

Cocoa Bean-Extract Mediated Graphene Quantum Dots as Antimicrobial, Anticancer and Plant Growth Regulators

Anooj E.S, Praseetha P. K

Abstract: A facile method has been developed for the first time for the synthesis of graphene quantum dots using *Theobroma cacao* (cocoa seeds) by hydrothermal method. The structure and morphology of ensuring product was characterized by UV, FTIR, X-ray Diffraction (PXRD) and TEM and SAED pattern. Then anti-microbial, anti-oxidant and anti-cancer activities of *T. cacao* mediated graphene quantum dots were analyzed. Further investigations were made on the use of *T. cacao* mediated graphene quantum dots for growth enhancement in green gram (*Vigna radiate*). CSE-GQDs (Cocoa seed extract-Graphene quantum dots) showed greatest antimicrobial activity against the *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Salmonella sp*, *Escherichia coli*, *Microsporium gypseum*, *Trichophyton sp*. The results of antioxidant activity of the CSE- GQDs evaluated using DPPH scavenging assays indicates that the CSE- GQDs possess a higher scavenging activity. The cytotoxicity studies showed that the synthesized CSE- GQDs exhibited a dose dependent toxicity on human breast cancer (MCF-7) cell lines. The IC_{50} value for MCF-7 cells is found to be 31.2 μ g/ml of CSE- GQDs, where a 50% noticed cell death is observed. The germination rate of green gram seeds significantly increased after treatment with higher CSE-GQDs. Our investigations open up the opportunity to use graphene quantum dots as plant growth regulators that can be used in a variety of other food plants for high yield.

Keywords— green gram; XRD, SAED, anti-cancer, anti-bacterial, anti-microbial, plant growth regulators

I. INTRODUCTION

Over the last two decades, nanotechnology has formed the most promising discipline within science likely to present solutions for the growing challenges in biomedical applications such as drug delivery, therapeutics and diagnostics [1]. Synthesis of metal nanoparticles like Silver, gold, platinum, copper, selenium, quantum dots etc, with a particular shape, size and morphology is an important area of modern research [2]. Graphene quantum dots (GQDS), the zero-dimensional graphene nanoparticles with unique

properties such as biocompatibility and photoluminescence have attracted remarkable interest in the field of nano-science [3]. Due to their excellent optical and electrical property, low-toxicity, and non-pollution, this nano-material is promising to replace semi-conductor quantum dots (SQDs) in solar cells, drug delivery, photocatalysts [4].

So far, various methods have been utilized to prepare GQDs. GQDs are commonly produced by methods involving top down and bottom-up approaches, which are demanding in terms of time, energy and materials. The bottom up approach involves chemistry solutions [5-6], carbonization, a complex procedure which include use of exotic precursors and/or high quantities of organic solvents, which lead to large volume generation of waste [7]. However, GQDs produced by bottom-up procedures are of low standards and exhibit poor stability. On the other hand, the top-down approach, reported techniques involving hydrothermal cutting [8], electrochemical exfoliation [9], electron beam lithography [10] and surface passivating agents [11]. As the approach for the synthesis of GQDs, is referred to involve green synthesis, which would enable us a higher degree of control of the particle properties and have an environmental benign potential. Many studies, have proved top down approach, Hydrothermal Flow Synthesis technique to be an appropriate and green synthesized technique for production of Nanomaterials, as it involves continuously mixing streams of supercritical water and water soluble precursor(s) together, leading to the controlled and green manufacture of a wide range of applications [12]. Hence it has made an interest to us to postulate the utilizing the hydrothermal flow synthesis for synthesis of GQDs nanoparticles, using hydrazine hydrate capping agent and reducing agent.

The tropical tree *Theobroma cacao*, known as cacao or cocoa, belongs to the family *Sterculiaceae*, order *Malvales*. Cocoa beans are grown primarily as an intercrop in Andhra pradesh, Tamil nadu, Karnataka, and kerala, as the tree requires about 40-50% shade. Cocoa was used by ancient peoples as medicinal plants for treating various disorders [13]. The cocoa beans contain a large number of phytochemicals and physiologically active compounds. It is also rich in polyphenols, xanthine and anthocyanins [14]. The consumption of cocoa, which possess high anti-oxidant activity, could be beneficial in decreasing damage caused by

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genotoxic and epigenetic carcinogens and inhibit the complex process leading to cancer. All these desirable biological potentials of the chemical components of cocoa beans motivated the present investigation into its nano-biotechnological potential in green chemistry [13]. In this investigation, we report for the first time the synthesis of graphene quantum dots using cocoa seed extract as a reducing and capping agents, as well as demonstration of its anti-microbial, antioxidant and anticancer activities.

