PRODUCTION OF BIOACTIVE COMPOUNDS THROUGH *IN VITRO* TECHNOLOGIES OF CRITICALLY ENDANGERED HERB *SWERTIA CHIRAYITA* AND EXPLORATION OF ITS BIOLOGICAL ACTIVITIES

Thesis submitted in fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

By

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"Production of bioactive compounds through *in vitro* technologies of critically endangered herb *Swertia chirayita* and exploration of its biological activities"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Hemant Sood**. I have not submitted this work elsewhere for any other degree or diploma.

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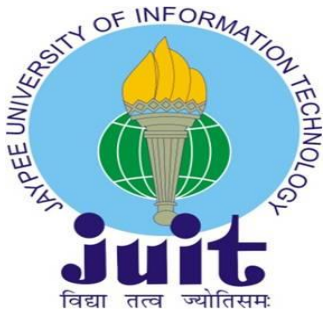
SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Production of bioactive compounds through *in vitro* technologies of critically endangered herb *Swertia chirayita* and exploration of its biological activities**", submitted by **Rolika Gupta** at **Jaypee University of Information Technology, Waknaghat**, **India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.



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Rolika Gupta

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2,4-D	2,4-Dichlorophenoxyacetic acid
AA	Ascorbic acid
AAC	Ascorbic acid content
ABTS	2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ADT	Prephenate dehydratase
AMPK	Adenosine monophosphate – activated kinase
ANOVA	Analysis of variance
ANS	Anthocyanidin reductase
AP2	APETLA 2
APRX	Alkaline metalloprotease
ATCC	American Type Culture Collection
BA/BAP	Benzyladenine or Benzylaminopurine
Bax	Bcl-2-associated X protein
Bcl-2	B-cell leukemia/lymphoma-2
bHLH	basic-helix-loop-helix
BHT	Butylated hydroxytoluene
C4H	Cinnamic acid 4-hydroxylase
CAB	Chlorophyll a/b-binding protein
CCA	Compact callus aggregate
CCC	Compact callus cluster
CDK-B	Cyclin-dependant kinase-B
CIF	Callus induction frequency
CLSI	Clinical & Laboratory Standards Institute
CM	Chorismate mutase
CNS	Central nervous system
CRD	Completely randomized design
CS	Chorismate synthase
DAD	Diode array detector
DAHPS	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase
DHAR	Dehydroascorbate reductase
DHQS	3-dehydroquinate synthase
DMAPP	Dimethylallyl diphosphate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DRSA	DDPH free radical scavenging assay
DT	Doubling time
DW	Dry weight

eIF-2a	Eukaryotic initiation factor 2A
EOMT	Eugenol O-methyltransferase
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ERF	Ethylene-responsive factor
F3H	Flavanone 3-hydroxylase
FGP	Field grown plants
FLS-2	Flavonoid 3'-hydroxylase
FRAP	Ferric Reducing Antioxidant Power
FTC	Ferric thiocyanate
FT-IR	Fourier transform infrared
FW	Fresh weight
GAE	Gallic acid equivalent
GDPS	Geranyl diphosphate synthase
GES	Geraniol synthase
GI	Growth index
GIOH	Geraniol 10-hydroxylase/8-oxidase
GME	GDP-D-mannose-3',5'-epimerase
GPP	Geranyl diphosphate
GSH	Glutathione
HAT	Hydrogen atom transfer
HIF-1	Hypoxia-Inducible Factor-1
HFRI	Himachal forest research institute
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HPA	Hypothalamic–pituitary–adrenal
Hsp70	Heat shock proteins70
IBA	Indole-3-butyric acid
ICH	International Conference on Harmonisation
IS	Iridoid synthase
ISG	Interferon-stimulated gene
IPP	Isopentenyl diphosphate
IUCN	International union for conservation of nature
KN	Kinetin
IVP	*In vitro* grown plants
LC-MS	Liquid chromatography–mass spectrometry
JA	Jasmonic acid
LEDs	Light emitting diodes
MAPK	Mitogen-activated protein kinase
MBC	Minimum bactericidal concentration
MDA	Malondialdehyde
MDA5	Melanoma differentiation-associated protein 5
MDAR	Monodehydroascorbate reductase
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant Staphylococcus aureus
MS	Murashige and Skoog

MYC2	Myelocytomatosis 2
NAA	Naphthaleneacetic acid
NIST	National Institute of Standards and Technology
NBPGR	National Bureau of Plant Genetic Resources
NMPB	National Medicinal Plant Board
NOX	NADPH oxidases
PAL	Phenylalanine ammonia lyase
PAT	Aspartate–prephenate aminotransferase
PGRs	Plant growth regulators
PDA	Photodiode-Array
PKC	Protein kinase-C
PKR	Protein kinase R
PPs	Protein phosphatises
PTFE	Polytetrafluoroethylene
QE	Quercetin equivalent
RGB	Red:Green:Blue
RIG1	Retinoic Acid Inducible Gene 1
ROS	Reactive oxygen species
RP-HPLC	Reverse Phase High Performance LiquidChromatography
RSD	Relative standard deviation
SA	Salicylic acid
SAK	Shikimate kinase
RT	Room temperature
SOD	Superoxide dismutase
SEM	Scanning electron microscopy
SPSS	Statistical package for the social sciences
STAT-3	Signal Transducer and Activator of Transcription 3
STRS	Streptomycin sulphate
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TEAC	Trolox Equivalent Antioxidant Capacity
TF	Transcription factor
TFC	Total flavonoid content
TLC	Thin-layer chromatography
TPC	Total phenolic content
TDZ	Thidiazuron

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ABSTRACT

Swertia chirayita, an indigenous ethno medicinal herb of the temperate Himalayas, which has been in use since ages in the Indian subcontinent. *S. chirayita* is a rich reservoir of phytochemicals mainly Amarogentin and Mangiferin, which are used in various herbal formulations as well as to cure cancerous and diabetic disorders. These important biomarker compounds are of great interest to the pharmaceutical industries. The population of *S. chirayita* has declined drastically in the natural habitat as a result the species has been, categorized as critically endangered herb by the IUCN. Accordingly, the current study focused on development of tissue culture platform to counter extinction concerns and meet the rising demand for medicinally and industrially significant biomarker compounds of *S. chirayita*.

Using the RP-HPLC method, the contents of bioactive compounds (Amarogentin and Mangiferin) were determined in *S. chirayita* at two different temperatures, 15 ± 1˚C and 25 ± 1˚C, during various phases of development. Each developmental stage of tissue cultured *S. chirayita* was explored, beginning with the leaf discs and progressing to callus induction and complete shoot regeneration. Amarogentin and Mangiferin had maximum concentrations of 5.79 and 15.56 µg/mg at 15 ± 1˚C, respectively. On completion of 80 days, the regenerated shoots grown in the full strength MS media provided with 3 mg/l IBA and 1 mg/l KN showed the greatest concentration of bioactive components. Tissue cultured grown shoots showed 8.51 folds higher Amarogentin and 4.09 folds higher Mangiferin, as compared to the field grown plants, respectively. As a result, for the first time, distinct spotting of developmental stages with biosynthesis of biomarker compounds at each stage was identified.

Given that there is no information available yet, on the use of distinct light treatments on the synthesis of medicinal metabolites (Amarogentin and Mangiferin) in the *S. chirayita* tissue culture system, this study examined the impact of various lighting scenarios (Red, Blue, Green, White fluorescent light and RGB) on the synthesis of bioactive compounds and explored their other releated activities in the tissue raised *S. chirayita* cultures. The present findings illustrated 1.01 folds higher biomass accumulation in the Red light, in comparison to the control (WFL), also higher growth parameters were observed in the Red light. The shoot cultures exposed to Blue light showed higher accumulation of Amarogentin (1.06 folds

higher than control), phenolics (1.86 folds higher than control), flavonoids (1.45 folds higher than control) and higher antioxidant activity (1.29 folds higher than control) after 30 days of culture. As a result, these findings present fresh avenues for the synthesis of important biomarker compounds in *S. chirayita* shoot cultures.

The tissue grown culture of *S. chirayita* has been explored for alternative methods like artificial seed production combined with cryo techniques for longer term storage of plant material as the population of the herb is on the verge of extinction. This study revealed the production of artificial seeds and their cryopreservation for long-term storage. For the very first time, cryopreserved artificial seeds of *S. chirayita* were revived and 93.3% of them were regenerated into shoots on the MS medium provided with 1mg/l IBA, 2mg/l KN, 3mg/l GA_3. Such research opens up new possibilities for cryopreservation of many endangered plants.

The competence of *in vitro* grown plants was analyzed with the field-grown plants of *S. chirayita* on the basis of their phytochemical analysis and their potential for pharmacology. As there is a hike in the industrial demand for biomarker compounds, cell culture can provide its worth as a substitute source to wild plants. This work illustrated the higher antioxidant (DPPH IC_{50}= 0.06 µg/ml, ABTS IC_{50}= 0.065 µg/ml) and antidiabetic (IC_{50}= 0.048 µg/ml) in the tissue culture raised *S. chirayita* in comparison to the field grown plants. However pronounced results of antibacterial and anti-cancerous activities were observed using the extracts of *in vitro* raised plants of *S. chirayita*. The *in vitro* raised plants contained more bioactive components than the field grown plants, according to LC-MS and FTIR analyses. This study has provided a conclusive comparison of the tissue raised plants with the field grown plants on various parameters. These findings would help in conserving this precious herb and provide grounds for meeting the demands of biomarker compounds in the medicinal industries through the cell and tissue culture systems of *S. chirayita*.

CHAPTER 1

INTRODUCTION

Swertia chitayita, commonly called as (chiretta), is prime herb of the family: Gentianaceae. It is broadly distributed in high temperate regions of India mainly in Kashmir, Nepal and Bhutan [1]. Mainly in India, this herb dwells along the temperate regions in the high altitude (1200-2100 m) and mostly prefers moist and shady hills (Figure 1) [2]. *S. chiaryita* is abundantly used in Ayurveda, Unani, Siddha, Tibetian medicine systems for treating various ailments like febrifuge, stomach disorders, antiperiodic, and blood purifier [3]. Pharmacological examination of this herb revealed that *S. chirayita* extracts have been utilized for Anti-viral, Anti-malarial, Anti-leshmanial, Anti-hepatitis, Anti-microbial, Anti-carcinogenic, Anti-oxidant and hepato-protective properties [4]. The pharmaceutically distinguished characteristics of *S. chirayita* have been accredited to terpenoid compounds, including Amarogentin, Mangiferin, Swertiamarin, Swerchirin, Sweroside, Amaroswerin, Gentiopicrin, Syringaresinol, flavonoids and phenolic acids [5, 6]. Amarogentin (secoiridoid glucoside) and Mangiferin (xanthone C- glucoside) are major biomarker compounds of *S. chirayita*, which are utilised as significant constituents in various herbal formulations, for curing diabetes, menstrual disorders and influenza [7, 8, 9]. These biomarker compounds possess high pharmaceutical importance, including anti-HBV infection, cardio stimulant, and anti-malarial properties [10]. These biomarker compounds have enormous scientific as well as industrial importance [18].

Figure 1.1 *S. chirayita* in three conditions: a) Wild, b) Green house, c) Tissue cultured.

S. chirayita is rich source of amino acids, dietary minerals, glycosides, chiratanin and palmitic acid; all these together make it a potent herb [5, 6]. *S. chirayita* is used in several pharmacological formulations [11]. The medicinal use of *Swertia* species is widely registered in the Indian Pharmacology Codex, American and British pharmacopeias [7]. From the years, Unani, Chinese, Tibetian and other conventional medicinal frameworks have used *Swertia* species as one of their chief therapeutic herbs due to its adaptogenic properties [12, 13].

Prior, *S. chirayita* roots and rhizomes were utilised for therapeutic purposes by common people, as a result, commercial interest in *S. chirayita* has expanded significantly [14]. The natural synthesis of these biomarkers isn't possible because of complex structures and high commercial stakes. As a result, these constraints necessitate the development of numerous biotechnological perspectives in order to ensure a consistent supply of *S. chirayita* biomarker compounds [15, 16]. *In vitro* culture technologies give a sustainable and eco framework for mass production of these highly therapeutic biomarker compounds [17]. Such biotechnological interventions have been instrumental in the production of various economically significant drugs including vinblastine, paclitaxol and camptothecin, and so forth [18]. Synthesis and production of bioactive components from tissue technologies has several advantages over conventional and chemical based methods, such as, consistent production and independent climatic and geological factors [20]. *In vitro* cultures provide vital source of numerous biomarker compounds with noticeable biological activity ranging from antioxidant, antimicrobial, anti-inflammatory and anti-cancerous activities [21, 22]. *In vitro* culture provides a functional system for production of numerous therapeutic biomarker compounds. Biosynthesis of these biomarker compounds using cell cultures provides magnificent way to explore biosynthetic pathways, under highly controlled environment; moreover cell culture conditions would help to enhance the accumulation of biomarker compounds too [23, 24]. *In vitro* system offers remarkable substitute medium for the synthesis of therapeutic biomarker compounds in hairy root cultures, cell suspension, shoot culture and callus among numerous plant varieties [25]. To develop its significant secondary products through tissue cultures, it is essential to have a thorough understanding of production and how it accumulates during various developmental phases. This will allow for the identification of the developmental stage that is the most appropriate for the tissue culturing. Synthesis of secondary products mainly occurs in the roots and shoots of *S. chirayita*. Due to the herb's seasonal dependence and high altitude growth, it is difficult to understand the proper biology for synthesis bioactive metabolites in the outer environment.

However, in the controlled biological environment of cell culture, where distinct developmental stage can be regulated by altering the concentration of growth regulators; there is a tremendous synthesis of pharmacologically significant secondary metabolites [26, 27]. However till date now, no study has been done where different developmental stages were explored for the synthesis and buildup of biomarker substances.

Plants are able to synthesize a variety of biomarker compounds in response to different environmental factors [25]. Elicitation is frequently utilised to improve the cell culture system's ability to produce bioactive chemicals [28]. Elicitation causes several induced defence systems in plants to activate secondary metabolite production [29]. Numerous studies have demonstrated that the synthesis of bioactive metabolites is induced by the stimulation of a metabolic network by both biotic and abiotic elicitors [29]. In the *in vitro* cells, light is thought to be a potent abiotic inducer of secondary metabolism [30]. The various types of plant photoreceptors, such as cryptochromes, phytochromes, phototropins, and zeitlupes regulate a variety of physiological responses, such as photomorphogenic reactions, secondary metabolism, growth, and development through specific signalling networks. For *in vitro* systems, LEDs have proved to be a good lighting source [31]. Now days LEDs are frequently used as practical source of lighting in tissue culture experimentation, due to their benefits over traditional light sources, including their high luminous efficacy, high fluence rate, high wavelength specificity, less heat emissions, low energy consumption, compact design, and longer shelf life [32, 33]. LEDs hold tremendous promise for increasing biomass yield and the synthesis of medicinal constituents in tissue raised plants [34]. In *Prunella vulgaris* [35] and *Artemisia absinthium* [36] enhanced synthesis of bioactive metabolites was reported by the use of various spectral lights (blue, green, white, and red). However, there is no study available so far that checks the influence of light quality on biomarker compound production under the culture conditions customised for *S. chirayita*.

Numerous *S. chirayita* species have gone extinct as a result of the illegal uprooting of the plant due to a recent increase in the demand for its biomarker compounds [37]. Although, the native resources of *S. chirayita* have declined dramatically, this is primarily due to habitat destruction, overuse for medicinal purposes, and the herb's low seed viability rate [38, 39]. The HP State Biodiversity Govt. of India has classified *S. chirayita* as critically endangered. To solve these issues, a great solution that will aid in preventing the extinction of this significant herb is plant cell culture combined with synthetic seed technologies. Synthetic seed technology is the development of rapid micropropagation technologies which could

have number of advantages over old approaches where environmental constrains limits the year around production of quality rich herbal raw material so such strategies aid in the conservation of germplasm along with quality production of herbal raw materials without harming and misbalancing the natural population [16, 29]. Synthetic seed technology, along with cryopreservation of artificial seeds, provides vast scope for the protection of endangered herbs and availability at any place under any set of conditions. However, so far there has been no information reported on the production of artificial seeds along with their cryopreservation in medicinal herb *S. chirayita*.

The inclusion of major bioactive components, including amarogentin, swerchirin, swertiamarin, mangiferin, and other metabolites that precisely impact mankind welfare, further increases *S. chirayita* appeal in the local as well as global market [7]. Because of a growing understanding of the value of wild herbs in improving human health, their use and significance are growing on the global market. In the Himalayan hills, many wild plant species particularly those with medicinal value are in danger of going extinct. In order to improve the species, wild populations need to be strengthened and managed slowly [40]. As the field grown population needs the pre-evaluation potential of the plant to generate required secondary compounds in a natural habitat, *in vitro* approaches enable the maintenance and optimal supply of plant products throughout the year [41]. The synthesis of biomarker compounds in medicinal plants using *in vitro* techniques has recently been reported by various researchers [42]. Differentiated plant cultures, however, accumulate more biomarker substances than mother tissues [25]. These results demonstrate that *in vitro* raised plants can serve as viable alternatives to wild plants and can be scaled up to an industrial level. To confirm such reports concluded comparative studies of phytochemical analysis, antioxidant, anti-diabetic, anti-inflammatory, antibacterial and anticancer capacities of *in vitro* and field-grown plants of critically endangered herb *S. chirayita* was performed for the very first time.

Thus, the present study focused on the optimization of culture conditions, different developmental stages, elicitation and synthetic seed technology for the continuous supply of quality rich shoots enriched with biomarker compounds. RP-HPLC was employed to measure the main biomarker substances. The culture conditions, along with different light intensities, were tested in the present study to enhance the generation of biomarker compounds (Amarogentin and Mangiferin) in tissue cultures of *S. chirayita*. Biological capacities (antibacterial, antioxidant, anti-diabetic and anticancer) were studied in ≈3 month's old

shoots of *S. chirayita* in comparison to the field grown plantlets. LC-MS and FTIR analysis were performed to ascertain the chemical constituents and biomarker compound potential in tissue cultures of *S. chirayita*.

Taking into consideration the pharmaceutical and ethno-traditional value of the critically endangered Himalayan herb, *S. chirayita*, as well as the utilisation and scientific validation of quality rich *in vitro* raised plants for commercialization, the following objectives were established for the current study:

- **Objective 1:** Optimization of culture conditions for biosynthesis and accumulation of bioactive compounds in different developmental stages of *S. chirayita.*
- **Objective 2:** Enrichment of shoot biomass and bioactive compounds in *S. chirayita* by using LED lights.
- **Objective 3:** Production of artificial seeds in *S. chirayita* by using somatic embryos.
- **Objective 4:** Comparative analysis of phytochemical contents, antioxidant, antimicrobial, anti-diabetic and anticancer activity in methanolic extracts of *in vitro* and field grown *S. chirayita.*

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

One of the prime requisites for primary health care to be successful is the appropriate use as well as availability of suitable drugs. Traditional medicine, till date holds the position of being the most affordable and easy to access means of treatment pertaining to various ailments in primary health care system. Developing world is still very much dependent on medicinal plants as the only and potential source of medicine may it be preparations in their traditional form or its bioactive constituents in pure form. The genus *Swertia*, belongs to the Gentianaceae family, included nearly about 135 different species of annual as well as perennial herbs. These species form one of the most common constituent of a variety of herbal preparations. Out of 40 Indian *Swertia* species [43, 44], *S. chirayita* has been recognized to be the most important species owing to its febrifuge, anthelmintic, tonic and laxative properties. *S. chirayita*, also commonly known as 'Chiretta' is an indigenous ethnomedicinal herb which has been recognizes to be critically endangered. It grows on high altitudes of temperate and sub temperate regions of Himalayas within the range of 1200 – 2100m above the sea level and bears a fragmented distribution from Kashmir to Nepal to Bhutan [45] on the shady and moist hills [46].

S. chirayita is popular by many names region wise (Chirrato or Chiraita in Nepal, Sekhagi in Burma) [7] or on the basis of language (In Sanskrit: Chiratitka, Kairata Anaryatikta, Bhunimba, in Arabi: Qasabuzzarirah and in Urdu called as Farsi, Chiaravata) [47]. Also it is mentioned in literature with names like *Agathotes chirayita* Don, Buch-Ham, *Gentiana floribunda* Don, *Ophelia chirata* Grisebach and *Gentiana chirayita* Roxburgh [48]. *S. chirayita* has been reported to be annual, biennial or pluri – annual by different authors [44, 49, 50]. Widespread use as a traditional medicine has led to it's over exploitation. In addition, factors like destruction of habitat have accelerated the extinction status of the wild species. Over exploitation, habitat destruction, constricted geographical incidence [51] along with unattended genetic and hereditary issues pertaining to viability and germination of seed [7, 52] have led to need for immediate actions for alternate propagation and conservation approaches to be developed and executed with the aim to have sustainable supply of this precious herb species and to revert down its ultimate extinction [10, 53, 54, 55, 56]. The present review is an attempt to identify and fill in the existing gap in existing knowledge

pertaining to documented ethno medicinal uses, pharmacological properties and role of plant biotechnology, propagation, conservation, production of medicinal compounds and its safety evaluation was carried out extensively. A comprehensive documentation of medicinal uses, phytochemical properties and many more is attempted in the present work. Future considerations like potential conservation approaches for sustainable supply for local as well as international markets along with technologies for production of medicinal compounds and their pharmacological importance have been highlighted.

2.2 Brief History

Earliest documented use of *S. chirayita* has been quoted in Charak Samhita Sutra in $3^{rd} – 2^{nd}$ century BCE for being used for plummeting fever and purification of breast milk in lactating mothers [57]. Its registered medicinal use has been quoted in the Indian Pharmacology Codex, American and British pharmacopeias [7]. Another very popular common name of *S. chirayita* is Kirata tikta. Chirata, the name of the species has been derived from Sanskrit word Kirata which is considered to be the name of mountain hunter tribe who inhabited high mountains. Tikta refers to bitter, pungent medicinal plant and thus the ultimate meaning comes out to be “a bitter plant of the Kiratas”. The word Kirata has been mentioned as per on the names of Lord Shiva in the available literature [58].

Since ages, some of the oldest systems of medicine like Ayurveda, Unani, Siddha, Tibetian and may other regional and conventional folk medicine systems have been using *S. chirayita* as one of the main medicinal herb to cure large number of ailments. It is Nepal’s highest foreign exchange revenue generating species of medicinal plants [59]. Countries like India, Nepal, Bhutan, China and Japan have recognized species of *S. chirayita* for their anthelmintic, anti – inflammatory and anti – carcinogenic properties, thus justifying a high rank in conventional medicinal system for the management of malaria, liver ailments, gastric troubles, jaundice, hepatitis and other disorders [60].

2.3 Botanical Description

Figure 2.1 Botanical description of *S. chirayita*.

S. chirayita is a 0.6 – 1.5m tall annual [66] or biennial [67] herb having an erect stem which is 2 – 3 ft tall with cylindrical shape in the center shaft part and a quadrangular shape of the upper part having a characteristic line at each angle of the quadrant (Figure 2.1). The stem color ranges from orange brown to purplish characterized by large yellowish pith in a continuous manner [7]. *S. chirayita* has lanceolate type of leaves appearing in opposite pairs with no stalks. Also the leaves are acuminate with cordate base and sessile. They bear about 5 – 7 nerves and are 4cm in length [58]. Features of roots include simple, yellowish and oblique to geniculate roots with tapering ends and are about 7 – 8cm in length while ½ inch in width. Small sized, large number of flowers having tetramerous and large leafy pinnacles is the characteristic feature of *S. chirayita.* Colour of the flowers may range from greenish yellow with purple to green tinge and bear minute white hair on the surface [7, 58]. *S. chirayita* has a gamophyllous, four lobed calyx. The corolla and the lobes are four each in number which are twisted and superimposed with a union at the base having a pair or nectarines on each lobe. These lobes are covered with long hairs on the surface. *S. chirayita* has four stamens which are situated just opposite to the base of corolla and lobe [47]. Ovary is unilocular having laminal ovules along with presence of two stigmas. An egg shaped pericarp which bears 2 valved capsules having transparent to light yellowish color and small, dark brownish seeds are characteristic to *S. chirayita.*

Two types of pollination modes i.e. self pollination and cross pollination have been reported [68, 69]. Cross pollination is majorly supported by the presence of multicoloured corolla and

nectarines [70]. Reproduction occurs through large number of seeds produced by the plant. Flowering peaks start from the mid of September to early October which is followed by fruiting season from late October to November. Species associated with *S. chirayita* in south – eastern hills include mixed forests of Acer and Quercus. At the herbaceous layer level the species that appear alongside *S. chirayita* have been reported to be *Polygonum amplexicaule*, *Anaphalis triplinervis*, *Stachys sericea* and *Anemone obtusiloba* [71]. While the species on the north or north west facing hilly regions of Bhutan tend to be different and include *Oxalis corniculata*, *Artemisia vulgaris*, *Anaphilis triplinervis*, *Cynodon dactylon*, *Fragaria indica* and *Digitaria adecendens* [72].

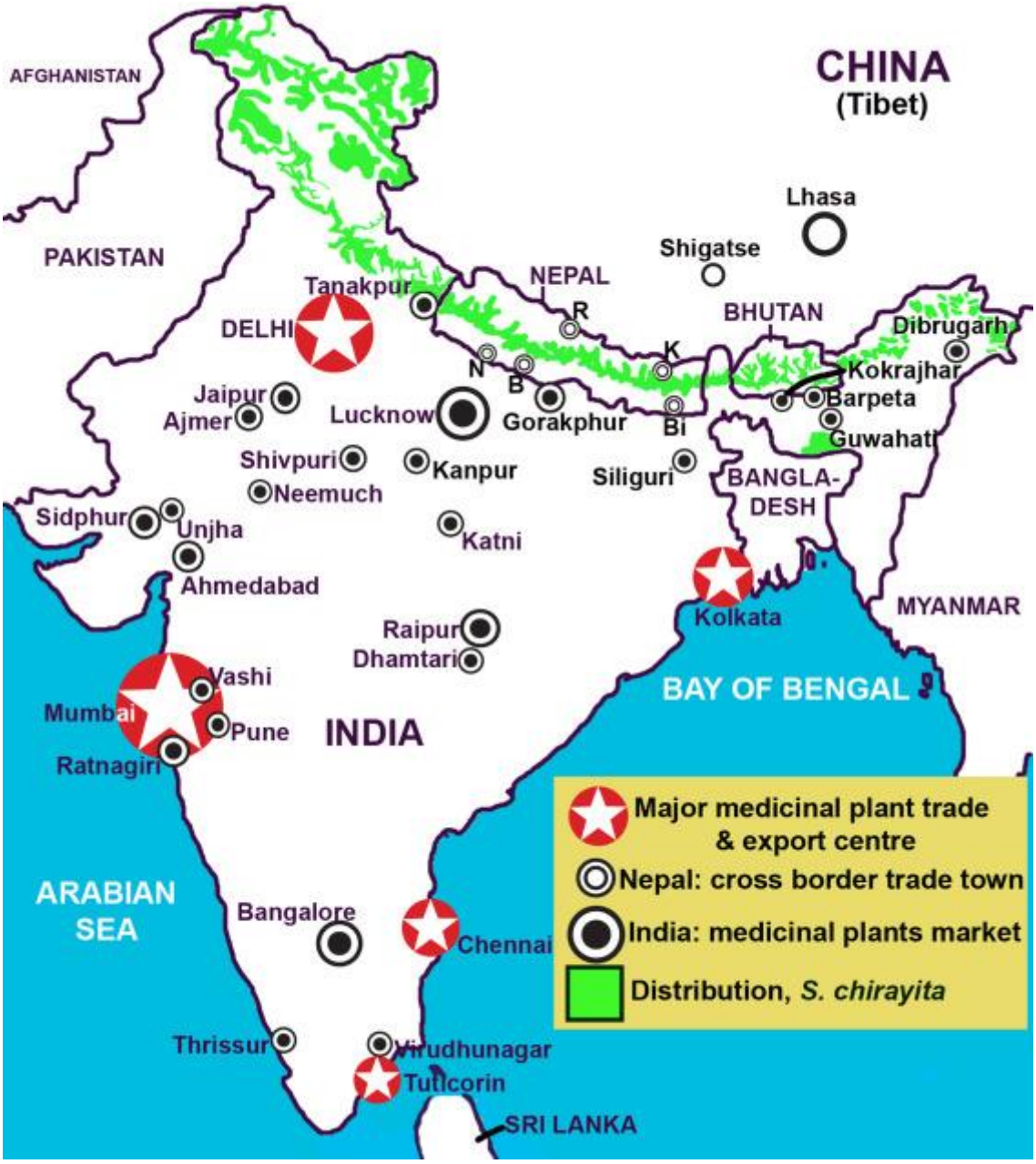

Figure 2.2 Distribution of *S. chirayita. Courtesy: Science Direct.*

Low densities of *S. chirayita* are reported in population studies conducted in Himachal Pradesh and Uttrakhand states of India [71] the range at Kalaseer site was reported to be 1.65 plant/m^2 and at the site of Kanchula was 2.35 plant/m^2 (Figure 2.2). Similar results were reported in another study conducted in North – eastern state of Arunanchal Pradesh [73]. Demographical studies reveal the annual – triennial reseeding character of *S. chirayita* which thus, emphasizes on sexual reproduction and soil – seed bank development and maintenance [59]. Seed germination success rates are also determined by its micro habitat and eventually the seeds belonging to open habitat are less viable than those collected from the closed

canopy forest bed. Studies revealed the stored seed viability to be nearly one year at room temperature which starts to decline after 18 months to up to 45 - 55% of the actual germination rate [57].

