



Facile Synthesis of Biologically derived Fluorescent Carbon Nanoparticles (FCNPs) from an Abundant Marine Alga and its Biological Activities

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ABSTRACT

Biologically derived carbon-based nanomaterials have been gaining attention in the recent years due to its various potential medical applications. In this study, fluorescent carbon nanoparticles (FCNPs) derived from an abundant alga, *Cladophora vagabunda*, is first reported. FCNPs with an average diameter of ~43 nm was synthesized via hydrothermal process, a simple and green method. The synthesized FCNPs exhibited absorbances in the ultraviolet to visible regions and emissions at 600 and 650 nm. Phytochemical screening and bioactivity tests were conducted to determine various bioactive compounds present in the FCNP surface and its antibacterial, antioxidant, and cytotoxic properties. Results showed that FCNPs from *C. vagabunda* contains tannin, saponin, flavonoids, and phenolic compounds which are known for their antimicrobial property as supported by the result of agar well diffusion method using a *Gram-positive* *S. aureus* and *Gram-negative* *E. coli*. Moreover, FCNPs shows inactive cytotoxicity using brine shrimp lethality test at ratio as high as 1:1 in 40% (v/v) ethanol. The results suggest the potential use of FCNPs from *C. vagabunda* as a fluorescent material with various biological activities.

Keywords: Marine algae, Facile synthesis, Carbon nanoparticles, Optical properties, Biological activities.



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INTRODUCTION

For the last few decades, the development and application of nanomaterials continue to progress due to their excellent properties and tremendous potential applications in various research areas such as electronics, chemistry and biomedical sciences¹⁻². For biomedical applications, quantum dots (QDs) turned out to be successful for various applications, such as biological imaging and labeling, biosensing, drug delivery and cancer diagnostics, due to their luminescent property, narrow emission, broad excitation, high quantum yield and excellent photostability under harsh environment³⁻⁴. These QDs are composed of a semiconductor core, such as CdSe and CdTe, enclosed in a shell, such as ZnS, to enhance its electrical and optical properties as well as to reduce the core metal leaching⁵. However, the growing potential of QDs in biomedical applications has raised considerable concerns regarding their toxicological impact. Indeed, in a recent *in vivo* studies using a mice subjects suggest that CdSe:ZnS quantum dots have high toxicity in the male reproductive system, can decrease in lamina propria, triggers the destruction of interstitial tissue, deformation of seminiferous tubules, and reduction of spermatogonia, spermatocytes, and spermatids⁶⁻⁷. Therefore, the search of alternative CdSe:ZnS nanomaterial for biomedical purposes must be explored.

Carbon nanoparticles (CNPs) is a potential substitute for quantum dots. Unlike semiconductor QDs, which uses heavy metal elements, this nanoparticle is generally small oxygenous carbon nanoparticles, which imparts easy modification, excellent water solubility, better biocompatibility, low toxicity, high chemical stability and low environmental hazard⁸⁻¹¹. For these reasons, CNPs were successfully used for bioimaging¹², DNA biosensing¹³, drug delivery¹², photocatalysis¹⁴ and fluorescence probing¹⁵. Fundamentally, CNPs can be produced from carbon-enriched materials using the top-down or bottom-up approach. Top-down approach involves the cutting of carbon sources, which includes the use of laser ablation, arc discharge, electrochemical exfoliation and oxidative acid treatment, whereas bottom-up approach uses microwave/ultrasonic-assisted and hydrothermal carbonization⁸.

