INCREASING SOME FLAVONOIDS COMPOUNDS FOR *ECHINACEA PURPUREA* L. USING COPPER OXIDE NANOPARTICLES *IN VITRO*.

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ABSTRACT

This study was aimed to increase some flavonoids active compounds production in *Echinacea purpurea* seedlings through plant seeds treatment with different concentrations of copper oxide nanoparticles that daignosed and characterized using AFM technique. This research was implemented at plant tissue culture labrotary of College of biotechnology - Al Nahrain University, during the period of 2021 and 2023. The experiment designed factorial within CRD using three factors and ten replicates (3X3). Sodium hypochlorite concentration (S1, S2, S3, S4) (0.0, 1, 2, 3%) represented the first factor, treatment duartion time (T1, T2, T3) (5, 10, 15min) represented the second factor and copper oxide nanoparticles concentrations (C1, C2, C3, C4) (0.0, 25, 50, 75mg/ml) represented the third factor. Results showed that the full reduction in the contamination rate of the selected *E. purpurea* explant recorded in 3% sodium hypochlorite at 10 and 15min. The results also showed that there were a significant increase in the shoots numbers in 50mg/ml CuNPs that recording the highest shoots numbers, the shoot length increased significantly within the 25mg/ml recording 13.5cm then decreased in 50 and 75 mg/ml and the seedlings dry weight increased significantly up to 50mg/ml CuNPs that recording the highest seddlings dry weight, then the seedlings dry weight also decreased significantly in 75mg/ml CuNPs. Also, all the analyzed flavonoids compounds using HPLC device as Echinolone, Humulene, Coumarin, Myricetin, Heperidin and Naringin concentrations were significantly increased in 50 and 75 mg/L^{-1} Cu ONPs, except the Humulene and Coumarin compound that significantly decreased in 75 mg/L^{-1} Cu ONPs in comparsion to the control.

Key words: Tissue culture; Plant medicinal compounds; Active flavoniods, Agricultural applications of nanoparticles.

زيادة بعض المركبات الفلافينويدية لنبات الاكنيسيا باستخدام جسيمات اوكسيد النحاس النانوية CuONPs خارج الجسم الحي

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المستخلص

تم تنفيذ هذا البحث في مختبر زراعة الأنسجة النباتية بكلية التقنيات الاحيائية – جامعة النهرين اثناء المدة من 2021 إلى 2023. كانت التجربة باستخدام تصميم عاملي كامل العشوائية مع ثلاثة عوامل وعشرة مكررات (3X3). يمثل تركيز هيبوكلوريت الصوديوم (S1، S2، S3، S4) (0.0، ، 2، 3٪) العامل الأول، ويمثل المدة الزمنية بالمعاملة (T1, T2, T3) (5، 10، 15 دقيقة) العامل الثاني وويمثل تراكيز جسيمات أكسيد النحاس النانوية (C1, C2, C3, C4) (0.0 ، 25 ، 50 ، 75 ملغ / مل) العامل الثالث. هدفت هذه الدراسة إلى زيادة إنتاج بعض مركبات الفلافونويد النشطة في شتلات نبات الاكنيسيا من خلال معالجة البذور النباتية بتراكيز مختلفة من جزيئات أكسيد النحاس النانوية التي تم تمييزها وتشخيصها باستخدام تقنية مجهر القوة الذرية AFM. أوضحت النتائج أن الانخفاض الكامل في معدل التلوث اثناء انبات بذور نبات الاكنيسيا سجل فى 3٪ هيبوكلوريت الصوديوم فى 10 و 15 دقيقة، كما أظهرت النتائج أن هناك زيادة معنوية فى أعداد البراعم بتركيز 50 مجم / مل من جسيمات اوكسيد النحاس النانوية، زاد طول النبيتات بشكل ملحوظ في 25 مجم / مل مسجلاً 13.5 سم ثم انخفض في 50 و 75 مجم / مل وزاد الوزن الجاف للشتلات بشكل ملحوظ حتى 50 مجم / مل من جسيمات اوكسيد النحاس النانوية التي سجلت أعلى وزن جاف للشتلات ، ثم الشتلات انخفض الوزن الجاف أيضًا بشكل ملحوظ في 75 ملجم / مل من جسيمات اوكسيد النحاس النانوية. أيضًا، حصلت زيادة بتراكيز جميع مركبات الفلافونويد التي تم تحليلها باستخدام جهاز HPLC مثل Echinolone و Humulene و Coumarin و Myricetin و Heperidin و Naringin بشكل ملحوظ في 50 و 75 مجم/لتر من جسيمات اوكسيد النحاس النانوية، باستثناء مركب Humulene و Coumarin الذي انخفض بشكل ملحوظ في 75 مجم. / L^{-1} Cu ONPs مقارنة بعنصر التحكم.

