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Original Research

Hydrothermal synthesis of highly fluorescent and non-toxic carbon dots using *Stevia rebaudiana* Bertoni

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Abstract: Herein, green, environmental-friendly and economical synthesize of luminescent carbon dots (CDs) was reported using the plant-based material, *Stevia rebaudiana* Bertoni, as a herbal bio-sweetener. In this regard, hydrothermal carbonization of Stevia was performed under mild conditions and without any chemical oxidizing agents. Exploring the morphological aspect, surface groups, chemical composition and structure of Stevia-based CDs have been carried out systematically using SEM-EDS, TEM, fluorescence, FT-IR, UV-Vis, and XRD techniques. UV–Vis spectra displayed signature absorption of CDs at 240 nm related to the π–π^* transitions of C=C. Fluorescence spectra showed characteristic emission at the peak wavelength of 431 nm with a quantum yield of approximately 17.5%. Moreover, there was no living cell cytotoxicity for the as-prepared CDs confirmed by confocal microscopy. These results indicated that these plant-based CDs are ideal non-toxic promising markers for cellular bio-imaging.

Key words: Carbon dot; Stevia; Hydrothermal; Bio-imaging.

Introduction

Carbon dots (CDs) are a new class of discrete, quasi-spherical carbon-based nanomaterials with sizes less than 10 nm in diameter (1). These novel fluorescence-based nanomaterials offer a non-toxic, more stable and cheaper promising substitute to other metal-based fluorescent nanoparticles like semiconductor Quantum Dots (QDs) (1-3). CDs exhibits high aqueous solubility, facile synthesis, tunable and high up-converted photoluminescence, robust chemical inertness, lack of blinking, low environmental toxicity, easy functionalization, good fluorescence photostability and biocompatibility (4-6). They have tremendous application prospects in metal sensing, probes for biomedical applications and bio-imaging, drug delivery, biological labelling, optoelectronic devices, biosensors and catalysis (1, 3, 7-9). The preparation of photoluminescent CDs was performed using many established protocols like laser ablation of graphite, microwave mediated synthesis, electro-oxidation of graphite, thermal cracking of organic compounds, and oxidation of candle soot (4, 6, 10-13). The majorities of these preparation methods need expensive raw materials, severe experimental conditions, a large amount of strong acid and have a complex manipulation (14). The thermal cracking or hydrothermal carbonization method as a widely used physical method possess some fascinating features such as needing to simple experimental setup, facile and one-step process, mild experimental conditions and avoids using highly toxic chemicals (14, 15). Biomass (orange peels, coffee grounds, and grass), proteins, amino acids, citric acid, and carbohydrates biopolymers (chitin, chitosan, cellulose, hyaluronic acid, cyclodextrin and dextran) were used as a molecular precursor for the formation of CDs (16-19). According to exploring a new natural precursor for preparation of CDs, Stevia, a plant material was selected. A small perennial shrub Stevia is a natural high-potency bio-sweeteners and dietary supplement. This white crystalline compound is over 100-300 times sweeter than table sugar and can be used as a non-caloric sugar substitute. Stevia has been used in various fields such as a natural sweetener in seafood, soft drinks, and candies (20), a natural control for diabetes (21), to help control weight in obese persons (22) and a non-caloric sweetener (23).

This work demonstrated the preparation of CDs using facile one-step hydrothermal carbonization of a herbal material (Stevia) as the carbon source. The structural and morphological characteristics of the as-prepared CDs were analyzed by various experimental approaches.

Materials and Methods

Reagent

All chemicals were analytical reagent grade and used as received.

Synthesis of CDs

In a typical procedure, 0.5 g of Stevia was dispersed into 10 mL water kept at room temperature overnight under stirring. Then, the mixture was transferred into a 25 mL Teflon-lined autoclave and heated at 180 °C for a period of 1 h. Afterwards, the autoclave cooled to room temperature naturally. The resulted CDs solution

was filtrated with 0.22 µm membrane and washed two times with dichloromethane followed by centrifugation at 8000 rpm for 15 min. Finally, the supernatant was gathered and freeze-dried for future use.

