TO STUDY THE EFFECT OF POLYCHROMATIC LIGHT EMITTING DIODES ON MICROPROPAGATED SHOOTS OF *SWERTIA CHIRAYITA* AND *PICRORHIZA KURROA*.

Dissertation submitted in fulfillment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

BY

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DECLARATION

We hereby declare that the work reported in the B.Tech Biotechnology thesis entitled **"To Study the effect of polychromatic LED's on Micropropagated shoots of *Swertia chirayita* and *Picrorhiza kurroa*"** 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**. We have not submitted this work elsewhere for any other degree or diploma.

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Date:

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in B.Tech. - Biotechnology project report entitled, **"To study the effect of Polychromatic LED's on micropropagated shoots of *Swertia chirayita* and *Picrorhiza kurroa*"** submitted by **Rhythem Sharma (141812) and Satyam Singh (141849)** at **Jaypee University of Information Technology, Waknaghat, India** is the bonafide record of their own work carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

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Date:

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RHYTHEM SHARMA **SATYAM SINGH**

LIST OF ABBREVIATIONS

26S	26S rRNA
DXPS	1-Deoxy-D-xylulose-5-phosphate synthase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GS	Geraniol synthase
G10H	Geraniol-10-hydroxylase
HMGR	3-Hydroxy-3-methylglutaryl-CoA synthase
HPLC	High Performance Liquid Chromatography
IBA	Indole-3-butyric acid
ISPE	4-(Cytidine-5-diphospho)-2-C-methylerythriol kinase
KN	Kinetin
LED	Light Emitting Diode
P-I	Picroside-I
P-II	Picroside-II
P. kurroa	*Picrorhiza kurroa*
PAL	Phenylalanine ammonia lyase
PMK	Phosphomevalonate kinase
P_W	*P. kurroa* incubated under white LED light
P_R	*P. kurroa* incubated under red LED light
P_{WFL}	*P. kurroa* incubated under white fluorescent light
S. chirayita	*Swertia chirayita*
WFL	White fluorescent light

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ABSTRACT

Swertia chirayita and ***Picrorhiza kurroa*** are medicinal plants of Alpine Himalayas having various medicinal properties and listed endangered species by IUCN. *Swertia chirayita* is antipyretic, hypoglycemic, antifungal, anticancer, antidiabetic, antioxidant and antibacterial; whereas *Picrorhiza kurroa* is hepatoprotective, antimicrobial, antioxidant, cardio-protective, antimalarial and antidiabetic. *Swertia chirayita* is an indigenous to temperate Himalaya, found at an altitude of 1200–3000 m (4000 to 10,000 ft) from Kashmir to Bhutan, and in the Khasi hills at 1200–1500m (4000 to 5000 ft); whereas *Picrorhiza kurroa* is found in the Himalayan region from Kashmir to Sikkim at an elevation of 2700-4500 m and in Nepal. Both medicinal herbs have great commercial importance. In order to see the effect of different lights on the morphological characters of the *in vitro* grown plants and for the accumulation of marker medicinal compounds the present study was conducted. The *in vitro* grown micro-shoots were taken and cultured on optimized solid MS media supplemented with growth hormones and were put under polychromatic LED's. The *in vitro* grown micro shoots of *P. kurroa* incubated under red and white LED's gave best results whereas, *S. chirayita* gave best excellent results in green LED's as compared to the shoots grown at optimised WFL.

HPLC quantification of marker medicinal compounds in *P. kurroa* and *S. chirayita* have been carried out where 0.318 mg/g Amarogentin and 1.818 mg/g Mangiferin is accumulated in shoots of *S. chirayita* incubated under green LED whereas, 1.458 mg/g P-I is accumulated in shoots of *P. kurroa* incubated under white LED and 0.788 mg/g P-I and 0.0389 mg/g P-II is accumulated in shoots of *P. kurroa* incubated under red LED. G10H gene involved in iridoid pathway of Picroside production showed increase in gene expression by 1.2834 fold in shoots of *P. kurroa* incubated under white LED and 1.90 fold expression in shoots of *P. kurroa* incubated under red LED as compared to shoots incubated under WFL.

So this is reported for the first time that LED's have significant effect on growth and secondary metabolite accumulation.

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 *Swertia chirayita*

Swertia chirayita is an annual/biennial herb of family Gentianaceae. It is also known as Chirayata, Charaita, Chirata (Hindi), Charayatah, Chirayita (Marathi), Nilavembu, Shirattakuchi, Anariyatittam (Tamil), Kiriyatta, Kiriyattu, Nilaveppa, Uttarakiriyattu (Malayalam), Nelavemu (Telgu), Nelabevu (Kannada), Chiraita shireen (Urdu), Anaryatikta, Ardhatikta, Bhunimba, Chiratika (Sanskrit).

1.1.1 Geographical distribution

Swertia chirayita is an indigenous to temperate Himalaya, found at an altitude of 1200–3000 m (4000 to 10,000ft) from Kashmir to Bhutan, and in the Khasi hills at 1200–1500m (4000 to 5000ft), it occurs in some parts of China, Tibet and Nepal. In India it is commonly found in Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Arunachal Pradesh, Meghalaya and Sikkim.

1.1.2 Morphology

S. chirayita is an erect, annual/biennial herb and reaches up to height of 0.6–1.5 m.

STEMS: Its stem is orange brown or purplish in color with length of 2-3 ft. Its stem has large continuous yellowish pith.

LEAVES: Leaves are generally lanceolate with length of 4 cm and is five to seven nerved It has no stalks, present in opposite pairs and is sessile.

ROOTS: Plant has simple yellow and somewhat oblique roots with short and tapering ends. Length of roots is about 7–8 cm. Roots is the most bioactive part of plant.

FLOWERS: Plant has green-yellow small, numerous, tetramerous flowers with large leafy panicles and tinged with purple and green or white hairs.

SEEDS: There are very small numerous and dark brown seeds of this plant.

1.1.3 Indigenous uses

Swertia chirayita is a medicinal herb with various medicinal properties such as it is antipyretic, hypoglycemic, antifungal, anticancer, antidiabetic, antioxidant, antibacterial. The wide range of medicinal uses include the treatment of chronic fever, malaria, anemia, bronchial asthma, hepatotoxic disorders, liver disorders, hepatitis, gastritis, constipation, dyspepsia, skin diseases, worms, epilepsy, ulcers, scanty urine, hypertension, melancholia, and certain types of mental disorders, secretion of bile, blood purification, and diabetes. Herbal formulations such as Ayush-64, Diabecon, Mensturyl syrup, and Melicon V ointment contain *Swertia chirayita* extract in different concentrations for its antipyretic, hypoglycaemic, antifungal, and antibacterial properties.

1.1.4 Chemical constitution

***Swertia chirayita*:** Major phytoconstituents are amarogentin, swertiamarin, mangiferin, swerchirin, sweroside, amaroswerin, and gentiopicrin. These compounds are responsible for the pharmacological activities of *Swertia chirayita.*

1.1.5 Pharmacological activity

***Swertia chirayita*:** According to previous researches *S. chirayita* extracts exhibit a wide range of biological activities, such as antibacterial, antifungal, antiviral, anticancer, anti-inflammatory, and others like antidiabetic and antioxidant activities. Therefore various *in vitro* and *in vivo* test systems has been used to evaluate the pharmacological properties of *S. chirayita* which proved that aqueous, alcoholic and methanolic extracts of *S. chirayita* possess a large number of pharmacological properties. The whole plant of *S. chirayita* have been reported to be used as antileishmanial, larvicidal, antidiabetic, anticarcinogenic, antihelmintic, antibacterial and antifungal. The whole plant is also used for treating CNS problems and is also anti-hepatitis B virus. Roots of the plant are antiinflammatory, antipyretic and analgesic whereas the leaf and stem portion of plant serves as antiviral, analgesic, antimalarial.

The classification of Swertia chirayita is as follows:

Kingdom: Plantae

Division: Angiosperm

Sub division: Dicotyledon

Class: Gamopetallae

Order: Gentionale

Family: Gentianaceae

Genus: *Swertia*

Species: *chirayita*

Figure 1.1 Plant morphology of *S. chirayita* (a) Plant in nature (b) Flowers

1.2 *Picrorhiza kurroa*

Picrorhiza kurroa is the perennial herb of family Plantaginaceae. It is also known as Kutki, Katuka, Tikta, Katui (Hindi, Sanskrit), Hu huang lian (China), Titka kul (Ayurvedic), Kadu (Gujarati).

1.2.1 Geographical distribution

Picrorhiza kurroa is found in the Himalayan region from Kashmir to Sikkim at an elevation of 2700-4500m, South-East Tibet, West China, North Burma, Pakistan and in Nepal between altitudes of 3500m and 4800m. In India it is mainly found in Himachal Pradesh, Kashmir, Kumaun and Garhwal region of Uttarakhand.

1.2.2 Morphology

Picrorhiza kurroa is a bitter, small trailing and perennial herb with rhizomes of 15-25 cm height.

STEM: *P. kurroa* has small, weak, erect with slightly hairy stem.

LEAVES: Plant has 5-15 cm long, radical, sharply serrate green leaves.

FLOWERS: Plant has 5-10 cm blue or violet colered flowers. These are subcylindric and obtuse, flowering occurs from June to August.

ROOTS: Roots are about 5–10 cm long and contains number of glycosides.

RHIZOMES: Plant has 15-25 cm long, woody, greyish-brown, irregularly curved rhizomes.

SEEDS: There are many, white seeds with oblong curved raphae.