Since its discovery in 2004 by Scriven's group¹⁶, various carbon-based materials, such as, cellulose¹⁷, lignin¹⁸, fungus¹⁹, animal meat²⁰, citrate²¹, and ascorbic acid²² have been reported as starting material for CNP synthesis. However, CNPs derived from green natural materials containing heteroatoms have shown promising carbon source to replace chemical reagents in obtaining low toxicity nanomaterial via bottom-up method¹⁶. For example, Zhen Ngu *et.al.*,²³ demonstrated that carbon nanoparticle with fluorescence property can be derived from waste rice husks using thermal-assisted carbonization at 120 °C with the aid of sulfuric acid. The produced carbon nanoparticle exhibits blue luminescence which can be quenched by adding various metal ions. Moreover, Guo *et.al.*,²⁴ showed the potential of microalgae biomass as a source of FCNPs using the hydrothermal method. The product produced showed emission peak at 435 nm using 360 nm excitation wavelength and negligible toxicity at concentrations as high as 400 µg mL⁻¹. In addition, microalgae are considered as a sustainable source of carbon-based materials because of their high photosynthetic efficiency and faster growth rate even at wastewater²⁵. Also, unlike the conventional biomasses, microalgae can be cultivated on marginal lands and cloned to double their weight within 24 h because they are single-celled organisms²⁶⁻²⁷. Therefore, microalgae can be a potential candidate for a sustainable source of FCNPs using a facile and low-cost method.

In the present study, we demonstrated for the first time the possibility of using an abundant alga, *Cladophora vagabunda*, as a raw material for synthesizing fluorescent carbon nanoparticles (FCNPs) using hydrothermal carbonization. The FCNPs were characterized with respect to its particle size, functional groups, and optical properties. This study also aims to identify the phytochemical compounds present in the surface of FCNPs, determine its cytotoxic activities, and its antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* for possible medical applications.

MATERIALS AND METHODS

Synthesis of Fluorescent Carbon Nanoparticles (FCNPs)

Fresh *Cladophora vagabunda* samples were randomly collected from three sites [Figs. S1

and S2 of Supplementary Information]: shore of Bubu Island, shore of Napayung Island, and coastline of San Sebastian Island, Batangas, the Philippines using a mesh net. Samples were identified and authenticated by the Botany Division of the National Museum of the Philippines. *Cladophora vagabunda* were thoroughly washed, shade-dried for five hours, and pulverized using a blender. Finally, samples were sieved through a 212 μ m mesh to achieve uniform particles.

The synthesis of FCNPs was patterned based on the work of Paikaray *et.al.*,²⁸ with few modifications. First, 0.5 g of fine *C. vagabunda* powder was mixed with 150 mL of 40% (v/v) ethanol solution in an Erlenmeyer flask. The solution was then poured into a 300 mL flask, covered with aluminum foil and heated at 200 °C in a furnace for one hour. After cooling down to room temperature, the as-prepared FCNP solution was centrifuged at

6000 rpm for 20 min. and the supernatant was collected.

Characterization of FCNPs

The ultraviolet-visible (UV-Vis) spectrum of as-prepared FCNP solution was determined using HITACHI U-2900 Spectrophotometer. Functional groups present in the as-prepared FCNP solution was identified using Nicolet 6700 FT-IR Spectrometer. Fluorescence spectra of FCNP solution were gathered using PerkinElmer LS45 Fluorescence Spectrometer. Lastly, the particle size distribution was determined using Malvern-Zetasizer DLS.

Phytochemical Screening

Phytochemical screening analyses were carried out to determine the presence of various bioactive compounds in the FCNPs using standard methods as shown in Table 1.

Table 1: Phytochemical screening analyses carried out in this study

Phytochemical present	Screening analysis	Literature
cardiac glycoside	Killer-Kallani test	[29]
anthraquinone	Anthraquinone test	[30]
terpenoid and steroid	Copper acetate and Salkowski test	[30]
tannin	Ferric chloride test	[31]
saponin	Foam test	[32]
flavonoid	Alkaline reagent test	[33]
phenol	Lead acetate test	[30]
reducing sugar	Fehling's test	[34]

Antibacterial Assay

The agar well-diffusion method was used to assess the antibacterial activities of the as-prepared FCNPs³⁵. The antibacterial activity of the produced FCNPs was tested against *Gram-positive Staphylococcus aureus* (ATCC25923) and *Gram-negative Escherichia coli* (ATCC25922). Adjustments on the turbidity were first conducted by optimizing bacterial cultures to 0.5 McFarland standards. Then, 100 μ L of the standardized cell culture was smeared onto four Mueller-Hinton agar plates. Four petri dishes of *Gram-negative Escherichia coli* were tested against FCNPs with four varying concentrations (as-prepared FCNP solution in 40% ethanol): 1:1, 1:2, 1:4, and 1:8. The same procedure was used for four petri dishes with

Gram-positive Staphylococcus aureus cultures. Each plate bore three wells that were filled with 20 μ L of the FCNP-ethanol solutions. The negative control was set to 40% (v/v) ethanol while positive control was set to ampicillin. The plates were incubated for 24 hours. Then, the zone of inhibition was measured for each plate and recorded.

