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Copper- or Zinc-Fortified Nutrient Solution in Vertical Farming System Enriches Copper or Zinc and Elevates Phenolic Acid and Flavonoid Contents in *Artemisia annua* L.

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Abstract: *Artemisia annua* L. is a well-known therapeutic herb that is widely used in folk medicine in Asian and African countries. *A. annua* can alleviate fever, wounds, and inflammation and is also popular as an anti-malarial agent. Cu and Zn are essential nutrients for human wellness and are vital to plants; they sometimes act as elicitors and induce stress mechanisms in plants to stimulate the production of secondary metabolites, which have bioactivities. Therefore, we added Cu or Zn to a nutrient solution and cultivated *A. annua* to enhance the Cu or Zn content. The Cu or Zn treatment during *A. annua* cultivation elevated their accumulation, and Zn showed a dramatic accumulation level in harvests. The aerial part of Zn16X contained 35 times higher Zn content than that of the control. Although the Cu or Zn contents were elevated, the plant height and yield were not affected, indicating the absence of toxic effects. The Cu or Zn treatment decreased the artemisinin content; however, these treatments increased the amounts of phenolic acids and flavonoids in *A. annua*. In particular, Zn4X showed a notable increase in the phenolic acids and flavonoids amounts. Moreover, the contents of certain types of caffeoylquinic acids were also highly elevated in Zn4X. Overall, Cu or Zn treatment in *A. annua* increased Cu or Zn accumulation and stimulated phenolic acid and flavonoid synthesis, which may have enhanced the therapeutic efficacy of *A. annua*.

Keywords: *Artemisia annua* L.; zinc; copper; nutrient solution; phenolic acid; flavonoid; vertical farm

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1. Introduction

Artemisia annua L. is a well-known ethnopharmacological herb belonging to the Asteraceae family and has been traditionally used to treat fever, tuberculosis, wounds, hemostasis, and malaria in Asian and African countries [1,2]. Owing to the effectiveness of *A. annua*, it has been documented in the International Pharmacopoeia and has received attention from the World Health Organization [3]. *A. annua* contains a wide range of bioactive compounds, including phenolic compounds, flavonoids, coumarins, purines, steroids, and terpenoids [4]. Artemisinin has high potency for treating malaria; therefore, artemisinin and its derivatives have been developed as therapeutic drugs [5]. *A. annua* is also rich in phenolic compounds, and its secondary metabolites have versatile biological activities that are attributed to its strong anti-oxidative properties [6]. Moreover, owing to its unique aroma, *A. annua* is sometimes used as a fragrant herb and its essential oil has strong health effects with high amounts of monoterpenoids, monoterpene alcohols, and sesquiterpenoids [7]. The habitat of *A. annua* is widespread worldwide and is distributed from the subtropical to cold regions of North America, Europe, and Asia, and *A. annua* is a weed that is easily propagated in nature [8]; however, geological characteristics and climate conditions can

alter its chemical constituents and efficacy [9]. Therefore, artificial cultivation has been proposed to obtain more standardized harvests and various agricultural techniques have been examined [10].

Cu is an essential mineral for all types of organisms, including plants and humans, and is primarily used as a cofactor for certain enzymes associated with redox reactions [11]. Despite the adverse effects associated with excess consumption, inadequate Cu intake can cause metabolic disorders [12], neutropenia [13], and cardiovascular disease [14]. According to the National Health and Nutrition Examination Survey (NHANES) 2011–2012 data, 29% of the US population consumed less Cu than the recommended dietary intake (RDI), and 14% of the respondents did not fulfill the estimated average requirement (EAR) [12]. Zn is a trace essential mineral for humans that mediates the functions of several enzymes and proteins and affects cellular metabolism, growth, and differentiation [15]. Zn shortage in the body reduces physical growth and reproductive function, and Zn deficiency prevalence is of particular concern in low- and middle-income countries, where it is estimated that over 25% of the population does not consume the required level of Zn [16]. Recently, Zn has received attention because of its immune-regulatory and antiviral functions. In patients infected with coronavirus disease 2019 (COVID-19), Zn deficiency aggravated the symptoms and led to more complications [17]. Inadequate intake of Cu and Zn was more prevalent in the US population that did not consume dietary supplements, signifying that their mineral intake was not sufficient from food sources alone [18,19]. Therefore, fortification with Cu and Zn has been attempted in various food sources, including agronomic sources such as Brassica species and soybean sprouts [20,21].

The adoption of Cu and Zn in cultivation systems has not only increased their contents in harvests but also enabled them to act as elicitors, causing alterations of miscellaneous attributes in plants. An elicitor is a molecule that generates phytoalexins in plants; however, its meaning has broadened to include molecules that stimulate any type of plant defense system [22]. Elicitors are found in both biotic and abiotic sources and a wide variety of chemicals induce stress and defense responses in plants [23]. Excessive stress impedes the growth and development of plants and deteriorates their quality and yield; however, the activation of defense signaling in plants concurrently accelerates the synthesis of secondary metabolites [24]. Secondary metabolites are not essential for plant development; however, they modulate responses to extrinsic stimulators and many secondary metabolites exhibit functional activities. Phenolic compounds, terpenes, and alkaloids are representative secondary plant metabolites and well-known bioactive compounds involved in human wellness [25]. The use of certain amounts of Cu or Zn in *Nostoc linckia* cultivation induced stress and elevated antioxidative activity and the contents of tannins and flavonoids [26], and Cu supplementation has also been tested in diverse plants, such as *Belamcanda chinensis* and *Withania somnifera*, which resulted in an increase in the amount of phenolic compound [27]. Studies on the effects of metal (Ag, Cu, Se, and Co) stress on *A. annua* cultivation have reinforced bioactive compound production [28–30]; however, studies on the effects of Cu and Zn on *A. annua* are lacking. Therefore, we aimed to verify whether Cu or Zn treatment could improve the secondary metabolite content in *A. annua*.

