Effect of LEDs on Micropropagated Shoots of *Picrorhiza Kurroa* Vis-À-Vis Biosynthesis of Picroside-I and Key Gene Expression

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Abstract: - Picrorhiza kurroa is a critically endangered medicinal species with various medicinal properties. The present study was conducted in order to see the effect of different lights on the morphological characters of the in vitro grown plants and for the accumulation of Picroside-I compound so that its natural population can be reclaimed by providing these quality rich shoots for industrial demand. The culture conditions for the micro-propagation of *P. kurroa* were optimised in MS media supplemented with IBA (3 mg/l) and Kinetin (1 mg/l). The novel part of study is use of monochromatic LED's (Green, Red, Blue and White) in comparison to standardised white fluorescent light for the period of 30 days and in results we observed 4.72 fold increase in number of shoots and shoot area and 100% survival percentage in red and white LED's as compared to other lights. So this is reported for the first time that LED's have significant effect on growth and secondary metabolite accumulation. Conditions for the hardening and acclimatisation for in vitro grown plantlets along with LED's and natural source i.e. sunlight were tested to observe the survival percentage of hardened plants in the greenhouse conditions.

HPLC quantification of marker medicinal compound Picroside-I in cultured shoots of *P. kurroa* have been carried out where 1.458 mg/g and 0.788 mg/g P-I is accumulated in shoots of *P. kurroa* incubated under white and red LED respectively. 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 as compared to key gene (26S gene) and 1.90 fold expression in shoots of *P. kurroa* incubated under red LED as compared to shoots incubated under WFL.

Key Words: - *Picrorhiza kurroa*, LED, Micropropagation, HPLC, Expression Analysis

Abbreviations

26S	26S rRNA
DXPS	1-Deoxy-D-xylulose-5-phosphate synthase
G10H	Geraniol-10-hydroxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GS	Geraniol synthase
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. kurroa	*Picrorhiza kurroa*
PAL	Phenylalanine ammonia lyase
P-I	Picroside-I
P-II	Picroside-II
PMK	Phosphomevalonate kinase
P_R	*P. kurroa* incubated under red LED light

P_W	*P. kurroa* incubated under white LED light
P_{WFL}	*P. kurroa* incubated under white fluorescent light
WFL	White fluorescent light

1 Introduction

Picrorhiza kurroa is the perennial herb of family Scrophulariacea. It is commonly known as Kutki and Katuka. *Picrorhiza kurroa* is found in the Indian Himalayan region from at an elevation of 2700-4500m, South-East Tibet, West China, North Burma, Pakistan and in sone parts of Nepal between altitudes of 3500 m and 4800 m. In India it is mainly found in Himachal Pradesh, Kashmir, Kumaun and Garhwal region of Uttarakhand. *P. kurroa* is an important traditional medicinal herb which is valued due to its medicinal properties like hepatoprotective, anti-malarial, anti-microbial, anti-inflammatory, anti-oxidant, anti-allergic, anti-anaphylactic, immune modulator and is also used for the treatment of fever, asthma, jaundice, leukoderma, snake bite, scorpion sting, gastrointestinal, and urinary[1]. The root of *P. kurroa* 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 weight, respectively[18]. 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], [3]and[4]. Some herbalists described *P. kurroa* as liver herb [5]. Micropropagation of *P. kurroa* is already otimised by [6]. Biosynthesis of Picroside-I has been studied by [6] and [8].

The major factor responsible for plant growth and development is light. LED is an alternative source of light. Light provide nearly all the benefits of sunlight. Most importantly, they produce the wavelengths that a plant needs to grow and develop as plants have various photoreceptors that helps plant to absorb light from blue wavelength to red wavelength. Various receptors are responsible for absorption of blue light such as cryptochromes and phototropins whereas phytochromes absorbs red light [10]. The main advantages of using LED's reported so far are that LED's have lower energy costs, are durable, longer lifetime of light source, lower heat generation and provides targeted wavelength. 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 [19]. According to Folta et al [15] green light more than 50% cause reductions in plant growth, whereas combinations including up to 24% green enhances growth for some species. Goins et al. [12] in 1997 reported that plant growth under red light (720 nm – 750 nm) is more optimal than blue light. Roel C. Rabra et al. [13] reported that root growth and seedling growth and development is more under red light than blue light. There are reports of using Fytoscope where different LED's have been tested for growing plants but in this study we have replaced WFL with simple LED's and their effect on plant growth and secondary metabolite synthesis and accumulation have been studied.

