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Green synthesis of copper oxide nanoparticles using *Juglans regia* leaf extract and assessment of their physico-chemical and biological properties

<https://doi.org/10.1515/gps-2019-0025>

Received November 8, 2018; accepted March 27, 2019.

Abstract: Copper oxide nanoparticles (CuO NPs) were green synthesized using walnut leaf extract. Effects of three synthesis parameters namely; amount copper salt (1–4 g), amount of walnut leaf extract (10–40 mL) and furnace temperature (300–500°C), on the particle size as manifested in broad absorption peak (λ_{max} , nm), concentration (absorbance), antioxidant activity and antibacterial activity as minimum inhibitory concentration (MIC) of the fabricated CuO NPs were studied using response surface methodology, based on Box behnken experimental design. The spherical and crystalline monodispersed fabricated CuO NPs with mean particle size of 80 nm, were achieved using optimum synthesis parameters including 1 g copper salt, 14 mL walnut leaf extract and 490°C of furnace temperature. The fabricated CuO NPs at these conditions had maximum antioxidant activity of 83.64% and minimum MIC value of 1.78% w/v against *E. coli*, with λ_{max} and absorbance values of 226 nm, 4.44% a.u., respectively.

Keywords: copper oxide nanoparticles; walnut leaf extract; green synthesis; antioxidant activity; minimum inhibitory concentration

1 Introduction

Among metal oxide, copper oxide has gained more attention in the last decade, due to its distinctive properties such as high temperature superconductivity, spin dynamics and electron correlation effects, which

those increase widely its applications as heterogeneous catalysts, antioxidants, drug delivery agents, and imaging agents in field of biomedicine [1,2]. Furthermore, copper oxide is inexpensive antimicrobial agent when compared to inorganic bactericidal agents such as silver and gold, and has longer shelf life as compared to other organic antimicrobial agents [1]. All the mentioned properties of copper oxide drastically increase when it fabricates in nano-size scale. In fact, copper oxide nanoparticles (CuO NPs) due to their extremely unusual crystal morphologies and high surface area to volume ratio, have unique physical, chemical and biological properties [1,3].

Development of a non-toxic, clean, reliable, cost-effective, eco-friendly and biocompatible processes to NPs fabrication is one of the great interests of researchers in the last decade to replace them with common physical and chemical synthesis methods, which those are high energy consuming with intensive capital cost and utilize toxic chemicals and non-polar solvents in their processes [4]. Green synthesis of NPs with plants and their derivatives extract, have gained more attentions during last years, due to the presence of the numerous natural reductants (i.e., polyphenols, flavonoids, tannins, ascorbic acids and sugars) and stabilizers (i.e., proteins, carbohydrates, gums and pectic substances) [5–7]. Numerous researches have been done on green synthesis of CuO NPs with plant extract such as tea leaf, coffee powder and *Eichhornia crassipes* extracts [8,9].

Walnut (– *regia* L.) leaf contains numerous main bioactive compounds such as, malic acid, 3-*O*-caffeoylquinic acids, quercetin *O*-pentoside, sucrose, disaccharide, α -tocopherol and tocopherol isomer, and phenolic compounds which those can be effectively utilized as reducing and stabilizing mediators in the NPs production such as silver NPs [10].

Present study focused on i) possible application of the walnut leaf extract in the CuO NPs fabrication, ii) optimization of the synthesis process using response surface methodology to achieved CuONPs with appropriate physico-chemical attributes, and iii) valuation of antibacterial activities of the formed CuO NPs using walnut leaf extract.

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2 Materials and method

Walnut fresh leaves, same in size and colour, and free of microbial damages, were collected locally from a walnut garden in Tabriz, Iran. Copper nitrate, $\text{Cu}(\text{NO}_3)_2$, Dimethyl sulfoxide (DMSO), methanol, ethanol, nutrient agar and Mueller Hinton broth were provided from Merck Company (Merck, Darmstadt, Germany). Resazurin reagent was purchased from (Sigma-Aldrich, United State of America). *E. coli* (PPTCC 1270) was obtained from the microbial Persian type culture collection (PTCC, Tehran, Iran). All the solutions in the present study were prepared using deionized double distilled water (DI).