II. MATERIALS AND METHODS

A. Collection and processing of sample

Fresh cocoa seeds were collected and the seeds were then washed, air-dried, then crushed and milled into powder with the aid of electric bender. Cocoa seed extract was obtained following the methods of Lateef et al. [15-17] and later filtered and then centrifuged at 4000 rpm to get the final cocoa seed extract (CSE). Then, the extract was stored at 4°C for further use.

B. Green synthesis of CSE-GQDs and characterization

CSE-GQDs was produced from a reaction mixture containing 1 ml of hydrazine hydrate and 0.1 g CSE dissolved in 10 ml of double distilled water and then ultra-sonicated for 30 min. Then, the solution was transferred into a 25 ml teflon lined stainless autoclave. The sealed autoclave was heated to 150–200 °C in an electric oven and kept for additional 6–10 h. After cooled to room temperature, the product containing water-soluble GQDs was filtered through a 0.22 mm microporous membrane to remove insoluble carbon product, and further dialyzed in a dialysis bag for 2 days to remove unused small molecules. The purified black GQDs were dried at 80 °C. The formation of CSE-GQDs was confirmed by measuring its absorbance spectrum using UV-visible spectrophotometer. The identity of the biomolecules that took part in the green synthesis was determined by FTIR, while the size, morphology and elemental composition of CSE- GQDs was unraveled by XRD, Transmission electron microscopy (TEM) and SAED pattern [18]

C. Antimicrobial activities of the synthesized CSE- GQDs

The antibacterial and antifungal property of the synthesized CSE-GQDs was investigated using the agar well diffusion method. Clinical isolates of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Salmonella* sp, *Escherichia coli*, *Microsporium gypseum* and *Trichophyton* sp obtained from the Department of Microbiology, VIT, Vellore were used as test organisms. Overnight grown cultures were used to seed the plates of Muller-Hinton agar and Sabouraud Dextrose agar medium. The plates were then bored using a cork borer (7mm) to create wells. The wells were irrigated with 100µl of different concentrations (30µl, 60µl and 90µl) of CSE-GQDs prepared by dispersion in sterile distilled water. The plates were thereafter incubated at 37°C for 24 h and 28°C for 48 h.

Standard used were Streptomycin and Ketaconazole. At the end of incubation, the plates were examined for the zones of inhibition, which were then measured [19].

D. DPPH free radical assay

A procedure similar to that reported by Molyneux, 2004 was carried out, where the concentration of DPPH was monitored by UV-Vis absorption spectroscopy at 515 nm. In the presence of antioxidants in the darkness DPPH changes its colour from purple to yellow. Freshly prepared 1 ml of (100µM) methanolic DPPH solution was mixed with 1 ml of various concentrations (100, 200, 300, 400 and 500 µg/ml) of GQDs and incubated in the dark for 1 hr. The radical scavenging activity of each GQDs at different concentrations was evaluated from the absorbance decrease at 515 nm for GQDs-DPPH mixtures with respect to the absorbance of the control. Ascorbic acid (5 µg/ml) was used as standard. Measurements were replicated three times [18].

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_c - A_{\text{GQDs}}}{A_c} \times 100$$

E. Anticancer activities of the synthesized CSE- GQDs

Cell culture

MCF-7 cells were cultured in high glucose Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO₂ humidified atmosphere. The medium was changed every two days during the incubation. Cells were made quiescent by starvation after growth to 70-80% confluence and then treated with experimental reagent [20].

F. Cell viability assay

Cells (1 × 10⁵/well) were plated in 24-well plates and incubated in 37°C with 5% CO₂ condition. After the cell reaches the confluence, the various concentrations of the CSE-GQDs were added and incubated for 24hrs. After incubation, the CSE-GQDs was removed from the well and washed with phosphate-buffered saline (pH 7.4). 100µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC₅₀) was determined graphically. The experiment was carried out in triplicates. The % cell viability was calculated using the following formula: % Cell viability = A₅₇₀ of treated cells / A₅₇₀ of control cells × 100 Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments [20].