2.4 Traditional medicinal usage

This is one of the prime traditional Ayurvedic herb used in multiple ways by several indigenous populations for different medicinal purposes. Although the making of traditional remedies includes the whole plant, however root is the part with highest bioactivity among all the parts of plant as mentioned by [7]. The roots form the major constituent of the tonic prepared in curing fever, weakness, joint pain cough, common cold and asthma. In Indian context *S. chirayita* is used as a tonic, to cure asthma, fever and liver related disorders, in managing stomach haemorrhage when administered in combination with sandalwood paste [74]. According to the American and British pharmacopoeias, it is utilised as tinctures and infusions [7].

Whole herb is useful in hepatitis treatment, digestive ailments and inflammation [71]. It can be effectively used in curing a wide range of ailments [75, 76, 77, 78, 79]. It is also useful in curing certain types of mental disorders too. In recent studies properties which help in curing hepatitis B virus (anti - HBV) infections have been reported [10]. Decoctions of *S. chirayita* have properties like antidiarrheal, antibacterial, antimalarial, anthelmintic, antifungal, hypoglycemic, cardiostimulant, antifatigue, antiaging are also competent in maintaining blood pressure [80]. *S. chirayita* is used as one of the major constituents in herbal preparations like Ayush-64, Mensturyl syrup, Diabecon and so on [8].

The whole plant as mentioned in the traditional and indigenous systems like Ayurveda, Siddha and Unani [44] is used as a remedy in curing headache and blood pressure. For this majorly the chopped leaves and stem are dipped in water overnight and churned to make a paste and is administered with water once a day or once in 2 – 3 days as per requirement [81, 82]. *S. chirayita* is easy to digest and its concoction along with cardamom, turmeric and kutki is administered in case of gastrointestinal infections, while when taken with ginger, helps in controlling fever [81]. Similarly, decoction of the whole plant is used to cure malaria [83] while the paste prepared from the whole plant along with neem, manjishta and gotu kola is applied on the skin to cure various skin problems like pimples and eczema [82]. Kidney diseases, ulcers, hiccups and vomiting can also be well cured using whole plant extracts of *S.*

chirayita [44]. Also it can be combined with other medications in curing the patients bitten by scorpion [7, 84]. Its utility in curing excessive vaginal discharge has also been reported [85].

Leaves of *S. chirayita* can be used as poultice. These are mildly heated and a simple paste is prepared with mustard oil and administered in the treatment of boils and scabies [86]. Seeds find their utility of being an astringent or refrigerant. It can also be used as a diuretic remedy, in ophthalmic formulation, as an appetizer or tonic and even as a water purifier. Bark extracts are used to as a remedy in cholera while root extracts can help cure Leprosy and liver ailments and instant relief in joints pain [87]. Some of the Ayurvedic formulations which have extracts of *S. chirayita* in combination with other drugs are Kabdeen which is used in the treatment of hepatitis, Mahasudarsana churna, Sudarshan churna, Bhunimbadi kvatha, Kiratatiktadai kvatha, and Kiratadi taila for the treatment of fever, Chandra Prabati is administered to the patients suffering from cancer, Palas abijadi churna which has anthelmintic properties and Dermafex oil which is used in skin problems and as hair vitalizes [57].

2.5 Phytochemistry

Owing to its widespread applicability in a broad spectrum of medical treatments, not only in traditional medicine, *S. chirayita* has been adopted and commercialized in the modern systems of medicine as well. For commercial purposes, there is a dire need to identify the active phytochemical compounds present in *S. chirayita* which are responsible for various biological activities. This has accelerated the scientific exploration and research in this regard [88, 89, 90]. This herb in wild can be identified by its bitter taste, which is imparted due to the occurrence of certain biochemical components which include compounds like amarogentin, swerchirin, swertiamarin and other bioactive constituents which have a direct bearing on human health welfare [7]. Amarogentin has been recognized as the bitterest compound which has been ever isolated till date. *S. chirayita* has a broad spectrum of biological activities attributed to an array of secondary metabolites having pharmacological importance. This herb shows similar properties with hops with respect to it's the bitterness potential possessed by this plant which can be accredited to occurrence of amarogentin and chiratin. Amarogentin hold the status of the bitterest compound ever extracted or known and chiratin hydrolysis to yield two bitter by products called ophelic acid and chiratin [63].

Another similarity is the antimicrobial property [64, 65]. These secondary metabolites come from a wide range of classes like xanthones, derivatives of xanthones, flavinoids, secoiridoid glycosides, iridoids and terpenoids [61]. The major bioactive constituents include swertiamarin, mangiferin and amarogentin which have high therapeutic values [62]. These three compounds in combination impart the characteristic bitter taste and are xanthone C – glucoside derivatives [9]. A diverse series of pharmacologically active compounds present in *S. chirayita* are responsible for this wide spectrum of biological activities. These bioactive compounds are classified as xanthones, derivatives of xanthones, alkaloids, terpenoids, iridoids, flavonoids, secoiridoids and many more (Figure 2.3). Along with these certain other compounds have also been reported like chiratin, ophelic acid, stearic acid, palmitic acid and oleic acid [61]. Some of the important phytochemically active compounds and their structures have been depicted in (Figure 2.3). First ever dimeric xanthone isolated from different parts of *S. chirayita* was chiratanin. Major phytochemical constituents present in *S. chirayita* which are attributed to major biological activity of this important herb are depicted in (Figure 2.3) [6, 91].

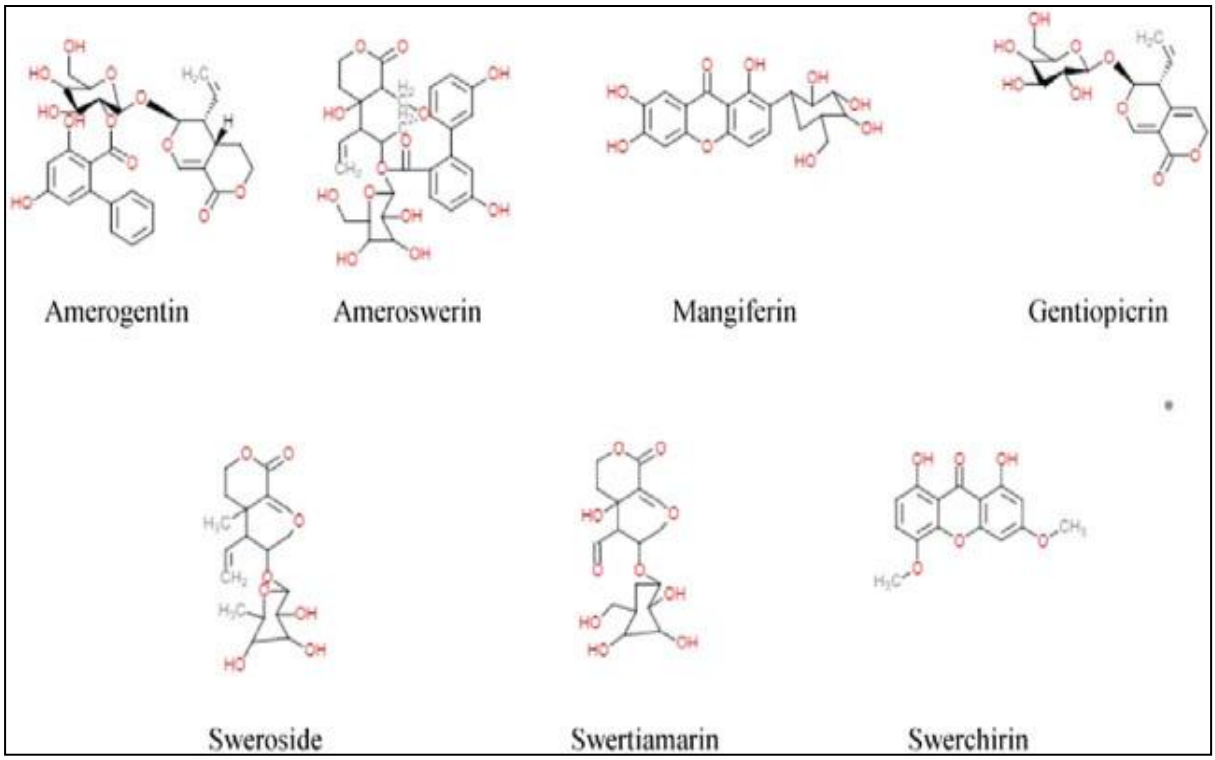

Figure 2.3 Phytochemical structures of some bioactive compounds present in *S. chirayita. Courtesy: Researchgate* [47]

Padhan et al., (2015) [62] reported higher accumulation of secoiridoids majorly Sewrtiamarin (2.8%) and Amarogentin (0.1%) in root tissues while higher levels of Mangiferin (1.0%) were observed in floral parts of *S. chirayita*. Both *in vitro* and *in vivo* bioactivities of majority of these phytochemical compounds have been and are being studied in the field of chemistry, biology and plant biotechnology for the evaluation and development of their novel structures and bioactivities to contribute in various fields of science and human health welfare.

2.6 Biosynthesis of medicinal compounds

In the present study, biosynthesis and accumulation of medicinal compounds (Amarogentin and Mangiferin) was focused extensively so the below mentioned pathway gives clear illustrations of different enzymes and substrates involved in their synthesis (Figure 2.4 (a) and (b)). Amarogentin and Mangiferin is biosynthesized from the common MVA/MEP/Phenylpropanoid route. Amarogentin originate from Swereoside as an intermediate, which is biosynthesized from GPP, which is a byproduct of IPP from MVA Pathway and DMAPP from MEP Pathway. Further they are transformed to the end products through cascade of reactions in which Amarogentin is biphenyl-carboxylic acid derivate of Swereoside [92]. However, Mangiferin completely follows Phenylpropanoid pathway where it is biosynthesized from the Phenylalanine by using two enzymes PAL and ACC [93].

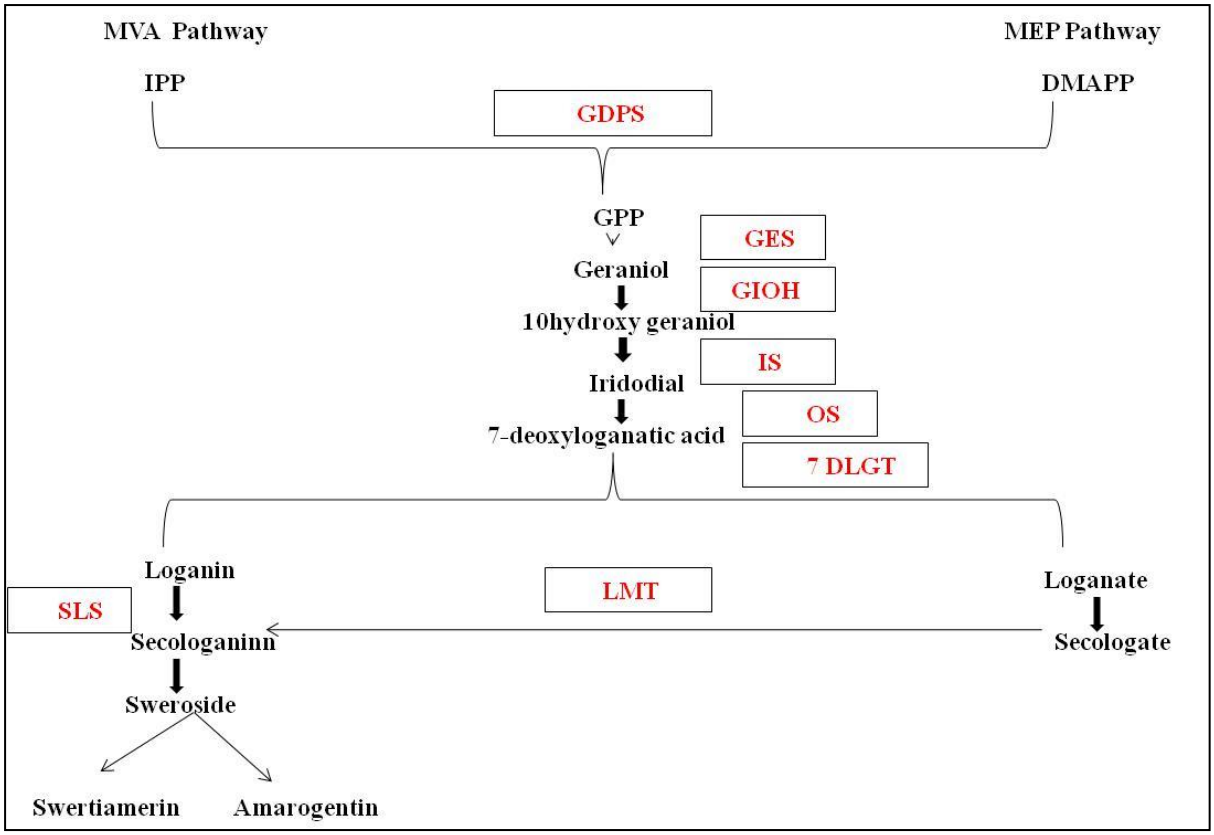

Figure 2.4 (a) Biosynthetic pathway of Amarogentin

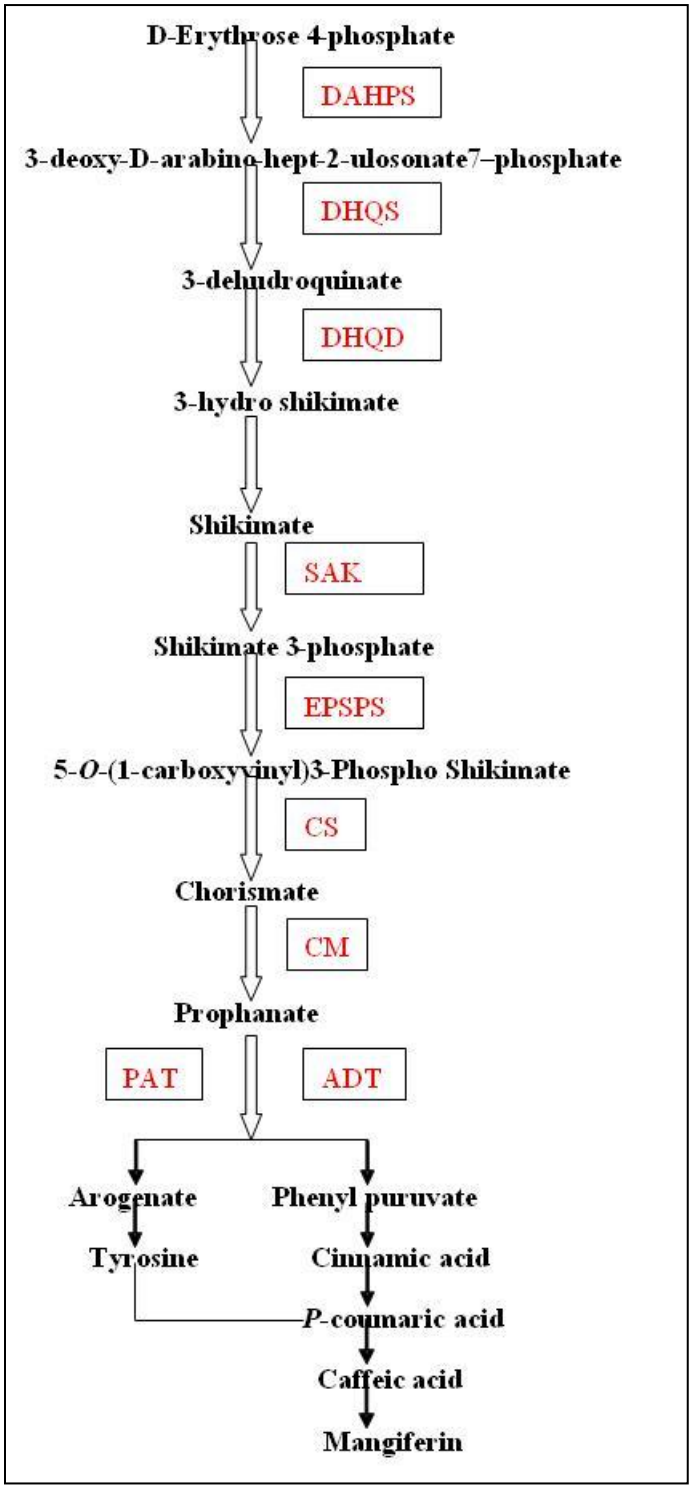

Figure 2.4 (b) Biosynthetic Pathways of Mangiferin

2.7 Pharmacological Activity

Various pharmacological examinations have been conducted on *S. chirayita* due to its distinct ethnobotanical utilities. Wide variety of biological activities exhibited by *S. chirayita* extracts like antibacterial, antiviral, anti-inflammatory, antifungal, antidiabetic, antioxidant and many more have been reported by many researchers [78, 94, 95, 96, 97]. A wide array of test systems have been developed for the pharmacological evaluation, which indicates promising characteristic properties in alcoholic, methanolic and aqueous extracts of *S. chirayita*. Many researchers have reported promising anti bacterial and antifungal properties possessed by *S. chirayita* whole plant through evidence based laboratory testing [96, 97, 98]. Anti-inflammatory and hypoglycemic activity of the whole plant was reported in various studies [76, 100, 101, 102, 103]. Using a 70% ethanolic extract of *S. chirayita*, reducing power and beta-carotene assays revealed significant antioxidant capabilities. [78]. It showed high levels of DPPH hunting activity i.e. IC_{50} = 267.80µg/mL. In Table (2.1, 2.2) herbal extracts under different solvent system were testified and reports were also generated for different *in vivo* models for its pharmacological significance.

Table 2.1 Pharmacological study of *S. chirayita* plant extracts under different solvent system.

Solvent[a]	Test Organism	Control	Bioactivity evaluated	References
EtOH	*P. vulgaris* ATCC 6380, *E. coli* ATCC 26922	Ciprofloxacin	Antibacterial	[98, 99]
MeOH	*B. subtilis* (ATCC 6133), *P. aeruginosa* (ATCC 17843), *E. faecalis* (ATCC 24505), *S. aureus* (ATCC 1538), *S. typhi* (ATCC 13028).	Ceftriaxone, Ciprofloxacin, Gentamycine, Levofloxacin, Metronidazole, Tranexamicacid Ceftriaxone sodium Cefuroxine	Antibacterial	[105]
MeOH	*B. subtilis* MTCC 736, *B. polymyxa*.	Gentamycin	Antibacterial	[96]
DCM; EtOH	*S. aureus*.	Kanamycin 30 µg/disc	Antibacterial	[102]

EtOH	*S. aureus, B. subtilis*.	Chloramphenicol 30 µg/disc	Antibacterial	[105]
MeOH	*A. niger* MTCC 1881, *A. flavus* MTCC 1883.	Amphotericin	Antifungal	[96]
95% EtOH	*L. donovani* UR6		Antileishmanial	[106]
MeOH	*L. donovani* AG83		Antileishmanial	[107]
Water; MeOH	*H. contortus*	Levamisole 0.55 mg/ml	Antihelmintic	[108]
MeOH; PE	*P. falciparum* FCK 2	Parasitized RBC's and 10 µCi of [35S]-methionine	Antimalarial	[109]
HEX; MeOH	*A. aegypti, C. quinquefasciatus*	Tween-20	Egg hatchability and larvicidal	[110]
40% EtOH	HepG2 cells line	Amphotericin	Anti-hepatitis B virus	[10]
85% EtOH HEX	Diabetic albino mice	Metformin (100 µg/kg)	Antidiabetic	[111]
EtOH; HEX; Chloroform	Diabetic albino mice	Metformin (100 µg/kg)	Antidiabetic	[95]
Water	Iinduced Hyperexia	Paracetamol (150 mg kg−1)	Antipyretic	[94]
Water	Herpes simplex virus type-1	Acyclovir (1.5 mg/mL)	Antiviral	[113]

Solvent[a]: N/A, not applicable, EA, ethyl acetate, HEX, hexane, PE, petroleum ether, EtOH, ethanol, MeOH, methanol.

Table 2.2 *In vivo* pharmacological studies of *S. chirayita*.

Solvent[a]	Control	Bioactivity evaluated	References
Petroleum	Diclofenac treated Mice (10 mg/kg)	Antiinflammatory	[13]
95% EtOH	Diclofenac (25 mg/kg)	Antiinflammatory	[100]
95% EtOH	Mice treated with vehicle	Hypoglycemic	[101]
EtOH	Glibenclamide (5 mg/kg)	Hypoglycemic	[102]
EA; EtOH	Glibenclamide (5 mg/kg)	Hypoglycemic	[103]
HEX	9,10-dimethyl benz(a)anthracene (DMBA)	Anticarcinogenic	[79]
EtOH	Diclofenac sodium (25 mg/kg)	Analgesic	[102]
EtOH	Aminopyrine (50 mg/kg)	Analgesic	[100]
70% EtOH	Paracetamol (150 mg/kg)	Hepatoprotective	[100]

2.7.1 Antioxidant activity

Pharmacologists, biochemists and health professionals show keen interest in certain antioxidants as these components help narrow down the oxidative damage and provide protection towards active free radicals that cause harmful oxidation of the body cells [113]. Natural antioxidants are gaining popularity among various groups like food scientists, nutritionists and common people as well, as these lower down the potential risk of various chronic ailments as well as to remain fit and healthy [114]. Various in vivo and *in vitro* tests to check the antioxidant properties include superoxide test, ferric reducing antioxidant power assay, nitric oxide test, metal ion chelating activity assay, 2,2'-azino-bis(3-ethylbenzothiazoline- 6- sulphonic acid), 1,1-diphenyl-1-picrylhydrazyl or 2,2- diphenyl-1-picrylhydrazyl, chemi-luminescence method, hydroxyl radical scavenging activity assay and anti-lipid peroxidation method. Satisfactory IC_{50} Values have been reported by various authors (Table 2.3). Mangiferin and Oleanolic acid [115] extorted from *S. chirayita* have been reported to show the antioxidant properties. The specific xanthones structures along with the atypical catecholic moiety and a complex as well as conjugated system are the basic reason of a promising antioxidant property exhibited by *S. chirayita* [116]. Moderately good

results have been reported against different types of free radicals, however, more comprehensive studies are required specifically on the antioxidant property, activity and mechanisms to identify and utilize the active compounds for human welfare.

Table 2.3 Antioxidant activities of *S. chirayita* in different solvent systems.

Part of plant	**Solvent**	**Antioxidant assay**	**IC_{50} Values**	**RSA%**	**Bio – active compound**	**References**
Leaves	80% Methanol	DPPH		85.33 ± 0.58	Phenols, Flavonoids	[117]
Plant	Chloroform	DPPH	76.18 ± 1.14 µg/ml		Decussatin	[116]
	Ethyl acetate		57.92 ± 6.16 µg/ml		Swertiamarin	
	MeOH		64.15 ± 8.77 µg/ml		Bellidifolin	
	Acetone		34.12 ± 3.84 µg/ml		Isobellidifolin	
	MeOH: Water (8:2)		55.54 ± 2.71 µg/ml		Amarogentin	
	Acetoene:Water (8:2)		31.52 ± 1.21 µg/ml		Swertianolin	
	Water		55.52 ± 0.78 µg/ml		Mangiferin	
	Decussatin		45.52 ± 0.78 µg/ml			
	Bellidifolin		27.15 ± 0.27 µg/ml			
	Isobellidifolin		15.24 ± 5.75 µg/ml			
	Amarogentin		20.13 ± 1.05 µg/ml			

	Swertionolin		30.18 ± 1.84 µg/ml			
	Mangiferin		9.11 ± 1.03 µg/ml			
	Swertiamnin		12.56 ± 0.18 µg/ml			
	Water	DPPH		59.8		[118]
Plant powder	Methanol	DPPH		87.58±0.37		[119]
				71.67±1.72		
				25.05±0.48		
				45.51±1.24		
Whole plant	MeOH	DPPH		27.70		[120]
Whole plant	MeOH	DPPH	222.74 µg/ml			[121]
Whole plant	70% EtOH	DPPH	267.80 µg/ml			[53]
		β – carotene	1.502± 0.20µg/ml			
		ABTS	6.50 µg/ml			
Whole plant	MeOH	DPPH	551.26 µg/ml			[120]
	EtOH		557.61 µg/ml			
	ACE		551.96 µg/ml			
	Water		559.05 µg/ml			
Leaves	Water	DPPH	86 µg/ml			[55]
Plant	12% EtOH	DPPH	156.62 g/ml			[9]

2.7.2 Antibacterial activity

Antibacterial activities are usually examined by the use of IZD test (inhibition zone diameter) or MIC test (minimum inhibitory concentration). For targeting specific set of harmful bacteria, the antibacterial compounds are required to be identified and extracted from *S. chirayita* which may lead to more accurate results. Swertiamarin [122] and Sweroside [123] have been recognized to show considerable antibacterial activity. Many *in vitro* studies using different extraction solvents like EtOH [98, 105], MeOH [96, 104], DCM and EtOH [97] have been reported in the literature. High antibacterial activity was reported by the ethanolic extract of *S. chirayita* in comparison to the aqueous extract as described by Rehman et al., (2012) [98] and congruent outcomes were seen by Alam et al., (2009) [97]. All these reports emphasize on the recognition and obtaining of specific antibacterial bioactive substances present in *S. chirayita* to be used against specific target bacteria groups for more fruitful results.

2.7.3 Antimicrobial activities

Oleanolic acid, Ursolic acid [124] and β – Amyrin (Vazquez et al. 2012) were reported to show specific antimicrobial activities among the bioactive compounds isolated from *S. chirayita* till date. Alam et al., (2009) [97] reported potential use of *S. chirayita* to be effective against skin infections. The flavonoids in the plant, which are in charge of shielding it from microbial diseases and UV radiation, have reportedly been linked to the bioactivity in this regard. The flavonoids are extracted more effectively in the ethanolic solvents as compared to aqueous solvents and thus show higher potency against microbial infections.

2.7.4 Antidiabetic activity

Presence of flavonoids and secoiridoids impart the hyperglycemic properties to *S. chirayita* [125]. Amarogentin [9] Swertiamarin [126], Mangiferin [127], Swerchirin [128, 129, 130], Bellidifolin and Isobellidifolin [131] are the bioactive compounds derived from *S. chirayita* which have been reported to exhibit antidiabetic activities. Kavitha and Dattatri (2013) [132] conducted the antidiabetic assessment of aqueous concentrate and revealed significant commitment of mangiferin, amarogentin and swertiamarin to be available in aqueous concentrate and 12% ethanolic concentrates of all aspects of *S. chirayita*. Among all the above mentioned bioactive compounds showing positive antidiabetic activities, mangiferin has been identified to be the most potent phytochemical against diabetes and in lowering

down the blood lipid profile pertaining to diabetes [133, 134]. Modes of action shown by mangiferin may be attributed to its ability of stimulating β – cells for the release of insulin or may be due to lowering down the absorption of glucose in the intestine [135]. It causes enhanced release of glycolytic enzymes to stimulate glycogenesis [136] along with enhanced peripheral utility of glucose in the body [137]. It inhibits α – glucosidase, maltase, sucrose and osomaltase [138] and increases glycogen content in liver and muscles along with dipeptidyl peptidase IV mediated glucagon degradation [136].

Aqueous extracts of roots, leaves and inflorescence contain Swertiamarin which also shows considerable antidiabetic activity [128]. Rehman et al., (2011) [98] concluded a dose of aqueous extract equivalent to 200mg/kg of body weight to be effective as an antidiabetic drug however the activity is comparatively less marked than standars diabetic drugs. Swerchirin belongs to the xanthone group extracted from *S. chirayita* hexane fraction [128, 130] which stimulates the islets of Langerhans to release insulin [137] for the management of blood sugar levels. It shows higher potency in blood sugar regulation as compared to standard drugs as reported by [128]. The studies support the antidiabetic activity shown by the phytochemical compounds found in *S. chirayita*.

Sensitivity and effectiveness of antidiabetic property needs to be validated and standardized in humans. Various methods like OGTT (oral glucose tolerance test), FPG (fasting plasma glucose test) and RCBG (random capillary blood glucose) can be employed for the same [11]. In addition to RCBG, AST (aspartate aminotransferase test) and ALT (Alanine aminotransferase test) can reveal liver conditions of the subject too. This is necessary for the confirmation of positive effectiveness of the plant extract over the β – cells activity pertaining to normal secretion of insulin. However, the exact mechanism of this activity has not been clearly understood and needs further considerations along with standardization of the extract compositions of *S. chirayita* used for testing.

2.7.5 Anti-carcinogenic activity

Cancer treatments through radiation therapy or surgery are considered to be temporary resort and better drugs and methods are the prime requisite of the hour. Phytochemically active compounds present in certain plants may contribute towards better drug treatment for this dreadful disease along with other infections and chronic ailments as well. Plant based drugs

show no or minimal side effects on the patient. Crude extracts and their purified components have been reported [136] to be having effective anti-carcinogenic properties, however specific activity of the bioactive compounds and their modes of action need to be analyzed and standardized through research. Bioactive compounds present in *S. chirayita* like Amarogentin [139, 140], Swerchirin [141, 142] Mangiferin [143] and Swertiamarin [144] were reported to exhibit anti cancer properties. Saha and Das (2010) [136] reported study on skin cancer stimulated by dimethylbenz (a) anthracen (DMBA) in mice and reported (2.5mg/mouse) crude *S. chirayita* extract and a (0.2mg/mouse) purified amarogentin extract to have significant suppression of target site apoptosis and inhibition of cell growth.

