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Effect of LEDs in phytochemical accumulation and biomass production in callus cultures of *Podophyllum hexandrum*

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Callus cultures; Phytochemicals; Elicitor

ABSTRACT:
Different *Podophyllum hexandrum* explants have been used to establish callus cultures. Different levels of important medicinal substances with high pharmacological value such as those with total phenolics, total flavonoids, total antioxidants and anti-microbial activities are found in *Podophyllum hexandrum*. Although the presence of podophyllotoxin had already been reported but the micropropagation technique for plant growth and plant regeneration is not yet optimized. Few reports available which are non-reproducible and podophyllotoxin production under *in vitro* condition is not showing demarcated significance. So the sustained growth and regeneration of callus tissue were successfully accomplished using a fully defined medium composed of Murashige and Skoog salts and supplemented with GA_3 (0.5mg/L), IAA (2mg/L), TDZ (1mg/L), 2,4-D (1mg/L) and BAP (1mg/L). In the current study, a variety of stimulating chemicals (elicitors) were used to increase the synthesis of podophyllotoxin as well as biomass growth. Flavonoids, terpenes, saponins, and glycoside chemicals were detected in the phytochemical screening of a variety of methanolic extracts. The methanolic extract of red+green light treated callus produced the best results in terms of total phenol, total glycoside, anti-oxidant activity (DPPH assay), and Well diffusion and MIC techniques.

Introduction

Podophyllum hexandrum a member of the Berberidaceae family. The temperate zones are ideal for the growth of this priceless medicinal plant, which is in high demand on both the domestic and international markets because it includes a substance known as podophyllotoxin that is used to cure some types of malignancies [1]. The herb *Podophyllum hexandrum* is erect and succulent, growing to a height of about 30 cm. Its smooth, elongated, tangled rhizomes grow horizontally and rest near to the ground. Simple, leaves without a top one or two stems. Alternate, palmate, the leaves have 3-5 lobes that are broadly split, toothed margins, and prominent purple dots that can reach a diameter of 25 cm. Flowers with a diameter of 4 cm and colours ranging from white to pinkish occur in the stem's fork. Three petaloid sepals make up each sepal. Usually six stamens. When ripe, the ovoid, 5 cm long, pulpy fruits turn scarlet [2]. The plant prefers humus-rich, well-drained soil and does well in subtropical and subalpine climates. It thrives in both woodland settings and as undergrowth. It was first discovered across the Himalayan region, more especially in Uttarkashi, India. *Podophyllum hexandrum* is found growing naturally in sub-Himalayan regions of India, Afghanistan, Bhutan,

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Pakistan, Taiwan and China, particularly in Uttarakhand, Jammu and Kashmir, as well as certain areas of northeastern India[3],[4]. Seeds and rootstocks are used to multiply the plant. To fulfil the growing need of pharmaceutical companies, the species has been illegally collected from the wild and as a result, it is in grave danger because the rate of regeneration is lower than the pace of harvesting. As a result, this species need immediate preservation and conservation [5]. To lessen the severe biotic pressure on the natural habitat, it is urgently necessary to design for its growth, in situ and ex situ cultivation, user-friendly packing and adhesives [6].

The major goal of the herb industry is the generation of significant yields of secondary metabolites. For the production of phytoconstituents numerous techniques have been used recently including synthetic seed technology, micropropagation and elicitor's treatments. Elicitation is clearly the best biotechnological technique for boosting the generation of bioactive chemicals in plant tissue cultures [7], [26]. Important bioactive chemicals are inspired to be created by light which acts as an abiotic elicitor. For indoor culture, the majority of breeders use conventional fluorescent bulbs but these sources are ineffective because of their high operating temperatures, uneven spectral distribution and high energy requirements for plant growth [8], [27]. Instead of using conventional illumination, LEDs have the potential to increase the efficiency of the synthesis of biomarker compounds through modifications to the primary and secondary metabolic processes of plants, increased light usage, and the delivery of certain wavelengths designed for the best plant growth. Additionally using LEDs has several benefits, like being economical providing a sustainable source of energy and producing less heat than a typical light source throughout the course of its lifetime [9], [25]. The effect of various LEDs on the rise of bioactive compounds in therapeutic plants including *S. chirayita*, *P. ginseng* and *R. imbricate* has been the subject of several investigations, however none of these research investigations involved *in vitro* raised plants or *Podophyllum hexandrum* callus cultures [5], [18], [19].
Therefore, the current objective of this work was to enhance the synthesis of important secondary metabolites while accelerating the biomass and antioxidant, anti-microbial properties in callus cultures of *Podophyllum hexandrum* using LED lighting.

