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SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF NITROGEN-DOPED CARBON DOTS

Wiwit Sepvianti, Suherman Suherman, Bambang Purwono

The aim of our work *is to synthesize N-CD and to evaluate its antibacterial activity against Gram-positive and Gram-negative bacteria.*

Material and Methods. *N-CDs were synthesized from citric acid and urea using a bottom-up approach using microwave-assisted treatment. The nitrogen doping in the CDs structure was studied on an FTIR (Shimadzu IR Prestige-21). The optical properties of N-CDs were detected using fluorescence spectroscopy (Shimadzu RF-6000) and UV-Vis absorption spectroscopy (Shimadzu 1800). The size of N-CDs nanoparticles was confirmed on a TEM (JEOL-JEM 1400). Elemental analysis was performed on an ELEMENTAR vario EL cube. The antibacterial activity of N-CDs was investigated using the agar disk diffusion and dilution method against S.aureus and E.coli bacteria.*

Results. *The structure and characteristics of N-CDs were confirmed through several stages. The size of N-CDs based on TEM images ranged from 2.4 to 2.6 nm with a fairly uniform size distribution. The success of nitrogen doping was confirmed through fluorescence spectra, UV-vis absoption spectra, FTIR spectra and elemental analysis. The antibacterial activity tests showed that N-CDs were able to inhibit the growth of Gram-positive bacteria (S. aureus, clear zone = 9.93±0.2 mm, MIC = 50 μg/mL, IC50 = 272 μg/mL) and Gram-negative bacteria (E. coli, clear zone = 8.29±0.3 mm, MIC = 50 μg/mL, IC50=339 μg/mL) which makes it a broad-spectrum antibacterial candidate.*

Conclusions: *N-CD was successfully synthesized and exhibited broad bacterial inhibitory activity. However, its inhibitory performance against Gram-positive bacteria was much better, as seen from the clear zone diameter and IC50 values*

Keywords: *antibacterial, bactericides, carbon dots, S. aureus, E. coli*

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1. Introduction

Pathogenic bacteria can cause persistent and widespread infectious diseases in both humans and animals. They are found in food, water, soil, and other objects and can spread through direct contact [1]. Various diseases caused by bacterial infections are frequently reported, such as tuberculosis, typhus, diarrhea, dysentery, diphtheria, cholera, pneumonia, gonorrhea, tetanus, syphilis, and leptospirosis [2, 3]. Challenges faced in handling bacterial infections include environmental changes, antibacterial resistance and the emergence of new pathogens. Therefore, various research initiatives and the development of bactericides continue to be encouraged. One of the promising potential materials as an antibacterial is carbon dots (CDs) [4].

CDs, one kind of nanomaterial, widely known for their excellent optical properties and widely used as biosensors, apparently have promising potential as antibacterials because with extremely small size (under 10 nm), CDs provide the opportunity to open a wide interaction area with the bacterial cell wall [5, 6]. In contrast to traditional bactericides, CDs have excellent solubility, biocompatibility, a large surface area, and low cytotoxicity, making CDs appropriate for superficial disinfection and daily sterilization. The basic mechanism of CDs in paralyzing bacteria is thought to be through membrane damage and activation of reactive oxygen species (ROS). Even though bacteria have a defense mechanism against ROS, excessive ROS will damage DNA, proteins and mitochondria of bacteria, resulting in cell damage that leads to death [7, 8].

The antibacterial activity of CDs can be enhanced by doped heteroatoms or surface modification with persistent antibiotics. Nitrogen and Fluorine-codoped CDs (N,F-CDs) of o-phenylenediamine and 2,3,5,6-tetrafluoroterephthalic acid were reported to have antibacterial activity against *E. coli* and *S. aureus* [9]. Other studies also reported the antibacterial activity of S,N-doped CDs against *S. aureus*, *Enterococcus sp., P. aeruginosa,* and *E. coli* [10, 11]. In addition to being active as an antibacterial, doped CDs are also reported to be active as antioxidants, antivirals, anticancer, and antifungal [8, 12].

In this study, nitrogen-doped CDs (N-CDs) were synthesized using inexpensive and abundant raw materials, citric acid and urea. The synthesis of N-CDs was carried out with a low-power, environmentally friendly household microwave. Meanwhile, the antibacterial properties of N-CDs were observed using the dilution method and agar disk diffusion to determine their antibacterial abilities quantitatively and qualitatively. The test bacteria used were *S. aureus* (Gram-positive) and *E. coli* (Gram-negative), both of which were chosen to investigate the character of N-CDs as narrow or broad antibacterial spectrum.