Amarogentin (a secoiridoid glycoside) prevents progressive liver carcinogenesis. It upregulates the Bax-Bcl2 ratio, activates the cleavage of capase – 3 and poly – ADP ribose polymerase [140] thus, significantly inducing the process of apoptosis, making it a potent phytochemical for liver cancer treatment. Highest quantities of amarogentin are reported to be present in aqueous and ethanolic leaf extracts while the lowest in aqueous and ethanolic root extracts [145]. Vailanka et al., (2015) [146] studied anticancer activity shown by methanol extract derived from leaf and stem and revealed higher cytotoxicity of leaf extract then stem extracts in same concentrations. Verma et al., (2008) [94] reported *S. chirayita* use as an antiviral agent against herpes simplex virus and observed positive outcomes against cancer causing agents particularly viruses like human papilloma virus. In traditional Indian Ayurvedic treatment for liver disorders, *S. chirayita* has been reported to be the oldest herb to be administered [136]. The literature, reports the anticarcinogenic potential of *S. chirayita* and its extracts in early prevention of breast, brain and colon cancer [145]. It holds the status of both preventive and therapeutic remedy in cancer prevention. Alkaloid content of the plant is high due to presence of Amarogentin, Swertiamarin and Mangiferin along with other bioactive compounds which are helpful in reduceing cell proliferation in case of MCF – 7, KELLY and CACO – 2 cell lines. However, the actual mode of action and detailed investigations are recommenced.

2.7.6 Anti – hepatitis and Hepato-protective activity

S. chirayita is one of the oldest Ayurvedic herb used to cure bronchial asthma and liver disorders [147]. Swertiamarin [148], Swerchirin [149], Sweroside [150, 151] and Syringaresinol [152] derived from *S. chirayita* were recognized to show anti hepatitis and hepato-protective activity. Zhou et al., (2015) [10] reported anti-hepatitis properties against

HBV activity in the *in vitro* examination of 50% EtOH extract of entire plant and hepato-protective properties in 70% EtOH extract of aerial portions of the plant in – vivo by Nagalekshmi et al., (2011) [56]. Zhang et al., (2017) [153] examined how amarogentin affected CCl4 – induced liver fibrosis in mice and concluded its potentiality in anti – fibrotic activity by improvement observed in liver function and histopathological status of liver tissue. It works on an anti – oxidative mechanism to suppress MAPK signalling pathway. It shows suppressive effect on hepatic fibrosis and may serve as a therapeutic supplement in liver fibrosis treatment. Reen et al., (2001) [147] have reported anti – hepatotoxic activity of *S. chirayita* extracts against CCl_4 and paracetamol toxicity in rat hepatocytes. Hexane and methanol extracts gave promising results as hepato protective agents [48]. Butanol extracts of *S. chirayita* reported considerable toxicity effects in H4IIEC3/G cell lines of rat Reuber hepatoma. Oleanolic acid tablets made from *S. chirayita* extracts are used frequently as liver protection drugs in liver and as anti – tuberculosis drugs [154]. *Andrographics paniculata* (Family - Acanthaceae) shows almost comparable hepato protective and hepato – stimulative properties as *S. chirayita* owing to similar therapeutic modes of action [56].

2.7.7 Anthelmintic activity

Anthelmintic activity shown by aqueous and methanolic extracts are attributed to the phytochemical activity of medicinal constituents like amarogentin along with other secoiridoid glycosides namely amaroswerin and sweroside [108]. Kshirsagar et al., (2019) [48] reported the same on live Haemonchus contortus. High concentrations of the crude extracts have been recommended *in vivo* (3g/kg wt.) than *in vitro* (25mg/ml settings). Methanolic extracts showed complete inhibition of mobility in case of isolated worms. Egg reduction rate was 58.8% and 58.2% of methanolic and aqueous extracts respectively. Results reveal loss of potency in aqueous extracts over methanolic extracts on prolonged use while the anthelmintic properties were far lower than that of standard anthelmintic agent levamisole exhibiting the moderate anthelmintic activity of *S. chirayita*. However, this property needs to be explored in a more comprehensive way.

2.7.8 Antileishmanial activity

This has been studied on the hamster model by Medda et al., (1999) [107] and amarogentin was reported to be associated with this property. Its efficacy was tested in three forms namely free, liposomes and niosomes. The mode of action shows inhibitory activity upon

topoisomerase I in *Leishmania donovani*. However, further research is recommended to be carry forward for better designing of effective leishmaniasis drugs using *S. chirayita* as well as other *Swertia* species.

2.7.9 Miscellaneous activities

Bioactive compounds present in *S. chirayita* like Swertiamarin [128], Gentianine [155] and 1 – hydroxyl – 3 – 5 – 8 – trimethoxyxanthone [156] show good antimalarial properties. Swertiamarin is also known for its CNS depressant property [157] along with cardio – protective [126], anti – atherosclerotic and anti – arthritic [159] properties. Mangiferin has been reported to show anti – viral [24], anti – HIV, antitumor [159] and anti – Parkinson [160] activity. Sweroside is a common preventive bioactive compound in osteoporosis [161]. Mangiferin [162], Oleanolic acid [115], Swertanone [163], β amyrin (Vazquez et al.; 2012) and Chiratol [13] show anti–inflammatory activities. All these activities need further validation and may contribute as a potential and alternative source of treatment with lesser side effects and affordability. Complete description for its pharmacological significance is illustrated in for highlighting the significance of bioactive compounds present in *S. chirayita*.

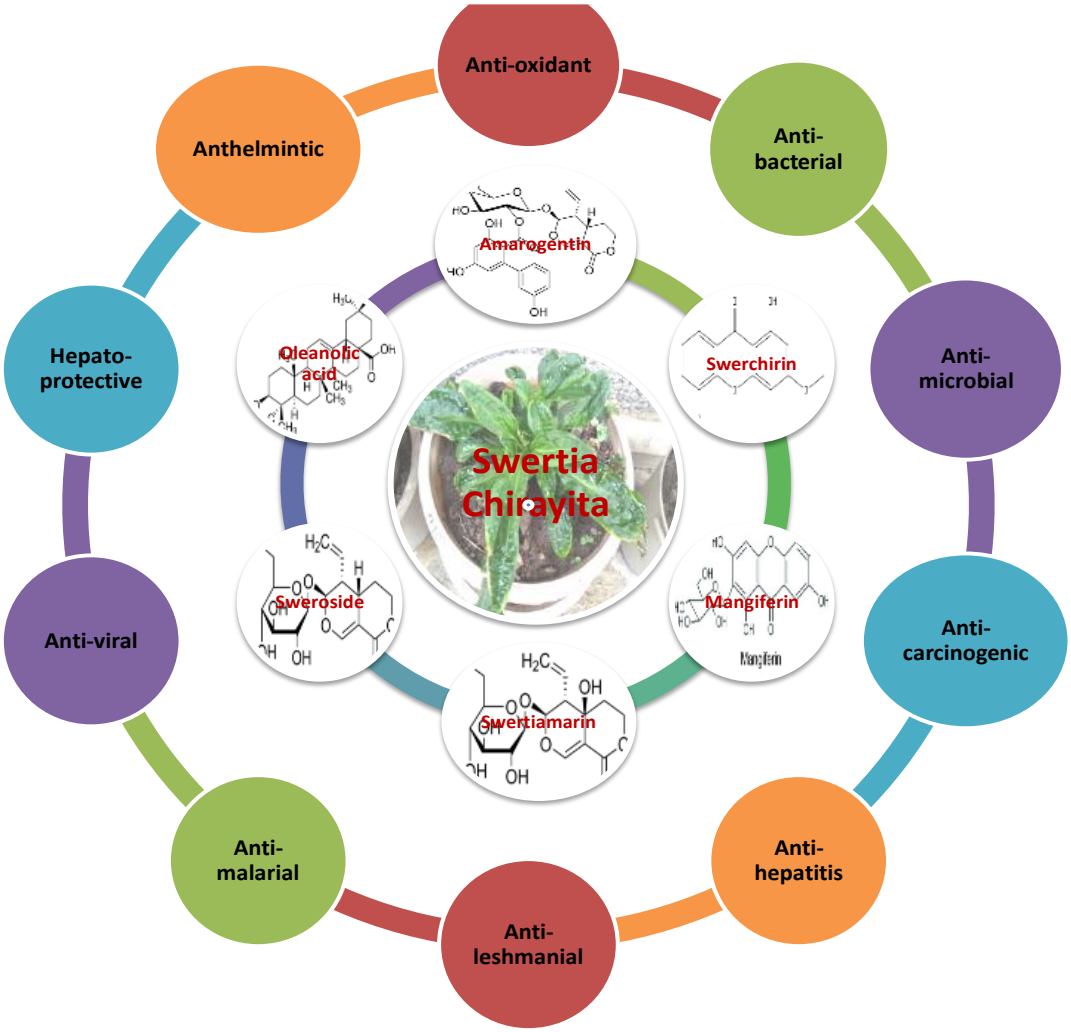

Figure 2.5 Potential of bioactive compounds of *S. chirayita*

2.8 Toxicology and Safety

Age old history of medicinal herbs apart from their positive attributes; have also been reported with some safety concerns as issues to be catered by the pharmaceutical industries. Notion of traditional medicines having no ill effects is not all true. Many studies have revealed mutagenic and cytotoxic effects of some commonly used medicinal plants over long history of herbal use [164, 165]. The traditional formulations are incompetent in providing safety over prolonged use. Only a few herbal formulations are tested clinically for toxicity with very limited data availability over *S. chirayita* and its bioactive compounds [166]. In recent times, there has been increased reporting of toxicity in crude extracts as well as

isolated compounds. Thus, a comprehensive pharmacological safety profiling is required on acute, sub – acute and chronic toxicity of *S. chirayita* before it can be clinically tested and used at a commercial scale.

In a toxic evaluation study conducted on ethanolic extract of *S. chirayita* [53] no toxic symptoms were reported in male KM mice when exposed with 2000 mg/Kg body weight. Another study on sub – acute toxicity also revealed negative cytotoxic effects on female swiss albino mice, orally administered with amarogentin (0.2 mg/kg body weight) [140]. *S. chirayita* has been mentioned to be non – toxic throughout literature. This ethno – medicinal herb revealed no obvious ill effects in mice with equivalent body weight and body temperature of the test as well as control groups [100, 102]. Liposomal as well as niosomal forms of amarogentin, have also been reported to show non – toxicity [107]. The studies majorly support the non – toxic nature of *S. chirayita* and its derivatives, however, the literature is inadequate and more stringent research including toxicological and mutagenic tests are required for foolproof validation and safety from *S. chirayita*.

2.9 Propagation and conservation practices

Plant resources are destructed through overutilization, interference 100 – 1000 folds [180]. Development in the hilly areas of Himalayas has caused the destruction of many wild plants with medical value and many of these species have been reduced to critically endangered threshold including *S. chirayita*. Its use as a medicinal drug has exposed it as a valuable trade object. Nepal's half of the GDP depends on *S. chirayita* trade as it has high demands in both national and international markets. Along with these issues, another major concern is an inappropriate data related to annual harvest and trade of *S. chirayita*. IUCN has categorized *S. chirayita* to be a critically endangered species [7]. The NMPB, Government of India, has enlisted 32 medicinal plants with high priority of which *S. chirayita* is one such medicinal herb plant.

Extinction of this valuable herb will have its implications in not only the genus loss required for plant growth but also on lack of biosynthesis pertaining to new and useful compounds along with the irretrievable loss of pharmaceutically and nutraceutically important novel compounds present in *S. chirayita*. For this situation to recover, cultivation of this species is required to be escalated to meet the national and international demands. Plant biotechnology

may prove to be a boon in this regard as certain studies have reported the limitations pertaining to seed propagation, less viability and little germination percentage [70, 99]. Biotechnological approaches may help establish genetically uniform plants and guarantee sufficient supply irrespective of constrains which are imposed by the environmental conditions. It may also be helpful in lowering down the harvesting pressure exerted on wild populations. Techniques like micropropagation, somatic embryogenesis and acclimatization may help generation of uniform availability of *S. chirayita* all through the year [54, 181]. At commercial level production of *S. chirayita,* adventitious roots, hairy root technology in addition to other contributing factors for root biomass and phytochemical compounds production are required. Micropropagation under controlled environment helps to prevent the plant diversity issues and contribute in improvement of secondary metabolite quality. Various studies in this regard have been reported and are enlisted in (Table 2.4).

Table 2.4 Micropropagation using different explants of *S. chirayita*.

Explant used	**Methodology**	**Inferences**	**References**
In vivo axillary bud/ shoot apices	Micropropagation	Swift *in vitro* propagation	[182]
In vitro shoot tip	Micropropagation	Enhanced shoot propagation	[183]
In vivo leaves	Somatic embryogenesis	Rapid micrpropagation system	[184]
In vivo stem with node	Regeneration	Superior revival from the nodal explants	[185]
In vitro leaves	Direct shoot multiplication	Improved propagation protocol	[186]
Seeds	Regeneration	Restoration from juvenile seed culture	[187]
Seedling-derived nodal explants	Axillary multiplication	Enhanced shoot proliferation	[7]
Node	*In vitro* regeneration	Rapid *in vitro* propagation	[188]
In vivo shoot tip	Efficient Regeneration	Competent shoot propagation	[54]
In vitro root	Shoot Organogenesis	Improved plant regeneration protocol	[90]

In vitro root	Callus culture	Plant regeneration via indirect organogenesis	[90]
Axillary bud	*In vitro* flower production	*In vitro* flowering and effective regeneration protocol	[120]
In vivo leaves	Direct shoot regeneration	*In vitro* shoot regeneration	[189]
Seeds	Regeneration	Adventitious shoot rejuvenation from root explants	[190]

Several studies advocate the use of synthetic seed technology showing enormous potentiality towards ease of handling micropropagation and germplasm conservation by the use of cryopreservation method [191, 192, 193, 194]. In case of secondary metabolites which get concentrated in roots [195], the harvesting process causes destruction, hairy root technology using *Agrobacterium rhizogenes* have gained popularity and has opened newer scopes in applied research. This method has important biotechnological applications on extensive synthesis of important compounds using hair root cultures [196, 197].

Somatic embryogenesis is the process by which somatic cells to develop into somatic embryo under lab conditions (*in vitro*). Somatic embryogenesis is an alternative strategy to enhance the number of endangered medicinal plants in the tissue culture conditions. As it retains genetic integrity and can promote the development of germplasm conservation, such as through the cryopreservation of *in vitro* tissues, this technique has various benefits over older and has more conventional ways of propagation [13, 198, 199]. Ara et al., (2000) [191] reported synthetic seed technology to be useful in mass propagation followed by storage of genetically uniform clones for very less period of time through *in vitro* tissue culture systems which enhance the possibility of transformation of seed into complete plantlets. On the other hand, *in vitro* preservation can preserve germplasm, especially when used in combination with cryogenic techniques [200, 201]. With advancements, cryopreservation can be utilised to preserve plant tissues from the tropics to the Himalayan temperate zones. It has been employed for long-term seed storage. Even though various conservation methods have been

used, cryopreservation of somatic embryos through encapsulation and dehydration method have not yet been used for tissue cultured *S. chirayita*.

2.10 Metabolite production and elicitation

Elicitation is a beneficial technique to encourage the synthesis of medicinal constituents in plant cell culture [167]. Elicitors can be an abiotic and biotic medium, cause various molecular, morphological, physiological, and biochemical changes in plants [168]. Abiotic elicitors comprise of physical and chemical triggers like sodium acetate, JA, SA, silver nitrate, cobalt nitrate, heat shock, hydrostatic pressure, and hyperosmotic stress [169]. The biotic elicitors comprise elicitors originating from plants, fungi, animals, bacteria, and algae, including chitosan, pectin, yeast extract, alginate, and fungi-derived cerebosides, among others [169]. Numerous studies have demonstrated that both biotic and abiotic elicitors cause the creation of secondary products by activating metabolic networks [170]. Elicitors are a crucial biotechnological tool for researching the synthesis of both well-known and unexplored secondary metabolites [169]. JA, arachidonic acid, vanadyl sulphate, lanthanum salts, use of fungal extracts and SA increase the *in vitro* synthesis of taxane in *Taxus* species. [171]. The kind and specificity of the elicitors, the time period of exposure and the timing of elicitor addition, the composition of the medium, the secondary metabolite of interest, and the cell line all have a role in elicitation success [167].

2.10.1 Light as abiotic elicitor

In vitro cultures have long benefited from the use of light to promote growth, development, and the production of bioactive substances [172]. As in the *in vitro* cultures light quality plays an important abiotic factor for stimulating the synthesis of biomarker compounds [172]. The molecular and physiological basis of photo induced processes has been thoroughly documented by a number of authors [173]. LEDs have developed as a commercially sustainable source for *in vitro* cultures [31]. LEDs are semiconductors that work on the electroluminescence principle [31]. A number of studies have shown how LEDs stimulate plant growth and secondary metabolism [31]. Numerous researchers have studied the many benefits of LEDs in plant *in vitro* growth in great detail [174]. Different light spectrums

sensed by various photoreceptors in plants regulate physiological and metabolic activities [175]. Plant morphology, photosynthesis, and other physiological activities are significantly influenced by light quality [176]. Sunlight emits a combination of UV (100-400 nm), far-red (690-760 nm), and photosynthetically active radiation (400-700 nm) [177]. Through the selective activation of photoreceptors like phytochromes by red and far-red light and cryptochromes and phototropins by blue light, the light quality controls plant development and physiological processes [178]. Because it controls the gene expression of different metabolic pathways, light quality has an impact on the production of amino acids, carbohydrates, phytohormones, and nucleic acids [178]. Through the activation of various light-responsive metabolic pathways, light can variably regulate the secondary metabolism of plants [45, 179, 236]. Although different lighting conditions have been utilised on large scale to enhance the production of bioactive compounds in some plant cell cultures, these parameters have not yet been applied to upgrade the biosynthesis of bioactive substances in tissue cultures of *S. chirayita*.

2.11 Conclusion and Future perspectives

Recently a new trend has been observed worldwide with respect to natural medicines derived from plants or its parts. About 40% of the western countries are using naturally derived pharmaceuticals [202]. Owing to the diverse climatic conditions apparent in India, there exist a huge variety of medicinal plants and herbs. Plants obtained from their natural habitat are the major source of pharmaceutical companies and these resources are depleting at an alarming rate. Many promising projections including both traditional as well as modern medicine are offered by *S. chirayita*. It serves as a potential herbal remedy in large number of ailments. Till date no toxic effects have been reported, however this aspect needs to be confirmed with respect to humans. Biological activities exhibited by *S. chirayita* are so versatile that it also provides a huge scope in research. These activities and their modes of action are still a grey area to be explored as we have a limited knowledge. Toxicological and mutagenic effects of different bioactive compounds need to be properly validated. The efficacy of *S. chirayita* to be used as a medicine is required to be established through clinical trials. Both national and international markets have huge demand for this ethno – medicinal herb due to its multiple uses. However, this is also the reason behind over exploitation of this precious plant species up to the verge of extinction in collaboration with habitat destruction. Research pertaining to

proper conservation practices and an uninterrupted supply of *S. chirayita* is in critical demand in the present scenario for its successful commercialization. Biotechnological intervention, use of innovative tools for its conservation and enhancing the commercial production are required. Synthetic seed germination technique may also prove to be useful in this context. However, detailed studies pertaining to maximum frequency of germination attained through synthetic seed and soil factors essential for plant growth are required to meet the commercial demands. In addition, hairy root technology is a futuristic approach which can be applied as a model tool in plant biotechnology to improve quality and levels of important phytochemicals of *S. chirayita*.

The literature showed the establishment of micropropagation, is still in developing stage and due to scarcity on studies focused on seed biology and improvement strategies pertaining to bioactive secondary metabolites of *S. chirayita* and further studies in this regard may pave the path for its commercialization. There is a dire need for quality control strategies for the prevention of misidentification and adulteration of *S. chirayita*. These recommendations may enhance the therapeutic importance of this medicinal herb with collaboration with novel biotechnological tools and strategies.

It will not be wrong to conclude that, plants being the natural nutraceuticals, produce numerous phytochemical compounds which are essential for their proper functioning or metabolites produced as by products which are beneficial for human health maintenance too along with prevention and treatment against large number of diseases. In this regard *S. chirayita* has gained special attention since long. Various aspects have been discussed above; however mechanisms of action related to most of the bioactive compounds are still unknown and need to be determined. *S. chirayita* and its biochemical compounds do not have a scientific confirmation and require further investigation pertaining to prove age old beliefs, explore new medicinal properties and to enhance its potential pharmaceutical applications. Quality rich tissue culture plants would have been studied for exploring their pharmacological effects so that the natural population could be rescue from its further deterioration and tissue raised planting material would be utilized as the best alternate to the field ones.

The literature review of *S. chirayita* has revealed the following research gaps:

- Less exploration available on developing *in vitro* techniques for carrying out quality rich production of *S. chirayita*.
- A promising alternative platform for the production of bioactive compounds is provided by plant cell culture. However, no thorough investigation into the *in vitro* production of bioactive compounds in *S. chirayita* has been reported as of yet.
- In many plant cell cultures, the stimulating influence of light quality on the synthesis of medicinal constituents has been extensively documented; however, yet not reported in *S. chirayita*.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Plant selection and its propagation

Plants of the *S. chirayita* were procured from the "HFRI", located in Shimla, H.P., India (20˚76'N, 67˚12'E). Plants have been submitted to the "NBPGR" in Delhi with the accession number IC-594053 for the purpose of conducting additional research, and tissue-raised plants have been verified by "Dr. Y.S.P. University" in Himachal Pradesh, India, using UHF-Herbarium No. 13570. At JUIT in Waknaghat, Solan, India (1400 m altitude), and plants were grown in a greenhouse with regulated illumination (1300-4700 W m^{-2}), humidity (74%), and a photoperiod of 16 hours of daylight and 8 hours of light. Shoot apices were selected as an explant and after surface sterilization with Bavistin and Mercuric chloride, axenic cultures were established and *in vitro* shoot proliferation was carried out under the optimized tissue culture conditions in plant tissue culture laboratory JUIT, Waknaghat, India. *S. chirayita* shoots were kept in tissue culture room of JUIT lab. Shoots were raised in MS media [203] that had Indole-3-Butyric Acid (IBA) 3 mg/l and Kinetin (KN) 1 mg/l growth hormone concentrations. On completion of 30 days, plants were regularly sub cultured and different tissues were used for further experimentation as mentioned below.

3.2 Quantification through RP-HPLC

3.2.1 Plant sample preparation

All the required samples were shade dried and further placed into 100 ml of 85% MeOH. Vortexed samples were used and kept for some time. Same day, the samples were further sonicated for ten minutes with a 2 second pulse at 30% amplitude. Following sonication, 10,000 rpm centrifugation was carried out for ten minutes. The leftover pellet was discarded and the supernatant was saved for further use. The following day, 0.22 µm syringe filters were used to filter the supernatant. After being diluted to a concentration of 10X, the filtrate was injected onto the column and utilised to quantify Amarogentin and Mangiferin.

3.2.2 RP-HPLC procedure

Agilent 11,200 series HPLC system, HPLC Pump C18 (5 m) Waters column, and PDA detector (Waters 2996) were used to quantify the bioactive compounds. Solvent A (Trifluoroacetic acid 0.1%) and Solvent B (a combination of acetonitrile and H_2O in the proportion of 70:30), were utilised as the solvent systems. Amarogentin and mangiferin were isocratically eluted from the column at a flow rate of 1.0 ml/min and a wavelength of 270 nm. At 25°C, the cycle lasted 30 minutes. On the basis of their retention time, Amarogentin and Mangiferin were examined using Chromadex, Inc. criteria.

3.2.3 RP-HPLC procedure confirmation by using calibration curves

Stock solutions of Aamarogentin and Mangiferin (1mg/ml) were prepared in 85% methanol and further five concentrations were diluted from the stock solution i.e. 10, 20, 30, 40, 50µg/ml (Standard Amarogentin and Mangiferin).

3.3 Establishment of different developmental stages of tissue cultured *S. chirayita* at two different temperature 15 ± 1˚C and 25 ± 1˚C

Different developmental stages were established in tissue cultured *S. chirayita* by optimizing nutrient media conditions to know maximum accumulation of major medicinal constituents in these respective stages.

3.3.1 Initiation of callus establishment stage

The leaf discs were used for initiating callus formation. In order to de-differentiate into calluses, leaf discs were excised from tissue raised shoots that had been cultured *in vitro* for four weeks. The de-differentiation media contained MS + 2,4-D (1 mg/l), 6-BAP (0.5 mg/l), TDZ (0.5 mg/l), 0.8% agar-agar, 3% w/v sucrose, and pH 5.7. MS medium was then transferred into a 250 ml flask (Borosil) and around thirty ml of media poured in the culture jars. The bottles were then incubated under (White fluorescent light (WFL) at 3000 lx intensity, 15 ± 1˚C and 25 ± 1˚C temperature, humidity (74%) with 16 hour of day and 8 hour of light) in the tissue culture chamber at JUIT. A set of 6 explants were used in each experiment, which was conducted three times. Following the inoculation of explants, a green colour callus started to grow after two weeks, and a callus mass started to form after four weeks.

3.3.2 Complete regeneration of plant

After achieving the complete callus stage, for re-differentiation, callus mass was also transferred to shoot generating medium. The sole difference in the nutritional medium's basic composition was the concentration of growth hormone. For carrying out re-differentiation, IBA 3 mg/l and KN 1 mg/l were used, which leads to development of quality rich shoots.

3.3.3 RP-HPLC quantification of bioactive compounds at various developmental phases

Samples at different developmental stages were collected and bioactive compounds (Amarogentin and Mangiferin) were quantified in triplicates by using a previously validated RP-HPLC method **(Section 3.2.2).**

3.4 Elicitation experimentation using light emitting diodes (LED's)

3.4.1 Establishment of shoot cultures under controlled tissue culture conditions by using LED lighting

The shoot cultures were established under controlled tissue culture conditions **(Section 3.3.2)** by using various LEDs: red (100%, ≈ 660nm wavelength), blue (100%, ≈ 460nm wavelength), green (100%, ≈ 550nm wavelength), "RGB: 40% Red, 40% Green and 20% Blue" at 15˚C ± 1 inside culture room. The White fluorescent light (WFL) was used as control under controlled tissue culture conditions **(Section 3.1)**. For 30 days, different time intervals were used to measure the amount of biomass andto quantify bioactive compounds accumulating in *S. chirayita* shoot cultures (0, 5, 15, 21, 25 and 30th day).

3.4.2 Analytical methods

3.4.2.1 Growth determination of shoot cultures

Shoots were taken out of the media bottles, the remaining water was pressed onto Whatman^{TM} filter paper, and the weight of the shoots was measured to calculate their fresh weight (FW). Shoots were simultaneously dried using oven at 40˚C, weighed, and the dry weight (DW) of the medium employed was expressed as (g/l). After that, on the 30th day, the growth index (GI) of shoot cultures was deliberated in accordance with Ketchum et al. [205].

$$\mathbf{GI = W_f - W_i / W_i}$$

W_f, Final dry weight of shoots on 30^{th} Day

Wi, Initial dry weight on first day

Different growth parameters like biomass of the plant, shoots and roots length (cm) and number were examined on day 30.

3.4.3 Phytochemical analysis of light treated plant extracts

3.4.3.1 Plant sample preparation and extraction

Every plant sample was processed and extracted utilising a previously proven method **(Section 3.2.1)**.

3.4.3.2 Quantification of bioactive compounds through RP-HPLC

Bioactive compounds were measured using an RP-HPLC technique that has previously been verified **(Section 3.2.2).**

3.4.3.3 Determination of TPC and TFC using plant extracts

3.4.3.3.1 Total phenolics content

The TPC of extracts from *S. chirayita* incubated under various LED lights was assessed using a modified version of the Kim et al. [205] methodology. In this process, 0.2 ml of extract from plant and 0.3 ml of distilled water were combined, and the sample also received 0.15 ml of FC reagent. After that it was properly mixed and then incubated for 5 minutes at room temperature. 0.5 ml of 20% Na_2CO_3 was then added afterward. After gentle mixing, sample was further incubated for an hour in a darkened area. A UV-visible spectrophotometer was used to detect absorbance at 750 nm. A calibration curve was plotted using Gallic acid (100-500 mg/ml) as standard. The following regression equation, $y = 0.003x - 0.046$, $r^2 = 0.996$, was used to compute the amount of phenolics was termed as (mg GA/g DW).

3.4.3.3.2 Total flavonoid content

The TFC was deliberated using Ebrahimzadeh et al. [206] method by using the extract of *S. chirayita* plants incubated under various light treatments with certain modifications. 0.1 ml of extract from plants was mixed 0.1 ml of 10% AlCl2, and 0.4 ml of methanol. The final volume was then increased to 4 ml using distilled water before being given 0.1 ml of 1M sodium acetate. After that, it was incubated at RT for 30 minutes. The absorbance was measured at 415 nm. The calibration curve was produced using quercetin as the reference (100–500 mg/ml, Sigma-Aldrich). After determining TFC using the regression equation ($y = 0.001x - 0.019$, $r^2 = 0.993$), TFC was represented as (mg QE/g DW).

