Substantially Improved Antioxidant Activity of Modified Polymeric Nanostructure Entrapping Curcumin

Deni Rahmat[1], Wahyu Widowati[2,*], Etik Mardliyati[3], Eny Kusrini[4], Abdi Wira Septama[5], Yati Sumiyati[1], Mita Restinia[1], Sjaikhurrizal El Muttaqien[3], Cintani Dewi Wahyuni[6], Hanna Sari Widya Kusuma[6], Muhammad Aldi[6], Tri Handayani[6] and Rizal Rizal[6,7]

[1]*Faculty of Pharmacy, Pancasila University, Jl. Srengseng Sawah, Jagakarsa, Jakarta Selatan, 12640, West Java, Indonesia*

[2]*Faculty of Medicine, Maranatha Christian University, Jl. Surya Sumantri no 65, Bandung 40164, West Java, Indonesia*

[3]*Pharmacy Technology and Medic Center of BPPT, Kawasan Puspiptek Serpong Tangerang Selatan, Banten, Indonesia*

[4]*Faculty Engineering, Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, Depok 16424, West Java, Indonesia*

[5]*Chemistry Research Center, LIPI, Kawasan PUSPITEK, Serpong, Tangerang Selatan, 15314, Banten, Indonesia*

[6]*Biomolecular and Biomedical Research Center, Aretha Medika Utama, Jl. Babakan Jeruk 2 no 9, Bandung 40163, West Java, Indonesia*

[7]*Biomedical Engineering, Department of Electrical Engineering, Faculty of Engineering, Universitas Indonesia, Depok 16426, West Java, Indonesia*

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Abstract: BACKGROUND: Chronic and degenerative diseases due to free radicals cause oxidative stress in the body. The body requires natural antioxidants to cope with the negative effects of free radicals. Curcumin is a compound that has been shown to have pharmacological potential, such as antioxidant, anti-inflammatory, and anti-tumor properties. In recent years, the nanoparticle system for drug administration has become one of the most frequently studied methods of treating the disease. OBJECTIVE: This study aimed to formulate nanocurcumin (NC) to enhance its antioxidant activity. METHODS: The antioxidant activity of Curcumin and NC was evaluated using 2,2-diphenyl-1-pycrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS), H2O2, NO scavenging activities and ferric reducing antioxidant power (FRAP) assay. RESULTS: The results showed that the median Inhibitory Concentration (IC50) for DPPH, ABTS, H2O2, NO scavenging activities of NC was 0.68; 15.59; 24.98; 19.61 µg/mL, respectively. While the IC50 value for curcumin was 3.20; 18.54; 38.40; 24.94 µg/mL, respectively. The FRAP activity of NC and curcumin was 502.92 and 256.50 µM Fe(II)/µg, respectively, at the highest concentration of 50 µg/mL. CONCLUSION: The antioxidant activity of the NC was higher than that of curcumin alone. Thus, the nanoparticle system may enhance the antioxidant activity of curcumin.

1 INTRODUCTION

Free radicals are unstable and highly reactive molecules due to the lack of electron pairs in the atomic orbits (Yildiz, 2020). To become stable, free radicals can either accept electrons or give electrons to other molecules. This results in the target molecule losing electrons and becoming free radicals, which triggers a chain reaction and ultimately harms living cells (Phaniendra, et al., 2015). Free radicals can be produced in the body through metabolic processes or occur due to environmental factors such as X-ray exposure, smoking, air pollution, and industrial chemical (Zubieta-Calleja & Zubieta-DeUrioste, 2017).

*Corresponding author

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An imbalance between antioxidants and free radicals in the body can cause oxidative stress, which will lead to various diseases such as heart disease, stroke, gastric ulcers, hypertension, preeclampsia, and neurological disorders (Alzheimer's disease and Parkinson's disease) (Zubieta-Calleja & Zubieta-DeUrioste, 2017).

