

Synthesis and Characterization of Graphene Quantum Dots from Nutmeg Seeds and its Biomedical Application

Anooj E. S, Praseetha P. K

Abstract:The present investigation was suggested to the synthesis of graphene quantum dots using Nutmeg seed by green synthesis and hydrothermal method. The structure and morphology of ensuring the product would have represented by UV, FTIR, X-ray Diffraction (PXRD), TEM and SAED pattern. Then anti-microbial, anti-oxidant and anti-cancer activity, MIC (Minimum Inhibition Concentration) and phytotoxicity of Nutmeg seeds mediated graphene quantum dots were analyzed. The formation of GQDs are confirmed using the noticeable UV absorption peaks 320nm, XRD pattern shows the average crystallite size of the GQDs is 6.1nm, TEM results revealed most of the particle size possessed spherical shapes with an average particle size range of 7.2 nm. CSE-GQDs (Nutmeg seed extract-Graphene quantum dots) showed greatest antimicrobial activity against several bacteria species such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Salmonella sp*, *Escherichia coli*. The results of antioxidant activity of the CSE- GQDs evaluated using DPPH scavenging assays indicate that the CSE- GQDs possess a higher scavenging activity. The germination rate of Nutmeg seeds significantly increased after treatment with higher CSE-GQDs. Our examinations develop the chance to utilize graphene quantum dots as plant development controllers that is possible to utilize in an assortment of different food plants for highly efficient.

Index Terms: XRD, SAED, anti-cancer, anti-bacterial, anti-microbial, plant growth regulators

I. INTRODUCTION

Quantum dots (QDs) are a special type of semiconductor nanocrystals which have high brightness and high photo stability. Diameters ranging from 2 nm to 10 nm in these QDs, with their electronic characteristics depending on their size and shape [1]. These quantum dots produced by conventional methods are highly toxic and are not stable [2]. These QDs are commonly used to study intracellular processes, tumor targeting, in-vivo observation of cells, high resolution of cell imaging and cell cycle diagnostics [3]. QDs are also better than conventional organic dyes owing to its high yield, photo-stability and high emission spectrum which are tuned. The brightness of such spectrum is generally 100 times steadier and 20 times brighter than the ordinary fluorescent dyes [4]. This high photo-stability of QDs makes them ideal for highly sensitive cell imaging systems. These QDs also have the ability to specifically target cells or proteins using peptide mechanism, antibodies or ligands and to study their behavior. They are also used in delivering the siRNA gene-silencing tools [5]

But due to its large size when compared with the biomolecule, the resistance created by it in biological applications are very high [6]. Graphene Quantum Dots (GDs) and Carbon Quantum Dots (C-Dots) are as known to be far superior to the conventional QDs. Graphene Quantum Dot is a nano-sized crystalline structure of size about 2-20 nm. These GQDs closely parallel to the structure of single or multi-layered graphene incorporate by Pan et al., in 2010 [7]. The largest ever synthesized GQD was measured to be around 60 nm in size [8]. GQDs are usually 2D single-layered with sp² hybridized carbons which are arranged in a hexagonal lattice manner [9, 10]. These GQDs have high fluorescence property which is owing to quantum confinement [9]. They have a large number of applications in biomaterial and biosensor production owing to its property of fluorescence, low toxicity, large surface area and ability to cross-link with polymer matrix [11, 12].

GQDs have gathered a lot of consideration in recent times owing to its high-quality optical-electronic and its advanced biocompatibility, distinctive photostability, biocompatibility, small size, electro chemiluminescence, extreme high tunable photo-luminescence, and its unique fictionalization with biomolecules and chemical inertness [13, 14]. All these properties together are utilized for the applications in biomedical fields. GQDs have already been used in several fields like drug delivery, stem cell, near-infrared (NIR) light-

induced photo-thermal therapy [15]. The distinct fluorescence signal emitted by these GQDs plays a vital role in identifying the anti-cancer drug release and response mechanism [16]. Owing to the structural capability and synergic composition of graphene-based QDs; they are also used in the manufacture of several immune-sensing devices [17]. Few of the fields where GQDs have already marked their presence are bio-imaging markers [18], antibacterial [19], disinfection systems [20], anti-biofouling [21], humidity and pressure sensors [22], and photovoltaic devices [23], and light - emitting diodes (LEDs) [24], fluorescent polymers [25].