الكلمات المفتاحية: زراعة الأنسجة، مركبات نباتية طبية، الفلافونيدات النشطة، التطبيقات الزراعية للجسيمات النانوية.

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INTRODUCTION

Currently, more than 100,000 natural compounds have been isolated and identified from plants classified as terpenes, phenols and alkaloids, as these compounds such as flavonoids are part of a large group of phenolic compounds and these compounds are an essential component of plant cells (10). Many medicinal plants have been used to obtain flavonoids for use in the treatment of many diseases that affect humans, and recently the increasing demand, especially industrial ones, for biologically active natural compounds available in different parts of medicinal plants, resorting to methods of culture systems in the laboratory to provide and produce large quantities of active and active secondary substances. The most important characteristics of this technique is to avoid destroying the natural sources of plants, cutting off the roots of those plants or parts of them underground, or by over-exploiting rare wild plant species (14). Flavinoid compounds are effective secondary plant compounds that have gained great importance in industry as well as in biotechnology. This importance comes from their unique properties related to their great role in health. These compounds are found in various growing plant parts and are responsible for the pigmentation of flowers, fruits and leaves. Also, those flavonoid compounds provide plant protection such as tannins, anthocyanins, and phytotoxins that provide plant protection from ultraviolet radiation, pathogens and herbivores. In addition to these applications and the great importance of these compounds, they are considered to have a wide range of health-promoting effects and are indispensable in nutritional, pharmaceutical, medical and cosmetic applications. These compounds also provide anti-tumor, anti-allergic, anti-inflammatory, antiviral, anti-hepatotoxic, anti-oxidant, anti-osteoporosis and anti-bacterial activity, in addition to their cardioprotective activity, which is characterized by those compounds isolated from various botanical parts of different plants (13). Among the modern strategies used to increase and enhance the yield is elicitation by adding external substances as a kind of stress on the plant cells that stimulate the defense of the plant body and stimulate the secondary metabolism process and thus increase the production of secondary compounds of important nutritional, industrial and medicinal value. This may also positively affect plant growth and yield. (6). Al-Ziyadi and Hussein (4) also recommend the addition of date palm pollen, as well as the addition of pumpkin extract and the use of nano-fertilizer with natural fertilization to improve the effectiveness of the herbicide used on the one hand, as well as to ensure good nutrition on the other hand for a group of studied cultivars. The studies of different educators showed through their results that nanoparticles (NPs) have great potential to act as catalysts to increase the production of flavonoids in different types of plants. Different types of nanoparticles were used as strong and effective catalysts to increase the production of flavonoids in laboratory cultures of different parts of various medicinal plants (5). Nanoparticles of various classes of NPs have been extensively studied including metallic nanoparticles such as copper and silver, metal oxides such as zinc oxide, copper oxide, iron and silicon dioxide (16). Where it has been proven that flavonoids have a very large role in the mechanism of plant adaptation to withstand various environmental conditions and can directly affect the change in the effectiveness of secondary metabolism in metabolism through those nanoparticles as stressors or through their interference in the metabolic pathway of daughters and their direct impact on the Crop quality and agricultural production. The pharmacological properties of many medicinal plants are attributed to crude extracts or decoctions rather than individual compounds. Therefore, any change in the secondary metabolism of medicinal plants will affect their pharmacological potential, market and industrial value. Therefore due to the importance of this field, this study aimed to increase some flavonoids from *E. purpurea* L. using copper oxide nanoparticles *in vitro.*

MATERIALS AND METHODS

1- Plant sample collection

Seeds of *E. purpurea* collected from the local market in Baghdad city, calssified according to the National Herbal Commission / General

Commission for Agricultural Research, Daghdad, Iraq.

2- Preparation of copper oxide nanoparticles solution and detection

Copper oxide nanoparticles (CuO.NPs) was obtained from Segma Company. The solution of nanomaterial was prepared by dissolving 100 mg of the material in 1000 ml of distilled water with continuous stirring using a hot plate magnetic stirrer to ensure its solubility, an ultrasonic probe was used at a frequency of 60Hz for 15min by placing a small drop of these samples on a glass slide ($cm1cm^{-1}$) and, these samples left at the room temperature to used later for other experiment purposes. The drying sample of nanomaterial was examined with an (AFM) Atomic Force Microscope type Angstrom Advanced (AA) 2000 made in the United States of America, and the examination pattern used was the contact pattern and at room temperature.