1.2.3 Indigenous uses

Picrorhiza kurroa is an important traditional medicinal herb which is used to treat liver and upper respiratory conditions, bronchial asthma, chronic diarrhea, constipation, dyspepsia and jaundice. It has several medicinal properties such as it is hepatoprotective, antimicrobial, antioxidant, cardio-protective, antimalarial, antidiabetic, anti-inflammatory, antiallergic; modulates the immune system and liver enzyme levels. It is also helpful as a remedy for a number of auto-immune diseases such as vitiligo and psoriasis. Traditionally it was also used for treating scorpion stings and snake bites.

1.2.4 Chemical constitution

Picrorhiza kurroa: The root contains very bitter glycosides including kutkin which is a mixture of Picroside (I, II) and kutkoside. Picroside I and II are present at 1.611% and 0.613% of the roots dry biomass, respectively. Some other components such as androsin, α-methoxy substituted catechol Apocynin, structurally similar to vanillic and ferulic acids and nine cucurbitacin glycosides based on cucurbitacin B and dihydrocucurbitacin B are also present in plant.

1.2.5 Pharmacological activity

Picrorhiza kurroa: There are various properties of *P. kurroa* such as it shows antioxidant, anti-inflammatory, immunomodulatory activities bit the most valued property is that it is hepatoprotective. Some herbalists described *P. kurroa* as liver herb. Today researchers estimated few active constituents which serves hepatoprotective property of plant. After isolation of kutkin from plant, various *in vitro* tests were conducted on rats to observe the hepatoprotective activity of it, and it was proved that kutkin shows significant curative avtivities. Rhizomes of plant treats indigestion. A phenolic glycoside known as androsin reduces allergic reactions. Ethanol, ethyl acetate and butanol extract of *P. kurroa* leaves showed antioxidant activity.Roots of *P. kurroa* contains Iridoids which exhibits a wide range of pharmacological activities such as cardiovascular, hepatoprotection, hypoglycaemic, antimutagenic, antispasmodic, anti-tumour, antiviral, immunomodulation and purgative effects.

The classification of *Picrorhiza kurroa* is as follows:

Kingdom: Plantae

Division: Angiosperms

Class: Dicotiledonea

Subclass: Asteride

Order: Scorphulariales

Family: Scorphulariaceae

Genus: *Picrorhiza*

Species: *kurroa*

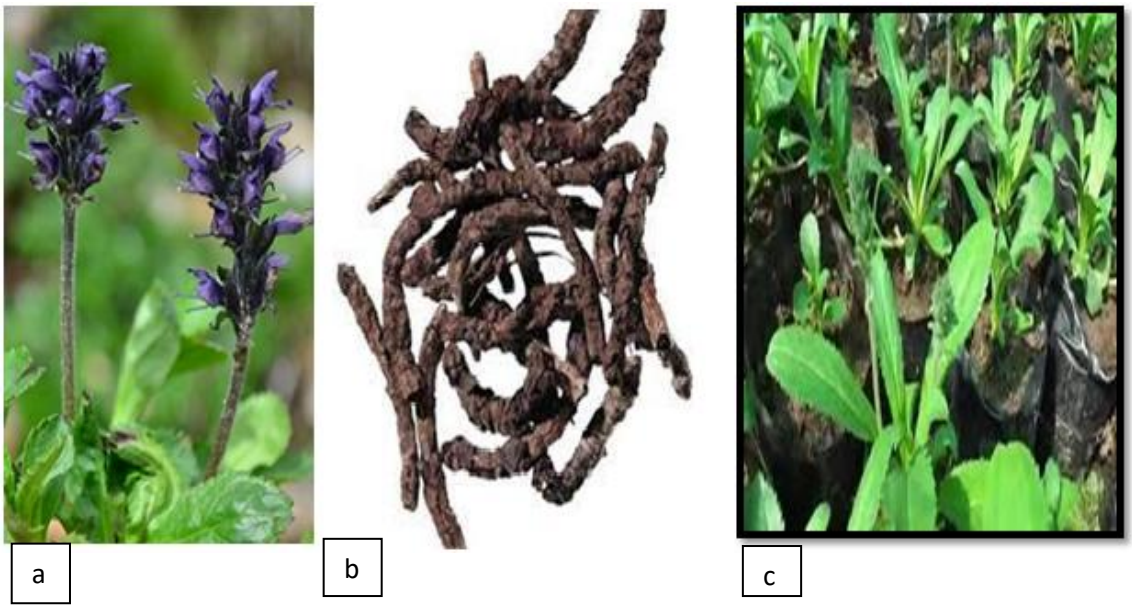


Figure 1.2 Plant morphology of *P. kurroo* (a) Flowers (b) Rhizomes (c) Plant in nature

1.3 Effect of LED light on Plants

The major factor responsible for plant growth and development is light. As plants are autotrophs so they are reliable on some sort of light source for the production of food, energy and for maintaining morphology. For outdoor plants the greatest source of light is sun. However, for indoor plants any other source of light can be helpful. LED's provide nearly all the benefits of sunlight. Most importantly, they produce the wavelengths of light that a plant needs to grow and develop.

Light-emitting diodes (LED's) are solid state conductor that generate photons by using few properties of metals and likewise convert electricity into light. In 1980s LED's were used in photobiology research and in 1990s use of LED's for plants were evaluated for space flight. Over the past few years LED technology has developed and is also used for photobiology.

The applicative uses of LED's are increasing day by day. In Japan lettuce is commercially produced by using LED's, light fixtures for intracanopy lighting are developed, few reports shows that some secondary metabolite production is increased by using LED's, some reports shows rapid growth and root formation in plants.

The main advantages of using LED's reported so far are that LED's are lower energy costs, durable, longer lifetime of light source, lower heat generation and provides targeted wavelength **[26]**; Morrow 2008). The predicted lifetime of LED's is about 50-100 hours without drop in efficiency and even consumption of energy is 40% lower than traditional fluorescent tubes. It's even reported that LED's provide high quality and intensity light than traditional florescent tubes. Plant response varies for each targeted wavelength LED light. According to some reports better growth is reported in plants that are not exposed to Ultraviolet light (200 nm - 380 nm). Schuerger et al. (1997) reported that leaf thickening and number of chloroplasts per cell is more depended on blue light than the red: far red ratio. According to Schwartz and Zeiger, (1984) blue light plays various photomorphogenic roles in plants such as helps in stomatal control which further affects carbon dioxide exchange, water relations. Cosgrove (1981) reported in the studies that blue light helps in stem elongation, whereas Blaauw and Blaauw-Jansen (1970) reported the use of blue light in phototropism. According to few

researchers green light (495 nm – 570 nm) help in enhancing chlorophyll production. According to Kim et al. (2004) green light more than 50% cause reductions in plant growth, whereas combinations including upto 24% green enhances growth for some species. Source from NASA indicates that yellow light (570 nm – 590 nm) does not contribute to photosynthesis. Goins et al. in 1997 reported that plant growth under red light (720 nm – 750 nm) is more optimal than blue light. Roel C. Rabra et al. (2017) reported that root growth and seedling growth and development is more under red light than blue light.

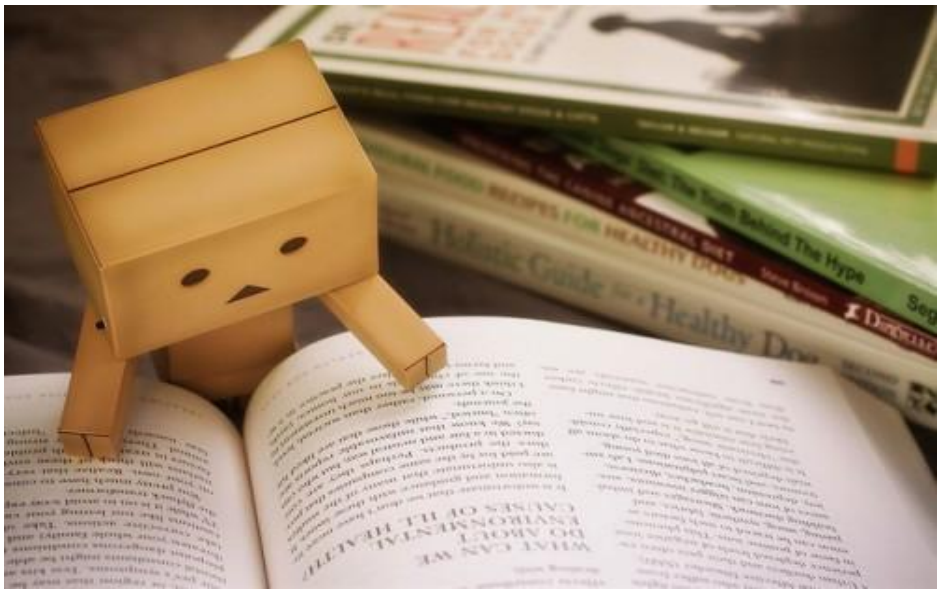
1.4 Rationale of work

1. Under culture conditions phytopharmaceutical compounds are produced in very less concentration when compared to field grown plants, so we would like to see the effects of polychromatic LED's on these two commercial important medicinal plants as a model system.
2. As there are no reports on using these polychromatic LED's for improving the medicinal & growth status of *Swertia chirayita* and *Picrorhiza kurroa.*

1.5 The objectives are followed:

1. To study the effect of LED's on morphology and phytochemical composition in micropropagated shoots of *Swertia chirayita* and *Picrorhiza kurroa*
2. To study the gene expression for Picroside-I production.

