Original Research Article

Effect of Copper Sulfate Induction on *Phyllanthustenellus*Roxb. Total Phenolic (TPC) and Flavonoid (TFC) Content

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ABSTRACT

Aims: Plants need an appropriate amount of nutrients such as copper for growth and development. However, excess of copper may interrupt plant development and cause stress that led to biochemical compounds being synthesized. The influence of a high copper sulfate concentration on phenolic and flavonoid content in *Phyllanthustenellus* plants was investigated.
Place and Duration of Study: The experiment was conducted in a government compound at MARDI Serdang, Selangor, Malaysia (2° 59' 31.7292" N 101° 41' 56.706" E), from April 2021 to Jun 2021.
Methodology: The experiment was conducted using a vertical column planting system under a side-netted rain shelter. The plants were subjected to 0.5 M copper sulfate sprayed after 60 days of planting and harvested 0.5, 1.5, 3, 6, 12 and 24 h after sprayed for further analysis. Total phenolic content was calculated as mg gallic acid equivalent and total flavonoid content were measured as quercetin equivalent.
Results: Highest total phenolic and flavonoid content was detected after 0.5 hours of copper sulfate application and started to decrease towards 24 hours after sprayed. Treated samples showed 1.18-fold increase in total phenolic content and 1.4-fold increase in total flavonoid content compared to control untreated samples after 0.5 hours of sprayed. Control samples showed stability in both total phenolic and flavonoid content throughout the harvesting periods. Phenolic is the major secondary metabolites in *Phyllanthustenellus* plants.
Conclusion: Data revealed that application of 0.5 M copper sulfate able to enhance total phenolic and flavonoid content in *Phyllanthustenellus* plants. Study suggested that optimum harvesting time is 0.5 hours after copper sulfate application.

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Keywords: Phyllanthustenellus, copper sulfate, phytochemical, antioxidant, induction

1. INTRODUCTION

Phyllanthus have been investigated and many molecules have been isolated and identified phytochemically and pharmacologically. Many classes of medicinal organic compounds have been reported, including alkaloids, flavonoids, lactones, steroids, terpenoids, lignans and tannins in this genus. *Phyllanthustenellus*Roxb. is a member of the Phyllanthaceae family, widely distributed in most tropical and subtropical countries. This species has been investigated as a source of medicinal herbs used as antibacterial and to treat kidney and urinary bladder disturbances, to dissolve renal stones, diabetes and intestinal infections [1]. This herb, native to tropical regions, is known for its medicinal properties and rich phytochemical composition. Calixto et al. (1997) also proposed that the phenolic compounds of these plants have antinociceptive properties. The cultivation of *Phyllanthustenellus* had gained increasing attention due to their rich phytochemical composition and potential applications in various industries.

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Plant secondary compound alteration is one of the major area of plant science studies that associated with plant defense mec and increase secondary metabolites production when they are affected by biotic and abiotic stress [3]. These secondary me are of particular interest due to their association with antioxidant and medicinal qualities. Abiotic and biotic stresses had show degree of complexity in plant responses, as the responses to these factors are largely controlled by different signaling path plants [4, 5]. Plant metabolic and signaling pathways involved in the response of plants to stress including antioxidant mechanisms, pathogen responses, hormone signaling, osmolyte synthesis transcription factors and photosynthesis [6, 7]. Many studies had been conducted to uncover the responses of plants to different types of stresses involving abiotic and biotic stresses [8, 9, 10].

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Heavy metal induction as an abiotic elicitor is a technique in plant biology research that has been employed to investigate its effect on the antioxidant content in plants [11]. This innovative approach involves the exposure of plants to heavy metals such copper sulfate to

stimulate a plant's defense mechanisms and enhance the production of antioxidants. The rationale behind this technique is rooted in the idea that oxidative stress triggered by heavy metals prompts plants to synthesize higher levels of antioxidants as a protective response [12]. Application of copper sulfate spray led to microlesions formation and phytoalexins synthesis in punctuated regions, a type of hypersensitive reaction symptom similar induced by pathogen attack[13]. This action producing phenolic substances induced in a plant's defence response to abiotic stress [14]. Therefore, the present investigation was conducted to study the effects of copper sulfate induction influences the accumulation of TPC an TFC in *Phyllanthustenellus*Roxb.