G.CSE-GQDs treatment with green gram seeds

Green gram (*Vigna radiata*) seeds of approximately 30 seeds were soaked in distilled water with CSE-GQDs (also referred as control medium or growth medium) and stirred for 3 hrs for hydration. Approximately 10 seeds of the same size were selected and placed about 3 cm deep in soil and watered every day. The pots were placed at room temperature and 55% relative humidity. Germination begins after 4 days of sowing and the number of germinated seeds was recorded every 24 hrs [21].

III. RESULTS AND DISCUSSIONS

A.Green synthesis of CSE-GQDs and characterization

The optical properties of the synthesized CSE-GQDs were studied using UV-vis spectroscopy (Fig.1). From the UV-visible spectrum, a distinct absorption peak around 275 nm was observed. In agreement with these findings, absorption peak around 227 nm was observed by Jiang et al. [22]. Fig.2. reveals the FTIR spectrum of CSE-GQDs. The transmittance peaks at the spectrum are displayed at 3343.81, 2973.56, 2952.82, 2595.83, and 1713.31. The peak at 3343.81 corresponds to strong alcoholic OH stretch and the peak at 2973.56 and 2952.82 corresponds to strong carboxylic acid OH stretch and weak -C-H stretch. The peak at 1713.31 shows strong C=O amide stretch. The XRD pattern of CSE-GQDs exhibited peaks at 18.25, 32.42, 33.45, 37.65, 42.98, 45.87 which can be assigned the (111), (220), (311), (222), (400), (331) planes of CSE-GQDs (Fig.3). The morphology, composition and structural properties of CSE-GQDs studied using TEM. Figure.4. shows the TEM image of CSE-GQDs which reveals that they are planar nanocrystals. The average size of the resultant GQDs decreases from 12 to 3.4 nm with increasing scan rates, as verified by their TEM images. Fig.5. shows the SAED pattern with diffraction rings corresponding to the (102), (110), (112), (200), (210) planes of CSE-GQDs. The TEM data is thus in good agreement with XRD.

B.Antimicrobial activities of the synthesized CSE- GQDs

The antimicrobial activity of CSE- GQDs was determined by well diffusion method. The results indicated that the CSE-GQDs showed the greatest antimicrobial activity against the tested organism (Table 1). There are three different concentrations (30 μ l, 60 μ l and 90 μ l) which were taken to kill the pathogenic microorganisms.

C.Anti-oxidant activities of the synthesized CSE- GQDs

The antioxidant activity of the CSE- GQDs was evaluated using DPPH scavenging assays. As shown in table 2, a significant difference was observed among the respective values obtained. The DPPH values were increased in a dose dependent manner. The recorded value for the lowest concentration of the CSE- GQDs (100 μ g/ml) was 43% and

this value was increased to 84% when the concentration was increased to 500 μ g/ml. These results indicated that CSE-GQDs possessed a higher scavenging activity. This indicates a strong hydrogen donor behavior as a result of their large amount of functional groups at edge sites for the free electron delocalization and stabilization. Previous reports by Ruiz et al.[23] demonstrated the great potential of antioxidant activity of GQDs. The results showed an remarkable influence of the chemical composition of a series of GQDs on their antioxidant and free radical scavenging activity. The GQD exhibited an extraordinary high antioxidant activity, with inhibition effective concentrations for ROS considerably lower than those of ascorbic acid or other carbon nanomaterials.

D. Anticancer activities of the synthesized CSE- GQDs

The cytotoxicity studies showed that the synthesized CSE-GQDs exhibited a dose dependent toxicity on human breast cancer (MCF-7) cell lines. It is found that the cell viability is decreasing with increase in the concentration of the CSE-GQDs. The IC₅₀ value for MCF-7 cells is found to be 31.2 μ g/ml of CSE- GQDs, where a 50% noticed cell death is observed (Fig 6 and Table 3). However, it is not clear why GQDs do not affect normal cells, though it is a good sign. Previous report by Sui et al [20] also demonstrated that combination of GQDs with cisplatin effectively enhance the cytotoxicity, cell cycle arrest and DNA fragmentation.

E.CSE-GQDs treatment with green gram seeds

The germination rate of green gram seeds significantly increased after treatment with higher CSE-GQDs. The germination percentage (%) of green gram seeds was found to be 80% when treated with CSE-GQDs and untreated showed only 20% germination. The length of shoot and root were also significantly affected by the untreated seeds rather than the treated one. The plumule and radical length of green gram seeds was represented in Table 4 and Fig.7.