1. Materials and Methods

1.1. Selection of plant source

The (mother plant) callus samples were procured from Himalayan Forest Research Institute, Himachal Pradesh, India (20.76´N, 67.12´E). Additional attention was given to the plants in a culture room of the PTC lab at JUIT, Waknaghat, Solan, India (1400m altitude), with regulated light intensity of (10,000 lux) and lighting that was modified in terms of the amount and photoperiod duration. The temperature was kept between 15°C and 30°C, and the humidity ranged from 20-98% [11].

1.2. Subculturing and regeneration of callus samples

Subculturing and regeneration of callus samples were successfully accomplished using a fully defined medium composed of Murashige and Skoog salts and supplemented with GA_3 (0.5mg/L), IAA (2mg/L), TDZ (1mg/L), 2,4-D (1mg/L) and BAP (1mg/L). Callus samples were regularly subcultured 30 days later.
In vitro sub-cultured callus were employed as inoculums for conducting additional experiments while the callus cultures were being maintained under LED lights. Freshly subcultured callus cultures of *Podophyllum hexandrum* (≈7gm weight) were moved to MS media (50mL in glass jars) containing TDZ (1mg/L) + BAP (1mg/L) + GA_3 (0.5mg/L) and IAA (2mg/L) + BAP(1mg/L) + 2,4-D (1mg/L) and 0.8% agar-agar and white florescent light (WFL) at 3000 µmol m^{-2} s^{-1} intensity, 15 ± 1ºC temperature, humidity (74%), 16 h day and 8 h light photoperiod and aseptic conditions were used to maintain pH at 5.6 and 3% w/v sucrose.
Calluses from the following subculture were used in LED bulb elicitation experiment.

2.3.Light setup with growth conditions

Different colored 12-watt LED bulbs with light colours of red, green, and yellow (Tejas and Empire brand), as well as WFL as a control, were employed to elicit the presence of medicinal constituents. The bulbs were purchased from Amazon www.amazon.com (accessed on August 10th, 2022) using an online manner. Two separate light-emitting components were used in our experiment. A controllable direct supply of current made up the second component, whereas the first was a simply removable LED electric circuit. *Podophyllum hexandrum* callus were spread out at random among several LED treatments in the plant tissue culture lab, which was kept at 15°C and an 8-hour photoperiod [11], [12], [25], [26]. The following conditions applied to the LED lineup:

a) (Control: WFL at temp. 15 °C ± 1 with intensity 3000 µmol m^{-2} s^{-1} (8 h of dark photoperiod and 16 h of light).
b) Red: 100% red LED at ~660nm wavelength with 20 nm of bandwidth at ½ peak height.

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c) Green: 100% green LED at ~550nm wavelength with 20 nm of bandwidth at ½ peak height.
d) R+G: 60% red, 40% green LEDs at 15◦C± 1 inside plant tissue culture room. [12]

By removing the callus from the culture jars under sterile circumstances after 30 day cultivation period, growth parameters, total flavonoid, total phenolic and total antioxidants, anti-microbial activity by Well diffusion and MIC techniques were assessed.