2- Preparation the concentrations of CuNPs.

Different concentrations of nanomaterial were prepared (0.0, 25, 50 and 75mg/ml) using sterilized distalled water for dissolving and dilution, then its kept in vials with dark situations at the room temperature for the purpose of using it in stimulation experiments later, including shoot induction, increase or decrease the concentration of secondary metabolites compounds and test their effect on the molecular level.

3- Sterilization of *E. purpurea* plant seeds.

E. purpurea seeds chosed randomly, that they have the same size, running water used for the plant seed washing, then they soaked in Ethanol alcohol (90%) for 5sec, and, 2.5% sodium hypochlorite solution (NaOCl) used for further surface sterilization for about 20min with a continuous stirring using magnetic stirrer instrument, then the seeds were washed five times with sterilized $DDWH_2O$ for 5-10min each time with continuous (1).

4- Plant treatment with copper oxide nanoparticles.

E. purpurea seeds were cultured in Murashige and skooge media supplemented with 2 mg. L^{-1} of BA in growth chamber (1000 lux light) (10) in ten replicates for each seeds sapmle cultured in each CuNPs concentration (0.0, 25, 50 and 75mg/ml). Shoot length was determined about six weeks after seed germination under stress, also, dry wights of the dried treated seedlings (drying in an over in 45 °C) were obtained using sensetive balance.

5- Qualitative and quantitative determination of active substances using the HPLC device

The active compounds of echinacea plant extract were detected in several stages and under different conditions as follows:

A - Echinolone, Coumarin, Humulene.

- Device type: HPLC
- Manufacturer and model: Shimadzu 10 AV-LC
- Column dimensions: 50x2.0mm
- The particle size: 3µm
- Mobile phase: (Mobil phase) (A) 0.1% H_2SO_4 in 70% methanol
- The flow rate of the device: 1.0 ml. min-1
- Detector: ultraviolet (UV) radiation at a wavelength of 245 nm
- Temperature: 35°C

B - Myricetin, heperidin, Naringin compounds

- Device type: HPLC
- Manufacturer and model: Shimadzu 10 AV-LC
- Column dimensions: 50 x2.0mm
- The particle size: 5µm
- Mobile phase: (Mobil phase) (A) 0.1% Formic acid in 70% Acetonitrile
- The flow rate of the device: 0.7 ml. min-1
- Detector: ultraviolet (UV) radiation at a wavelength of 245 nm
- temperature: temperate room

According to the concentration of each sample using the following equation (7).

$$\text{Concentration of the unknown } (\mu g/g) = \frac{\textbf{Model package space}}{\textbf{The area of the standard solution package}} \textbf{ X}$$

the concentration of the standard solution X the number of times of dilution.

6- Experimental design and statistical analysis: The statistical program Genstat was used for analyzing of the resultued data according to the factorial experiment. The experimental design was Completely Randomized Design (CRD) with 10 replicates for each treatment (p=0.05).

RESULTS AND DISCUSSION

Hot plate magnetic stirrer used in the preparation of Cu NPs samples for completely homogenize the solution and an ultrasonic probe was used to ensure the dispersion and disintegration of the nano-elements and ensure that they did not agglomerate and return them to their normal size. Also the results in Figure 1 exhibit the 3D examination of nanomaterials for copper oxide nanoparticles measured by the Atomic Force Microscope (AFM) that measure the surface topography, particle size, diameter and roughness of copper oxide nanoparticles, three-dimensional images of the surface topography of copper nanoparticles, where the size of the copper oxide nanoparticles is 51.81 nm, and the same figure shows the homogeneous distribution of copper nanoparticles on the surface. As for the accumulation of copper oxide nanoparticles, it is noticed that the average diameter of the copper oxide particles is 31.22 nm and the RMS surface roughness of the copper oxide nanoparticles was 4.96 nm. These results aws agreed with those obtained by Al-Jubouri *et al*. (2) who reported The atomic force microscope (AFM) examination was used in several researches to study the surface properties, size of nanoparticles and surface roughness of copper nanoparticles (CuNPs). The two- and three-dimensional images of all measured nanoparticles showed that they were all of regular and uniform shape and size. Data in Table 1 shows a significant decrease in the contamination rate of *E. purpure*a seeds in the concentration of 1, 2 and 3% sodium hypochlorite recording 83, 38 and 3.4 respectively compared to the control that recording 100% contamination rate and the lowest rate recorded in the concentration of 3% sodium hypochlorite, according to the treatment duration time, also a significat decrease were obtained with the increasing of treatment duration time recording 45.3, 53.6 and 69.8 through 5, 10 and 15 min respectively. While the interaction between the duration time of treatment and sodium hypochlorite conc. exhibited that the full reduction in the contamination rate recorded in 3% sodium hypochlorite at 10 and 15min recording 0.0 contamination rate respectively, for that the *E. purpure*a seeds were treated using 3% sodium hypochlorite for 15min to ensure 100% sterilization of *E. purpure*a seeds during germination period. Masoud *et al*. (17), in their study, they mentioned that the nanoparticles manufactured *in vitro*, as well as the essential oils of the clove plant in its normal or nano-processed form, showed an anti-fungal effect against *A. solani*, *F. solani* or *F. oxysporum*. Also, laboratory treatments with laboratory-purified flavonoids or nanoparticles manufactured from green sources led to an increase in the production of polyphenol oxidase in all fungi used in the experiment. Also Alwash *et al*. (3), they demonstrated in their study that M-CuNPs are selective as an antifungal treatment against Saprolegniasis, where they (M-CuNP) were used as a fair alternative treatment to chemotherapeutic compounds used in aquaculture feeding to achieve a cost-effective, hygienic and environmentally friendly benefit.