2. Planning (methodology) of research

The research methodology was based on two experimental stages. The first stage was the optimization of urea content in N-CDs and the investigation of its structure and characteristics. The second stage included the study of the antibacterial activity of N-CDs. In this stage, two methods were used: agar disk diffusion and dilution. The agar disk diffusion method was used as the initial stage of observing the bacterial inhibition ability of N-CDs against Gram-positive and Gram-negative bacteria. Next, the dilution method investigated the minimum inhibitory concentration (MIC) and the concentration to inhibit 50% growth (IC_{50}). The research results were interpreted and conclusions were drawn.

3. Material and methods

3. 1. Synthesis of N-CDs

The N-CDs were synthesized according to [10] with some modifications. Synthesis used citric acid and urea as the source of C and N with microwave-assisted synthesis (Fig. 1). The 0.2 g of citric acid was mixed with urea with various mass ratios of urea to citric acid of 0, 25, 50, 75, 100 and 125%. A mixture of citric acid and urea dissolved in 10 mL of aquabidest was placed in a Teflon chamber (PTFE Chamber). The solution was then heated with a microwave oven (450 W, Medium Heat, Sharp) for 25 min. The solution turned into light brown caramel indicating the formation of CDs.

The crude products were dissolved in 20 mL phosphate-buffered saline (PBS). The purification products were performed by centrifugation at 10.000 rpm for 30 min to remove the large solids, followed by filtration via a 0.1 µm filter membrane. The filtrate obtained was characterized.

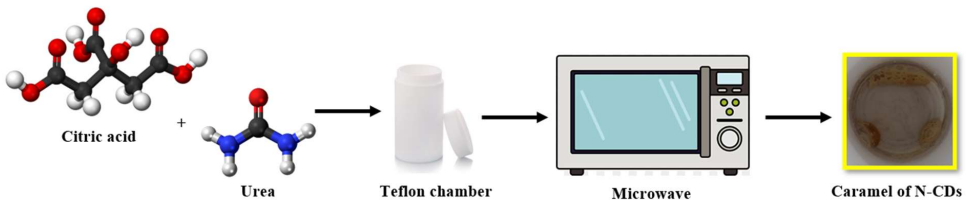


Fig. 1. Schematic to synthesize the N-CDs

3. 2. Characterization of the CDs

The optical properties of the synthesized carbon dots were measured using fluorescence spectroscopy (Shimadzu RF-6000) and UV-Vis absorption spectroscopy (Shimadzu 1800). The insights into particle size and uniformity were investigated using a transmission electron microscope (TEM) (JEOL-JEM 1400). The nitrogen doping in the CDs structure was studied through analysis using Fourier transform infrared spectroscopy (FTIR) (Shimadzu IR Prestige-21) which can detect the presence of marker functional groups on N-CDs. Elemental Analysis was carried out using an elemental composition analyzer (Elemental-Vario Macro Cube).

3. 3. Antibacterial activity assay

Agar disk diffusion method. The antibacterial activity test of N-CDs using agar disc diffusion is a preliminary test to determine the potential for inhibiting N-CDs bacteria. A paper disc dipped in N-CDs solution for 10 minutes was placed on the solidified agar media containing *S. aureus* and *E. coli*. The N-CDs solvent, phosphate buffered saline (PBS, pH= 7.40), served as a negative control, while amoxicillin served as a positive control. Observations of the bacterial inhibition zone were carried out after 18 hours of incubation. The diameter of the inhibition zone formed was measured manually using a vernier caliper, the difference between the diameter of the clear zone and the diameter of the paper disc (diameter 6 mm) [13].

Dilution methods. Dilution methods are the most appropriate ones to determine the MIC and IC_{50} values, since they offer the possibility to estimate the concentration of the tested antimicrobial agent in liquid medium (macrodilution or microdilution). A total of 100 µL of bacterial suspension in saline solution containing 10^4 CFU/mL of bacteria (*S. aureus* and *E. coli*) was put into each 96-microwell plate and 100 µL of N-CDs solution with a series of concentrations of 25; 50; 100; 200; 400; 800 µg/mL was added. As a media control and bacterial control, 100 µL of saline media and bacterial suspension were added to the wells. The culture that had been given the test material was incubated for 18 hours at 37°C in an incubator. After the incubation process was completed, the absorbance was read using a microplate ELISA reader at λ 405 nm. The MIC value is expressed in µg/mL or mg/L as the lowest concentration that inhibits the growth of microorganisms. The IC_{50} value was determined by a linear regression equation of the dose concentration of the test compound versus the percentage of bacteria [13].

