Cumhuriyet Sci. J., 45(1) (2024) 73-79
DOI: https://doi.org/10.17776/csj.1333574

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Fluorescence Cell Imaging by Carbon Quantum Dots Derived from the Medicinal Plant *Malva Sylvestris*

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Research Article

History
Received: 27/07/2023
Accepted: 31/01/2024

ABSTRACT

Carbon quantum dots (CQDs) derived from biological samples exhibit low cytotoxicity and are often used as fluorescent agents for bioimaging cells or bacteria. In this study, fluorescent CQDs derived from *Malva sylvestris,* a wild edible medicinal plant (common mallow), were used to visualize the human epithelial cells (PNT1A). CQDs with strong blue photoluminescence were synthesized by microwave irradiation of dried *M. sylvestris* in water. CQDs' structural, morphological, surface features and photoluminescence properties were evaluated. *In vitro,* cytotoxicity assays revealed that carbon quantum dots from *M. sylvestris* have no cytotoxic effects on human epithelial cells. Non-cytotoxic and high photoluminescent CQDs from *M. sylvestris* can be exploited in bioimaging applications as a fluorophore agent.

Keywords: Carbon dots, *Malva sylvestris*, Cell viability, PNT1A

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Introduction

There is a growing interest in *in vitro* cellular imaging with fluorescent nanomaterials. Fluorescent CQDs are attracting attention as an alternative to organic or inorganic fluorophore agents due to their multicolor luminescence and biocompatibility [1]. Recent studies have demonstrated their high potential to be used in bioimaging and bio-sensing applications as an alternative fluorophore agent [2, 3].

Fluorescence emission of CQDs is ascribed to their surface defects and sp^2/sp^3 hybridized surface functionalities. CQDs exhibit multicolor luminescence depending on the excitation wavelength [4]. Their surface functionalities are also responsible for the solubility of CQDs in aqueous solutions. The water solubility of carbon quantum dots is particularly important for biological studies requiring bodily fluids or simulated bodily fluids [5]. Hydroxyl, amino or carboxylic moieties on the CQDs surface are key in interacting with cells, tissues, or biological molecules [6]. Biocompatibility low/non-cytotoxicity of carbon quantum dots is often related to the surface moieties [7].

CQDs derived, particularly biological samples, exhibit low cytotoxicity and are often called green and biocompatible nanoparticles. Their size, biocompatibility, and water-dispersible properties make it possible to use these fluorescent nanoparticles as fluorophore probes for bioimaging cells or bacteria [8]. Numerous literature reports have demonstrated the potential and suitability of CQDs derived from natural sources for fluorescence imaging of live cells. Cabbage-derived CQDs, for example, were used to visualize human keratinocyte cancer cells [9]. Green fluorescent carbon dots from coriander leaves were evaluated for *in vitro* cell imaging of normal and cancerous cells [10]. In another study, blue/green/red emissive CQDs from onion wastes were used as fluorophore probes for multicolored imaging of HeLa cells [11]. In a report by Jeong et al. (2014), CQDs from mango fruit exhibited blue, green, and yellow emission colors. They were administered intravenously to a mouse to evaluate their suitability for bioimaging applications [12]. The distribution of the carbon dots in a nude mouse was assessed with reference to their multicolor luminescence.

CQDs have tunable/modifiable surfaces. For instance, the intensity of their photoluminescence emission can be increased by surface passivation, a form of surface modification. Surface functionalities are effective in the chemical modification of the surface [13]. Numerous recent literature reports revealed that CQDs could be evaluated in drug delivery studies as vehicles [14-16].

Furthermore, CQDs can be synthesized by carbonization of plants, mushrooms, or biopolymers using laser energy, electrochemical oxidation, and thermal and microwave irradiation [17, 18]. Compared to other techniques, microwave-assisted methods do not require longer operational time, the use of organic solvents, and higher temperatures [19].

Malva sylvestris is an edible wild plant commonly used in pharmaceutical ethnobotany. It grows wild in North Africa, Europe, and Asia. It contains phenolic compounds, flavonoids, carotenoids, tocopherols, and unsaturated fatty acids [20]. This medicinal plant is being extensively studied due to its therapeutic properties and nutrient

contents [21-23]. In recent studies, *M. sylvestris* extracts were used to synthesize nanoparticles such as CuO [24], Fe_3O_4 [25], and Ag/AgCl [26].

