Copper sulfate nanoparticles enhance growth parameters, flavonoid content and antimicrobial activity of *Ocimum basilicum* Linnaeus

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Abstract: Nanotechnology has provided new insights in Phytomedicine. We hypothesized that copper sulfate nanoparticles (CuSO4-NPs) enhance growth parameters, flavonoid content and antimicrobial activity of sweet basil (*Ocimum basilicum* L.). In order to test our hypothesis, the impact of copper sulphate versus CuSO4-NPs on sweet basil was tested. The treatment with 5, 10 and 15 µM/LCuSO4-NPsenhanced the growth parameters (fresh weight, root and shoot length) where 5µM was found to be optimal. Flavonoids were present in all tested samples. The group treated with CuSO4-NPs showed the highest levels of flavonoid content calculated as quercetin equivalent as compared to other control groups. TheCuSO4-NPs also exhibited higher antimicrobial activity against the 13 tested microorganisms included in this study. The *Klebsiella pneumonia*, was most sensitive to the CuSO4-NPstreatment. The optimum CuSO4-NPs concentration for the flavonoid content and antimicrobial activity was10µM. To the best of knowledge, this is the first *in vitro* CuSO4-NPs application of *Ocimum basilicum L.* to enhance its pharmaceutical performance characteristics. In conclusion, CuSO4-NPs can improve growth parameters, flavonoid content and antimicrobial activity in *Ocimum basilicum L.*

[Ezzat A. Genady, Shreen S. Ahmed, Ashraf. H. Fahmy and Rehab M. Ashour. **Copper sulphate nanoparticles enhance growth parameters, flavonoid content and antimicrobial activity of *Ocimumbasilicum* Linnaeus.** *J Am Sci* 2017;13(4):108-114]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). http://www.jofamericanscience.org. 14. doi:10.7537/marsjas130417.14.

Key words: Copper Sulfate Nanoparticles (CuSO4-NPs), *In vitro*, *Ocimum basilicum*, Growth, Antimicrobial Activity, Flavonoid Content.

1. Introduction

Nanotechnology holds a great promise in the development and design of many kinds of novel products used in medicine. The development of nano-devices and nanomaterials play a vital role in the expansion of novel applications in plant biotechnology and agricultural industries[1]. Applications of nanomaterials can facilitate faster plant germination, production of improved plant to biotic and abiotic stress, efficient nutrient utilization and plant growth enhancements, with a reduced environmental impact compared to traditional approaches[2]. The discovery of novel nanotechnology applications is required to target specific delivery of chemicals for crop genetic transformation[3]. Also, nanotechnology has large potential to provide an opportunity for plant science and other field researchers. The development of new tools for the incorporation ofnanoparticles into plant is of great importance as it could add new functions or improve existing ones[4]. *Ocimum basilicum*L. (sweet basil) belongs to family Lamiaceae. It is native to Africa, India and Asia[5]. It is known as king of herbs, great basil and the royal herb. The name "basil" comes from Greek basilikón which means royal plant[6]. It shows high variation in morphologic traits among the ecotypes[7]. Several studies have reported the phytochemical constituents of sweet basil, it is rich in essential oil, flavonoid, phenolic acids, steroids, anthocyanins and vitamins [8-10]. Traditionally, the whole plant has been used against various human conditions worldwide. The essential oil is used in aromatherapy and as additive in cosmetics and pharmaceutical preparations[5]. It is also used as a medicinal plant in treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney dysfunction [11]. Methanolic extract of the herb was found to possess antileishmanial and antimicrobial activities[12]. Other numerous biological activities were reported such as hepatoprotective, antidiabetic, antispasmodic, carminative, digestive, stomachic, antimicrobial, antioxidant, anticancer and antiviral[5, 13, 14]. The development of new antimicrobial drugs from phytochemicals present in plant extracts is of great importance to overcome the resistance of pathogenic microorganisms[15]. The aim of the study was to evaluate the *In vitro* effect of copper sulfate and copper sulfate nanoparticles on the growth parameters, flavonoid content and antimicrobial activity of *Ocimum basilicum* L.

2. Material and methods

Plant material

Mature fresh plant of *Ocimum basilicum* L. were collected from botanical garden at Al-Monofia governorate, Egypt. The botanical identification was confirmed by Mrs. Therese Labibconsultant of Egyptian flora, Orman Garden, Giza, Egypt. Ripe fruits containing seeds were collected at the end of April 2016 and storedat (25 - 27°C) in a cool dry place. Seeds were collected as needed. Voucher sample was deposited in herbarium of faculty of Pharmacy, Cairo University.