2.1 Preparation of walnut leaf extract and synthesis of CuO NPs

Provided walnut leaves were washed three time using DI water to remove their surface contaminations and shade dried at room temperature (30°C) during a week. A domestic miller (MX-GX1521; Panasonic, Tokyo, Japan) was utilized to prepare walnut dried powder and in order to prepare walnut leaf extract, 5 g dried powder was added into 100 mL of boiling DI water for 1 h, and after cooling the mixture solution, it was filtered (Whatman No. 40 filter paper) and the extract was then collected in dark bottle and kept in the refrigerator.

In order to prepare CuO NPs, 1 to 4 g of the copper nitrate was added into the defined amount of the prepared walnut leaf extract (10-40 mL) and the reaction solutions were mixed using a heater-stirred, adjusted at 500 rpm and 70°C, for 15 min and finally the samples were put in an electric furnace (FM4P, Fanazma Gostar Co., Alborz, Iran) adjusted at 300 to 500°C for 2 h. The obtained powders, as farmed CuO NPs, were then used for further studies.

2.2 Physico-chemical properties of the synthesized CuO NPs

Formation of the CuO NPs can be easily confirmed using UV-Vis spectrophotometry (UV-3600 Plus, Shimadzu Co., Tokyo, Japan) because of the surface Plasmon resonance (SPR) of the formed NPs. Due to the SPR of the fabricated CuO NPs, the solution containing CuO NPs indicated the broad absorption peak (λ_{max}) in the wavelength ranged 200-300 nm [8]. Furthermore, fabrication of CuO NPs can be easily verified by X-Ray Diffractometry (XRD: D5000, Siemens Co. Germany) using $\text{Cu K}\alpha$ radiation and compression of the obtained pattern with the standard

XRD pattern for CuO NPs. Scanning electron microscopy (SEM, CamScan MV 2300, Tescan, Czech Republic) was utilized to assess of the structural properties and morphology of the formed CuO NPs. Antioxidant activity of the fabricated CuO NPs using walnut leaf extract had been assessed according to the scavenging ability on 2,2-diphenyl-2-picrylhydrazyl (DPPH) method, which that was described by Vahidi et al. [11].

2.3 Antibacterial activity assess of the formed CuO NPs

Antibacterial activity of the formed CuO NPs was assessed using MIC test according to the clinical and laboratory standards institute (CLSI) protocol [12]. In order to obtain the MIC values, serial dilutions of CuO NPs with the concentrations of 100, 50, 25, 6.25, 3.12, 1.56, 0.78 and 0.39% (W/V) were provided in a 96 well-cell plate. After that 100 and 30 μL of the sterilized MHB and Resazurin reagent were added into the wells, respectively. Finally, 100 μL provided bacteria suspension (*E. coli*) adjusted to 0.5 McFarland standards have been added into the wells. The turbidity of the poured wells were measured at wavelength of 625 nm by UV-visible spectroscopy measurements (250-800 nm, Perkin Elmer, Germany), at the beginning of the test and after 24 h incubation of the 96 well-cell plate at 37°C and the MICs were determined.

2.4 Design of experiments and data analysis

Response surface methodology (RSM) has numerous advantages as compared to other statistical techniques due to its potential to generate valuable data using minimum experiment runs and assessment of their different effects such as linear, quadratic and interaction on the responses [13-16]. Therefore, Box behnken design, using a block, with RSM were utilized to experimental design and optimize of the CuO NPs synthesis parameters namely; amount of copper ion concentration (X_1 , 1-4 g), amount of walnut leaf extract (X_2 , 10-40 mL) and electric furnace temperature (X_3 , 300-500°C), on the λ_{max} (Y_1 , nm), absorbance at λ_{max} (Y_2 , % a.u.), MIC (Y_3), and anti-oxidant activity (Y_4 , %) of the mixture solutions containing CuO NPs. Response variables, as function of the synthesized parameters, were followed a second-order polynomial Eq. 1, which in this equation, B_0 is a constant and B_i , B_{ij} and B_{ij} are the coefficients of the linear, quadratic and interaction terms.

Table 1: Box behnken design and response variables for synthesis of CuO NPs using walnut leaf extract.

