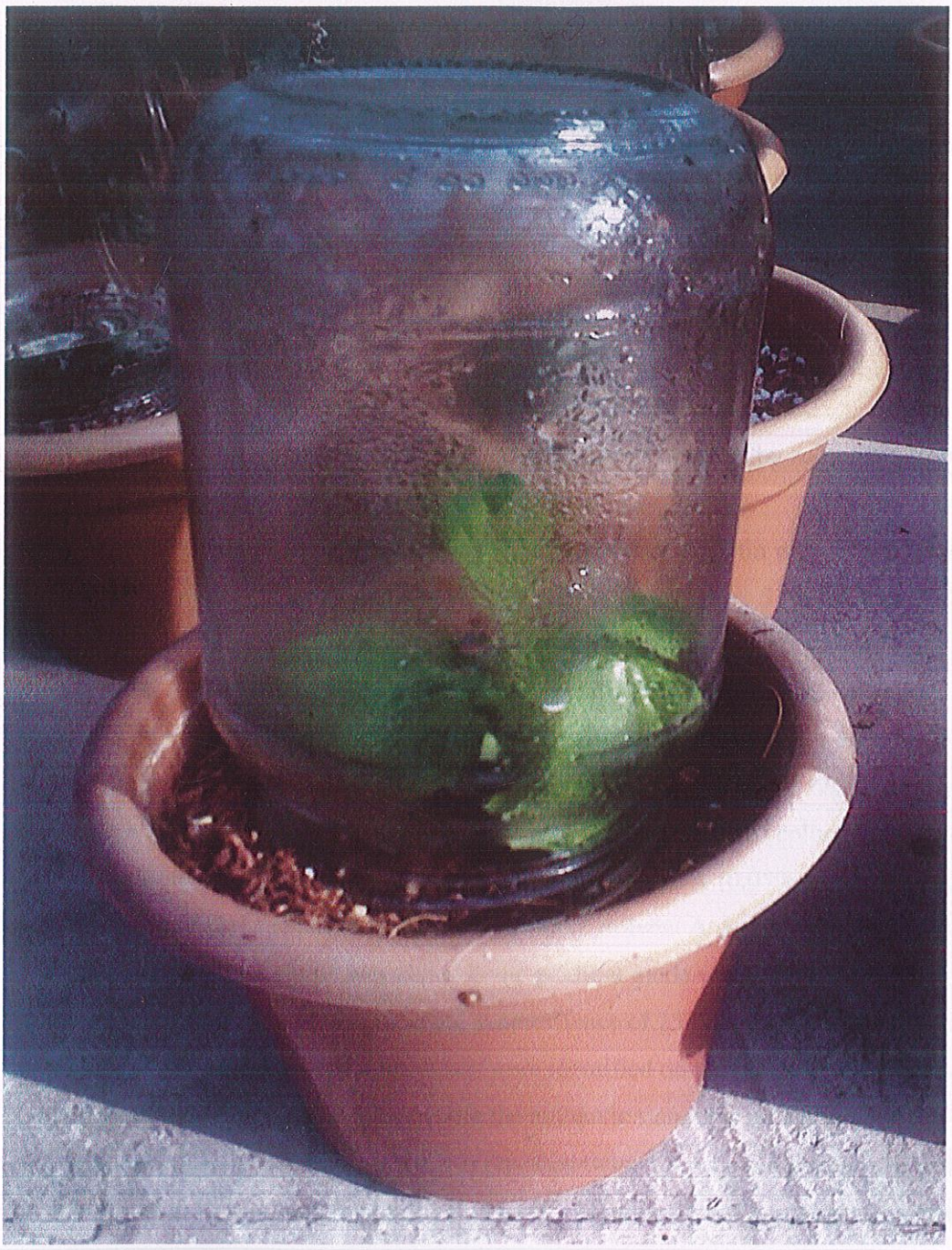
The shoots formed from *in vitro* grown cultures of axillary shoot tips were transferred on MS media supplemented with different concentrations and combinations of IBA (0 – 3 mg/l), IAA (0-1mg/i) and NAA (0 – 2 mg/l) for root induction. Data were recorded for number of roots/ shoot and per cent shoots forming roots. Root induction was best observed in MS + IBA (2mg/l) + KN (2mg/l) + IAA (2mg/l) and occurred in 9-10 days of culturing with 80% of shoots forming roots. The cultures were incubated again at both 25±2o C and 15±2o C with the same 16/8 hr light/dark cycle.