3.4.4 Determination of total antioxidant activity

3.4.4.1 DPPH Free-radical scavenging assay

Using a modified version of the method deliberated by Yesmin et al. [207], *S. chirayita* shoot culture extracts was assessed using the DPPH free radical scavenging assay under various LED lights. Shortly, 3 ml of methanolic DPPH (0.004%) solution was added to 50 µl of plant extract. After that, it was incubated at ambient temperature for 30 minutes in dark. Using a UV-Visible spectrophotometer, the absorbance was taken at 517 nm. With BHT (Sigma-Aldrich) as the standard, the free RSA of several extracts placed under various LED lights were compared. Readings were recorded in triplicates. The DRSA (%) was calculated as follows:

DRSA (%) = (^{Abs} control - ^{Abs} Sample)/^{Abs} control*100

^{Abs} control: DPPH absorbance

^{Abs} sample: DPPH absorbance with extract

3.4.4.2 ABTS activity

ABTS was deliberated using Re et al. [208] method, with slight modifications, the antioxidant activity of *S. chirayita* extracts were assessed by ABTS assay under various LED lights. Sample extract mixed with ABTS+ solution is added with ethanol for dilution and PBS for plasma antioxidants at pH = 7.5, absorbance 0.7 (± 0.02) at 734 nm at 30˚C. Addition of 10 µl sample extract wt. 1.0 ml of dilute ABTS+ solution (Ab 734 nm = 0.700±0.020) in

ethanol or PBS is carried out followed by absorbance reading at 30˚C between 1minute – 6 minutes of initial mixing. Readings were recorded in triplicates.

3.4.5 Acclimatization of LED elicited plants

The plants were transferred from the culture conditions to the greenhouse containers that contained cocopeat, sand and vermiculite in the proportion of (1:1:1 v/v). Plants successfully acclimatized in the outer environment.

3.5 Establishment of somatic embryogenesis and their cryopreservation

3.5.1 Plant selection and callus induction

Plant selection and callus induction were done using previously published methods (**Section 3.1 and 3.3.1**).

3.5.2 Establishment of somatic embryogenesis

To obtain the optimal growth of somatic embryos, the established 4 week old callus cultures were once more sub cultured to the media (MS + 1 mg/l 2,4 D + 0.5 mg/l BAP + 0.5 mg/l TDZ) for an additional 3 weeks. Somatic embryos were removed from the culture as they reached the torpedo stage, dried, and dissected into minute pieces. Under a dissecting microscope, a little speck of tissue was placed on the slide. Torpedo-shaped structures with a yellowish green colour and heart-shaped structures were visible, making them easy to distinguish.

3.5.3 Scanning electron microscopy

Somatic embryos were fully dried and dehydrated before being dissected into the necessary number of little pieces for the experiment. The samples were then deposited on the rotating discs of the Joel fine coat ion sputter (JEOL, JFC 1600), where embryos were given a coating with colloidal gold, and specimen stubs were attached to them using carbon conductive adhesive tape. Images were then captured at various magnifications while the specimen stubs

were mounted to the specimen holder of a scanning electron microscope (JEOL JSM 6390LV) that was kept at an accelerating potential voltage of 20 kV.

3.5.4 Establishment of artificial seeds with osmoprotection and dehydration protocol

Encapsulation technique was used to produce artificial seeds. Torpedo-shaped somatic embryos were collected and then dissolved in a solution containing sodium alginate at different concentrations (1%–5% in 100 ml of water). To accomplish encapsulation, selected somatic embryos were mixed in the sodium alginate mix in the beaker. For the encapsulation of complete embryos, sodium alginate (1-5%) and 0.85 M of sucrose solution with the embryos was drop wise introduced into the (100 mM) calcium chloride which was placed onto the magnetic stirrer so that beads will attain the proper round shape for around 30-35 minutes. Encapsulated somatic embryonic beads were then transferred to liquid MS media to remove the unwanted calcium chloride, and the excess water was subsequently removed by drying the beads on filter paper. 15 somatic embryos that had been encapsulated were shifted to open petri plates and dehydrated for at least an hour in sterile air in a laminar airflow cabinet.

3.5.5 Determination of moisture content

Encapsulated somatic embryos were kept in 0.85 M sucrose under slow agitation for at least 40 minutes in order to measure the moisture content. At each time-interval, the fresh weight of the encapsulated embryos was recorded. These were then dried for at least an hour in a cabinet with laminar airflow. Dehydrated embryos were dried under oven at 40˚C for 15 hour of time period and weighed.

% of moisture content = Initial weight - Dry weight

3.5.6 Establishment of cryopreservation and regeneration of encapsulated seeds

Dehydrated encapsulated seeds were placed in sterile cryovials (2 ml) containing 4 encapsulated seeds each, and the cryovials were then placed straight into liquid nitrogen for an hour to perform cryopreservation. Cryovials containing frozen encapsulated seeds were rapidly transferred to water bath for rewarming at 30˚C for 2-3 minutes. After being taken out of the cryovials, these somatic embryonic seeds were then placed to MS media for shoot

regeneration. Encapsulated seeds were then transferred to MS media containing different growth regulators MS + 1mg/l IBA+ 2mg/l KN + 3mg/l GA_3, 2mg/l BAP+ 1mg/l GA_3, 3mg/l IBA+ 1mg/l KN, 1mg/l NAA+ 0.5mg/l KN, 0.5mg/l NAA+ 1mg/l BAP+ 1mg/l GA_3, 1mg/l KN+ 0.5mg/l BAP+ 1mg/l NAA for regenerating into shoots.

3.5.7 Quantification of bioactive compounds through RP-HPLC

Regenerated shoots from somatic embryos were utilized for quantification of Amarogentin and Mangiferin to crosscheck the effect of cryotreatments on the accumulation of bioactive compounds. Using a previously validated RP-HPLC method, the Amarogentin and Mangiferin were quantified **(Section 3.2.2).**

3.5.8 Acclimatizatio and transplantation of cultured plants to fields

Using a previously acclimatization protocol plants were transferred to the outer environment **(Section 3.4.2.2.5).**

3.6 Comparative analysis of *in vitro* raised plants (IVP) with field grown plants (FGP)

3.6.1 Plant sample preparation and extraction

For carrying out comparative analysis, tissue samples (leaves, shoots) were procured from ≈3 month old IVP and FGP. After the collection of required samples, shade dried and subjected to methanolic extraction by soxhlet apparatus. Until their next use, extracts were kept at 4˚C.

3.6.2 Phytochemical screening

≈3 month old IVP and FGP were tested for phytochemical screening as mentioned below:

3.6.2.1 Qualitative analysis

a. **Test for carbohydrates** – 1ml of H_2O is added to 0.5 mg of leaf extract. Formation of yellow color upon addition of aqueous NaOH solution shows presence of glycosides [209].
b. **Test for protein** – A few drops of HNO_3 are added to the leaf extract and yellow color formation shows the presence of proteins [209].
c. **Test for alkaloids** – In 10 mg of leaf extract, add one or two drops of Wagner's reagent. Alkaloids presence was confirmed by reddish brown colour formation [209].
d. **Test for flavonoids** – Drops of 8% lead acetate solution when mixed with 10 mg of leaf extract, if forms yellow colored precipitates. This indicates occurrence of flavonoids [209].
e. **Test for phenols** – Addition of 5 ml water and 5% aqueous ferric chloride into the leaf extract, if the leaf extract is blue or green in colour. This is a sign that phenols are present [209].
f. **Test for tannins** – 0.5 ml of 5% $FeCl_2$ is added to 5 mg of leaf extract. Dark bluish black color shows occurrence of tannins in the extract sample [209].
g. **Test for sterols** – 5 mg leaf extract dissolved in 2l chloroform is treated with concentrated sulphuric acid with careful addition along the sides of the test tube. Red precipitate formation shows occurrence of sterols [210].

3.6.2.2 Quantitative analysis

3.6.2.2.1 Carbohydrate estimation

Using the Anthrone method, carbohydrates were estimated by adding 1 ml of plant extract to 4 ml of anthrone reagent and incubating the mixture for approximately 8 minutes in a boiling water bath. The absorbance against a reagent blank is recorded at 630 nm in triplicates. Results are displayed as mg/g DW samples [211].

3.6.2.2.2 Protein estimation

The Lowry method was used for estimating protein which involves mixing 2 ml of plant extract with 5 ml of fresh alkaline copper reagent, incubating the same at RT for 10 minutes.

Fresh FC reagent, 0.5 ml, is now added, and it is once more incubated for 30 minutes at room temperature in a dark area before being well mixed. Appearance of blue colour at 660 nm is recorded in triplicates [212].

3.6.2.2.3 Determination of total phenolics and total flavonoid content

The TPC and TFC were estimated using above mentioned method **(Section 3.4.3.3.1 and 3.4.3.3.2).**

3.6.3 Determination of total antioxidant activity

The ABTS and DPPH free RSA was determined using above mentioned method **(Section 3.4.4).**

3.6.4 Physiochemical characterization

Physiochemical characterization of ≈3 month old *in vitro* and field derived leaf extracts of *S. chirayita* were screened for various parameters. The following parameters were carried out on the extracts as per the British Pharmacopoeia [213]:

a. Determination of Moisture Content

Moisture content determination was conducted using 10 g powdered leaf material in a pre-weighed watch glass/moisture box over night in an oven within the temperature range between 100 – 105˚C. The sample was cooled at room temperature and desiccated before observing the residual weight. The weight loss in the sample depicts the moisture content [22]. Calculation was done by the formula mentioned below:

% Moisture = (Total weight – Final weight) / Weight of the sample x 100

b. Total ash

3g of each field grown and *in vitro* powdered leaf extracts of *S. Chirayita* were weighed and subjected to a pre ignited and weighed silica crucible. A fine layer of the powder was evenly spread at the bottom. The powder was burned with a

gradual increase in temperature to obtain a dull red hot appearance. The crucible was cooled and weighed repeatedly to obtain a constant weight. Total ash percentage was calculated [23].

c. Acid insoluble ash

The ash produced by the aforementioned technique was heated in 2N HCl for 5 minutes. The insoluble ash was collected on ash-free filter paper, washed with hot water, and then placed in a crucible for cremation. The residue was then weighed, and calculations were made using the air-dried sample as a guide [24].

d. Water soluble extractive

100ml of distilled water and 1gm of sample were macerated in a closed flask for 24 hours with regular shaking before being filtered. 25ml of filtrate was decreased by evaporation in a shallow dish with a flat bottom and tar, and then it was further dried at 100˚C and weighed. The water soluble extractive percentage was then determined using the medications that had been air dried [214].

e. Ethanol soluble extractive

Similarly 1 gm of sample was macerated with 100ml ethanol for 24 hours in closed flask. Rapid filtration to avoid loss of ethanol was done. Filtrate (25ml) was then evaporated, dried (100˚C), weighted and calculated in reference to air dried drug [214].

3.6.5 Determination of biological activities by the following test

3.6.5.1 Antimicrobial activities

The antimicrobial activities were examined against *Staphylococcus aureus* (MTCC3160), *Salmonella typhi* (MTCC98), *Bacillus subtilis* (MTCC121) and *Esherichia coli* (ATCC25922) using agar gel diffusion method and determining MIC.

3.6.5.1.1 Agar gel diffusion assay

Anti bacterial properties was tested using Agar well diffusion method [215]. A 100 µl test samples (10^6-10^8 cells/ml) were spread on nutrient agar plates. A (30 mg/ml) of 50µl was

added in each agar wells made by cork borer (6 mm diameter). Than after 24 hours zone of inhibition (mm) was measured DMSO and Ampicillin was used as controls.

3.6.5.1.2 MIC (Minimum inhibitory concentration) assay

To carry out MIC assay CLSI guidelines were followed [216, 217]. A 2X diluted plant extracts (30 mg/ml) was added in the microtitre plates followed by 1:1 serial dilution, until lowest dilution was reached. Turbidity was observed after incubated for 24 hours at 37˚C. Resazurin dye was used to check viability of bacterial cells.

3.6.5.2. Anti – inflammatory activity

Using the Gunathilake et al. [218] protocol with certain changes, Anti – inflammatory activity (Human red blood cell (HRBC) membrane stabilization test) was assessed. Whole human blood was collected and centrifuged at 3000 rpm for 5 minutes in heparinized centrifuge tubes, followed by three times washing with normal saline solution. Thereafter, 10% v/v erythrocyte suspension was reconstituted with normal saline. The 3 ml reaction mixture consisted of 0.05 ml of plant extract, 0.05 ml of blood cell suspension and 2.9 ml of phosphate buffer saline (7 pH). Then incubation of 20 minutes at 54℃ was given to the reaction mixture, followed by centrifugation at 2500 rpm for 3 minutes and the absorbance of the supernatant was recorded at 540 nm. For the positive control, aspirin was used. The level of protection was delibrated as follow:

% protection = [(^{Abs} of control – ^{Abs} of sample) / ^{Abs} of control × 100]

3.6.5.3 Anti - diabetic activity

Using the Mahindrakar et al. [219] technique with certain changes, anti-diabetic activity (Alpha-Amylase Inhibitory Assay) was assessed. Plant extract (500 µl) along with Acarbose (standard, at the range of 10 – 100 µg/ml) were added to alpha amylase enzyme (2 units/ml) solution and incubated for 10 minutes at 28˚C. Starch solution made in sodium phosphate

buffer was added to above at incubated again for 10 minutes at 28˚C. Finally 1 ml of DNS reagent is added at 100˚C for 10 minutes to stop the reaction. Absorbance was determined at 540 nm. % α- amylase inhibition is determined by using the equation:

$$\% \text{ inhibition} = \frac{^{Abs}\text{ Control} - {}^{Abs}\text{ Sample} * 100}{^{Abs}\text{ Control}}$$

3.6.5.4 Anticancer activity

Plant extracts were tested against HepG2 (liver) and MCF-7 (breast) cancer cell lines in addition to WI38 as normal cell line using DMSO as vehicle control. Anti cancer activity was tested using the following:

3.6.5.4.1 To check Cell Viablity using PrestoBlue Test

HepG2 and MCF-7 cell lines were seeded in 24 well plates (50,000 cells/well) using EMEM + 10% FBS and DMEM + 10% FBS media, respectively, and the vitality of the cells was then tested by incubating the sample for 24 hours at 37 ℃ with 5% CO2. After 48 hours, plant extracts at a gradient concentrations (0, 25, 50, 100, 250, and 500 µg/ml) were added. The cell activity was measured using the PrestoBlue method (Invitrogen, A13261). For this, each well received 50 µl of PrestoBlue, which was then left to incubate for 20 minutes at 37 ℃ with 5% CO2. Finally the absorbance was taken at 570nm using ELISA plate reader (USA). Plant extract concentration that lessens the number of viable cells into half i.e. IC_{50} was checked by using OriginPro 8.5 Software.

3.6.5.4.2 Annexin V–FITC/AAD Apoptosis detection

HepG2 cells were seeded at $5*10^4$ cells/ml in 6 well plate incubated with 2 ml EMEM + 10% FBS for 24 hours. Then, these cells were given treatment with 88 (IC_{50}) and 44 µg/ ml of extract for 48 hours after which they were twiced washed in cold 1XPBS and then reconsituted in 100 µl of 1X Annexin binding buffer. Annexin V – FITC (5 µl) and 7 – AAD (5 µl) was used for staining cells using Biologend Kit, 640922. For this, treated cells were

incubated for 15 minutes in dark and readings were taken using flow cytometer at ACEA 3000 USA.

3.7 FTIR analysis

3.7.1 Sample preparation for FTIR analysis

Tissue cultured and field grown plant sample were processed and extracted utilising a previously proven method **(Section 3.1)**.

3.7.2 FTIR method

The FTIR is regarded as one of the most potent analytical instruments for the identification of chemical functional groups in unidentified compounds. Methanolic plant extract powders were used for the FTIR analysis. Pallet sample discs made of 10 mg of powdered extract enclosed in 100 mg of GBR were loaded onto an FTIR spectroscope (Shimadzu, IR Affinity - 1, Japan) with a scan range of 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

3.8 LC-MS analysis

3.8.1 Sample preparation for LC-MS analysis

Tissue cultured and field grown plant sample were processed and extracted utilising a previously proven method **(Section 3.1)**.

3.8.2 LC-MS method

The identification of the bioactive chemicals found in plant extracts was done using LC-MS analysis. Direct injection mode was used in addition to Electron Spray Ionization (ESI) probe at 28°C capillary temperature, 8µl/ min of sample flow rate within the mass range of 50 – 1000 m/z. The factors were optimized for the most favourable ionization. The bioactivity of phytochemicals evaluated by LC-MS was examined using the phytochemical database.

3.9 Statistical analysis

Data were provided as mean SD (Standard Deviation) and all tests were carried out in triplicates. The Duncan multiple range test was used with one-way ANOVA to examine differences in group averages. P values less than 0.05 were regarded as significant. All of the statistical analysis was conducted using SPSS Software (SPSS 20.0, USA). To produce the figures, MS Office (Window Version 10, USA) was utilised.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Callus induction and formation of different developmental stages

Tissue culture techniques were used to achieve different developmental stages passing through various morphogenetic events, such as the de-differentiation of leaf segment into callus mass and the subsequent re-differentiation of callus into shoot primordia and fully grown and elongated shoots. For *S. chirayita*, leaf discs segments (1 cm in daimeter) responded to the induction of callus in all provided media combinations at 25 ± 1˚C and 15 ± 1˚C. This may be explained by how various plant species and plant parts react to internal plant growth regulators in similar tissue culture conditions. Our results showed formation of green callus within 10-15 days after inoculation of leaf explants on MS media supplemented with different growth hormone combinations 2,4D, 6BAP and TDZ at 25 ± 1˚C and 15 ± 1˚C. Further callus mass observed in 16-30 days on MS media supplemented with 2,4D, 6BAP and TDZ at 25 ± 1˚C and 15 ± 1˚C (Figure 4.1). These callus cultures were then added to shoot regeneration medium including MS basal media in a range of BAP and KN concentrations. Shoot initiation and multiple shoot formation were observed best in lower concentration of KN. Patial et al. also reported for the use of KN at low levels for the start of shoots in *in vitro* cells. Shoots were afterwards moved to rooting media, namely MS basal medium enriched with IBA (3 mg/l) and KN (1 mg/l), to aid in rooting. Fully elongated shoots with complete growth were observed at 80^{th} day. A set of 6 explants per treatment was cultured on media bottles and every experiment was repeated for at least three times. Elongated shoots and thicker leaves were observed better at 15 ± 1˚C in comparison to 25 ± 1˚C. This might be explained by the low temperature-induced formation of hemicelluloses that promotes the growth as well as help in the thickening of leaves [220].

	Temperature	15±1℃	25±1℃
	Developmental Stage	Leaf Segment	Leaf Segment
1.	Plant Segment as leaf disc explants (0 Days) (2,4D = 1mg/L, 6BAP = 0.5mg/L, TDZ = 0.5mg/L)		
2.	Callus initiation (1-15 Days) (2,4D = 1mg/L, 6BAP = 0.5mg/L, TDZ = 0.5mg/L)		
3.	Callus mass development (16-30 Days) (2,4D = 1mg/L, 6BAP = 0.5mg/L, TDZ = 0.5mg/L)		
4.	Shoots Primordia development (31-50 Days) (BAP = 2 mg/L, KN = 1 mg/L)		
5.	Manifold shoot formation (51-65 Days) (BAP = 2 mg/L, KN = 1 mg/L)		
6.	Shoot elongation with complete growth (66-80 Days) (IBA = 3mg/L, KN = 1mg/L)		

Figure 4.1 *In vitro* morphogenic stages of *S. chirayta*, Scale bar = 1cm.

4.2 RP-HPLC method for quantification of bioactive compounds

Synthesis of amarogentin and mangiferin were observed at different developmental phases of *S. chirayita* at two varying temperatures 25 ± 1˚C and 15 ± 1˚C. The estimation of amarogentin and mangiferin accumulation was done by using RP-HPLC method (Figure 4.2, 4.3). At the initiation, leaf explants were used as first stage, where accumulation of

Amarogentin was 4.72 µg/mg, 4.41 µg/mg and Mangiferin was 15.54 µg/mg, 9.70 µg/mg at 15 ± 1˚C and 25 ± 1˚C reported (Table 4.1).

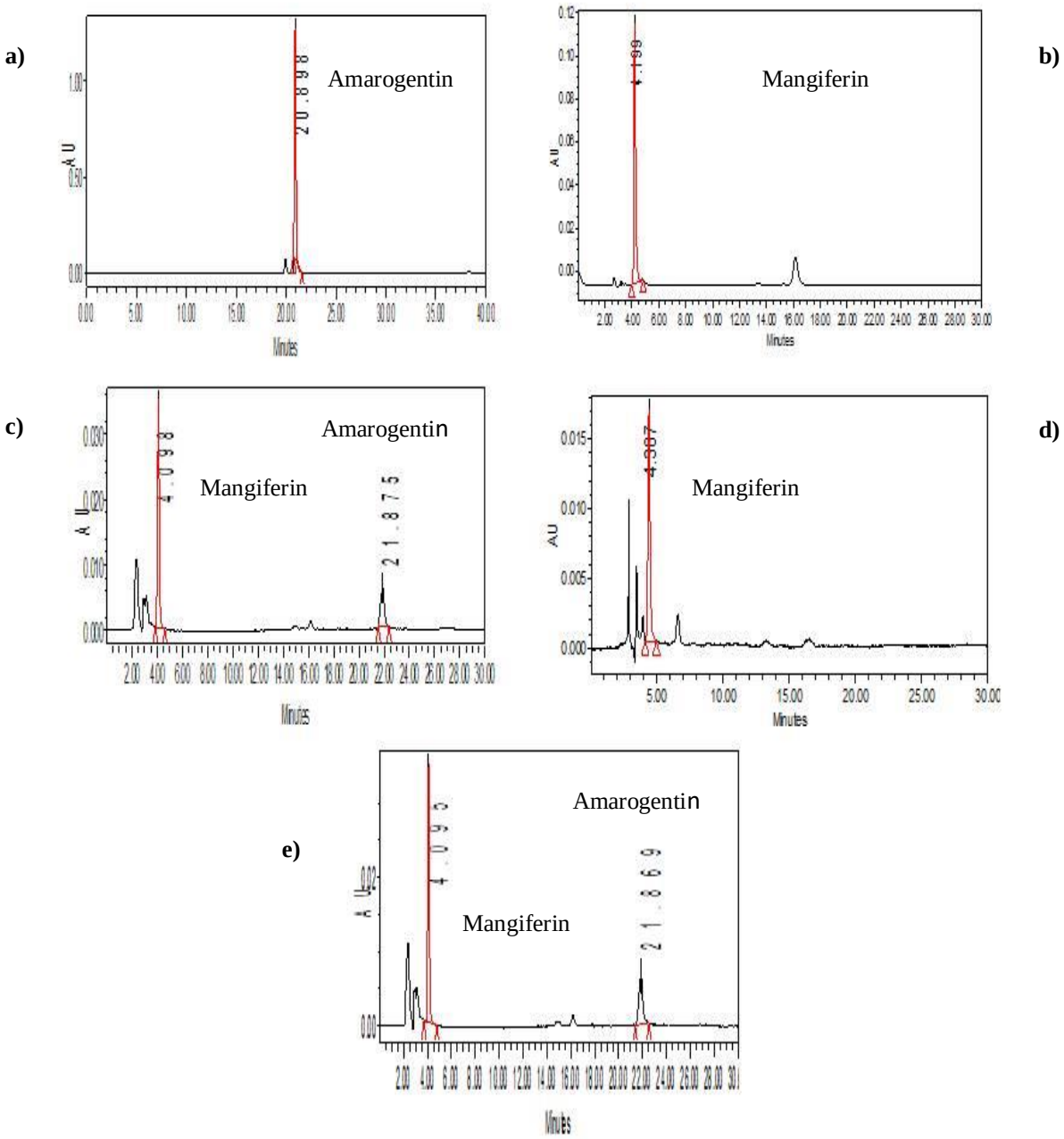


Figure 4.2 HPLC Chromatogram representing peaks at **15±1˚C** a) Amarogentin standard, b) Mangiferion standard, c) Leaf segment, d) callus cultures, e) Elongated shoots.

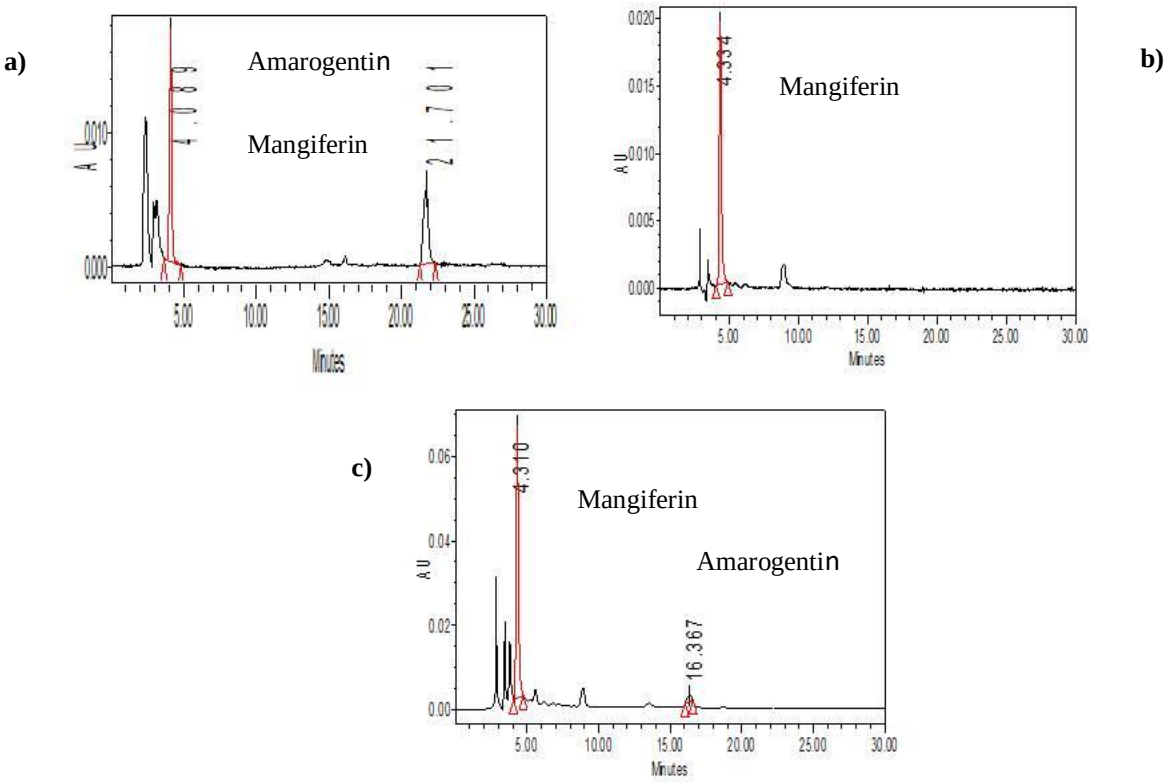


Figure 4.3 HPLC Chromatogram representing peaks at **25±1˚C** a) Leaf segment, b) Callus cultures, c) Elongated shoots.

Table 4.1 Amarogentin and Mangiferin quantified at developmental stages of tissue raised *S. chirayita*.

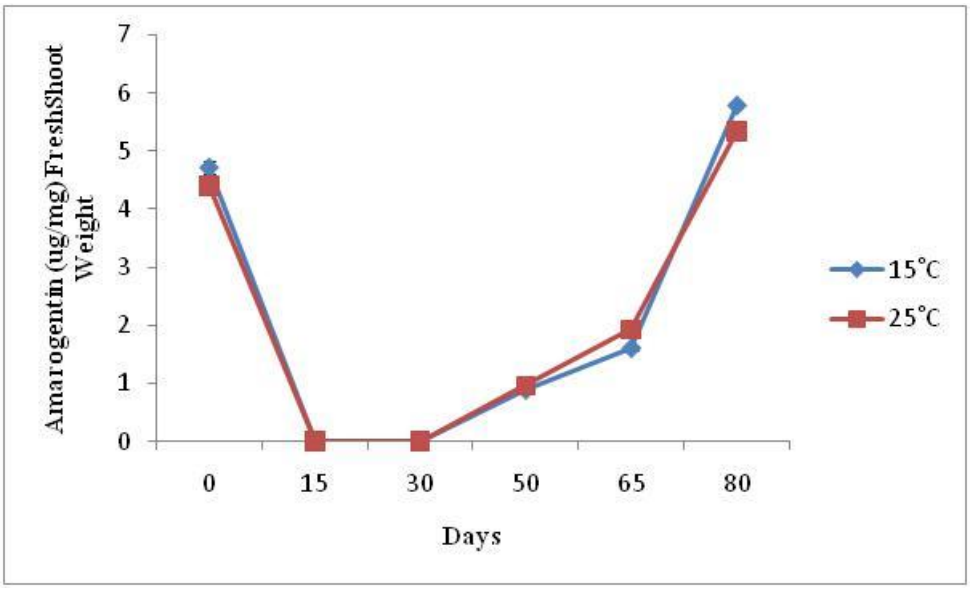
Amarogentin Content (µg/mg)			Mangiferin Content (µg/mg)	
15±1˚C	25±1˚C		15±1˚C	25±1˚C
4.72±0.11	4.41±0.02	1 Plant Segment (0 Day)	15.54±0.05	9.70±0.32
0.00±0.00	0.00±0.00	2 Initiation of callus formation (0-15 Days)	4.67±0.03	5.67±0.06
0.00±0.00	0.00±0.00	3 Callus Mass development (16-30 Days)	8.57±0.03	8.93±0.02
0.89±0.04	0.96±0.02	4 Shoot Primordia (31-50 Days)	11.32±0.05	9.10±0.17
1.61±0.03	1.93±0.03	5 Mainifold shoot formation(51-65 Days)	14.34±0.02	9.84±0.01
5.79±0.05	5.35±0.05	6 Complete growth (66-80 Days)	15.56±0.05	13.15±0.04

As the leaf explants starts to de-differentiate into callus, the accumulation of bioactive compounds also start declining (Figure 4.4, 4.5). The amount of Amarogentin was almost negligible and Mangiferin content was also less 8.57 µg/mg, 8.93 µg/mg at 15 ± 1˚C and 25 ± 1˚C, but higher in comparison to Amarogentin. As per the completion of almost 40-50 days, callus mass undergone to re-differentiation to form shoot primordial. Further the non-detectable content of the Amarogentin started to increase during shoot primordial formation along with hike in the Mangiferin content was also observed. On completion of 80th day of the culture, leaf discs were transformed into fully developed plants with the elongated shoots, almost equal content of Aamrogentin and Mangiferin was evaluated 5.79 µg/mg, 5.35 µg/mg and 15.56 µg/mg, 13.15 µg/mg at 15 ± 1˚C, 25 ± 1˚C (Table 4.1). Bioactive compounds

accumulate in disparate manner in shoots, which may reflect changes in their development and patterns of growth.

