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Biyoteknolojik Uygulamalar İçin Limon Suyundan Karbon Noktalarının Mikrodalga Tabanlı Sentezi

Erkan MOZİOĞLU[1*]

[1]Department of Medical Biotechnology, Institute of Health Sciences, Acibadem Mehmet Ali Aydinlar University, İstanbul, Türkiye

[1]https://orcid.org/0000-0002-3027-5166
*Sorumlu yazar: erkan.mozioglu@acibadem.edu.tr; erkanmozioglu@yahoo.com

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Anahtar Kelimeler:
Karbon noktalarının sentezi (karbon dot sentezi)
Mikrodalga yöntemi
Bovin Serum Albumin (BSA) sönümleme/saptama
Antimikrobiyal aktivite.

ÖZ

Bu çalışmada, doğal bir sitrik asit ve azot/kükürt kaynağı olan limon suyundan karbon noktalarının (CD'ler) sentezi için basit bir mikrodalga yöntemi kullanılmıştır. Bilim yazınında, limon suyundan CD sentezi için hidrotermal temelli yöntemler kullanılmıştır. Daha basit ve hızlı olması açısından bu çalışmada mikrodalga yöntemi kullanılarak karbon noktalarının sentezi araştırılmıştır. Hidrotermal bazlı diğer yöntemlerle karşılaştırıldığında, bu yöntemle elde edilen CD'lerin kuantum verimi oldukça düşük bulunmuştur (%0.74). Bununla birlikte, sentezlenen CD'lerin, biyoteknolojide kullanılan sığır serum albüminin (BSA) floresans ışımasını sönümlediği gösterilmiştir. Ayrıca, *S. epidermidis*, *C. albicans*, *S. aureus*, *P. aeruginosa*, *E. coli*, *E. feacalis*, *K. pneumonia*, *A. baumanii* gibi klinik olarak önemli hastalık etkenleri üzerinde mikrop-kıran (*antimikrobiyal*) etkilere sahip olduğu bulunmuştur.

Microwave-Based Synthesis of Carbon Dots From Lemon Juice For Biotechnological Applications

Research Article

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ABSTRACT

In this study, a simple microwave method was used for the synthesis of carbon dots (CDs) from lemon juice as a natural source of citric acid and nitrogen/sulfur. In the synthesis of CDs from lemon juice, hydrothermal-based methods were used in the literature. In terms of being simpler and faster, the synthesis of carbon dots from the microwave method was investigated in this study. Compared to the hydrothermal-based methods, the quantum yield of CDs obtained by this method was found to be quite low (0.74%). However, the synthesized CDs showed quenching features over bovine serum albumin (BSA) proteins, which can be used in biotechnology. Moreover, it has been found to have antimicrobial effects on clinically important pathogens such as *S. epidermidis*, *C. albicans*, *S. aureus*, *P. aeruginosa*, *E. coli*, *E. feacalis*, *K. pneumonia*, and *A. baumanii*.

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1. Introduction

Carbon dots (CDs) have been used in many different applications such as biosensors, drug delivery, antimicrobial/antiviral/cancer therapies in medical biotechnology, since they are synthesized environmentally friendly and cheaply as well as they have very unique optical properties (Pramanik et al., 2018; Huo et al., 2020; Leong et al., 2020). Natural CDs are new alternatives and they can be

obtained from natural sources such as fruits, flowers, eggs, fruit juice, carrot roots etc. (Pramanik et al., 2018; Huo et al., 2020; Nair et al., 2020). For today, natural CDs have low fluorescence intensities, however studies to improve their fluorescence features have been going on (Huo et al., 2020). For these purposes, studies have been focused on synthesis methods such as the use of different solvents or compounds, surface modifications, heteroatom addition etc. (Huo et al., 2020).

Natural products are used as sulfur and nitrogen-rich sources which are very important for the synthesis of CDs (Monte-Filho et al., 2019). Because of the carbonization temperature, the use of citric acid is also proper for producing of CDs (Tadesse et al., 2018). As a natural source of citric acid, lemon juice has been already used for the synthesis of fluorescent water soluble CDs by using a hydrothermal method at 280°C for 12h or 200°C for 3h (Tadesse et al., 2018; Hoan et al., 2019). Besides hydrothermal methods, microwave, pyrolisis, ultrasonic, laser ablation, and chemical oxidation methods have been used for the synthesis of CDs from other natural sources (Nair et al., 2020).