In this study, the plant was used to synthesize carbon-based nanoparticles for the first time. Fluorescent CQDs were obtained by microwave-assisted carbonization of dried aerial parts of *M. sylvestris* without using any chemicals. In this study, the synthesis of carbon quantum dots from *M. sylvestris* was explored for the first time. The synthesis process was refined to produce CQDs with heightened photoluminescence emission. Various analytical tools, including TEM, XRD analysis, FT-IR spectroscopy, XPS, UV-visible absorption spectrophotometry, and fluorescence spectroscopy, were used to examine the structural and photophysical features of the CQDs. The fluorescence quantum yield of the CQDs was also calculated. In addition, the paper reports on the cytotoxic effects of the CQDs on the PNT1A human prostate epithelial cell line.

Experimental

Materials

M. sylvestris specimens were collected in Sizma (Selcuklu, Konya, Turkey) and identified by the Faculty of Pharmacy, Department of Pharmaceutical Botany (Selcuk University, Konya, Turkey). The roots of the plants were removed, and the remaining parts, including the leaves, stems, flowers, and leafy flowered stems were rinsed with water and dried. *M. Sylvestris* specimens were ground using a metal blade grinder and stored in powder form in sealed glass bottles at +4°C. Quinine hemisulphate salt monohydrate was purchased from Sigma.

Optimization of Experimental Parameters

CQDs were synthesized by microwave irradiating dried *M. sylvestris* powder in water. The experimental parameters, i.e., the microwave power levels, the microwave irradiation time, and the mass of *M. sylvestris* powder were studied as follows.

The microwave power level: 0.25 g of *M. sylvestris* in 10 mL of distilled water was exposed to microwave irradiation (CEM Mars 5) for 20 min (at 400 W). The crude solution was centrifuged (at 3260 x *g*), and the supernatant was recovered and filtrated using a microfilter (0.22 µm). The filtrate was dialyzed against distilled water. The dialysis medium was refreshed three times overnight. The fluorescence emission of the CQDs in water was recorded (λ_{ex} = 365 nm) on Perkin Elmer LS 55 instrument. The same procedure was followed for 800 and 1600 W power levels. The CQDs solution with the highest fluorescence emission was recorded as the optimum power level (Figure 1, top). The carbon quantum dots in the solution were recovered by lyophilization.

The exposure time: The microwave exposure time was optimized at the specified power level. The same procedure was followed at varying exposure times (Figure 1, middle).

The mass of the plant: Finally, the mass of *M. sylvestris* was optimized at the specified power level and the exposure time. The same procedure was followed at varying plant masses (Figure 1, bottom).