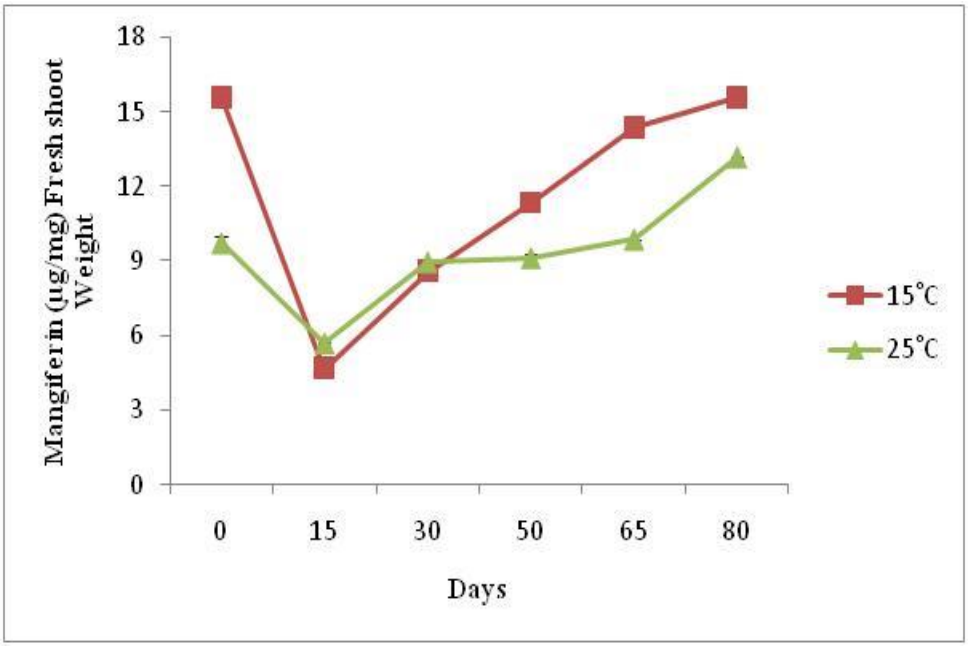
From such results, we can conclude that defined phase during the development of plant under the tissue culture condition is the key to controlling the synthesis of secondary metabolites. Since some stages during the process of plant's development are not very clear so, we have highlighted here distinct developmental phases, starting with the explants and continuing through plant shoot growth. The current study is relevant to the reports given by Sood and Chauhan et al. [221]. Numerous studies [221, 222, 223, 224] have shown that the production and concentration of these important secondary metabolites varied from plant to plant and can change at various developmental phases. Only the shoot cultures of *Salvia officinalis* contained the carnosic acid, not the callus cultures [223]. Vindoline accumulation in *Catheranthus roseus* is found in shoot cultures but not in callus mass cultures [224]. Similar outcomes were shown in our results, where the majority of metabolites accumulated in shoot cultures and were barely detectable in callus. Amarogentin is absent in undifferentiated callus cultures and Mangiferin levels are lower in *S. chirayita* callus cultures, which may be due to a lack of cell machinery programming and balanced cell organisation required for metabolite synthesis in tissue culture conditions. Kumar et al. [54] reported, 1.03 µg/mg of Amarogentin and 2.99 µg/mg of Mangiferin in media fortified with IBA and KN, however in our study ≈5.62 and ≈5.20 folds higher levels of bioactive compounds was reported in tissue raised shoots. As such reports suggest that there is no synthesis of Amarogentin in the callus stage, whereas Mangiferin accumulates both in the de-differentiation as well as re-differentiation stages. As reported by Pradhan et al. [225] Amarogentin belongs to class of monoterpenes which is a secoiridoid glycoside and Mangiferin belongs to class of xanthones, wherein tissue culture system organogenesis favour monoterpene production but unable to produce it in the un-differentiated stage of callus [226].

As temperature is one of key factors that affect the synthesis of biomarker compounds, similarly in our study accumulation of bioactive compounds is found to be comparitively higher at 15 ± 1˚C than at 25 ± 1˚C (Table 4.1). This suggests that 15 ± 1˚C is much appropriate temperature for the growth of *S. chirayita* shoot cultures [227]. Similar findings show that picroside-1 level is higher at 15˚C than it is at 25˚C [228]. This could be attributed to the lower temperatures which can up-regulates the genetic expression of the secondary metabolites production, thereby enhancing the quality rich shoots [228].



Callus induction medium 0-30 Days **Shoot induction medium 30-80days**

Figure 4.4 Accumulation of Amarogentin at developmental phases of *S. chirayita*. Mentioned values are mean ± SD of three replicates.



Callus induction medium 0-30 Days **Shoot induction medium 30-80days**

Figure 4.5 Accumulation of Mangiferin at developmental phases of *S. chirayita*. Mentioned values are mean ± SD of three replicates.

4.3 Quantification of bioactive compounds in the field grown and tissue cultured plants

As the tissue cultured shoots of *S. chirayita* (≈3 months old) accumulated maximum amount of bioactive compounds at 15 ± 1˚C, so to confirm the quantified metabolites, further comparison was evaluated with the field grown *S. chirayita* (≈3 months old) through RP:HPLC analysis. It was reported from our findings that tissue cultured shoots of *S. chirayita* (≈3 months old) accumulated 5.79 µg/mg Amarogentin and 15.56 µg/mg Mangiferin, whereas field grown *S. chirayita* (≈3 months old) accumulated 0.68 µg/mg Amarogentin and 3.80 µg/mg Mangiferin respectively (Figure 4.6). According to the above findings, Amarogentin concentrations were 8.51 times higher and Mangiferin concentrations were 4.09 times higher in *in vitro* grown shoots (≈3 months old) than in field grown shoots (≈3 months old).

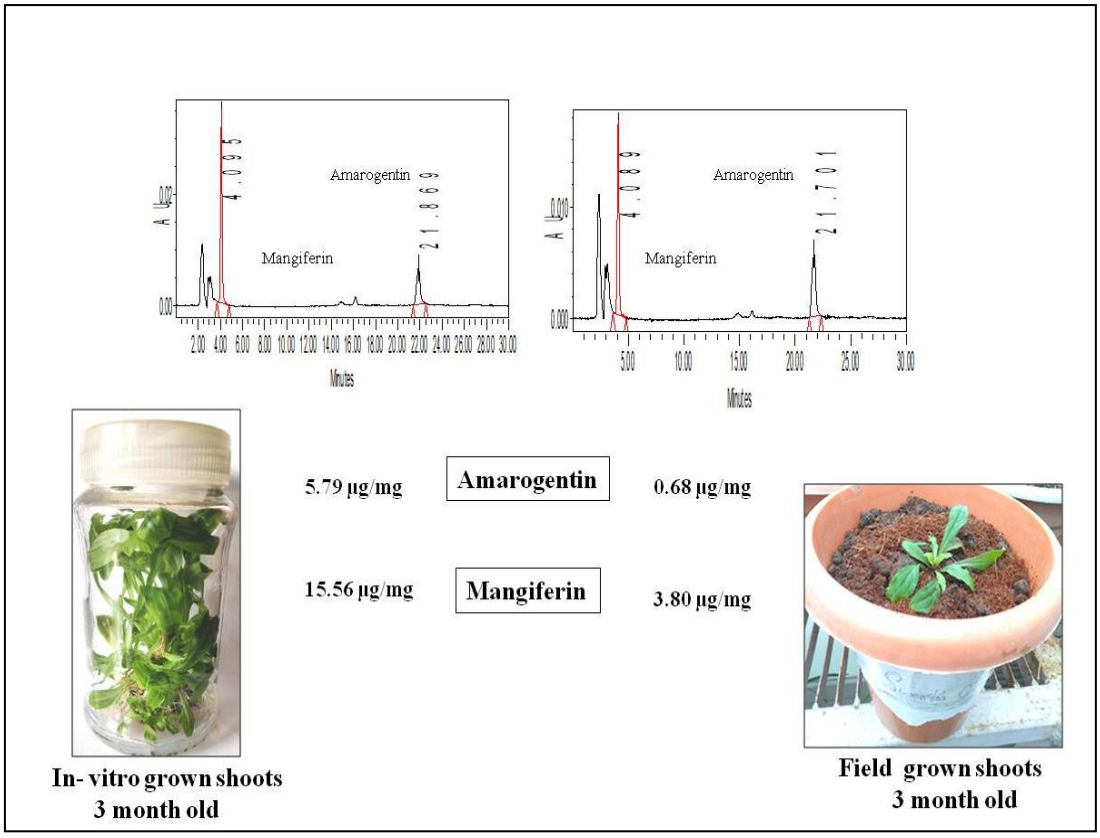


Figure 4.6 Comparative analyses of bioactive compounds in field and tissue grown plants of *S. chirayita*.

Therefore, the above mentioned results for the very first time reveal the detailed distinctive developmental stages in tissue cultured shoots of *S. chirayita* along with the accumulation of bioactive compounds in each stage. As per the identification of optimum development stage further can be scaled up to the bioreactor level. Additionally, the increased number of metabolites that accumulate in tissue cultured shoots compared to shoots that are grown in the field will undoubtedly open up new avenues for the sale of high-quality, potent herbs to the pharmaceutical sectors. Additionally, it will offer year-round access to a rich supply of bioactive compounds for the development of herbal medicines.

4.4 Effect of LED lighting on *S. chirayita* shoots culture

Source of light regulates the growth of the plant by specific photoreceptor activation, such as that of the "cryptochrome, phytochrome, UV-B receptors" by blue, red, and far-red light, and ultraviolet light, respectively [229]. Till now, no information known about the light spectrum that will best promote the growth of shoot cultures in *S. chirayita*. Therefore, in this work we have examined the impact of varying light sources on the growth parameters in shoot cultures of *S. chirayita*. The cultures placed at different LED lighs conditions exhibit similar pattern for growth phases. However, there were significant variations in growth characteristics, morphology, dry weight and growth index under various LED lighting conditions (Figure 4.7, 4.8 and Table 4.2). On day 21, shoot cultures under Red LED incubation display the highest accumulation of biomass (3.03 0.01 g/l DW), in comparison to the other LED lighting treatments (Figure 4.7). The maximum increase in the shoots and roots length (6.13 ± 0.91cm, 3.09 ± 1.33cm) along with the rise in number of shoots as well as roots (5.51 ± 0.82, 1.94 ± 1.18) were observed in Red LED and followed up by Blue, WFL, RGB and Green LED's (Table 4.2). Slightly variations in the morphological patterns were noted in the shoot cultures incubated under various LED lighting conditions (Figure 4.7). The maximum GI was unveil by Red LED shoot cultures (3.06 ± 0.01) as compared to other LED lighting conditions (Figure 4.8). It is conceivable that red light increased the synthesis and activity of growth-related enzymes through the physiologically active form of phytochrome (Pfr) to boost biomass accumulation in shoot cultures [230]. Additionally, this might potentially be related to improved photosynthetic and photochemical efficiency [231]. Numerous studies [232] have also noted the significance of red light for chlorophyll synthesis and chloroplast

growth. Red light impact the development in *Picea abies* via controlling the synthesis of gibberellins, as shown by Ouyang et al., (2015) [233]. The above findings could assure the sustainable reservoir of pharmaceutically important bio resources in environment-amicable way.

Table 4.2 Growth parameters illustrated under different light qualities in *S. chirayita*.

Light Quality	Shoot length (cm)	Root length (cm)	No. of shoots/ explants	No. of roots/ Shoot
Red LED	6.13 ±0.91^{a}	3.09 ±1.33^{a}	5.51 ±0.82^{a}	1.94±1.18^{a}
Blue LED	4.14 ±0.61^{b}	2.71 ±1.34^{ba}	4.91 ±0.90^{a}	1.70 ±0.83^{a}
RGB LED	3.70 ±0.35^{cb}	1.18 ±0.65^{ba}	2.61 ±0.25^{b}	1.42 ±0.72^{a}
Green LED	2.61 ±0.37^{cb}	0.00 ±0.00^{b}	2.19 ±0.37^{b}	0.00 ±0.00^{a}
WFL (Control)	3.96 ±0.33^{cb}	2.11 ±1.12^{ba}	3.91 ±0.93^{a}	1.59 ±0.54^{a}

Experiment was repeated thrice and mentioned values were represented as mean ± SD with in a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan Multiple range test.

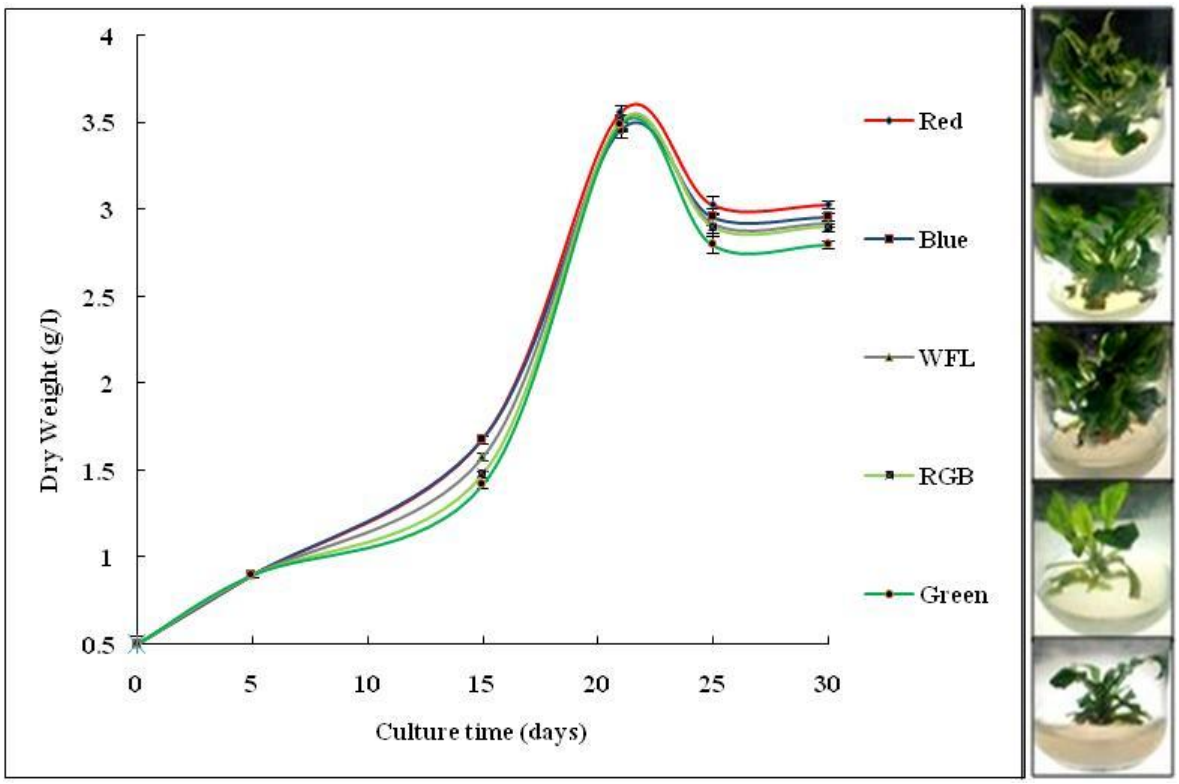


Figure 4.7 Effect of LED lighting on biomass accumulation. Mentioned values are mean ± SD of three replicates.

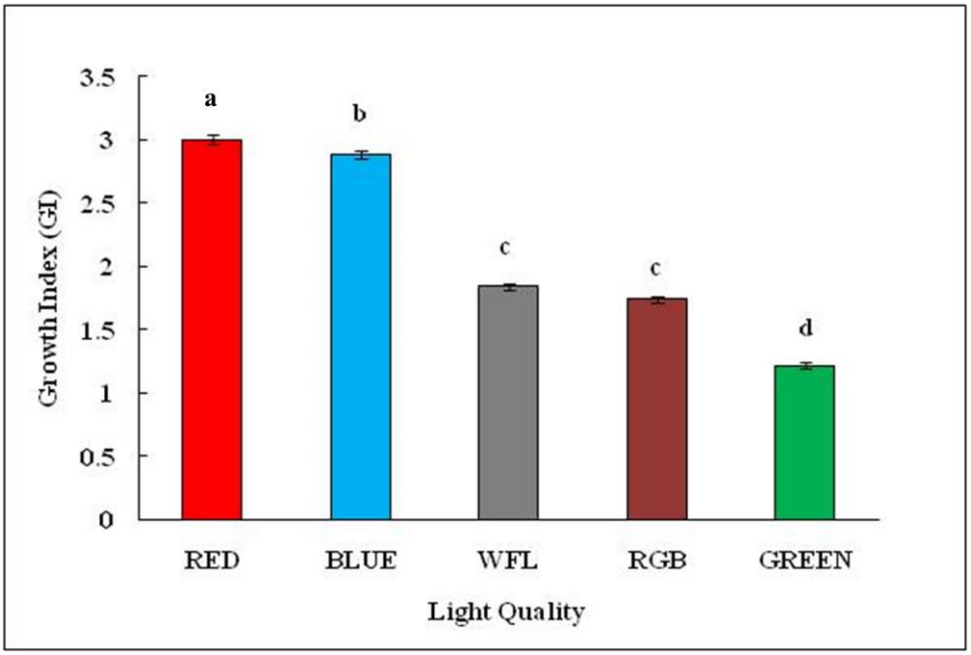


Figure 4.8 Effect of LED lighting on *S. chirayita* shoot cultures GI. Mentioned values are mean ± SD of three replicates in which means with similar letters are not significantly different at $p < 0.05$ according to Duncan's post-hoc test.

4.5 Influence of LED lighting on production of bioactive compounds in shoot cultures of *S. chirayita*

As an abiotic inducer, light efficiently controls the secondary metabolism of plants [234]. Numerous defense-related genes connected to the generation of secondary metabolites are controlled by light quality in cell cultures [235]. Previous studies have verified the effect of various lighting conditions on the production of secondary metabolites in various medicinal plants under tissue culture conditions [236, 237, 238, 239, 240, 241]. The significant fluctuations in amarogentin levels in the present finding were discovered by the RP-HPLC analysis of shoot cultures subjected to varied LED illumination settings. Amarogentin, however, was not found in shoot cultures that had RGB lighting applied to them. Moreover, Mangiferin was also lacking in all of the shoot cultures except WFL used as control, which calls for further investigations. The shoot cultures subjected to various spectral regimes exhibit comparable production trends. Between days 5 and 21, the Amarogentin accumulation rose exponentially, then from day 30, it gradually decreased. The shoot cultures treated with Blue LED showed maximum accumulation of Amarogentin (8.035 ± 0.04 µg/mg DW) on 21st day, in comparison to the other LED lighting conditions (Figure 4.9). The production of Amarogentin was observed to be growth-related in the shoot cultures of *S. chirayita*. However, Mangiferin was not produced in any treatments needs further investigations.

These results revealed for the first time a distinct effect of Blue light on the accumulation of Amarogentin in *S. chirayita* cell cultures. This might be explained by the significant levels of active phytochrome (Pfr), which controls the production of bioactive compounds biosynthetic genes [242].

It might also act as a defense against high ROS levels [242]. According to Ouyang et al. [233], Blue light increases the expression of multiple genes in the biosynthetic pathway of *Picea abies*, which in turn stimulates the production of phenolics. *In vitro* cultures of the *Saussurea medusa* showed increase in the jaceosidin accumulation under Blue light incubation [243]. Zhao et al. and Matsumoto et al. [243, 244] reported similar findings about the accumulation of cyanidin and chlorogenic acid when treated with the Blue light conditions. These results indicate how effectively light spectrum can control the production of Amarogentin in shoot cultures. According to the findings, Blue LED can actively be used as substitute medium to enhance the biotechnological production of Amarogentin in *S.*

chirayita tissue cultures. This elicitation technique holds powerful impact on both the therapeutic as well as pharmaceutical applications.

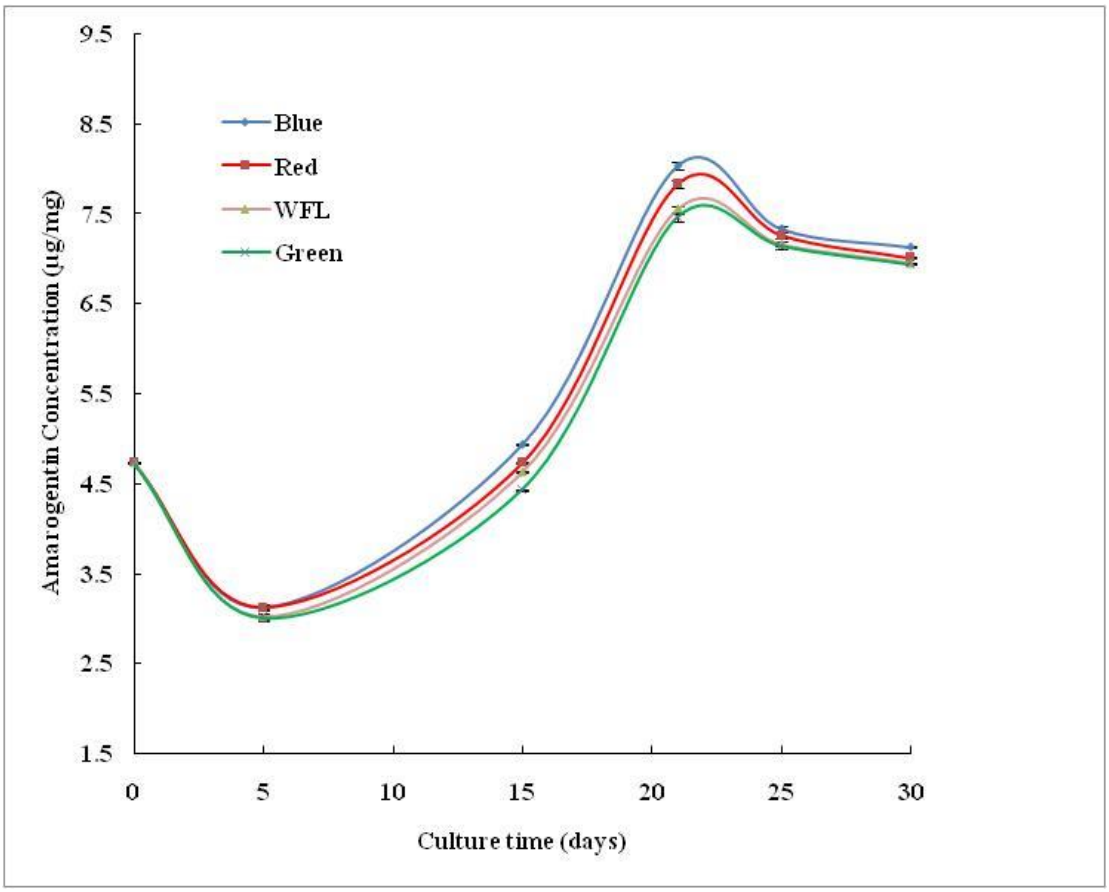


Figure 4.9 Effect of LED lighting on production of Amarogentin in tissue cultures of *S. chirayita*. Mentioned values are mean ± SD of three replicates.

4.6 Effect of LED lighting on TPC and TFC in shoot cultures of *S. chirayita*

Phenolic compounds are widely distributed throughout the plants. The primary class of phenolic compounds which help in scavenging oxygen free radicals is flavonoids. Due to their multiple biological defences as antiproliferative agents, antioxidants, antimutagens, antiatherogenic, and defenders against cardiac diseases, they have acquired tremendous relevance [245, 246, 247, 248]. The biological features and health benefits of phenolic compounds are numerous [249, 250, 251, 252]. A number of researchers have noted the significant influence of light treatment on the production of phenolics in the *in vitro* cultures [253], although other authors have shown that this impact is species-specific in phenolics synthesis [254]. In the present findings, shoot cultures treated with different light treatments

showed significant variations in TPC and TFC. The TPC in shoot cultures vary from (4.33-22.33 mg GA/g DW) whereas, TFC in shoot cultures vary from (13-29 mg QE/g DW of plants). Shoot cultures treated with Blue LED light conditions exhibit maximum total phenolic content (22.33 ± 1.05 mg GA/g DW) and total flavonoid content (29 ± 1.03 mg QE/g DW) in comparison to other LED's treatment followed up by Red, WFL, RGB and Green (Figure 4.10, 4.11). Recent investigations have demonstrated the extraordinary impact of blue LED on the increased synthesis of phenolics in *S. chirayita* shoot culture. According to various studies, exposure to blue light boosts the expression of the PAL, 4CH, CHS, CHI, F3H, FLS-2, UFGT, ANS, and MYBA1 genes via cryptochrome and phototropins [255, 256, 257]. This results in an increase in the creation of phenolic compounds. According to several reports jasmonic acid production was induced by Blue light treatment which uplifted the phenolics synthesis [258]. Additionally, Blue light can modulate the secondary metabolism to offer defence against biotic and abiotic challenges [31]. On the synthesis of phenolics in the cell cultures of *Saussurea medusa* [241], *Scutellaria lateriflora* [259], *Schisandra chinensis* [260], and *Stevia rebaudiana* [261], several researchers have shown a significant influence of Blue light treatments. These findings suggest that this elicitation strategy may significantly enhance the medicinal utility of cell culture systems. These findings suggest that in shoot cultures of S. chirayita, a certain type of light increases the production of phenolic chemicals. The results show for the first time that the application of Blue LED in the shoot culture system of *S. chirayita* can improve the synthesis of biologically active phenolic compounds that are simultaneously advantageous to health and valuable from an industrial viewpoint.

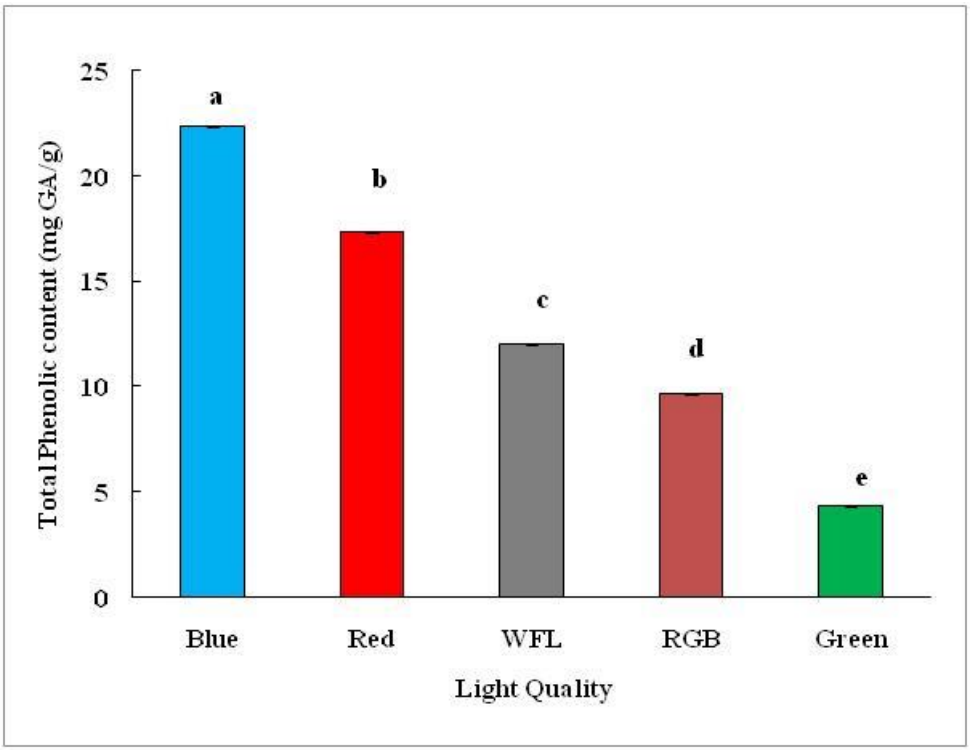


Figure 4.10 Effect of LED lighting on TPC in *S. chirayita* shoot cultures. Mentioned values are mean ± SD of three replicates in which means with similar letters are not significantly different at $p < 0.05$ according to Duncan's post-hoc test.