Brine Shrimp Lethality Assay

Cytotoxic activities of the FCNPs were evaluated by using the Brine Shrimp Lethality Assay (BSLA)³⁶. *Artemia salina* cysts were placed in a beaker containing 4% saline solution and placed under a lamp for 24 h allowing the cysts to hatch. After hatching, active nauplii were collected for the assay. In a 96-well microtiter plate, ten (10) nauplii

were placed on each well with the addition of the as-prepared FCNPs of varying concentrations (1:1, 1:2, 1:4, 1:8 and 1:16 in 40% (v/v) ethanol). A controlled setup and a negative control of 40% ethanol were also included in the assay. All setups were left for two hours with a light source at room temperature. The number of nauplii that survived was then counted and % mortality was calculated.

RESULTS AND DISCUSSION

Synthesis and characterization of carbon nanoparticles (FCNPs)

Figure 1 illustrates the procedure employed for the synthesis and characterization of FCNPs. *Cladophora vagabunda* is known to contain sterols, terpenoids, unsaturated fatty acids and lipids, such as glycolipids, triacylglycerols, phoquinovosyl diacylglycerol, galactolipids, phospholipids, etc.³⁷⁻³⁸ which can be a potential

source of P, S, O and C. FCNPs produced from *Cladophora vagabunda* is pale green in color under normal lighting conditions and emits red luminescence under UV light as shown in the left inset of Figure 2.

To confirm the functional groups present in synthesized FCNPs, the IR spectrum was collected as shown in Fig. 3(A). The characteristic absorption peak at 3427 cm^{-1} can be assigned to O-H stretching vibrations¹⁰. Moreover, the peaks at 2924 cm^{-1} , 1403 cm^{-1} , and 653 cm^{-1} can be associated to stretching vibrations of C-H, C=C, and C=C-H out of plane bending, respectively³⁷. The peak at 1700 cm^{-1} indicates the presence of symmetric/asymmetric stretching vibrations of C=O¹⁰. Also, the bands at $1208\text{--}1370\text{ cm}^{-1}$ correspond to the C-N group while the stretching vibration bands of C-O and P-O at approximately $1038\text{--}1170\text{ cm}^{-1}$ are due to the presence of carboxyl and other oxygen-containing functional groups¹⁶.

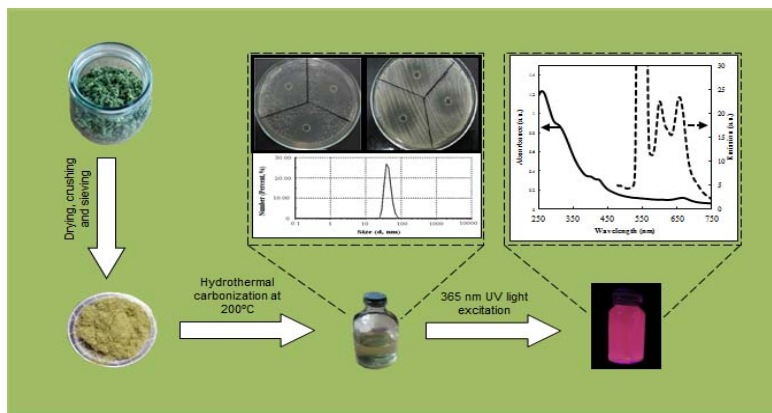


Fig. 1. (Color online) Overview of synthesis and characterization of FCNPs produced from *C. vagabunda*