CHAPTER 2



REVIEW OF LITERATURE

2.1 *Swertia chirayita*

S. chirayita is the crucial herb with large number of medicinal properties. Roxburgh in 1814 firstly described *S. chirayita* as *Gentiana chyrayta* **[1]**. Common name of *S. chirayita* is "Chiretta" which is a critically endangered herb, grown at high altitudes of Himalayas between 1200 and 2100 m altitudes **[2]**. *S. chirayita* is bitter in taste because of presence of Amarogentin which is the most bitter compound isolated till date. Various other bioactive compounds such as swerchirin, swertiamarin, mangiferin and many more are also isolated from *S. chirayita*. There are various medicinal properties of *S. chirayita* which includes treatment of asthma, gastritis, hepatitis, epilepsy, hypertension, dyspepsia, malaria, anemia **[3]**. Micropropagation of *Swertia chirayita* was carried using different plant growth hormones. They discovered BAP at 1.0 mg/l and KN, 0.1 mg/l induced highest multiple shoots per explants. However, highest frequency of multiple roots were in culture which was supplemented with NAA, 0.1 mg/l **[8]**. Best shoot regeneration was obtained in Murashige and Skoog's basal medium supplemented with 0.44 µM 6-benzylaminopurine and 4.65 µM 6-furfurylaminopurine. The highest number of shoots were regenerated in media fortified with 10 mM $KNO_3$and 75 mg l^{-1} of casein hydrolysate **[12]**.

Elicitation of major pharmaceutical metabolites in *Swertia chirayita* was conducted and it was discovered that media containing *Agrobacterium rhizogenes* showed best result when compared with other elicitors like Methyl Jasmonate, Salicylic Acid, Vanadylsulphate, Yeast extract, and Hoagland solution **[9]**. Quantification of marker compounds of *S. chirayita* was done by researchers by using HPLC and they found that retention peaks for swertiamarin, mangiferin and amarogentin were observed at 8.5 min, 13.6 min and 21.7 min. They quantified 2.99 µg/mg of mangiferin and 1.03 µg/mg of amarogentin in shoots of *S. chirayita* on MSM medium with IBA and KN as growth hormones. **[9]**. There is a report in which scientists have reported that content of mangiferin, amarogentin is high in tissue cultured plants over callus culture and field grown plants of *S. chirayita* **[10]**.

2.2 *Picrorhiza kurroa*

P. kurroa is a perennial herb which is grown in Himalayan regions at 3000 – 4300 m altitudes from Kashmir to Uttarakhand **[11]**. Various phytoconstituents (total 132) have been reported in *P. kurroa* from various plant parts such as leaf, stem, roots, seeds and rhizomes **[13]**. Chemical compounds such as iridoid glycosides (P-I, P-II), cucurbitacins, phenolics and phenylethanoids have been isolated from *P. kurroa* **[14]**. There are various medicinal properties of *P. kurroa* such as it is used for treatment of fever, asthma, jaundice, leukoderma, scorpion sting, snake bite and urinary problems, anti-inflammatory, hepatoprotective, immunmodulatory **[13]**. Few reasearchers have found that *P. kurroa* has anti-anaphylactics, anti-allergic properties **[17]**. P-I is biosynthesized in shoots and P-II is biosynthesized in roots or stolons **[13]**. Vegetative propagation of *Picrorizha kurroa* using different growth hormone was conducted by researchers. It was discovered that for root multiplication 50.0 μM IBA and for shoot multiplication 1.0 μM BAP is best **[21]**. Hemant Sood et al. discovered IBA and KN are the best PGR for propagation of *Picrorhiza kurroa*. IBA (2 mg L^{-1}) + KN (3 mg L^{-1}) is the best medium for multiple shoot formation which was upto 86.3%. This study was focused on low cost medium combination for shoot propagation of *Picrorhiza kurroa*. Researchers concluded that P-I is accumulated in shoot cultures and not in callus cultures. P-I content reported in leaf discs, stem and root segments was 1.9, 1.5 and 0.04 mg/g. P-II was not detected by them **[18]**. Molecular expression of genes responsible for the production of P-I in *P. kurroa* was done by researchers and they found that there is up-regulation and high transcript abundance of seven genes i.e. HMGR, PMK, GS, G10H, PAL, DXPS, DAHPS **[20]**.

2.3 Effect of light on plants

Researcher carried out study on effects of different wavelength of sunlight obtained at different time on field-grown *Nicotiana tabacum*. He discovered end-of-day light i.e. far red light caused development of longer internodes, which were light green in colour and had thinner leaves as compared to plants that received red light. Plants which received red light had branches from axils of lower leaves which were not developed in far red-irradiated plants **[22]**. For the growth of plant, different wavelengths of light are

required. After reviewing different researches which were conducted on different plants concluded that at different growth stage of plant it requires different wavelength which can be most effectively controlled by the use of LED lights. They also conclude that many research needs to be done on this aspect as to find optimal combination of LED lights of different wavelength for maximum growth and development for large scale industrial use. **[24]**. Cryptochrome 1 (CRY1) is flavin-type blue light receptor of *Arabidopsis thaliana* which causes development of dwarf plants but had increased accumulation of anthocyanin. This study infers that light has effect on plant growth and development, and in addition to it light effects can be seen on different metabolite production and accumulation **[24]**. Chitose Kami et al. studied light regulated plant growth and development. They found that there are many photoreceptors that are structurally unrelated to each other. They concluded that even all the photoreceptors induce light mediated protein-protein interactions but the end result of all the interactions is different on plant growth and development **[25]**. Researchers discovered the use of LED light for horticulture. Study suggests that LED's are potential source of lighting system for horticulture. At NASA's Kennedy Space Centre they are focused on finding out optimum proportion of colour light from LED's that give maximum growth and development **[26]**. Franka Seiler compared phenotypic and molecular traits of *Arabidopsis* thaliana under different colour LED lights. According to analysis it can be inferred that growth behaviour can be easily influenced using LED's. Fluorescent lights have disadvantage that they produce heat at high intensity of light which is eliminated while using LED's thus provides stable environmental factors in each colour of light **[27]**. Blue light has detrimental effect on plant growth. Researchers used eight blue and green lights at two photosynthetic photon fluxes (PPF; 200 and 500 μmol m^{-2} s^{-1}). Plants used were tomato, cucumber, pepper, radish, soybean, lettuce and wheat. Out of these seven plants tomato, cucumber, and pepper showed reduced growth with increasing intensity of blue light. Green light showed no effect on plant growth and development even after the 93 % treatment **[28]**. Combinations of didderent LED's were also tested with white light in which people found that red LED in combination with blue light has same effect as white light. Triticum aestivum was used to study the effect of red LED, red LED with blue fluorescent lamp, and white fluorescent on photomorphogenesis, photosynthesis, and seed yield in plant. Red LED is preferred in this study due to its safety, small volume, wavelength specificity, and longevity. After experimentation it

was discovered that alone red LED has detrimental effect, but in combination with blue fluorescent lamp the growth was same as white light **[29]**. Effect of polychromatic LED's was observed on different plants. LED's have lifetime from 50 hrs to 100,000 hrs without drop in efficiency of bulbs. Additionally the bulbs consumed 40% less energy than the traditional illumination technology. Effect of polychromatic LED's were studied on *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Glycine max*, *Solanum tuberosum* and *Brassica napus*. Study showed that effect of polychromatic LED's was different on every plant **[30]**. People discovered the effect of spectral quality of Monochromatic LED lights on growth of artichoke seedlings and reported that red light increased about 97% seedling growth and development in three artichoke genotypes. They even reported the increase in all plant characters including growth in red light than the other lights **[31]**.

CHAPTER 3

MATERIALS

AND

METHODS

MATERIAL AND METHODS

3.1 Plant Source

Swertia chirayita and *Picrorhiza kurroa* cultures are maintained in the culture room of Department of Biotechnology and Bioinformatics, JUIT, Waknaghat.

3.2 Mass propagation of plants on defined media

Shoot explants of *Swertia chirayita* and *Picrorhiza kurroa* were cultured and subcultured after every 30 days on MS media containing IBA (1mg/L), KN (3mg/L), sucrose (30 g/L) and agar (8 g/L) with pH 5.7 followed by autoclaving the media at 121℃, 15 lb $inch^{-2}$ pressure for 20 minutes in order to carry out mass multiplication of the plants.

3.3 Use of LED lights

Different colored 12 Watt LED's were bought through online shopping website named Amazon. Blue, red and green colored LED's are of brand Empire whereas, white colored LED is of Tejas company. Light that is used as control i.e. WFL is of Philips.

Table 3.1 Types of LED's used in experiment

S. No.	COLOR	BRAND & POWER
1	White	Tejas – 12 Watt
2	Blue	Empire – 12 Watt
3	Red	Empire – 12 Watt
4	Green	Empire – 12 Watt

3.4 Preparations of LED setup at 15℃ ± 1 and 25℃ ± 1

LED's were set in Plant tissue culture room at 15℃ ± 1 and 25℃ ± 1 and the respective chambers were covered with black chart papers, so that plants do not expose to any other light. 25 replicates of both plants were placed under each setup along with the control (WFL) (Figure 4.2)

3.5 Data collection

Plant samples of *S. chirayita* and *P. kurroa* were collected from all LED setups after 30 days of interval. Various parameters were noted which included length of root (cm), length of shoot (cm), number of roots, number of shoots, adventitious roots, number of browned leaves, plant biomass.

3.6 Hardening of plants

Well grown shoots of *S. chirayita* and *P. kurroa* were gently uprooted from the culture jars followed by washing of the plant under running tap water and transferred to pots containing sand and soil mixture (control) and to potting mixture (1:1:1 mixture of perlite : vermiculite : cocopeat). *S. chirayita* and *P. kurroa* were even planted in different mixtures like Perlite + Vermiculte (1:1); Perlite + Cocopeat (1:1:1); Cocopeat + Vermiculte (1:1). In order to avoid dessication, the plantlets were covered with glass jars for 15-20 days and in order to acclimatize the plantlets to the external environment, glass jars were removed from the plantlets for 1-2 hours every day. Data was recorder in order to observe the survival rate of plants.

