Article

Effects of different colored LEDs on the enhancement of biologically active ingredients in callus cultures of *Gynura procumbens* (Lour.) Merr.

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Abstract: Conventional fluorescent lamps used in tissue culture are costly light sources showing excessive wavelength emission-bandwidth that must be replaced by alternative, less costly and much lower power-consuming energy sources. The use of Light-Emitting Diodes (LEDs) is the best option due to their potential role as elicitors of secondary metabolite production in many plant models. *Gynura procumbens(G. procumbens)* is widely used for treating various diseases. Here, leaf explants were cultivated in MS medium supplemented with 0.5 mg/L of NAA and 2.0 mg/L of BAP for 30 days under white, blue, and red LEDs. Secondary metabolites were analyzed by HPLC and LC-MS. Blue LEDs elicited the highest antioxidant activity, total flavonoid, and phenolic content. Furthermore, the content of cyanidin-monoglucosides increased significantly increased under blue light.

Keywords: LEDs, elicitor, *Gynura procumbens*, callus, HPLC, LC-MS, cyanidin-monoglucosides

1. Introduction

Light is one of the most crucial environmental factors that affect the developing plant and regulate its behavior[1] . Generally, fluorescent lamps, high-pressure sodium lamps and metal halide lamps are used as light sources for *in vitro* culture. However, they contain unnecessary radiation wavelengths that lead to low quality radiation for the stimulation of growth and reportedly are responsible for as much as 65% of the total electricity consumed in tissue culture laboratories [2-4]. Recently, LEDs light has been widely used in agriculture as an alternative light source for plant growth and photosynthesis as they conveniently show specific wavelength and bandwidth, long-life and minimum heating, in addition to their small mass and volume [5, 6].

Research has shown that it is mostly white, red, and blue light wavelengths which increase signal transduction and betalain biosynthesis [7]. It is estimated that nearly 90% of plant development and physiology is influenced by the absorption of blue and red light (LEDs) [8, 9]. In tissue culture studies, LED colors (wavelengths) or color combinations (wavelength combinations) commonly used include white, red, blue, and blue-red mixtures. Blue light plays a major role in chlorophyll biosynthesis, photosynthesis, stomatal opening, and maturation of chloroplasts [10]. LEDs are preferred in *in vitro* growing environments due to their durability, small size, low heat emission and energy consumption; all of which traits make them ideal for *in vitro* plant propagation work [11]. Generally, anthocyanins are induced by visible and ultraviolet light [12]. Anthocyanins are phenolic molecules that cater natural colors to fruits and vegetables [13] and are influenced by pH, temperature and light [14]. Anthocyanins have nutraceutical potential and are used as active pharmaceutical ingredients; indeed, some of them are used as ancient practice for treatment of several diseases. As a nutraceutical, the bioavailability of anthocyanin is crucial for maintaining good health and preventing disease [15]. The major pigment in different types of berry species is cyanidin, which has a natural reddish-purple (magenta) pigment as in red sweet potato and in purple corn [16, 17]. The application of high light intensity or hormonal combinations induce morphological changes, such as purple [18] or pink [19] calluses in *in vitro* callus culture.

Gynura procumbens (Lour.) Merr. (Asteraceae) is a well-known traditional medicinal plant in southeast Asia, whose leaves are succulent, elliptic and glossy purplish; it is about 10-25 cm high [20]. The leaves have served for food for more than many years, generally served raw as salad. *G. procumbens* is widely applied for the treatment of inflammation, high cholesterol levels, high blood pressure, diabetes, kidney discomfort and cancer [20]. This plant is especially well-known for its antioxidant activity [21-23], and for its antihyperglycemic and antihyperlipidemic properties [24]. The advantages of using *G. procumbens* in the traditional manner have been supported by the identification and isolation of various medicinally important chemical constituents including, phenolic compounds, polyphenols, flavonoids, saponins, tannins, terpenoids, and essential oils [25, 26]. Adding elicitors to *in vitro* plant cell, tissue and organ cultures, is a common practice which increases the production or induction of *de novo* synthesis of secondary metabolites, as for example, anthocyanins, which are water-soluble pigments found in most plants [27, 28].

To date, anthocyanin has not been reported in *Gynura procumbens*. As LED technology offers a possibility to enhance various compounds using different wavelengths, we interestingly observed the presence of anthocyanin in calli extracts. Hence, the experiment continued to explore the induction of higher anthocyanin content under different wavelengths. This is the first report of the effects of LEDs on the production of anthocyanin.