2 Material and Methods

2.1 Plant Material and Culture Conditions

Picrorhiza kurroa cultures are maintained in the culture room of Department of Biotechnology and Bioinformatics, JUIT, Waknaghat under 1400 Lux light intensity, 15±2℃ and 25±2℃ temperature and 16/8 hours photoperiod. Shoot explants of *Picrorhiza kurroa* were cultured and sub cultured 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 as optimized by [6].

2.2 Establishment of Culture Conditions using 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.

LED's were set in Plant tissue culture room at 15±2℃ and 25±2℃ having photoperiod of 16/8 hours and the respective chambers were covered with black chart papers, so that plants do not get expose to any other light. 25 replicates of both plants were placed under each setup along with the control (WFL) (Fig. 3).

2.3 Data Collection

Plant samples of *P. kurroa* were collected from all LED setups at 15±2℃ 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 and quantification of Picroside-I and statistical analysis were carried out.

2.4 Hardening of Plants

Well grown shoots of *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). Some plantlets of *P. kurroa* were even planted in different mixtures like Perlite + Vermiculte (1:1); Perlite + Cocopeat (1:1:1); Cocopeat + Vermiculite (1:1). In order to avoid desiccation, 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 under greenhouse conditions [6].

2.5 Quantification of Marker Compounds by HPLC

After 30 days of incubation in LED's fresh shoots of *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* were gently uprooted from glass jars and shoots were grinded in liquid nitrogen which led to the formation of powdered sample, 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 content by following the method described by Sood et al. [6]. Quantification is done on basis of retention time of marker compounds. Retention time of P-I is 14.154 (Fig. 1). 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 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 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.

2.6 Expression Analysis

2.6.1 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).

2.6.2 Quantitative Real Time-PCR (QRT-PCR) Analysis

There are various metabolic pathways involved in the production of P-I, 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 1). The housekeeping genes, 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 monochromatic LED's.

3 Results and Discussions

3.1 In Vitro Shoot Multiplication

In vitro shoots of *Picrorhiza kurroa* were cultured and sub cultured in optimized MS media with growth hormones (IBA + KN; 3:1) incubated for 4 weeks under optimized conditions as done by

Sood et al. [6] and Neha et al. [8] were used for further experimentation (Fig. 2). Sood et al. [6] reported that 15±2℃ is best for in vitro growth of the plants and provides best culture conditions for the growth of and shoot biomass formation, so control plantlets grown in accordance to that study whereas, in vitro shoots under different LED's also behaved positively with 4.72 fold improvement in shoot length and biomass.

3.2 Incubation of in Vitro Shoots in Monochromatic LED's.

Cultured in vitro shoots of *P. kurroa* were incubated at same culture conditions as of control (WFL) but in different (monochromatic) LED's (Fig. 3), which include incubation in white LED, red LED, blue LED and green LED. After 30 days of growth plant samples were collected for observing various growth parameters. 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 (Fig. 4(a)). Maximum increase in number of shoots (7.67) was reported in plants incubated under red (Fig. 4(b)) and white light as compared to other LED lights. Maximum increase in shoot length (9.5 cm), white colored adventitious roots and browning in leaves were reported in plants incubated under blue light (Fig. 4(c)). In plants incubated under green light maximum increase in number of roots (11.67) was observed (Fig. 4(d)) (Table 2).

3.3 Hardening of Plants of *P. kurroa*

Tissue cultured plants of *P. kurroa* were kept under specific LED lights for 15 days for hardening (Fig. 5) and after15 days plants were transferred to greenhouse (Fig. 6). Survival rate recorded for *P. kurroa* was 85% whereas, according to Neha et.al (2015) the survival rate of *P. kurroa* grown under normal light was 80%.

3.4 Quantification of Marker Compound (Picroside-I) by HPLC

3.4.1 Effect of LED on P-I Production in *P. kurroa*

Sood et al. [9] reported in their study that after four weeks of in vitro growth of *P. kurroa*, 2.0 mg/g, 1.5 mg/g and 1.0 mg/g fresh wt. Picroside-I content is present in the fully developed shoots from callus cultures of leaf segments, stem and root segments, respectively. Neha et al. [8] reported in their study that 3.65 µg/mg and 2.87 µg/mg P-I content is present in shoots developed from leaf and root segments, respectively at 15 ±2℃.

