Elevation of secondary metabolite production by using light-emitting diodes illumination in Mulberry (*Morus* spp.)

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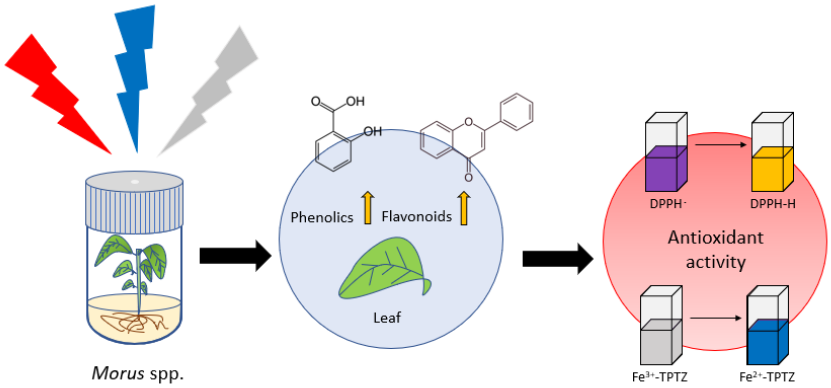
Abstract: Mulberry leaves have long been used in traditional medicine and are associated with several impressive health benefits. Recently, light-emitting diode (LED) is widely used as a light source in plant growing systems. Therefore, the objectives of this study were to investigate the effect of LEDs on secondary metabolite production in mulberry leaves and evaluate their antioxidant activities using ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays. Mulberry plants cultured in vitro were exposed to red, blue, and white LEDs for 7 days. Lighting experiments were performed under controlled conditions (PPFD - 100 μmol m^{-2} s^{-1}; 16/8 h photoperiod; 25 ± 2 °C). Blue light illumination resulted in the greatest phenolic content (9.13 ± 1.2 mg gallic acid equivalent (GAE) g^{-1}) measured by the Folin-Ciocalteu reagent method. Total flavonoid contents were found to be in the range of 8 - 13 mg quercetin equivalent (QE) g^{-1}), as determined by the aluminium chloride colorimetric method. However, there was no significant change in total flavonoid content between red, blue, white LEDs, and fluorescent light used as a control. Furthermore, the most potent antioxidant activity, measured by FRAP assay, was in the plants treated with blue light, suggesting that phenolic compounds might be associated with this effect. All extracts could inhibit, but not significantly, the DPPH radicals approximately at 70 - 80%. Taken together, the results of this study support that LEDs might be utilized as an alternative light source to improve the production of some valuable secondary metabolites in mulberry leaves.

Graphical abstract:



Keywords: antioxidant; light-emitting diode; mulberry; secondary metabolite; tissue culture

1. Introduction

Mulberry (*Morus* spp.) belongs to the Morus genus of the Moraceae family. It is a fast-growing plant under cultivation in many temperate world regions. Leaves, fruits, barks, and branches of mulberry are traditionally used in the treatment of joints, hyperglycemia, dyslipidemia, and hypertension. Mulberry leaves contain several bioactive compounds, such as phenolic acid, alkaloid, terpenoid, and flavonoid [1]. Light is one of the essential environmental factors that regulate plant growth and secondary metabolite production. A previous study demonstrated that deficient or excess levels of light increase bioactive compounds such as phenylpropanoids and phenolics [2]. Light-emitting diode (LED) is a more efficient artificial light source than fluorescent lamps generally used in plant micropropagation. LEDs have several advantages, including narrow band spectra of wavelengths, least thermal effect, better longevity, and energy-saving [3]. This system is also proven to be an optimal and effective tool to study the effect of wavelength on plant physiological and biochemical responses [4]. Here, we focused on the impact of LEDs illumination on secondary metabolite accumulation in mulberry leaves for future utilization in food and cosmetic industries.

2. Materials and Methods

2.1 Reagents and chemicals

2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Gallic acid and quercetin standards were purchased from Sigma-Aldrich (Sigma, USA). Folin-Ciocalteu reagent was obtained from (Merck, Germany). All solvents were of reagent grade.

2.2 Plant Material

Mulberry cv. Kamphaeng Saen-MB-42-1 obtained from the Department of Biochemistry, Faculty of Science, Kasetsart University, Thailand, was used in this study. The shoot of explants was sterilized with 0.1% mercury (II) chloride ($HgCl_2$) (Ajax, Australia) for 10 minutes after being immersed in 70% ethanol (Macron, USA) for 1 minute. Then, the explants were washed with distilled water and transferred into Murashige and Skoog (MS) medium (PhytoTechnology, USA) containing 30 g of sucrose and 8 g of agar powder. The pH of the MS medium was adjusted to 5.6 using 1 M sodium hydroxide (NaOH) (Fisher Chemical, Belgium). All plant tissue samples were initially grown in a tissue culture room at 25 ± 2 °C under fluorescent white light (light/dark period of 16/8 hours and ~75% RH) for 6 weeks.