4.6Transplantation and Hardening of in vitro plantlets:

The survival rate of the *in vitro* generated plantlets was observed to be the best in the potting mixture containing sand: soil:vermiculite: perlite: coco peat in the ratio 1:1:1:1:1 . This combination of potting mixtures was then used to transfer the plantlets of appropriate statistics. Initially, the plantlets were covered with poly bags or jars, for 10-15 days, to provide sufficient humidity and avoid desiccation till the plantlets showed new growth. During the hardening process, poly bags were taken off every day for 10 – 15 mins so as to acclimatize the plantlets to external environment, gradually increasing the length of exposure over time. Hardening was successfully done with a survival rate of 62 %

Composition of Potting Mix (Ratio)	**Percentage Survival of Plants (%)**
Sand : Soil : Vermiculite (1 : 1 : 1)	58
Sand : Soil : Perlite (1 : 1 : 1)	49
Sand : Soil : Cocopeat (1 : 1 : 1)	50
Sand : Soil : Vermiculite : Perlite : Coćopeat (1 : 1 : 1 : 1)	67

Figure 4.6.1: Plants hardened till date



4.6.2: Hardened plant

Metabolic analysis

- The plants of *Swertia chirayita* were regularly monitored for a month to analyze the growth of the plant. Parameters such as number of leaves, plant height, leaf blade area, root length, were noted along with the number of daughter plants arising from the mother plants. Shoots which were tested for elicitation were regularly collected at 7 days intervals and stored at -80C.

- **Estimation of Swertiamarin , Mangiferin and Amarogentin**
- 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 10mins. 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 254 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 repeated twice for each sample and the data were interpreted in terms of amount of Swertiamarin , Mangiferin and Amarogentin present.

Table 4.7: Metabolite content for field grown 1.5-2 yr old plants was analyzed by HPLC.

Sample name	µg/mg		
Plant from glass house	**Swertiamarin**	**Mangiferin**	**Amarogenitin**
Callus with small shoots and some roots	0.71	0.08	0.00
Callus initiation started in leaves	0.97	0.10	0.35
Fully grown callus	0.32	0.12	0.11
Callus regenerated shoots	0.40	0.13	0.72
Callus having only roots	0.31	0.28	0.00
Shoots having pigmentation	0.35	3.02	0.78

CHAPTER 5 – CONCLUSION

The current study reports the protocols for development of *in vitro* shoot and root cultures with the help of micropropagation. *Swertia chirayita* is an endangered plant with important medicinal properties, so it is important to develop various techniques for its *in vitro* micro propagation and its successful transfer to fields. The same objective has been achieved in the present study. Due to its endangered status, it is difficult to get *Swertia* plants for establishing *invitro* cultures, so we have developed the technique for generating shoot cultures through *in vitro* propagation.We have applied successful techniques for its micro propagation with maximum 30 shoots proliferating on the MS medium containing KN (2mg/l) + IBA (2mg/l) + GA3(2mg/l) + sucrose 3% (w/v) *in vitro* induction was achieved with good growth of plantlets. Callus cultures were established from leaf discs with 70 % success rate. And high callus regeneration was seen on medium containing 2, 4-D (3mg/l) + IAA (3 mg/l) + sucrose 3% (w/v) +agar-agar (0.9%). As we have regenerated shoots and micro propagated plantlets and successfully transferred them to green house. So this complete process is used as an alternative to conserve this medicinally important herb by growing it in large number in lab and then transferring to field. These could then act as a source of pure and collectible metabolites and provide regular support to pharmaceutical. This novel technique of *in vitro* conservation and micro propagation can help in conservation and production of a large number of disease-free, true-to-type plants. *S. Chirayita*enjoys a good domestic and international market. *S chirayita* extracts are used in a number of medicinal formulations and it is required in large amounts by the pharmaceuticals companies. So if these micro propagation techniques are 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 homogeneity of herbs.

APPENDIX

STOCKS	CHEMICALS	ORIGINAL STRENGTH (mg/l)	STOCK (g/l)	FINAL VOLUME
A - 10X	KNO_3	1900	19	}
	$MgSO_4 .7H_2O$	370	3.7	100ml/l
	KH_2PO_4	170	1.70	
B - 20X	NH_4NO_3	1650	33	50ml/l
C - 100X	$CaCl_2 .2H_2O$	440	44	10 ml/l
D - 100X	Na_2EDTA	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	}
	$MnSO_4 .4H_2O$	22.3	2.23	
G - 100X	m-INOSITOL	100	10	} 10ml/l
	GLYCINE	2	0.2	
H -100X	PYRIDOXINE-HCl	0.5	0.05	}
	NICOTINE ACID	0.5	0.05	} 10 ml/l
	THIAMINE	0.i	0.01	

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