Preparation of Copper Sulfate Nanoparticles ($CuSO_4$-NPs) suspension

Copper sulfate nanoparticles were synthesized using a chemical reduction method involving the reduction of copper sulfate by sodium borohydride and the stabilizing agent being polyethylene glycol 6000. The four step preparation scheme for copper nanoparticles was started by dissolving 0.01 M CuSO4.5H2O, in distilled water to obtain a blue solution. Next, 0.02 M PEG 6000 was dissolved in water and added to the aqueous solution containing the copper salt while vigorously stirring. In this step, the solution changed from blue to white. In the third step, ascorbic acid (0.02 M) and NaOH (0.1 M) was dissolved in water and added to the synthesis solution. Color change occurred in the aqueous phase from white to yellow. Finally, a solution of NaBH4 (0.1 M) in distilled water was prepared and added to the solution under continuous rapid stirring. An instant color change occurred in the aqueous phase from yellow to blackish green which indicated the presence of copper nanoparticles[16]. $CuSO_4$-NPs (1g) were dissolved in 50 ml distilled water to prepare different concentrations of $CuSO_4$-NPs (5, 10 and 15 µM/L). Prior being added to culture media, nanoparticle suspensions were centrifuged and filtered to avoid particles aggregation.

Surface Sterilization

Basil seeds (*Ocimum basilicum* L.)were surface sterilizedunder aseptic condition in laminar flow hood (NUAIRE™, USA) with 0.1% fungicide for 10 min followed by soaking for 2 min in 70 % (v/v) ethanol, then washed once with sterile distilled deionized water (D.D. H_2O), then seeds were rinsed with 20% (v/v) commercial Clorox® (5.25% Sodium hypochlorite) supplemented with few drops of Tween 20for 20 min, followed by soaking for 2 min in sterile D.D. H_2O, then rinsed five times in sterile D.D. H_2O.

Culturing of explants

Seeds were cultured onto germination medium, which basically contains Murashige and Skoog salts[17] enriched with 30g sucrose, 8g agar as a solidifying agent. Two groups were included in the experiment with three levels compared to control. Group one was treated with 5 µM/L, 10 µM/L and 15 µM/L$CuSO_4$ while group two was treated with 5 µM/L, 10 µM/L and 15 µM/L$CuSO_4$-NPs. Cultured seeds were then incubated in dark for two days in controlled growth chamber (Shel-Lab, USA) at 25°C and then transferred to a 16 hours photoperiod. After 25 days the seedlings were collected and the rate of germination, fresh weight and length of shoot and root were measured.

Statistical Analysis

Data obtained were exposed to the proper statistical analysis of complete randomized design [18]. Experiments were carried out in three replicates. Means obtained were differentiated using Duncan's new multiple range test [19]

Plant extraction

20 grams of plant was placed in 100 ml of 70%methanol in a conical flask, plugged with cotton and then kept on a rotary shaker at 180-200 rpm for 24 h. After 24 h, it was filtered through filter paper and centrifuged at 5000 x g for 10 min. The supernatant was collected and theextraction step was repeated twice and both extracts were pooled and evaporated.

Flavonoid content determination

The total flavonoid content of the 70% methanolic extract of all treatments of *Ocimum basilicum* L were determined using colorimetric modified method [20]. 75 µl of 5% $NaNO_2$ solution was added to 1.5 ml of 70% methanolic extract. After 6 min, 150 µl of 10% $AlCl_3.6H_2O$ was added to the mixture and kept at room temperature for another 6 minutes, followed by the addition of 0.5 ml of 1M NaOH then deionized water was added to reach 2.5 ml total volume. The resulting solution was mixed and immediately, the absorbance was measured at 510 nm on a UV spectrophotometer. For blank preparation, the extracts were replaced with an equal volume of deionized water. The total flavonoid content was determined in triplicate. A calibration curve (R^2=0.9988) was prepared using quercetin standard (Sigma Co., St. Louis, MO, USA) at concentrations (0-40 mg/ml). Results were expressed as quercetin equivalents (mg QE/ g extract).