2.3 LEDs treatment on mulberry tissue culture

LEDs were employed on 6-week-old mulberry tissue culture to study the effects of different wavelengths of light on secondary metabolite production. The plants were irradiated with white light (430-780 nm), red light (600-700 nm) with a peak of 635 nm, and blue light (400-500 nm) with a peak of 452 nm. The spectra range of each LED treatment was detected by Spectral Colour Illumino meter (Hangzhou Hopoo, China). The light intensity at the container surface of each treatment was controlled at 100 μmol m^{-2} s^{-1}. The plants were grown at 25 ± 2 °C with an average of 16/8-hour light/dark and 70 ± 2% relative humidity for 7 days. The leaves were harvested, dried using a freeze dryer, and ground into a fine powder. All samples were stored at -20 °C until analysis. In this experiment, the plants grown under florescent white light were included as controls.

2.4 Extraction procedure

Lyophilized leaves (5 mg) of mulberry cultured in vitro were extracted with 1 mL of different solvents, including ethanol, methanol, ethyl acetate, hexane, and water. Then, the samples were treated with an ultrasonic bath and incubated for an hour at room

temperature. After centrifugation at 13,000 rpm, 4°C for 10 minutes, the upper phase was collected and kept at -20 °C for further experiments.

2.5 Total phenolics and flavonoids contents

Quantification of phenolics compounds was determined using the Folin-Ciocalteau method as previously reported [5]. Briefly, 20 μL of the extract solution was combined with 100 μL of 10% (v/v) Folin-Ciocalteau reagent in a 96-well plate and incubated at room temperature for a few minutes. After that, 80 μL of 1 M sodium carbonate was added and incubated for 20 minutes in the dark. The absorbance of the mixture was measured at 760 nm. The total phenolics contents in the extracts were calculated as gallic acid equivalent (GAE) from a calibration curve (0 – 200 μg mL^{-1}). The total phenolics contents were expressed as μg of gallic acid equivalent per 1 g of dry weight plant tissue (μg GAE g^{-1} DW). All measurements were performed in triplicate.

Total flavonoid contents were determined spectrophotometrically following a previously reported method with slight modifications [6]. The samples (50 μL) were mixed with 10 μL of aluminum chloride ($AlCl_3$), 150 μL of 60% ethanol, and 10 μL of 1 M potassium acetate. After 15-minute incubation at room temperature, the absorbance was measured at 415 nm using a Spark™ 10M multimode microplate reader (Tecan, Switzerland). Total flavonoid contents were calculated as quercetin equivalent (QE) using a calibration curve (0 – 400 μg mL^{-1}) and expressed as μg QE g^{-1} DW. All measurements were performed in triplicate.

2.6 Antioxidant assays

For Ferric reducing antioxidant power (FRAP assay), the method is based on the reduction of Fe^{3+} TPTZ complex (colorless complex) to Fe^{2+} tripyridyl triazine (blue colored complex) at low pH [7]. Briefly, 20 μL of extract solution was added into a 96-well plate followed by 280 μL of FRAP working solution. The solutions were then mixed, covered, and allowed to incubate at 37°C in the dark for 30 min. The absorbance at 593 nm was used to measure, and ascorbic acid (0 – 1 mM) was used as a standard. The FRAP value showed as mM of ascorbic acid equivalent per gram of sample.

For DPPH radical scavenging assay, the samples were estimated according to previous studies with some modifications [3]. Briefly, 25 μL of samples were added into a 96-well plate followed by 200 μL of DPPH solution (150 μM). After that, the solutions were mixed, covered, and allowed to react in the dark for 30 min. The absorbance at 517 nm was used to measure, and ascorbic acid (0 – 80 mg mL^{-1}) was used as a standard to generate a calibration curve. The capability to scavenge the DPPH radicals was calculated using the following equation. Each sample was performed in triplicates.