2. Results

2.1. Callus induction

Optimum callus induction was observed after three weeks of culture on Murashige Skoog (MS) basal medium containing a combination of 0.5 mg/L of naphthaleneacetic acid (NAA) and 2.0 mg/L of benzylaminopurine BAP (Supplementary Table S1). This condition showed that the highest antioxidant activity (57.90 ± 2.32), total flavonoid content (TFC, 0.29 ± 0.14 µg/g) and total phenol content (TPC, 0.97 ± 0.03 mg/g) were observed in the MS basal medium supplemented with the same combination, at a statistically significant level (Supplementary Figure S1). In subsequent

experiments, the same conditions which yielded optimum callus induction were used to induce the accumulation of anthocyanin from calli using LEDs of different wavelength.

The cultivation of callus under different colors (wavelengths) caused morphology transformation of callus into brown, dark-green and pinkish-brown by applying dark, blue, white, and red LEDs, respectively (Figure 1). The effect of different light spectra on the phenotype of calli grown under darkness appeared paler or brown instead of green or red, compared to calli grown under light. Overall, visual inspection can reveal the effect of LED wavelength, as calli did not show any organ development.

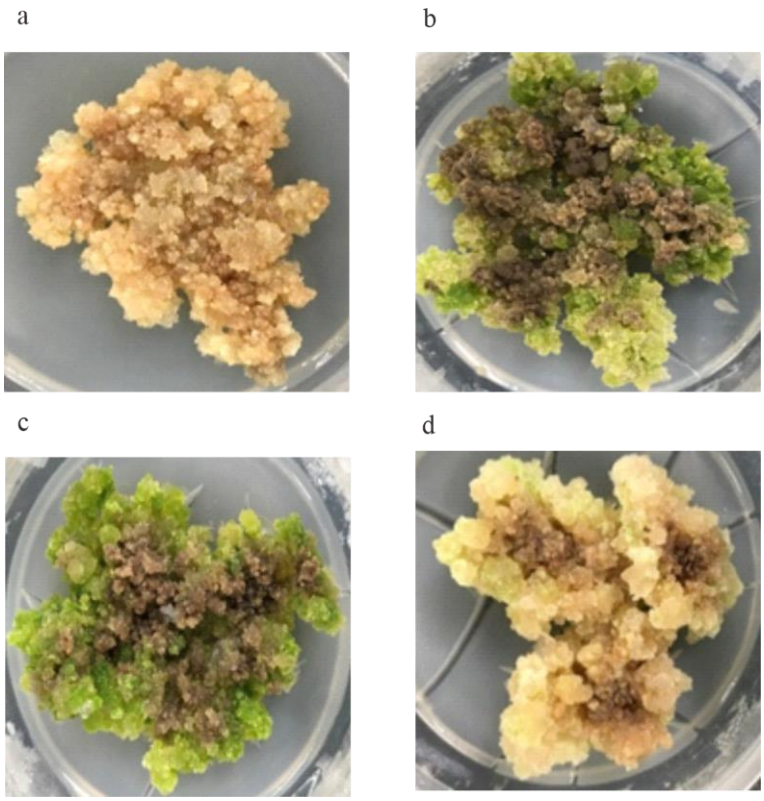

Figure 1. *Gynura procumbens* callus after 30-day culture under different LED lights. (A) Dark (B) Blue, (C) White (D) Red

2.2. Effects of LED lights on antioxidant activity, TFC, TPC and total anthocyanin content of G. procumbens calli

LED effects on radical scavenging as determined by the DPPH assay are shown in Figure 2a, and supplementary Table S2. Comparing the various LED lights tested, *G. procumbens* calli cultured under blue LEDs showed the highest radical-scavenging activity ($P<0.05$) (Figure 2a). Thus, the expression of radical-scavenging activity can be sequenced as Blue > White > Red > Darkness (Supplementary Table S1). The development of antioxidant activity in calli grown under blue LEDs compared to calli grown in darkness might attributed to morphology differences between them, as the calli grown under blue LED light developed a dark-green color, while gray calli were induced in the dark (Figure 1). Phytochemical compounds may be present in plant extracts, which are capable of donating hydrogen ions to a free radical scavenger [29]. A major role of phenolic compounds in preventing various chronic diseases is due to their properties as antioxidant, anti-carcinogenic and

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anti-inflammatory compounds that have attracted the attention of many researchers for their health benefits, especially the antioxidant activity [30]. The maximum content of phenolic compound was observed in the *G. procumbens* callus grown under blue light ($P<0.05$), followed by cultures grown under red light, white light and darkness (Figure 2b, Supplementary Table S2).