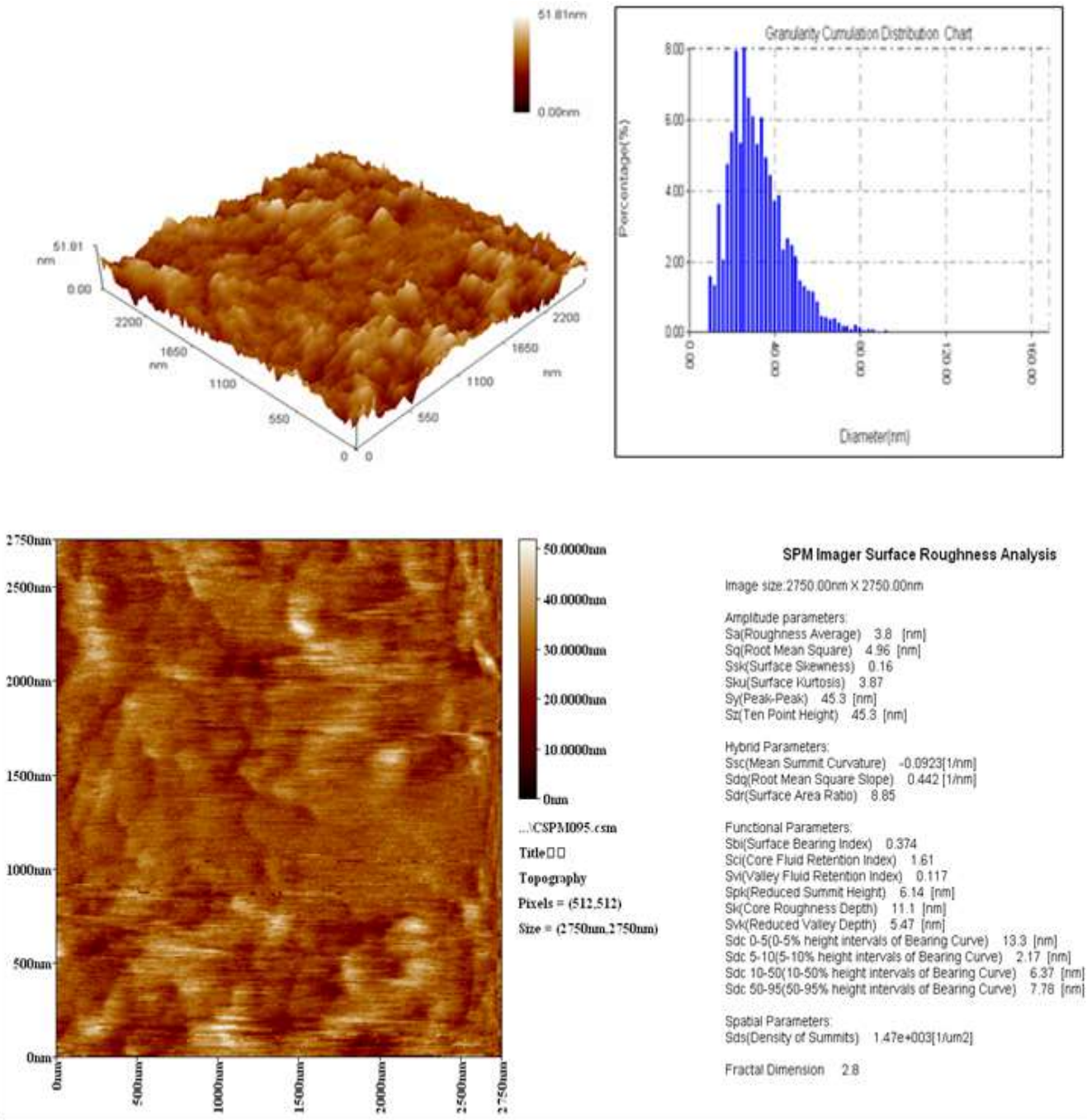

Figure 1. Three dimentional image of the surface morphology for CuNPs using AFM

Table 1. Effect of sodium hypochlorite concentrations on the contamination rate of *E. purpurea* seeds grown in MS medium for a period of seven days. n=10

Sodium hypochlorite concentration %	Treatment duration time (min) Contamination rate			Mean
	5	10	15	
0	100	100	100	100
1	100	83	67	83
2	69.0	31	14	38
3	10.0	0.0	0.0	3.4
L.S.D. (0.05)		3.35		1.93
Mean	69.8	53.6	45.3	
L.S.D. (0.05) time		1.67		

Results in Table 2 and Figure 2 indicate a significant increase in the shoots numbers up to 50mg/ml CuNPs that recording the highest shoots numbers (7.6) then its decreased significantly in 75mg/ml CuNPs (2.4) compared to the value obtained in the concentration of 50mg/ml. But the shoot length increased significantly in 25mg/ml recording 13.5cm then decreased in 50 and 75 mg/ml recording 11.90 and 9.60cm respectively. While the seedlings dry weight increased significantly up to 50mg/ml CuNPs that recording the highest dry weight 197 mg, then the seedlings dry weight also decreased significantly in 75mg/ml CuNPs (82.8mg), and these results were disagreed with those obtained by Yang *et al*. (23) who recommended that 62.5, 125 or 250mg/L CuO-NPs dissolved in nutrient solution (Yoshida's) could negatively affect the rice seedlings growth and the contents of chlorophyll in rice plants. Aslo, oxidative effect was also observed in rice shoots that exposed to CuO-NPs at different concentrations, at the time, and the electrical conductivity or the content of MDA significantly upregulated incomparasion to the control, that the activity of SOD was significant in rice seedlings roots that exposed to 125 mg/L CuONPs. This differences in the results could be due to the small concentrations used in our study. Also it was shows that with increasing the