$$\% \text{ DPPH inhibition} = 1 - A_{control}/A_{sample} \times 100$$

2.7 Statistical analysis

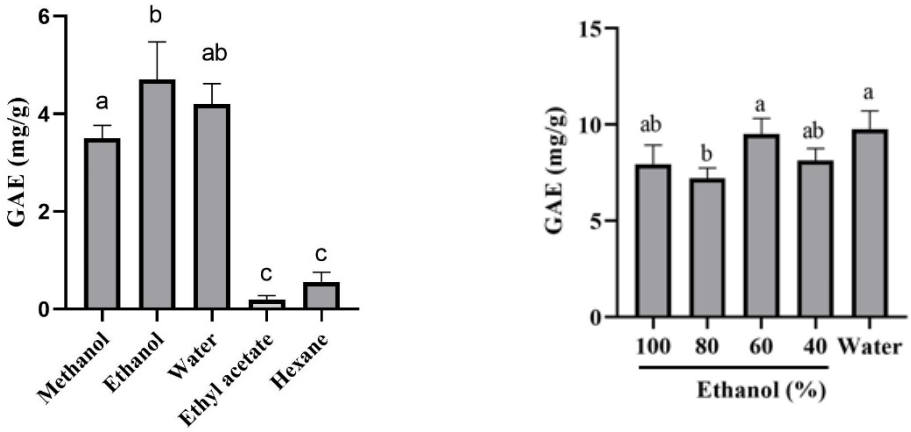
All data were presented as the means with standard deviation of 3-5 biological replicates. Analyses of variance were performed by ANOVA test and significant differences between the means were determined by Tukey's multiple comparisons test at the level of p-values < 0.05.

3. Results

3.1. Comparison of extraction solvents

To find the most suitable method for phenolics extraction, leaves were extracted with different solvents (ethanol, methanol, ethyl acetate, hexane, and water). The total phenolic content was determined by the Folin-Ciocalteu method (Figure 1a). The result showed that ethanol (4.7 ± 0.77 mg g^{-1}) and water (4.2 ± 0.42 mg g^{-1}) gave the similar amount of

total phenolics (gallic acid equivalents), followed by methanol (3.5 ± 0.27 mg g^{-1}), hexane (0.56 ± 0.20 mg g^{-1}), and ethyl acetate (0.21 ± 0.08 mg g^{-1}), respectively. The optimum concentration of ethanol was further investigated (0%, 40%, 60%, 80%, and 100 %). However, no significant difference was found among the concentrations tested. Previous studies demonstrated that 60% ethanol was the most efficient for extracting phenolic compounds in guava leaves [8]. Therefore, this concentration was chosen for further analysis.

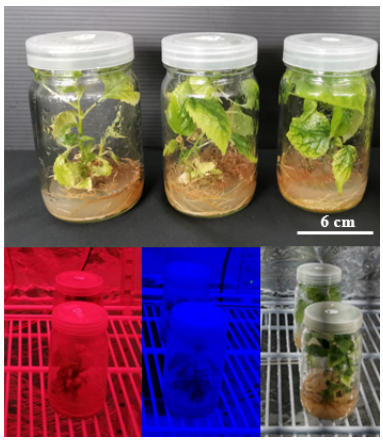


(a) (b)

Figure 1 (a) Total phenolic contents of different solvent extracts from mulberry leaves. (b) The effect of ethanol concentration on phenolic compounds extraction. Differences between solvents analyzed using one-way ANOVA were statistically significant ($p < 0.05$). Error bars were calculated from the results of 3 replicates.

3.2 Determination of total phenolic and flavonoid contents

The mulberry plants were exposed to different light conditions (red, blue, and white LEDs) and the total phenolics and flavonoid contents were determined (Figure 2a). The result showed that blue light treatment significantly induced phenolic compound accumulation compared to red, white, and fluorescent lamps used as a control (Figure 2b). For flavonoid contents, no statistical change was detected (Figure 2c). This result suggested that the light conditions (quality and intensity) used in this study did not affect flavonoid accumulation in the leaves of mulberry.



(a)

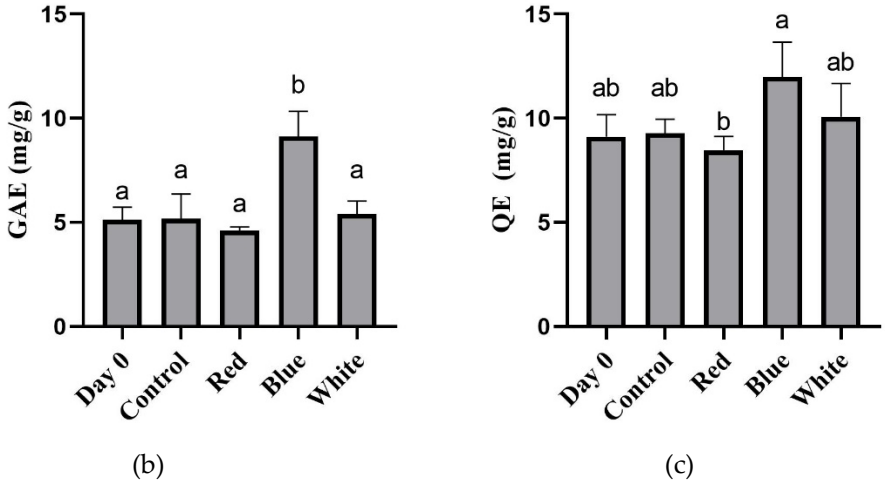


Figure 2 (a) The mulberry plants grown under different light conditions; (b) Total phenolics and (c) total flavonoid contents in the leaf extracts by spectrophotometric determination. Error bars were calculated from the results of 4 replicates. Differences analyzed using one-way ANOVA were statistically significant ($p < 0.05$).