Flavonoid plant pigments consist of a group of secondary metabolites that have gained increasing attention because of their potential effects as helpful antioxidants for cancer and cardiovascular diseases, pathological disorders of gastric and duodenal ulcers, vascular fragility, allergies and anti-viral and anti-bacterial activities [31]. Blue LEDs light showed the highest TFC ($P<0.001$), followed by red ($P<0.001$), and white ($P<0.001$) LEDs (Figure 2d, Supplementary Table S2). In comparison, *G. procumbens* callus culture under dark conditions exhibited the lowest TFC among all culture extracts.

Anthocyanins are water-soluble glycosides and acylglycosides of anthocyanidins, a class of naturally occurring phenolic compounds. Naturally, 3-O-glycosides or 3,5-di-O-glycosides of cyanidin, delphinidin, peonidin, petunidin, pelargonidin and malvidin are reportedly the most common anthocyanidins found in fruits and vegetables [32]. Anthocyanins have potential antioxidative, antiangiogenetic, anticancer, antidiabetic, antimicrobial, anti-obesity and neuroprotective health benefits; additionally, they may help prevent cardiovascular disease and improve visual health [33]. The results on the detection of total anthocyanin by a pH differential method showed that blue light was the most effective in increasing anthocyanin accumulation with ($P<0.005$) (Figure 2c, Supplementary Table S2). Therefore, *G. procumbens* calli demonstrated that blue light was the best option compared to darkness, red or white light (Figure 2).

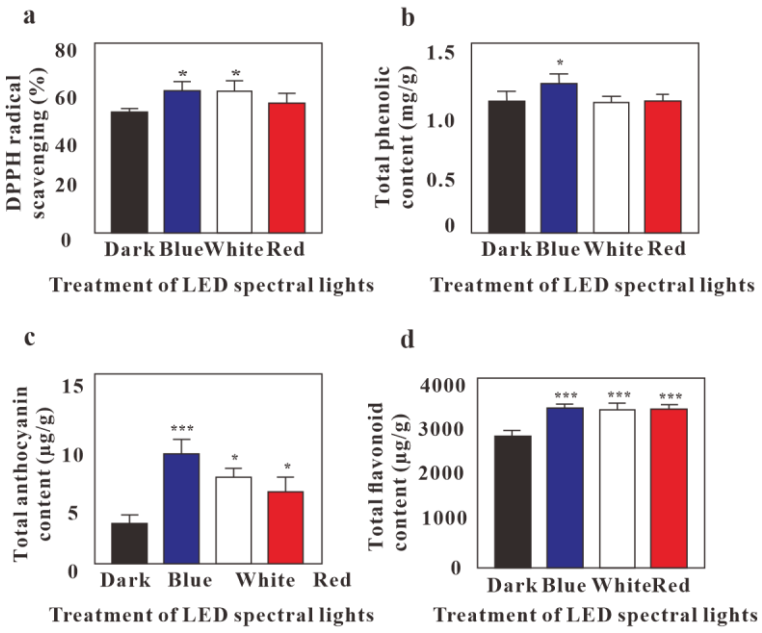

Figure 2. Effect of LEDs on callus culture for (a) DPPH free-radical savaging activity, (b) TPC, (c) total anthocyanin content, and (d) TFC in callus culture of *G. procumbens*. Values are means ± standard deviation (SD) from three replicates (n=3). Ordinary one-way ANOVA followed by Dunnett's multiple comparisons test were performed, where $P<0.05$, $P<0.01$, $P<0.001$, $P<0.0001$, are represented as *, **, *** and ****, respectively.