Characterization

The Fourier transform infrared (FT-IR) spectroscopy experiments were conducted using a Shimadzu spectrophotometer in the form of KBr pellets. Transmission electron microscopy (TEM) was carried out on a Hitachi H600 electron microscope (Tokyo, Japan) using an acceleration voltage of 100 kV. High-resolution transmission electron microscopy (HRTEM) images were taken by a JEOL-2010 electron microscope. The scanning electron microscopy (SEM) images were obtained using FEI-quanta 200 (at 20 kV) with energy dispersive X-ray spectroscopy (EDS) analyses. X-ray diffraction (XRD) pattern was collected by an Inel Equinox 3000 diffractometer, France, 30 kV, 20 mA using Cu Kα radiation (λ=1.5418 Å). The absorption spectrum of the as-prepared CDs was recorded by UV–Vis Spectroscopy (Shimadzu, Japan). The photoluminescence spectrum was taken on a fluorescence spectrometer (Hitachi, Japan). The fluorescence quantum yields of CDs were computed based on the following equation (24):

$$Q_{CDs} = Q_R \frac{I_{CDs}}{I_R} \frac{A_R}{A_{CDs}} \frac{\eta_{CDs}^2}{\eta_R^2} \quad (1)$$

where, Φ_{CDs} and Φ_R represent the quantum yields of CDs and reference respectively; I_{CDs} and I_R denote integrated fluorescence intensities of CDs and reference respectively, A_R and A_{CDs} refer the absorbance of the reference and CDs at excited wavelengths, η_{CDs} and η_R are refractive indices of the CDs medium and reference medium respectively.

In vitro cytotoxicity evaluation

Cytotoxicity of the Stevia-based CDs was examined using MTT assay on mesenchymal cells. These cells were seeded in 96 well culture plates and allowed to grow and further incubated for 48 h at 37℃ in a 5%CO_2 humidity incubator. After incubation, the medium was aspirated and cells were treated with increasing concentrations of CDs. Control wells of untreated cells were also included. After 24 h of further incubation, the medium was removed and replaced with 0.1 ml of MTT (1 mg ml^{-1}) solution followed by dissolving of the purple formazan crystals in DMSO (0.1 ml). Absorbance was determined on an ELISA plate reader (Biotek, H1 M) with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain a sample signal (OD570–OD630).

Results

The presence of the surface functional groups on the as-prepared CDs after hydrothermal treatment was identified by FTIR (Fig. 1). The absorption peaks at 3421, 2954, 1620, 1454, 1091 cm^{-1} in FTIR spectra were attributed to the asymmetric and symmetric stretching vibrations of hydroxyl, methyl, C=O, C–O–C and C-O groups, respectively (24-26).

Figure 2 displayed the typical XRD image of CDs. The intense peaks centred at 28º, 40º, 50º and 58º were

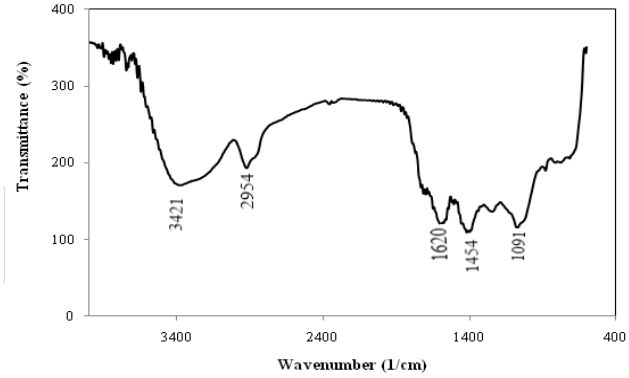


Figure 1. FTIR spectra of Stevia-based CDs.

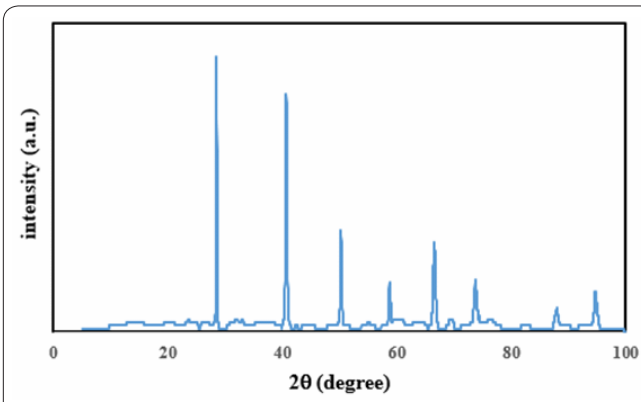


Figure 2. X-ray diffraction pattern of CDs synthesized from Stevia.

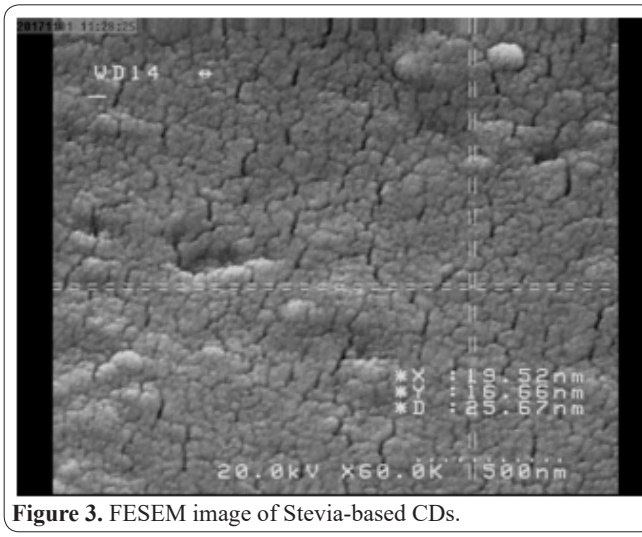


Figure 3. FESEM image of Stevia-based CDs.

assigned to the (002), (101), (102) and (103) diffraction patterns respectively which showed the presence of turbostratic and graphitic carbon in the CDs (24).