Curcumin is one of the compounds contained in the rhizomes of *Curcuma longa*. Curcumin is an extremely potent antioxidant that has been studied by many scientists over the world. Antioxidant activity was related to the phenolic groups that are presence in curcumin (Sokmen & Khan, 2016). The U.S. Food and Drug Administration (FDA) has stated that curcumin is a safe compound, with a daily intake of curcumin at a dose of 0.10003 mg/kg BW. However, the use of curcumin is limited because curcumin has poor absorption, a short half-life, and rapid metabolism in the digestive system (Ghosh et al., 2015).

Nanocurcumin, chitosan-coated curcumin, liposome-encapsulated curcumin, cyclodextrin encapsulated curcumin and polylactic-coglycolic acid encapsulated curcumin are structural modifications of curcumin that have been tried to increase the bioavailability of curcumin (Ghosh et al., 2015). In a study conducted by Aditya et al. (2015), curcumin in the form of nanosuspension could increase the bioavailability of curcumin. Nanoparticles containing curcumin compounds can also be a promising strategy for delivering drugs to target organs and increasing antidiabetic activity. This has been proven in research by Rahmat et al. (2020), which stated that nanoparticles containing curcumin could reduce blood sugar levels in Alloxan-induced diabetic rats. The objective of this study was to determine the antioxidant activity of nanoparticle curcumin (NC) by comparing it to curcumin alone. This research was a preliminary study, the continued research evaluated Curcumin and NC as anti-inflammatory and antioxidant potential on acute lung injury rat model.

2 MATERIALS AND METHODS

2.1 Preparation of Nanoparticles

The preparation of nanoparticles was carried out as described by Rahmat et al. (2020) with modifications. A 1 g of chitosan was dissolved in 100 mL of glacial acetic acid 1% (v/v) to obtain 1% chitosan. A 1 g of ethylcellulose was dissolved in 100 mL of 96% ethanol to obtain 1% ethylcellulose. Curcumin was dissolved in the final concentration of 60 mg/mL in a mixed solvent containing 20 mL of 10% dimethyl sulfoxide (DMSO), 20 mL of 70% ethanol, and 20 mL of polypropylene glycol (PPG). Subsequently, the curcumin solution was added by 40 mL of chitosan solution. The mixture was then stirred for ten mins. A 40 mL of ethylcellulose was added to the preparation. Sodium tripolyphosphate 0.2% was added per drop to the final mixture up to 3 mL while being stirred.

2.2 DPPH Scavenging Assay

Briefly, 50 µL of samples, 200 µL DPPH solution (Sigma Aldrich, D9132) were added to 96-well. microplate. The plate then incubated for 30 mins in dark condition at room temperature. A microplate reader was used for measuring the absorbance at 517 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific) (Widowati et al., 2017; Prahastuti et al., 2019; Prahastuti et al., 2020). The DPPH scavenging activity was calculated using the equation below:

$$\%Scavenging\ activity = \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} x\ 100 \quad (1)$$

2.3 ABTS Reducing Activity Assay

To obtain ABTS•+ solution, 14 mM ABTS (Sigma Aldrich, A1888) was mixed with 4.9 mM potassium persulfate (Merck, EM105091) with a volume ratio of 1:1. The solution was incubated in dark condition at room temperature for 16 h. The solution was added by 5.5 mM Phosphate Buffered Saline (PBS) at pH 7.4 until the solution's absorbance was 0.70±0.02 at 745 nm. A 198 µL of ABTS•+ solution and 2 µL of samples was added into several well in 96-well microplate. The plate then incubated at 30°C for 6 mins (Widowati et al., 2017; Prahastuti et al., 2019; Prahastuti et al., 2020). ABTS reducing activity was calculated using the equation below:

$$\%Scavenging\ activity = \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} x\ 100 \quad (2)$$