concentration of nanoparticles, the readings begin to decrease. Also, the reason for the significantly increasing in the shoots number or shoots length, and the stem dry weight is due to the short time periods during which the seeds were treated with CuO NPs. However, root length, germination rate, and biomass decreased, while Cu uptake was increased in roots and shoots with high concentrations of CuO NPs. It was also observed that copper nanoparticles (CuO NPs) aggregated in plant cells especially in chloroplasts, and was accompanied by fewer thylakoids per granome. The photosynthetic rate, stomatal conductance and transpiration rate and the maximum quantitative yield of PSII photochemistry and photosynthetic pigment contents decreased significantly (9). Also Faraz *et al*. (11) reported that plants treated with CuO NPs recorded an increase in growth and biomass over their control. Among the different concentrations of CuO NPs (0, 2, 4, 8, 16 mg/L) had a clear effect on the plant as 8 mg/L proved to be the optimal treatment using the foliar spray method and increased the chlorophyll content, proline content, and assimilation rate. net photosynthesis in leaves, and antioxidants as enzyme activity. We concluded that CuO NPs interact with dystrophic cells triggering conductive biochemical pathways to enhance growth traits.

Table 2. Effect of different concentrations of copper oxide nanoparticles mg/ml on shoots number, shoots length, and dry weight of *E. purpurea* grown on MS medium supplemented with 2 mg. L^{-1} of BA four weeks after cultivation. n=10

Concentrations of copper oxide nanoparticles (mg/ml)	Shoots number	Shoots length (cm)	dry weight (mg)
0	**1.90**	**10.80**	**15.30**
25	**3.50**	**13.50**	**91.40**
50	**7.60**	**11.90**	**197.00**
75	**2.40**	**9.60**	**82.80**
Mean	**3.85**	**11.45**	**96.60**
L.S.D. (0.05)	**1.05**	**1.46**	**21.48**