Proteins like serum albumin are very important to transport biologically active compounds in the blood (Wang et al., 2009). The bovine serum albumin (BSA) is used for drug targeting studies because human serum albumin and BSA are homologous proteins (Nair et al., 2020). In biotechnology, interactions of BSA and some fluorogenic compunds such as acridine orange are used for their quenching features over these proteins (Nair et al., 2020).

In this study, it was aimed to synthesize natural carbon dots from lemon juice by using a simple microwave method. Besides their optical properties, quenching features over BSA were studied. Also, their antibacterial and antifungal effects on clinically important pathogens such as *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus feacalis* ATCC 29212, *Klebsiella pneumonia* ATCC 700603, *Acinetobacter baumanii* BAA717 were studied. According to our knowledge, these findings about BSA quenching effects and antimicrobial activities of lemon-based CDs are the first for the literature.

2. Material and Method

Lemon for synthesis of the fluorescent CDs were purchased from a local market in İstanbul, Türkiye. Fouirer Transform Infrared Spectrometer (FTIR) measurements were performed by Perkin Elmer FT-IR Spectrometer. Fluorescence and absorbance spectra was obtained by Varioscan Spectrophotometer (Thermofisher, USA). Transmission Electron Microscopy (TEM) images were recorded by TALOS L120 C.

2.1. Synthesis of CDs

Carbon dots (CDs) were synthesized by a one-step microwave-heating process (Stefenakis et al., 2014). 10 mL of pulp-free lemon juice were transferred into a 50 mL of erlenmeyer flask and was

heated in a domestic microwave oven (800 W) for 5 minutes since this time was enough to obtain brownish black solid materials as suggested in the literature (Stefenakis et al., 2014). Then, the product was dispersed in 2 mL of double distilled water and centrifuged for 2 min at 17,000xg. Then, the supernatant was transferred into a clean tube and it was filled to a final volume of 10 mL with double distilled water; it was filtered by using syringe filters having pore size, 0.2 μm to remove aggregates. To calculate the amount of CDs, 500 μL of the liquid was evaporated by Thermo SpeedVac Vacuum Concentrator and the dried product was weighted. It was found that the concentration of CDs in the liquid was 52 μg/μL.

2.2. Characterization

Absorbance spectra from 200 nm to 800 nm and the photon-induced light emission for different excitation wavelengths from 200 nm to 260 nm were obtained by Varioscan Spectrophotometer (Thermofisher, USA). Fluorescence measurements were conducted in black colored 96-well plates (Ratiolab, Germany) by using Varioskan Flash (Thermo Scientific, USA); the excitation/emission wavelengths were 240/390 nm. To obtain fluorescence spectra of CDs in different pHs, potassium buffer solutions (pH 1-12) were used. FT-IR spectra were collected by Perkin Elmer FT-IR Spectrometer, USA. For the TEM measurement, the sample was dropped on a carbon-coated grid and TEM images were recorded by TALOS L120 C.

2.3. Bovine Serum Albumin (BSA) Quenching Assay:

Different concentrations of CDs were suspended in the water including 0.5% BSA and their fluorescence spectra at 240 nm excitation wavelength were obtained by using Varioscan Flash (Thermofisher, USA).

2.4. Antimicrobial Activites of CDs

Minimal inhibitor concentrations (MIC) of concentrated lemon juices were published as 50, 25, and 12,5 μg/mL for *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively (Oikeh et al., 2016). CDs obtained from lemon-juice in this study were tested against *S. aureus* ATCC 25923, *E. feacalis* ATCC 29212, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *C. albicans* ATCC 10231, *S. epidermidis* ATCC 12228, *A. baumanii* BAA717, *K. pneumonia* ATCC 700603 for the determination of MIC and minimal bactericidal concentrations (MBC) (Okullu et al., 2020). For this purpose, fresh cultures of microorganisms were prepared on LB agar media from the stock culture at -80^{o}C. Then, suspensions of 0.5 McFarland of microorganisms were prepared in LB broth media. In sterile 96-well plates, serial dilutions of CDs in 100 μL of LB broth were done as 2600, 1300, 650, 325, and 163 μg/μL; then 5 μL of microorganisms (0.5 McFarland) were added into the wells. As the negative control, some wells did not include any CDs. 96-well plates were incubated at 37^{o}C overnight. Turbidity was used to

determine MIC values. Then, 20 µL of media from wells not including any turbidity were taken and dropped on LB agar media to determine MBC.