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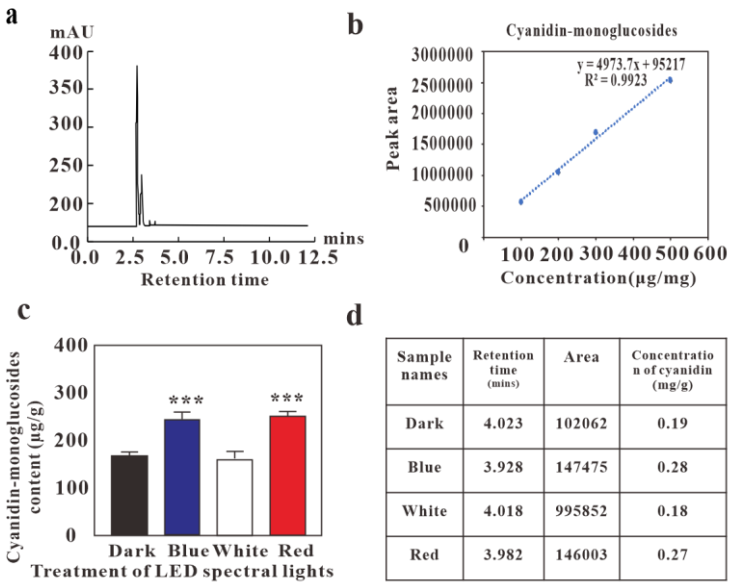
2.3. HPLC and LC-MS analysis on the effects of LED lights on cyanidin-monoglucosides accumulation in callus

Cyanidin-monoglucoside is one of the most common anthocyanins found in plants. We found cyanidin-monoglucoside in leaves of *G. procumbens* and determined its concentration by HPLC and LC-MS in calli. Extracts were analyzed by comparing peak cyanidin-monoglucoside concentrations to the standard compound at 4.0 min, under dark at 4.023 min, under blue light at 3.928 min, under white light at 4.018 min, and under red light at 3.982 min (Figure 3, Supplementary Figure S2). HPLC results showed that the extract from calli grown under blue light showed the highest accumulation of cyanidin-monoglucoside (0.28 mg/g), followed by calli cultured under red light, followed by calli cultured under white light, and finally, the lowest content in cyanidin-monoglucoside was detected in calli kept in darkness (Figure 3c).



Sample names	Retention time (mins)	Area	Concentration of cyanidin (mg/g)
Dark	4.023	102062	0.19
Blue	3.928	147475	0.28
White	4.018	995852	0.18
Red	3.982	146003	0.27

Figure 3. HPLC profile for the effect of LED lights for cyanidin-monoglucoside content in *G. procumbens* callus (a) peak of cyanidin-monoglucosides (b) standard curve (c) HPLC results (d) retention time and area of cyanidin-monoglucosides. Values are means ± standard deviation (SD) from three replicates (n=3). Ordinary one-way ANOVA followed by Dunnett's multiple comparisons test were performed, where P<0.05, P<0.01, P<0.001, P<0.0001, are represented as *, **, *** and ****, respectively.

The cyanidin-monoglucoside concentration in *G. procumbens* calli cultured under blue light increased significantly using LC-MS, compared to the anthocyanin concentration observed under dark conditions. In contrast, red light had a relatively weak effect on anthocyanin production (Figure 4d). The composition of cyanidin-monoglucosides obtained using LC-MS under blue, red and white light was 6.29, 4.80, and 5.98 ng/g, respectively, and significantly more strongly affected than calli grown under dark conditions (4.26 ng/g, P<0.05). The application of LEDs in tissue culture ensures numerous advantages for the production of secondary metabolites, especially blue light, which proved the most effective in inducing anthocyanin accumulation compared to red, white or dark (Figure 3c). These data demonstrate that light, especially of specific wavelength is an essential

environmental factor for anthocyanin biosynthesis and may therefore be helpful for a faster and higher yield in industrial anthocyanin production. Further research still needs to be conducted on the effects of cross-talk or interaction among different light wavelengths on the biosynthetic pathway of anthocyanin in calli of *Gynura* species.