4. Results

The effect of Nitrogen atom incorporation into CDs was carried out by varying the contents of urea to citric acid with mass ratio variations in urea from 25% to 125%. The addition of Nitrogen atoms aims to increase the fluorescence of CDs. In Fig. 2, the addition of urea significantly increased the CD fluorescence at 50% urea mass. The escalation of N-CDs fluorescence intensities could be attributed to the presence of the high electronegativity of Nitrogen atom (3.04 on the Pauling scale) and the presence of a lone pair of electrons that can be transferred to the phi orbital of the sp^2 carbon structure [14, 15]. The transferred electron could change the electronic structure of the carbon dot, thus changing the photophysical properties of the carbon dot, such as enhanced photoluminescence, photoluminescence shift, and changes in light absorption [16].

The UV–vis absorption spectrum (Fig. 3, *a*) represents an absorption band at 263 nm, which indicates the π–π* transition of the C=C bond. In addition, a broad band was observed at 335 nm, corresponding to the *n*–π* transition of the C=O bond [17]. These results confirmed

the formation of carbon dot nanoparticles, as reported in previous studies. N-CDs exhibit blue fluorescence under 254 nm UV light (Fig. 3, *b*), which is thought to be a quantum confinement effect.

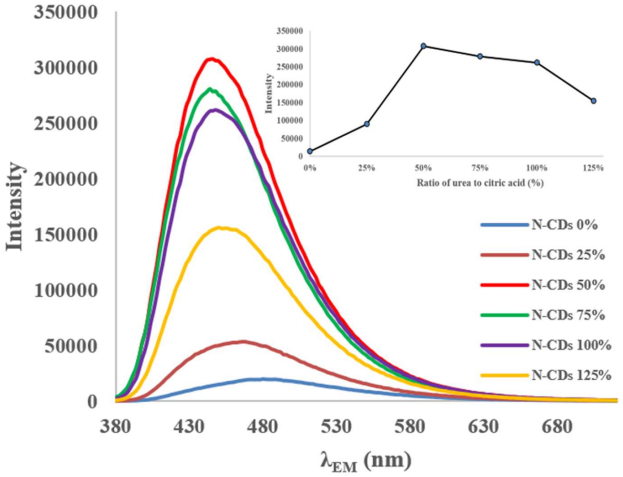
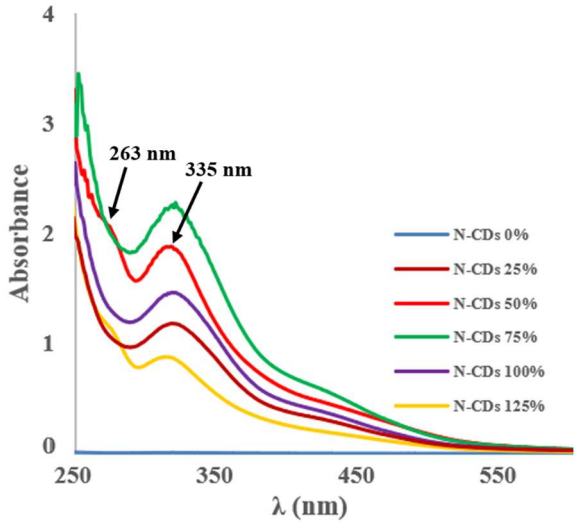


Fig. 2. Fluorescence spectra of varied N-CDs



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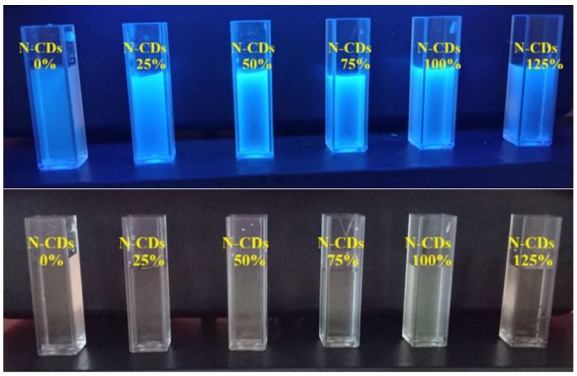
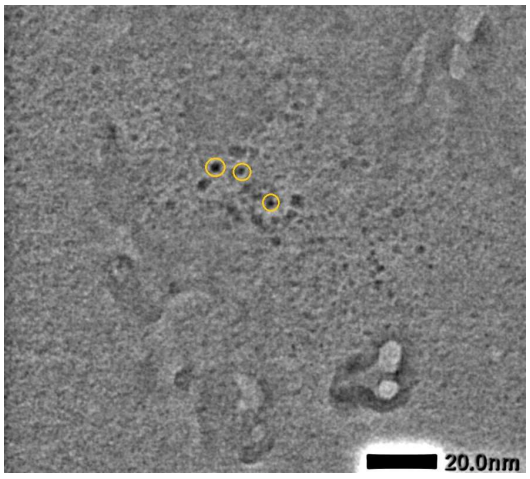