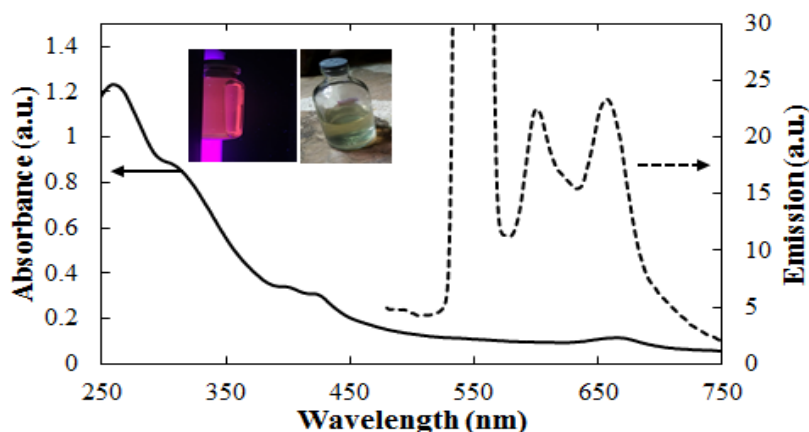


Fig. 2. (Color online) UV-Vis (solid line) and fluorescence (dashed line) spectra of as-prepared FCNP solution

Dynamic light scattering (DLS) was employed to determine the particle size of synthesized FCNPs. As shown in Fig. 3(B), the as-prepared FCNP solution contains nanoparticles with an average diameter of 42.78 nm. This confirms that carbon nanoparticles (CNPs) were successfully synthesized from *Cladophora vagabunda* using the hydrothermal method.

Optical properties of as-prepared FCNPs

Optical properties, such as fluorescence, are important parameters for nanomaterials to be used for bio-imaging applications. The UV absorption, fluorescence excitation and emission spectra of the FCNPs are shown in Fig. 2. The absorption spectrum exhibits a peak at 260nm and a shoulder band at around 305 nm, which can be

ascribed to the typical absorption of an aromatic π system or the $n \rightarrow \pi^*$ transition of a carbonyl group^{16, 37-38}. This observation is consistent with the IR results. Moreover, FCNPs produced from *Cladophora vagabunda* has two distinct emission spectra at around 600 nm and 650 nm when excitation energy of 550 nm was used which could be due to the presence of two chromophores with different energy levels. According to Das *et al.*,⁵⁴ single carbon dots could possess multiple chromophoric units associated with the carbon nanodot core. The same observation was reported by Ju *et al.*,⁵⁵ where they have demonstrated that full-color carbon dots have different color under the same excitation wavelength resulting to a broad emission band from blue to red region.

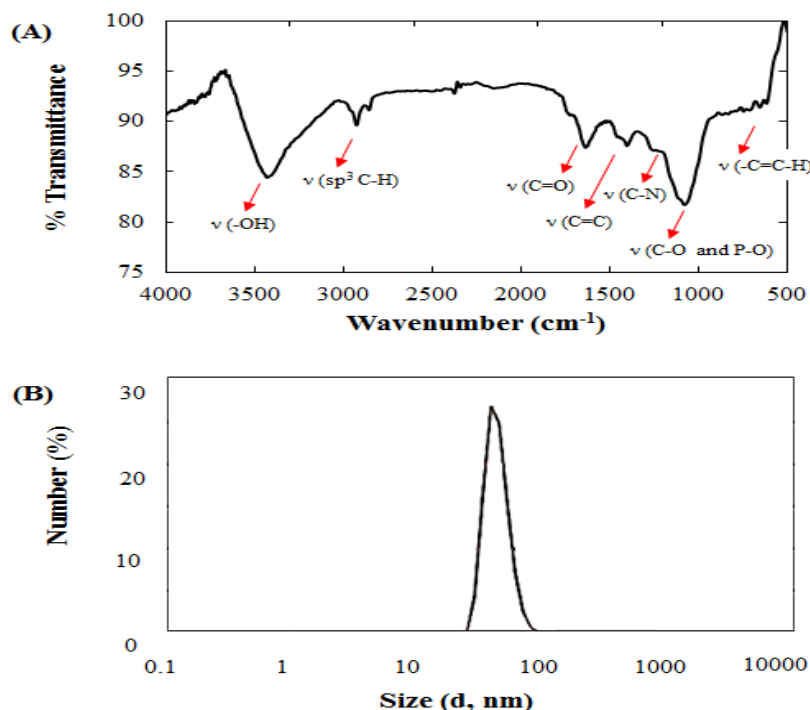


Fig. 3. (Color online) FTIR (A) and DLS (B) spectra of as-prepared FCNP solution

Phytochemistry of as-prepared FCNPs

Several studies emphasized that algal species are rich sources of phytochemicals, such as flavonoids, saponins, tannins, alkaloids, etc. which have been a particular interest in food and pharmaceutical field because of their potential to reduce the incidences of coronary heart disease, diabetes, cancers, and other chronic diseases³⁹⁻⁴⁰. Moreover, the biological nature of *C. vagabunda*

poses lesser chances of side effects on human health rather than synthetically-based products.