3.7 Quantification of Marker compounds by HPLC

After 30 days of incubation in LED's fresh shoots of *S. chirayita* and *P. kurroa* were used for estimation of marker medicinal compounds or secondary metabolites by using High Performance Liquid Chromatography (HPLC).

Fresh plantlets of *P. kurroa* and *S. chirayita* were gently uprooted from glass jars and shoots were crushed in liquid nitrogen which led to the formation of powdered sample, the 100 mg of this powdered sample was mixed with 10 ml of 80% methanol. The samples were vortexed followed by sonication for 10 minutes with 2 seconds pulse at

30% amplitude, then the samples were centrifuged at 10,000 g for 10 minutes followed by filtration of supernatant using 0.22 µm filter (Millipore). The filtrate of *P. kurroa* was used for estimation of P-I and P-II content by following the method described by **[18]** and the filterate of *S. chirayita* was used for the estimation of Amarogentin and Mangiferin content by following the method described by **[9]**. Quantification is done on basis of retention time of marker compounds. Retention time of P-I is 14.154 (Figure 3.1), P-II is 8.626 (Figure 3.2) , Amarogentin is 18.658 (Figure 3.3) and Mangiferin is 4.617 (Figure 3.4). Quantification was done on Waters HPLC System equipped with Waters 515 HPLC pumps, Waters 717 autosampler, Waters 2996 photodiode array detector and Empower software. Waters Spherisorb reverse phase C18 column (4.6 mm x 250 mm, 5 µm) was used as the stationary phase and 20 µL of the sample was injected into it with the mobile phase for the analysis of the secondary metabolite content. The mobile phase used for the analysis of P-I and P-II in *P. kurroa* was solvent A (0.05% trifluoro-acetic acid in water) and solvent B (1:1 methanol : acetonitrile mixture). Isocratic method was used as ratio of Solvent A : Solvent B was 70:30 (v/v) throughout the process with a flow rate of 1 ml/min at detection wavelength of 270 nm. The cycle time of analysis was 30 min at 30℃. The mobile phase used for the analysis of Amarogentin and Mangiferin content in *S. chirayita* was solvent A (0.1% TFA) and solvent B (acetonitrile : water in ratio of 70:30). The linear gradient at a flow rate of 1.0 ml/min was start with 15% B; 20% B in next 5 min, 70% in next 25 min, hold for 5 min; 15% B in next 5 and equilibrated for 5 min at 240 nm UV wavelength. The presence of specific compounds in the sample was identified on the basis of the retention time and comparison of UV spectra with specific standards of both species procured from Chroma Dex, Inc. and calculated in µg/mg.