The purpose of this study was to fortify two trace minerals (Cu or Zn) in *A. annua* to increase its nutritional value. Moreover, we tested whether Cu or Zn, as elicitors, could elevate the secondary metabolites in *A. annua*. To elucidate the characteristics of *A. annua* after Cu or Zn treatment, the growth parameters, yields, Cu and Zn content, and secondary metabolite content were verified. Moreover, we investigated the yield and chemical constituents of *A. annua* roots, as well as the epigeal parts, to determine the availability of the roots. In this study, we aimed to develop a novel agricultural technique for *A. annua* cultivation and reveal its multiple implications by multifarious ways.

2. Materials and Methods

2.1. Plant Materials

This study was conducted using commercial *A. annua* seeds (Danong; Gyeonggi, Republic of Korea) and *A. annua* was cultivated in a Smart U-FARM at the Korea Institute of Science and Technology (Gangneung, Republic of Korea). *A. annua* seeds were soaked in a 5% sodium hypochlorite solution for 15 min, followed by inoculation on filter paper in a Petri dish with sufficient moisture. The Petri dishes were placed on cultivation shelves, 25 cm from fluorescent lamps (TL5 14W/865; Philips, Amsterdam, The Netherlands) under 150 ± 12 μmol/m^{2}s of light intensity on a 14:10 h light/dark cycle, at 18–26 °C and 50–80% relative humidity conditions under closed and controlled cultivation conditions.

At 21 days after sowing (DAS), *A. annua* had two true leaves. The plants were transplanted to moist Rockwool cubes (W × L × H, 25 × 25 × 40 mm; Grodan Co., Roermond, The Netherlands) located on the same cultivation shelves. Modified Hoagland solution with 0.8 dS/m of electrical conductivity (EC) was supplied as nutrient solution. The compositions of the nutrient solutions were 210, 31, 235, 160, 64, and 49 ppm of N, P, K, Ca, S, and Mg (macronutrients), respectively, and 3.00, 0.27, 0.11, 0.13, 0.03, and 0.05 ppm of Fe, B, Mn, Cu, Zn, and Mo (micronutrients), respectively.

At 49 DAS, *A. annua* had 7.5 true leaves. The plants were transplanted into a hydroponic system in a vertical farming room and supplied EC 1.5 dS/m of the nutrient solution. Temperature in the vertical farming room was set to 18 ± 1 °C (night) and 23 ± 1 °C (daytime), which was controlled by air conditioning (RNW0720T2S; LG electronics, Seoul, Republic of Korea) and circulation fans (UCR, Anyang, Republic of Korea). LED lamps (H22P; APACK Inc., Daejeon, Republic of Korea) were used with a light intensity of 284 ± 7.3 μmol/m^{2}s at plant canopy. The relative humidity was 60 ± 10% during plant growth, and carbon dioxide (CO_2) was supplied at 800 ppm during the day.

At 63 DAS, the *A. annua* plants were randomly divided into nine groups (each group consisted of three replicates with 12 plants arranged in a completely randomized design). The nutrient solutions for each of the nine groups were replaced with the modified Hoagland solution (CON) or modified Hoagland solutions with increased concentrations for 2, 4, 8, and 16 times of Cu and Zn, respectively. Samples were designated Cu##X or Zn##X, where each mineral was provided ## times higher comparing to the control nutrient solution (modified Hoagland solution containing 0.13 and 0.03 ppm of Cu and Zn).

Three random *A. annua* L. plants from each replicate were harvested at 63 DAS (week 9; before replacing the nutrient solution), 70 DAS (week 10), and 77 DAS (week 11) were used for analysis.

2.2. Growth Parameters and Yield

The plant growth rate was measured to compare the growth of *A. annua* subjected to different Cu and Zn concentration treatments. Plant height was measured using a ruler. The fresh and dry weights of the aerial and underground parts were measured using a digital balance (W-200; CAS Crop., Yangju, Republic of Korea). Dry weight was measured after freeze-drying.

2.3. Preparation of Dried Samples and Their Extracts

The harvested *A. annua* samples were segregated into two parts, the epigeal and root, and lyophilized (PVTFD 300R; Ilshin BioBase, Dongducheon, Republic of Korea). The dried samples were pulverized using a mixer (HR-2172; Philips, Amsterdam, The Netherlands) and mixed with an 80% ethanol solution (1:10, w/v). The extraction was conducted for 1 h at 40 °C in a sonicator (JAC-5020; Kodo Technical Research Co., Whaseong, Republic of Korea) and centrifuged at 3000× g (Combi-514R; Hanil Science Industrial, Daejeon, Republic of Korea). The supernatant was filtered through Whatman No.1 filter paper (Whatman, Buckinghamshire, UK). The remaining powder was extracted twice and the collected supernatants were evaporated using a rotary evaporator (RE111; Büchi, Flawil, Switzerland).

2.4. Chlorophyll Contents of *A. annua*

Lyophilized *A. annua* powder (1 g) and 5 mL 80% acetone solution were mixed for 1 h at 40 °C in a shaker. The supernatant was transferred to a cuvette and its absorbance was determined at 645 and 663 nm (Optizen 2120 UV; Mecasys, Daejeon, Republic of Korea). The chlorophyll a and b contents were calculated using the equation provided by Wellburn [31].