b

Fig. 3. UV-vis absorption spectra and optical images of varied N-CDs: *a* – UV-vis spectra; *b* – optical images under UV light (254 nm) and daylight

The basic physical properties of N-CDs, including size and morphology, were characterized using TEM. The TEM image exhibits that the size of N-CDs is in the range of 2.4–2.6 nm with a fairly uniform size distribution (Fig. 4, *a*– *b*). This confirmed that obtained N-CDs are nanomaterials because they have a diameter under 100 nm [18, 19]. Meanwhile, the size of N-CDs is an advantage that allows for a larger reaction area between the N-CDs and target cells [7]. This size also opens up opportunities for N-CDs to be applied as biosensors [14, 15], drug delivery and antibacterial materials [7, 17].

FTIR spectra of synthesized N-CDs reveal the surface functional groups of N-CDs and assert their chemical composition (Fig. 5). Broad absorption bands from 3000-3500 cm^{-1} correlate with stretching vibrations of hydroxyl (O-H) and amine (N-H) groups [16]. The wavenumbers of 1720 and 1630 cm^{-1} are assigned to the stretching of C=O and C=C [20, 21]. Stretching vibrations of the N-H band show at 1580 cm^{-1}, C-N reveals at 1404 cm^{-1} and C-O bending vibration exists at 1203 cm^{-1}. These represent the integration of nitrogen into the sp^2 carbon framework that is assigned to N-CDs [22]. The presence of hydroxyl and carboxyl functionalization on the N-CDs surface confirmed its water solubility [21]. This property is a privilege of N-CDs which makes it easier to measure antibacterial properties using both agar disk diffusion and dilution methods.



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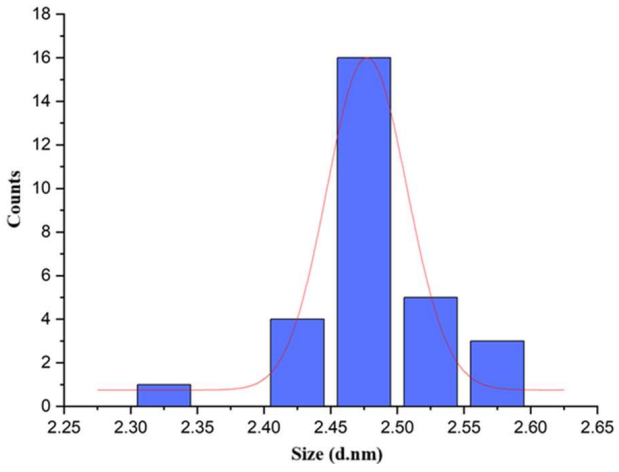

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Fig. 4. Physical properties of N-CDs: *a* – TEM image; *b* – histogram of N-CDs size distribution

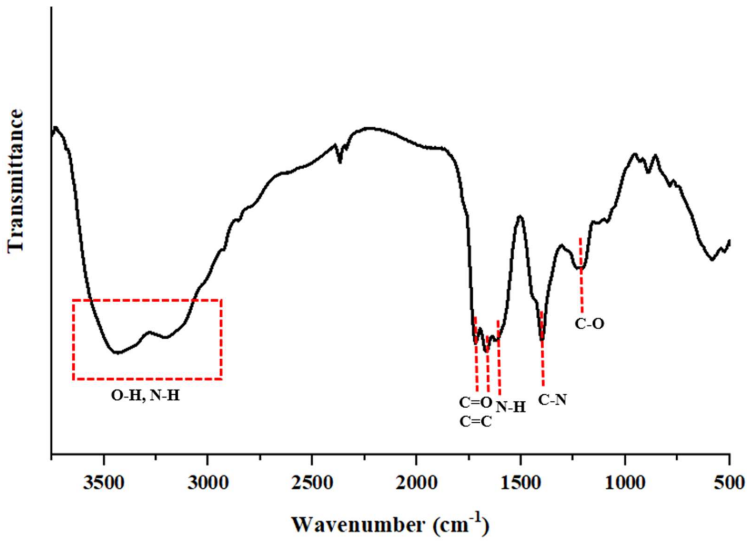


Fig. 5. FTIR spectrum of N-CDs

Meanwhile, the elemental analysis (Table 1) provides additional structural information on N-CDs that indicate an increase in nitrogen content from CDs to N-CDs [23].