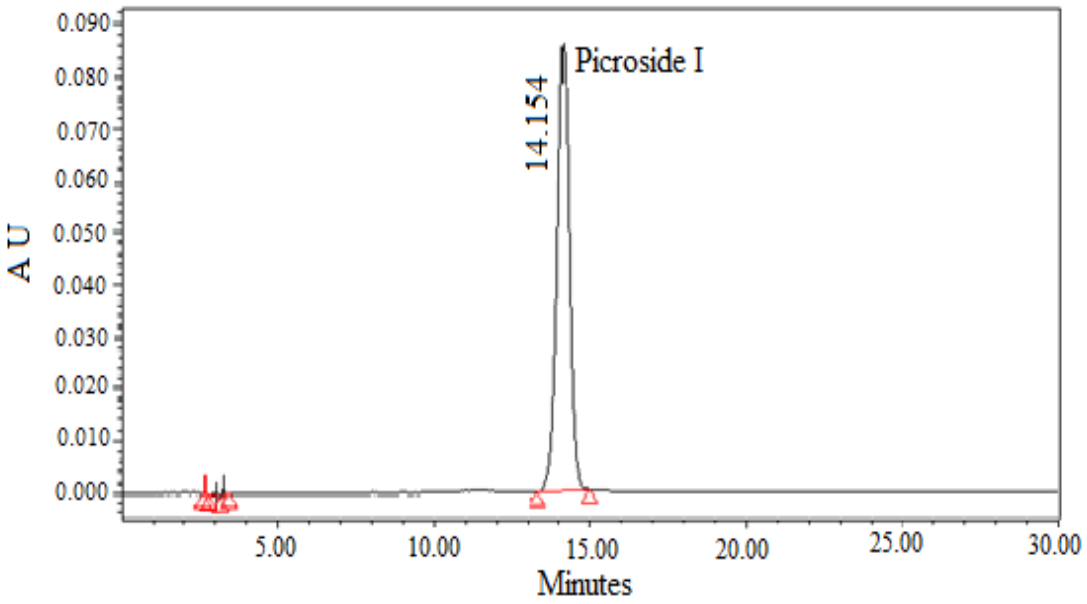


Figure 3.1 HPLC chromatogram of P-I standard of *P. kurroa*

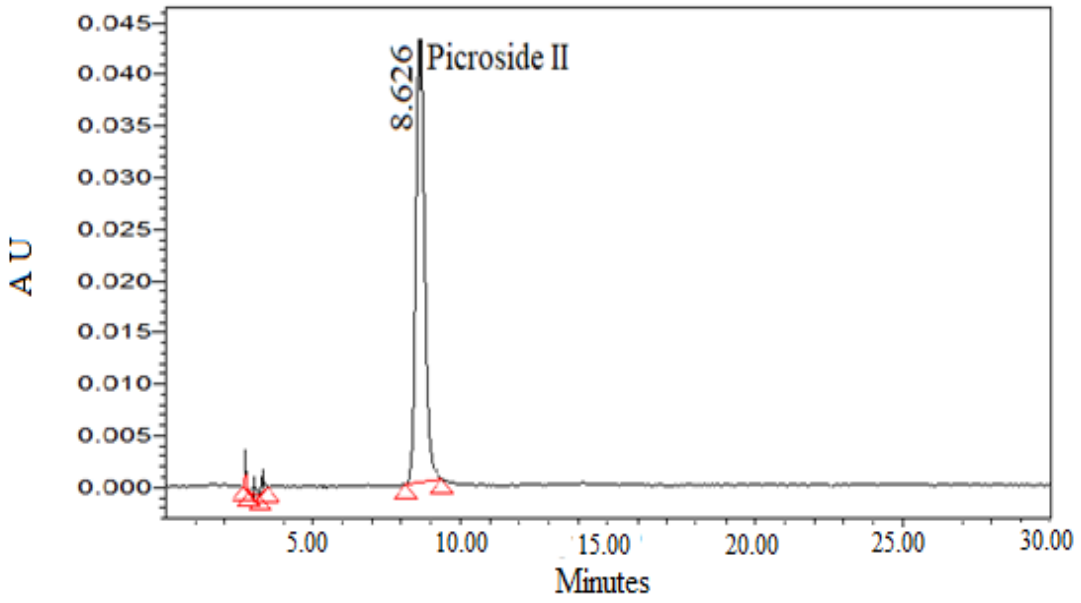


Figure 3.2 HPLC chromatogram of P-II standard of *P. kurroa*

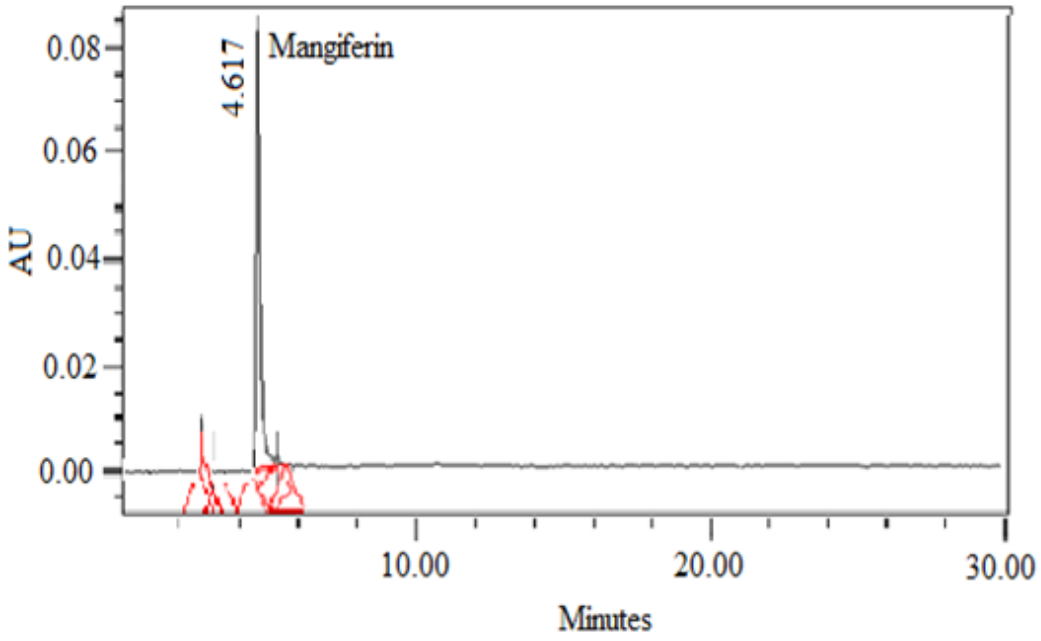


Figure 3.3 HPLC chromatogram of Mangiferin standard of *S. chirayita*

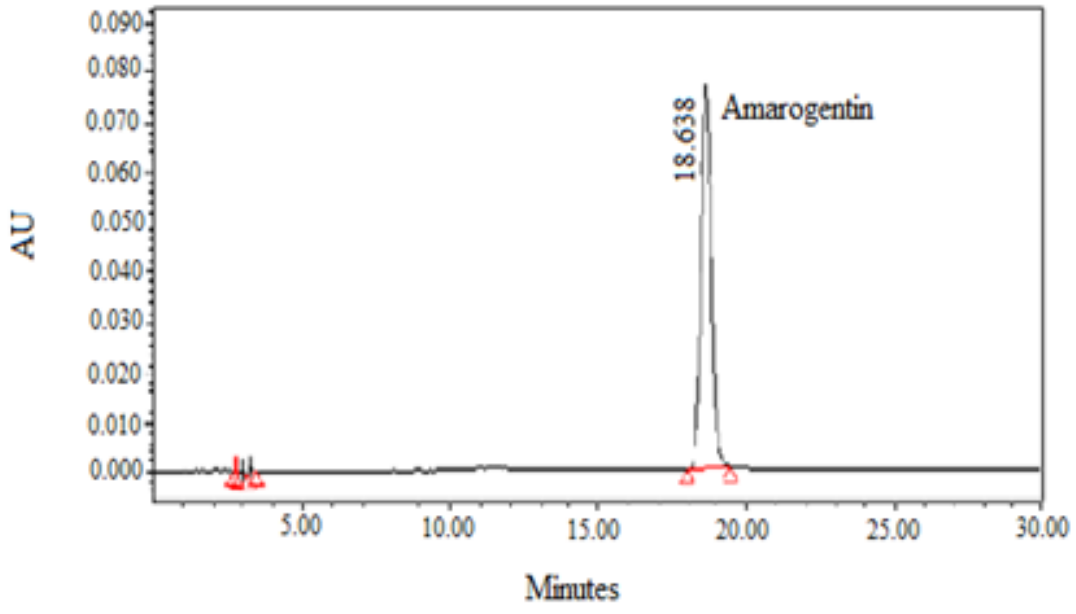


Figure 3.4 HPLC chromatogram of Amarogentin standard of *S. chirayita*

3.8 Expression Analysis

3.8.1 RNA isolation and preparation of cDNA

RNA was isolated from the fresh samples of P_W, P_R and P_{WFL} using RNA isolation kit of QIAGEN. The isolated RNA was quantified using spectrophotometeric (A260 and A280) measurements (NanoDrop, Thermo Scientific, USA). Further integrity of the RNA was assessed on agarose gel stained with 1% (w/v) ethidium bromide. The isolated total RNA was used to prepare cDNA using Verso cDNA synthesis kit (Thermo Scientific).

3.8.2 Quantitative real time-PCR (qRT-PCR) analysis

There are various metabolic pathways involved in the production of P-I and P-II, which mainly includes MVA pathway, MEP pathway, Phenylpropanoid pathway/Shikimate pathway and Iridoid pathway. Two different genes from the MVA pathway (HMGR, PMK), two genes from MEP pathway (DXPS, ISPE), two genes from iridoid pathway (GS, G10H) and one gene from Shikimate pathway (PAL-1) were finalized based on the previous conducted studies. Primer pairs for HMGR, G10H, PAL-1, GS, PMK, DXPS, ISPE were procured from **[21]**(Table 3.2). The housekeeping gene, 26S was used as an internal control for the calculation of transcript abundance. Gene expression analysis in samples P_W, P_R and P_{WFL} of *P. kurroa* was done in order to verify the physiological changes observed in plants due to the effect polychromatic LED's.

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')	Fragment Size (bp)	Annealing Temperature (?)
G10H	TATCGAGCTTTTCA GTGGAT	GATGTGAGTCCTGT CGATTT	136	52
PAL-1	GCAAGATAGATAC GCTCTAA	GTTCCTTGAGACGT CAAT	136	49
ISPE	TTCATCTAGATAAG AAGGTGCCAAC	CCTCTACCAGTACA ATAAGCAGCTC	110	55
HMGR	CGTTCATCTACCTT CTAGGGTTCTT	GACATAACAACTTC TTCATCGTCCT	100	60
DXPS	ACATTTAAGTTCAA GTCTGGGAGTG	ATGTGCACTCTCTT CTCTTTTAGGA	110	55.9
GS	TGGGTAGATTAGAA GCCAGA	CTGGTGATTTCTAC CAGCTC	139	52
PMK	TGGATGTTGTCGCA TCAGCACCTGG	GTAATAGGCAGTCC ACTCGCTTCAA	100	58

Table 3.2 List of primer sequences used in qRT-PCR based expression analysis

CHAPTER 4

RESULTS AND DISCUSSIONS

RESULTS AND DISCUSSION

4.1 *In vitro* shoot multiplication

In vitro shoots of *Swertia chirayita* and *Picrorhiza kurroa* were cultured and sub cultured in MS media with growth hormones (IBA + KN; 3:1) at 15℃±1 and 25℃±1 and it was observed that plants at 15℃±1 showed best growth (Figure 4.1(a), 4.1(b)).

4.2 Incubation of *in vitro* shoots in polychromatic LED's.

Cultured *in vitro* shoots of *S. chirayita* and *P. kurroa* were incubated at same culture conditions as of control (WFL) but in different (polychromatic) LED's (Figure 4.2), which include incubation in white LED (Figure 4.3), red LED (Figure 4.4), blue LED (Figure 4.5) and green LED (Figure 4.6). After 30 days of growth plant samples were collected for observing various growth parameters.

In *S. chirayita* best results were observed in green LED light where maximum shoot length (5.67 cm), root length (0.67 cm), number of shoots (6.67) and number of roots (21.67) compared to other LED's (Figure 4.7). Under blue light incubation adventitious roots were observed (Figure 4.8[a], 4.8[b]). Maximum increase in plant biomass (13.875 g) was observed in plants incubated under red LED as compared to other LED's (Figure 4.9). Plants incubated under white LED showed increase in number of leaves (37.34) and formation of adventitious roots only (Figure 4.10[a], 4.10[b]) (Table 4.1).

In *P. kurroa* maximum increase in root length (0.74 cm), number of leaves (46) and plant biomass (1.709 g) was observed in plants incubated under white LED light as compared to other lights (Figure 4.11). Maximum increase in number of shoots (7.67) was reported in plants incubated under red and white light as compared to other LED lights (Figure 4.12). Maximum increase in shoot length (9.5 cm), white colored adventitious roots and browning in leaves were reported in plants incubated under blue light (Figure 4.13). In plants incubated under green light maximum increase in number of roots (11.67) was observed (Figure 4.14) (Table 4.2)

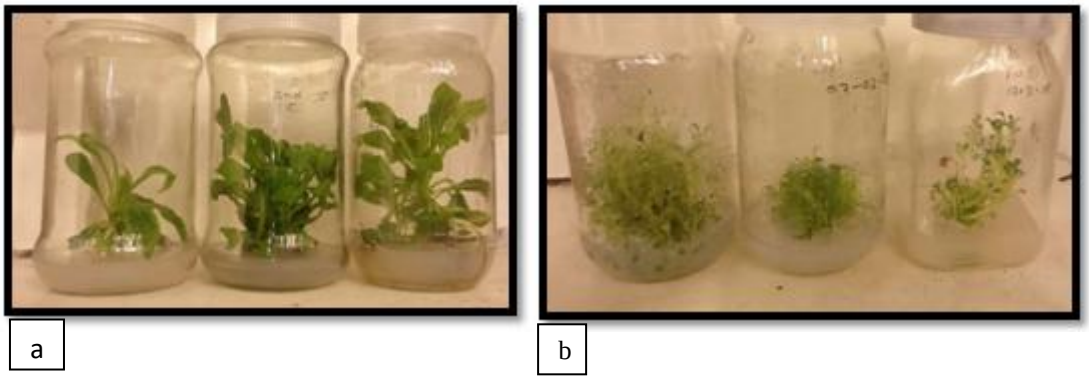


Figure 4.1 *In vitro* micro propagated shoot in MS medium of (a) *Swertia chirayita* (b) *Picrorhiza kurroa*

Figure 4.2 Fabrication of LED Setup in Plant Tissue Culture Room at 15℃ ± 1

Figure 4.3 *Swertia chirayita* and *Picrorhiza kurroo* under White LED light at 15℃ ± 1

Figure 4.4 *Swertia chirayita* and *Picrorhiza kurroo* under Red light at 15℃ ± 1

Figure 4.5 *Swertia chirayita* and *Picrorhiza kurroo* under Blue light at 15℃ ± 1

Figure 4.6 *Swertia chirayita* and *Picrorhiza kurroo* under Green light at 15℃ ± 1

Table. **4.1** Growth and developmental parameters observed under LED's and standarised white fluorescent light (WFL) in *S. chirayita*

GROWTH PARAMETERS	Incubated *in vitro* shoots under WFL	Incubated *in vitro* shoots under GREEN LED light	Incubated *in vitro* shoots under RED LED light	Incubated *in vitro* shoots under BLUE LED light	Incubated *in vitro* shoots under WHITE LED light
Shoot length (cm)	**3.00**	**5.67**	**5**	**5.17**	**4.16**
Root length (cm)	**0**	**0.67**	**0.65**	**0.4**	**0**
No. of shoots	**1.34**	**6.67**	**4**	**4**	**4.34**
No. of roots	**0**	**21.67**	**20.34**	**10.37**	**0**
No. of leaves	**5**	**29.67**	**29**	**21**	**37.34**
Adventitious Roots	**0**	**0**	**0**	**4.34**	**8.67**
Browned leaves	**0**	**0**	**0**	**0**	**0**
Plant Biomass (g)	**0.098**	**2.11**	**13.785**	**1.819**	**1.815**

Table. **4.2** Growth and developmental parameters observed under LED's and standarised white fluorescent light (WFL) in *P. kurroa*

Growth Parameters	Incubated *in vitro* shoots under WFL	Incubated *in vitro* shoots under GREEN LED light	Incubated *in vitro* shoots under RED LED light	Incubated *in vitro* shoots under BLUE LED light	Incubated *in vitro* shoots under WHITE LED light
Shoot length (cm)	**2.72**	**8.17**	**6.67**	**9.5**	**7.67**
Root length (cm)	**0**	**0.43**	**0.4**	**0.5**	**0.74**
No. of shoots	**1.34**	**5**	**7.67**	**1.67**	**7.67**
No. of roots	**0**	**11.67**	**3.67**	**1.67**	**8.67**
No. of leaves	**7.27**	**16**	**40.67**	**29**	**46**
Adventitious Roots	**0**	**0**	**0**	**7**	**0**
Browned leaves	**0**	**4.67**	**1.67**	**6.67**	**0**
Plant Biomass (g)	**0.158**	**0.781**	**1.223**	**0.26**	**1.709**

Figure 4.7 Cultured micro shoots of *Swertia chirayita* grown under green LED light

Figure 4.8 [a] Cultured micro shoots of *Swertia chirayita* grown under blue LED light.