2.5. Mineral Analysis

The Cu and Zn contents of *A. annua* samples were analyzed following the method described by Son et al. [32] with slight modifications. Lyophilized *A. annua* powder (200 mg) was placed in a beaker and 40 mL of 60% HNO_3 solution was added. The solution was boiled for more than 4 h at 150 °C until it became transparent. The solution was then filtered through Whatman No.42 filter paper (Whatman, Buckinghamshire, UK), and its volume was adjusted to 50 mL by adding distilled water. The prepared sample solution was filtered again using a 0.20 µm syringe filter and analyzed using an inductively coupled plasma-optical emission spectrometry (ICP-OES) (OPTIMA 5300 DV; PerkinElmer, Waltham, MA, USA). Standard solutions of Cu and Zn were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Determination of Artemisinin Content

Quantification of artemisinin in *A. annua* extract was conducted using reversed-phase high-performance liquid chromatography (HPLC). The C18 ODS-AQ column (4.6 × 150 mm, 5 µm; YMC, Meridian, ID, USA) was equipped to Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) and 20 µL of *A. annua* 80% ethanol extract was injected into column. Water (A) and acetonitrile (B) were used as the mobile phases. The gradient condition of solvent was 90% of A, 0–3 min; 87% of A, 20 min; 78% of A, 35 min; 50% of A, 40 min; 30% of A, 50 min; 10% of A, 55 min; 90% of A, 60 min. The flow rate of mobile phase was 1 mL min^{-1} and the absorbance was detected at 210 nm. The column temperature was maintained at 35 °C.

2.7. DPPH Radical Scavenging Activity, Total Phenolic Contents, and Total Flavonoid Contents

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined as previously described with some modifications [33]. Briefly, 200 µM DPPH solution in 80% methanol was prepared and 160 µL of DPPH solution was mixed with 40 µL of each sample extract in a microplate. The plate was kept for 30 min at 37 °C and the absorbance was measured at 517 nm (Synergy HT; BioTek Instruments, Winooski, VT, USA). The same procedure was used for Trolox, and the DPPH radical scavenging activity of *A. annua* samples was presented as the Trolox equivalent antioxidant capacity (TEAC).

Total phenolic content (TPC) of *A. annua* was examined using a modified version of the method described by Singleton and Rossi [34]. The *A. annua* extract (300 µL) was mixed with Folin–Ciocalteu phenol reagent (250 µL) and 750 µL of saturated Na_2CO_3 solution. After addition of 200 µL of distilled water, the samples were kept for 2 h in a dark room. After centrifugation, the supernatant was transferred to a cuvette and the absorbance was measured at 765 nm (Optizen 2120 UV; Mecasys, Republic of Korea). Gallic acid was used as the standard compound, and the TPC of *A. annua* was expressed as gallic acid equivalent (GAE).

The analysis of total flavonoid contents (TFC) was conducted by referring to the method of Meda et al. [35] with slight modifications. Each *A. annua* extract sample (150 µL) was mixed with 400 µL of distilled water, and 45 µL of $NaNO_2$. After 5 min, 45 µL of 10% $AlCl_3$ solution was added and kept aside for 6 min. Then, 300 µL of 1 M NaOH solution and 360 µL of distilled water were added. The absorbance of the samples was analyzed at 510 nm, and the TFC was calculated as the catechin equivalent (CE) using catechin as a standard.

2.8. Determination of Caffeoylquinic Acid Family Compounds

Four types of caffeoylquinic acids (CQAs), neochlorogenic acid, chlorogenic acid, 3,5-dicaffeoylquinic acid (3,5-DCQA), and 4,5-DCQA, were analyzed using a reversed-phase HPLC system (Agilent 1260; Agilent Technologies, Santa Clara, CA, USA). For this, 80% ethanol extract of *A. annua* (20 µL) was injected and separated in a ODS-AQ column (4.6 × 150 mm, 5 µm; YMC, USA) at 35 °C. Water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) were used as mobile phase. The gradient conditions were the same as those used for the artemisinin analysis. The flow rate was 1 mL min^{-1} and the absorbance of the CQA compounds was detected at 280 nm.

2.9. Statistical Analysis

The means and standard deviations (SDs) of the results were calculated using the SPSS Ver. 26 (IBM Corp; Armonk, NY, USA). Statistical differences among the sample groups were determined using one-way analysis of variance (ANOVA) and Duncan's multiple range test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of EC Condition on Growth Parameters and A. annua Yield

The shoot height and fresh and dry weights of the *A. annua* at 9–11 weeks are presented in Figure 1. The Cu- and Zn-supplemented groups did not show significant differences in the shoot height compared to that in CON at 11 weeks, signifying that the growth of *A. annua* was not hindered or expedited by the Cu and Zn treatments. However, in the Zn-supplemented *A. annua* groups, Zn2X showed the highest shoot height and yield in the aerial parts, which were significantly different from those of Zn16X ($p < 0.05$). Although a certain concentration of Zn (Zn2X) elevated the growth of the aerial parts in the *A. annua*, Cu treatment did not affect the growth of the aerial parts. In comparison with the aerial part, the highest level of Cu supplementation (Cu16X) resulted in the highest yield for the root part, although Cu16X did not show significant differences with the other groups. Therefore, these two minerals are anticipated to regulate different local sites in *A. annua*.

Chlorophyll a, b, and their sum in *A. annua* are shown in Figure 2. At 10 w, Zn16X showed a significantly lower chlorophyll a content than the CON group ($p < 0.05$). The Zn16X group also showed the lowest chlorophyll b and sum of a and b contents at 10 weeks; however, no significant differences in the chlorophyll content between the CON and Zn16X groups were found at 11 weeks ($p > 0.05$). However, the Zn4X sample showed a significant difference from CON in the chlorophyll content ($p < 0.05$), but the other sample groups did not show significant differences in chlorophyll a, b, and their sum amounts compared to those of CON.