2.4. Extract Preparation

In order to make the callus extract, the calluses were taken out of the culture medium that was subjected to five different light treatments: white, red, green, yellow and a combination of red+green, the additional water was pressed out of them using Whatman TM filter paper (GE Healthcare, UK Limited), and they were weighed (Citizon, cg-203) in an aseptic environment [11].
After drying with liquid N_2 and grinding into a fine powder by a mortar-pestle, it was freeze-dried using a lyophilizer. The resulting powder was then dissolved in 100mL of 80% methanol, and a shaker was used to extract the powder with methanol. A rotary vacuum evaporator was used to concentrate the obtained extracts, and the resultant supernatant was then filtered via PVDF syringe filters (0.22 µm). For later use, the leftover extract was chilled to 4ºC.

2.5. Phytochemical Screening of Callus Extracts

Phytochemical testing involves both qualitative and quantitative analysis. Qualitative analysis focuses on determining the presence or absence of specific phytochemicals in a callus sample, while quantitative analysis measures the concentration or amount of a particular phytochemical present. Preliminary phytochemical screening is typically the first step in this process before conducting a quantitative analysis. Standard methods, as outlined by Sofowora (1993), Trease and Evans (1989), and Herborne (1973), were utilized to perform both quantitative & qualitative analyses of various phytoconstituents in *Podophyllum hexandrum* methanolic extracts [10], [17], [21].

2.5.1. Preliminary qualitative test :

Alkaloid test, Amino acid test, Saponin Test, Terpenes Test, Glycoside Test, Phenolic Test and Flavonoid Test were preliminary performed for the qualitative analysis of callus cultures [10], [15], [20], [28].

2.5.2. Methods for Quantitative Analysis 1.‘

Determination of Total Flavonoid Content: Total Flavonoid Content of callus extracts of *Podophyllum hexandrum* grown under different LED bulbs was determined by using the protocol of Gupta R. et al. (2023), [11], with some modifications. A standard curve was created by preparing various concentrations of gallic acid in methanol, while quercetin was dissolved in 100.0 ml of methanol to produce a final concentration of1% (10 mg/ml) quercetin. Then, 150.0µl of Al_2Cl_3 was added, and the mixture was incubated for 5 minutes before adding 200µl of NaOH. The absorbance at 510 nm was measured, and the flavonoid content was estimated in terms of quercetin equivalents [14], [22].

2. Determination of Total Phenolic Content:

Total phenolic content of callus extracts of *Podophyllum hexandrum* grown under different LED bulbs was determined by using the method of Ainsworth et al. (2007), [28], with some modifications. The total phenolic content was determined through a spectroscopic method. A reaction mixture was created by combining 1 ml of callus extracts (1 mg/ml), 1 ml of 10% Folin-Ciocalteu's reagent, and 5 ml of 7% Na_2CO_3 solution in 13 ml of deionized water. The mixture was allowed to react for two hours at room temperature in the dark, after which the absorbance was measured at 760 nm using a spectrometer. The mean absorbance value was obtained after three repetitions of the experiment, and the total phenolic content was calculated using a calibration curve created with a gallic acid solution. The total phenolic content was reported as gallic acid equivalent (mg GAE) per gram of the dried sample used as the unit of measurement. [10], [13], [15], [16], [28].

2.5.3.Anti-oxidant activity of *Podophyllum hexandrum*:

The antioxidant properties of callus culture extracts produced from *Podophyllum hexandrum* was evaluated using the DPPH free radical scavenging assay. The methodology described in Yesmin et al. [24], study from 2008 was used to conduct this evaluation utilizing a variety of LED lights, with some changes.

DPPH Assay: A fresh solution of 0.002% (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) was created by dissolving it in the solvent methanol. The absorbance of this solution was recorded at 515.0nm to establish a standard value. Gallic acid was dissolved in methanol to produce solutions of varying concentrations (0, 2, 4, 6, 8, and 10). To test for antioxidant activity, 50µl of plant extract (at a concentration of 1.0 mg per milliliter) was added to 3 ml of DPPH, followed by a 15-minute incubation period at room temperature. The absorption was then recorded at 515 nm to determine the antioxidant capacity of the plant extract.