2. MATERIAL AND METHODS

2.1Study Area

The experiment was conducted in a government compound at MARDI Serdang, Selangor, Malaysia (2° 59' 31.7292" N 101° 41' 56.706" E), from April 2021 to Jun 2021. A side-netted rain shelter of 30 m long x 10 m wide x 4.5 m high was used in the study. All structures were made of galvanized steel frames and insect repellent net (0.1 x 0.1 mm2) side cladding. The entrance into the shelter was through double doors to reduce the chance of insect entry.

2.2 Planting materials

*Phyllanthustenellus*Roxb. seeds were used as planting materials in the experiment. Seeds were collected from plants and germinated using peat moss. Plants about 7 cm tall with 2-3 stalks were used as seedlings.

2.3 Treatments and Experimental Design

The experiment was conducted using a vertical column planting system under a side-netted rain shelter. Fourteen days after germination, the seedlings were then transplanted into a vertical column planting system and coir dust as growth substrates. The planting distance between plants in columns was 15 cm and 15 cm between rows. Each vertical column can be fixed with 18 plants which gave a plant density of 108 plants per m^2. After 60 days of cultivation, plants were sprayed with an aqueous solution of 0.5 M copper sulfate and 0.05 % (v/ v) Tween-20, and plants were collected at 0.5, 1.5, 3, 6, 12 and 24 h after being sprayed for further analysis. Control plants were treated with 0.05 % (v/v) Tween-20 in sterile distilled water and the plants were collected at the same time points. The experiment was arranged in a randomized complete block design (RCBD) with 3 replications.

2.4 Nutrient supplementation

The nutrient solutions were prepared in a 200 L tank. Stock A and stock B were added into the tank at a 1:1 ratio until the needed electricity conductivity (EC) was achieved. The EC of the fertigation solution was between 1800 µS/cm and 2400 µS/cm. Nutrient solutions were applied twice per day throughout the cultivation periods. The daily nutrient solution volumes per plant were 150 ml in the first three weeks and 500 ml after four weeks until the end of the cultivation periods. If necessary, routine horticultural practices for pest, disease, and weed control were done using biopesticides.

2.5 Extraction

Whole plants except root were harvested, rinsed and air-dried for few minutes at room temperature. The plant material was cut into smaller pieces and ground with liquid nitrogen prior to freeze-drying. 1g of freeze-dried powder was extracted with 40 mL of water extracting buffer (20 mMdiethyldithiocarbamic acid, 0.5% formic acid) and mixed thoroughly for 30 minutes. The extract was then centrifuged at 8900 rpm, 4°C for 5 mins and filtered with WHATMAN No. 40. The residue was resuspended in 20 mLof water and the same extraction protocol was repeated twice to obtain a total of 80 mL of crude extract which was then subjected to the antioxidant activity assay. Same extraction protocol was applied for methanol extraction.

2.6 Determination of total phenolic content (TPC)

A slight modification of the total phenolic content assay was adopted from previous study [15]. A standard calibration curve was plotted using different concentrations of gallic acid and the absorbance was recorded at 765 nm. 0.3 mL of aliquot extract (with appropriate dilution, if necessary) was mixed with 1.5 mL Folin-Ciocalteu (previously diluted 10x with water) followed by 1.2 mL sodium carbonate. The mixture was then vortexed thoroughly and kept in dark at room temperature for 30 minutes. Subsequently, the absorbance was measured at 765 nm using Perkin Elmer Lambda 25 UV/ VIS Spectrophotometer. 3-hydroxyphenylacetic acid (100 µg/mL) was used as positive control and the water extracting buffer as the negative control. Results were calculated as mg gallic acid equivalent per g dry weight plant (mg GAE/ g dry weight plant). All assays were carried out in triplicates.