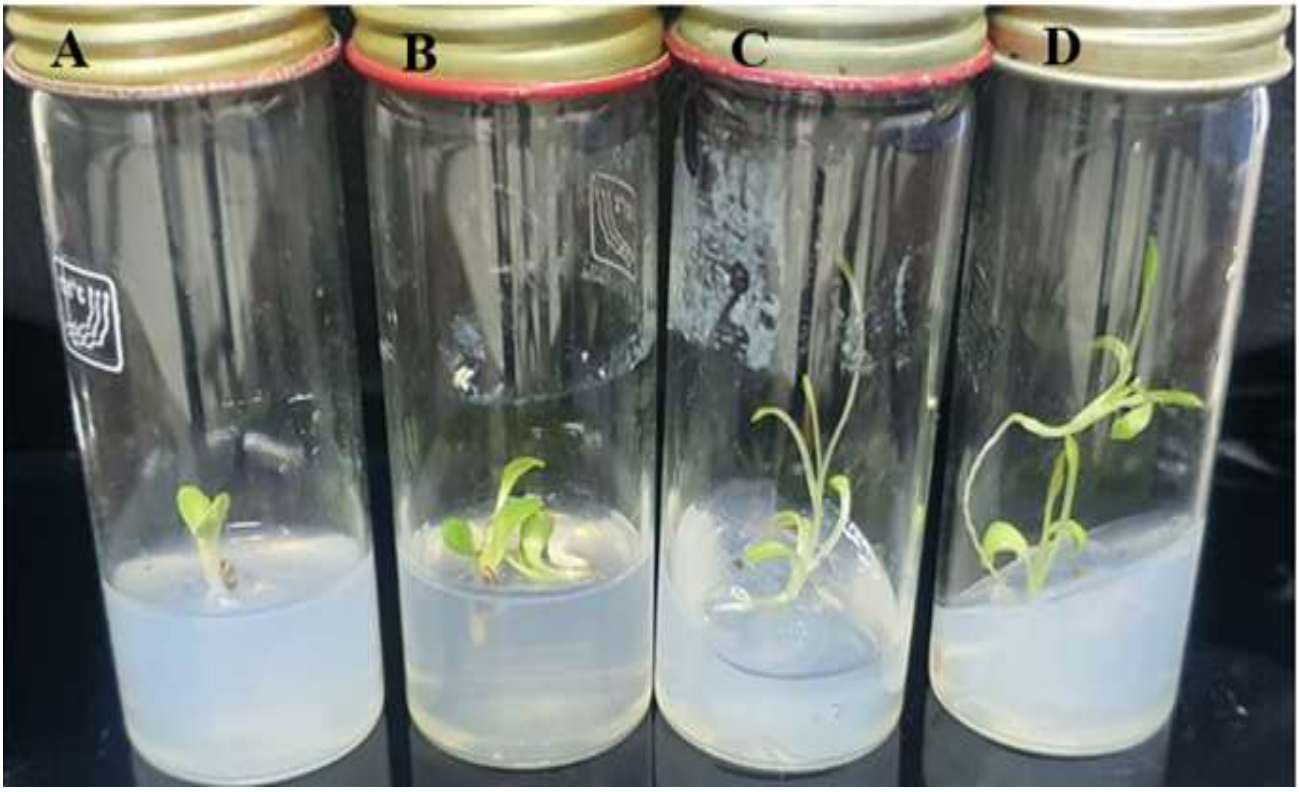

Figure 2. Effect of different concentrations of copper oxide nanoparticles on *E. purpurea* growth on MS medium supplemented with 2 mg. L^{-1}, A=0.0 mg/ml, B= 25mg/ml, C=50mg/ml and D= 75mg/ml CuONPs.

While the results in Table 3 and Figure 3 exhibited that the highest concentration of the analyzed active compounds was Echinolone that recorded 21.380 µg. g^{-1} compared to Humulene, Coumarin, Myricetin, Heperidin and Naringin that recorded 8.081, 3.843, 0.507, 1.1623 and 3.925 µg. g^{-1} respectively. Also, the data shown that there was a significant increase in Echinolone concentrations with the increase of copper oxide nanoparticles concentrations recording 20.697, 22.640, 23.025 µg. g^{-1} in 25, 50 and 75 mg. L^{-1} CuNPs respectively compared to the control (9.156 µg. g^{-1}), while the Humulene concentration increased significantly in 50 mg. L^{-1} CuNPs compared to the control and 25 mg. L^{-1} CuNPs (7.769 and 7.772 µg. g^{-1} respectively), and decreased significantly in 75 mg. L^{-1} CuNPs recording 7.673 µg. g^{-1}. The Coumarin active compound concentration increased significantly in 25, 50 mg. L^{-1} CuNPs (3.933 and 4.402 µg. g^{-1} respectively) and decreased significantly in 75 mg. L^{-1} CuNPs recording 3.224 µg. g^{-1} compared to the control (3.812 µg. g^{-1}), a significant increase in Myricetin concentrations obtained in all CuNPs concentrations recording 0.503, 0.536, 0.500 µg. g^{-1} in 25, 50 and 75 mg. L^{-1} CuNPs respectively compared to the control (0.488 µg. g^{-1}), while Heperidin concentration was decreased significantly in 25 mg. L^{-1} CuNPs (0.6008 µg. g^{-1}) and significantly increased in 50 and 75 mg. L^{-1} CuNPs recording 1.0099 and 2.1799 µg. g^{-1} in comparsion to the control (0.8589 µg. g^{-1}) and a significant increase in the Naringin concentration was recorded in 50 and 75 mg. L^{-1} CuNPs (4.167 and 4.095 µg. g^{-1} respectively) compared to control an 25 mg. L^{-1} CuNPs that recording 3.726 and 3.713 µg. g^{-1} respectively. These results were on line of those by Nuraniye *et al*. (19) who reported that HPLC examination revealed that more than 18 standard phenolic compounds with different amounts and concentrations were found in various extracts from different botanical parts of different plants. According to the results of the experiments obtained in this aspect, the methanolic extract obtained from the different parts of the plant contained high concentrations of flavonoids and phenols. While Fierascu *et al*. (12This study concluded that the use of low concentration aqueous and