DPPH free radical scavenging activity (%RSA) was determined as follows:

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%RSA= {(Ab. of control − Ab. of Sample)/Ab. of control} × 100

Where, Ab. is absorbance. To determine the IC50 value of the extracts, which is the concentration required to scavenge 50% of DPPH, a calibration curve was generated. The curve was constructed using varying concentrations of gallic acid and the corresponding percentage of inhibition. Each experiment was performed in triplicate. [10], [23], [24].

2.5.4.Anti-microbial activity of *Podophyllum hexandrum*:

Preparation of Inoculum

The stock cultures were kept at a temperature of 4°C on nutrient agar slopes. For the experiments, active cultures were created by transferring a small amount of cells from the stock cultures to test tubes containing Mueller-Hinton broth (MHB) and then incubating them at 37°C or 25°C without any movement for 24 hours. A volume of 0.2ml of the culture was then inoculated into 5ml of MHB and left to incubate until it reached the same level of cloudiness as the standard 0.5 McFarland solution at 600nm, which is equivalent to a concentration of 106-108 CFU/ml.

i. Well Diffusion Method:

The purpose of this study was to determine whether extracts from plants or microbes possess antibacterial properties. The first step involved culturing bacteria in Muller Hilton broth and adjusting the turbidity to meet the 0.5 McFarland criterions, which meant that the broth contained 106 cells. Next, the bacteria were spread on Muller Hilton agar plates, which were left to dry and then sterile cork borers, were used to puncture wells. To test for antibacterial activity, different concentrations of plant extracts (in 50µl amounts) were added to each well, while a positive control consisting of an antibiotic and a blank consisting of DMSO were also used. Following that, the Petri plates were kept inside an incubator with the temperature of 37°C for 24 hours. After this period of incubation, the zones of resistance against various microbes were measured [29], [30].

ii. MIC (Minimum Inhibitory Concentration):

The minimum inhibitory concentration (MIC) is the smallest quantity of any anti microbial agent required to halt the growth of the tested microorganism. In this experiment, 100µl of Muller Hilton broth was added to each of the 96 wells in a plate, and 3*106 bacterial cells and 100µl of plant extract were added to each well. The plate was then incubated for 24hours at 37°C. Resazurin dye was added to each well, and the color transition from green to pink was observed. This was used to determine the MIC for the plant extract against the microorganism being tested [31], [32].

3. Results and Discussion

3.1. Impact of LED lightning on growth and biomass production

A constantly changing group of photoreceptors termed phytochromes, which (red light) are found in plants, and they aid in their ability to react to various photo-environmental situations. A change in callus morphology was caused by the culture of callus under various wavelengths, with yellow, green, white & red LEDs resulting in brown, lightgreen, and creamish white-brown callus, respectively. The effect of light spectra on callus phenotype was observed as paler or brown in the absence of light compared to green or red callus grown under light [25], [26]. The use of LED lights was found to increase the biomass, total phenolic content, and antioxidant activity total glycoside content, total flavonoid content in callus.

Figure 1: Effect of LEDs on *In vitro* callus cultures of *Podophyllum hexandrum*

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Fig.2: *In vitro* callus cultures of *Podophyllum hexandrum* incubated under LED lighting i.e. Red, green and yellow.

3.2. Results of Qualitative and Quantitative Analysis

Qualitative test done for the *Podophyllum hexandrum* plant extract and the results obtained for Phenols, Terpenes, Saponins, Flavonoids and Glycosides was positive and negative results were obtained for Amino acids, tannins and alkaloids.