GQDs are generally produced by top-down and bottom-up methods that are more time, energy and material consuming process [26, 27]. The bottom-up procedure involves several complex processes which use high-cost chemicals and also leading to the production of a huge number of by-products and also the quality of such products are poor [28].

Revised Manuscript Received on December 22, 2018

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On the other hand, the top-down approach involves methods such as hydrothermal cutting [7], electrochemical exfoliation [29], electron beam lithography [30] and surface passivating agents [31]. All these methods are referred to as green-synthesis methods and would give full control over the GQDs particle properties. One of such top-down methods is Hydrothermal Flow Synthesis technique which is best suited for the synthesis of nanomaterial's as it involves thorough mixing of supercritical water streams along with water-soluble precursor materials. This would lead to the controlled growth of nanoparticles which would have several applications in biomedical fields [32].

In the current study, endeavors were made to orchestrate GQDs from a natural source such as Nutmeg seeds owing to several properties of it such as antibacterial [33], antiviral [34] and antioxidant properties [35]. Hydrothermal Flow Synthesis methodology was used in synthesizing Nutmeg seed based GQDs using hydrazine hydrate as a capping agent because of its antibacterial property [36] and reducing property [37]. The synthesized GQDs were subjected to several characteristic studies, phytochemical analysis and later subjected to test the antimicrobial activity, minimum inhibitory concentration (MIC), anticancer activity studies and bio-imaging studies.

II. METHODOLOGY

Hydrothermal synthesis methodology was performed to synthesize GQDs from nutmeg seeds, a natural source. These synthesized GQDs were later analyzed for its structural characterization, phytochemical analysis, antimicrobial activity, minimum inhibitory concentration (MIC), anticancer activity studies and bio-imaging studies.

A. Synthesis of GQDs

The nutmeg seeds have been cleaned, crushed and arid in a hot-air oven at 80 °C. After drying, 0.1 g of nutmeg seed sample is taken with 1 ml of hydrazine hydrate also dissolved in 10 ml of water in an ultrasonic water bath around half an hour. This solution was transferred to a 25 ml Teflon lined stainless autoclave. This was then heated between 150-200 °C in an electric oven and kept for 6-10 h additionally. This water-soluble GQD product sample was cooled to 37°C and then drained via 0.22 mm micro-porous membrane to expel the insoluble carbon products from the sample. Further, these samples were dialyzed using a dialysis bag for 2 days to expel the unfused small molecules from the sample. These purified black colored GQDs were later dried at 80 °C with a yield of nearly 33% and moreover utilized for basic portrayal and property measurements.

B. Phytochemical Analysis

Phytochemical investigation was performed utilizing standard conventions (Sofowora, 1993 and Trease and Evans., 1997). Subtleties of the diverse tests that were led on the samples are as per the following:

Alkaloids: Methanolic extract (1ml) were separated and also 2 ml of 1% fluid HCl was added. This was included for around 2 minutes and afterward, 2 drops of Dragondroff's reagent was included. Reddish brown hasten with turbid nature portrays the alkaloid's presence.

Flavonoids: 5 ml of methanolic extract was separated and 1 ml of 10% NaOH solution was combined with it. Then, 2 drops of concentrated HCl was added onto the sides

of the beaker. Yellow color solution turning to a colorless solution is an indicator of the existence of flavonoids.

Saponins: Methanolic extract (1 ml) were drained and also 2 ml(1%) Sodium Bicarbonate was added and shook vigorously. Lather like formation which is persistent for a period of time depicts the presence of saponins.