3. Results

3.1. Characterization of CDs

CDs were synthesized by heating in a microwave for 5 minutes. Due to the nature of lemon, it contains citric acid and therefore their three carboxylic groups might be used as a source to form the core of CDs, while carbohydrates in it might act as chiral scaffolds for CDs (Stefenakis et al., 2014; Bourlinos et al., 2008; Hill ve Galan, 2017). According to Energy Dispersive X-Ray (EDX) analysis in Figure 1, CDs were including 31% C, 51% O, 9% K, 3% Ca, 3% P, and 2% Mg (Figure 1).

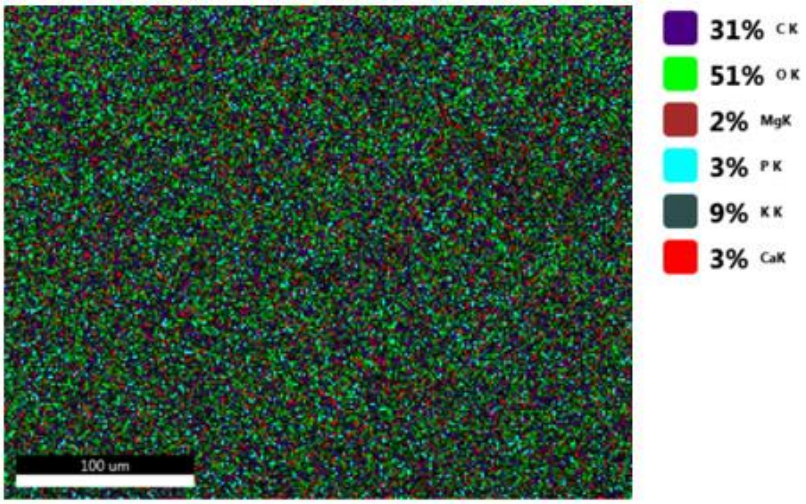

Figure 1. EDX analysis

TEM analysis was performed for CDs and results were given as Figure 2. According to TEM images, CDs were carbon nanoparticles exist in clusters in range 20 nm to 700 nm (Figure 2A and 2B). When looked at these clusters closer, CDs in average 2 nm were seen (Figure 2C and 2D).