Figure 4.8 [b] Cultured micro shoot of *Swertia chirayita* grown under blue LED light showing adventitious roots.

Figure 4.9 Cultured micro shoots of *Swertia chirayita* grown under red LED light

Figure 4.10[a] Cultured micro shoots of *Swertia chirayita* grown under white LED light

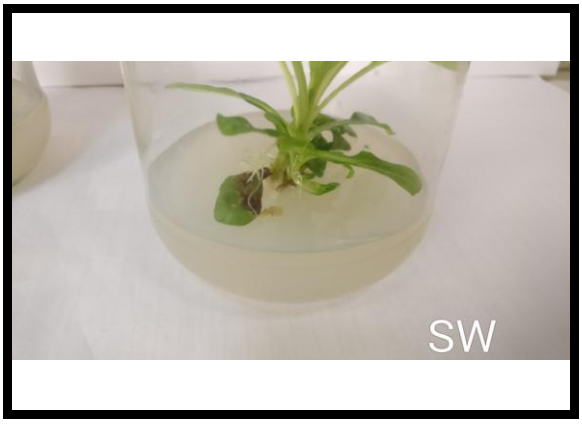


Figure 4.10[b] Cultured micro shoots of *Swertia chirayita* grown under white LED light showing adventitious roots.

Figure 4.11 Cultured micro shoots of *Picrorhiza kurroa* grown under white LED light

Figure 4.12 Cultured micro shoots of *Picrorhiza kurroa* grown under red LED light

Figure 4.13[a] Cultured micro shoots of *Picrorhiza kurroa* grown under blue LED light

Figure 4.13 [b] Cultured micro shoots of *Picrorhiza kurroa* grown under blue LED light showing adventitious roots.

Figure 4.14 Cultured micro shoots of *Picrorhiza kurroa* grown under green LED light.

4.3 Hardening of plants of *S. chirayita* and *P. kurroa*

Tissue cultured plants of *S. chirayita* and *P. kurroa* were kept under specific LED lights for 15 days for hardening (Figure 4.15) and after15 days plants were transferred to greenhouse (Figure 4.16). Survival rate recorded for *S. chirayita* was 60% whereas, for *P. kurroa* survival rate was 70%.

Figure 4.15 *S. chirayita* and *P. kurroa* for hardening at 15℃ ± 1 under (a) red LED light (b) blue LED light (c) green LED light (d) white LED light.

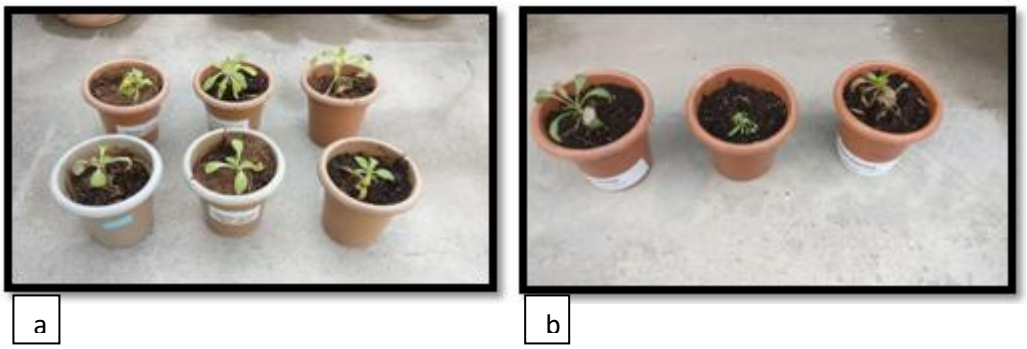


Figure 4.16 (a) *Swertia chirayita* and (b) *Picrorhiza kurroa* in green house for hardening.

4.4 Quantification of Marker compounds by HPLC

4.4.1 Effect of Green LED on Amarogentin and Mangiferin production in *S. chirayita*

HPLC analysis of one month old plantlets of *S. chirayita* grown in MS media and incubated under green LED at 15℃ ± 1 was done to study the effect of green LED on production of amorogentin and mangiferin. Our results showed increased production of mangiferin by 1.818 folds and amarogentin by 0.318 folds (Figure 4.18) compared to *S. chirayita* plantlets grown under WFL (Figure 4.17) (Table 4.3, 4.4).

4.4.2 Effect of White LED on P-I and P-II production in *P. kurroa*

HPLC analysis of one month old plantlets of *P. kurroa* grown in MS media and incubated under white LED at 15℃ ± 1 was done to study the effect of white LED on production of P-I and P-II. Our results showed increased production of P-I by 1.458 folds (Figure 4.20) compared to *P. kurroa* plantlets grown under WFL (control) (Figure 4.19) (Table 4.5, 4.6).

4.4.3 Effect of Red LED on P-I and P-II production in *P. kurroa*

HPLC analysis of one month old plantlets of *P. kurroa* grown in MS media and incubated under red LED at 15℃ ± 1 was done to study the effect of red LED on production of P-I and P-II. Our results showed increased production of P-I by 0.788 folds and P-II by 0.0389 folds (Figure 4.21) compared to *P. kurroa* plantlets grown under WFL (control) (Figure 4.20) (Table 4.5, 4.7).

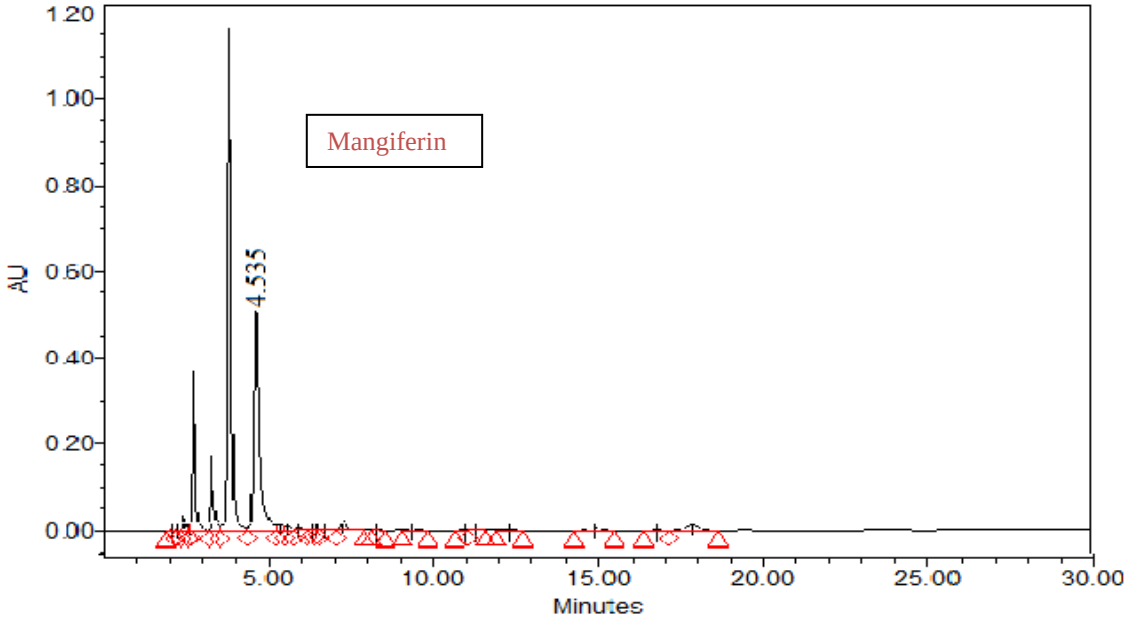


Figure 4.17 HPLC chromatogram showing peaks of standards of *S. chirayita* incubated under WFL (control) at 15℃ ± 1

Table 4.3 HPLC analysis for amarogentin and mangiferin in *S. chirayita* incubated under WFL (control) at 15℃ ± 1

Standard	**Retention Time for S1(wfl)**	**Peak Area**	**%Area**	**Concentration in S(wfl) (mg/g)**
Amarogentin	0	0	0	0
Mangiferin	4.535	6091400	31.37	4.545