Antimicrobial activity determination

Antimicrobial susceptibility test was done at Regional Center for Mycology and Biotechnology (RCMB), Cairo, Egypt using the diffusion agar technique (6.0 mm diameter). The susceptibility tests were performed according to the recommendations of Clinical and Laboratory Standards Institute (CLSI). Screening tests regarding the inhibition zone were carried out by the disc diffusion method[21]. The inoculum suspension was prepared from tested colonies i.e. Gram negative bacteria: *Enterobacter cloaca* RCMB 001 (1) ATCC 23355, *Escherichia coli* (RCMB 010052) ATCC 25955, *Klebsiella pneumonia*

RCMB 003 (1) ATCC 13883, *Proteus vulgaris* RCMB 004 (1) ATCC 13315 and *Salmonella typhimurium* RCMB 006 (1) ATCC 14028; Gram positive bacteria: *Staphylococcus aureus* (RCMB010010), *Bacillus subtilis* RCMB 015 (1) NRRL B-543, *Streptococcus mutans* RCMB 017 (1) ATCC 25175, *Micrococcussp.* RCMB 028 (1), *Streptococcus epidermtitis* RCMB 009 (2) and Fungi: *Aspergillusniger* (RCMB 002005), *Candida albicans* RCMB 005003 (1) ATCC 10231, *Penicilliumaurantiogriseum* IMI 89372(Table 3) grown overnight on agar plates, and inoculated into Mueller-Hinton broth medium. A sterile swab was immersed in the colonies suspension to inoculate the Mueller-Hinton agar plates. Plant extract samples and control were dissolved in dimethyl sulfoxide (DMSO) at 5 mg/ml. The inhibition zone was measured (mm) around each extract and control well using DMSO after 24h at 37°C.

3. Results and Discussion:

Effect of $CuSO_4$ and CuSO4-NPs on growthof *Ocimum basilicum* L.

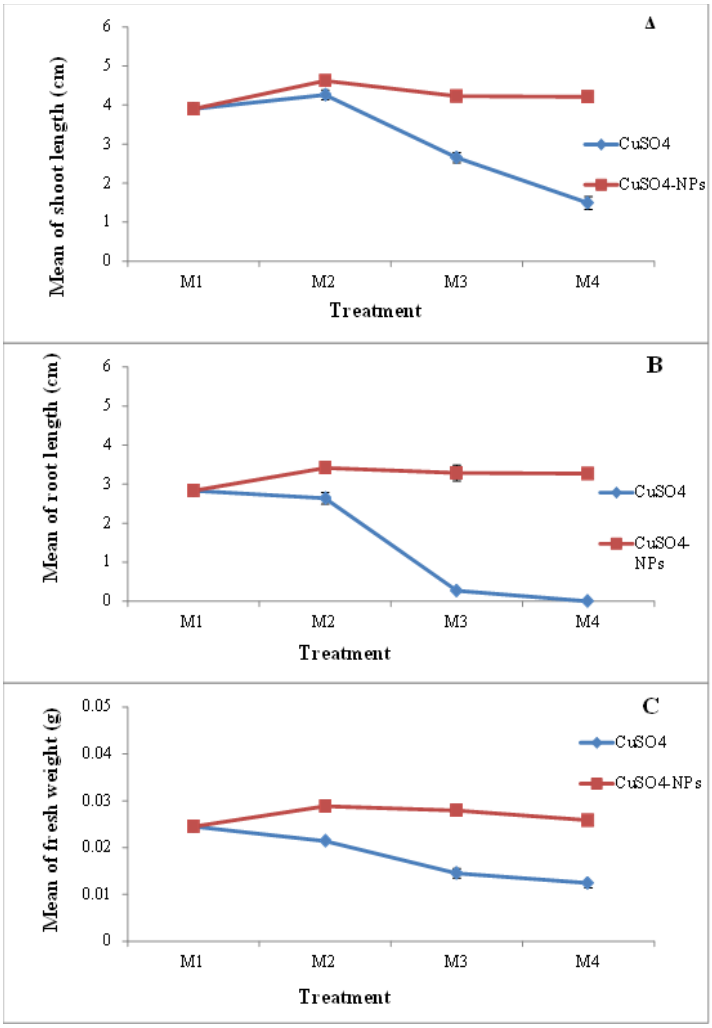

Figure (1): Effect of different concentrations of $CuSO_4$ and $CuSO_4$-NPs on growth parameters of *Ocimumbasilicum* L.