Steroids: 100 µl of methanolic extract was filtered and taken in a test tube. Around 400 µl of acetic anhydride was added to it. At that point, 1 or 2 drops of conc.sulphuric acid were included. In the periphery of the mixture contains brownish ring concludes that the presence of steroids.

Glycosides: 100 µl of methanolic extract was filtered and taken in a test tube. Around 400 µl of acetic anhydride was added to it. At that point, 1 or 2 drops of concentrated sulphuric acid were added to it. In the boundary of the mixture contains Blue-Green color conclude the presence of glycosides.

Tannin: About 1 g of the sample was mixed with 100 ml of refined water and mixed well. This was boiled and cooled and then filtered. Than 1% of ferric chloride were mixed drop wise to this filtrate. In the boundary of the green-black precipitate depicts the presence of tannin.

C. Phytotoxicity Study (Seed Germination test)

The effect of different samples at the concentration of 500 µg and 1000 µg was evaluated on germination of seeds of green gram (*Vignaradiata*). The seeds were germinated in pots containing 3 g of paddy field soil. Three sets of 10 green gram seeds were treated with the sample in different concentration (500µg and 1000µg) and then one control without the sample treatment. All pots were kept under shade near sunlight for a certain period for incubation. The seed growth was noted for every 24 h for all pots.

D. Minimal inhibition concentration (MIC)

The isolated colonies were selected from an 18-24 hours blood agar plate (nonselective medium) for making a broth suspension to prepare an inoculum. The suspension was changed in accordance with accomplish turbidity equal to a 0.5 McFarland turbidity standard so that the suspension include approximately 1-2×10⁸ CFU/mL (colony forming units) for bacterial cultures viz., *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus Mutants*. At that point, the inoculum tube was contrasted also 0.5 McFarland standards across a card on a white background and conflicting black line. About an optimal time of 15 minutes, the adjusted inoculum suspension in broth was diluted by using diluting the 0.5 McFarland suspension to 1:150 ratio concluding in a tube incorporate generally 1×10⁶ CFU/mL, so as to have approximately 5×10⁵ CFU/ml in each tube after inoculation.

In MIC investigation, the sample has been prepared through mixing 1 mg of test in 1 ml of DMSO to acquire the consolidation of 1mg/ml. 1ml of sterile LB broth was appropriated for each tube were kept in an autoclave below consistent pressure in the temperature of 121°C. Subsequently, the broth achieves normal room temperature include 1 ml of diluted sample in tube 1. Transferring 1 ml from tube 1 to tube 2. The process was repetitive progressively until the 7th tube. 100µl of bacterial culture is



added to each tube.

Incubation was done at 37 °C for 24 h. Subsequently the incubation period, the turbidity was checked. MIC is the consolidation of higher dilution tubes in which the nonappearance of bacterial growth occurs.

E. Antimicrobial studies

The stock cultures also kept up at 4°C on nutrient agar slant. From the stock culture, a loop full of culture was transferred into the test tubes which contain nutrient broth is the active cultures which are incubated at 24 h at room temperature. Muller Hinton agar medium was used for disc diffusion method also used to analyses the assay. The medium was poured into the Petri plate and left for some time. After that inoculum was spread on the solidified Muller Hinton agar plate, moistened with the bacterial suspension using a sterile cotton swab. 20 µl of the sample (Conc. 1000µg, 750µg, and 500 µg) also added to the disc which placed in MHA plates, the incubation period is 37 °C for 24 hours. The presence of zone inhibition was confined the antimicrobial activity

F. Anticancer activity studies

Anti-cancer assay (MTT assay) (Mosmann, 1983) was performed against Helacell lines. Cells were plated in 24 well microplates with DMEM at 1×10^5 cells/ well. The plates were incubated at 37°C temperature, 5 % CO₂ condition. Then the plates were incubated to allow for cell attachment. After incubation, the cell lines were washed with PBS and then serum-free DMEM medium. 100 ml of 5mg/ml 3-(4, 5-(dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) solution was added into each well and kept for 4 hrs in the incubator. After incubation, 1000 ml of DMSO was mixed properly. Spectrophotometrical absorbance was measured at 570 nm. Measurements were accomplished and the graph was plotted between percentage cell inhibition and log concentration and IC₅₀ value were determined. The viability of the cell(%) was estimated according to the following formula:

$$\% \text{ Cell viability} = (A(570) \text{ of treated cells}) / (A(570) \text{ of control cells}) \times 100$$

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.