3.2. High Concentration Treatments of Cu or Zn to A. annua Immensely Elevated the Zn Content in Aerial Parts

The Cu and Zn contents of *A. annua* were analyzed in the aerial and underground parts, respectively (Figure 3). The Cu content of the aerial parts increased numerically when the Cu concentration in the nutrient solution was elevated, but the increase was not statistically significant at week 11 ($p > 0.05$). However, interestingly, the Zn16X group showed a significant increase in the Cu content in the aerial part of *A. annua* at 11 weeks compared to that in the CON group ($p < 0.05$). The effect of Cu addition was clearly observed in the roots of *A. annua*, and all the Cu-treated groups showed a significant increase in the Cu content at week 11 ($p < 0.05$). The root of Cu16X contained the highest amount of Cu, with 164.23 ± 19.08 µg/g dry basis (DW) and it was three times higher than that in CON. In the roots, Zn treatment did not show any change in the Cu content at week 11. Compared to Cu, the Zn content of *A. annua* changed dramatically in the treatment groups. The Zn treatment significantly increased the Zn content in the aerial parts in a dose-dependent manner, and the high-level Cu treatment also elevated the amount of Zn in the aerial parts of *A. annua*. Cu8X, Cu16X, and Zn16X showed high Zn contents in the aerial parts

of *A. annua*. In the *A. annua* roots, the Zn treatment at a high concentration significantly increased the Zn content, and Zn16X exhibited a Zn content of 310.21 ± 12.71 μg/g DW, which was approximately six times higher than that of CON. The Cu treatment slightly increased the Zn content in the *A. annua* roots, and its effect was marginally dissimilar to that of the aerial parts.

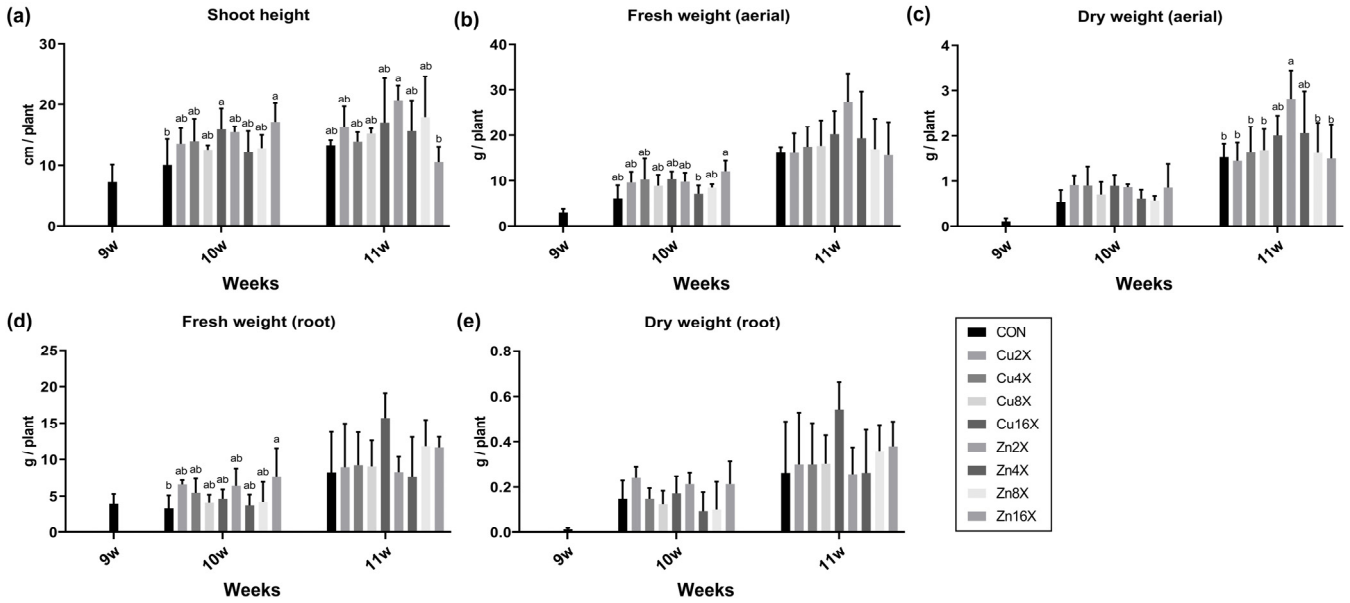

Figure 1. Changes in growth and yield parameters of *A. annua* after Cu or Zn treatment. Shoot height (**a**), fresh and dry weight of aerial parts (**b**,**c**) and roots (**d**,**e**) were determined. Results are represented as mean ± SD. Different letters indicate statistical differences among *A. annua* samples collected during the same week. CON received modified Hoagland solution with 0.13 and 0.03 ppm of Cu and Zn. ##X signifies that Cu or Zn was provided ## times higher compared to the control nutrient solution.

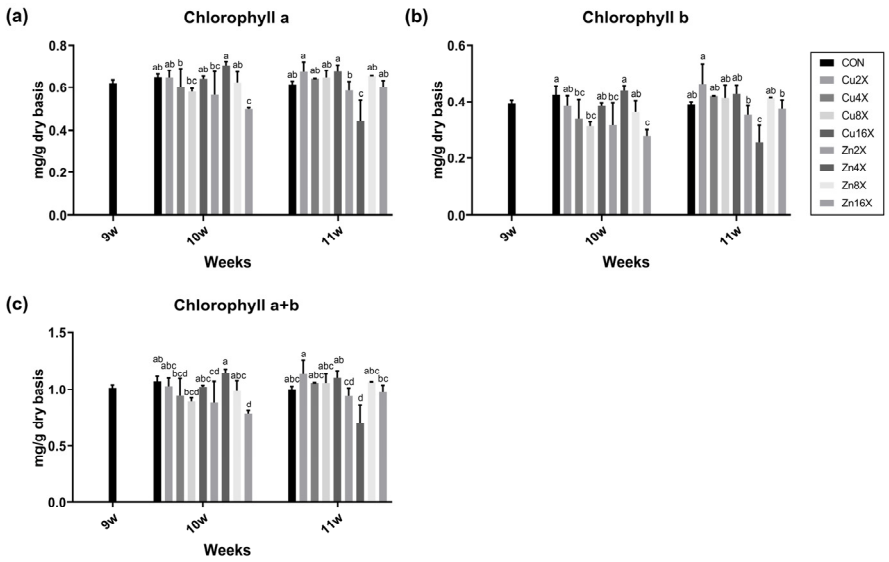

Figure 2. Chlorophyll contents of *A. annua* after Cu or Zn treatment. The contents of chlorophyll a (**a**) and chlorophyll b (**b**) were analyzed, and their sum amounts were calculated (**c**). Results are represented as mean ± SD. Different letters indicate statistical differences among *A. annua* samples collected during the same week. CON received modified Hoagland solution with 0.13 and 0.03 ppm of Cu and Zn. ##X signifies that Cu or Zn was provided ## times higher compared to the control nutrient solution.