alcoholic extract of Echinacea, which was prepared using the traditional method and with moderate heat, resulted in the manufacture of nanoparticles with dimensions as small as less than 10 nanometers, when compared to biologically manufactured nanoparticles from extracts with high concentrations. The study also showed that the developed nanomaterials showed improved antioxidant effects and antimicrobial properties as well, compared to the original extracts. Also, Chung *et al*. (8) reported that a 48-h treatment with a concentration of 3 mg/L CuO NPs resulted in the largest yields of GA II, total phenols, and flavonoids. The cultures also showed clear antioxidant, anti-inflammatory, antidiabetic, antifungal, antibacterial and anticancer activities. The use of CuO NPs (3 mg/L) also resulted in a nine-fold increase in the yield of GA II and PC compared to unmodified CSC, Siddiqi *et al.* (21) reported that CuONPs are also examined for their effect on the growth and yield of agricultural crops, as the growth of mung bean roots and shoots increased with the use of concentrations of CuONPs. While these nanoparticles reduced sprout growth in wheat plant, through enhancing the grain yield by increasing the tolerance to stress through the hydrolysis of starch, and the treated seedlings with CuO-NPs showed a decrease in the content of carotenoids, chlorophyll and sugar, while treating seedlings of Brassica rapa increased proline and anthocyanins. While Nazir *et al*. (18) reported that callus tissue grown on MS media supplemented with 10 mg/L CuO-NPs resulted in the highest biomass accumulation (FW/ 172.8 g/L, DW/ 16.7 g/L), phenolic contents (TPC: 27.5). mg/g DW), flavonoid contents (TFC/9.1 mg/g DW) along with antioxidant activities (DPPH: 94%, ABTS: 881 µM TEAC, FRAP: 386 µM TEAC) compared with MnO-NPs and control. Also, the activities of superoxide dismutase (SOD/1.28 nM/min/mg FW) and peroxidase activities (POD/0.48 nM/min/mg FW) were observed to be maximum in cultures of CuO-NPs elicited from MnO-NPs and control. Furthermore, the results of the HPLC assay showed that rosmarinic acid (11.4 mg/g DW), eugenol (0.21 mg/g DW), and chicoric acid (16.6 mg/g DW) were optimally found in the cultures at 10 mg/L of CuO-NPs. Overall. It can be concluded that CuO nanoparticles can be effectively used as a catalyst for green biosynthesis of nanoparticles from metabolites in callus cultures of *O. basilicum* (20). Also, Yaaqoob *et al*., (22) The study concluded that 0.01 mg/L of titanium nanoparticles showed that it is the best concentration for increasing the production of prodigiosin by *S. marcescens*, as well as for the production of phenazine by *P. aeruginosa*. Thus, it can be recommended that titanium nanoparticles are highly effective in stimulating the activity of producing secondary metabolites using different industrial microorganisms and from different environments.