FCNPs produced from *C. vagabunda* were screened for the presence of phytochemicals as shown in Table 1. A positive result for ferric chloride test suggests the presence of tannins. Tannins are natural polyphenolic compounds and plant secondary metabolites that are associated with

plant defense mechanism⁴¹. For this reason, tannins are known to have biological activities, such as antimicrobial, anti-parasitic, antioxidant, anti-viral and anti-inflammatory properties⁴². FCNP from *C. vagabunda* is also positive for foam test which indicates the presence of saponin. Saponins are plant-derived biosurfactant⁴³ and a biologically active glycoside consisting of a sugar moiety linked to a hydrophobic aglycone with a triterpenoid or a

steroid structure⁴⁴. Moreover, saponins have plasma cholesterol lowering effect in humans, which is important in reducing the risk of many chronic diseases and shows a strong cytotoxic effect against cancer cell lines⁴⁵. Also, FCNPs from *C. vagabunda* showed positive results for flavonoids and phenolics which are known to have anticancer, anti-inflammatory, antioxidant and antimicrobial properties as well⁴⁶⁻⁴⁸.

Table 2: The number of *Artemia salina* that survived in control, negative control and increasing concentrations of FCNPs

Replicate		Trial 1			Trial 2			Trial 3		
		1	2	3	1	2	3	1	2	3
A.	Controlled set-up	10	10	10	10	10	10	10	10	10
B.	Negative control (40% EtOH)	8	9	8	8	8	9	9	8	9
C.	1:16 as prepare FCNP solution in saline solution	10	10	10	10	10	10	10	10	10
D.	1:8 as prepare FCNP solution in saline solution	10	10	10	10	10	10	10	10	10
E.	1:4 as prepare FCNP solution in saline solution	10	10	10	10	10	10	10	10	10
F.	1:2 as prepare FCNP solution in saline solution	10	10	10	10	10	10	10	10	10
G.	1:1 as prepare CNP solution in saline solution	10	10	10	10	10	10	10	10	10

3.4 Bioactivity of as-prepared CNPs

Antibacterial assay against *S. aureus* and *E. coli*, and cytotoxic activity using brine shrimp lethality test were conducted to determine the biological activity of synthesized FCNPs from *C. vagabunda*.

A range of laboratory techniques has been developed in order to make assessments in terms of *in vivo* antimicrobial activities. One specific method is the so-called agar well diffusion routine which is the official method used in assessing the antimicrobial properties of plants extracts in many clinical laboratories⁴⁹. As shown in Fig. 4, the zone of inhibition for both *Gram-positive S. aureus* and *Gram-negative E. coli* is dependent on the concentration of FCNPs. This suggests that FCNPs from *C. vagabunda* has antimicrobial activity against both *Gram-positive* and *Gram-negative* bacteria. This result is consistent with the study of Ristic *et al.*,⁵⁶, where GQD nanoparticle showed a concentration-dependent cytotoxic effect against *S. aureus* and *E. coli* when treated under UV light. However, in this study, the bioactivity of produced

FCNPs were active despite the absence of UV light. Also, the zone of inhibition is higher for *S. aureus* compared to *E. coli* at the same concentrations of FCNPs which shows its higher efficacy against the *Gram-positive* bacteria. The antibacterial property of FCNPs can be attributed to the presence of phytochemicals as previously discussed.

The brine shrimp lethality test was conducted to predict the preliminary toxicity which could provide a possible indication of cytotoxic properties of produced FCNPs⁵²⁻⁵³. Three replicates with three trials each were conducted, which is composed of controlled set-up, negative control (40% EtOH) and increasing concentrations of FCNPs in saline solution as shown in Table 2. Surprisingly, no brine shrimp died for concentrations ranging from 1:1- 1:16 ratio of FCNPs in 40%(v/v) ethanol, while the negative control shows an average of 15.6% mortality. This suggests that the as-prepared FCNPs from *C. vagabunda* exhibit inactive cytotoxicity and can be potentially used for bio-imaging and drug delivery.