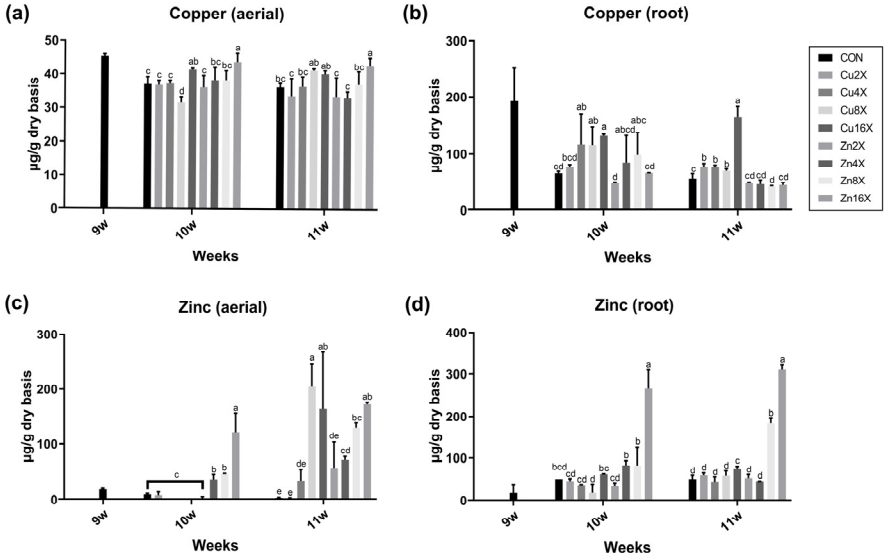

Figure 3. Cu or Zn treatment fortified their amount in *A. annua* plants. Cu amounts in aerial parts (**a**) and roots (**b**) of *A. annua* were verified using ICP analysis. Zn content in aerial parts (**c**) and roots (**d**) of *A. annua* was also examined. Results are represented as mean ± SD. Different letters indicate statistical differences among *A. annua* samples collected during the same week. CON received modified Hoagland solution with 0.13 and 0.03 ppm of Cu and Zn. ##X signifies that Cu or Zn was provided ## times higher compared to the control nutrient solution.

3.3. Cu or Zn Treatment Decreased the Artemisinin Amount in A. annua

Figure 4 represents the artemisinin content in the aerial parts and roots of the *A. annua* after the Cu or Zn treatment. We found that the aerial parts and roots of the *A. annua* contained similar amounts of artemisinin, which were maintained for 9–11 weeks. The effect of the Cu or Zn treatment on artemisinin content was negative at all treatment levels at week 11 ($p < 0.05$). In the aerial parts, the treatment groups presented approximately half the amount of artemisinin compared to that in the CON sample. Cu or Zn treatment significantly decreased the artemisinin content in the *A. annua* roots at week 11. The decrease in the artemisinin content because of treatment was not dose-dependent and the lowest level of treatment also significantly decreased the artemisinin content in the aerial parts and roots (Figure 4). However, at 10 weeks, treatment with Cu or Zn for 1 week did not result in a severe decrease in the artemisinin levels in *A. annua*. Moreover, the roots did not show any changes in the artemisinin content after one week of treatment.

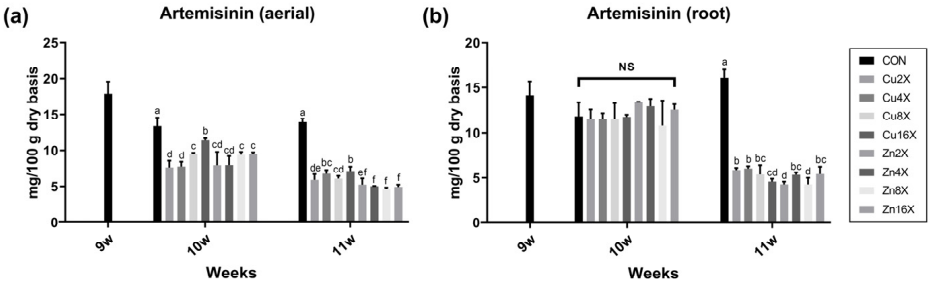

Figure 4. Artemisinin content in Cu- or Zn-treated *A. annua*. *A. annua* was separated into aerial parts (**a**) and roots (**b**) and their artemisinin contents were examined using HPLC. Results are represented as mean ± SD. Different letters indicate statistical differences among *A. annua* samples collected during the same week. CON received modified Hoagland solution with 0.13 and 0.03 ppm of Cu and Zn. ##X signifies that Cu or Zn was provided ## times higher compared to the control nutrient solution.

3.4. Cu or Zn Treatments Elevated the Contents of Phenolic Acids and Flavonoids in A. annua

The DPPH radical scavenging assay was used to verify the changes in the antioxidative activity of *A. annua* following Cu or Zn treatment (Figure 5a,b). For the aerial parts of *A. annua*, at 11 weeks, the Zn4X group showed the highest radical scavenging activity (11.96 ± 0.43 mg TEAC/g DW) and Zn4X only showed significant differences with CON ($p < 0.05$). The DPPH radical scavenging activity was relatively low in Zn8X and Zn16X compared to that in Zn4X at week 11. Therefore, we concluded that Zn4X may be the optimal concentration to increase the antioxidative activity of the epigeal parts of *A. annua*. Meanwhile, both the Cu and Zn treatment reduced the DPPH radical scavenging activity of the *A. annua* roots. Analogous with the result of DPPH radical scavenging activity, the aerial part of Zn4X possessed the highest TPC and TFC at week 11 (11.17 ± 2.28 mg GAE/g DW and 19.51 ± 3.95 mg CE/g DW) (Figure 5c–f). Although a higher concentration of Zn treatment than that for Zn4X decreased the TPC and TFC, Cu16X showed the highest TPC and TFC among the Cu treatment groups, and these values were significantly higher than those of the CON group ($p < 0.05$).