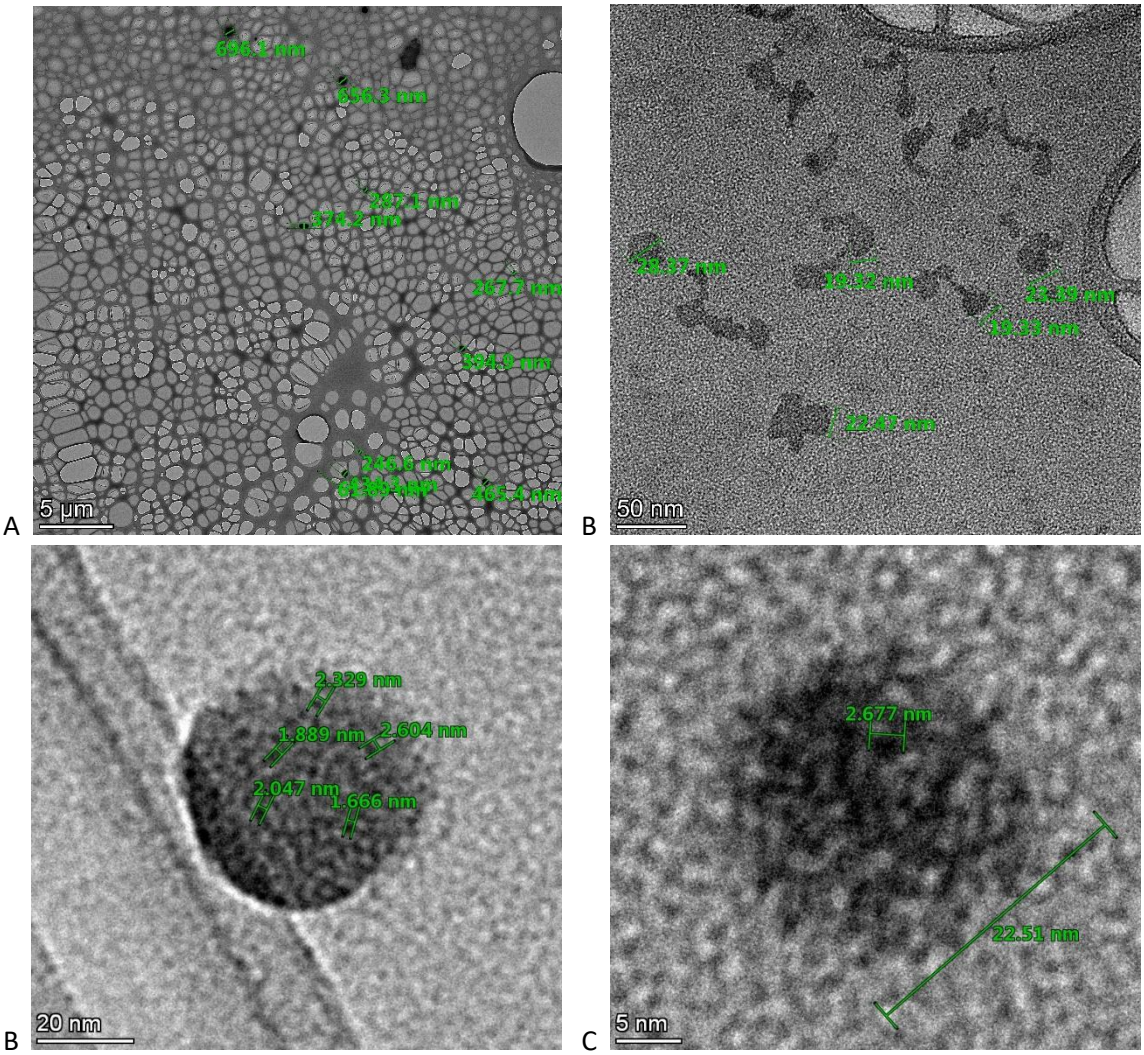

Figure 2. TEM image of CDs at 50 nm (A), 20 nm (B), and 5 nm (C) magnification.

In the FTIR analysis as Figure 3, peaks at 3300, 1634, and 1226 cm−1 were obtained, which were attributed to stretching vibrations of ≡C-H; C=C alkene, and C-O-C respectively (Hoan et al., 2019; Infrared Spectroscopy Table, 2021).

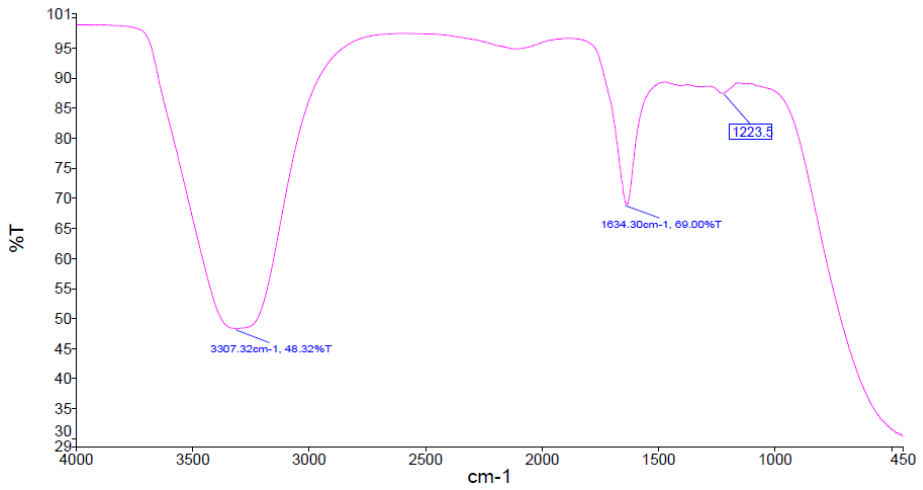

Figure 3. FTIR

The absorbance spectra of CDs in distilled water were scanned from 250 nm to 800 nm (Figure 4A). When measured dilutions of CNDs at 280 nm, absorbance values were obtained in a linear range as expected (Figure 4B).