Whereas, in our study, HPLC analysis of one month old plantlets of *P. kurroa* grown in MS media and incubated under different monochromatic LED's at 15±2℃ was done to study the effect of LED's on production of P-I. Our results showed increased production of P-I in *P. kurroa* incubated under white LED by 1.458 folds (Fig. 7 (b)) and under red LED by 0.788 folds (Fig. 7 (c)) compared to *P. kurroa* plantlets grown under WFL (control) (Fig. 7 (a)). We obtained highest amount of 0.36 mg/g P-I content in *P. kurroa* incubated under White LED and amount of 0.0197 mg/g P-I content in plants incubated under Red LED as compared to 0.25 mg/g P-I content in plants incubated under WFL (control) at 15±2℃.

3.4.2 Gene Expression Analysis

Pandit et al. [21] observed the expression pattern of fifteen genes of non-mevalonate (MEP) pathway and menalonate (MVA) pathway in different tissues of *P.kurroa*. It is reported in their study that P-I is present only in shoots of field grown plants. They also revealed that in shoots four genes of MEP pathway (DXPS, ISPD, ISPE and MECPS) and one gene of MVA pathway (PMK) showed elevated transcript level whereas in roots two genes of MEP pathway (HDS, DXPR) showed higher expression. In 2016, Sharma et al. [8] revealed that gene expression is higher in samples of shoot primordia, multiple shoots and fully developed *P. kurroa* as compared to callus culture. Therefore in our research we prefered to study the gene expression of full developed plants of *P. kurroa*. Sharma et al. [8] also analysed quantitative expression of secondary metabolism genes at two temperatures and revealed that seven genes HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed high transcript abundance (32-87-folds) in fully developed stage derived from leaf and root segments at 15 °C compared to 25 °C in *P. kurroa* therefore all these seven genes were taken to observe whether LED's also 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. From MVA pathway two genes (HMGR, PMK) were 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 (Fig. 8).

In *P. kurroa* as maximum increase in plant biomass is reported in plants incubated under white LED light, so in order to increase the production on large scale and for enhancement in production of P-I plants should be exposed to white LED light. MJ Kasperbauer (1971) reported growth of long branches under leaves in red light. As in *P. kurroa* adventitious roots were observed under blue LED light which clearly indicates that blue component of light is responsible for formation adventitious roots in *P. kurroa*. Maximum increase in number of leaves was observed in plants under white LED 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 LED light, this shows that for increase in such parameters multiple lights of different wavelengths are required. Roel C. Rabra (2017) also reported red light enhances growth of plant and even in our study it is observed that plants incubated under red LED light showed best growth results and increase in production of secondary metabolites. 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.

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 *P. kurroa* and increase in gene expression of plants of *P. kurroa* incubated under white and red LED's.

4 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 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 *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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Author Contribution Statement:

RS and SS conducted tissue culture experiments and molecular experiments. HS conceived and designed research. RS, SS and HS analyzed data. RS and SS wrote the manuscript. All authors read and approved the manuscript.

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Tables

Table 1: List of primer sequences used in qRT-PCR based expression analysis

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')	Fragment Size (bp)	Annealing temperatures (°C)
26S	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	500	58
G10H	TATCGAGCTTTTCAGTGGAT	GATGTGAGTCCTGTCGATTT	136	52
PAL-1	GCAAGATAGATACGCTCTAA	GTTCCTTGAGACGTCAAT	136	49
ISPE	TTCATCTAGATAAGAAGGTGCCAAC	CCTCTACCAGTACAATAAGCAGCTC	110	55
HMGR	CGTTCATCTACCTTCTAGGGTTCTT	GACATAACAACTTCTTCATCGTCCT	100	60
DXPS	ACATTTAAGTTCAAGTCTGGGAGTG	ATGTGCACTCTCTTCTCTTTTAGGA	110	55.9
GS	TGGGTAGATTAGAAGCCAGA	CTGGTGATTTCTACCAGCTC	139	52
PMK	TGGATGTTGTCGCATCAGCACCTGG	GTAATAGGCAGTCCACTCGCTTCAA	100	58