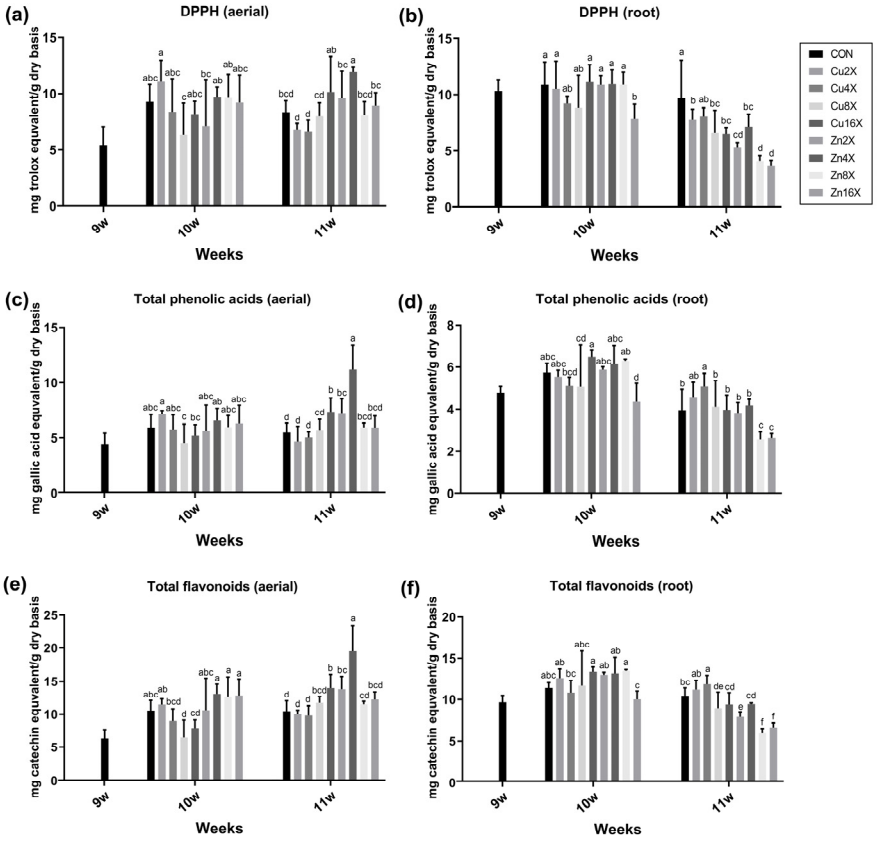

Figure 5. Changes in anti-oxidative activity and phenolic compounds contents in *A. annua* after Cu or Zn treatment. DPPH radical scavenging activity (**a**,**b**), total phenolic acid contents (**c**,**d**), and total flavonoid contents (**e**,**f**) were analyzed for the aerial parts and roots of *A. annua*. Results are represented as mean ± SD. Different letters indicate statistical differences among *A. annua* samples collected during the same week. CON received modified Hoagland solution with 0.13 and 0.03 ppm of Cu and Zn. ##X signifies that Cu or Zn was provided ## times higher compared to the control nutrient solution.

3.5. Contents of Each CQA Compound in A. annua Were Changed by Treatment with Cu or Zn

Four types of CQA compounds (neochlorogenic acid, chlorogenic acid, 3,5-DCQA, and 4,5-DCQA) were quantified using HPLC, and the results are presented in Figure 6. Among the aerial samples of *A. annua* collected at 11 weeks, Zn4X contained the highest amount of all types of CQAs analyzed in the present study. This result corresponded with

the TPC and TFC results, which may have affected the increased DPPH radical-scavenging activity of Zn4X. The effect of the Cu treatment on the CQA content in the epigeal part of *A. annua* was lower than that of the Zn treatment, and the amounts of neochlorogenic and chlorogenic acids did not change in the Cu-treated groups. However, the Cu treatment elevated the levels of 3,5-DCQA and 4,5-DCQA in the epigeal part of *A. annua* in a dose-dependent manner, and Cu16X showed significantly higher levels than CON at week 11 ($p < 0.05$). Meanwhile, Cu16X significantly increased the contents of neochlorogenic acid and chlorogenic acid in *A. annua* roots at week 11 ($p < 0.05$), but did not increase the contents of 3,5-DCQA and 4,5-DCQA in *A. annua* roots. Similarly, Zn treatment of *A. annua* did not increase the content of CQAs in the root but decreased the amounts of 3, 5-DCQA and 4,5-DCQA in *A. annua* roots.