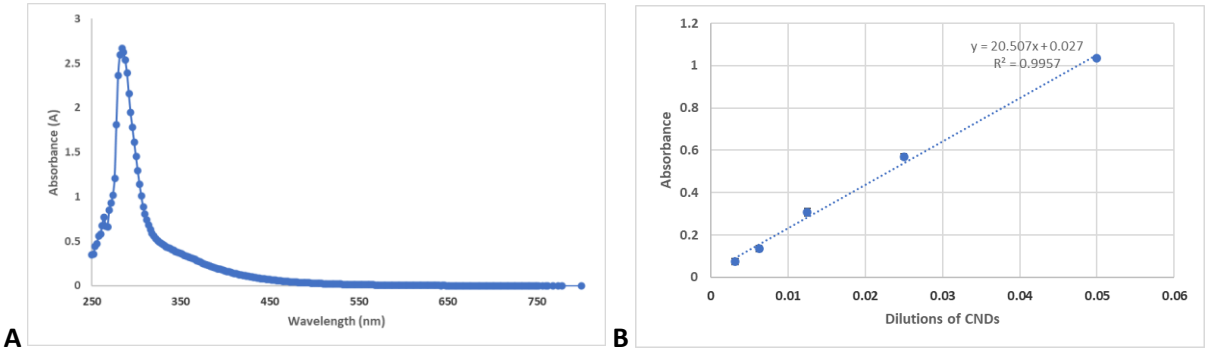

Figure 4. Absorbance spectrum of CDs (A) and absorbance values in different concentrations of CDs (B) at 280 nm.

CDs were excited at different wavelengths in range between 200-260 nm and their emission spectra were scanned as Figure 5. According to this data, the highest emission was at 390 nm when excited at 240 nm.

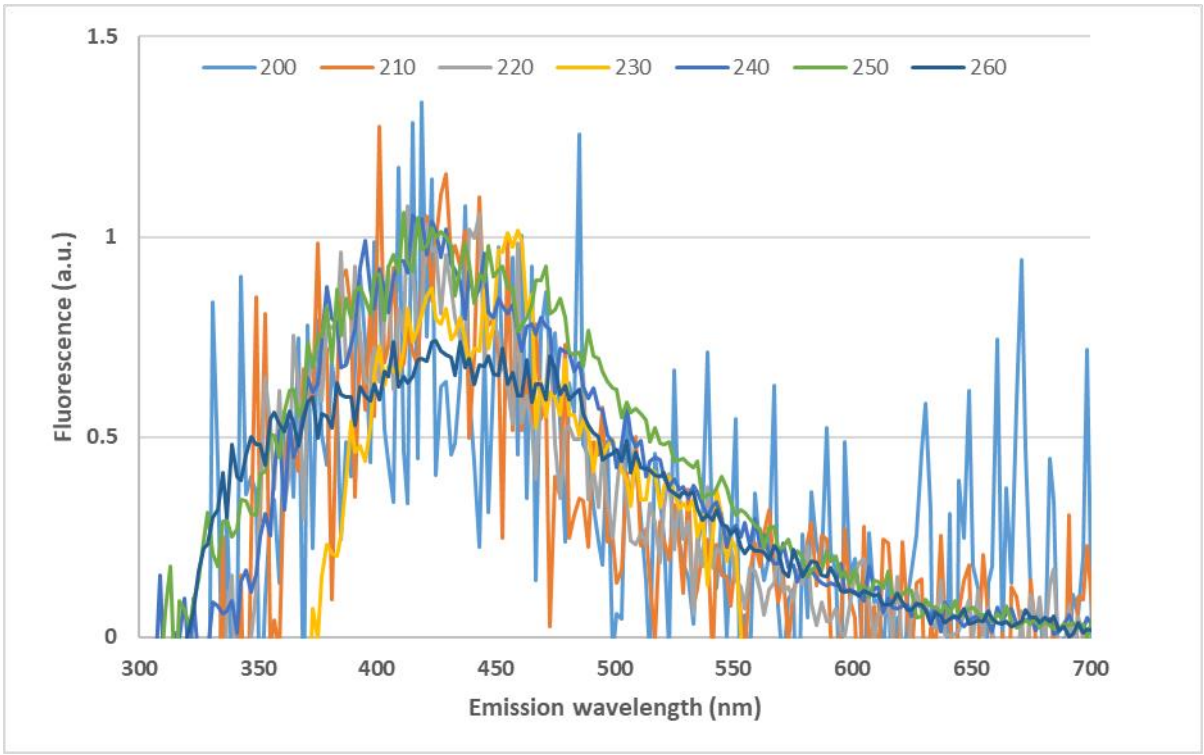

Figure 5. Fluorescence spectra of CDs exciting at different wavelengths

Different concentrations of CDs in range between 52 to 0.13 µg/µL were excited at 240 nm, their spectra were obtained as Figure 6. Over 13 µg/µL, CDs were quenched by theirselves. Fluorescence intensities were proportional in between 13-0.13 µg/µL concentrations of CDs as expected.