Table 2: Growth and developmental parameters observed under LED's and standarised white flourocent 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 ±0.5**	**8.17 ±0.6**	**6.67 ±0.8**	**9.5 ±0.7**	**7.67 ±0.6**
Root length (cm)	**0**	**0.43 ±0.54**	**0.4 ±0.84**	**0.5 ±1**	**0.74 ± 0.5**
No. of shoots	**1.34 ±0.05**	**5 ±0.6**	**7.67 ±0.8**	**1.67 ±0.2**	**7.67 ±0.4**
No. of roots	**0**	**11.67 ±0.6**	**3.67 ±0.29**	**1.67 ±0.47**	**8.67 ±0.59**
No. of leaves	**7.27 ±0.51**	**16 ±0.94**	**40.67 ±0.41**	**29 ±0.97**	**46 ±0.35**
Adventitious Roots	**0**	**0**	**0**	**7 ±0.4**	**0**
Browned leaves	**0**	**4.67 ±0.51**	**1.67 ±0.21**	**6.67 ±0.32**	**0**
Plant Biomass (g)	**0.158 ±0.51**	**0.781 ±0.45**	**1.223 ±0.56**	**0.26 ±0.44**	**1.709 ±0.47**

Figures

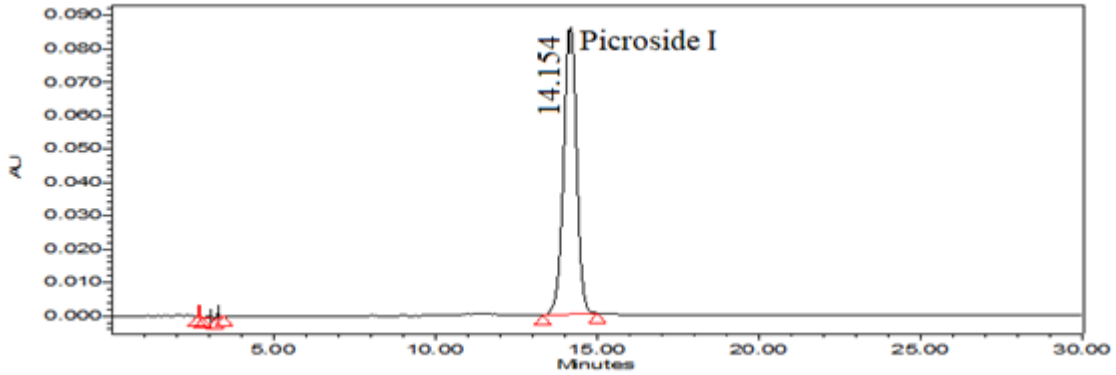

Fig. 1: HPLC chromatogram of P-I standard of P. kurroa.

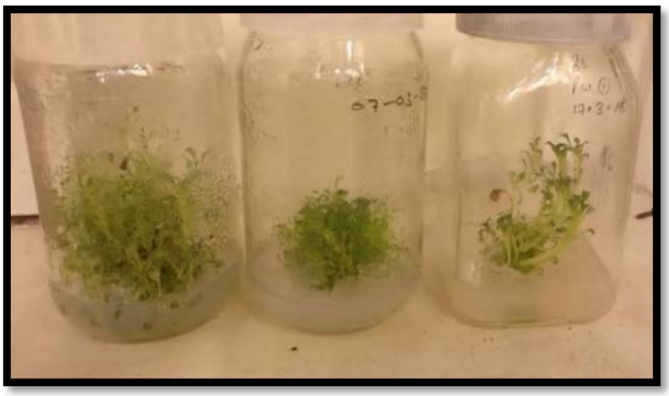

Fig. 2: In vitro micro propagated shoot in MS medium of *Picrorhiza kurroa.*

Fig. 3: Fabrication of LED Setup in Plant Tissue Culture Room at 15℃ ± 2.

Fig. 4: Cultured micro shoots of *Picrorhiza kurroa* grown under (a) white LED light, (b) red LED light, (c) blue LED light and (d) green LED light.

Fig. 5: *P. kurroa* for hardening at 15℃ ± 2 in (a) red LED (b) blue LED (c) green LED (d) white LED.