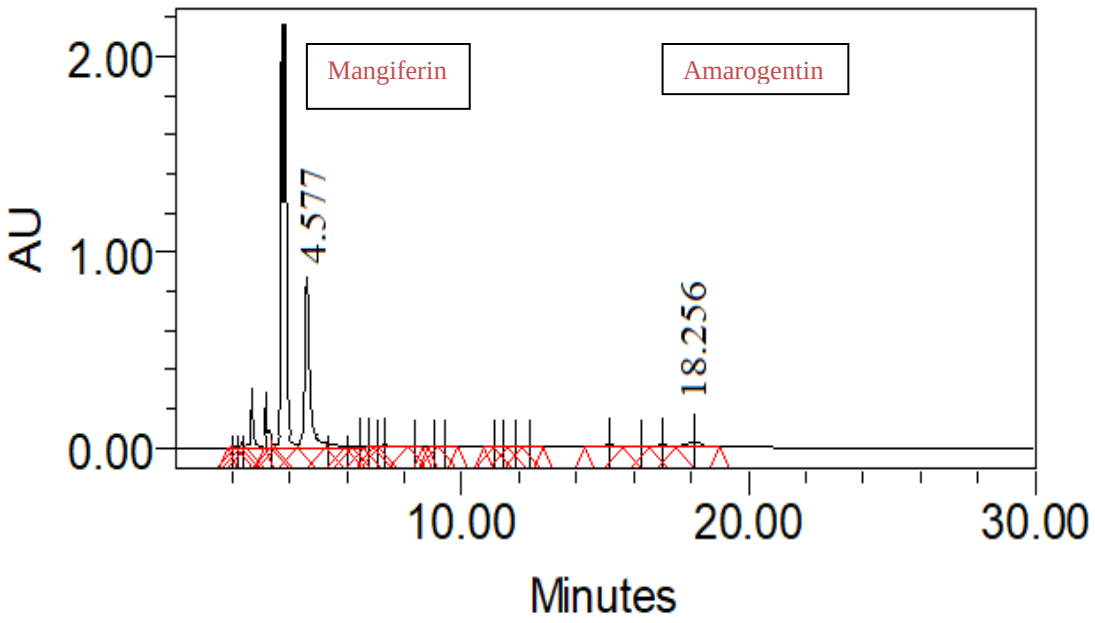


Figure 4.18 HPLC chromatogram showing peaks of standards of *S. chirayita* incubated under Green LED light at 15℃ ± 1

Table 4.4 HPLC analysis for amarogentin and mangiferin in *S. chirayita* incubated under Green LED light at 15℃ ± 1

Standard	Retention Time	Peak Area	% Area	Concentration(mg/g)
Amarogentin	18.256	1037015	3.12	0.318
Mangiferin	4.577	11075518	33.36	8.265

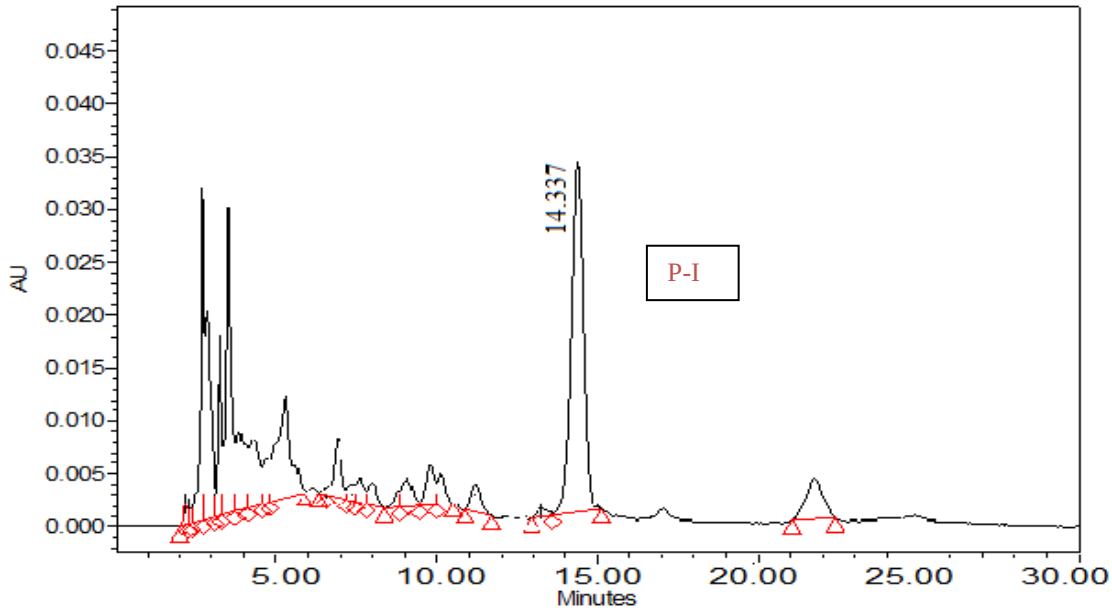


Figure 4.19 HPLC chromatogram showing peaks of standards of *P. kurroa* incubated under WFL (control) at 15℃ ± 1

Table 4.5 HPLC analysis for P-I and P-II in *P. kurroa* incubated under WFL (control) at 15℃ ± 1

Standard	**Retention Time for P(wfl)**	**Peak Area**	**% Area**	**Concentration in P(wfl) (mg/g)**
Picroside I	14.337	708239	16.54	0.25
Picroside II	0	0	0	0

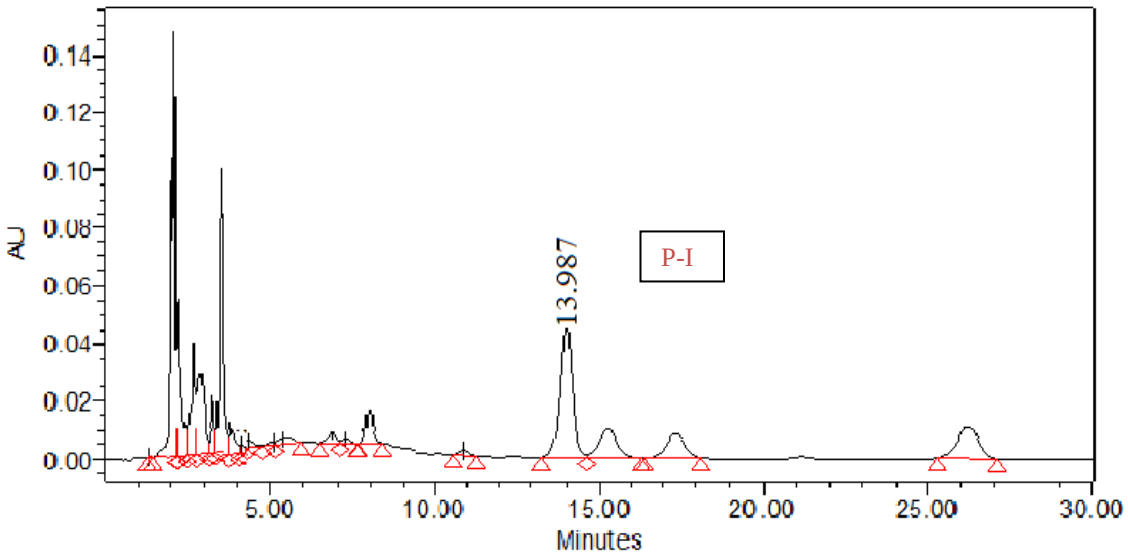


Figure 4.20 HPLC chromatogram showing peaks of standards of *P. kurroa* incubated under White LED light at 15℃ ± 1

Table 4.6 HPLC analysis for P-I and P-II in *P. kurroa* incubated under White LED light at 15℃ ± 1

Standard	**Retention Time**	**Peak Area**	**% Area**	**Concentration (mg/g)**
Picroside I	13.987	1201046	19.79	0.3646
Picroside II	0	0	0	0

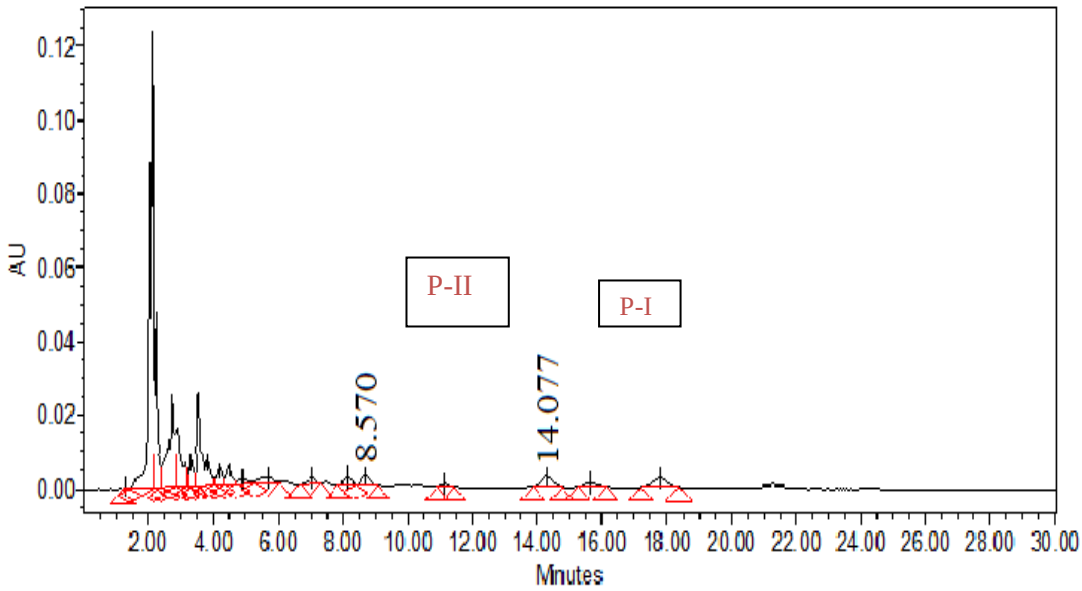


Figure 4.21 HPLC chromatogram showing peaks of standards of *P. kurroa* incubated under Red LED light at 15℃ ± 1

Table 4.7 HPLC analysis for P-I and P-II in *P. kurroa* incubated under Red LED at 15℃ ± 1

Standard	Retention Time	Peak Area	% Area	Concentration (mg/g)
Picroside I	14.077	65180	2.70	0.0197
Picroside II	8.570	48270	2.00	0.0389

4.5 Gene Expression Analysis

There are various pathway genes responsible for the production of P-I, out of all these genes only seven key genes **[21]** were taken to observe whether LED's contribute to the change in gene expression. Gene expression of two genes (G10H, GS) from iridoid pathway were analysed, in which it was observed that G10H gene expression has increased by 1.2834 fold in PW as compared to P_{WFL} and 1.90 folds in PR as compared to P_{WFL} whereas, GS gene expression has increased by 0.181 folds in PW and 0.105 folds in PR as compared to P_{WFL}. Gene expression of key gene from shikimate pathway

phenylpropanoid pathway i.e. PAL-1 gene was also analysed, in which it is observed that gene expression of PAL-1 gene in PW has increased by 0.44 folds as compared to P_{WFL} and 0.061 folds increase was observed in PR. In addition, gene expression of two genes from MEP pathway (ISPE and DXPS) were analysed. Gene expression of ISPE gene has increased by 0.120 folds in PW and 0.118 folds in PR as compared to P_{WFL} whereas, comparatively less increase in gene expression of DXPS gene has been observed. Expression of genes from MEP pathway (HMGR, PMK) were also analysed and it was found that expression of HMGR gene in PW was increased by 0.171 folds and in PR by 0.058 folds as compared to P_{WFL} whereas, in PMK gene 0.077 folds increase is observed in PW and 0.078 folds gene expression has increased in PR as compared to P_{WFL}. Gene expression in plants incubated under blue and green LED's was less than the plants kept under white and red LED's but was higher than the plants incubated under WFL (Figure 4.22).