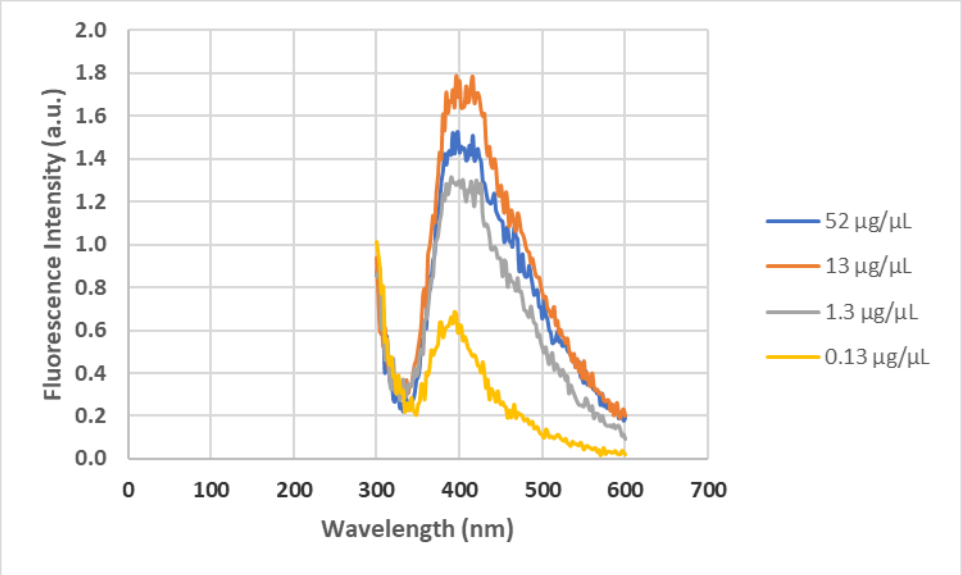

Figure 6. Fluorescence spectra of CDs in different concentrations.

Quantum yield was calculated by InstaNano Calculator (InstaNano, 2021; Chen, 1967). The calculator is used this equation: QY-sample = (QY-std x PL-sample x Abs-std x RefrativeIndex-sample^2) / (PL-std x Abs-sample x RefrativeIndex-std^2). As the standard sample, fluorescein was used. Quantum yield was calculated as 0.74%.

When suspended in buffer solutions in different pHs, their intensities were the same and emission wavelength was not shifted (Figure 7).

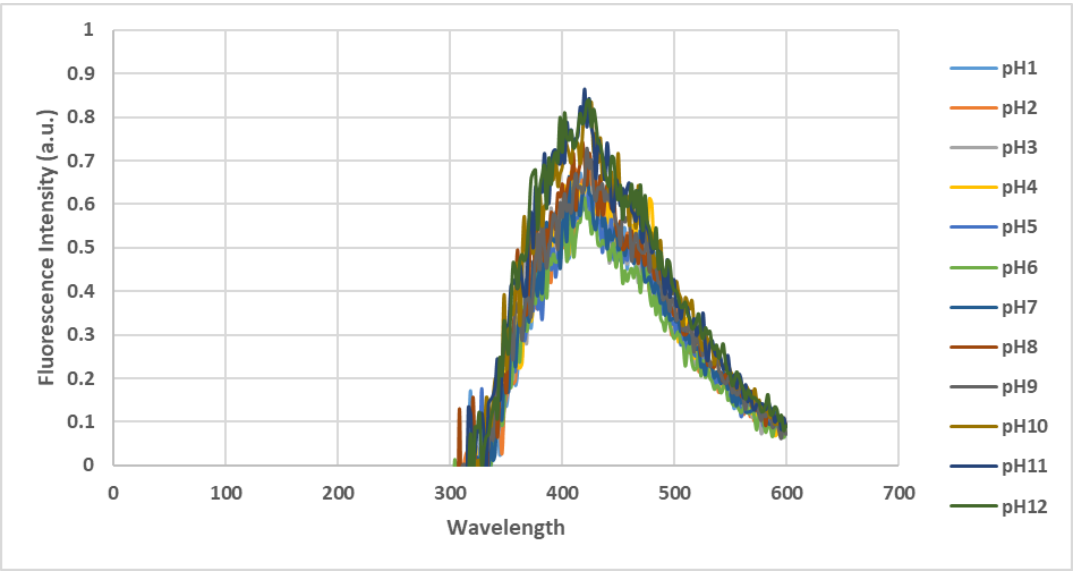

Figure 7. Fluorescence spectra of CDs in different pHs.

Bovine Serum Albumin (BSA) Quenching Assay:

When mixed 0.5% of BSA and different concentrations of CDs in a range between 10.4-0 µg/µL fluorescence was quenched with proportional of CD concentrations when excited at 280 nm (Figure 8).