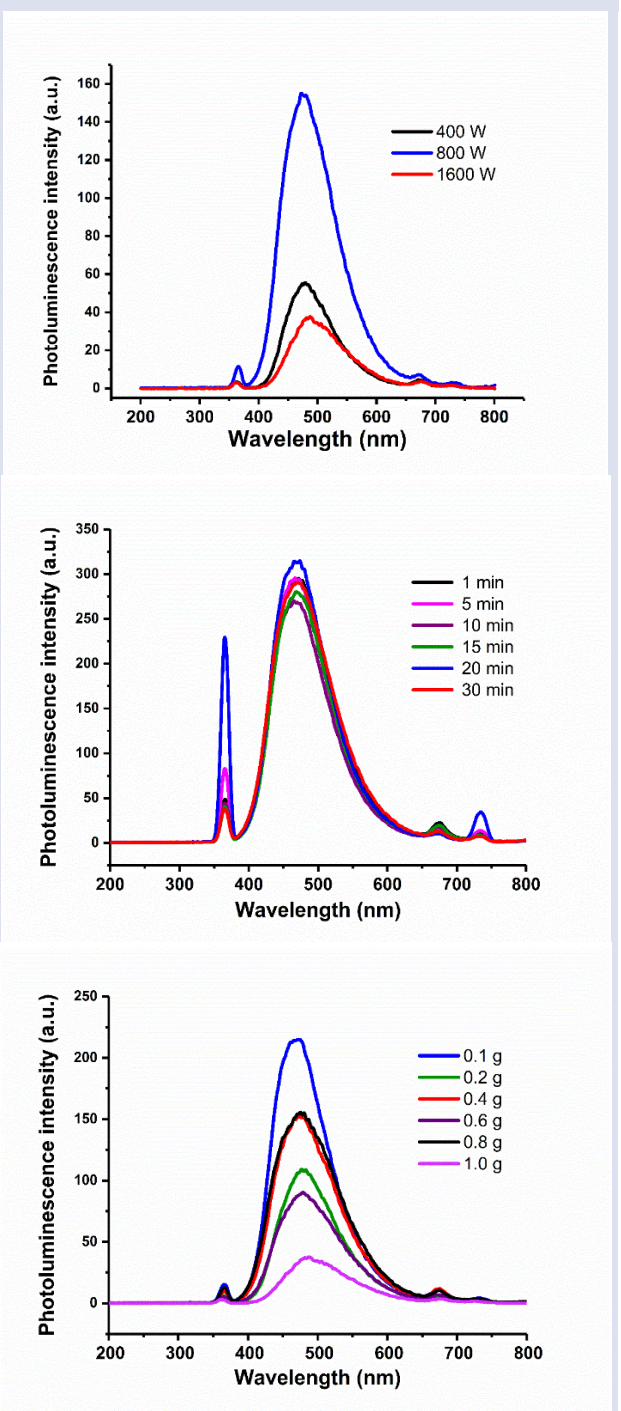

Figure 1. Optimization of operational parameters for synthesizing CQDs from *M. sylvestris* through microwave irradiation (Microwave power level: 800 W, exposure time: 20 min, the mass of *M. sylvestris* plant powder: 0.1 g and the volume of distilled water: 10 mL).

Preparation of CQDs from M. sylvestris

The optimized method mentioned above was used to obtain CQDs from *M. sylvestris*. In short, 0.1 g of *M. sylvestris* was mixed with 10 mL of distilled water and exposed to microwave irradiation at 800 W for 20 minutes. The mixture was then centrifuged, filtered with a syringe filter, and dialyzed against distilled water. Finally, the solution was recovered through lyophilization, as shown in Figure 2.

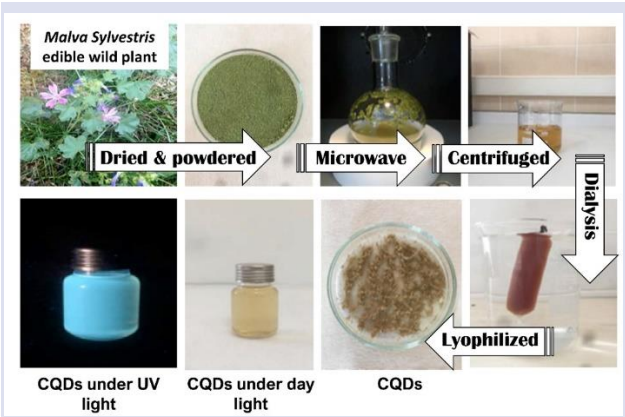

Figure 2. Synthesis route of carbon quantum dots from edible wild plant *M. Sylvestris*.

Characterization of CQDs

Structural properties of CQDs: The visualization of CQDs was accomplished through TEM (JEOL JEM-2100, UHR- Ultra High-Resolution instrument). The transmission electron microscopy (TEM) images gave insights into the surface and structural properties of CQDs. The X-ray diffraction pattern for CQDs was obtained using a Bruker D8 Advance instrument (2θ angle, 0°-90°, scan rate 3° min^{-1}) to reveal the core structure and the crystallinity of CQDs.

Surface characterization of CQDs: FT-IR spectroscopy (Bruker Vertex 70 FT-IR spectrometer, in the range of 4000-500 cm^{-1}) and XPS (PHI 5000 VersaProbe Thermo Scientific K-alpha X-ray Photoelectron Spectrometer) were used to examine surface atomic groups of CQDs.

Photophysical features of CQDs: CQDs' luminescence was analyzed using (UV-vis Shimadzu UV1800) absorption spectrophotometer and fluorescence emission spectra.