Table1: Results obtained on phytochemical analysis of callus cultures of *Podophyllum hexandrum.*

Phytochemical Test	Result	Observance
Phenol (Ferric chloride test)	Present (+)	Red, green, green or purple color was observed
Terpenes Test	Present (++)	Reddish brown color interface was observed.
Saponin (Foam Test)	Present (++)	Foam formation was observed.
Flavonoids (Alkaline reagent Test)	Present (++)	Greenish Yellow Precipitates were observed.
Glycoside (Salkowski test)	Present (++)	Reddish color was observed.

Impact of LED Lighting on Total Flavonoid Content

The total flavonoids present in the various methanolic plant extracts were assessed in the current investigation using the Gupta R. et al. (2023), [11], method and given as quercetin equivalents. The red+green light treated methanol extract of callus from *Podophyllum hexandrum* had the most flavonoids of any of the four methanolic extracts from the plant. The results of earlier investigations on the contents of flavonoids have been published in a similar manner. The range of the total flavonoid concentrations in callus cultures was 5.33 to 25.33 mg QE g^{-1} DW of callus. In comparison to other red, green, and yellow LEDs used as controls (20±0.06 mg QE/g DW), callus cultures maintained under red+green LED demonstrated the highest amount of total flavonoid (25.33±1.05 mg QE g^{-1} DW). In callus cultures incubated under WFL LED, the least amount of flavonoid accumulation (5.33±0.03 mg QE g^{-1} DW) was noted. The diverse extraction techniques and solvent polarity could be to blame for the inconsistent outcomes. Some volatile active chemicals may be lost or evaporated from the samples during processing and drying.

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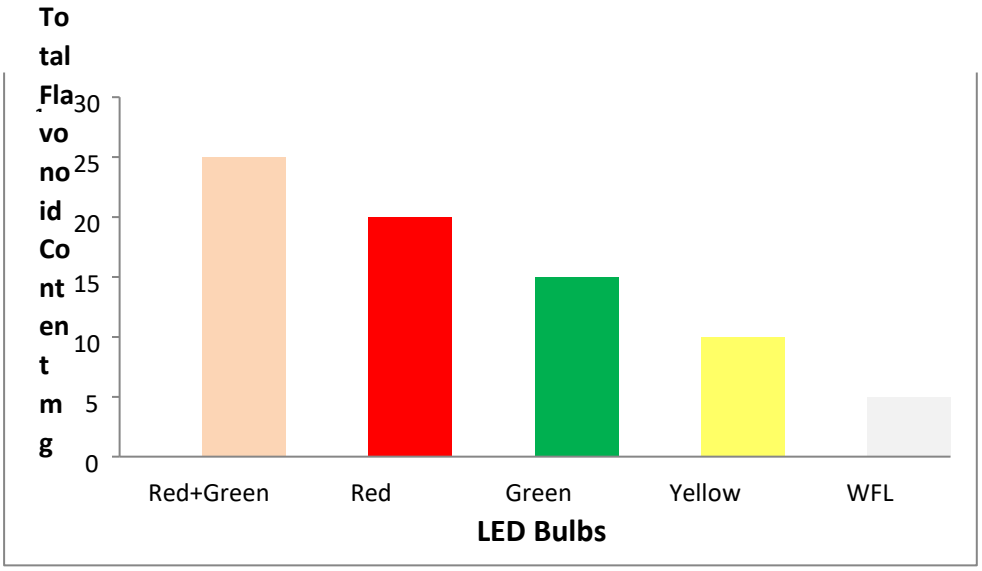

Fig.4: Impact of LED lighting on total flavonoid content on callus cultures of *Podophyllum hexandrum* after 30 days. Experiment was conducted 3 times and data was recorded as mean ± SD, with R+G representing the highest, red representing the mild while green, yellow and wfl was representing the least.