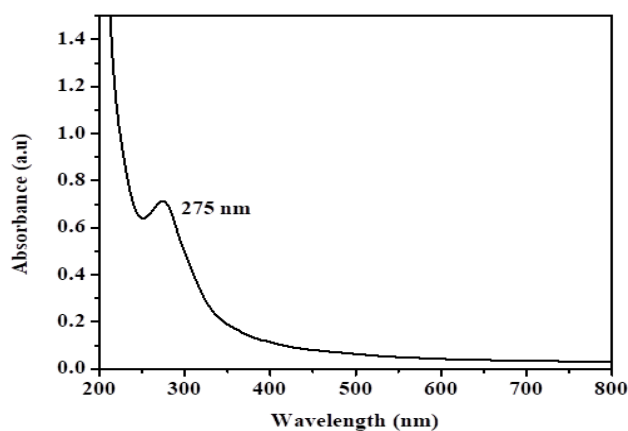


Figure 1 UV-Vis absorption spectrum of cocoa seed extract-grapheme quantum dots (CSE- GQDs)

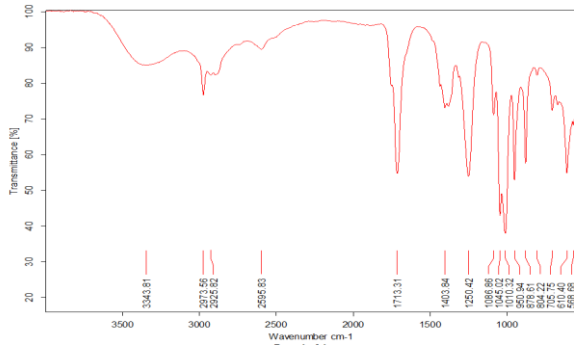


Figure. 2 FT-IR spectrum of cocoa seed extract-grapheme quantum dots (CSE- GQDs)

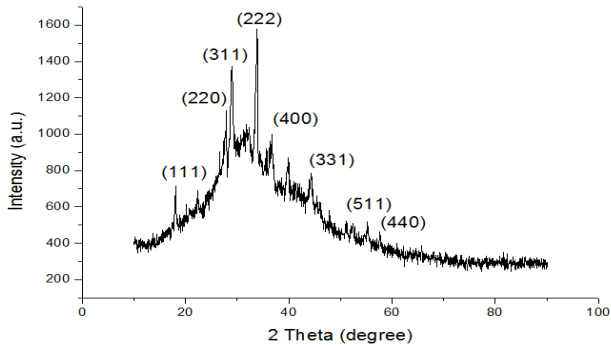


Figure. 3 XRD pattern of cocoa seed extract-grapheme quantum dots (CSE- GQDs)

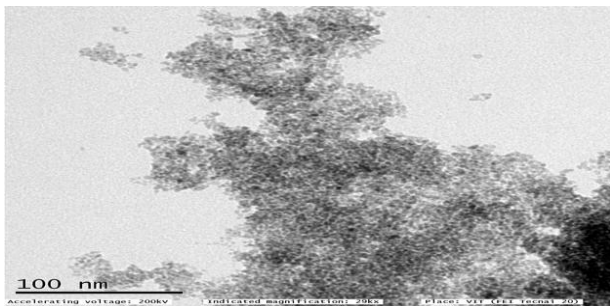


Figure.4 TEM analysis of cocoa seed extract-graphene quantum dots (CSE- GQDs)

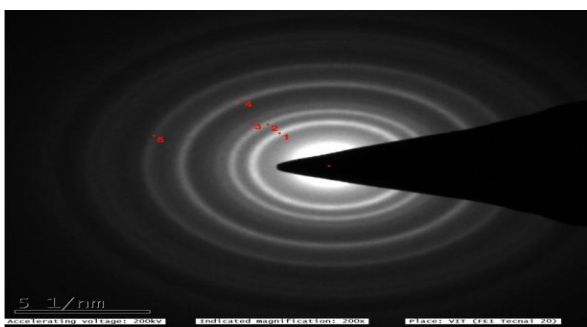
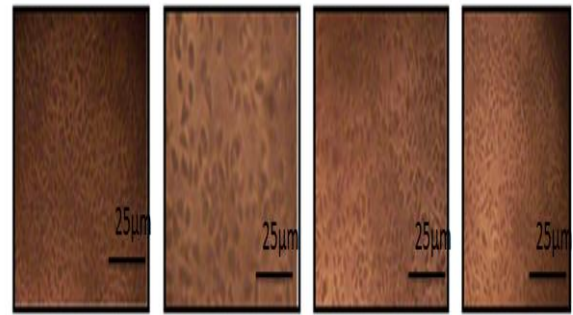