G. Antifungal activity studies

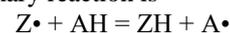
The inoculum was prepared and the stock culture also kept up at 4°C on Sabouraud Dextrose Agar slant. Stock cultures were transfer into the test tubes which contain Sabouraud Dextrose broth are the active cultures which are incubated at 48 h at room temperature. Sabouraud Dextrose agar medium was used for disc diffusion method also used to analyses the assay. Then the medium was poured into the Petri plate and left for some time. After that inoculum was spread on the solidified Sabouraud Dextrose agar plate, moistened with the fungal suspension using a sterile cotton swab. Two different microorganisms were used for this assay namely *Microsporium gypseum* and *Trichophyton*, also Amphotericin-B is used as a positive control. 20 µl of the

sample and positive control were added into the disc which placed in SDA plates then incubated at 28 °C for 24 h. By measuring the diameter of the zone inhibitions were confined antifungal activities.

H. Antioxidant Activity study

The free radical scavenging activity of the sample was measured by DPPH assay (Molyneux, 2004). By the delocalization of the spare electron over the molecules. So molecule will not dimerize. It gives deep violet color in ethanol solution at 520nm. DPPH was mixed with the substance that will give a hydrogen atom. During the period it will reach to the diminished form (Blois, 1958) of violet color.

Defining the DPPH radical by Z• and the donor molecule by AH, the primary reaction is



Where ZH is the decreased form and A• is free radical generated in this initial step. This latter radical will at that point, further responses that control the general stoichiometry, that is, the number of a molecule of DPPH diminished (decolorized) by one molecule of the reductant.

I. Chemicals used:

The chemicals used for synthesis are 1, 1 – diphenyl -2-picrylhydrazyl (DPPH), Dimethylsilphoxide (DMSO) and BHT (standard)-1.6mg/ml in methanol. The samples desired concentration is from 1 mg /ml –max of 5mg / ml (in /DMSO)

J. Procedure:

In all the test tubes containing the aliquot level of absolute methanol of 3.7 ml and 3.8ml of absolute methanol was also used as a blank. Adding 100µl of BHT to a tube that were marked as standard and 100µl of respective samples of other tubes were marked as tests. In all the test tubes were added 200µl of DPPH reagent including blank. Then each tube was incubated at 37°C in dark condition for 30 minutes.

K. Structural characterization

The UV study of the synthesized GQDs nanoparticles revealed that the natural source Nutmeg seeds exhibit the stable synthesis of GQD nanoparticles. The UV spectrum graph (Fig. 1) shows the maximum absorption of the synthesized GQDs nanoparticles at about 320 nm.

L. X-ray powder diffraction (XRD) with EDX

XRD pattern (Fig. 2) with sharp peaks at $2\theta = 32.36, 45$ and 71.69 corresponding to representing the face-centered cubic structure of carbon was obtained. The standard crystallite size predicted using the Debye-Scherrer formula was observed to be about 6.1 nm. This is identified with the polycrystalline nature of the nanoparticles. XRD pattern exposed the FCC structure of the synthesized GQDs.

M. Antimicrobial studies

The Antimicrobial studies of biosynthesized GQDs of Nutmeg seeds have proved a potential against several bacteria species. The readings of the antibiotic activity of biosynthesized GQDs of Nutmeg seeds with different bacterial species is given in Table 4.



III. RESULT

A. Phytochemical analysis

The results obtained by different phytochemical analysis are given in Table 1.