3.3. Impact of LED Lighting on Total Phenolic Content

Gallic acid equivalents are used to report the total phenol content of the methanolic extracts as assessed by a recognized method. The most phenolic chemicals were found in the red+green light treated methanolic extract, which was one of the four methanolic extracts. The results of the literature search indicate that there has never been any research on the total phenol content of this *Podophyllumhexandrum*. The range of the total phenolic concentrations found in callus cultures was 5.33 to 24.33 mg GA g^{-1} DW of callus. In comparison to other red, green, and yellow LEDs used as controls (16±0.06 mg GA/g DW), callus cultures maintained under red+green LED demonstrated the highest amount of total phenolics (24.33±1.05 mg GA g^{-1} DW). In callus cultures incubated under WFL LED, the lowest phenolic accumulation was noted (4.33±0.03 mg GA g^{-1} DW). Comparing our findings to other reported findings on total phenol reveals significant differences.

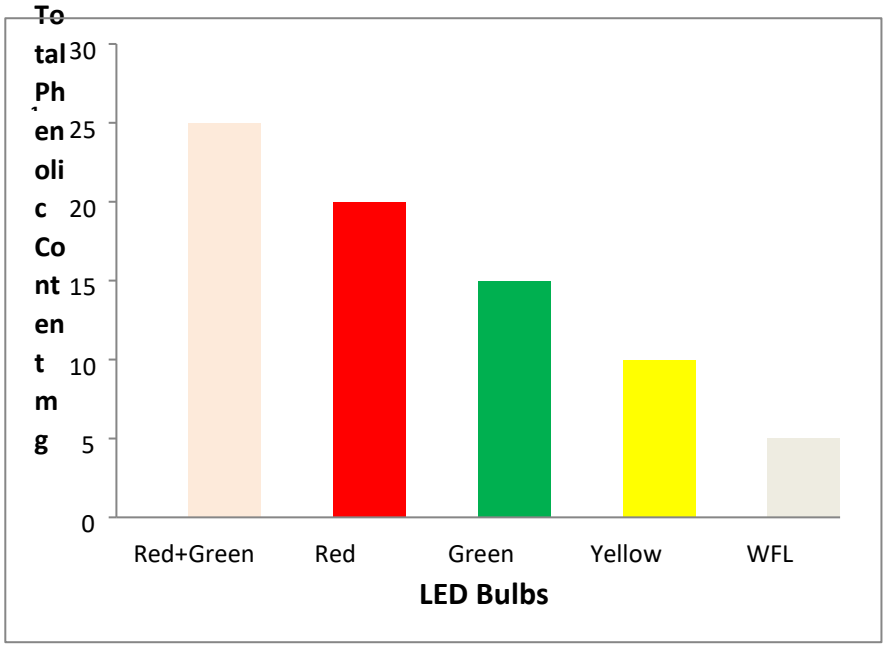

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Fig.5: Effect of LED lighting on the total phenolic content of *Podophyllum hexandrum* callus cultures after 30 days. The experiment was carried out three times, and the data were recorded as means with standard deviations, with R+G standing for the highest value, red for the mildest, and green, yellow, and wfl was representing least.

3.4. Impact of LED Lighting on DPPH Activity

On performing Anti-oxidant activity on plant *Podophyllum hexandrum*, the results were obtained by performing DPPH assay. Antioxidant synthesis may have risen in callus cultures to guard against oxidative stress produced on by reactive oxygen species, as seen by the greater concentration of antioxidants seen in red and red+green LEDs. In callus cultures, the RSA% ranged from 30.45 to 55.40% (Table 2). Mild in red (41.02 ± 0.11%) and highest RSA in R+G LED (55.40 ± 0.15%). In callus cultures in yellow and WFL, the lowest RSA (30.45 ± 0.19%) was noted. Therefore, these can be employed as a replacement for currently accessible antioxidants, which are highly appreciated in the food and pharmaceutical industries.