Fluorescence quantum yield of CQDs: To determine the fluorescence quantum yield of CQDs, the method described in a previous report [27] was followed.

Cytotoxicity and cell imaging

PNT1A cells (Epithelial cells, ATCC) were cultured in RPMI 1640 medium with 2 mM L-glutamine and 1% penicillin. Then, the cells were incubated for 24 hours at 37°C with 5% CO_2. Next, the cells were exposed to a solution of CQDs ranging from 0 to 1000 µg mL^{-1}, maintained at a temperature of 37°C. Following the 48 h-incubation, the cell proliferation assays were performed using the Alamar Blue Method, and IC_{50} of the carbon quantum dots solution was calculated from a sigmoidal plot of cell viability [28]. PNT1A cells were grown in RPMI-1640 growth medium containing 10% FBS (Foetal Bovine Serum) and 2 mM L-Glutamine. This medium was also used to dilute the carbon quantum dots solutions. Once the cells reached the confluency, they were washed with phosphate buffer (10 mM), detached with the trypsin-EDTA solution, and transferred to 96-well plates. The wells' cells were subjected to CQDs solutions ranging from 0-1000 µg mL^{-1} before being incubated at 37°C and 5% CO_2 for 24 hours. In the assay, the absorbance values depend on the amount of the strongly fluorescent product, resorufin, formed by cellular reduction of resazurin, a blue-colored weak fluorescent dye. A microplate reader spectrophotometer was used to analyze the fluorescence of Resorufin. Then, the cell viability percentage was calculated.

The cytotoxic effect of CQDs was determined from the sigmoidal graph of the viability ratio, and IC_{50} values were calculated.

Bio-imaging studies were performed using a fluorescent microscope (ZOE, Bio-rad, USA). The cells ($1x10^5$) were placed on a 3-well chamber microscopy glass slide (ibidi, USA) and then incubated at 37°C for 24 hours. Next, the old growth medium was substituted with a new growth medium containing 50 µg/mL of CQDs and left to incubate at a temperature of 37°C for a duration of 45 minutes. Finally, the slides were rinsed with 10 mM PBS to remove excess carbon quantum dots solution and examined under a fluorescence microscope.

Results and Discussion

Optimization studies

The effects of experimental design manipulation on photoluminescence emission of the CQDs were studied at varying microwave power levels, microwave exposure time, and mass of dried *M. sylvestris*. The synthesis of CQDs from *M. sylvestris* was optimized by measuring the photoluminescence intensity of the resulting aqueous solutions of the CQDs (Figure 3). The optimum synthesis conditions were recorded as follows: Microwave power level: 800 W, exposure time: 20 min, the mass of *M. sylvestris* plant powder: 0.1 g, and the volume of distilled water: 10 mL.

Figure 3. The image of carbon quantum dots from *M. sylvestris* dissolved in water (left) and blue fluorescence emission of the CQDs under UV light (at 365 nm) (right).

Structural and Physical Properties of CQDs

CQDs from *M. sylvestris* were visualized using TEM. The TEM and HR-TEM images provided information on CQDs' morphology, size, and crystalline structure (Figure 4 a-d). The CQDs had a crystalline core, and their size varied in the ~10–50 nm (Figure 4 b, c). Figure 4d displays the crystal lattice of the graphitic core. The lattice spacing of CQDs was found to be similar to that of graphite, around 0.35 nm. This suggests that the CQDs derived from *M. sylvestris* have a crystalline structure. In TEM images, some long rod-like structures were also observed (Figure 4 a).