Figure. 5 SAED pattern of cocoa seed extract-grapheme quantum dots (CSE- GQDs)



Normal MCF 7 Cell line Toxicity- 1000 µg/ml Toxicity- 31.2 µg/ml Toxicity- 7.8 µg/ml

Figure.6 Anticancer activity of cocoa seed extract-grapheme quantum dots (CSE- GQDs) on MCF 7 cell line

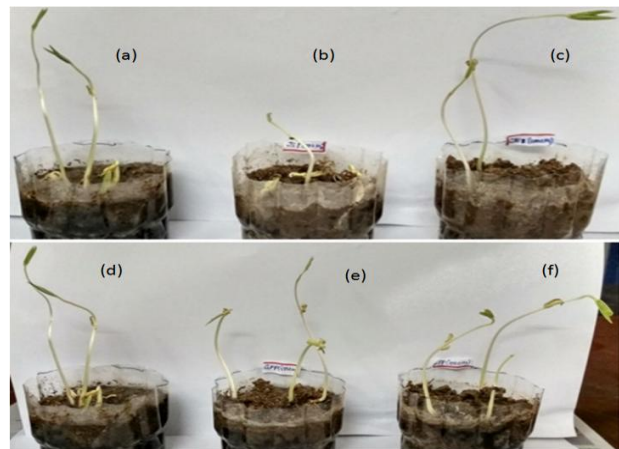


Figure 7. Phytotoxicity study of cocoa seed extract-grapheme quantum dots (CSE- GQDs) (a) Control (b) Untreated seeds (c) Treated seeds (25µl of CSE- GQDs) (d) Treated seeds (50µl of CSE- GQDs) (e) Treated seeds (75µl of CSE- GQDs) (f) Treated seeds (100µl of CSE- GQDs)

Table.1 Antimicrobial activities of the synthesized CSE- GQDs

Microorganisms	Concentration of CSE- GQDs (µl)	Zone of inhibition (mm)
<i>Staphylococcus aureus</i>	30µl	21
	60 µl	24
	90 µl	29
<i>Pseudomonas aeruginosa</i>	30µl	14
	60 µl	23
	90 µl	24
<i>Streptococcus mutans</i>	30µl	15
	60 µl	17

	90 µl	20
Salmonella sp	30µl	16
	60 µl	18
	90 µl	20
Escherichia coli	30µl	21
	60 µl	22
	90 µl	28
Microsporium gypseum	30µl	11
	60 µl	15
	90 µl	17
Trichophyton sp	30µl	13
	60 µl	16
	90 µl	19

Table.2 Antioxidant activity

Concentration (µg/ml)	Test
100	43±0.9
200	51±0.8
300	67±0.1
400	79±0.0
500	84±0.6

Table.3 Anticancer activity of GQDs on MCF 7 cell line

Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability
1000	Neat	0.161	17.61±0.9
500	1:1	0.236	25.82±0.6
250	1:2	0.286	31.29±0.1
125	1:4	0.352	38.51±1.2
62.5	1:8	0.403	44.09±0.4
31.2	1:16	0.472	51.64±1.6
15.6	1:32	0.524	57.33±1.7
7.8	1:64	0.597	65.31±0.0
Cell control	-	0.914	100±0.0

Table.4 The germination (%) of Vigna radiata

Treatments	Vigna radiata	
	Plumule (cm)	Radicle (cm)
Control (Water)	30±1.4	11±0.5
Untreated Seed	6.8±0.51	5.4±2.1
Treated Seed (CSE-GQDs)	17±0.52	8.4±0.8

IV. CONCLUSION

The biosynthesized GQDs have been characterized by UV-Vis, FTIR, XRD and TEM. The CSE-GQDs showed excellent anti-microbial, anti-oxidant and anti-cancer activities. Also, we have demonstrated the use of GQDs as plant growth regulator in green gram seeds. It has been observed that the growth rates of plant were increased substantially in the presence of GQDs. The use of non-toxic GQDs in plant growth might plays an important role in the area of agriculture for enhanced production.

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