Sample runs	Ion concentration (g)	Leaf extract amount (mL)	Temperature (°C)	$\lambda_{\max}$ (nm)	Absorbance (% a.u.)	Antioxidant (%)	MIC (% w/v)
1	2.5	10	500	237.5	3.000	86.0	4.100
2	1.0	10	400	245.0	2.410	88.7	2.310
3	4.0	25	500	260.0	1.100	88.1	1.470
4	2.5	10	300	246.0	1.314	86.3	2.040
5	4.0	25	300	250.0	0.500	85.7	3.000
6	2.5	25	400	241.0	1.066	85.9	1.140
7	2.5	40	500	260.0	2.300	83.4	1.100
8	2.5	40	300	258.0	1.188	78.8	1.520
9	4.0	40	400	260.0	2.009	90.0	1.670
10	1.0	25	500	225.0	5.010	80.0	1.095
11	1.0	40	400	260.0	4.338	79.7	1.390
12	2.5	25	400	290.0	1.070	88.0	1.410
13	1.0	25	300	267.0	1.100	83.7	1.250
14	2.5	25	400	263.0	1.000	87.3	2.030
15	4.0	10	400	*	*	*	*

* Out of range.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Analysis of variance (ANOVA) based on coefficient of determination (R^2) and the terms p-value ($p < 0.05$) and F ratio was employed to significance determinations of the obtained reduced models [17]. In the reduced models, those terms which had insignificant effects on the responses were removed [18,19]. Two-dimensional contour plots, based on the generated models, were established only for significant ($p < 0.05$) interaction effects of the synthesis parameters. Numerical multiple response optimization was utilized to obtain exact values of the optimum synthesis parameters [20]. Obtained optimum synthesis parameters were used three times to synthesis CuO NPs for verification of the generated reduced models accuracy [21]. Minitab v.16 statistical software (Minitab Inc., PA, USA) was used for the design of experiments, RSM, ANOVA and optimization procedures.

3 Results and discussion

3.1 Generation of response surface models

Based on the attained values for the responses (Table 1) the reduced models for the $\lambda_{\max}$, absorbance, antioxidant activity and MIC of the prepared CuO NPs, as function of copper salt, walnut leaf extract and furnace temperature were generated. Table 2 indicates regression coefficients

Table 2: Regression coefficients, R^2 , adjusted R^2 (R^2 -adj) and probability values for the final reduced models.

Regression coefficient	$\lambda_{\max}$ (nm)	Absorbance (% a.u.)	Antioxidant (%)	MIC (% w/v)
β_0 (constant)	283.29	0.512	87.060	1.445
β_1 (main effect)	9.13	-0.448	2.690	0.683
β_2 (main effect)	NS	NS	-2.365	-0.931
β_3 (main effect)	-14.19	0.475	0.375	NS
β_{11} (quadratic effect)	NS	NS	NS	0.636
β_{22} (quadratic effect)	NS	NS	NS	0.573
β_{33} (quadratic effect)	-19.72	1.838	-3.060	NS
β_{12} (interaction effect)	NS	NS	2.295	-0.793
β_{13} (interaction effect)	13.00	-1.350	1.525	NS
β_{21} (interaction effect)	NS	NS	NS	NS
β_{23} (interaction effect)	NS	NS	1.225	NS
R^2	0.8828	0.8440	0.9522	0.8665
R^2 -adj	0.8359	0.7815	0.8964	0.7831
Lack of fit	0.331	0.602	0.503	0.604

NS: Non-significant.

1: Ion concentration (g); 2: Leaf extract amount (mL);

3: Temperature (°C).

of the models terms including main, quadratic and interaction, R^2 and R^2 -adj, and lack of fit for the fitted models. As can be observed in Table 2, higher values of the R^2 (> 0.88), R^2 -adj (> 0.78) and lack of fit p-value ($p > 0.05$) for the generated models, verified their proper precision [22,23]. The significance probability of the regression coefficients for the final reduced models presents in Table 3. As can be shown in Table 3, main effect of the copper salt had significant ($p < 0.05$) effects on the all studied responses. Furthermore, main and quadratic effects of furnace temperature and its

Table 3: The significance probability (p-value) of regression coefficients for the final reduced models.