2.7 Determination of total flavonoid content (TFC)

The total flavonoid content was measured according to Zhishen et al., 1999 with modification. One hundred micro litres of extract were added to 4 ml of distilled water. Then, 0.3 ml 5% sodium nitrite was added. After 5 min, 0.3 ml of 10% aluminium chloride was added. In 6 min, 2 ml of 1 M sodium hydroxide was added to the mixture. Immediately, the mixture was diluted by the addition of 3.3 ml distilled water and mixed thoroughly. The absorbance was determined at 510 nm versus a blank. Results were calculated as mg quercetin equivalent per g dry weight plant (mg GAE/ g dry weight plant). All assays were carried out in triplicates.

2.8 Statistical Analysis

Data obtained were subjected to statistical analysis using analysis of variance (ANOVA) procedures to test the significant effect of all the variables investigated using the SAS application (version 9.2. SAS Institute, Cary, NC, USA) [12]. Means and standard deviation (SD) of replications were determined using analysis of variance (ANOVA) and Tukey HSD test was used to determine the significant differences between treatments. The statistical significance level was set at 0.05 for all tests.

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3. RESULTS AND DISCUSSION

3.1 Total Phenolic Content (TPC)

There was a downward trend in total phenolic content (TPC) starting from 0.5 hours towards 24 hours of the harvest period (Table 1). Meanwhile, the TPC content of the control sample was stable throughout the harvest time in the range of 28.05 - 28.55 mg g^{-1}. The study showed that the application of copper sulfate reduced TPC in *Phyllanthustenellus*over time. However, copper sulfate spray was able to increase TPC in the treated samples compared to control samples. There was a 1.18-fold increase in TPC in treated samples compared to control samples after 0.5 hours of copper sulfate spray. It is suggested to harvest the plants 0.5 hours after copper sulfate to enhance the TPC. However, six hours is the maximum harvesting time as the TPC is higher compared to control samples. The decrease in TPC suggests that prolonged exposure to copper sulfate may have a negative impact on the phenolic content of *Phyllanthustenellus*. Phenolic is the major secondary metabolites in *Phyllanthustenellus* compared to flavonoid. The initial increase in TPC observed at 0.5 hours indicates that copper sulfate treatment may have induced a stress response in *Phyllanthustenellus*, leading to an accumulation of phenolic compounds. Phenolic compounds are known to act as antioxidants and can be produced in response to various stressors, including heavy metal exposure.

Previous studies on application of copper able to enhance total phenolic content on *Medicago sativa* using direct soil irrigation showed a significant increase in the total content of phenols [18]. An increase in secondary metabolite content could be due to reactive oxygen species (ROS), one of the mechanisms for plant interaction and stress induction due to biotic and abiotic factors [19]. The phytotoxicity properties of copper sulfate induced the simultaneous increase of Phenylalanine Ammonia-Lyase (PAL) and phenolic compounds characteristic of phytoalexins producing necrotic lesions in plant [20]. Plant synthesizes metabolites to mitigate and reduce stress factors caused by cellular damage, thus increasing antioxidant activity as observed in the experiment [21]. The subsequent decrease in TPC over time suggests that the initial stress response may have been followed by a metabolic adjustment or a detoxification process within the plant. However, prolonged exposure led to a decline in TPC, suggesting the need for careful consideration of treatment duration when aiming to optimize phenolic content in this plant species. This phenomenon is not uncommon and has been observed in other studies involving plant responses to stressors. Based on these results, it appears that the optimal treatment duration to maximize the TPC of *Phyllanthustenellus* leaves is around 0.5 hours. Longer exposure times, such as 24 hours, resulted in a significant reduction in TPC, which may not be desirable for applications where high phenolic content is desired. To better understand the underlying mechanisms of phenolic compound accumulation and degradation in response to copper sulfate treatment, further research is needed. This could involve investigating the specific phenolic compounds affected, as well as the gene expression patterns and enzymatic activities involved in their synthesis and degradation.

Table 1.Total phenolic content (TPC)of *Phyllanthustenellus* after treated with copper sulfate.

