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Anti-bacterial Potential of Carbon Dots derived from Coconut Waste

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ABSTRACT: Carbon dots (CDs) are emerging zero-dimensional carbon nanomaterials (<10 nm) with wide spectrum of applications. One of the best advantages of utilizing carbon dots in developing fields is its large scope to synthesize from various synthetic and agro-wastes like coconut wastes for its cost-effective production. The disposal issue of this waste shell has been solved by its use as a precursor for the synthesis of CDs with antimicrobial property. This study was attempted to synthesize valuable carbon dots from coconut waste (shell) and assess its antibacterial activity of CDs. CDs were synthesized by pyrolysis method followed by sonication and characterized through High resolution transmission electron microscope (HR-TEM) to confirm its average size as 7 nm. The synthesized CDs were subjected to multi-assay approaches to assess its toxicity potential against E scherichia *coli* (*E. coli*), a common indicator of water contamination. Resazurin test showed CDs @ 500 ppm as Minimum Inhibitory Concentration (MIC) against *E. coli*. Furthermore, MTT assay demonstrated dose dependent toxicity of CDs wherein the lowest dose (0.48 ppm) exhibited highest cell viability (97.9 %) and the highest dose (1000 ppm) recorded lowest cell viability (48.3%). Overall, the coconut shell derived CDs were found to be a potential anti-bacterial agent which has a great scope for wide range of environmental applications. Cytotoxic effect of CDs was assessed using MTT assay founds to observe reduction in cell viability to 48.34 % at 1000 ppm. Minimum inhibitory concentration (MIC) and cell viability assay (MTT) revealed that CDs showed toxicity against *E. coli* and it can be used in various disinfection systems.

Keywords: Antibacterial activity, Carbon dots, Coconut shell, Cytotoxicity, *E. coli*, MTT, Resazurin.

INTRODUCTION

Nanotechnology has immense potential to contribute for the development of various fields of sciences including medical, pharmaceutical and environmental clean-up (Kaur *et al*., 2021; Garg, 2021). Metal and metal oxide nanoparticles have been extensively used in all these scientific fields, but they have a number of disadvantages, including toxicity and non-biodegradability (Khayal *et al*., 2021). Carbon dots (CDs), the youngest member in carbon nanomaterial family were discovered by Xu *et al.* (2004) during the electrophoretic purification of single walled carbon nanotubes. These novel nanomaterials are considered as a viable substitute for metal-based nanoparticles due to their biocompatibility and feasibility (Wang *et al*., 2020). The synthesis methods of carbon dots can be divided into two main categories, top-down and bottom-up approaches (Wang and Hu 2014). Despite the fact that carbon dots can be synthesized from diverse biomasses, crop residues are considered as potential sources (Kang *et al.,* 2020; Kurian and Paul 2021). Among the various crop residues, coconut wastes have been exploited for synthesis of carbon dots (Chunduri *et al*., 2016; Chauhan *et al*., 2020; Abinaya *et al.*, 2021). Additionally, the use of coconut shell as a beginning precursor for the synthesis of CDs has been signified by the presence of cellulose, hemicelluloses, and lignin (Abinaya *et al.*, 2021). The disposal issue of this coconut waste has been solved by converting bulk carbonaceous materials into effective CDs, which has antibacterial properties (Chauhan *et al*., 2020). These CDs are widely exploited for bioimaging and biosensing applications (Su *et al*., 2020) and research is being focussed to explore the possibilities of applying CDs as antibacterial agents (Yang *et al*., 2016; Lin *et al*., 2019). Therefore, the development of more potent antibacterial drugs for long-term usage is widely used to combat bacterial contamination. Earlier reports showed that the CDs has the ability to inhibit and suppress various microorganisms (Jijie *et al.,* 2018). With this background, this study was designed to evolve a win-win strategy for effectively transforming coconut wastes into CDs and understand its potential as anti-bacterial agent. In this study, the antibacterial performance of CDs has been analysed using multi-assay approaches via *Escherichia coli* (*E. coli)*, the most common indicator for faecal contamination in drinking water (Ishii and Sadowsky 2008). Hence, this current work has proposed a novel and green synthetic methodology for synthesising carbon dots using coconut waste.