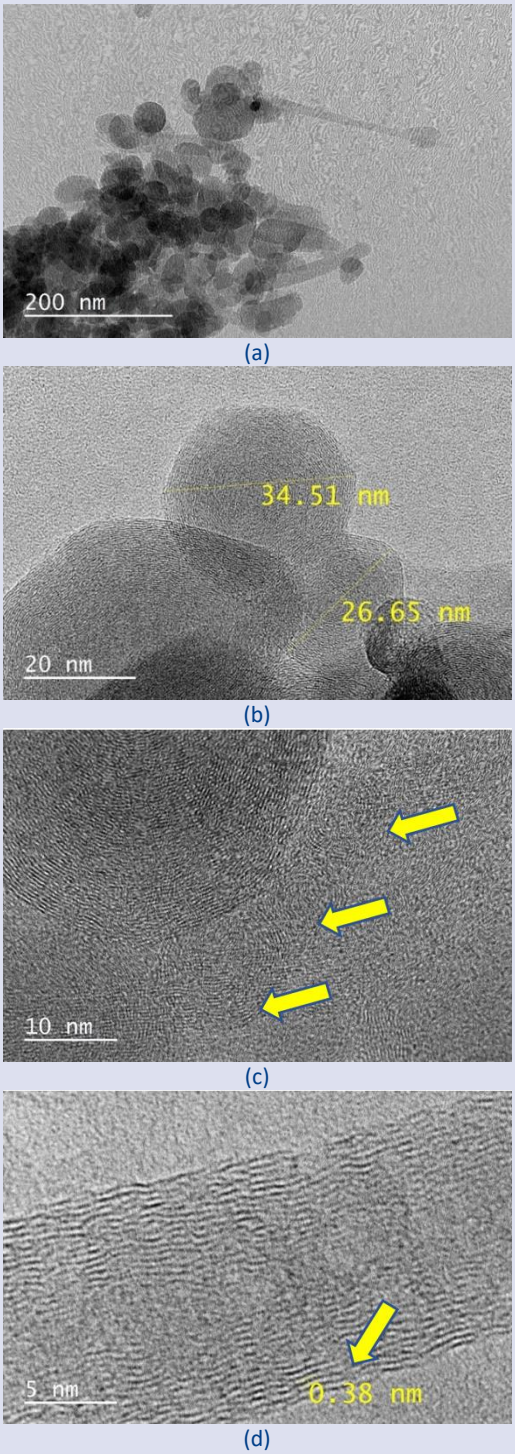

Figure 4. TEM (a) and HR-TEM (b, c, and c) images of CQDs synthesized from *M. sylvestris*.

Figure 5 displays the crystallinity of CQDs as demonstrated by the X-ray diffraction (XRD) pattern. Based on the diffraction peaks observed at around 22 and 26°, it can be concluded that the CQDs derived from *M. sylvestris* possess graphitic crystallinity. The presence of a weak diffraction peak around 15° may be due to the CQDs' amorphous characteristics [29, 30].

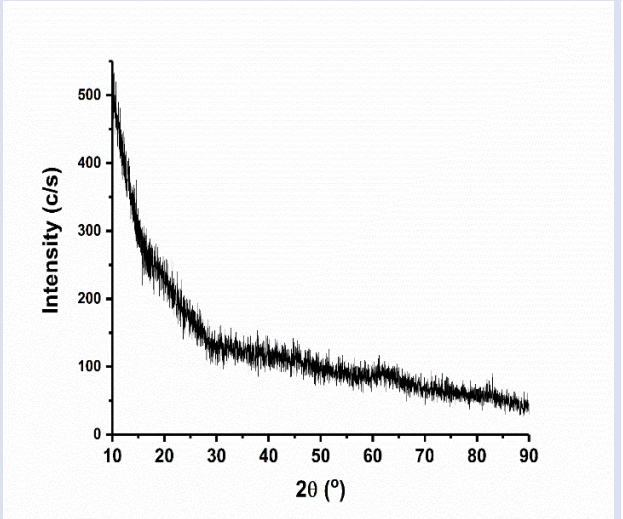

Figure 5. X-ray diffractogram of carbon quantum dots synthesized from *M. sylvestris*.

The analysis of the CQDs' surface functional groups was conducted using FT-IR spectroscopy (as shown in Figure 6). The spectrum analysis identified two vibrational bands: one at 3281 cm^{-1} for –NH and –OH stretching, and another at 2927 cm^{-1} for –C-H stretching. The bands at 1597 cm^{-1} corresponded to the C=C stretching of polycyclic aromatic hydrocarbons [31]. The CQDs were found to contain sp^2 hybridized C-H and C-O bonds, which were confirmed through vibrational bands at 1412 cm^{-1} and 1040 cm^{-1}, respectively. The existence of hydrophilic functional groups such as -COOH and -NH_2 implies that CQDs could be useful for biological purposes [32].