Table 1: Results of Phytochemical analysis of the biosynthesized GQDs from Nutmeg seeds

Phytochemical tests	Qualitative analysis	Results
Alkaloids	Wagner’s test	++
Carbohydrates	Benedict,s test	-
Proteins and amino acid	Ninhydrin test	++
Glycoside	Borntrager’s test	++
Saponins	Foam test	+++
Steroids and sterols	Salkowski test	+++
Phenolic	Lead acetate test	+
Tannins	Ferric chloride test	++
Flavanoids	Sodium hydroxide test	++
Terpenoids	Lieberman Test	++

B. Structural characterization

The UV study of the synthesized GQDs nanoparticles revealed that the natural source Nutmeg seeds exhibit the stable synthesis of GQD nanoparticles. The UV spectrum graph (Fig. 1) shows the maximum absorption of the synthesized GQDs nanoparticles at about 320 nm.

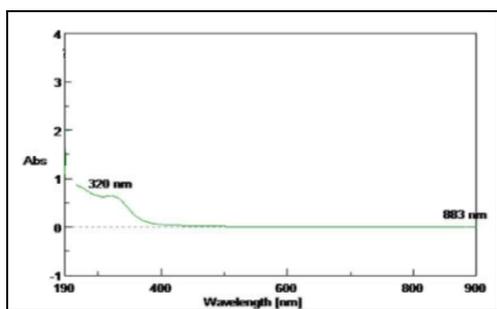


Fig 1: UV Spectrum Graph of the biosynthesized GQDs of Nutmeg seeds

C. X-ray powder diffraction (XRD) with EDX

XRD pattern (Fig. 2) with sharp peaks at $2\theta = 32.36, 45$ and 71.69 corresponding to representing the face-centered cubic structure of carbon were obtained. The average crystallite size predicted using the Debye-Scherrer formula was observed to be about 6.1 nm. This is identified with the polycrystalline nature of the nanoparticles. XRD pattern exposed the FCC structure of the synthesized GQDs.