The morphology and particle size of as-prepared CDs were identified using both SEM and TEM. It can be observed from the FESEM image that the synthesized CDs display spherical morphology with 20-30 mm in size (Fig. 3).

The hydrodynamic diameter of the Stevia-based CDs was explored through dynamic light scattering (DLS) technique and an average particle size of 35 nm was obtained. Also, the exact determination of particle size was possible from TEM images.

As evident from the Figure 4, the discernible lattice fringes with the crystal interplanar spacing of 0.21 nm agreeing well with the (100) in-planar lattice of graphite (27).

The components of the as-synthesized CDs were investigated through EDS analysis. Figure 5 exhibits

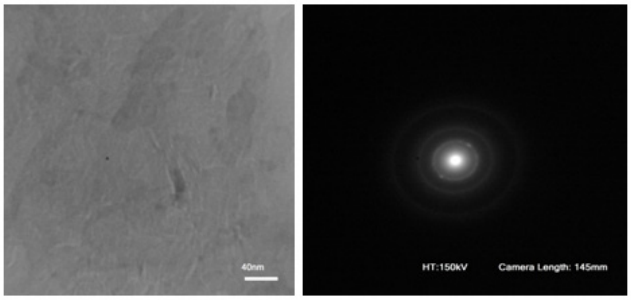
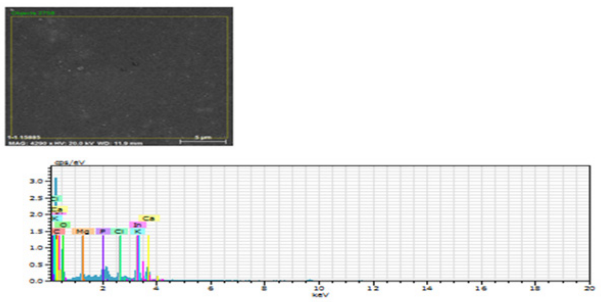


Figure 4. HR-TEM images of Stevia-based carbon dots.



El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
C	6	K-series	41.15	50.60	62.97	6.05
O	8	K-series	27.13	33.35	31.16	4.70
K	19	K-series	4.07	5.00	1.91	0.17
In	49	L-series	2.77	3.41	0.44	0.14
Ca	20	K-series	2.48	3.05	1.14	0.12
Mg	12	K-series	1.53	1.88	1.16	0.13
P	15	K-series	1.19	1.46	0.71	0.09
Cl	17	K-series	1.01	1.24	0.52	0.07
		Total:	81.33	100.00	100.00	

Figure 5. EDS analysis of Stevia-based CDs.

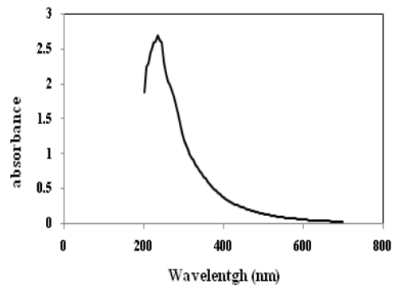


Figure 6. UV–vis spectroscopy of the Stevia-based CDs.

Table 1. Quantum yield (QY) of Stevia-based CDs as a function of temperature.

Temperature	QY(%)
100℃/30 min	13.2
150℃/30 min	17.5
200℃/30 min	18.1

the Stevia-based CDs mainly are composed of carbon (41.15 wt%) and oxygen (27.13 wt%). Other elemental compositions of CDs are including K, In, Ca, Mg, P and Cl. These findings suggest the presence of many functional groups containing carbon and oxygen in their structures (carboxyl, hydroxyl, etc).

Optical properties

The UV-vis absorption spectrum of Stevia-based CDs is shown in Figure 6. As seen in this Figure there was an obvious characteristic peak at 240 nm which corresponded to the π–π* transition of aromatic sp^2 domains (5).

To further investigate the optical properties of the Stevia-based CDs, the PL emission and absorption spectra of them are presented in Figure 7. As shown in Figure 7, the maximum fluorescence emission band was located at about 431 nm and maximum excitation is located at 320 nm. Moreover, the quantum yield (QY) of photoluminescent Stevia-based CDs were calculated by taking quinine sulfate as a reference material. The Stevia-based CDs showed a high photo-luminescent quantum yield of 17.5 % and can be considered as an effective bio-imaging reagent.