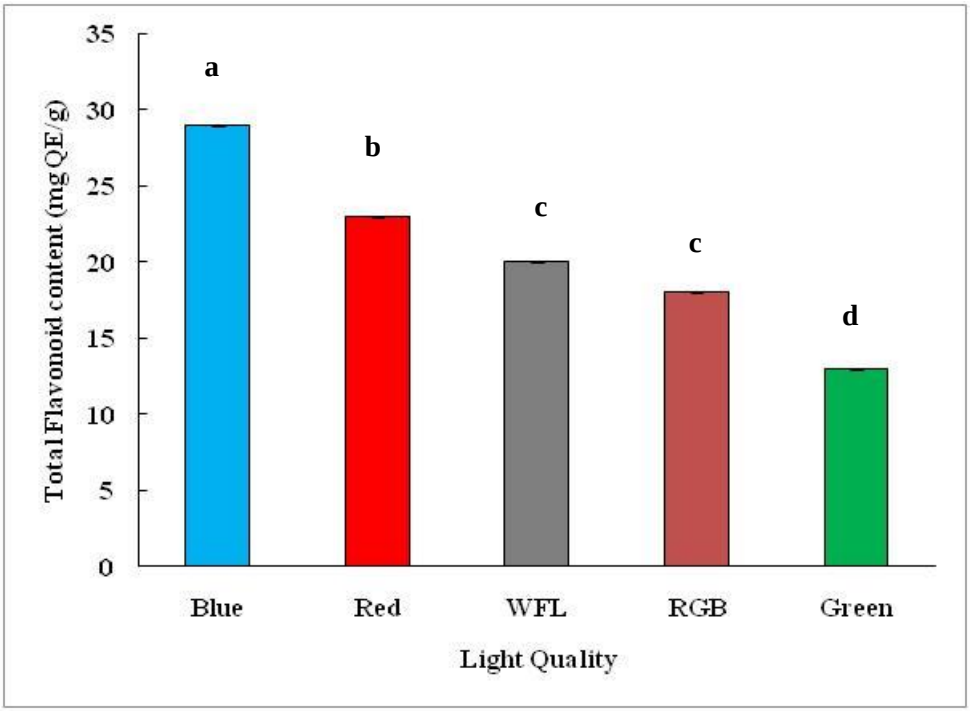


Figure 4.11 Effect of LED lighting on TFC in *S. chirayita* shoot cultures. Mentioned values are mean ± SD of three replicates in which means with similar letters are not significantly different at $p < 0.05$ according to Duncan's post-hoc test.

4.7 Effect of LED lighting on antioxidant activity in *S. chirayita* shoots cultures

The quest for natural antioxidants has become extremely important in light of the rise in oxidative stress related to health ailments [262]. Natural antioxidants are essential for reducing reactive oxygen species harmful effects [263]. The different LED light-spectra treated shoot cultures in this investigation showed a significant difference in DRSA. The DRSA% in cultures varies from 28.45-50.40 % (Table 4.3). The Blue LED treated shoot cultures exhibit maximum % of RSA (50.40 ± 0.15 %) in comparison to other LED's followed up by Red, WFL, RGB and Green LED's. By boosting the levels of natural antioxidants including ascorbate and flavonoids, blue light has been shown to increase antioxidant activity in plants [254]. The findings show that extracts from shoot cultures exposed to Blue LED have the highest antioxidant potential, which could be interpreted as a defence mechanism against rising ROS levels [264]. Because of their ability to donate hydrogen, ability to chelate metals, and redox characteristics, phenolic compounds have significant antioxidant activity [262]. Through the induction of endogenous protective enzymes and the regulation of gene expression, these compounds also demonstrate indirect antioxidant effect [262]. These findings imply that *S. chirayita* shoot cultures exposed to Blue LED treatment may serve as a source of natural antioxidants that could be utilized as a cancer, diabetes, and neurological disease treatment agent.

Table 4.3 Antioxidant activity is illustrated under different light qualities.

Antioxidant Activity	
Light Quality	**%RSA**
Blue	50.40 ± 0.15^{a}
Red	43.08 ± 0.06^{b}
WFL	39.02 ± 0.11^{c}
RGB	35.77 ± 0.05^{c}
Green	28.45 ± 0.19^{d}
Standard	
BHT	67.47 ± 0.05^{e}

Mentioned values are mean ± SD of three replicates in which means with similar letters are not significantly different at $p < 0.05$ according to Duncan's post-hoc test.

4.8 Impact of various growth hormones on callus induction

The callus culture system is an alternate source that has potential for producing useful bioactive metabolites [265]. Additionally, callus cultures are frequently used in both research and pharmaceutical industries. [266]. The best concentration and combination of growth regulators, explants, and plant species are largely responsible for the success of callus induction [267]. Therefore, in this finding the leaf explants from the tissue raised plant were cultured on the MS media provided with different growth hormones concentration 2,4D (1-.2.5 mg/l), BAP (0.5-1 mg/l), TDZ (0.5 mg/l) and KN (1 mg/l). Establishment of callus from leaf explants were observed in almost all of the concentrations of growth regulators (Figure 4.12). However the frequency of callus induction alters in respond to the different growth regulators concentration. The 1 mg/l 2,4D + 0.5 mg/l BAP + 0.5 mg/l TDZ revealed higher frequency of callus induction, in comparison to other growth regulator concentration. The lowest frequency of callus induction was observed in 1 mg/l 2,4D + 0.7 mg/l BAP. In the *in vitro* cultures of *Aquilaria malaccensis* [268], *Cynara scolymus* [269], *Scrophularia striata* [270], *Ecballium elaterium* [271], and *Rhodiola crenulata* [272], the synergistic combinations of 2,4D, BAP and TDZ likewise produced the highest callogenic frequency.

The endogenous production of growth regulators, which keeps the ratio that encourages callus formation, may be altered by externally administered growth regulators [273]. For the best callus induction, the auxin and cytokinin containing media often up regulates the expression of LBD16, LBD17, LBD18, and LBD29 [274]. The callus cultures induced from lower concentrations of 2,4D and BAP were yellowish-white friable, whereas the callus induced from higher concentrations of 2,4D, BAP and TDZ was green and compact. These morphological variations seen with various growth regulators treatments could be explained by the physiology of the specific explants. The above findings reveal that specific concentration of growth regulators is responsible for callus induction in tissue cultures of *S. chirayita*. The current finding illustrated that MS media provided with 1 mg/l 2,4D + 0.5 mg/l BAP + 0.5 mg/l TDZ was appropriate for formation of callus from leaf explants of tissue culture *S. chirayita*.

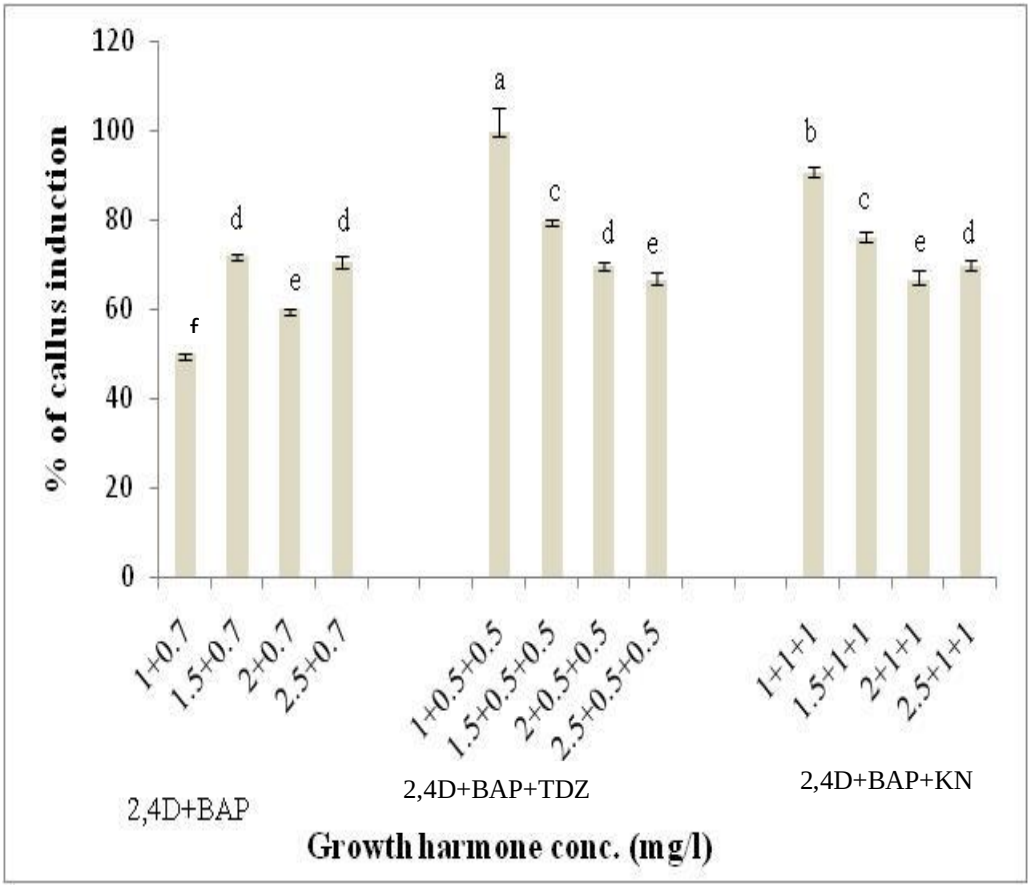


Figure 4.12 Effect of different growth hormone concentration on callus induction. Data is represented as mean ± SD of triplicates in which means with similar letters are not significantly different at $p < 0.05$ according to Duncan's post-hoc test.

4.9 Production of artificial seeds through encapsulation dehydration along with cryopreservation method

Somatic embryogenesis is an alternative medium to the micropropagation technique that provides an efficient way to conserve uniform clones over the years [275]. Encapsulation of these somatic embryos to form synthetic seeds provides new revenue to the existing plant biotechnology techniques [276]. As cryopreservation provides long term storage of the germplasm so storing synthetic seeds by cryopreservation methods helps in supply of endangered herbs and their precious bioactive compounds in sustainable manner [313]. Therefore, in the present study, the callus obtained from leaf explants of tissue culture *S. chirayita* were further sub cultured for 4 weeks to form embryogenic callus in the MS media

supplemented with 1mg/l 2,4D+ 0.5mg/l BAP+ 0.5mg/l TDZ. After achieving embryonic calli, they were transferred to the above mentioned media for another 30 days to achieve torpedo stage of somatic embryos (Figure 4.13). Auxins and cytokinins both are very important hormones to initiate somatic embryogenesis as well as important at every phase of somatic embryogenesis [7, 277].

For carrying out successful somatic embryogenic induction in the plants, combination of both auxin and cytokinin is required [278]. In *Gentiana* species from callus induction to embryo maturation 0.5mg/l 2,4D + 1mg/l KN media had been used [279]. In the present finding, SEM analysis was used to identify the different stages of somatic embryos and these studies help in selecting the right developmental stage of somatic embryo for formation of artificial seeds (Figure 4.14). The induction, growth, and maturation of somatic embryos in the leaf explants of *S. chirayita* in MS media supplemented with 1 mg/l 2,4D+ 0.5 mg/l BAP+ 0.5 mg/l TDZ were thus revealed by scanning electron microscopy images of somatic embryo surfaces. In the present study, encapsulated synthetic seeds were best observed using 3% sodium alginate with 0.85M of sucrose introduced in 100mM calcium chloride for 30-35 minutes. In the present findings 0.85M of sucrose was best suited as osmoprotectant; same observation was seen in Balavcik et al. [280] which reveal that high sucrose amount in preculture act as osmoprotectant and enhance the dehydration tolerance. Present findings illustrated that the moisture level of *S. chirayita* somatic embryos, after desiccation and before exposing them to liquid nitrogen was 18-20%. Similar reports on cryopreservation of *M. azedoroach* L. and *L. ledebourii* (Baber) Bioss, seeds revealed optimum moisture level was 15-20% [281], which was the ideal moisture level before exposing to liquid nitrogen (Figure 4.15).

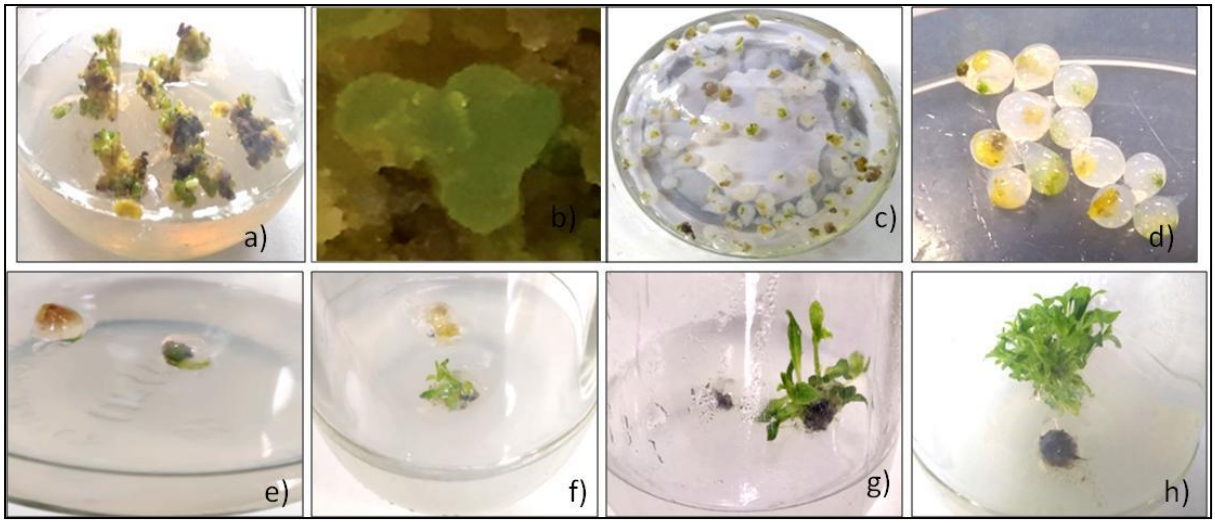


Figure 4.13 Artificial seeds production: a) Developed somatic embryos, b) Somatic embryo under high definition microscope, c) Artificial seeds encapsulated with sodium alginate and complexes with calcium chloride, d) Encapsulated somatic embryo cultured on MS media for germination, e) Cultured somatic embryos on MS media for germination, f) Shoot emergence from germinated artificial seeds after 7 days of culture, g) Shoot growth from germinated artificial seed after 21 days, h) shoot formation after 30 days of germination.

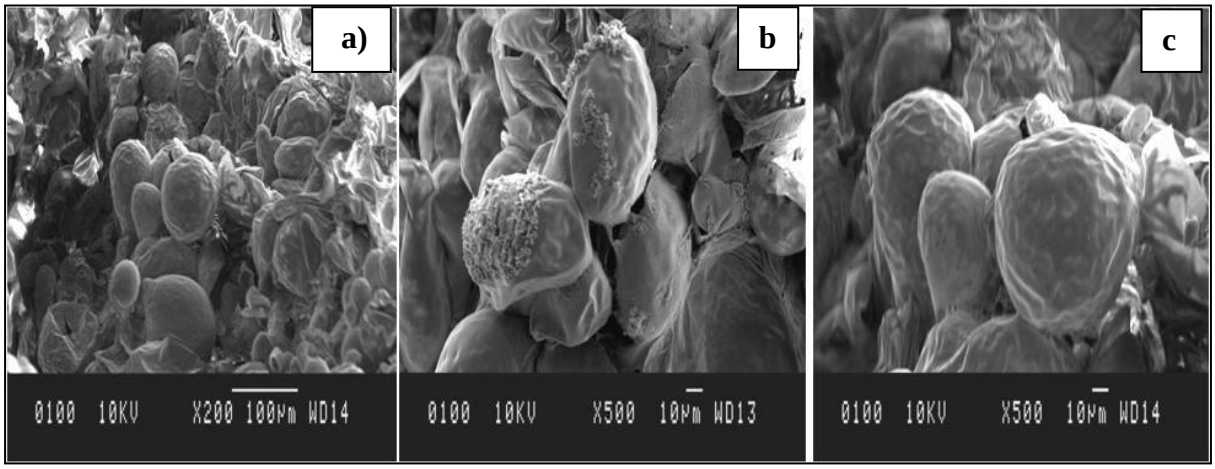


Figure 4.14 Somatic embryos images under SEM a) Cluster of globular shape, b) Heart shaped, c) Torpedo shaped.

Figure 4.15 Cryopreservation of artificial seeds: a) Preculture of artificial seeds in 0.85M sucrose and 100mM $CaCl_2$, b) Artificial seeds in cryovials after exposure to liquid nitrogen, c) Artificial seeds after rewarming in water bath, d) Germination of artificial seed on 3rd day, MS media provided with 1mg/l IBA+ 2mg/l KN + 3mg/l GA_3, e) Germination of artificial seeds on 7th day, f) Shoot formation after 30 days of germination, g) 45 days old plants used for quantification of metabolites, h) Hardening and acclimatization of *S. chirayita* plantlets from artificial seeds.

4.10 Regeneration vis-a-vis Amarogentin and Mangiferin production from artificial seeds of *S. chirayita*

In the cell culture system, the growth regulators have a significant impact on secondary metabolism [283]. However, the response is strongly influenced by both the plant and the concentration of growth regulators [283]. The regeneration of cryopreserved seeds into shoots of *S. chirayita* was therefore studied in the current study using various concentrations of growth regulators. The 1mg/l IBA+ 2mg/l KN+ 3mg/l GA_3 displayed highest percentage of regeneration frequency into shoots in comparison to the other concentration of the growth regulators (Figure 4.16). Surprisingly, 93.3% of successful regeneration was reported after 1-2 weeks in the MS media supplemented with 1mg/l IBA+ 2mg/l KN+ 3mg/l GA_3. Similar outcomes have been observed by *Clitoria ternatea Linn* [282].

The present finding illustrated that revived artificial seeds into tissue culture shoots of *S. chirayita* showed accumulation of Amarogentin 4.72±0.11 µg/mg and Mangiferin 14.54±0.05 µg/mg respectively (Figure 4.17). In the present finding revived plants were acclimatized to the outer environment where survival percentage rate of the plantlets was 85%. From the above findings we concluded successful cryopreservation and revival of artificial seeds from *in vitro* grown leaf explants of *S. chirayita.* The production of the medicinally valued *S.*

chirayita herb was hindered by a variety of issues, such as low seed viability and habitat degradation, therefore an appropriate solution that can be scaled up to large productions is synthetic seed synthesis. Therefore, this technique can be used to conserve this priceless, threatened herb.

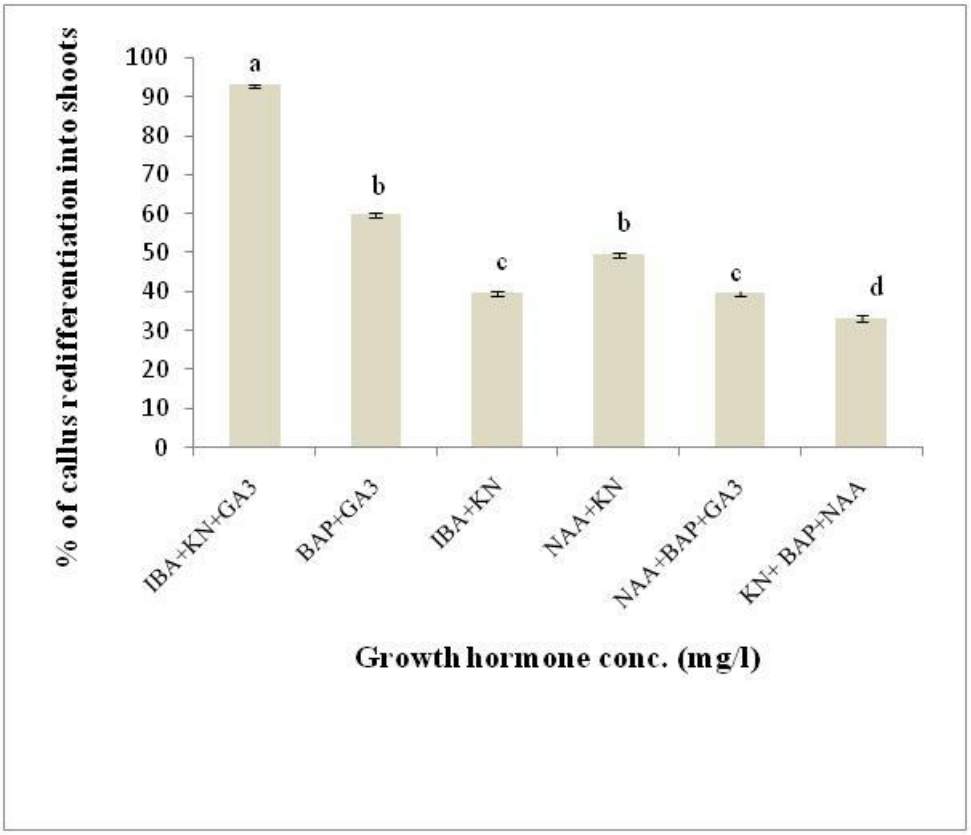


Figure 4.16 Effect of MS media supplemented with different growth hormones concentration to redifferentiate callus into shoots plantlets. Data is represented as mean ± SD of triplicates in which means with similar letters are not significantly different at $p < 0.05$ according to Duncan's post-hoc test.

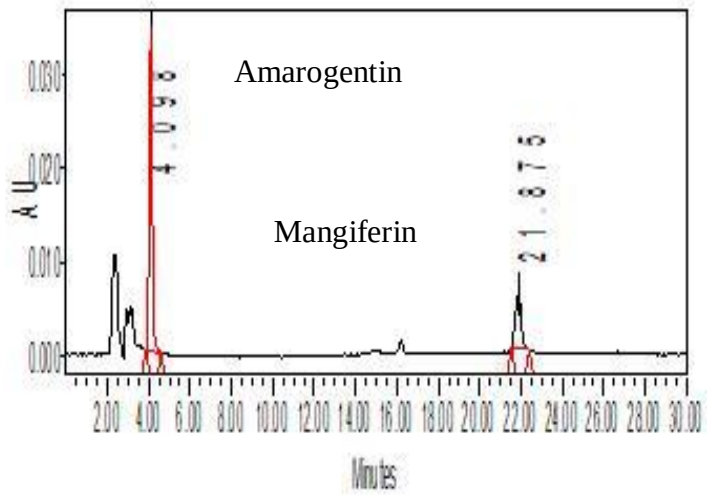


Figure 4.17 HPLC chromatogram of Amarogentin and Mangiferin from regenerated shoots of *S. chirayita* after cryopreservation.

4.11 Comparative analysis of phytochemicals and pharmacological potential of ≈3 month old *in vitro* grown (IVP) plants with ≈3 month field grown plantlets (FGP) of *S. chirayita*

For the pharmacological evaluation of field grown plants, a wide range of test systems have been developed. Alcoholic, methanolic, and aqueous extracts of *S. chirayita* have shown promising findings. In addition to anti-inflammatory and hypoglycemic action, numerous researchers have found promising anti bacterial and antifungal capabilities possessed by *S. chirayita* through evidence-based laboratory testing [13, 97, 98, 100, 102]. Although a thorough comparison of IVP with the FGP of *S. chirayita* in all the pharmacological aspect has never before been documented.

4.12 Qualitative analysis of methanolic extracts from IVP and FGP of *S. chirayita*

The qualitataive analysis of phytochemicals present in the methanolic extracts of IVP and FGP *S. chirayata* is represented in Table 4.4. The present finding revealed the positive incidence of phytoconstituents like carbohydrates, proteins, phenols, flavonoids, tannins, alkaloids and sterols in both the extracts of *S. chirayita*. Phytochemical screening gives a detailed description of the worth and potential of plants as medicines. Respective phytochemicals possess various medicinal properties of the plant. As flavonoids have antioxoidant activity as well as can anti-cancerous properties and phenolics on the other hand provides significant antimicrobial and anti-inflammatory properties [284, 285, 286]. Alkaloids and Tannins have antimicrobial as well as anti-cancerous properties [287, 288]. Steroids posses' cardio-tonic affects and helps to treat cholesterol related problems [289, 290].

Table 4.4 Qualitative phytochemical analysis of FGP and IVP of *S. chirayita.*

Metabolites/ Plants	IVP	FGP
Carbohydrates	+ve	+ve
Proteins	+ve	+ve
Alkaloid	+ve	+ve
Tannins	+ve	+ve
Flavonoids	+ve	+ve
Phenols	+ve	+ve
Sterols	+ve	+ve

4.13 Quantitative analysis of IVP and FGP extracts of *S. chirayita*

As per the results obtained from the qualitatitve analysis, quantitative analysis was also done on some major phytochemicals like carbohydrates, proteins, total flavonoid and TFC and TPC in both the extracts of *S. chirayita*.

4.13.1 Protein estimation

Proteins in plants play various structural as well as enzymatic roles like biosynthesis, transport and in some cases also act as storage medium for the growth of the plant. IVP and FGP extracts were examined for protein estimation in the current finding. Protein estimation was performed using Lowry method, where BSA was used as standard compound. Results revealed significant variations in the protein content of IVP and FGP extracts. *In vitro* produced plants had a protein level of 3.41% whereas field-grown plants had a protein content of 4.36% (Table 4.5). So, the above findings confirmed the higher percentage of proteins in the field grown sample. The plant's greater amount of protein raises the possibility that it has greater nutritional significance or that one day a protein-based bioactive a compound may be discovered [291].

4.13.2 Carbohydrate estimation

Carbohydrates in the plants play major role in the storage of energy as well as play role in skeleton system for the formation of organic compounds in the plants. In the present finding methanolic extracts of IVP and FGP were tested for the carbohydrate estimation. Carbohydrate estimation was performed using Anthrone method, where Glucose was used as standard compound. Results revealed significant variations in the carbohydrate content of IVP and FGP extracts. Carbohydrate content in the field grown plants was found to be 10.01% whereas carbohydrate content in the IVP was found to be 7.69% (Table 4.5). So, the above findings confirmed the higher percentage of carbohydrates in the field grown sample.

4.13.3 Total Flavonoid content

In the present finding methanolic extracts of IVP and FGP were tested for the TPC. TPC estimation was performed using Aluminum Chloride assay where the standard compound used was Quercetin. The total flavonoid concentration of extracts produced *in vitro* and in the field showed substantial differences, according to the results. In comparison to the FGP,

which had a flavonoid content of 32.06 mg QE/g DW, the IVP had a total flavonoid content of 44.03 mg QE/g DW (Table 4.5). So, the above findings confirmed the higher percentage of flavonoids in the *in vitro* grown sample. Similar to this, 12 plants from the Asclepiadaceae and Periplocaceae families were found to have different phenol and flavonoid amounts within the plants [292]. The current research demonstrates that external supply of various growth regulators during *in vitro* regeneration has a considerable influence on the *in vitro* synthesis of phenols and flavonoids. Similar effects of growth regulators on secondary metabolite synthesis from callus suspension culture have been noted in *Gymnema sylvestre* [293, 294]. Hence from the above findings we can conclude that *in vitro* grown plantlets of *S. chirayita* can serve as alternate source of antioxidant to the field grown plantlets.

4.13.4 Total phenolic content

In the present finding methanolic extracts of IVP and FGP were tested for the TPC. TPC estimation was performed using FC reagent where standard was used as Gallic acid. The total phenolic content of extracts produced *in vitro* and in the field showed substantial differences, according to the results. TPC was found to be 30.16 mg GA/g DW in the IVP, compared to 18.73 mg GA/g DW for phenolic content in the FGP (Table 4.5). The increased concentration of phenols in the *in vitro* produced sample was therefore verified by the aforementioned observations. The higher phenolic content may be due to the buildup of plant growth regulators. Many researches have shown how different growth regulators and elicitors are used to boost the production of secondary metabolites and other advantageous bioactive compounds, such phenol in olive trees [295]. Hence from the above findings we can conclude that *in vitro* grown plantlets of *S. chirayita* can serve as alternate source of antimicrobial and anti-inflammatory properties to the field grown plantlets.

Table 4.5 Total carbohydrates, proteins, phenols and flavonoids in extracts of *S. chirayita*.

Plants	Primary metabolites		Secondary metabolites	
	Carbohydrates (%)	Proteins (%)	Phenols (mg GA/g)	Flavonoids (mg QE/g)
In-vitro	7.69±0.01	3.41±0.02	30.16±0.24	44.03±0.50
Field grown	10.01±0.01	4.36±0.07	18.73±0.09	32.06±0.13

4.14 Physiochemical characterization of IVP and FGP extracts of *S. chirayita*

The physiochemical properties of the plants affirmed that plant was erect and tall with white flowers in the natural conditions. Although physiochemical characterization of plant has various importance like ash value in the plants indicates the content of minerals, acid value in the plants indicates the presence of siliceous matter, water soluble extractive value in the plants indicates acids, inorganic compounds, sugar presence and alcohol soluble extractive value in the plants indicates presence of polar compounds in the plant. Moreover lesser value of moisture in the plants helps in preventing microbial growth. In the present finding different properties of physiochemical were explored in the IVP and FGP extracts of *S. chirayita*. The results revealed overall, high concentrations of water and alcohol soluble extractives were obtained among both *in vitro* (Water extractive = 84.76%, Alcohol extractive = 81.5%) as well as field grown extracts (Water extractive = 88.23%, Alcohol extractive = 85.6%). However, average moisture (2.8%), ash (3.8%) and acid ash (1.21%) content in the *in vitro* extracts of *S. chirayita* was observed to be higher as compared to field grown extracts (moisture (2.7%), ash (3.5%) and acid ash (0.98%) as represented in Table 4.6. From the above findings we concluded that, Proximates derived from *in vitro* extracts showed slightly lower mean values as compared to field grown extracts with respect to water and alcohol soluble extractives and slightly higher value in moisture, ash, acid ash in comparison to the field grown plantlets.

Table 4.6 Preliminary analysis of IVP and FGP extracts of *S. chirayita*

Plants	Moisture Content (%)	Total Ash (%)	Acid insoluble ash (%)	Alcohol soluble extractive (%)	Water soluble extractive (%)
In vitro	2.8±0.2	3.8±0.2	1.21±0.17	81.5±0.64	84.76±0.25
Field grown	2.7±0.2	3.5±0.2	0.98±0.2	85.6±0.52	88.23±0.25

.