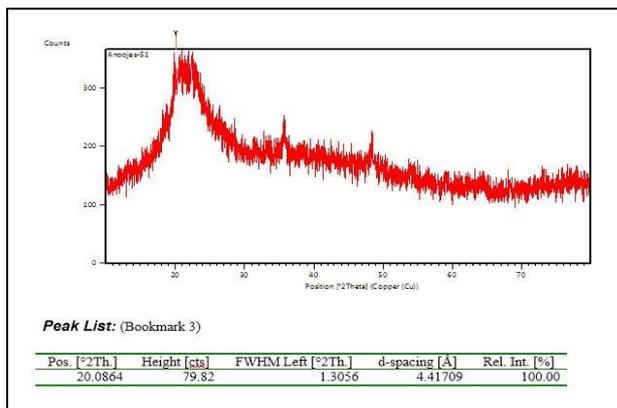


Fig. 2: XRD pattern of the biosynthesized GQDs of Nutmeg seeds

D. Phytotoxicity (Seed germination test)

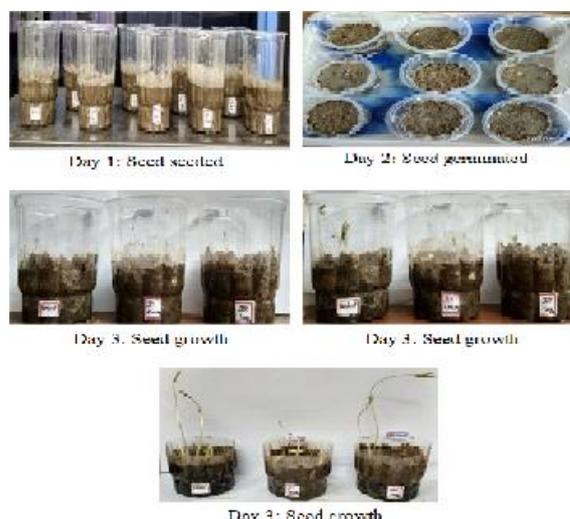


Fig. 3: Phytotoxicity Analysis of Biosynthesized GQDs of Nutmeg Seeds

Table 2: Phytotoxicity Analysis of biosynthesized GQDs of Nutmeg

Dates		Concentration of sample	No of Seed seed	No of Seeds Germinated	Measurement of growths in Cm				
Day I	JS1	500 µg	10	Seeding	-				
		1000 µg							
	Control								
Day II	JS1	500 µg	10	Seeds Germinated	-				
		1000 µg							
	Control								
Day III	JS1	500 µg	10	No Seed Germination	0.9				
		1000 µg			0.8	0.4	0.5		
	Control				2	0.4	0.5		
Day IV	JS1	500 µg	10	No Seed Germination	0.8				
		1000 µg			4	1.4	0.9	1.2	
	Control				4	5	0.8	0.5	
Day V	JS1	500 µg	10	No Seed Germination	1.5	2.4	1.8	2.9	0.9
		1000 µg			6	5.1	8.2		
	Control				4	10.4	9.3	4.6	0.8

TEM Analysis

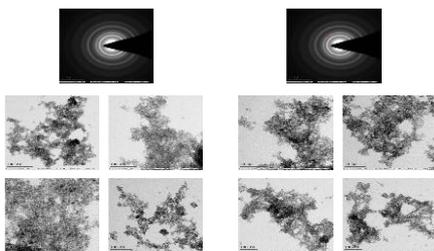


Fig. 4: TEM Analysis of biosynthesized GQDs of Nutmeg seeds

Table 3: TEM Analysis of the biosynthesized GQDs of Nutmeg seeds

Spot #	d-spacing (nm)	Rec. Pos. (1/nm)	Degrees to spot 1	Degrees to X-axis	Amplitude
1	0.3457	2.893	0	129.71	3324
2	0.2749	3.638	0.82	128.89	2297.89
3	0.2427	4.12	4.82	134.53	1335.47
4	0.181	5.525	3.67	126.04	1504.75
5	0.1464	6.828	32.77	162.48	868.81

Minimal inhibition concentration (MIC)



Fig. 5: Minimum Inhibitory Concentration(MIC) for biosynthesized GQDs of Nutmeg seed vs 5 Human pathogens

Table 4: Minimum Inhibitory Concentration(MIC) for biosynthesized GQDs of Nutmeg seed vs 5 Human Pathogens



Name of organism	Minimum Inhibitory Concentration
	(µg/ml)
Staphylococcus aureus	250
Pseudomonas aeruginosa	250
StrptococcusMutans	250
Salmonella spp	250
Escherichia coli	250

Antimicrobial studies

The Antimicrobial studies of biosynthesized GQDs of Nutmeg seeds have proved a potential against several bacteria species. The readings of the antibiotic activity of biosynthesized GQDs of Nutmeg seeds with different bacterial species is given in Table 4.

Table 5: Antimicrobial studies of biosynthesized GQDs of Nutmeg seeds

Organisms	Zone of Inhibition (mm)			Antibiotic Concentration (1mg/ml)
	Concentration (µg/ml)			
	1000	750	500	
Staphylococcus aureus	15	10	7	30
Escherichia coli	13	7	7	28
Pseudomonas aeruginosa	10	7	-	21
Salmonella spp	9	-	-	-14
Streptococcus Mutans	15	10	8	30



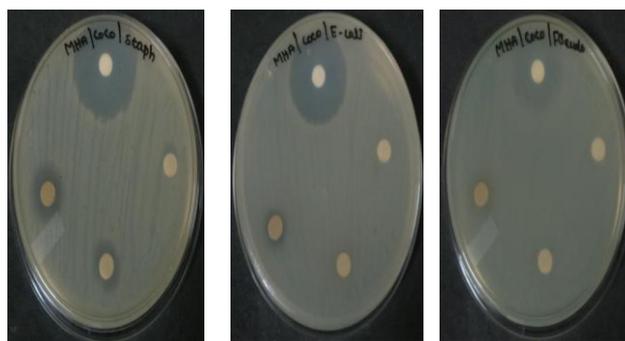
Normal MIC of 1000 µg/ml Toxicity-7.8 cell line µg/ml µg/ml µg/ml

Fig. 6: Antibacterial activity studies of biosynthesized GQDs of Nutmeg seeds with different microorganisms.