2.4 H2O2 Scavenging Activity

Briefly, 3 µL of 5 mM H2O2 (Merck 1,08597), 12 µL of 1 mM ferrous ammonium sulphate (Sigma Aldrich, 215406) were added to 96-well microplate. Subsequently, the mixture then added by 60 µL of samples, 63 µL of Dimethyl sulfoxide (DMSO) (Supelco, 1.02952.1000) was added in the control well and 90 µL in the blank well. The plate was put in

dark condition, room temperature for 5 mins. Amount 75 µL of 10-phenanthroline (Sigma Aldrich, 131377) was added to the mixture, and the plate was re-incubated for 10 mins. The sample absorbance was measured at 510 nm using a microplate reader (Utami et al., 2017; Prahastuti et al., 2020). The H2O2 scavenging activity was calculated according to the following equation:

$$\%Scavenging\ activity = \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} x\ 100 \quad (3)$$

2.5 NO Scavenging Activity Assay

The samples were mixed with 40 µL of 10 mM sodium nitroprusside (Merck, 106541) in PBS. (Gibco, 1740576). The mixture was then incubated for 2 h at room temperature. Into the mixture, add 100 µL Griess reagent containing 1% sulfanilamide [Merck 111799, Germany], 2% H3PO4 (Merck, 100573), and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Aldrich, 222488) was added. The absorbance was measured by using a microplate reader at a wavelength of 546 nm (Utami et al., 2018 Prahastuti et al., 2019; Prahastuti et al., 2020). NO scavenging activity was calculated using the following equation:

$$\%Scavenging\ activity = \frac{control\ abs - sample\ abs}{control\ abs} x100 \quad (4)$$

2.6 FRAP Assay

Briefly, 10 mL of 300 mM acetate buffer was mixed with 1 mL of 10 mM 2,4,6-Tris (2-pyridyl)-s- triazine (TPTZ) (Sigma Aldrich, T1253), and 1 mL of 20 mM ferric chloride hexahydrate (Merck 1.03943.0250) at pH 3.6 to obtain FRAP reagent. Amount 142.5 µL of FRAP reagent and 7.5 µL of samples were then added into several well in a 96-well microplate. The plate was then incubated for 6 mins at 37°C and the absorbance was measured at 593 nm (Prahastuti et al., 2019; Prahastuti et al., 2020).

2.7 Statistical Analysis

The data were statistically analyzed using Oneway ANOVA and Tukey's HSD Post-hoc test (IBM SPSS Statistics for Windows, version 20.0, Armonk, NY) at a significance level of $P < 0.05$. The results were expressed as mean ± standard deviation.

3 RESULT AND DISCUSSION

3.1 Preparation of Nanoparticles

The nanoparticles were generated by the ionic gelation method. The positively charged substructure of chitosan interacted with the negative charge of TPP ions. The resulting nanoparticles showed a diameter of 330 nm.

3.2 DPPH Scavenging Assay

The radical DPPH is typically used as a substrate for the observation the antioxidant activity. The DPPH is typically used to attribute the radical scavenging activity of plant extracts or organic compounds (Widowati et al., 2016; Widowati et al., 2018). When a hydrogen donor is present, it becomes paired and absorption at 517 nm will be reduced (Widowati et al., 2018). Stable DPPH radicals will be reduced to diphenyl picrylhydrazine (DPPH-H) during the DPPH assay. The concentration that allowed an antioxidant to scavenge 50% of the free radical of DPPH corresponds to the median inhibitory concentrations (IC50) value. The lower the IC50 value, indicating higher the antioxidant activity.

Table 1: The IC50 value of Antioxidant Activities of Curcumin and Nanocurcumin.

Sample	IC_{50} of DPPH (µg/mL)	IC_{50} of ABTS (µg/mL)	IC_{50} of H_2O_2 (µg/mL)	IC_{50} of NO (µg/mL)
Curcumin	3.20	18.54	38.40	24.94
Nano curcumin	0.68	15.59	24.98	19.61

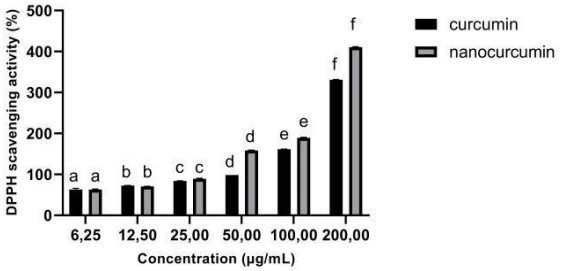

Figure 1: DPPH Scavenging Activity of Curcumin and Nanocurcumin. The data was presented as mean ± SD. Different letters (a,b,c,d,e,f) indicate a significant difference among concentration both curcumin and nanocurcumin based on Tukey's post hoc test ($p < 0.05$).