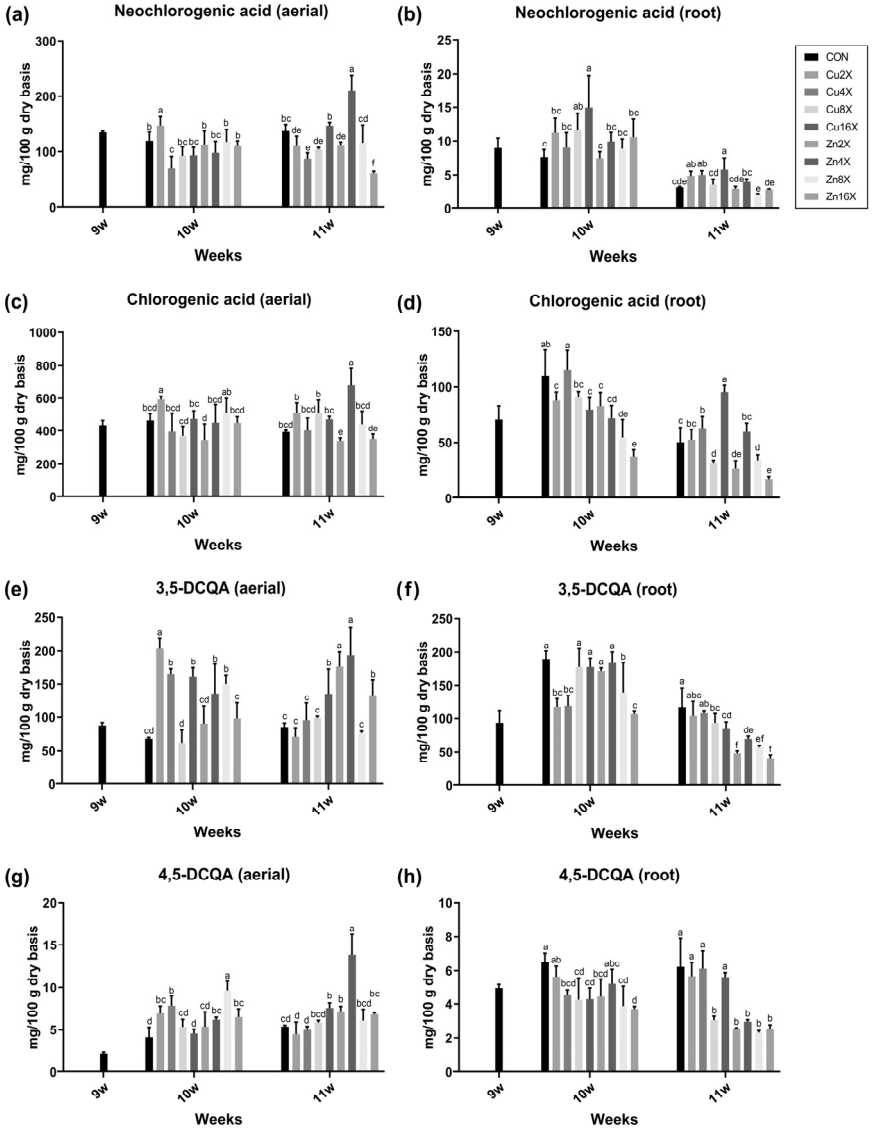

Figure 6. Contents of four kinds of caffeoylquinic acids (CQAs) in *A. annua* after Cu or Zn treatment. A total of four kinds of CQAs were analyzed with an HPLC analysis. Neochlorogenic acid (**a**,**b**), chlorogenic acid (**c**,**d**), 3,5-dicaffeoylquinic acid (DCQA) (**e**,**f**), and 4,5-DCQA (**g**,**h**) contents were determined for the aerial parts and roots of *A. annua*. Results are represented as mean ± SD. Different letters indicate statistical differences among *A. annua* samples collected during the same week. CON received modified Hoagland solution with 0.13 and 0.03 ppm of Cu and Zn. ##X signifies that Cu or Zn was provided ## times higher compared to the control nutrient solution.

4. Discussion

Although the required intake level is low, Cu and Zn are essential minor nutrients for humans and their inadequate consumption poses several health risks. It was estimated that approximately 30% of the US population struggled with insufficient intake, which was lower than the RDI, in 2011–2012, and 10% could have been risking insufficient Zn intake [12,36]. Cu or Zn deficiency is more prevalent in low-income countries; therefore, the use of nutraceuticals and biofortification as countermeasures has been proposed [37]. In this regard, an attempt to fortify the Cu and Zn content in *A. annua* was accomplished in the present study, and Zn, in particular, showed dramatic effects. The concentration of Zn in ordinary *A. annua* was about 5 and 50 μg/g DW for the aerial and root parts, respectively, but the Zn treatment elevated them up to about 175 and 310 μg/g DW. The favorable accumulation of Zn in *A. annua* has already been demonstrated in a previous study [38], and the absorbed Zn was mostly stored in the leaves, which is consistent with our results. The RDI of Zn for adults was established to be 11 mg and 8 mg for males and females older than 13 years, respectively [39], and the intake of 35 g or 26 g of Zn-fortified *A. annua* root satisfied the criteria. Recently, an association between Zn supplementation and COVID-19 pathogenesis has been reported, and ample intake of Zn hinders viral replication and the pathogenic progress of COVID-19 [40]. The efficacy of Zn is based on its immune-regulating functions as a signaling molecule, and the Zn supplementation inhibited nuclear factor kappa B (NF-κB) expression and upregulated antioxidative-related genes [41]. Moreover, *A. annua* is an herb that biosynthesizes artemisinin, a chemical compound with antimalarial activity [1], and Zn fortification in *A. annua* could enforce therapeutic activities against malaria. Related studies have disclosed the alleviation of morbidity and mortality due to malaria after micronutrient supplementation, and the combined consumption of vitamin A and Zn or Zn and Fe led to a reduction in malaria morbidity [42,43]. Therefore, the notable biofortification of Zn in *A. annua* may result in its elevated potency against immune diseases and malaria.

Both Cu and Zn are essential for human health, and they are also essential micronutrients for plants. Cu has a role in photosynthesis, respiration, and cell wall metabolism and modulates the activities of metalloenzymes, such as Cu/Zn–super oxide dismutase (SOD) [44]. Zn is a component of many plant enzymes and is involved in carbon and nucleic acid metabolism; therefore, it is required for the production of optimal fruits with proper carbohydrate content [45]. Despite the importance of Cu and Zn in plant physiology, their excessive accumulation can cause toxicity. For example, excessive Zn uptake can reduce the concentration of crucial macroelements, including K, and disturb mineral homeostasis in plants [46]. Therefore, in the present study, we investigated changes in the growth parameters and yields of *A. annua* after Cu or Zn treatments. Cu and Zn treatments did not have toxic effects on the growth and yield of *A. annua*, but they did not enhance the growth of *A. annua*. Only the Zn2X condition showed a significantly elevated dry weight for the aerial parts, but the other treatment groups did not result in an alteration of yields. Alternately, the addition of Cu or Zn to the nutrient solution of *A. annua* produced mineral-enriched harvests, but with consistent yields.