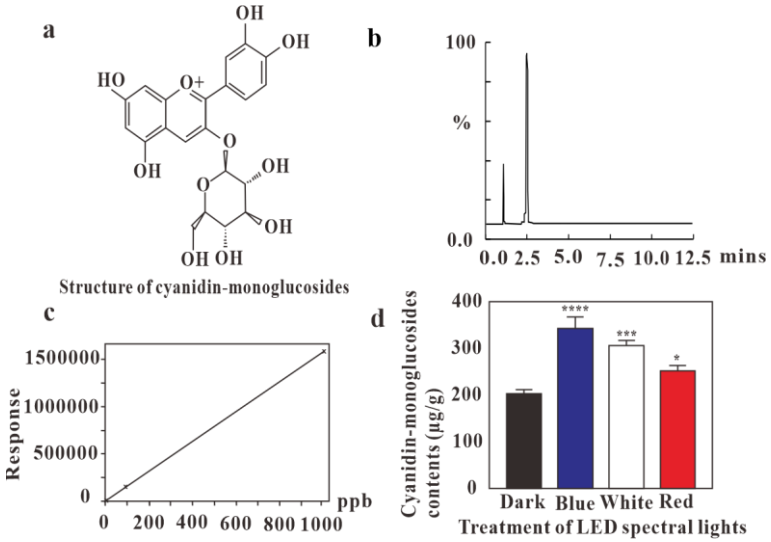


Figure 4. LC-MS profile for the effect of LED lights for cyanidin-monoglucoside content in *G. procumbens* calli. (a) Structure of cyanidin-monoglucosides, (b) peak of cyanidin-monoglucosides, (c) standard curve, and (d) effect of LEDs on cyanidin-monoglucoside content. Values are means ± standard deviation (SD) from three replicates (n=3). Ordinary one-way ANOVA followed by Dunnett's multiple comparisons test were performed, where P<0.05, P<0.01, P<0.001, P<0.0001, are represented as *, **, *** and ****, respectively.

4. Materials and Methods

4.1. Plant material

Wild *G. procumbens* plants were grown in pots (Plastic pots Planters; 17cm Diameter x 15cm x 12.3 cm Height). The stems were sterilized using 15% NaOCl for 15 min and rinsed four times using sterile distilled water. Sterile filter papers were used to absorb the remaining water so that the stems were completely dry. Next, stems were cut into 1.0-cm segments and planted on Murashige and Skoog (MS) solid basal medium in a Magenta box in order to obtain virus free plants. All media were adjusted to pH 5.8 by adding NaOH and HCl and then autoclaved (DAIHAN Scientific) at 121 °C for 15 mins. After autoclaving, the media were allowed to solidify on a clean bench until the experiment started. The nodal cultures were maintained in the culture room at 25 ± 2 °C.

4.2. Callus establishment

After six weeks of culturing, leaves were used as explants for callus induction. MS basal medium was supplemented with a combination of indolebutyric acid (IBA, 0.5, 1.0, 3.0, or 5.0 mg/L), naphthaleneacetic acid (NAA, 0.5, 1.0, 3.0, or 5.0 mg/L), 2.0 mg/L of Kinetin, and 2.0 mg/L of benzylaminopurine (BAP) for callus induction. Cultures were incubated under dark conditions for 30 days and observed twice a week under naked eye.

4.3. Spectral light treatments

Different LED (ODTech, Ltd., Korea) treatments were used as physical elicitors including: red LEDs (24-h, 660 nm), blue LEDs (24-h, 460 nm), white LEDs (24-h, 400–700 nm), or darkness (24-h, as a control). Leaf calli were then transferred to a new medium which included added NAA (0.5 mg/L) and BAP (2.0 mg/L). This was the optimum combination for callus induction. The calli were maintained in the culture room at 25 ± 2 °C under different spectral lights at 40 to 50 μMol m^{-2} s^{-1} photon flux measured with a Lux meter in the growth chamber (SU10, Jeiotech). After 4 weeks of culturing, the morphological differences among calli were observed under naked eye, and they were harvested for subsequent extraction and analysis.

4.4. Sample extraction

Induced calli were maintained and cultured on MS basal medium supplemented with a combination of 1.0 mg/L IBA and 2.0 mg/L Kn under different LED lights (red, blue, white) or darkness. After 30 days of culture, the calli were harvested, carefully separated from the medium and kept in a freeze-drier at -50 °C. Dried calli were ground and 1 g of each powder was soaked in 20 μL methanol 95% for 24 h before filtering through Whatman filter paper. The methanolic extracts were kept at -20 °C until analysis.

4.5. Analysis of total phenolic (TPC) and total flavonoid (TFC) contents

One of the oldest methods to determine TPC in vegetables, fruits, and medicinal plants is Folin-Ciocalteu's assay [34]. Briefly, 2% Na_2CO_3 and 50% of Folin-Ciocalteu reagent were prepared. Next, 100 μL of the sample and 2 mL of 2% Na_2CO_3 were mixed in a glass tube and left to stand for 3 min, after which, 100 μL of 50% FCR was added to the tube and left standing for another 30 min. Absorbance was measured at 700 nm with a micro plate reader (*Thermo Scientific™ Multiskan™ GO*). The protocol of Fazal *et al.* [35] was followed with minor modifications in order to evaluate TFC.