Table 1

CHN Elemental Analysis of N-CDs

Sample	N%	C%	H%
CDs	N/A	84.18	9.784
N-CDs	26.47	54.13	6.499

Note: N/A – not available.

Antibacterial activity test of N-CDs using agar disc diffusion was conducted as a preliminary test to determine the potential of bacterial inhibition by N-CDs [13]. At this stage, the concentration of N-CDs used was 20 mg/mL and Amoxicillin was chosen as a positive control. The results showed that N-CDs could produce a clear zone around the bacterial colonies (Fig. 6), indicating bacterial growth inhibition due to the presence of N-CDs on the agar plate [4, 24]. Compared with amoxicillin, a broad-spectrum antibiotic, N-CDs showed similar effects on both Gram-positive and Gram-negative bacteria (Table 2).

The results obtained are in line with several previous studies that CDs have broad-spectrum bactericidal activity. For instance, N,S-CDs synthesized from citric acid and cysteine has been reported to inhibit the growth of *S. aureus* (Gram-positive) and *P. aeruginosa* (Gram-negative) [10]. Furthermore, halogen and nitrogen-doped carbon dots also reported were able to inhibit the growth of *S. saprophyticus; S. aureus; L. ivanovii; L. innocua; E. faecalis* (Gram-positive) and *E. coli; S. enteritidis; E. aerogenes; C. freundii; S. typhimurium* (Gram-negative) [7].

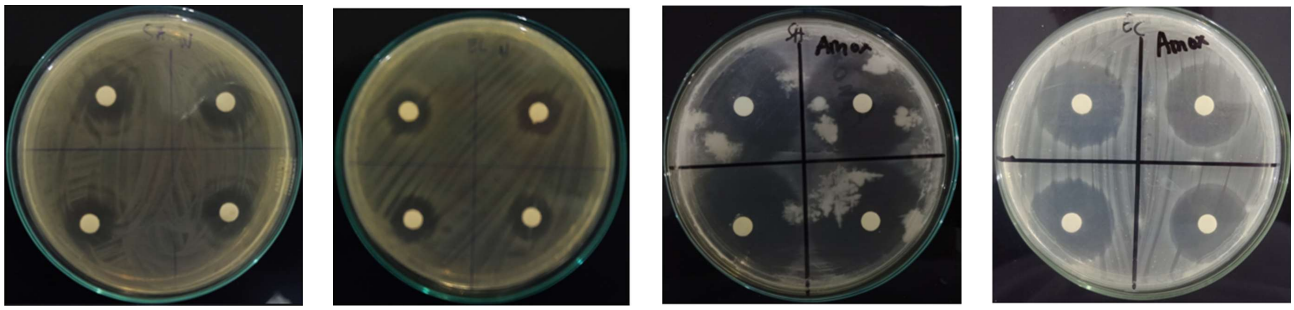
The antibacterial activity study of N-CDs was continued using a dilution test to investigate the minimum inhibitory concentration (MIC) and half-maximal inhibitory concentration (IC_{50}). The MIC value provides an estimate of the minimum inhibitory concentration of bacteria, while the IC_{50} value indicates the concentration required to inhibit 50% of bacterial cell growth [4, 10, 11]. MIC and IC_{50} values were measured through color changes resulting from the interaction of bacteria with N-CDs after 18 hours of incubation. These changes were measured using an ELISA microplate reader based on the intensity of light absorption passing through the sample [13] (Fig. 7).

Table 2

Antibacterial activity of N-CDs.