Terms	$\lambda_{\max}$ (nm)	Absorbance (% a.u.)	Antioxidant (%)	MIC (% w/v)
β_0 (constant)	0.000	0.046	0.000	0.000
β_1 (main effect)	0.007	0.032	0.001	0.002
β_2 (main effect)	NS	NS	0.002	0.000
β_3 (main effect)	0.000	0.028	0.042	NS
β_{11} (quadratic effect)	NS	NS	NS	0.013
β_{22} (quadratic effect)	NS	NS	NS	0.020
β_{33} (quadratic effect)	0.001	0.000	0.003	NS
β_{12} (interaction effect)	NS	NS	0.021	0.009
β_{13} (interaction effect)	0.007	0.002	0.036	NS
β_{23} (interaction effect)	NS	NS	0.045	NS

NS: Non-significant.

1: Ion concentration (g); 2: Leaf extract amount (mL);

3: Temperature (°C).

interaction with copper salt had significant ($p < 0.05$) effects on the $\lambda_{\max}$, absorbance and antioxidant activity of the prepared CuO NPs.

3.2 Effects of the synthesized parameters on the $\lambda_{\max}$ and absorbance of the samples

As can be realized in Table 1, $\lambda_{\max}$ and absorbance of the prepared solutions containing CuO NPs varied from 225–290 nm and 0.500–5.010% a.u., respectively. Obtained results revealed that CuO NPs with smallest particle size ($\lambda_{\max}$ of 225 nm) and maximum concentration (5.010% a.u.) were fabricated using synthesized parameters including amount of copper salt, amount of walnut leaf extract and furnace temperature of 1 g, 25 mL and 500°C, respectively. Furthermore, statistical analysis demonstrated that the interaction effects of the copper salt and furnace temperature had significant ($p < 0.05$) effects on the $\lambda_{\max}$ and absorbance of the prepared solutions containing CuO NPs (Table 3). Effects of the copper salt and furnace temperature on the $\lambda_{\max}$ and absorbance are indicated in Figures 1a and 1b, respectively. In the synthesis of organic NPs, the main aim is to form NPs with minimum particle size and maximum concentration which these two parameters can be manifested in minimum $\lambda_{\max}$ and maximum absorbance [7]. As can be seen in Figures 1a and 1b, CuO NPs with minimum $\lambda_{\max}$ and maximum absorbance could be achieved using minimum amount of copper salt and maximum furnace temperature. The obtained results can be explained by the fact that at minimum

ion concentration, due to higher concentration of bioreductant compounds of the plant extract, the nucleation rate was increased and NPs were fabricated rapidly and stabilized by the presented stabilizers of the plant leaf extract [4,5]. This result was in line with finding of Eshghi et al., they fabricated silver NPs using walnut leaf extract [10].

3.3 Effects of the synthesized parameters on the antioxidant activity of the samples

As can be seen in Table 1, antioxidant activity of the fabricated CuO NPs is varied from 78.8 to 90%. The achieved results demonstrated that maximum antioxidant activity of the fabricated CuO NPs was obtained using synthesis conditions including amount of copper salt, amount of walnut leaf extract and furnace temperature of 4 g, 40 mL and 400°C, respectively. In addition to, statistical analysis revealed that the interaction effects of all three synthesis parameters had significant ($p < 0.05$) effects on the antioxidant activity of the formed CuO NPs using walnut leaf extract (Table 3). Effects of the synthesis parameters on the antioxidant activity of the formed CuO NPs indicate in Figures 2a–c. As clearly observed in Figure 2a and 2b, at minimum amount of copper salt, by increasing the amount of walnut leaf extract and furnace temperature, the fabricated CuO NPs indicated maximum antioxidant activity, respectively. The obtained results can be explained by the fact that, at lower amount of copper salt, by addition of higher amount of walnut leaf extract and subjected the prepared reaction solution into the higher temperatures, the CuO NPs with minimum particle size and maximum concentration were fabricated, as can be seen in Figure 1, which those increased antioxidant activity of the formed NPs [11]. However, as can be seen in Figure 2c, the formed CuO NPs with highest antioxidant activity was produced using maximum amount of walnut leaf extract and minimum furnace temperature.