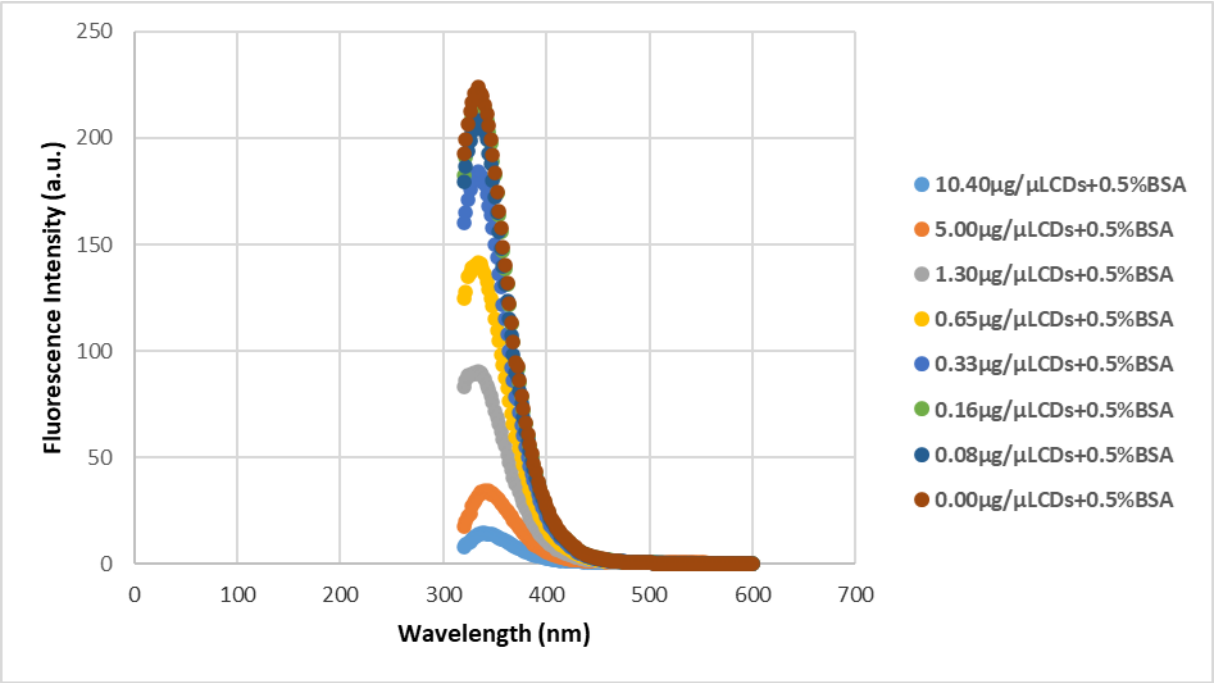

Figure 8. Fluorescence spectra of CDs and BSA.

Antimicrobial Activities:

MBC and MIC values were given in Table 1. MBC values of *S. epidermidis* ATCC 12228 and C. *albicans* ATCC 10231 were 2600 µg/mL, whereas other microoganisms tested in this study were bigger than 2600 µg/mL. The lowest MIC values as 650 µg/mL were obtained against *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228, whereas 1300 µg/mL for *P. aeruginosa* ATCC 27853 and 2600 µg/mL for *C. albicans* ATCC 10231, *E. coli* ATCC 25922, *E. feacalis* ATCC 29212, *K. pneumonia* ATCC 700603, *A. baumanii* BAA717.

Table 1. MBC and MIC values of CDs.

Bacteria	MIC (µg/mL)	MBC (µg/mL)
S. aureus ATCC 25923	650	>2600
S. epidermidis ATCC 12228	650	2600
C. albicans ATCC 10231	2600	2600
E. coli ATCC 25922	2600	>2600
P. aeruginosa ATCC 27853	1300	>2600
E. feacalis ATCC 29212	2600	>2600
K. pneumonia ATCC 700603	2600	>2600
A. baumanii BAA717	2600	>2600