Test N-CDs	*S. aureus*			*E. coli*		
	Clear zone (mm)	Average ± SD	Inhibitory activity (%)	Clear zone (mm)	Average ± SD	Inhibitory activity (%)
1	10.10	9.93 ± 0.2	34.72	8.84	8.29 ± 0.3	36.67
2	9.86			8.05		
3	10.08			8.18		
4	9.66			8.08		
(+) Amoxicillin	28.60 ± 1.13			22.61 ± 0.92		

Note: tests were performed at 20 mg/ mL.



a *a* *a* *a*

Fig. 6. Clear zones show the bacterial inhibition of: *a* – N-CDs against *S. aureus*; *b* – N-CDs against *E. coli*; *c* – Amoxicillin against *S. aureus*; *d* – Amoxicillin against *E. coli*

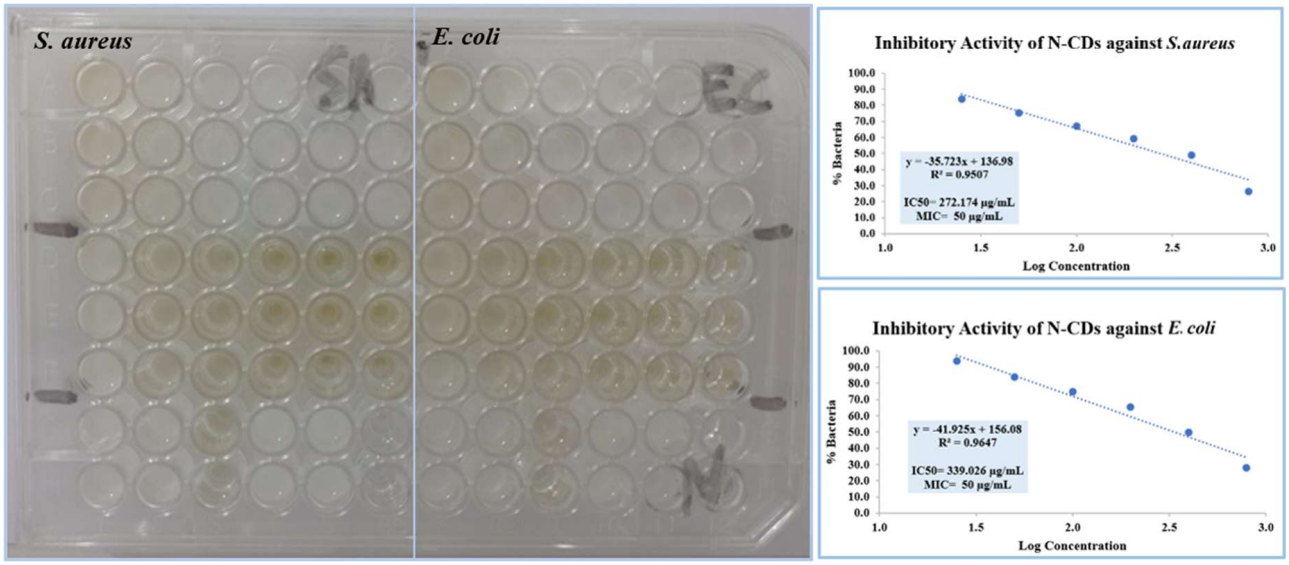


Fig. 7. MIC and IC_{50} value of N-CDs

The results showed that at 50 µg/mL of N-CDs (Fig. 7), there was a decrease in turbidity in *S. aureus* and *E. coli* bacteria of 24.5% and 16.0% respectively. Indicates that the minimum concentration of N-CDs to inhibit bacterial growth is approximately 50 µg/mL. Furthermore, with the linear regression, it was found that the effective concentration of N-CDs to inhibit 50% of bacterial growth was about 272.174 µg/mL; 339.026 µg/mL for *S. aureus* and *E. coli* bacteria, respectively. Based on observations using agar disks and dilution, N-CDs work effectively on both Gram-positive and Gram-negative bacteria, however, the N-CDs tend to show better performance against *S. aureus* (Gram-positive). Table 3 presents the comparison of MIC and IC_{50} value with some other previously reported papers based on the bacterial activity of CDs.

Fig. 8 illustrates the fluorescence intensity of N-CDs at different pH values, demonstrating their pH sensitivity due to the presence of surface functional groups, particularly carboxyl (–COOH) and amino ($–NH_2$) groups. Stability studies of N-CDs in various buffer solutions and citrated plasma were conducted to evaluate their response under biological environments. The fluorescence intensity was significantly reduced under acidic conditions (pH 4.01), reached an optimum at physiological pH (7.35–7.40), and decreased again under alkaline conditions (pH 9.18).