After sprayed (hour)	Total phenolic content (PFC)*	Control Total phenolic content (PFC)*
0.5	33.51[a]	28.38[a]
1.5	32.67[b]	28.13[a]
3	31.63[c]	27.99[b]
6	30.20[c]	28.05[ab]
12	28.62[d]	27.89[b]
24	27.03[d]	28.55[a]

Mean values in the same column followed by the same letter are not significantly different at p <0.05
*Equivalent to gallic acid per g dry weight (mg g^{-1})

3.2 Total Flavonoid Content (TFC)

Similar to the trend observed in the total phenolic content (TPC), the TFC of *Phyllanthustenellus* increased significantly following the application of copper sulfate at 0.5 hours (Table 2). At this time point, the TFC reached 15.59 mg g−1, whereas the control group had a TFC of 11.13 mg g−1. This initial rise suggests that copper sulfate treatment triggered a rapid accumulation of flavonoids, which are known for their antioxidant properties and their role in stress responses in plants. As time progressed, the TFC gradually declined. By 24 hours, the TFC had decreased to 11.08 mg g−1, which is even lower than the control group's TFC (11.16 mg g−1). This decreasing trend indicates that the prolonged exposure to copper sulfate may have adverse effects on the flavonoid content of *Phyllanthustenellus*. However, the TFC of the treated sample was higher throughout the harvesting time until 24 hours compared to control samples. TFC in control showed stability in the range of 11.01 – 11.25 mg g^{-1} throughout the harvesting time.

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The study showed that the application of copper sulfate reduced TFC in *Phyllanthustenellus*over time. Copper sulfate spray was able to increase TFC up to 12 hours in the treated samples compared to control samples. The optimum harvesting time was 0.5 hours after the copper sulfate was sprayed. There was a 1.4-fold increase in TFC in treated samples compared to control samples after 0.5 hours of copper sulfate spray. 12 hours is the maximum harvesting time as the TFC is higher compared to control samples. Longer exposure times, such as 24 hours, led to a significant reduction in TFC, which may not be desirable if high flavonoid content is desired for potential applications. *Phaseolus vulgaris* treated with cerium dioxide showed increased flavonoid in roots and leaves parts of the plant [22]. Formations of flavonoid compounds in plants are usually related to the plant defensive mechanism to abiotic factors [23]. Previous studies have shown that phenolic is a major compound in *Phyllanthus species compared* to flavonoid [24]. The study also revealed that phenolic is major secondary metabolites in *Phyllanthustenellus* compared to flavonoid as data shown that higher TPC compared to TFC. In summary, the study reveals the dynamic response of *Phyllanthustenellus* to copper sulfate treatment, with an initial surge in flavonoid content followed by a gradual decline. This highlights the importance of considering both the timing and duration of stressor exposure in efforts to optimize flavonoid production in this plant species.

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Table 2.Total flavonoid content (TFC) of *Phyllanthustenellus* after treated with copper sulfate.

After sprayed (hour)	Total flavonoid content (TFC)*	Control Total flavonoid content (TFC)*
0.5	15.59[a]	11.13[a]
1.5	14.97[b]	11.01[a]
3	14.74[b]	11.25[a]
6	12.48[c]	11.18[a]
12	12.30[c]	11.07[a]
24	11.08[cd]	11.16[a]

Mean values in the same column followed by the same letter are not significantly different at $p < 0.05$

*Equivalent to quercetin per g dry weight (mg g^{-1})

4. CONCLUSION

The results of the study revealed that copper sulfate induction had a significant impact on *P. tenellus*Roxb.'s TPC and TFC levels. The treated plants exhibited a notable increase in both TPC and TFC corresponding with harvesting time after copper sulfate sprayed with 0.5 hours was the optimum harvesting time for both TPC and TFC. Studies also suggested that copper sulfate induced a higher production of these valuable compounds. These findings hold promise for potential applications in the pharmaceutical and nutraceutical industries, as *P. tenellus*Roxb. could be a valuable source of phenolic and flavonoid compounds with enhanced medicinal and antioxidant properties.

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