Briefly, 5% $NaNO_2$, 10% of $AlCl_3 6H_2O$ and 1N NaOH were prepared separately. Next, 250 µL of the sample was added to 1 mL distilled water and 75 µL of 5% $NaNO_2$ and 15075 µL 10% of $AlCl_3 \cdot 6H_2O$ were added and the mixture was left standing for 6 min. After addition of 500 µL of 1N NaOH, the reaction was allowed to proceed for 11 min and absorbance was measured at 500 nm using a micro plate reader. Gallic acid and catechin standard calibration curves were used for TPC and TFC estimation, respectively. Values were calculated as Gallic acid (GE)/g and catechin (CAT)/g of dried weight (DW).

4.6. Determination of antioxidant activity

Measurement for free-radical scavenging potential was performed according to the method developed by Blois [36]. Briefly, 40 µl of extract mixed with 160 µl methanol were added to 1800 µl of DPPH solution and allowed to react 30 min in the dark. Absorbance was measured at 517 nm using a micro plate reader. DPPH solvent was used as control and methanol was used as a blank. The following equation was used to estimate free-radical scavenging activity:

% scavenging DPPH free radical = 100 × (1 AE/AD)

where, AE represents absorbance at 517 nm and AD is the absorbance of DPPH solvent as control.

4.7. Analysis of total anthocyanin content

Total anthocyanin content of the calli were measured using the pH-differential method. For making 1.0 pH buffer (potassium chloride, 0.025 M), we measured the weight of 1.86 g KCL in a beaker, then added DW 980 mL. We measured the pH and adjusted to 1.0 with HCL. To adjust the pH buffer to 4.5 (sodium acetate, 0.4 M), we weighed 54.43 g of $CH_3CO_2Na_3H_2O$ in a beaker and combined it with 960 mL of distilled water. After addition of 4.5 mL of pH=1.0 or pH=4.5 buffer solution to 0.5 mL of each sample (dilution factor, DF=10) absorbance was measured at 510 and at 700 nm. Distilled water was used as a reference. Detailed information is provided in the supplementary materials (S1).

4.8. High Performance Liquid Chromatography (HPLC) Analysis

Calli grown under different LED spectral lights and in the dark were analyzed by HPLC using a Shimadzu HPLC System (CBM-20A, LC-20A, SPD-20AD, and CTO-20A, Japan) with Nucleosil C18 reverse-phase (125-by-mm) column at 40 °C (Shimadzu, Japan). Detailed information is provided in the supplementary materials (S2).

4.9. Liquid chromatography-mass spectrometry (LC-MS) analysis

A XEVO-TQS#WAA250 triple quadrupole mass spectrometer (AB Sciex, USA) with ESI source coupled with UHPLC (UltiMate 3000 RS, Thermo Fisher Scientific, USA) and a nitrogen generator (Parker, USA) were used. Fresh calli grown under different LED lights or in darkness were harvested after 30 days and 1 g of each callus was extracted with 10 mL of 100% methanol. Detailed information is provided in the supplementary materials (S3).

4.10. Statistical analysis

All experiments were repeated thrice. Graph Pad Prism (Windows, v7.0) was used for statistical analysis and for making graphs. To define significant results, ordinary one-way ANOVA followed by Dunnett's multiple comparisons test to separate means were performed ($P<0.05$).

5. Conclusions

The optimum medium for callus induction was a combination of 0.5 mg/L NAA and 2.0 mg/L of BAP as it showed the highest antioxidant activity as well. The utilization of blue LED lights on *in vitro* callus cultures induced a high antioxidant activity and enhanced the accumulation of TPC, TFC, and TAC (Figure 2). The effects of different lighting conditions on *in vitro* cultures demonstrated that blue LEDs increased anthocyanin accumulation. The system is cost-effective and low energy requiring; furthermore, it rendered a higher quantitative and qualitative yield compared to the conventional method. Therefore, the utilization of LEDs satisfies the demand of various aspects in *in vitro* cultures involving commercial micropropagation and it can be part of a reliable protocol for developing new drugs in addition to drug supplements.

Author Contributions: T.L, S.Y.C, K.S.B and Y.J.K designed the study. T.T.L, K.S.B and Y.J.K carried out the experiments. T.T.L, S.Y.C, M.M.M, K.S.B, and Y.J.K performed data analysis and prepared the manuscript.

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Conflicts of Interest: Declare conflicts of interest or state "The authors declare no conflict of interest."

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Sample Availability: Samples of the compounds are available from the authors.