Copper is essential for plant growth and development, but it is extremely toxic at high concentrations. It results in growth inhibition and toxicity symptoms[22]. Copper sulfate increased seed germination by 14% and 16.6% at concentrations 5 µM/L and 10 µM/L, respectively more than control. Copper sulfate treatments had significant effect on tested sweet basil growth parameters (fresh weight, root and shoot length). Results were shown in table (1) & figures (1&2). Increment by 9% in shoot length was observed at 5 µM/L of $CuSO_4$ compared to control. It could be concluded that 10 µM/L and 15 µM/L copper sulfate concentrations adversely influenced shoot and root length. Copper sulfate concentration increments reduced seedlings fresh weight and scored 12% and 40% at 5 µM/L and 10 µM/L, respectively. The

highest level of copper sulfate tested (15 µM/L) was accompanied by maximum decrement in seed germination, seedling fresh weight, shoot and root length by (10%, 49 %, 38% and 100%, respectively) as compared to the control. This could be explained by the phytotoxicity caused by the presence of copper sulfate in the growth medium at high concentration. Copper sulfate affects directly the uptake of immobile nutrients such as Phosphorus needed for growth promotion. The shoot growth was directly influenced by the root growth reduction as the root plays a role in the transportation of nutrients and water to the aerial parts of the plant. In addition both shoot and root reduction affects fresh mass.

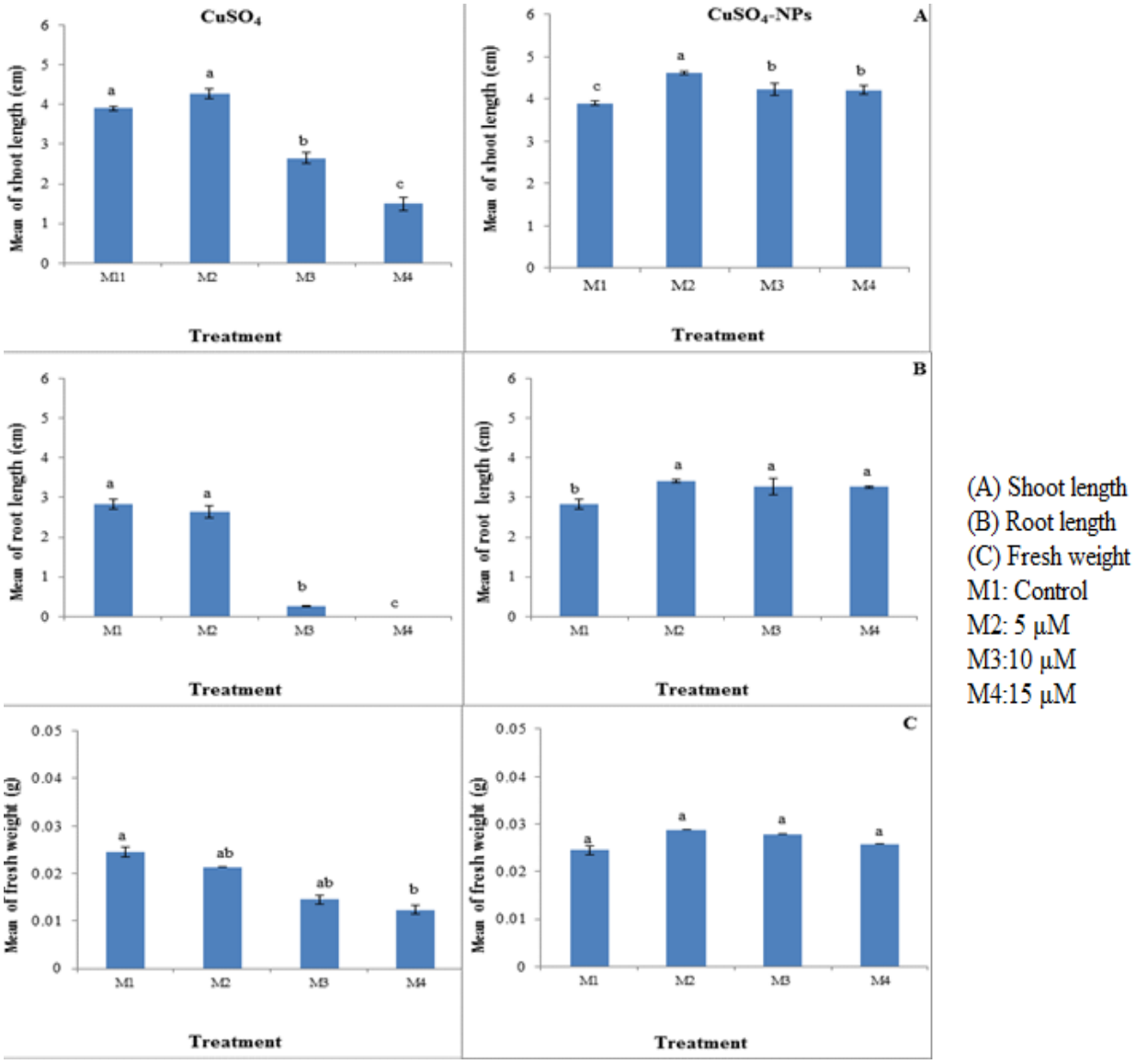

Figure (2): Effect of different concentrations of $CuSO_4$ and $CuSO_4$-NPs on growth parameters of *Ocimumbasilicum* L