3.4 Effects of the synthesized parameters on the MIC of the formed CuO NPs

As can be seen in Table 1, MIC of the formed CuO NPs is changed from 1.095 to 4.100% w/v. The attained results demonstrated that minimum MIC of the formed CuO NPs was gained using synthesis conditions including amount of copper salt, amount of walnut leaf extract

Figure 1: Contour plots for $\lambda_{\max}$ (a) and absorbance (b) of the formed CuO NPs as function of copper salt amount (g), amount of walnut leaf extract (mL) and furnace temperature ($^{\circ}\text{C}$).

and furnace temperature of 1 g, 25 mL and 500°C , respectively. In addition to, statistical analysis revealed that the interaction of amounts of copper salt and walnut leaf extract had significant ($p < 0.05$) effect on the MIC of the fabricated CuO NPs using walnut leaf extract (Table 3). Furthermore, as clearly indicated in Figure 3, minimum MIC was attained by minimum and low amounts of copper salt and walnut leaf extract, respectively. This result could be related to the smallest particle size of the CuO NPs using walnut extract at minimum amount of the copper salt. Due to higher surface to volume ratio of the fabricated small NPs, those effectively attached to the microbial membrane

and altered its permeability which in turn, caused microorganism death [14].

3.5 Optimization of processing parameters for the CuO NPs synthesis

The CuO NPs synthesized parameters were considered as optimum conditions when the formed NPs had minimum particle size (according to the $\lambda_{\max}$) and MIC, and maximum concentration (absorbance) and antioxidant activity. Figures 4a-c indicate graphical optimization area, white coloured surfaces, based on an overlaid contour

Figure 2: Contour plots for antioxidant activity of the formed CuO NPs as function of copper salt and walnut leaf extract amounts (a), copper salt amount and furnace temperature (b), and amount of copper salt and furnace temperature (c).

plot. Numerical multiple optimization also shown that the optimum synthesized conditions for achievement of CuO NPs with desirable properties were obtained using 1 g of copper salt, 14 mL walnut leaf extract and 490°C of furnace temperature. At these obtained synthesized parameters,

the values of $\lambda_{\max}$, absorbance, antioxidant activity and MIC of the fabricated CuO NPs were predicted as 226 nm, 4.44% a.u., 83.64% and 1.78% w/v, respectively. Statistical analysis indicated that there were non-significant differences between the experimental and predicted

Figure 3: Contour plots for MIC of the formed CuO NPs as function of copper salt amount (g), amount of walnut leaf extract (mL) and furnace temperature (°C).

Figure 4: (Continued).

Figure 4: (a-c) Overlaid contour plot CuO NPs λ_{max} , absorbance, antioxidant activity and MIC with acceptable levels as function of copper salt amount (g), amount of walnut leaf extract (mL) and furnace temperature (°C).

values of the responses at obtained optimum synthesized conditions. This result confirmed the adequacy of the generated reduced models.

3.6 Characteristics of CuO NPs at obtained optimum conditions

Formation of CuO NPs using walnut leaf extract could be evaluated based on their SPR, using UV-Vis spectral

analysis. Figure 5 shows the broad emission peaks (λ_{max}) of the synthesized CuO NPs using optimum conditions, which the peak was achieved at 226 nm. TEM analysis was done to monitor the morphology of the formed CuO NPs. TEM image of the synthesized NPs at obtained conditions indicated that the particles were monodispersed and spherical with mean particle size of 80 nm in diameter (Figure 6). XRD pattern of the formed CuO NPs using walnut leaf extract is shown in Figure 7. As clearly indicated in this figure, there were 11 peak positions with

Figure 5: UV-Vis spectra of the mixture solution including synthesized CuO NPs at obtained optimum synthesis conditions.

Figure 6: SEM image of synthesized CuO NPs at obtained optimum conditions.

Figure 7: XRD pattern of synthesized CuO NPs at obtained optimum conditions.

different 2θ values which were in line with the finding of Ahmed et al. [24] and confirmed the fabrication of a crystalline structure for the CuO NPs.

4 Conclusions

As conclusion, present study indicated that walnut leaf extract due to several natural and bioactive compounds which those are capable to reduce copper ions and convert them into the NPs and stable the fabricated CuO NPs, could be effectively utilized in green synthesis of CuO NPs. Furthermore, RSM based on the Box behnken experimental design, could significantly evaluate the effects of synthesis parameters on the selected responses of the synthesized CuO NPs. Finally, the resulted green synthesized CuO NPs indicated high antioxidant (83.64%) and antibacterial activities (MIC value of 1.78% w/v), which these properties make the fabricated CuO NPs more applicable in various fields such as medicine and food packaging.

Acknowledgements: The authors appreciate the supports of Islamic Azad University-Tabriz branch to accomplish this paper.

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