3.3 Effects of LED on antioxidant activity

The antioxidant activity was determined using ferric reducing antioxidant power (FRAP) and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging assays. The effect of extracts obtained from plants exposed to different types of light on the scavenging activity is shown in Figure 3. The sample exposed to blue LED light had the highest FRAP value and significantly differed from the others. There were no differences between the samples exposed with red, white, and the control (Figure 3a). For DPPH scavenging activity, there were no differences among the samples or even compared with the control. All extracts could inhibit, but not significantly, the DPPH radicals approximately at 70 - 80% (Figure 3b).

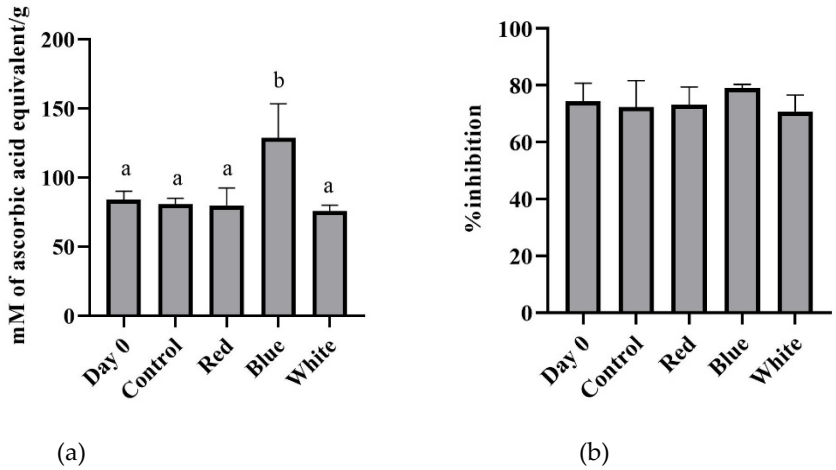


Figure 3 Antioxidant activity of mulberry leaves (a) Ferric ion reducing antioxidant power (FRAP) assay; (b) DPPH radical scavenging capacity. One-way Analysis of Variance (ANOVA) was used in analysis ($p < 0.05$). Different letters on bars mean significantly different. Error bars were calculated from 4 replicates.

4. Discussion

In vitro cultures play a promising role in producing plant secondary metabolites that can be used in pharmaceutical and cosmeceutical applications. Different light sources have variable effects on plant growth and secondary metabolite production. Previous studies showed that red and blue LEDs elevate the accumulation of some secondary metabolites in various plants [9-10]. Phenolic compounds are the most widely distributed secondary metabolites found in plants. Our results revealed that solvents with high polarity, such as ethanol and water, are the most efficient solvent. However, the concentration of ethanol/water solvents also affect the extraction efficiency.

It has been reported that light enhances the phenolic contents by improving the malonyl-CoA biosynthesis pathway [11]. In this study, the stimulation of phenolic synthesis was predominantly enhanced by blue light after 7 days of illumination. This result agrees with the reports showing that blue light had stimulatory effect on phenolic compounds production [12-13]. It is worth noting that more work is required to examine the effect of LEDs on flavonoid production, such as light intensity and the duration of the treatment.

Furthermore, the antioxidant capacity of the leaf extracts was evaluated. The results showed that the extract containing the highest amount of phenolics was the most potent reducing agent (high FRAP value), indicating that phenolic compounds might be associated with this effect. This result is corresponding with the previous studies reporting that blue light significantly increased the total phenolic compounds and the highest FRAP of pea sprouts [4].

5. Conclusions

In this work, we quantified phenolic acids and flavonoids extracted from the leaves of mulberry (*Morus* spp.) cultured in vitro after exposed to LEDs. Blue light significantly stimulated the phenolic compound production. However, no significant difference was observed in total flavonoid content. These findings suggested that LEDs might be utilized as an alternative light source to improve the production of some valuable secondary metabolites in mulberry.

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Conflicts of Interest: The authors declare no conflict of interest.

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