Based on the results, both curcumin and nanocurcumin were successful to show antioxidant

activity by DPPH scavenging assay. The results of this study were in line with some previous studies. Curcumin's antioxidant activity has been revealed in biological models by many researchers. There is several scientific evidence that proven curcumin's free radicals trapping capability on living cells (Rafiee et al., 2019). The IC50 DPPH scavenging activity of curcumin was 3.2 µg/mL, whereas the IC50 for nanocurcumin was 0.68 µg/mL. According to Widowati et al. (2017), the smaller the IC50 of a sample, the better the sample's ability to trap free radicals. The DPPH scavenging activity of curcumin and nanocurcumin were categorized very active when the IC50 value < 50 µg/mL (Marjoni and Zulfisa, 2017).

It was discovered that nanocurcumin was more active in the DPPH scavenging activity than curcumin because it provided the lower IC50 value (Table 1). Nanocurcumin has a better reduction activity than curcumin (Figure 1). This finding was was in line with the previous study, which concluded that the antioxidant activity of nanocurcumin was improved over that of curcumin. Moghaddasi et al. (2018) tested the synthesis of the nanocurcumin system (Nano- CUR) using the O/W nanoemulsion method. The antioxidant activities of Nano-CUR have more potential than its native curcumin and it has a potent candidate for treating chronic diseases (Moghaddasi et al., 2018). In another study, Hosseini et al. (2019) studied the effect of curcumin and nanocurcumin on the oxidant and antioxidant system of liver mitochondria using an aluminum phosphide (AIP)-induced toxicity model in rats. It was found that nanocurcumin enhanced the oxidative stress factors and protected the liver against the adverse effects of AlP by scavenging the free radicals and stabilizing the oxidative status (Hosseini et al., 2019).

Several physico-chemical properties considered to make nanocurcumin more effective than native curcumins are surface area, particle size, hydrophobicity, and surface charge. Previous studies have demonstrated that these properties can enhance solubility, bioavailability, and active targeting (Biswas et al., 2014). The reduction in particle size greatly increases the efficiency of nanocurcumin and makes it superior to curcumin. Nanocurcumin is an ideal drug compared to normal curcumin because of its larger surface area (Flora et al., 2013; Rahmat et al., 2020).

3.3 ABTS Reducing Activity Assay

ABTS is generated by the reaction between ABTS salt and a strong oxidizing agent (potassium permanganate/potassium persulphate). An ABTS reduction activity test was performed to measure the relative capacity of antioxidants to trap the ABTS produced. The reduction of the ABTS solution by antioxidants is measured by the spectrum of long-wave absorption. The effectiveness of the 50% trapping activity (IC50) is the concentration required for the sample to trap 50% of the ABTS radical. The lower the IC50 value, the higher the antioxidant activity. An ABTS reduction activity test in this study use the sample with the final concentration of 50 µg/mL; 25 µg/mL; 12.5 µg/mL; 6.25 µg/mL; 3.13 µg/mL; and 1.56 µg/mL. The ABTS IC50 reduction values are given in Table 1. The results of curcumin and nanocurcumin ABTS reduction activities are presented in Figure 2.

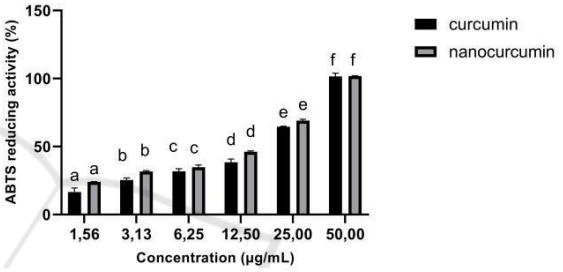

Figure 2: ABTS Reducing Activity of Curcumin and Nanocurcumin. The data was presented as mean ± SD. Different letters (a,b,c,d,e,f) indicate a significant difference among concentration both curcumin and nanocurcumin based on Tukey's post hoc test ($p < 0.05$).