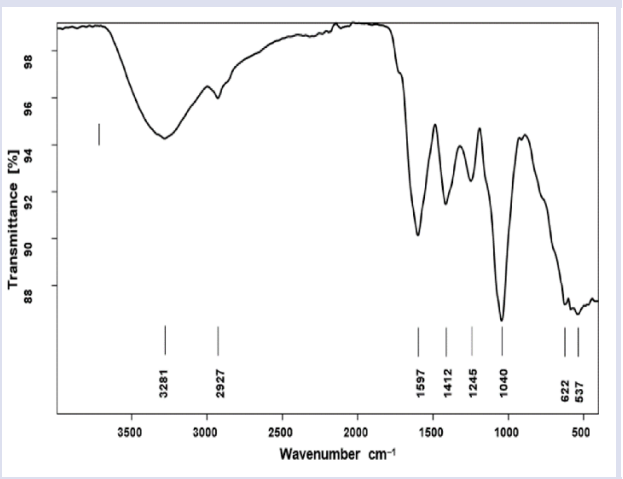

Figure 6. FT-IR spectrum of carbon quantum dots synthesized from *M. sylvestris*.

The surface atomic groups of the CQDs were analyzed using X-ray photoelectron spectroscopy. XPS spectrum showed two major signals corresponding to C1s and O 1s and minor signals for N 1s and Ca 2p (Figure 7).

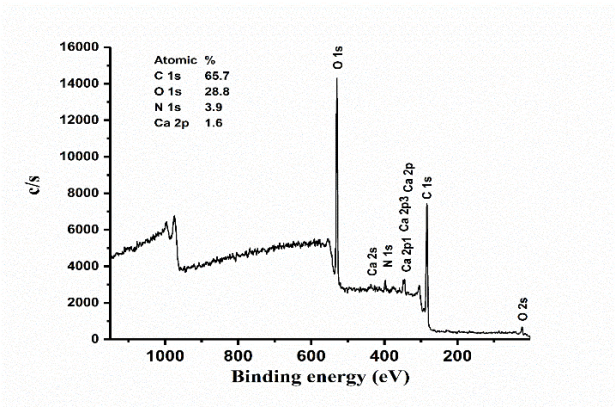

Figure 7. XPS survey spectra of CQDs from *M. sylvestris*.