4.15 Determination of antioxidant activity in IVP and FGP extracts of *S. chirayita*

4.15.1 DPPH assay

Recently, the food industry and the field of medicine have become quite interested in plant-based antioxidants [296]. In order to keep oxidative stress at manageable levels, antioxidant compounds scavenge excessive ROS [31]. A number of *in vitro* based assays can be used to quickly determine the antioxidant activity of plant extracts [297]. Because the antioxidant activity cannot be thoroughly assessed using just one method, we have used ABTS and DPPH assay in the current work to determine antioxidant activity in the extracts of *in vitro* and field grown extracts of *S. chirayita*. The results shows variable sensitivity levels by different concentrations towards the tested sample of field grown extracts and *in vitro* extracts. The DPPH scavenging activity for *in vitro* plants was observed to be within 26.61± 0.21% to 74.60± 0.21 at various concentrations (Figure 4.18). Maximum inhibition effect was observed at 0.1 mg/ml concentration while minimum inhibition effect was seen at 0.025 mg/ml concentration gradient. The field grown extract showed lower inhibition range within 20.14±0.36% to 67.52±0.21% at 0.1 mg/ml and 0.025 mg/ml concentrations respectively. At 540 nm absorbance, IC_{50} values in control, field grown and *in vitro* sample groups (IC_{50} = 0.06 µg/ml; IC_{50} = 0.071 µg/ml, IC_{50} = 0.06 µg/ml respectively) were observed. Comparable antioxidant activity was recorded among all the three sample groups at various concentrations. However, lower antioxidant activity was recorded among field grown samples as compared to *in vitro* samples at various concentration gradients.

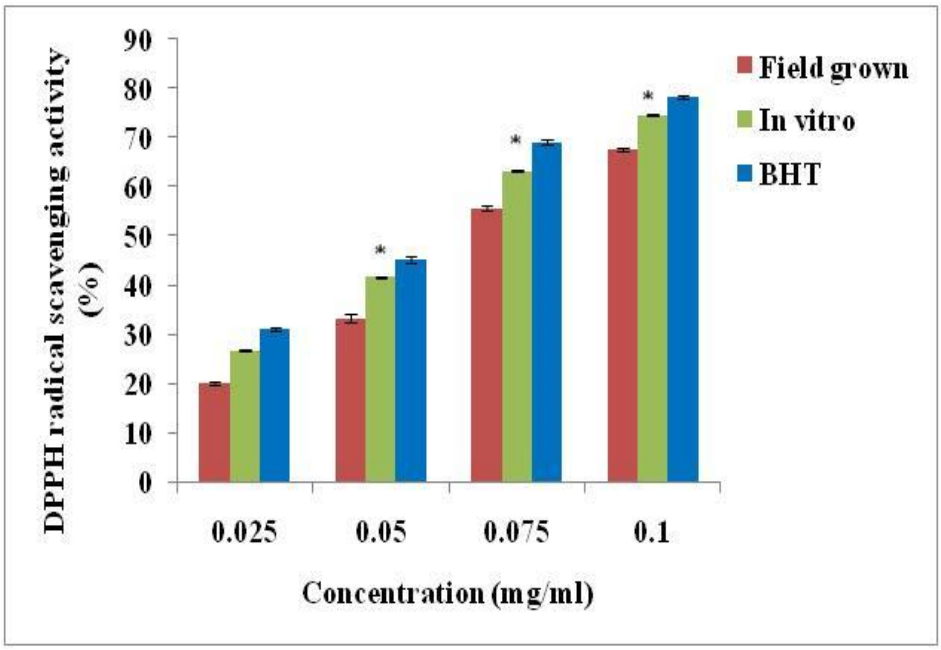


Figure 4.18 DPPH radical scavenging activity of IVP and FGP extracts of *S. chirayita*. Data is represented as mean ± SD and the significant differences from control are indicated by * $p < 0.05$.

4.15.2 ABTS assay

Similarly the results of ABTS are depicted. The range of ABTS activity for *in vitro* plants was observed to be within 23.38±0.08% to 73.47±0.08% at various concentrations. Maximum inhibition effect was seen at 0.1 mg/ml concentration while minimum inhibition effect was seen at 0.025 mg/ml concentration gradient. The field grown extract showed lower inhibition range within 69.04±0.29% to 19.47±0.04% at 0.1 mg/ml and 0.025 mg/ml concentrations, respectively (Fig 4.19). At 754 nm absorbance was taken, IC_{50} values in control, field grown and *in vitro* sample groups (IC_{50} = 0.048 µg/ml; IC_{50} = 0.07 µg/ml, IC_{50} = 0.065 µg/ml respectively) were observed. Antioxidant activity of *in vitro* extracts was recorded to be highest than field grown ectracts at different concentration gradients. Mangiferin and Oleanolic acid [115] present in *S. chirayita* have been reported to show antioxidant properties. Using a 70% ethanolic extract of *S. chirayita*, reducing power and beta-carotene assays revealed significant antioxidant capabilities [53]. Methanol extracts have phenolic and flavonoid levels that are relatively greater than that of other solvent extracts. This may be attributed to high DPPH free radical scavenging activity and $ABTS^{+}$ cations antioxidant activity at all concentrations in the extracts of *S. chirayita*.

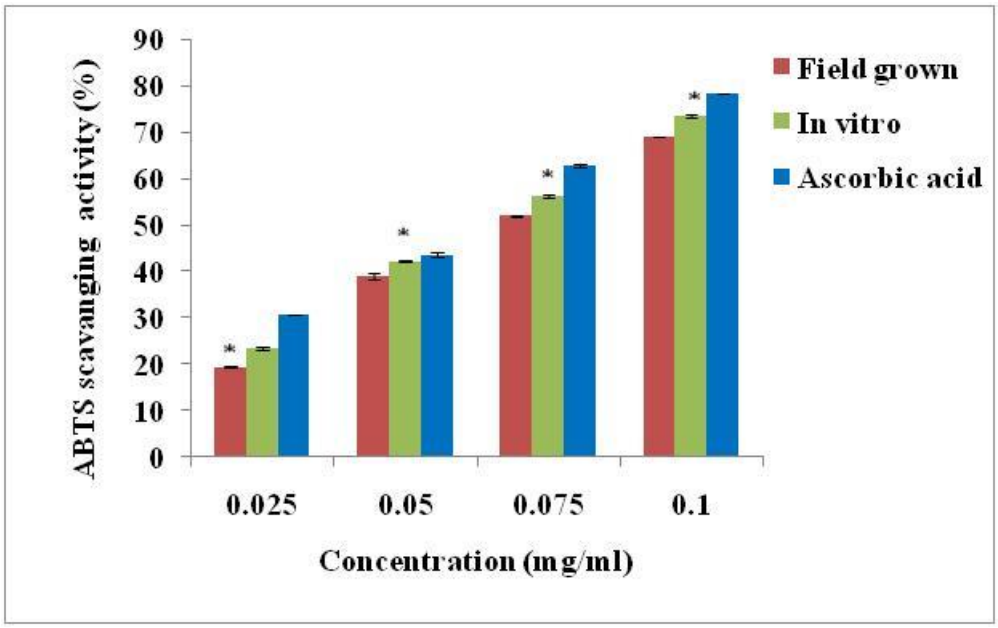


Figure 4.19 ABTS scavenging activity of IVP and FGP extracts of *S. chirayita*. Data is represented as mean ± SD and the significant differences from control are indicated by * $p < 0.05$.

4.16 Antimicrobial activity of IVP and FGP extracts of *S. chirayita*

The prevalence of microbial resistance has dramatically increased due to the extensive usage of conventional antibiotics [298, 299, 312]. As a result, human population rates of morbidity and mortality have considerably increased [299]. This makes it even more urgent to find new medications to combat microbial illnesses [298]. An efficient alternative source of antibacterial chemicals is plant cell culture [300]. Numerous researches have recently shown an increase in the antibacterial activities of plant cell culture extracts [300]. Methanolic extracts of *in vitro* and field grown *S. chirayita* were investigated to evaluate their antibacterial activity against two strains of Gram positive bacteria *S. aureus* (MTCC3160) and *B. subtilis* (MTCC121)) and two strains of Gram negative bacteria *E. coli* (ATCC25922) and *S. typhi* (MTCC98) using agar well diffusion method. The results of the antibacterial examination using agar gel diffusion method are depicted in Figure 4.21. It shows different levels of sensitivity shown by different bacterial species (like *S. aureus*, *S. typhi*, *B. subtilis* and *E. coli*) towards the tested sample of field grown extracts and *in vitro* extracts. *In vitro* plants had a larger zone of inhibition than field-grown plants did. The range of diameter of zone of inhibition for *in vitro* plants lies within 24.4 mm to 27.25 mm whereas field grown extract showed lower inhibition range within 22.25 mm to 25.1 mm at concentration of 30 mg/ml (Figure 4.20). The range of MIC for *in vitro* plants was observed to be within 0.39 to

1.625 mg/ml. The range of MIC for field grown plants was observed to be within 0.781 to 3.125 mg/ml as represented in Table 4.7. This confirms the IVP have higher potential to act as antibacterial agents in comparison to the FGP. Maximum inhibition zone was observed towards *S. typhi* and *E. coli* while minimum inhibition effect was towards *B. subtilis*. Because they have more flavonoids and phenolics than plants cultivated in the field, *in vitro* plants have better antibacterial activity.

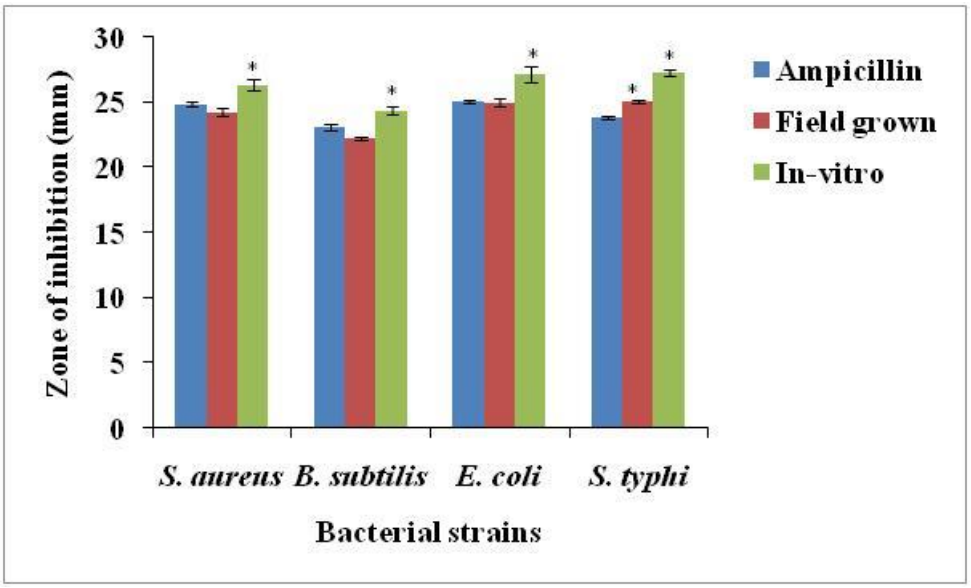


Figure 4.20 Zone of inhibition of IVP and FGP extracts of *S. chirayita* against Gram-positive and Gram-negative bacterial strains. Data is represented as mean ± SD and the significant differences from control are indicated by * $p < 0.05$.

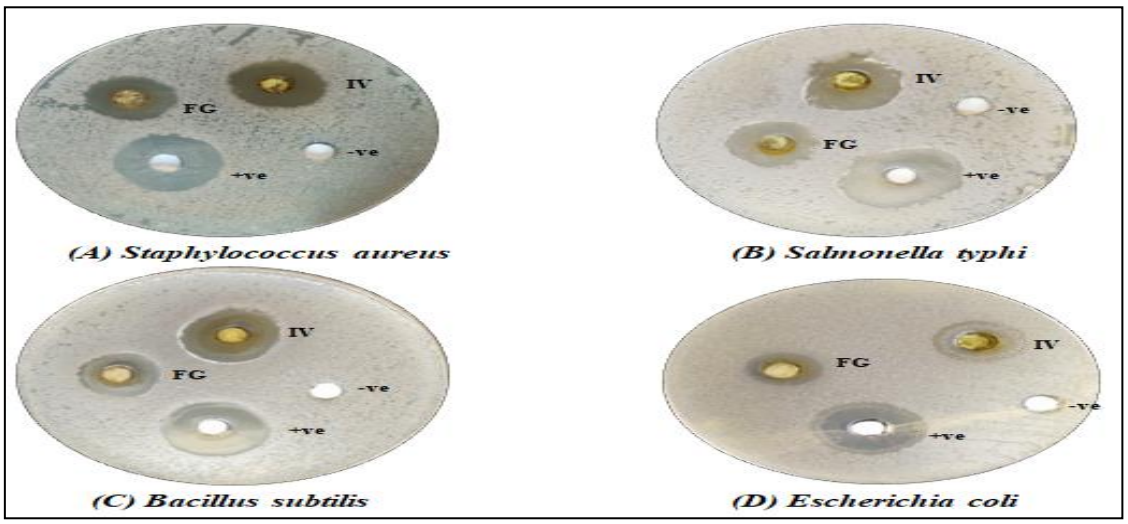


Figure 4.21 Zone of inhibition of IVP and FGP extracts of *S. chirayita* against Gram-positive and Gram-negative bacterial strains.

Table 4.7 MIC of IVP and FGP extracts of *S. chirayita* against Gram-positive and Gram-negative bacterial strains.

S. No.	Plant Extract	MIC (mg/ml)			
		S. aureus **(mg/ml)**	***S.typhi*** **(mg/ml)**	***B.subtilis*** **(mg/ml)**	***E.coli*** **(mg/ml)**
1	**In vitro**	**1.562****	**0.39**	**1.625****	**0.39**
2	**Field grown**	**1.562****	**0.781***	**3.125****	**0.781***
3	**Ampicillin**	**0.078**	**0.625**	**0.312**	**0.625**

Data difference was statistically significant at (* $p < 0.05$, ** $p < 0.01$).

4.17 Determination of anti-inflammatory activity in IVP and FGP extracts of *S. chirayita*

The results of the HRBC membrane stabilization test method shows variable sensitivity levels by different concentrations towards the tested sample of field grown extracts and *in vitro* extracts. The range of percentage inhibition for *in vitro* plants was observed to be within 27.28±0.66% to 63.72±0.44% at various concentrations (Figure 4.22). Maximum anti-inflammatory effect was seen at 0.1 mg/ml concentration while minimum inhibition effect was seen at 0.025 mg/ml concentration gradient. The field-grown extract had an anti-inflammatory activity at doses of 0.1 mg/ml and 0.025 mg/ml that ranged from 21.600.22% to 59.460.22%. At 540 nm absorbance, IC_{50} values in control, field grown and *in vitro* sample groups (IC_{50} = 0.14 µg/ml; IC_{50} = 0.18 µg/ml, IC_{50} = 0.16 µg/ml respectively) were observed in gradual decreasing order. Comparable anti-inflammatory activity was recorded among all the three sample groups at various concentrations. Bioactive compounds like mangiferin, swertanone, oleanolic acid, chiratol, β-amyrin and xanthones present in the extracts of *S. chirayita* are the major factors for the anti-inflammatory activity shown by the plant [47, 301]. The plant's ethanolic extracts have been suggested by a number of researches as having the best anti-inflammatory effects [302, 303]. *In vitro* extracts have been reported to have the highest anti-inflammatory impact when compared to field grown.

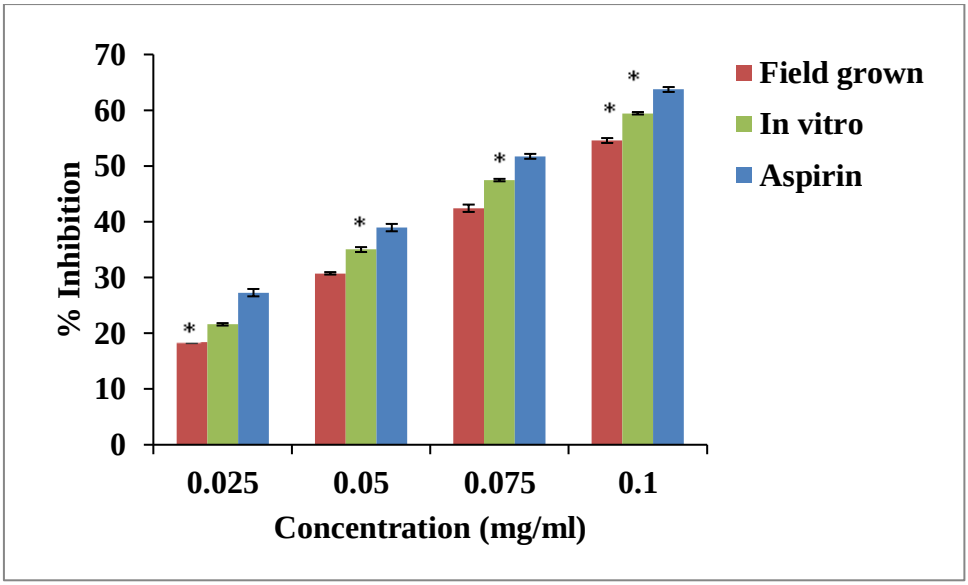


Figure 4.22 Anti-inflammatory activities of IVP and FGP extracts of *S. chirayita*. Data is represented as mean ± SD and the significant differences from control are indicated by * $p < 0.05$.

4.18 Determination of anti-diabetic activity in IVP and FGP extracts of *S. chirayita*

Numerous herbal extracts are utilised in Ayurveda to treat diabetes since they have been shown to have antidiabetic properties. Many contemporary medicines have been made with the use of herbal extracts, either directly or indirectly. The anti-diabetic properties of *S. chirayita* plants that were cultivated *in vitro* and in the field were investigated in the current study. The results of the α-Amylase inhibitory assay show variable sensitivity levels by different concentrations towards the tested sample of field grown extracts and *in vitro* extracts. The range of percentage inhibition for *in vitro* plants was observed to be within 33.95±0.17% to 68.88±0.34% at various concentrations (Figure 4.23). A concentration gradient of 0.025 mg/ml produced the least amount of α-Amylase inhibition whereas 0.1 mg/ml concentration produced the greatest amount.

The field grown extract showed α-Amylase inhibitory effect within 30.61 ±0.34% to 59.38±0.52%. At 540 nm absorbance, IC_{50} values in control, field grown and *in vitro* sample groups (IC_{50} = 0.048 µg/ml; IC_{50} = 0.08 µg/ml, IC_{50} = 0.06 µg/ml respectively) were observed. Anti-diabetic activity of *in vitro* extracts was recorded to be highest in comparison to the field grown plants. Presence of flavonoids and secoiridoids are responsible for the

hyperglycemic properties shown by *S. chirayita* [9, 11, 126, 127], Mangiferin and swertiamarin the most potent phytochemicals against diabetes and in lowering down the blood lipid profile with respect to diabetes [98, 133]. Hence *in vitro* raised plants showed higher anti-diabetic activity in comparison to field grown samples and have greater potential to become an alternate source with respect to pharmaceutical industry.

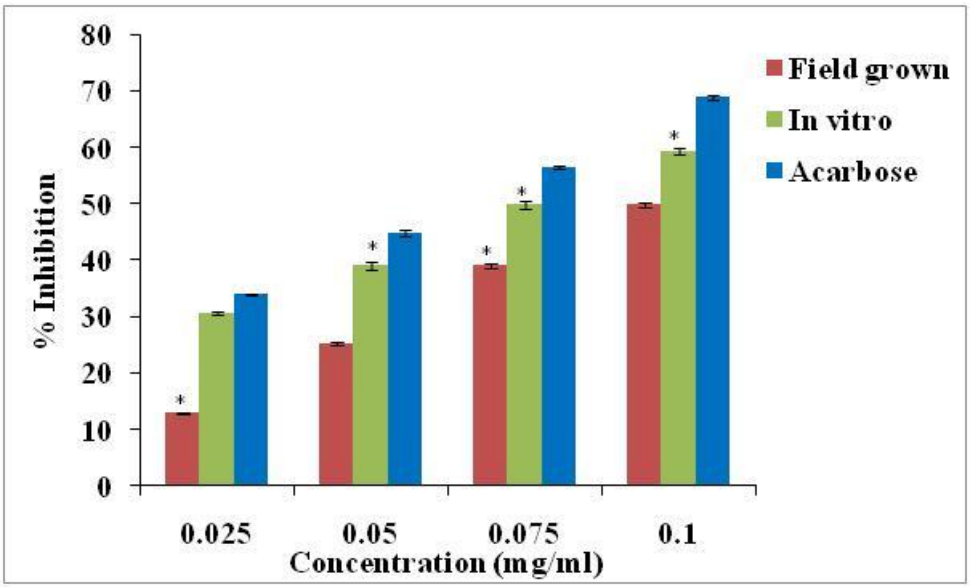


Figure 4.23 Alpha amylase activity of Acarbose and extracts of IVP and FGP of *S. chirayita*. Data is represented as mean ± SD and the significant differences from control are indicated by * $p < 0.05$.

4.19 Cytotoxic effect and induction of apoptosis in IVP and FGP extracts of *S. chirayita*

Since the beginning of known records, people have used plants as a source for medicinal purpose. With the present advancements in technology, the significance of plants as sources of therapeutic ingredients is becoming more and more recognized. Because of their low toxicological profile and elevated therapeutic index, plant-based substances have gained interest as alternative treatment methods in the fight against diseases around the world [304, 305]. Many current medications used in health context come from natural plants [306, 315]. *S. chirayita* is an important source of drug for traditional medicine to cure various ailments so present finding evaluated the anticancer activity of methanolic extracts from *in vitro* and field grown plantlets of *S. chirayita* in HepG2, MCF-7 cancer cell lines. In the present study HepG2, MCF-7 cancer cell lines were given different amounts of treatment of field grown

and *in vitro* grown *S. chirayita* methanolic extracts to check its cytotoxic capabilities and after that half inhibitory concentration IC_{50} were calculated for cancer cell lines. After completion of 48 h, significant values were determined using dose response-inhibition curve as represented in Table 4.8. Figure 4.24 and 4.25, depicts the cytotoxic effect of methanolic extracts of field grown and *in vitro* plants of *S. chirayita* against cancer cell lines. Both the extracts showed cytotoxic effect however HepG2 cancer cells were most sensitive in terms of IC_{50} value (IC_{50} = 88µg/ml), therefore, HepG2 cancer cells treated with methanolic extracts of *in vitro* grown plants were used for further assay.

Effect of *in vitro* grown *S. chirayita* methanolic extracts on HepG2 cells were analyzed using flow cytometry. Staining with Annexin V FITC and 7-AAD was used to confirm the apoptotic induction on HepG2 cell lines by using methanolic *in vitro S. chirayita* extract. Figure 4.26 depicts the results obtained from the experiment. Here, in the treated cells with (44 µg/ml) live cells were reduced to 32.72 ± 0.5% and an increase in early apoptosis was observed 67.02 ± 0.14%. Similarly, increase from 0% (early apoptotic cells from control) to 17.65 ± 0.5% and 70.36 ± 0.84% from early to late apoptotic cells was observed after treatment with (88 µg/ml) of sample extracts. Vailanka et al. [146] studied anticancer activity shown by methanol extract derived from leaf and stem. Alkaloid content of the plant is high due to presence of Amarogentin, Swertiamarin and Mangiferin along with other bioactive compounds which are helpful in reducing cell proliferation in case of MCF – 7, KELLY and CACO – 2 cell lines. The findings of the present study support higher efficiency of tissue raised plants over field grown plant extracts against cancer cell lines. While comparing the field grown and *in vitro* plant extracts of *S. chirayita* revealed that *in vitro* plants were showing higher efficiency over field grown extracts. Our findings indicate that *S. chirayita* extracts may play a role in triggering apoptosis through dose-dependent increases in the number of early and late apoptotic cells. This work offers early evidence that suggests *in vitro* grown extracts of *S. chirayita* showed anti-cancer activity by triggering apoptotic cell death. *In vitro* plants proved to be quality rich herbs in comparison to field grown samples and have greater potential to become an alternate source with respect to pharmaceutical industry.

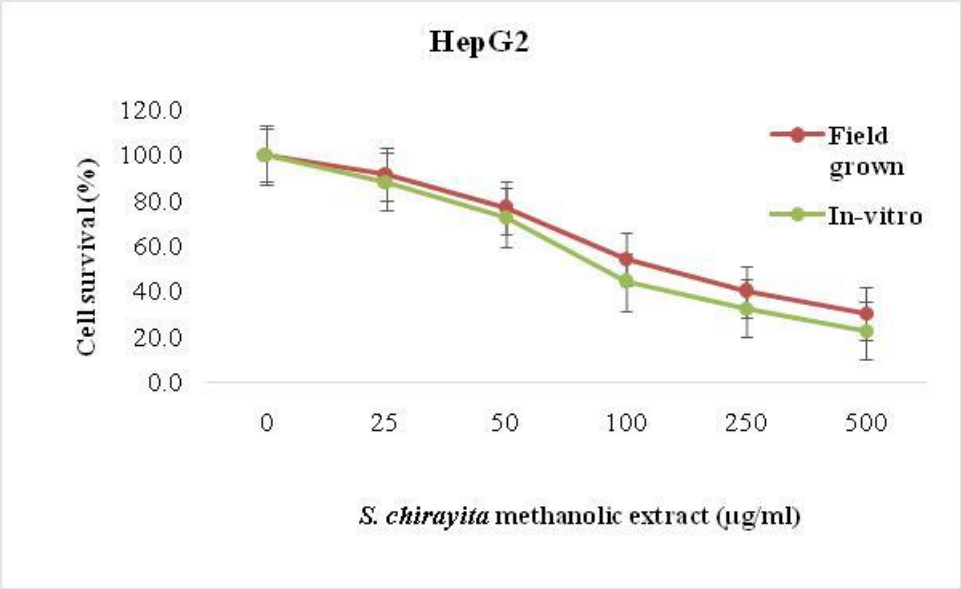


Figure 4.24 Cytotoxic effects of IVP and FGP *S. chirayita* methanolic extracts against HepG2 cell lines after 48 h of exposure.

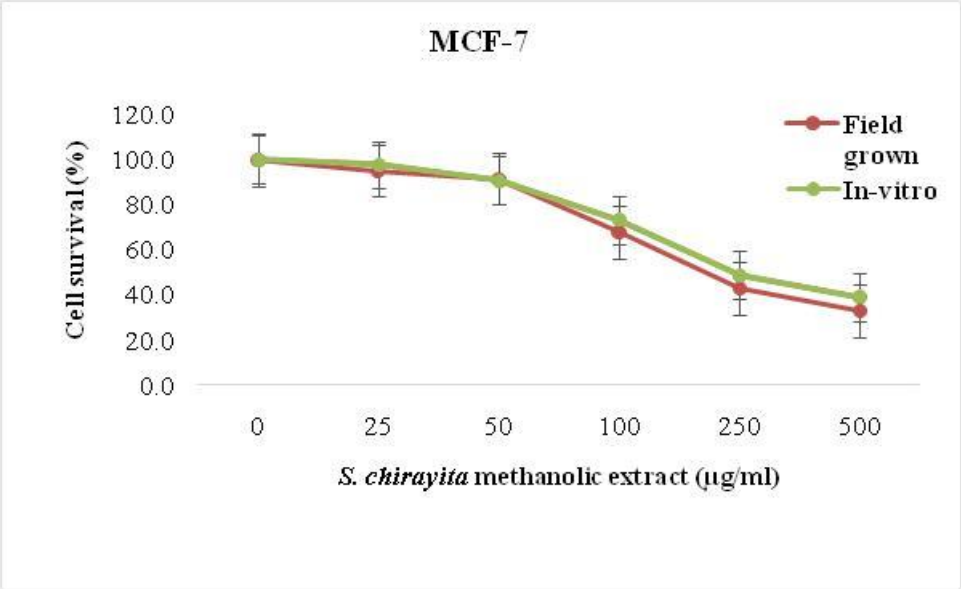
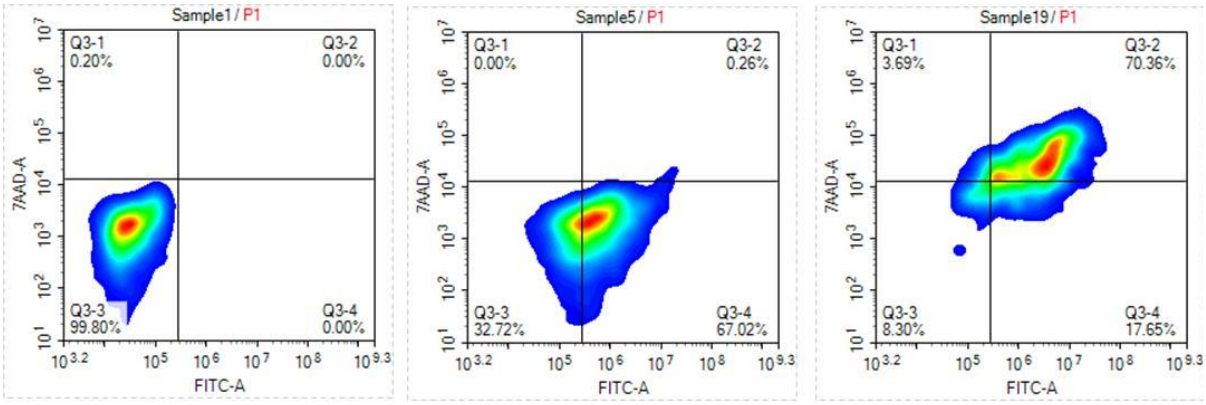


Figure 4.25 Cytotoxic effects of IVP and FGP *S. chirayita* methanolic extracts against MCF-7 cell lines after 48 h of exposure.