The results indicated that the IC50 for the ABTS reducing activity assay was 18.54 µg/mL, while the IC50 for nanocurcumin was 15.59 µg/mL. Both curcumin and nanocurcumin were categorized very active toward ABTS reducing activity (Marjoni and Zulfisa, 2017). Nanocurcumin has higher ABTS reducing activity compared to curcumin since it has a lower IC50 (Table 1). This finding is also consistent with other previous study that concluded that the antioxidant activity of nanocurcumin was higher than curcumin (Hosseini et al., 2019).

3.4 H2O2 Scavenging Activity

Hydrogen peroxide (H2O2) plays an important role in the production of energy such as phagocytosis, *in vivo* systems, cell growth control, intercellular signal transfer, and the synthesis of essential biological compounds. H2O2 is a byproduct of normal aerobic metabolism that has generated and increased during training, infections, and stressful conditions (Mukhopadhyay et al., 2016). The concentration

allowed by an antioxidant to scavenge 50% of the H2O2 free radical is the IC50 value. The smaller the IC50 value indicating higher the antioxidant activity. The IC50 value of the H2O2 radical scavenging activity of curcumin and nanocurcumin were presented in Table 1. The results of H2O2 reduction activities of curcumin and nanocurcumin are shown in Figure 3.

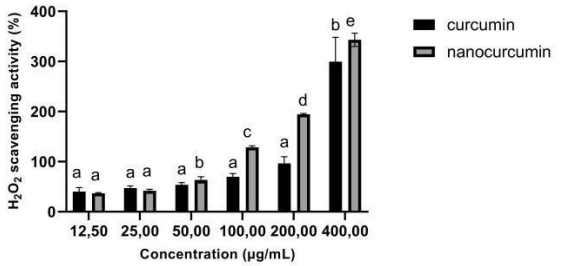

Figure 3: H2O2 Scavenging Activity of Curcumin and Nanocurcumin. The data was presented as mean ± SD. Different letters (a,b) for curcumin and different letters (a,b,c,d,e) indicate a significant difference among concentration based on Tukey's post hoc test ($p < 0.05$).

Based on the results, the IC50 of H2O2 scavenging activity for curcumin was 38.40 µg/mL, whereas the IC50 for nanocurcumin was 24.98 µg/mL. According to Widowati et al. (2017), the lower the IC50 for a sample, the higher the sample's ability to trap free radicals. Both curcumin and nanocurcumin were categorized as very active toward H2O2 scavenging activity (Marjoni and Zulfisa, 2017). Nanocurcumin was found to be more active in H2O2 scavenging activity than curcumin because it had the lower IC50 (Table 1). Nanocurcumin has a better reduction activity in comparison to curcumin (Figure 3). This finding was appropriate for an earlier study that concluded that the antioxidant activity of nanocurcumin was improved over curcumin (Hosseini et al., 2019).

3.5 NO Scavenging Activity

Nitric oxide (NO) is a potent signaling mediator in several cellular processes. This molecule acts as a mediator in the regulation of inflammation, neurotransmission, host defense mechanisms, and vascular tonus (Utami et al., 2018). In this study, NO scavenging activity was tested by using the sample with final concentration of 133.33 µg/mL; 66.67 µg/mL; 33.33 µg/mL; 16.67 µg/mL; 8.33 µg/mL; and 4.17 µg/mL. The IC_{50} value of NO radical scavenging activity of curcumin and nanocurcumin are presented in Table 1. The results of NO reduction activities from curcumin and nanocurcumin are shown in Figure 4.