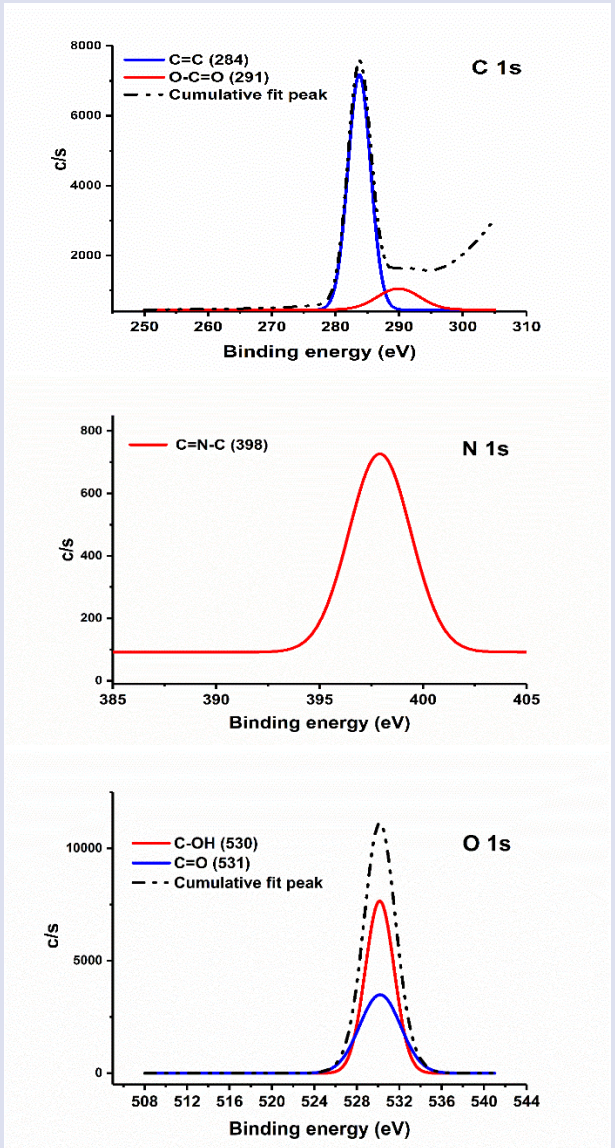

Figure 8. CQDs from M. sylvestris showed distinct C, N, and O bands in their XPS spectrum. The spectrum shows which functional groups correspond to different atoms on the surface of CQDs.

The atomic percentages were as follows; C 1s: 65.7; O 1s: 28.8; N 1s: 3.9, and Ca 2p: 1.6%. CQDs from *M. sylvestris* mainly comprised C, O, and N atoms.

In Figure 8, the high-resolution C 1s spectrum was deconvoluted and showed two peaks that corresponded to C=C and -O-C=O. In the deconvoluted high-resolution N 1s spectrum, C=N-C at 398 eV dominated the N 1s spectrum. The signal at O 1s was separated into two peaks at 530 eV and 531 eV. These peaks represent groups containing C-OH and C=O [30, 33].

The UV-visible absorbance spectrum of the CQDs displays a wide absorbance peak, reaching up to 600 nm. This peak can be attributed to the energy-absorbing groups found on the surface of the CQDs (Figure 9, top) [17]. In the UV–vis absorbance spectrum, two peaks could be corresponded to the π- π* and n- π* transitions. CQDs from *M. sylvestris* exhibited excitation-dependent photoluminescence, which is typical of CQDs (Figure 9, bottom). When the aqueous solution of CQDs was excited at 400 nm, it produced the strongest fluorescence emission. The fluorescence quantum yields (Φ) of CQDs were determined by comparing them to the quinine sulfate solution (Φ = 0.54). The quantum yield of CQDs was calculated to be Φ = 0.07.

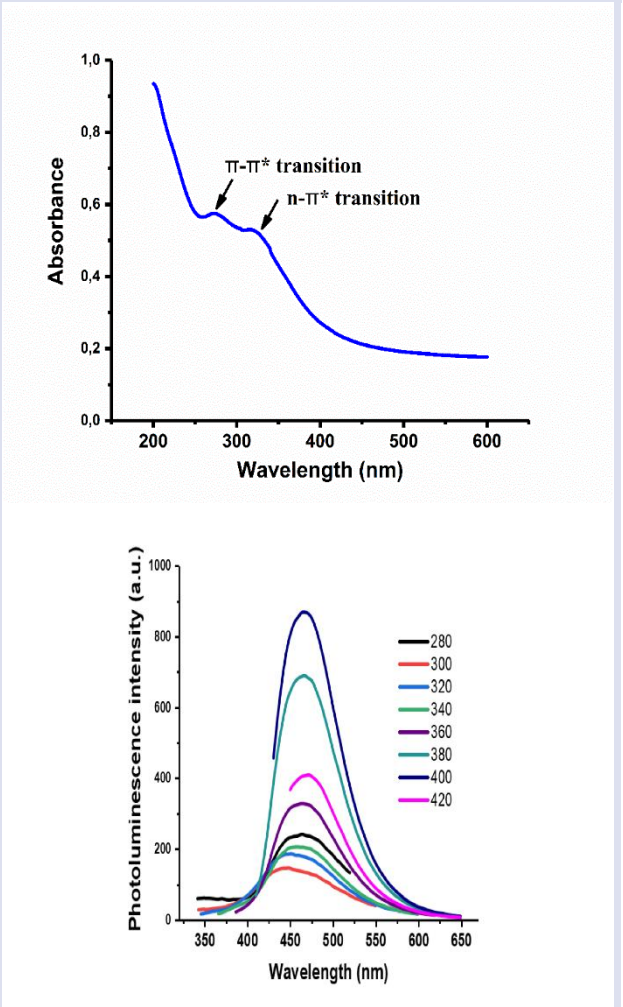

Fig. 9. UV–vis absorbance (top) and photoluminescence emission spectra (bottom) of CQDs from *M. sylvestris* (in distilled water).