4. Discussion

Carbon dots have been used in biotechnology because of their photon-induced light emission and fluorescence features. Synthesizing CDs from natural sources such as eggs, fruits, flowers etc. have been getting important because of biological compatibilities. There are many different methods to synthesize natural CDs and the simplest one is the microwave-based method. Although the synthesis CDs from only lemon juice were published, there are no studies with the microwave method. Any publications about uses of the synthesized CDs from the lemon as a quencher for BSA proteins and their antibacterial/antifungal activities were not available, according to our knowledge. Therefore, in this study, the simple microwave method was used to synthesize lemon-based CDs. They had fluorescence intensity at 390 nm when excited at 240 nm and also there are no shifts at the emission wavelength when they are dissolved in buffer solutions in the different pHs. The quantum yields were quite low as 0.74% when compared with the literature such as 24.89 and 10.2% from citrus lemon juice with hydrothermal heating methods (Tadesse et al., 2018; Hoan et al., 2019). Other methods such as heating in an autoclave for a long time might give a chance to form additional bonds to gain them more fluoroscence capabilities when compared to short reaction time with microwave-based method. Monte-Filho et al. (2019) used a mixture of onion and lemon juice for the synthesis of CDs via a microwave-based method and the quantum yield of the CDs was 23.6% (Monte-Filho et al., 2019). When compared to this microwave-based method, it is clear that the addition of another component like onion juice to the lemon juice helped to obtain a better quantum yield (Monte-Filho et al., 2019). TEM images indicated that CDs were in average 2 nm as expected. In FTIR, peaks at 3300, 1634, and 1226 cm−1 were obtained attributed to stretching vibrations of ≡C-H; C=C alkene, and C-O-C as compatible with data in the literature (Tadesse et al., 2018; Hoan et al., 2019). BSA is a fluorogenic protein because of its tryptophan amino acids and its quenching with some fluorogenic compounds like acridine orange have been used for drug targeting (Wang et al., 2009). However, acridine orange is a very toxic compound because it is bound to ssDNA, dsDNA, and RNA molecules. Instead of this kind of toxic compound, it might be better to use natural CDs as alternatives. To show quenching effects of synthesized CDs from lemon in this study, BSA and different concentrations of CDs (0-10.4 μg/μL) were mixed and fluorescence intensities were measured. Results showed that CDs acted as quenchers proportional as their concentrations and it is the first data from lemon-based CDs in the literature.

It has been showed that CDs have antimicrobial activities in different ranges (Dong et al., 2017; Ring et al., 2020; Devkota et al., 2021; Saravanan et al., 2021). When synthesized CDs from 2,2'-(ethylenedioxy)bis(ethylamine) (EDA), their MIC values were found as 64 μg/mL on *E. coli* and *B. subtilis* (Dong et al., 2017). Amine-coated CDS synthesized from glucosamine HCl and 4,7,10-trioxa-1,13-tridecanediamine inhibited *Pseudomonas* spp. at 500 μg/ml and other microorganisms such as *Agrobacterium*, *Salmonella*, *Pectobacterium*, and *E. coli* at 5000 μg/mL (Devkota et al., 2021). CDs obtained from natural leaves were effected on *E. coli* and *S. aureus* with MIC values in the

range between 150 to 5000 µg/mL, whereas MIC values of CDs synthesized from Oyster mushroom was 45 µg/mL (Saravanan et al., 2021). MIC values of curcumin-based CDs were 7.8, 15.6, and 62.5 µg/mL for *K. pneumonia*, *S. aureus*, and *P. aeruginosa* respectively (Ring et al., 2020). Oikeh and colleagues (2016) announced that different citrus juice concentrates also showed antimicrobial activities. According to their results, MIC values of concentrated lemon juices were 50, 25, and 12,5 µg/mL for *E. coli, S.aureus*, and *P. aeruginosa*, respectively (Oikeh et al., 2016). It was also effected on fungi such as *C. albicans, A. niger, Penicillum* spp and MIC values were 25, 50, and 100 µg/mL for them respectively (Oikeh et al., 2016). According to our knowledge, no any results is showing antimicrobial activities of lemon-based CDs. As our results are first for the literature, we can only compare them with CDs synthesized from other natural sources with MIC values in the range of 7.8 to 5000 µg/mL (Dong et al., 2017; Ring et al., 2020; Devkota et al., 2021; Saravanan et al., 2021). MIC values of the CDs on microorganisms studied here are in the range between 650 to 2300 µg/mL.

The first study using the microwave-based method to synthesize only lemon-based CDs showed that there is a need to improve the fluorogenic features of these CDs. However, they can be certainly used as quenchers for BSA proteins in biotechnological applications. Since they showed antimicrobial activities on microorganisms tested here, further studies would help our understanding whether they can be used as antimicrobial therapy alternatives in the future.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Contribution Statement

The author declares that he has contributed 100% to the article.

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