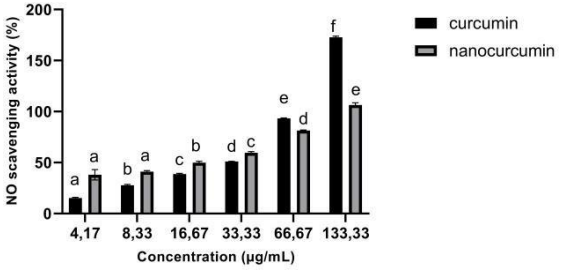

Figure 4: NO Scavenging Activity of Curcumin and Nanocurcumin. The data was presented as mean ± SD. Different letters (a,b,c,d,e,f) for curcumin and different letters (a,b,c,d,e) indicate a significant difference among concentration based on Tukey's post hoc test ($p < 0.05$).

The result indicated that the IC50 value of the NO reducing activity was 24.94 µg/mL, whereas the IC50 of nanocurcumin was 19.61 µg/mL. Both curcumin and nanocurcumin were categorized as very active toward NO scavenging activity (Marjoni and Zulfisa, 2017).

Nanocurcumin showed a higher NO scavenging activity compared to curcumin because it has a lower IC50 (Table 1). The reduction activity of this assay differed from that of the other assay. Curcumin had a trapping activity of 172.72±1.22%, higher than nanocurcumin trapping activity of 106.44±2.22%, especially at the highest concentration (133 µg/mL) (Figure 4). This finding is also in line with another previous study that concluded that nanocurcumin had higher antioxidant activity than curcumin (Hosseini et al., 2019).

3.6 FRAP Activity

FRAP assays are commonly used for antioxidant properties based on the electron transfer potential of the existing antioxidants. The FRAP method was based on the reduction of analog ferroin in an acid medium, the $TPTZ3^{+}$ in the colored Fe^{2+} complex of $Fe(TPTZ)^{2+}$ (greatly blue) by antioxidant (Widowati et al., 2018). A reduction in the tripyridyltriazine Fe(III) complex at 593 nm results from the absorbance of the Fe(II) complex. The results of FRAP reduction activities from curcumin and nanocurcumin are shown in Figure 5.

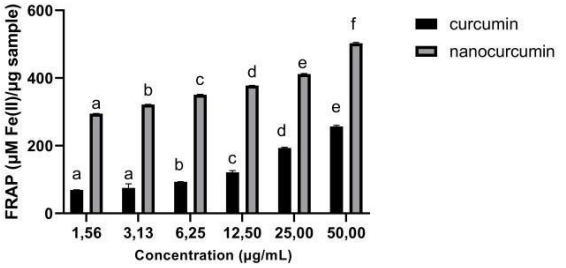

Figure 5: FRAP Scavenging Activity of Curcumin and Nanocurcumin. The data was presented as mean ± SD. Different letters (a,b,c,d,e) for curcumin and different letters (a,b,c,d,e,f) indicate a significant difference among concentration based on Tukey's post hoc test ($p < 0.05$).

Based on the results, nanocurcumin is more active in FRAP scavenging activity than curcumin because it provides a lower IC50 value (Table 1). Nanocurcumin has a better reduction activity than curcumin (Figure 5). At the highest concentration (50 µg/mL), nanocurcumin had a trapping activity of 502.92±2.55%, higher than the curcumin trapping activity of 256.50±3.68%.

This finding was appropriate for a previous study that concluded that the antioxidant activity of nanucurcuma was improved over curcumin (Hosseini *et al.,* 2019). Our current findings could demonstrate the antioxidant activity of curcumin and nanocurcumin. Nevertheless, this study has not been able to describe the effect of curcumin and nanocurcumin treatment on cells. Future research is expected to be able to test curcumin and nanocurcumin's effect on cells or *in vivo.*

4 CONCLUSIONS

Nanocurcumin was found to be more effective than native curcumins in the free radical scavenging activities of DPPH, ABTS, H2O2, NO, and FRAP. Nanocurcumin showed higher antioxidants reduction activity in DPPH, ABTS, H O, and FRAP compared to curcumin. Both curcumin and nanocurcumin have very active antioxidants.

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