Table 3. Effect of different concentrations of copper oxide nanoparticles (CuO NPs) on flavonoids compounds in plant seedlings, n=10

Concentration of CuO NPs (mg. L^{-1})	Number of Flavonoids (µg. g^{-1})					
	Echinolone	Humulene	Coumarin	Myricetin	Heperidin	Naringin
0	9.156	7.769	3.812	0.488	0.8589	3.726
25	20.697	7.772	3.933	0.503	0.6008	3.713
50	22.640	9.111	4.402	0.536	1.0099	4.167
75	23.025	7.673	3.224	0.500	2.1799	4.095
Mean	21.380	8.081	3.843	0.507	1.1623	3.925
L.S.D. (0.05)	0.2463	0.0937	0.1128	0.0667	0.06319	0.1368

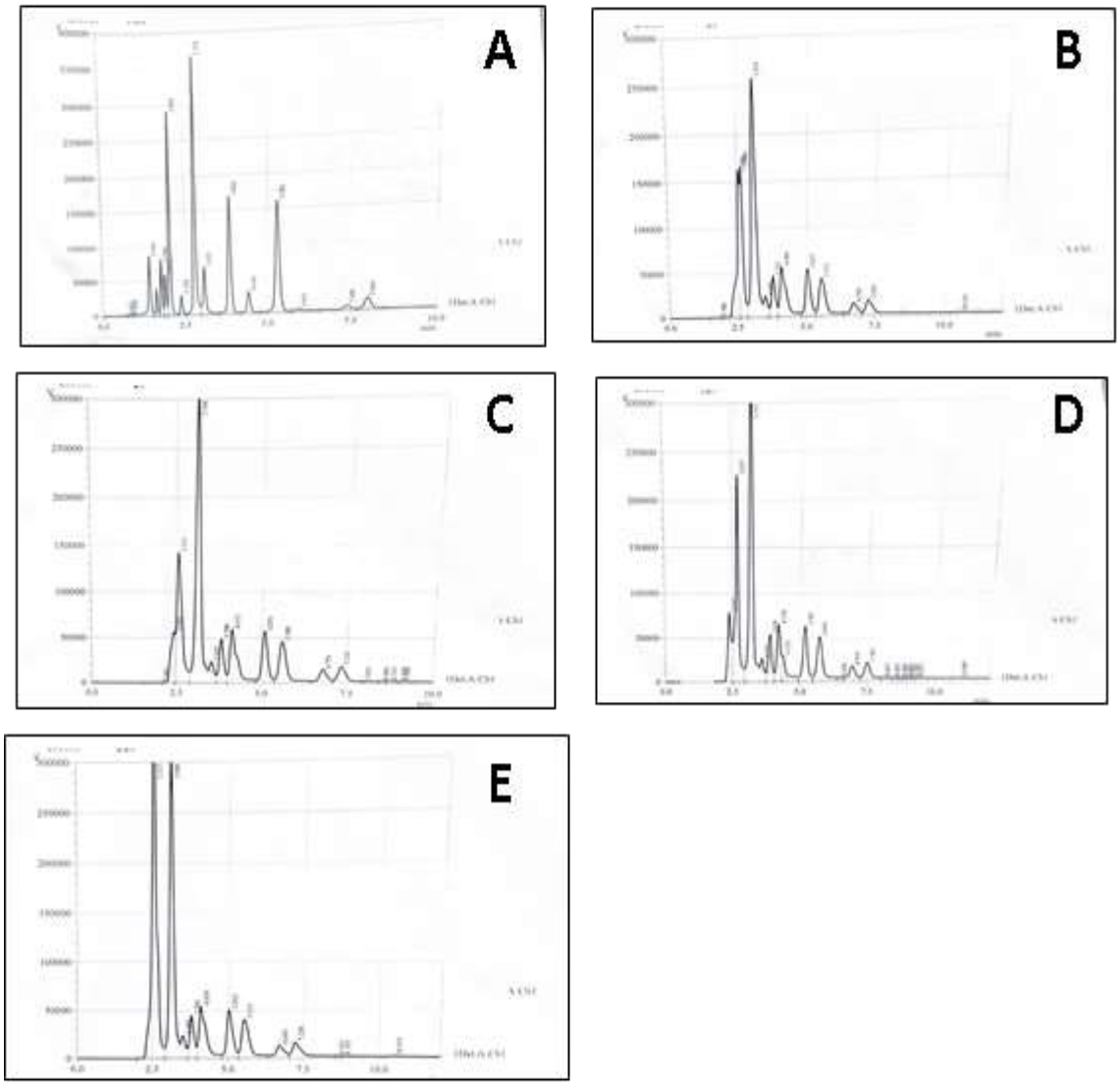

Figure 3. HPLC analysis for secondary metabolites Echinolone, Coumarin, Humulene, Myricetin, heperidin, Naringin and for cofactors A: 0.0 mg. L^{-1} NPs, B: 25mg. l^{-1} CuO NPs, C: 50 mg. L^{-1} CuO NPs, D: 75 mg. l^{-1} NPs CuO NPs. E: control

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