Table 2: Impactof LED lighting on anti-oxidant activity in callus cultures of *Podophyllum hexandrum*:

Anti-oxidant activity

Light Quality	%RSA
Red+green	55.40±0.15%
Red	41.02±0.11%
Green	34.02±0.11%
Yellow	32.04±0.11%
WFL	30.45±0.19%

Standard

BHT	67.47±0.05%

3.6. Results for Well Diffusion Method

Different plant extracts have been tested using the agar well diffusion method for their antibacterial effects against Gram-positive bacteria *Bacillus subtilis* (MTCC 121) and *Staphylococcus aureus* (MTCC3160), Gram-negative bacteria *Escherichia coli* (ATCC25922), and one antibiotic. In tests involving several microbes, the extracts demonstrated antibacterial properties. All of the bacterial strains that were examined responded favourably tomethanolic callus extract's antibacterial activity ($P<0.05$). Callus treated with red+green light
and then red, green, and yellow LEDs shown a notable antibacterial effect. The changes in internal pH (pHint) and membrane potential in *Escherichia coli* and *Staphylococcus aureus* cells following exposure to the plant extracts were examined to further our understanding of the antibacterial action mechanism of plant extracts. The results showed that both Gram-positive and Gram-negative bacteria's cell membranes were severely impacted by the plant extracts, as shown by the reduction in pH and the hyper polarization of cell membranes. In summary, plant extracts are extremely valuable as organic antimicrobials.

Table 3: Zone of Inhibition shown by *S. aureus*, *Bacillus subtilis*, *E. coli.*

	S. aureus	*Bacillus subtilis*	*E. coli*
Antibiotic	6 mm	10 mm	26 mm
Red Light	4 mm	4 mm	6 mm
Green Light	4 mm	6 mm	6 mm
Yellow Light	2 mm	4 mm	4 mm
Red+Green Light	8 mm	8 mm	8 mm

3.7.Results for MIC

The amount of an antibiotic required to completely stop bacterial growth is known as the minimum inhibitory concentration or MIC. The value found here is expressed in µg/ml, which is 1000 times smaller than mg/ml, proving that this was a successful study. The Podophyllotoxin efficiency in red+green LDE treated callus as a strong inhibitory agent is demonstrated by the MIC with the lowest concentration.

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Therefore, when MIC was calculated for different bacterial strains, the results obtained were:

Table 4: Minimum inhibitory concentration by *Bacillus subtilis* (MTCC 121):

Sample	Concentration (µg/ml)
Podophyllum hexandrum	6.25
Antibiotic	0.78

Table 5: Minimum inhibitory concentration by *S. aureus* (MTCC3160):

Sample	Concentration (µg/ml)
Podophyllum hexandrum	6.25
Antibiotic	0.39

Table 6: Minimum inhibitory concentration by *E. coli* (ATCC25922):

Sample	Concentration (µg/ml)
Podophyllum hexandrum	3.12
Antibiotic	0.390

4. Conclusion

In this paper, we worked on plant *Podophyllum hexandrum* callus extracts and find different qualitative and quantitative analysis in which terpenes, flavonoid, saponin and glycosides were found. This plant has shown anti-oxidant activity, which was proven by assays like DPPH method and antimicrobial activity, which was proven by Well diffusion and MIC method. Tissue culture techniques using plant material i.e. callus from different populations can help ensure high Podophyllotoxin content. To decrease pressure on the natural population, a sustained effort is needed, including callus sub-culturing and regeneration *(in vitro*) culture and estimation. The study assessed the podophyllotoxin concentration of callus, standardized medium for tissue culture, callus initiation, and generation, and raised *Podophyllum hexandrum* extract. The use of red and red+green LEDs as a source of elicitation offers a promising treatment for improving biomass accumulation and also aids in the enhanced manufacturing of phenolic content, total flavonoid, and antioxidant and antimicrobial activities in callus cultures of *Podophyllum hexandrum* in comparison to conventional fluorescent bulb. In the pharmaceutical sector, these significant secondary metabolites are crucial medicines. Future research on the biological effects of callus cultures extracts, including tests for anticancer, antifungal, and anti-diabetic properties, may be conducted.

This study may also be applied to commercially scale up the production of bioactive substances.

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