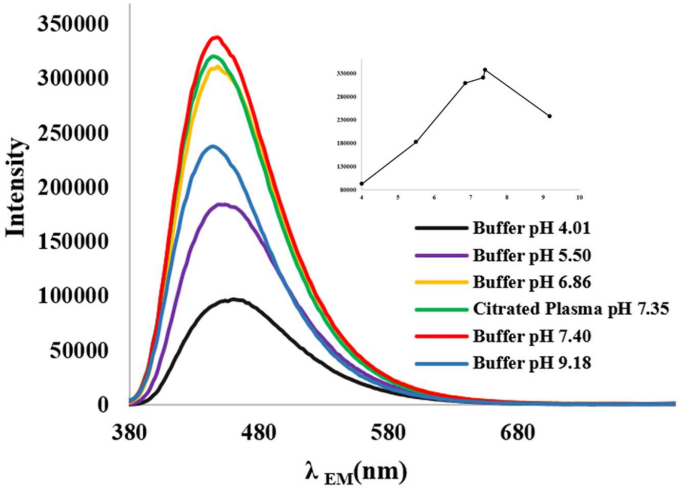


Fig. 8. Fluorescence spectra of N-CDs at various pH values

The pronounced decrease in fluorescence intensity at highly acidic pH can be attributed to the formation of hydrogen bonds among surface functional groups, leading to particle aggregation and subsequent fluorescence quenching. Under alkaline conditions, the reduction in fluorescence intensity is likely due to the deprotonation of carboxylic acid groups, resulting in the accumulation of negative charges on the N-CDs surface. This increase in surface charge enhances non-radiative recombination processes, thereby partially quenching the fluorescence emission [4, 27]. These findings indicate that N-CDs exhibit optimal fluorescence performance under physiological pH conditions, highlighting their potential suitability for biological applications.

Table 3

The comparative list of MIC and IC_{50} value of this work and previously reported

Material	Bacterial Species	MIC	IC_{50}	Reference
Citric acid, Polyethene pilyamine, L-glutathione	*S. aureus; P. aeruginosa*	15 µg/mL; 480 µg/mL	–	[25]
Tea Polyphenols, Ethylenediamine	*S. aureus*	375 µg/mL	–	[26]
Garlic	*E. coli*	50 µg/mL	–	[27]
Orange Juice	*E. coli*	0.1 ppm	–	[17]
o-phenylenediamine	*E. coli*	–	200 µg/mL	[4]
Glucose, Porphyrin	*P.aeruginosa*	16 µg/mL	–	[28]
Citric acid, Urea	*S. aureus; E. coli*	50 µg/mL	272 µg/mL; 339 µg/mL	This work

5. Discussion

During storage, the synthesized N-CDs should be stored in caramel rather than dissolved in water. Based on observations, N-CDs solutions are stable before

2 weeks of storage because after that, aggregation occurs which reduces their optical properties and bacterial inhibitory activity. This aggregation condition is thought to occur due to the weakening of the electrostatic repulsion between particles, causing Van der Waals attractive forces or hydrophobic interactions to become more dominant [18]. In addition, N-CDs also need to be dried before elemental analysis to obtain valid measurement results. Drying is carried out using the freeze-drying method for 48 hours.

The antibacterial activity tests showed that N-CDs were able to inhibit the growth of Gram-positive bacteria and Gram-negative bacteria, which makes them a broad-spectrum antibacterial candidate. The fundamental mechanism of N-CDs as antibacterials is thought to begin with disruption of the bacterial membrane area (Fig. 9). Despite the differences in structure and cell wall composition of Gram-positive and Gram-negative bacteria, both of them present an overall negative charge at the surface that makes electrostatic interaction with the positively charged of N-CDs [9]. This electrostatic interaction causes damage to the cytoplasmic membrane and disrupts the selective permeability of bacteria, allowing N-CDs to invade areas within the bacterial cell [6, 29]. After invading, N-CDs then induce the production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide $\left(\bullet O_2^-\right)$ and hydroxyl radicals ($OH^\bullet$). The presence of ROS ultimately triggers damage to cellular components such as lipids, deoxyribonucleic acid, proteins, and mitochondrial membranes. Resulting in cell leakage and impaired cellular function, leading to bacterial death [7, 30].