In a study by Luis Abreu [47], the author suggested that the abundant phenolic compounds and flavonoids in *A. annua* could be used as Zn ionophores and may result in the high synergistic potency of the Zn and *A. annua* mixture in humans. The relationship between Zn and certain phytochemicals in human health has been elucidated in several studies, and it was noted that the intake of these chemicals enhances the absorption, regulation, and bioaccumulation of Zn in the body [48,49]. Epigallocatechin-gallate (EGCG) is a representative phytochemical known for its biological action owing to its ionophore activity in liposomes [50]. Along with EGCG, quercetin also shows high potency, such as increased Zn metabolism and accumulation of intracellular labile Zn by regulating Zn transporters [51]. The presence of Zn cations also regulates the bioactivity and stability of EGCG. When Zn^{2+} was added to the EGCG solution, chelates were formed, which increased the stability of EGCG [52]. EGCG also formed a chelate complex with Cu ions,

and EGCG-Cu^{2+} showed increased anti-proliferative activity and efficacy in androgen-sensitive human prostate adenocarcinoma (LNCaP) cells [53]. Overall, the attempt to increase the Zn or Cu content in *A. annua* may improve the availability of Zn, Cu, and the intrinsic phytochemicals of *A. annua*.

A. annua contains numerous bioactive compounds of various chemical classes, including monoterpenes, sesquiterpenes, phenolic acids, flavonoids, tannins, coumarins, saponins, and phytosterols [3]. Among the diverse secondary metabolites of *A. annua*, studies on cultivation methods have primarily focused on improving artemisinin production, owing to its unique therapeutic use. Cell suspension culture and hairy root culture conditions have been introduced, and abiotic or biotic elicitors, such as fungal and bacterial extracts, have also been used to enhance artemisinin content in *A. annua* [54–56]. Some studies have shown increased artemisinin biosynthesis; however, some attempts decreased the amount of artemisinin. In a study by Irfan Qureshi et al. [57], the treatment of lead acetate and sodium chloride in the cultivation of *A. annua* to induce salt-associated oxidative stress increased the antioxidative activity and artemisinin content in short-term periods; however, they were lower than those in the control group when the treatment period was prolonged. Similarly, treatment with Cu or Zn for one week slightly decreased the artemisinin content, but longer treatment (2 weeks) considerably decreased the artemisinin levels in our study. Even though the artemisinin content was diminished, certain levels of Cu or Zn treatment intensified the antioxidative activity and contents of other secondary metabolites (TPC and TFC) in the aerial part of *A. annua* in the present study.

Phenols and flavonoids are abundant in *A. annua* and their strong antioxidative properties and biological activities could have synergistic effects with the diverse potencies of *A. annua* [58]. Among these elicitors, mild salt stress has been a popular approach to promote the synthesis of secondary metabolites in plants and is also applicable in *A. annua* cultivation. A certain degree of NaCl salinity enhances the TPC, TFC, and antioxidative properties of *A. annua* and modulates the terpenoid pathway to stimulate artemisinin synthesis [59]. Heavy metals are stress inducers that can hinder the growth and development of plants; however, they are possible elicitors that modulate the signaling pathways of secondary metabolites [60]. In particular, Artemisia species are highly tolerant of heavy metals; therefore, the toxic effects of heavy metals on growth are minimized, but they stimulate defense-related metabolic pathways [61]. Although we could not find the former application of Zn in *A. annua* cultivation, the proper amount of Cu treatment augmented the content of antioxidants in *A. annua* similar to our result [62]; and therefore, we concluded that treatment with Cu and Zn can increase the production of phenolic acids and flavonoids in *A. annua*. Furthermore, the Zn4X group showed a significantly increased content of CQAs, a family of quinic acid derivatives that are major constituents of *A. annua* [63,64], which could strengthen the anti-inflammatory and antiviral effects of *A. annua* [65].

5. Conclusions

Despite the importance of Cu and Zn in human nutrition, insufficient consumption is prevalent and threatens the health of the population, particularly in low-income countries. Moreover, interest in the immune-regulatory effect of Zn is increasing owing to the COVID-19 pandemic, and the adequate intake of micronutrients also assists the anti-malaria efficacy of *A. annua*. Therefore, in this study, the biofortification of Cu and Zn was tested in *A. annua*, a popular therapeutic herb with varied potencies, including antioxidative, antimalarial, and anti-inflammatory activities. Cu or Zn addition to the nutrient solution resulted in increased Cu or Zn content in *A. annua* with consistent growth rates and plant yields. The accumulation of Cu was more prominent in the root of *A. annua* but the accumulation of Zn appeared in both the aerial and root parts. In particular, the accumulation ratio of Zn was considerable (up to 35 times higher than non-treated *A. annua*), and it was anticipated that the enriched Zn would have a synergistic effect with bioactive compounds of *A. annua*. Although the artemisinin content decreased, Cu or Zn treatment upregulated

the production of phenolic acids and flavonoids, resulting in elevated antioxidative activity. Among the treatment groups, Zn4X showed the highest phenolic acid and flavonoid content, as well as high amounts of CQAs. Thus, Cu or Zn treatment of *A. annua* could elevate the accumulation of Cu or Zn in the plant body and lead to an increase in TPC, TFC, and antioxidative properties. The increased contents of polyphenols and flavonoids may have synergistic effects with other bioactive compounds in *A. annua*; therefore, the treatment of Cu and Zn could help the production of *A. annua* with higher potency.

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