With more studies using in-vitro approaches for toxicological assessments, a clearer understanding of the toxicological behaviour of nanomaterials is essential. Among the different assays, the resazurin assay is simple, quick, versatile, economical (Pereira *et al*., 2020), and has a strong correlation with other methods used to measure cytotoxicity (Riss and Moravec 2004; Breznan *et al*., 2015). Other commonly used assays like agar dilution is a labor-intensive and time-consuming quantitative procedure that is frequently employed to determine MIC values (Elshikh *et al*., 2016). Additionally, other tests, such as disc diffusion method, which is a qualitative measure of antimicrobial activity for test materials, can only produce only zone of inhibition indicative results. Resazurin assay is therefore viewed as a direct indicator of bacterial metabolic activity that may be used to ascertain the minimum inhibitory concentration (MIC) of substances (Sarker *et al*., 2007). Secondly, *in vitro* cytotoxicity can be done by assessing the cell viability and among the several assays, the MTT assay is mostly recognised as a rapid, quantitative and colorimetric assay (Bahuguna *et al*., 2017). Accordingly, in this study, CDs were subjected to two assays namely, resazurin and MTT to observe its minimum inhibitory concentration and cytotoxicity on *E. coli*.

MATERIALS AND METHODS

Synthesis of carbon dots. The carbon dots were synthesized from coconut shell through muffle furnace mediated synthesis as outlined by Chauhan *et al*. 2020 and further subjected to sonication process. The obtained sediment after centrifugation was oven dried at 80°C for 12 hours. The dried powder was ground using pestle and mortar and the resultant carbon dots were subjected for further characterization. The carbon dot suspension was homogenized by sonication prior to use in the following experiments.

Characterization. High resolution transmission electron microscope (HR-TEM) is an imaging mode of the Transmission Electron Microscope used for higher magnification studies of nano-materials at the atomic scale. The size, morphology and uniformity of CDs were measured with the help of HR-TEM (JEOL, Japan) with 200 kV and the image was further developed by using “Image J” programme (Das *et al*., 2019)

Microbial culture. *Escherichia coli* (*E. coli*) bacterial strain (MTCC 1652) was procured from Microbial Type Culture Collection, Chandigarh, India.

Determination of Minimum Inhibitory Concentration (MIC) of CDs by Resazurin test

Medium used for assay. Throughout the experiment, Muller Hinton medium (Himedia, India) was used as per the recommendation of NCCLS (National committee for clinical laboratory standard) for susceptibility testing.

Preparation of resazurin solution. The resazurin solution was prepared by dissolving 0.4 % resazurin sodium (Himedia, India) in sterile distilled water. Further, the prepared dye solution was mixed well using a vortex mixer to ensure the dye was well-dissolved.

MIC assessment. The minimum inhibitory concentration (MIC) assay was performed in a 96 well microplate using resazurin dye (Sarker *et al*., 2007). Initially, 100 µL of Mueller-Hinton broth was added to each well of a 96 well microplate. Subsequently, 100 µL of carbon dots was added by two-fold serial dilution 1000 ppm to 0.48 ppm concentration. Later, 10 µL of *E. coli* cell suspension was added to each well and then finally 10 µL of resazurin solution was added. The inoculated 96 well microplate was incubated at 37°C for 24h. Antibiotic ciproflaxin was used as positive control and was added to the Mueller-Hinton broth in serial dilution. Each test included a positive control (PC) antibiotic ciproflaxin and sterile control (BLK) (media alone). All tests were performed in triplicate and a negative control (NC) (without CDs) was added as an indicator to determine MIC (Chakansin *et al*., 2022).

Assessing the cell viability through MTT assay. EZcount^{TM} MTT cell assay kit was used to evaluate the cytotoxicity of CDs. The assay was carried out as per the manufacturer’s instructions with slight modifications. Briefly, *E. coli* cells at its early log phase (OD_{600} 0.07) was seeded in 96-well plate, then exposed to different concentrations of CDs (1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95, 0.97, 0.48 ppm) for 6 hours. The control group was kept as culture medium alone.10µl of MTT reagent (5 mg/ml concentration) was added and incubated for 3 hours and then 100µl of solubilizing agent was added. After 30 minutes, the optical density was measured at 570 nm by a spectrophotometer plate reader (BioTek, USA)

RESULTS AND DISCUSSION

HRTEM Characterization. In the present study, the coconut shell derived carbon dots were characterized using HR-TEM technique at 20 nm and 5nm scale magnification. At lower magnification scale (Fig. 1 a), HRTEM images of carbon dots derived from coconut shell were homogenous and spherical in shape, which is visible as dark spots. Surprisingly, the black dark spots were spotted as slanting lines at higher magnification scale, confirming the presence of carbon dots, and the average size of the carbon dots was observed as 7 nm (Fig. 1 b). The recorded size (less than 10 nm) of synthesized CDs in this study is similar to the reported size (3-5 nm) of carbon dots obtained from coconut shell (Chunduri *et al*., 2017).