Cell viability rate and cytotoxic effects of CQDs

The cytotoxic effects of CQDs on human epithelial cells were studied using the Alamar Blue assay, and the IC_{50} values were determined. In the cell proliferation experiments, the cells were exposed to the CQDs solutions at varying concentrations of 0-1000 µg mL^{-1}. Following the 48-hour incubation, the IC_{50} of CQDs was calculated as 600 µg mL^{-1}. The assay revealed that green synthesized carbon quantum dots from the medicinal plant *M. sylvestris* did not show cytotoxicity against human prostate epithelial cells (Figure 10-d). When CQDs from *M. sylvestris* were applied to the cells, the cell membrane and cytoplasm showed a blue-color emission (Figure 10 a-c). The CQDs were localized in the cytoplasm of PNT1A cells. This indicated that the high fluorescence properties of carbon quantum dots from *M. sylvestris* could be used for *in vitro* imaging of other mammalian cells.

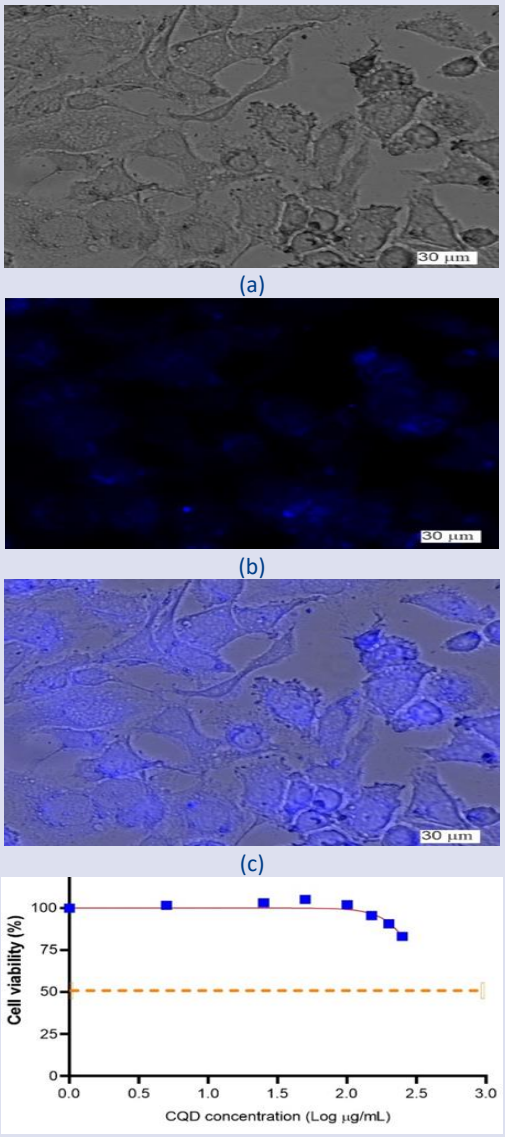

Fig. 10. Cytotoxicity and bio-imaging of CQDs from *M. sylvestris* in human epithelial cells. a) Bright field imaging of PNT1A cells, b) Fluorescence imaging of PNT1A cells, c) Fluorescence imaging of PNT1A cells (merged), and d) Cytotoxicity of CQDs.

Conclusions

The findings of this research highlight the ease of synthesizing biocompatible fluorescent CQDs from the medicinal wild plant *M. sylvestris*, without the need for organic solvents or chemicals. The CQDs exhibited no cytotoxicity against human prostate epithelial cells. The fluorescent CQDs demonstrated excellent cell permeability and are therefore ideal for utilizing in fluorescence imaging of mammalian cells, specifically PNT1A. Carbon quantum dots from *M. sylvestris* localized in the membrane and cytoplasm of PNT1A cells. CQDs from *M. sylvestris* in further studies should be tested in imaging bacterial, fungal, and plant cells. Carbon quantum dots derived from *M. sylvestris* also can be evaluated *in vivo* imaging and bio-sensing applications.

Acknowledgments

The authors thank Selcuk University Research Foundation for funding the study (BAP-19201057).

Conflicts of interest

The authors report no conflicts of interest in this work.

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