Fig. 6: *Picrorhiza kurroa* in green house for hardening.

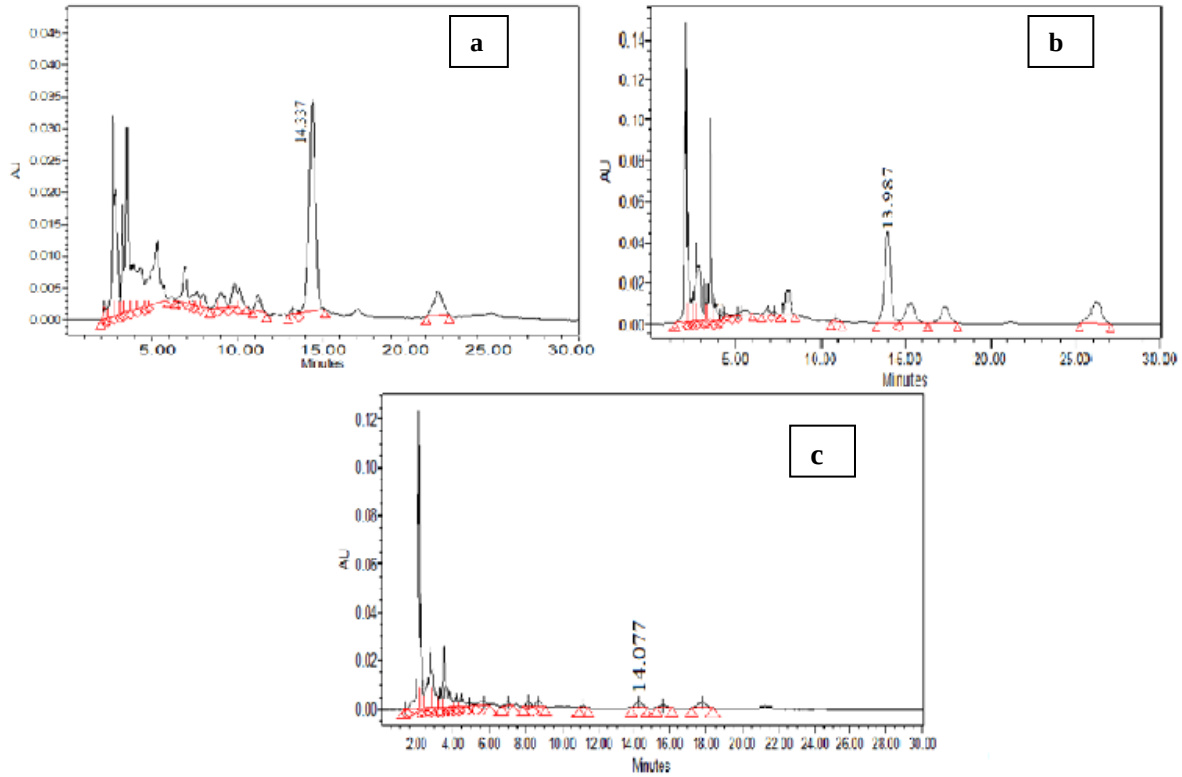

Fig. 7: HPLC chromatogram showing peaks of standards of *P. kurroa* incubated at 15±2℃ under (a) WFL (control) (b) White LED (c) Red LED.

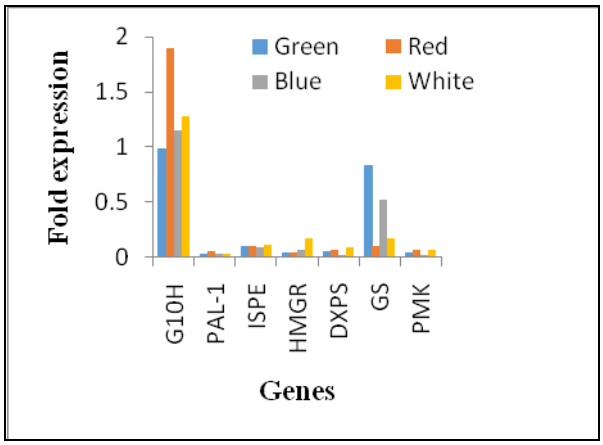

Fig. 8: Expression analysis of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways in *P. kurroa* plants incubated under different LED's as compared to P_{WFL} (control).