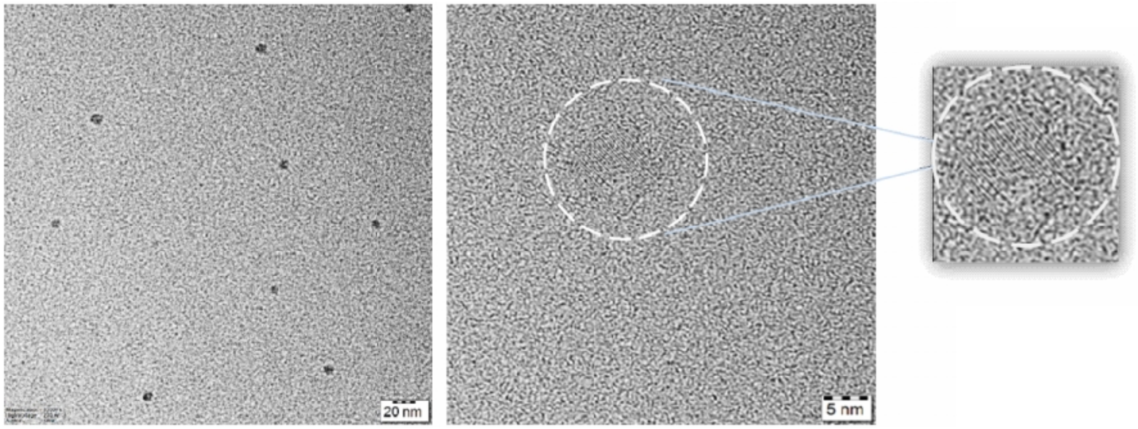
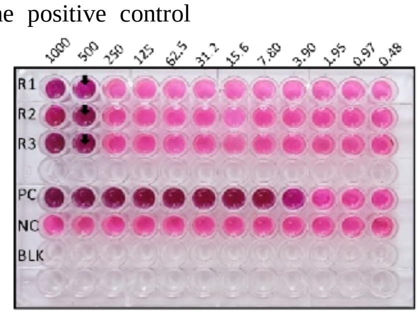


Fig. 1. HR-TEM images of Carbon dots from coconut shell. The scale bar represents (a) 20 nm scale and (b) 5 nm scale.

Determination of MIC of CDs by resazurin test. Carbon dots derived from coconut shell were tested for its cytotoxicity potential against *E. coli*. Although several cytotoxicity assays have been used to determine the cytotoxicity of nanoparticles, the resazurin dye-based method is one of the most effective and quick methods for visually determining the minimum inhibitory concentration of nanoparticles or any drugs of interest (Chakansin *et al*., 2022). Resazurin is a redox sensitive, non-fluorescent dye that is used to determine the cell viability. The non-fluorescent blue resazurin dye can be converted to the fluorescent pink resorufin by metabolically active cells. Non-living cells, on the other hand, do not reduce the resazurin, so the dye remains blue. As a result, the visible change in resazurin dye colour can indicate both viable and dead cells (Schmitt *et al*., 2013). In this work, the carbon dot at varying concentrations ranging from 1000 ppm to 0.48 ppm were tested against the *E. coli* (MTCC 1652) culture. The *E. coli* cell suspensions were seeded into the 96 well plate to carry out the MIC assay. After, 24 hours of the carbon dot and bacterial interaction, concentration dependent reduction in the dye intensity was observed. The carbon dots concentration, from 250 ppm to 0.48 ppm, did not significantly inhibit the *E. coli* cell growth and the resazurin blue dye was changed into pink color, indicating live cells (He *et al*., 2016), whereas at the higher concentrations of carbon dots at 1000 and 500 ppm, inhibition in the growth of *E. coli* cells was observed and that the dye retained its color. At end of 24 hours, the MIC of CDs against *E. coli* was observed as 500 ppm (Fig. 2). The positive control (Ciproflaxin) and negative control (*E. coli* culture + medium) were used as a reference point to measure the intensity of the dye's blue and pink colours.

Our results corroborate with the previous studies which has reported that CDs are potent antibacterial agent. The MIC of CDs synthesized from glucose and polyethyleneimine (PEI) against *E. coli* was observed as 64 ppm (Dou *et al*., 2015). Recently, MIC of CDs synthesized from oyster mushroom through hydrothermal carbonization and tested with resazurin assay against pathogenic bacteria *viz*., *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* was found to be 30 ppm for these three bacterial strains (Boobalan*et al*., 2020). In contradictory, Chauhan *et al*. (2020) reported that CDs derived from coconut waste (shell) through hydrothermal method and antibacterial test conducted through agar well diffusion method against *E. coli* showed no inhibition zone formation for any kind of bacterial species that were studied.