Figure (3): Effect of different concentrations of $CuSO_4$ and $CuSO_4$-NPs on growth of *Ocimumbasilicum* L.

Our results were in accordance with previously reported data [23] about the significant influence of low concentration of copper sulfate on growth promotion and its retardation at high concentrations. On contrary the $CuSO_4$-NPs treatments (Table 2) had positive effect on germination at all tested concentrations. The highest value was recorded at 10 µM/L with increment reached to 27.6% surpassing control. It was observed that by increasing $CuSO_4$-NPs concentration, the fresh weight, root and shoot length, also increased. However, 5µM/L$CuSO_4$-NPs was the optimum concentration for the growth parameters. Fresh weight, root and shoot length increased by 17.5%, 20% and 18.45%, respectively as compared to the control. In conclusion, all growth parameters were significantly higher than those of control plant sat all levels of CuSO4-NPs.

Total flavonoid content

The total flavonoids content calculated as quercetin was presented in Table (2). All tested samples were rich in flavonoid and scored its highest levels at $CuSO_4$-NPs applications surpassing $CuSO_4$, where highest amount of flavonoid was observed at 10 µM/L $CuSO_4$-NPs.

The results obtained of antimicrobial activity against thirteen pathogenic microorganisms (Table 3) *via* disc diffusion method indicated that *Klebsiella pneumonia*was more sensitive to $CuSO_4$-NPs more than control (gentamycin). $CuSO_4$-NPstreatment of 10µM/L concentration displayed a broad antimicrobial spectrum compared to the other concentrations of both $CuSO_4$ and $CuSO_4$-NPs.

Table (1): Effect of $CuSO_4$ and $CuSO_4$-NPs on seed germination of *Ocimum basilicum*L.

Treatment	$CuSO_4$	$CuSO_4$-NPs
Control	70	70
5µM	80	74.67
10 µM	81.67	89.33
15µM	60	79

Table (2) Total flavonoid content in different treatments of *Ocimumbasilicum* L. extracts.

Treatment	**Total flavonoid (mg QE /g extract)**	
	$CuSO_4$	**$CuSO_4$-NPs**
Control	38.3	38.3
5 µM	51.1	64.3
10 µM	28	79
15 µM	8.6	67

Antimicrobial Activity

Table (3): Antimicrobial activity for different treatments of *Ocimumbasilicum* L.

Sample / **Tested microorganisms**	**Plant control**	*Ocimumbasilicum* treated with						**Control**
		$CuSO_4$			$CuSO_4$ NPs			
		5 µM	10 µM	15 µM	5 µM	10 µM	**15**µM	
Gram Negative Bacteria								**Gentamycin**
Enterobacter cloaca	NA	4	NA	NA	14	20	17	26
Escherichia coli	5	7	NA	NA	12	21	16	29
Klebsiella pneumonia	1	2	NA	NA	5	11	8	4
Proteus vulgaris	3	6	4	2	10	19	14	26
Salmonellatyphimurium	6	8	NA	NA	13	21	17	28
Gram Positive Bacteria								
Staphylococcus aureus	4	6	2	NA	9	15	12	25
Bacillus subtilis	6	7	7	5	15	23	20	26
Streptococcus mutans	5	7	2	2	12	17	17	22
Micrococcussp.	4	4	NA	NA	9	13	10	21
Streptococcus epidermtitis	2	5	NA	NA	12	17	16	25
Fungi								**Ketoconazole**
Aspergillusniger	3	3	NA	NA	5	8	6	14
Candida albicans	7	9	4	2	12	16	15	21
Penicillium aurantiogriseum	8	10	5	NA	13	19	16	24

*NA: No activity

Conclusion

To the best of knowledge, this is the first*in vitro* CuSO4-NPs applicationof *Ocimum basilicum* L. It improved its growth parameters (fresh weight, root and shoot length), flavonoid content and antimicrobial activity.

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4/10/2017