The impact of reaction temperature on the QY of the synthesized CDs is presented in Table 1. The highest QY of CDs is observed at 150 °C reaction temperature. With increasing the reaction temperature up to 150°C, the QY of CDs also increased. When the temperature reached 200°C, no significant change of QY is observed.

Cell cytotoxicity and fluorescence microscopy imaging

To evaluate the potential of Stevia-based CDs for bio-imaging application in living systems; the cytotoxicity of them was investigated on mesenchymal cell line using MTT assays. Figure 8 displays the microscopic images of CDs (different concentrations) and its about 3% cytotoxicity.

Discussion

This work reported synthesizes and characterization of fluorescent carbon dots using cheap, abundant and commercial plant material. The bands appeared at FTIR spectra of the Stevia-based CDs prepared under hydrothermal conditions are corresponded to the presence of surface functional groups (hydroxyl, carboxyl and …) which allows the binding of CDs with targeted biomolecules. These results provide evidence that CDs contains $-CH_3$ and -OH groups which can be applied as linkers between CDs and therapeutic moieties like drugs for targeted delivery purposes. Findings of EDS analysis suggest the presence of many functional groups containing carbon and oxygen in their structures (carboxyl,

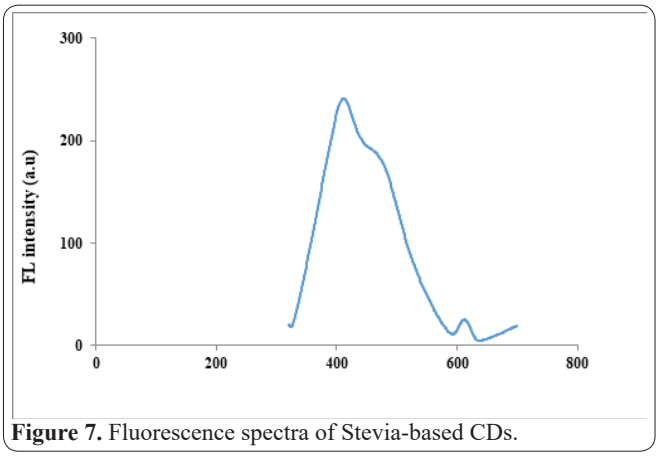


Figure 7. Fluorescence spectra of Stevia-based CDs.

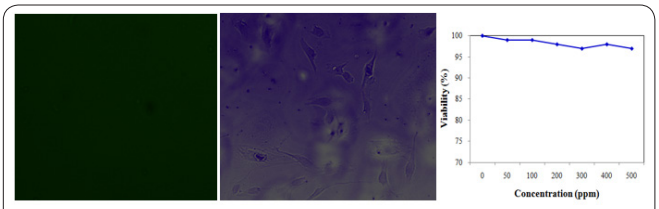


Figure 8. a) mesenchymal cells fluorescence image, b) mesenchymal cells treated with increasing concentrations of Stevia-based CDs at an excitation of 320 nm, c) mesenchymal Cells viability treated with increasing concentrations of Stevia-based CDs results.

hydroxyl, ...). The strong fluorescent, water-soluble and high-quality CDs possess low cytotoxicity and a high QY (about 17.5 %). Therefore, it can be concluded that an optimum reaction temperature of 150 °C could be considered. This result is consistent with previous studies (26).

As seen in Figure 8, the cell growth was not affected by CDs and the as-prepared CDs were non-cytotoxic to the mesenchymal cells at a wide concentration range from 50 to 500 ppm. Thus, an eco-friendly Stevia-based CDs can be applied in sensing and imaging applications. As with this results, in other studies, it has been observed on different kind of cells. Moradi et al in 2018 quantum dots based on chitosan and gum Tragacanth with fluorescence Properties were prepared and they examined their effect on cell toxicity. in this study in all carbon dots, no significant toxicity was observed in 500 ppm on HUVEC cell line (28).

The results of another study in 2016 by Liu show that carbon dots from rose-heart radish display low toxicity to the cells, which makes them a potential candidate for bio-imaging of live cells or other biomedical applications (29).

In this research, Stevia hydrothermal carbonation was carried out under mild conditions without any chemical oxidizing agent. The morphological aspects, surface groups, chemical composition and stevia-based CDs structure have been systematically studied using SEM-EDS, TEM, fluorescence, FT-IR, UV-Vis and XRD techniques. The UV-Vis spectrum reached the signature of the absorption of CDs at 240 nm. The fluorescence spectra represent a characteristic at a peak wavelength of 431 nm with a quantum yield of approximately 17.5%. In addition, there was no living cell cytotoxicity for prepared CDs approved by the confocal microscope. These results showed that these plant-based CDs are promising non-toxic markers for bio-imaging.

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