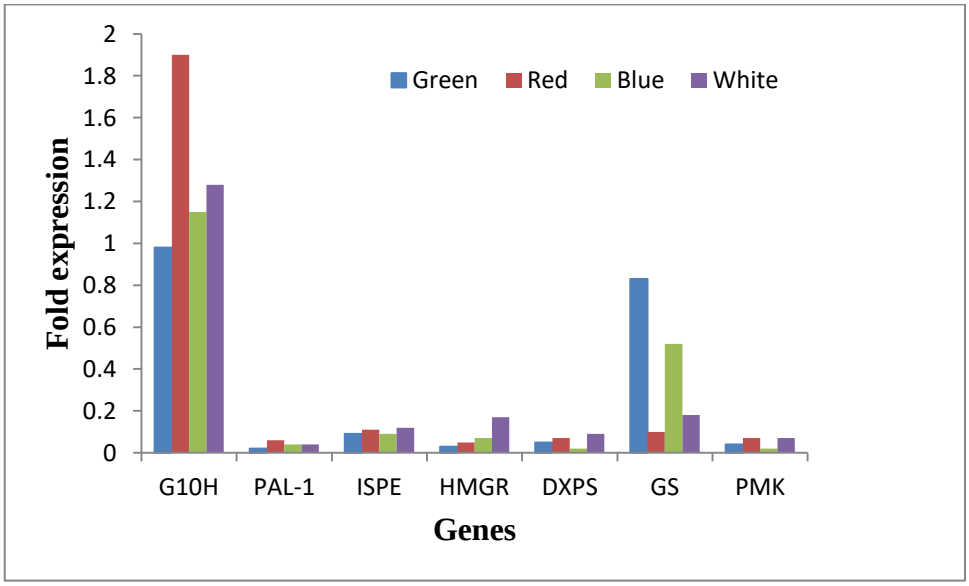


Figure 4.22 Expression analysis of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways in *P. kurroa* plants incubated under different LED's as compared to P_{WFL} (control)

4.6 Discussion

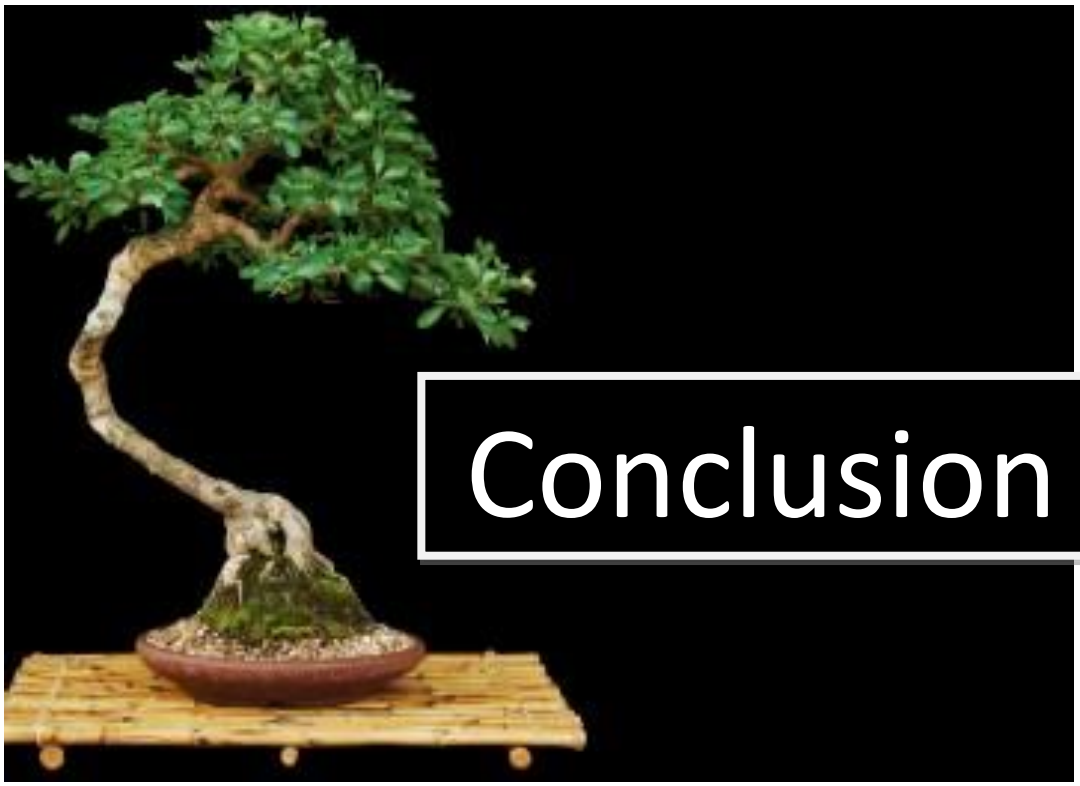
In *S. chirayita* as maximum increase in shoot biomass and root biomass is reported in plants incubated in green light, so in order to increase the production on large scale and for enhancement in production of marker compounds plants should be exposed to green light. Adventitious roots helps in propagation of the species and sometimes absorb nutrients, oxygen and make it available to the submerged roots. MJ Kasperbauer (1971) **[22]** reported growth of long branches under leaves in red light. As in *S. chirayita* adventitious roots were observed in white light and blue light which clearly indicates that blue component of light is responsible for formation adventitious roots. Maximum increase in number of leaves was observed in plants under white light, this shows that formation of leaves requires multiple wavelength lights.

In this study we observed maximum increase in root length, number of leaves and plant biomass in plants incubated under white light, this shows that for increase in such parameters multiple lights of different wavelengths are required. For enhancement in production of shoot biomass, plants should be incubated under red and blue light. Roel C. Rabra (2017) also reported red light enhances growth of plant. As roots of *P. kurroa* contains large number of glycosides, so in order to increase the production of glycosides plants should be incubated under green light.

In both plants as we can see effect of polychromatic LED's is varying. Martin Janda et al. (2015) also reported on the same lines that different species shows different results when exposed to polychromatic LED's.

As there are no reports on production of marker medicinal compounds and gene expression analysis of plants incubated under different LED's, so for the first time we have reported increase in production of secondary metabolites of *S. chirayita* and *P. kurroa* and increase in gene expression of plants of *P. kurroa* incubated under white and red LED's.

CHAPTER 5



CONCLUSION

The improvement of shoot biomass is perquisite for the commercial usage of medicinal plants along with accumulation of biomarker medicinal compounds, so we have reported for the first time where we have found 3.97 fold increase in number of shoots and the shoot area in plants of *S. chirayita* incubated under green LED and 4.72 fold increase in number of shoots and the shoot area in plants of *P. kurroa* incubated under red and white LED's. In addition to this amount of biomarker medicinal compounds accumulated in plants of *S. chirayita* (Amarogentin and Mangiferin) and *P. kurroa* (P-I) were also enhanced in appreciable quantity. Elevated gene expression in plants of *P. kurroa* incubated under red and white LED's was observed. So this would provide grounds for further explorations for improving the medicinal contents in other plant species so that quality rich planting material can be provided to the pharmaceutical for herbal drug development.

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ANNEXURE

Composition of Murashige and Skoog basal medium (MS MEDIUM)

STOCKS	CHEMICALS	ORIGINAL STRENGTH (mg/l)	STOCK (g/l)	FINAL VOLUME (ml/l)
A-10 X	KNO_3 $MgSO_4.7HO$ KH_2PO_4	1900 370 170	19 3.7 1.70	100
B-20 X	NH_4NO_3	1650	33	50
C-100 X	$CaCl_2.2H2O$	440	44	10
D-100 X	Na_2EDTA $FeSO_4.7H2O$	37.26 27.85	3.72 2.28	10
E-100 X	KI	0.83	0.083	10
F-100 X	H_3BO_3 $CoCl_2.6H_2O$ $ZnSo_4.7H_2O$ $CuSo_4.5H_2O$ $MnSo_4.4H2O$ $Na_2MbO_4.2H_2O$	6.2 0.025 8.6 0.025 22.3 0.25	0.62 0.0025 0.86 0.0025 2.23 0.0025	10
G-100 X	m-INOSITOL GLYCINE	100 2	10 0.2	10
H-100 X	PYRIDOXINE-HCL NICOTINE ACID THIAMINE	0.5 0.5 0.1	0.05 0.05 0.01	10

LIST OF PUBLICATION

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