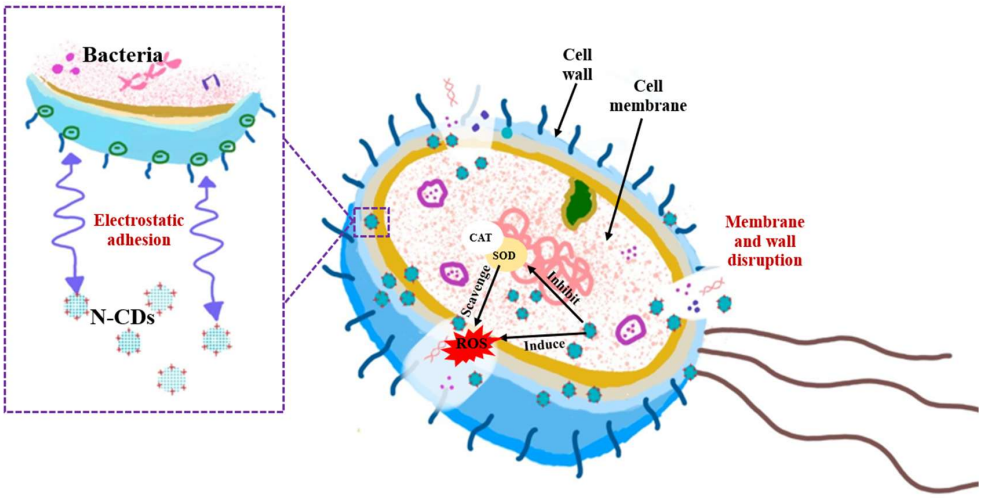


Fig. 9. N-CDs mechanism of antibacterial action

Practical relevance. The results obtained will contribute as an alternative new bactericidal agent that is environmentally friendly and derived from economical and abundant materials.

Research limitations. The study we planned was completed in full, and the results obtained are predictable and reproducible. A limitation of this study is that the antibacterial activity of N-CDs only covers *S. aureus* and *E. coli*.

Prospects for further research. The presence of functional groups on N-CDs, such as carboxylate, hydroxyl, and amine, provides a wider modification range for N-CDs. The surface of N-CDs can be modified with various active compounds and enhancing their pharmacological activity, because as nanomaterials, N-CDs have the excellent Potential to be drug delivery agents.

6. Conclusions

In this study, N-CDs were successfully synthesized by microwave-assisted treatment of citric acid and urea. TEM analysis showed that N-CDs nanoparticles had been formed with a size distribution of 2.4–2.6 nm. The structure of N-CDs was confirmed through UV-visible spectroscopy, FT-IR spectroscopy and elemental analysis. The UV-vis absorption spectrum captured the characteristic absorption of C=C and C=O bonds of N-CDs detected at wavelengths of 263 and 335 nm. Furthermore, the stretching vibration of the N-H band seen at 1580 cm^{-1} and C-N seen at 1404 cm^{-1} confirmed the integration of nitrogen into the sp2 carbon framework assigned to N-CDs. The presence of N atoms in N-CDs was also detected by the elemental analyzer with an abundance of 26.47%. Following this confirmation, the synthesized N-CDs showed broad antibacterial activity against both Gram-positive and Gram-negative bacteria. At a concentration of 20 mg/mL, N-CDs produced a clear zone of 9.93 ± 0.2 mm against *S. aureus* and 8.29 ± 0.3 mm against *E. coli*. Quantitatively, N-CDs also appeared to be more active in inhibiting the growth of *S. aureus* with a smaller IC_{50} value of 272 µg/mL compared to *E. coli* at IC_{50} = 339 µg/mL.

Conflict of interest

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Additional data will be made available on reasonable request

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies in creating the submitted work.

Authors' contributions

Wiwit Sepvianti: conception and design; acquisition and data; analysis and interpretation of data; drafting of the manuscript; and statistical analysis, **Suherman Suherman**: conception and design; critical revision of the manuscript for importanat intellectual content; and supervision, **Bambang Purwono**: conception and design; analysis and interpretation of data; critical revision of the manuscript for importanat intellectual content; and supervision.

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Wiwit Sepvianti, Assistant Professor, Department of Chemistry, Universitas Gadjah Mada, Yogyakarta, Indonesia, 55281, Department of Blood Bank Technology, STIKES Guna Bangsa Yogyakarta, Sleman, Indonesia, 55283
ORCID: https://orcid.org/0000-0003-1587-0044

Suherman Suherman, Professor, Department of Chemistry, Universitas Gadjah Mada, Yogyakarta, Indonesia, 55281
ORCID: https://orcid.org/0000-0002-3863-1057

Bambang Purwono*, Professor, Department of Chemistry, Universitas Gadjah Mada, Yogyakarta, Indonesia, 55281
ORCID: https://orcid.org/0000-0001-6284-7782

***Corresponding author:** Bambang Purwono, e-mail: purwono.bambang@ugm.ac.id*