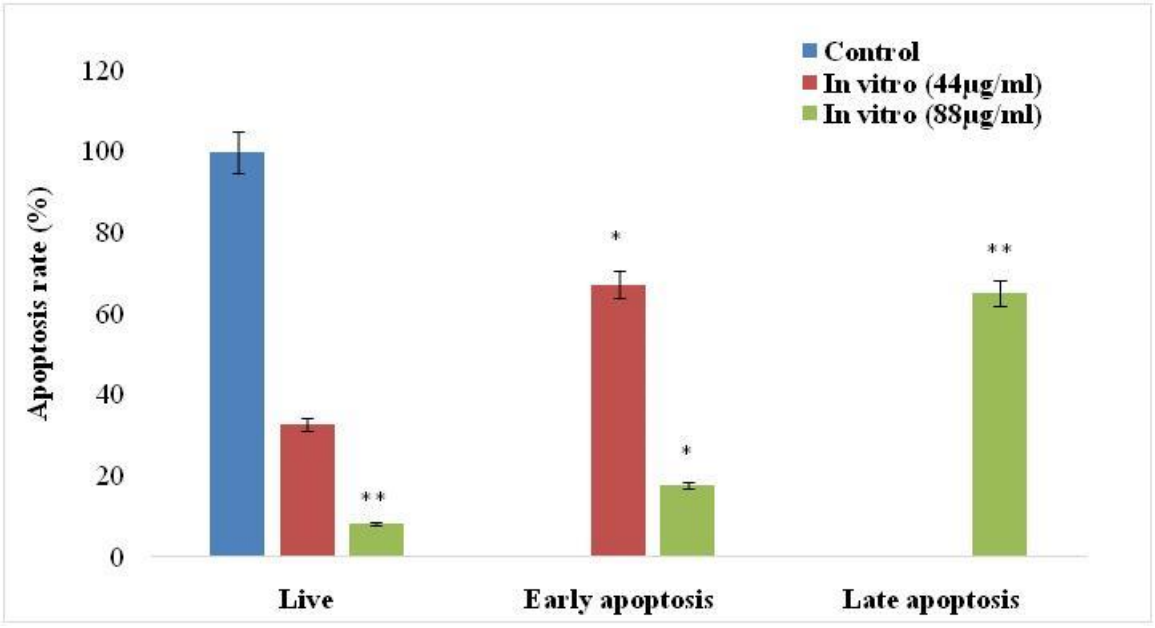
Table 4.8 Cytotoxic activities of IVP and FGP extracts of *S. chirayita* against cancer cells.

Cell lines and IC_{50} (µg/ml)		
Extracts	**Hep-G2**	**MCF-7**
In-vitro	88.93	260.74
Field grown	110.75	252.12



Control (HepG2 cells) **In-vitro (44µg/ml)** **In-vitro (88µg/ml)**

(a)



(b)

Figure 4.26 (a, b) Contour plot of ANNEXIN V-FITC stained HepG2 cells after 48 hr exposure to 44 and 88 µg/ml of *S. chirayita*. (Live cells= Q2-3; AnnexinV-negative, 7AAD-negative. Early apoptosis= Q2-4; AnnexinV-positive, 7AAD-negative. Late apoptosis= Q2-2; AnnexinV-positive, 7AAD-positive. Necrotic cells= Q2-1; AnnexinV-negative, 7AAD-positive) 20,000 events were read (n=3) and data is represented as mean ± SD and the data difference was statistically significant at (* $p < 0.05$, ** $p < 0.01$).

4.20 FTIR analysis of IVP and FGP of *S. chirayita*

FTIR spectrum used in identification of functional group of active compounds existing in the field grown and *in vitro* extracts of *S. chirayita* was based on peak values in IR radiation region which have been depicted in Figure 4.27 and 4.28. By passing the plant extracts through FTIR, respective functional groups were separated on the basis of their peak ratio. So, the result of FTIR analysis in the *in vitro* plant sample confirms the existence of N-H, O-H, C-H, C=O, S=O, C-O and C-Cl functional groups whereas field grown samples confirms the presence of C-H, C=N, O-H, S=O and C=C functional groups (Figure 4.27 and 4.28). The outcomes of FTIR study showed that secondary amine, carboxylic acid, alkane, aldehyde, δ - lactone, imine, sulphonyl chloride, sulfoxide, aliphatic ether, alkyl aryl ether and halo compound were present in the *in vitro* plants and alkene, alkane, imine/oxime, alkane, carboxylic acid, sulphone, sulphoxide and alkene compound was there in the field grown plants of *S. chirayita*. The biomolecular compounds of *in vitro* samples demonstrated existence of wide variety of functional groups as compared to field grown hence, enhancing the phytochemical activities of *in vitro* samples as compared to the field grown extracts.

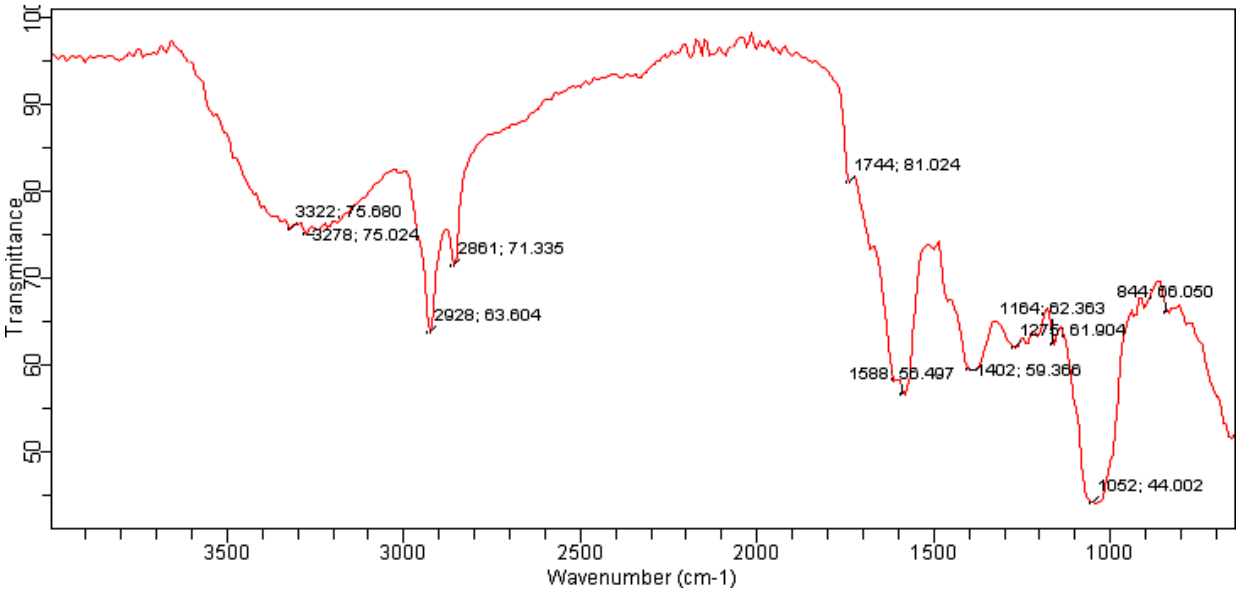


Figure 4.27 FTIR Spectrum of *in vitro* sample extracts.

Table 4.9 Functional groups in the *in vitro* sample extract

Peak observed (cm^{-1})	Group	Compound class
3322	N-H stretching	Secondary amine
3278	O-H stretching	Carboxylic acid
2928	C-H stretching	Alkane
2861	C-H stretching	Aldehyde
1744	C=O stretching	δ – lactone
1588	N-H bending	Amine
1402	S=O stretching	Sulphonyl chloride
1275	C-O stretching	Alkyl aryl ether
1154	C-O stretching	Aliphatic ether
1052	S=O stretching	Sulphoxide
844	C-Cl stretching	Halo compound

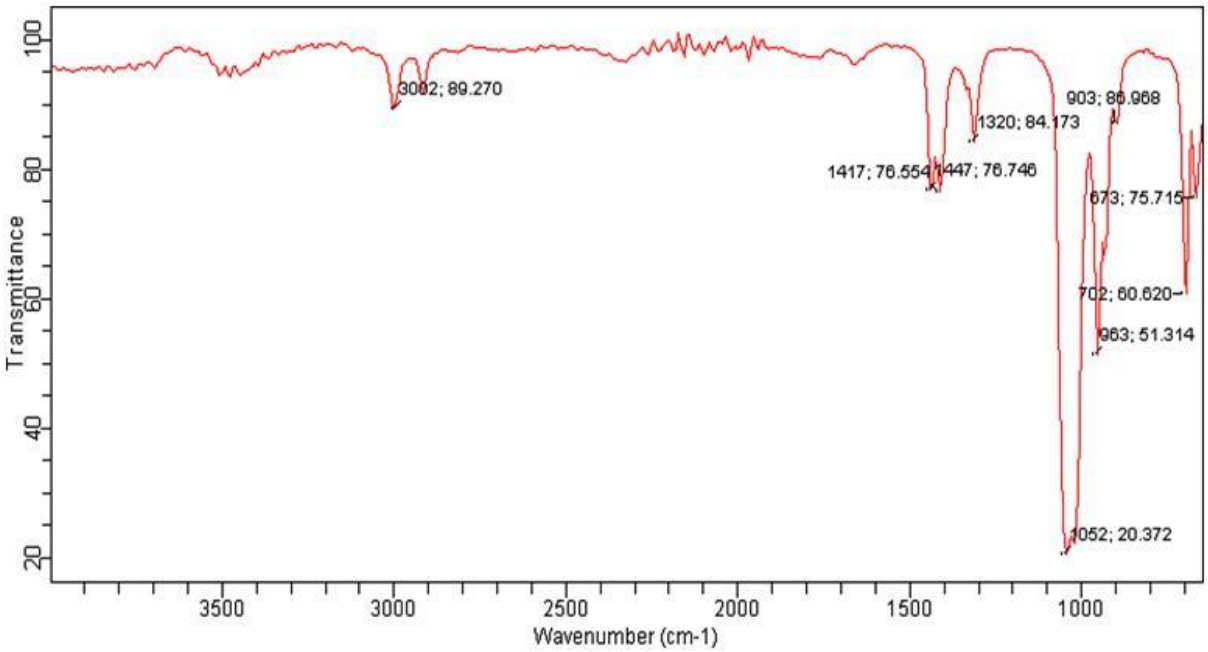


Figure 4.28 FTIR Spectrum of field grown sample extracts.

Table 4.10 Functional groups in the field grown sample extract

Peak observed (cm^{-1})	Group	Compound class
3002	C-H stretching	Alkene
2920	C-H stretching	Alkane
1640	C=N stretching	Imine/oxime
1447	C-H bending	Alkane
1417	O-H bending	Carboxylic acid
1320	S=O stretching	Sulphone
1030	S=O stretching	Sulphoxide
963	C=C bending	Alkene

4.21 LC-MS analysis of IVP and FGP of *S. chirayita*

The current study's goal was to use LC-MS to analyse the chemical makeup of *S. chirayita* plants produced *in vitro* and in the field. The extraction efficiency of various phytochemicals is influenced by a number of factors, including the extraction process, the matrix composition, the solute to solvent ratio, and the solvent polarity [310]. For the extraction of bioactive compounds, the right solvent selection is crucial [311]. Therefore, the plant samples were treated with methanol and subjected to liquid chromatographic-mass spectrometric analysis. The *in vitro* grown samples demonstrated the occurrence of 12 distinct phytochemicals whereas field grown samples demonstrated the occurrence of 9 distinct phytochemicals (Figure 4.29, 4.30). Identified phytochemicals in the *in vitro* grown samples are O,N-Permythylated N-Acetyllsine, Swertianin, 1-Cyclohexanone,5- [2{[1-[1,1-dimethylethy] -1,1-dimethylsilyl]oxy}-1-methylethyl] -2-methyl, Cis-13-Octadecenoic acid, methyl ester, Hexadecanoic acid, methyl ester, Benzoic acid-3-methoxy- 4-[(trimethylsilyl)oxy], Sweroside, Swertiamarin, Mangiferin, Amarogentin, Viminalol and Ameroswerin. Identified phytochemicals in the field grown samples are Swertianin, 1-Cyclohexanone,5- [2{[1-[1,1-dimethylethy] -1,1-dimethylsilyl]oxy}-1-methylethyl] -2-methyl, Cis-13-Octadecenoic acid, methyl ester, Hexadecanoic acid, methyl ester, Swertiamarin, Amarogentin, Viminalol and Ameroswerin as represented in Table 4.11. As per the best of our knowledge we have identified one chemical compound O,N-

Permythylated N-Acetyllsine in the *in vitro* grown samples which have the capabilities to treat various dermatological disorders, which was absent in the field grown samples [309]. Presence of such compounds can be explored further to check its potential in the *in vitro* and in vivo studies.

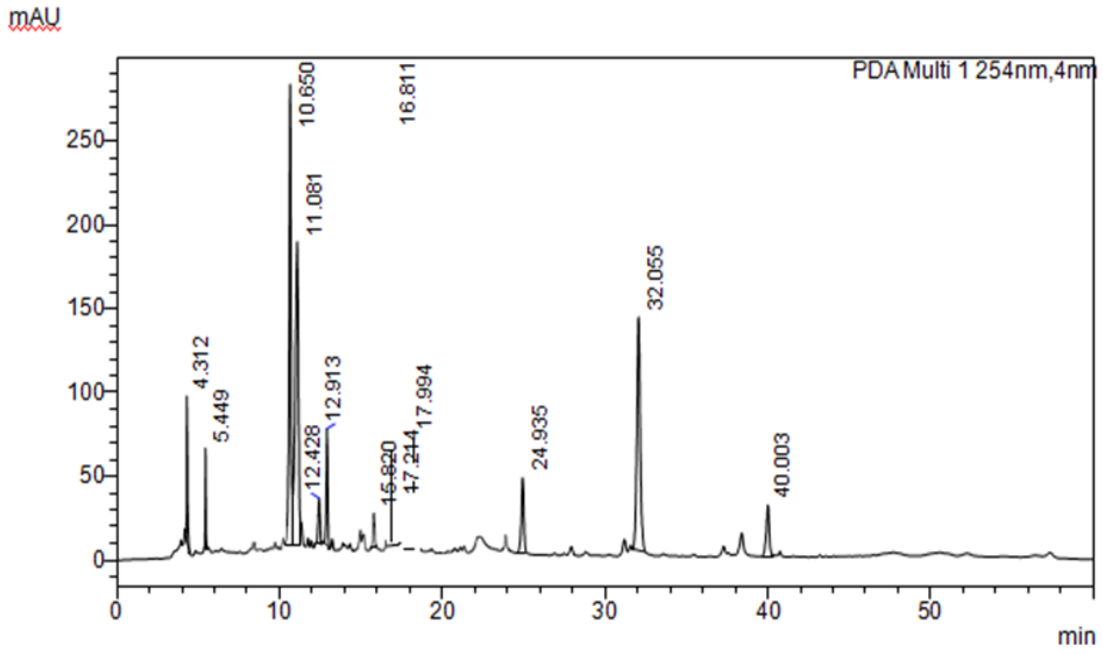


Figure 4.29 LC-MS chromatogram of *in vitro* extract of *S. chirayita*.

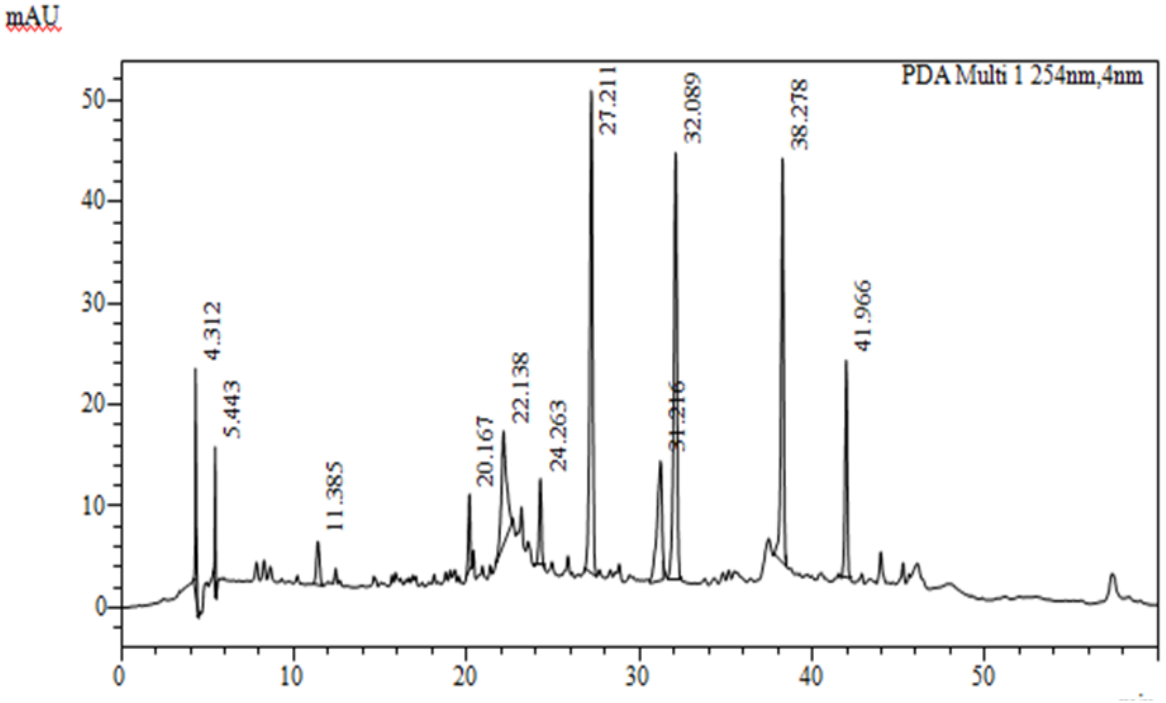


Figure 4.30 LC-MS chromatogram of field grown extract of *S. chirayita*.

Table 4.11 Retention time of *in vitro* and field grown *S. chirayita* samples (LC-MS Analysis)

Compound Name	Formula	MW	RT (FGP)	RT (IVP)
O,N-Permythylated N-Acetyllsine	$C_{13}H_{24}N_2O_4$	272		16.82
Swertianin	$C_{14}H_{10}O_6$	274.22	4.139	4.239
1-Cyclohexanone,5- [2{[1-[1,1-dimethylethy] -1,1-dimethylsilyl]oxy}-1-methylethyl] -2-methyl	$C_{16}H_{22}O_2$ Si	284	20.240	14.195
Cis-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	3.560	16.825
Benzoic acid-3-methoxy- 4-[(trimethylsilyl)oxy]	$C_{14}H_{24}O_4$ Si_2	312		31.168
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	328	18.686	14.196
Gentiopicrin	$C_{16}H_{20}O_9$	356.32	23.625	
Sweroside	$C_{16}H_{22}O_9$	358.34		6.268
Swertiamarin	$C_{16}H_{22}O_{10}$	374.34	31.811	14.195
Mangiferin	$C_{19}H_{18}O_{11}$	422		12.976
Viminalol	$C_{30}H_{50}O$	426	20.989	10.236
Amarogentin	$C_{29}H_{30}O_{13}$	586.54	18.686	3.935
Ameroswerin	$C_{29}H_{30}O_{14}$	602.5	26.161	14.195

As from the results we concluded presence of important groups like phenolics, flavonoids, terpenoids and esters, which shows various capabilities like antimicrobial, anticancer, antidiabetic, anti-inflammatory, and antioxidant. These results are in line with earlier studies in which ethanol based extracts of *S. chirayita* demonstrated that these phytochemicals were present in significant amounts [307, 308]. Overall, findings illustrated the higher number of compounds was identified at lower retention rates in the IVP as compared to FGP. This analysis revealed the presence of higher number of bioactive compounds in the IVP of *S. chirayita* in comparison to the FGP, so this provides *in vitro* grown plants as an alternate source of field grown plants in the era of pharmaceutical industries.

SUMMARY

Summary:

- Different developmental stages were identified for the production of major medicinal compounds (Aamrogentin and Mangiferin) in tissue cultures of *S. chirayita*.
- At 15 ± 1˚C, 1.08 folds increase in Amarogentin and 1.18 folds increase in Mangiferin was observed as compared to 25 ± 1˚C.
- 5.79 µg/mg of Amarogentin and 15.56 µg/mg of Mangiferin was reported highest in the fully grown shoots in MS media fortified with 3mg/l IBA and 1mg/l KN.
- 3 months old tissue culture plants accumulate 8.51 folds higher Amarogentin and 4.09 folds higher Mangiferin in comparison to the 3 months old field grown plants of *S. chirayita*.
- Maximum accumulation of bioactive compounds was achieved at shoot elongation with complete growth stage in the tissue raised plants under controlled gowth conditions (15 ± 1˚C in MS media fortified with 3mg/l IBA and 1mg/l KN).
- The Red LED illustrated higher propensity towards biomass accumulation (3.56 ± 0.0 g/l) in comparison to the other LED's on 21st day of culture.
- The Blue LED illustrated higher propensity towards Amarogentin (8.025 ± 0.04 µg/mg DW) accumulation on 21st day of culture.
- The highest phenolic content (22.33 ± 1.05 mg GA/g DW) and flavonoid content (29 ± 1.03 mg QE/g DW) was reported on the 30th day of culture in Blue LED.
- Highest antioxidant activity (50.40 ± 0.16%) was reported on 30th day of culture in Blue LED.
- Efficient establishment of somatic embryos on MS media fortified with MS + 1mg/l 2,4-D + 0.5mg/l BAP + 0.5mg/l TDZ.
- Artificial seeds were produced using 3% sodium alginate, 0.85 M sucrose and 100 mM calcium chloride.
- For better cryopreservation results, synthetic seeds were dehydrated and then immersed in liquid nitrogen for 1h.
- Successful cryopreservation, revival and germination of seeds were observed best in media provide with growth regulators MS + 1 mg/L IBA+ 2 mg/l KN + 3 mg/l GA3.
- Significant content of Amarogentin (4.72 ± 0.11 µg/mg) and Mangiferin (14.54 ± 0.05 µg/mg) was observed in the regenerated shoots and successfully acclimatized.

- *In vitro* raised plants showed highest antioxidant activity and antidiabetic activity in contrast to plants that are grown in fields.
- Promising antimicrobial activities were showed in the *in vitro* produced plants against Gram positive (*S. aureus* and *B. subtilis*) and Gram negative (*E. coli* and *S. typhi*) bacterial strains.
- Significant cytotoxicity was observed in HepG2 cancer cell lines (IC_{50} = 88.93 µg/ml) in *in vitro* raised plant extracts than in field grown plant extracts.
- Annexin VFITC and 7-AAD was used to confirm the apoptotic induction in the HepG2 cancer cells by using extracts of *in vitro* raised plants.
- This study provides preliminary data that proposes *in vitro* grown extracts of *S. chirayita* showed anti-cancer activity by triggering apoptotic cell death.
- LC-MS analysis and FTIR Analysis showed that there were more bioactive substances present in the *in vitro* raised plants hence indicating their potential role as in quality rich herbal materials for pharmaceutical industries.

FUTURE PROSPECTS

- Based on our principal findings, future studies can be prospectively directed towards large-scale production of industrially important bioactive compounds of *S. chirayita* in a bioreactor.
- Molecular exploration of biosynthetic pathways regulating bioactive compound production.
- Utilization of tissue culture plant extracts for the development of herbal formulations.

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PUBLICATIONS

Core thesis-oriented publications

1. **R. Gupta** and H. Sood, "Artificial seed production and cryopreservation by encapsulation dehydration for medicinal herb of Himalayan region, *Swertia chirayita*," *Cryoletters*, vol. 43 (5), pp. 295-302, 2022. [IF:0.83] (SCOPUS, SCI).

2. **R. Gupta** and H. Sood, "Optimizing nutrient media conditions for continuous production of shoot biomass enriched in major medicinal constituents, amarogentin and mangiferin of endangered medicinal herb, *Swertia chirayita*," *Vegetos*, Online (In Press), pp. 1-15, 2022. (SCOPUS).

3. **R. Gupta** and H. Sood, "Emerging technologies for the production of *in vitro* raised quality rich *Swertia chirayita* by using LED lights," *Sustainability*, vol. 15 (2), pp. 1-14, 2023. [IF:3.9] (SCOPUS, SCI).

4. **R. Gupta** and H. Sood, "Comparative phytochemical, anti-diabetic, antioxidant, antibacterial and cytotoxic analysis of *in vitro* and field-raised plants of critically endangered herb *Swertia chirayita*," *Plant cell tissue and organ culture,* [3.0] (SCOPUS, SCI). Communicated.

Other Publications

1. **R. Gupta** and H. Sood, "Micropropagation of critically endangered herb of Himlayan region: *Trillium govanianum* for *in vitro* shoot proliferation and production of Diosgenin," *Research Journal of Biotechnology*, vol. 16 (2), pp. 173-178, 2021. [IF: 0.45] (SCOPUS).

2. P. katoch, **R. Gupta** and H.Sood, "Effect of LEDs in phytochemical accumulation and biomass production in callus cultures of *Podophyllum hexandrum*," *Plant Science Today*, [0.9] (SCOPUS). Communicated.

3. **R. Gupta** and H. Sood, "Himalayan plant *Trillium govanianum* (nagchattri): Comprehensive review of botany, ethno medicinal use, agrotechnology and recent advancements," *Journal of Herbs, Spices and Medicinal Plants*, [2.4] (SCOPUS). Communicated.

Book Chapter

1. **R. Gupta** and H. Sood, "Trillium: An unexplored medicinal herb of Himalayan Region," *In Recent Advances in Pharmaceutical Sciences*, vol. 6, pp. 47-58, 2022, Bhopal: Innovare Academic Sciences. [ISBN : 978-81-952065-7-5].

Conferences:

1. **R. Gupta** and H. Sood. "Anti-diabetic and Antimicrobial activity of field grown and *in vitro* raised plants of *Swertia chirayita* – critically endangered herb of Himalayan Region" *Oral Presentation* in *International Conference on Recent Advances in Biotechnology*, Dr B R Ambedkar National Institute of Technology Jalandhar, December 2-4, 2022.

2. **R. Gupta** and H. Sood. "Biosynthesis and accumulation of Amarogentin and Mangiferin in different development phases of *in vitro* grown *Swertia chirayita*" *Oral Presentation* in *Proceedings of the 5th International Conference on Bioenergy, Environment and Sustainable Technologies (BEST2021)*, Arunai Engineering College, Tiruvannamalai, Tamilnadu, India, January 29-30th, 2021.

3. **R. Gupta**, D. Sharma and H. Sood. "Micropropagation of critically endangered herb of Himalayan region: *Trillium govaninanum* for *in vitro* shoot proliferation and production of Diosgenin" *Oral Presentation* in *Proceedings of the International Conference on Integrated Interdisciplinary Innovations in Engineering (ICIIE 2020)*, University Institute of Engineering and Technology (UIET), Panjab University, Chandigarh, August 28 – 30th, 2020.

4. **R. Gupta** and H. Sood. "Optimization of culture conditions for the production and germination of artificial seed in an important medicinal plant, *Swertia chirayita*" *Oral Presentation* in *International Conference on Technologies for Environmental Sustainability and Smart Agriculture Centre of Excellence in Sustainable Technologies for Rural Development [CESTRD]*, Jaypee University of Information Technology, Waknaghat, September 18-19th, 2020.

5. **R. Gupta** and H. Sood. "Biosynthesis and accumulation of medicinal compounds in different development stages of *Swertia chirayita* – the endangered medicinal herb of Himalyas" *Oral Presentation* in *International Conference on Recent Trends in Biotechnology and Bioinformatics*, Jaypee University of Information Technology, Waknaghat, August 1-3rd, 2019.

6. **R. Gupta** and H. Sood. "Direct organogenesis for rapid *in vitro* propagation of *Stevia rebudiana*" *Oral Presentation* in 3rd *Himachal Science Congress National, HIMCOSTE*, IIT Mandi, October 22-23rd, 2018.

7. D. Thakur, R. Kaur, **R. Gupta** and H. Sood, "Cryopreservation and regeneration of Picrorhiza kurroa" *Oral Presentation* in *Proceedings of the International Conference on Advances in Biosciences and Biotechnology*, Department of Biotechnology, JIIT,Noida, January 30th- February 1st, 2020.

8. S. Sharma, **R. Gupta**, A. Singh and H. Sood, "Effect of growth hormones on *in vitro* propagation and secondary metabolite production in *Stevia rebudiana*" *Oral Presentation* in *International Conference on New Horizons in Green Chemistry & Technology*, Uttaranchal college of Applied and Life Sciences, Uttaranchal University, Dehradun, November 27-28, 2018.

9. K. Thakur, P. Thakur, **R. Gupta** and H. Sood, "Optimization of Culture Conditions in Different Hydroponic Systems for Propagating and Hardening of *in vitro* grown medicinal plants" *Oral Presentation* in *National Seminar on Strategies for Conservation & Sustained utilization for Biodiversity*, Department of BT, School of Applied and Life sciences, Uttaranchal Univ, Dehradun and Uttrakhand Science Education and Research Centre, DST(Government Of Uttarakhand), October 18th -19th, 2019.

10. H. Sood, S. Singh, R. Sharma and **R. Gupta**, "Effect of Polychromatic Light Emitting Diodes on micropropagated shoots and metabolite production in *Swertia chirayita*" *Oral Presentation* in *Proceedings of the 2nd Global Conference on Plant Science and Microbiology Ecology*, Dubai, UAE, October 14th -16th, 2019.