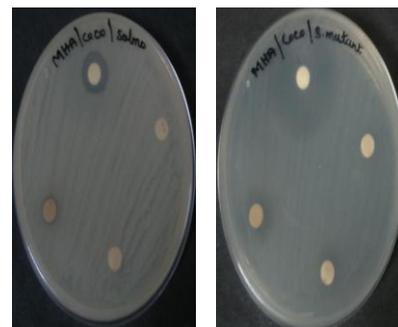
Anticancer activity studies

Table 6: Anticancer Activity Studies of biosynthesized GQDs of Nutmeg seeds

Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability
1000	Neat	0.213	23.3
500	1:01	0.268	29.32
250	1:02	0.326	35.66
125	1:04	0.399	43.65
62.5	1:08	0.461	50.43
31.2	1:16	0.512	56.01
15.6	1:32	0.584	63.89
7.8	0.086111	0.648	70.89
Cell control	-	0.914	100



Staphyloco Escherichi Pseudo



Salmonella Streptococ

Fig. 7: Anticancer Activity Studies Cell Lines of biosynthesized GQDs of Nutmeg seeds at different toxicity concentration

Antifungal Activity Assay

Table 7: Antifungal Activity

Organisms	Zone of Inhibition (mm)			Antibiotic Concentration (1mg/ml)
	Concentration			
	1000	750	500	
Microsporungypseu m	16	16	10	16
Trichophyton	14	12	10	11

Studies of biosynthesized GQDs of Nutmeg seeds



Microsporungyp Trichoph

Fig. 8 Antifungal Activity Studies Cell Lines of biosynthesized GQDs of Nutmeg seeds with different microorganisms



Antioxidant activity studies

Table 8: Antioxidant Activity Analysis of Biosynthesized GQDs of Nutmeg seeds

SAMPLE	Concentration (µg/ml)	O.D	DPPH activity (%)
JS1	25	0.835	53.24
	50	1.165	34.77
	75	1.29	27.77
	100	1.382	22.62
	125	1.563	12.48

IV. DISCUSSION

Despite the difficulties in the production of high-quality GQDs, Hydrothermal approach, using Hydrazine hydrate is considered to be one among the best methods owing to its high scale of production and easy purification methods. SuelaKellici et al. have described the synthesis of GQDs using continuous Hydrothermal Flow Synthesis (CHFS) which has proved to be an ecological and new approach for the synthesis of the large scale of GQDs [38]. Similarly, Parthik et al. have taken natural source extracts which have a high carbon and nitrogen contents have high effects in fabricating nitrogen doped GQDs [39].

GQDs synthesized with Nutmeg seeds in the present study showed maximum absorption at 320 nm, as illustrated in several studies which suggest that GQDs has strong optical absorption in the UV region which is 260-320 nm [40, 41]. Also, Xin Ting et al. stated that the absorption characteristics of GQDs can be successfully altered by functional groups and surface passivation [42]. Similar studies have also shown that GQDs are at an average range of 270-390 nm which exhibit a shoulder peak [43].

In this study, GQDs synthesized with Nutmeg seeds were evaluated for its phytotoxicity tests, antimicrobial, antiviral, antioxidant tests and anticancer properties which showed impressive results when compared with the conventional commercially available mycoplasma removal agents even at about 10 µgml-1 of small dose [42]. Similarly, methicillin-resistant Staphylococcus aureus and Escherichia coli were killed using photodynamic effects of GQDs. Ultimately; GQDs synthesized with Nutmeg seeds would exhibit similar properties.

Many research works are still in progress which is aiming to study the physical and medical properties of such GQDs synthesized with natural substitutes. This would definitely bring out the possible advantages of GQDs like drug or gene delivery, bioimaging, optical sensing, and theranostics.

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