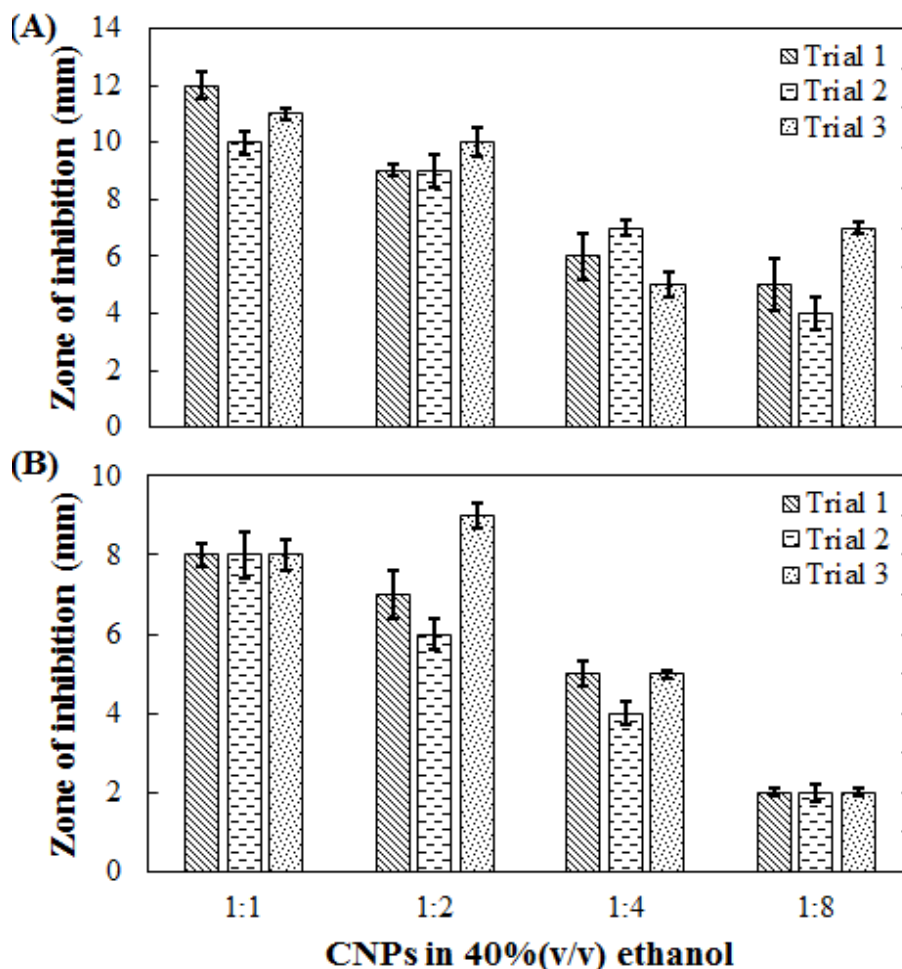


Fig. 4. Antibacterial activity of FCNPs against (A) *S. aureus* (B) and *E.coli* at different concentrations

CONCLUSIONS

We have demonstrated for the first time the synthesis of fluorescent carbon nanoparticles (FCNPs) from an abundant alga *Cladophora vagabunda*, using a green and facile method via the single-step hydrothermal process. The produced FCNPs absorbs light in the visible to UV regions and show emissions at 600 nm and 650 nm wavelengths. Moreover, phytochemical screening demonstrates the presence of tannin, saponin, flavonoid, and phenolic compounds which suggests its potential for future medical use. Furthermore, FCNPs produced from *Cladophora vagabunda* show antibacterial activity against *Gram-positive S. aureus* and *Gram-negative E. coli*,

and inactive cytotoxicity based on brine shrimp lethality test. These results suggest that FCNPs derived from *C. vagabunda* has the potential to be used as a fluorescent material with various biological activities.

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