Thus, this present study demonstrated that the coconut shell derived CDs had antibacterial property with MIC as 500 ppm against *E. coli*. The antibacterial effect of CDs might be due to the interaction of its surface functional groups with the cell membrane of the bacteria causing cell lysis. Dou *et al*. (2015) had reported that CDs were able to absorb onto the cytoplasmic membrane causing cell disruption of *E. coli*. Furthermore, Boobalan *et al*. (2021) had explained that antibacterial nature of CDs was due to its interaction with the cytoplasmic fluids inside the bacterial cell resulting in cell lysis including apoptosis.



Note: R1, R2 and R3 are three replications; PC-Positive control; NC- Negative control and BLK- Blank

Fig. 2. Determination of MIC of CDs against *E. coli* by resazurin test.

Assessing the cell viability through MTT assay. To evaluate the effect of synthesized CDs on the viability of *E. coli*, cell cultures were exposed to CDs at its exponential growth phase and the results of MTT assay showed a significant reduction in cell culture. Being one of the simplest cytotoxicity measurements, this assay utilizes 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a water-soluble yellow dye (Grela *et al*., 2018) that can be reduced to water-insoluble purple formazan crystals by the dehydrogenase enzyme of metabolicallyactive cells. The formazan crystals thus formed can be spectrophotometrically quantified by dissolution in a solvent, and the intensity is directly proportional to the number of metabolically active cells (Tunney *et al*., 2004). Cell cultures were exposed to CDs at its exponential growth phase and the results of MTT assay showed a significant reduction in cell viability. Looking closely at Fig. 3b, at the concentration of 1000 ppm, the cell viability was found to be 48.34 % and at 0.48 ppm it was 97.90 %.

Our results demonstrated dose-dependent cytotoxicity of CDs with significant decrease in cell viability at higher concentration. These results are comparable with the findings of Alsadooni and Obada (2020) who performed MTT assay and found that graphene quantum dots derived from coconut husk had cytotoxicity effects against MCF 7 cell line recording lower cell viability (20 %) at 1000 ppm in comparison with higher cell viability (70 %) at 7.8 ppm. Recently, Chauhan *et al*. (2022) conducted MTT assay for coconut husk derived CDs against macrophage cell lines and established that 1 ppm of CDs was able to reduce the growth of tested cells by 32 %.

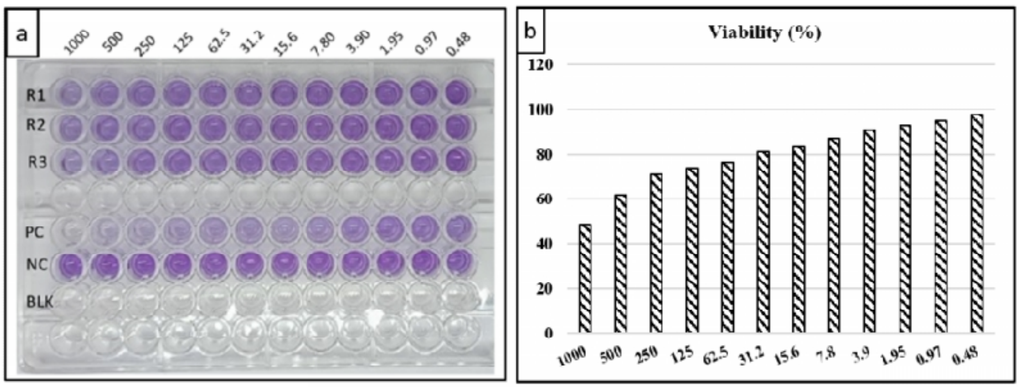


Fig. 3a. Determination of cell viability (%) of CDs against *E. coli* by MTT assay. R1, R2 and R3 are three replications; PC-Positive control; NC- Negative control and BLK- Blank; b. Graph showing cell viability (%) of CDs against *E. coli* by MTT assay.

CONCLUSION

Overall, the results revealed that carbon dots synthesized from coconut waste (shell) with an average size of 7 nm showed antibacterial activity against *E. coli* culture. Moreover, dose dependent cytotoxicity potential of CDs was also demonstrated in this study with MIC of 500 ppm against *E. coli*. In this study, the lowest dose (0.48 ppm) of CDs exhibited highest cell viability of 97.9 % and the highest dose (1000 ppm) of CDs recorded lowest cell viability of 48.3 %.

FUTURE SCOPE

The dataset generated in this study showed that coconut waste derived CDs possess antibacterial activity against *E. coli*, a best indicator of water contamination. Furthermore, the cytotoxicity assessment with two tests namely resazurin and MTT also showed dose dependent toxicity of CDs and thereby providing numerous avenues for developing several nanoproducts with different CDs concentration for wide range of applications. Such findings will greatly aid in evolving several applications of CDs as antimicrobial agents in general and water disinfectants in particular.

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Conflict of Interest. None.

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