**Preprint Title** Preparation of xylitol carbon dots and analysis of its antimicrobial potential in combination with other antimicrobial agents

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# Preparation of xylitol carbon dots and analysis of its antimicrobial potential in combination with other antimicrobial agents

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# ABSTRACT

**Background:** Bio-molecule based carbon dots (C-dots) have gained much attention in last few years due to their high biocompatibility, low toxicity and outstanding optical properties which can possibly be used as nano-carrier for drug delivery.

**Methodology:** To find out the best possible conditions for carbon dots preparation from xylitol different combinations of process conditions were evaluated. Synthesized carbon dots were purified and evaluated for their size, surface features, and luminescence by AFM, FT-IR and spectrophoto-fluorometry. Purified C-dots were loaded with conventional antimicrobial compounds and evaluated against clinical isolates of human pathogens.

**Results:** Xylitol and its C-dots were effective against *E. coli, S. pyogenes, C. albicans* and *Cryptococcus neoformans* while no activity was recorded against *Staphylococcus aureus, Klebsiella pneumonia, Listeria monocytogenes,* and *Salmonella typhi.* In contrast to this conjugates were more effective than conventional antimicrobials. MIC analysis with respect to the four selected pathogens showed that vary small concentration of Am-C dots is sufficient to inhibit the growth of pathogens as 0.01 mg/ml of Am-C dots was sufficient against *S. pyogenes* but 0.16 mg/ml xylitol and 0.08 mg/ml antimicrobial respectively were required. Similarly 0.16 mg/ml (*Escherichia coli*), 0.04 mg/ml (*Candida albicans* & *Cryptococcus neoformans*) were sufficient which is quite low in terms of concentration in comparison to crude form of antimicrobials and xylitol itself.

**Conclusion:** The results pertaining to current work further suggested that C-dots were not only found more effective but also improved the efficacy of conventional antimicrobials used against the pathogens. Such potential of this important low calorie sweetener can be exploited in variety of healthcare products after further R&D and clinical trials. The efficiency of xylitol C-dots and the conjugates with positive antimicrobials (tetracycline and ketoconazole) against several pathogens also exhibited the useful role of nanotechnology in healthcare.



**Keywords:** Sweet carbon dots, xylitol, antimicrobial, drug delivery, drug formulation.

## BACKGROUND

Nanotechnology is an exhilarating area in science & technology offering immense opportunities in different fields with special interest to medicine and pharmaceuticals. Manipulation in particle size to nanometer scale can alter the fundamental and biological activity to a great extent by:

- site specific targeted drug delivery
- altering/regulating the release of drug
- altering the solubility and blood retention time after consumption

Nano-structure shares diverse range of material having at least on dimension in the range of 1-100 nm. It also includes fluorescent particles, the newest member of nano structures known as carbon dots. These particles, prepared form variety of natural bio-molecules can become potential alternative to conventional metal-based quantum dots due to compatibility with biological systems [1]. Carbon dots (CDs) are recently added class of carbon materials that can be produced by ultrasonication, microwave and hydrothermal methods which can be used as coating material and carrier for drugs [2-4].

Besides conventional metal particles, CDs prepared from variety of biological materials such as fruits and vegetables juices and peels, grass and plant leaves, carbohydrates, organic acid (citrates) and other natural products [5] have gained much attention due to simpler synthesis route, stability, water solubility, high bio-compatibility and non-toxicity [6-9]. These C-dots have been widely used in process catalysis, light emitting devices and environmental monitoring but their applicability in drug delivery and bioimaging is commendable [10-11].

The xylitol, natural sugar alcohol is naturally present in certain vegetables and fruits. Because of its' equivalent sweetening power and lower calorie (40%) content, it has been

widely used in food, pharmaceutical and cosmetic industries. Previously it has been reported that xylitol can prevent the adherence of major otopathogens including *Haemophilus influenzae*, *S. pneumoniae* to nasopharyngeal epithelial cells and effectively inhibit their growth. It has also been reported that xylitol consumption reduces ovariectomy-induced bone resorption and increases the trabecular bone volume [12-13]. Keeping in view the importance of C-dots, current research work was designed to prepare C-dots from xylitol, produced from sugarcane bagasse by fermentation. Xylitol, purified from the fermentation broth was used for C-dots preparation and further evaluated for their antimicrobial potential.

**RESULTS**

For carbon dots preparation, xylitol was produced by fermentation of sugarcane hydrolysate at 1.5 l working volume in 2 l fermentor (New Brunswick Scientific, USA). Maximum conversion of 71.98 % (0.76 g/l.h) xylose to xylitol was recorded after 56 hr. Xylitol was recovered from the fermentation broth after removal of impurities and purification of desired products by activated charcoal treatment, cation & anion exchange treatment followed by vacuum evaporation. Maximum decolorization efficiency (upto 99.69 %) was recorded after treating fermentation broth with activated charcoal and ion exchange resins. Finally 48.49±0.45 g of xylitol crystals were recovered with (94.56% purity) after freeze drying (-20 $^{o}$C) overnight followed by washing with solvents. Purified xylitol crystals were further used for carbon dots synthesis.

**Preparation and characterization of Carbon dots:** 152.15 mg of xylitol crystals were dissolved in 10 ml of distilled water and mixed with 10µl of HCl and EDA. Contents were mixed by vigorous stirring for 2 min and heated in microwave oven for 2-5 minutes. After microwave treatment, content became yellow-brown thick gel like material which was dissolved in distilled water and purified by dialysis.

All the nano-crystals are represented as arrays of periodically arranged atoms in three-dimensional (3D), two-dimensional (2D) or one-dimensional (1D) nano-structures while in case of amorphous atoms are randomly assembled. Different morphological, physical and

chemical propertied of purified C-dots were determined using numerous techniques including X-Ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), U.V spectroscopy, fluorescence.

Thin layer of synthesized carbon dots was deposited on glass slide, dried overnight and analyzed by laser beam of 650 nm for topographical features like shape, size and roughness. Thorough analysis of the samples suggested that the particles were rough and irregular in shape. AFM micrograph revealed that the average size of carbon dots were 8.88±0.32 nm average size of carbon dots (Figure 1).

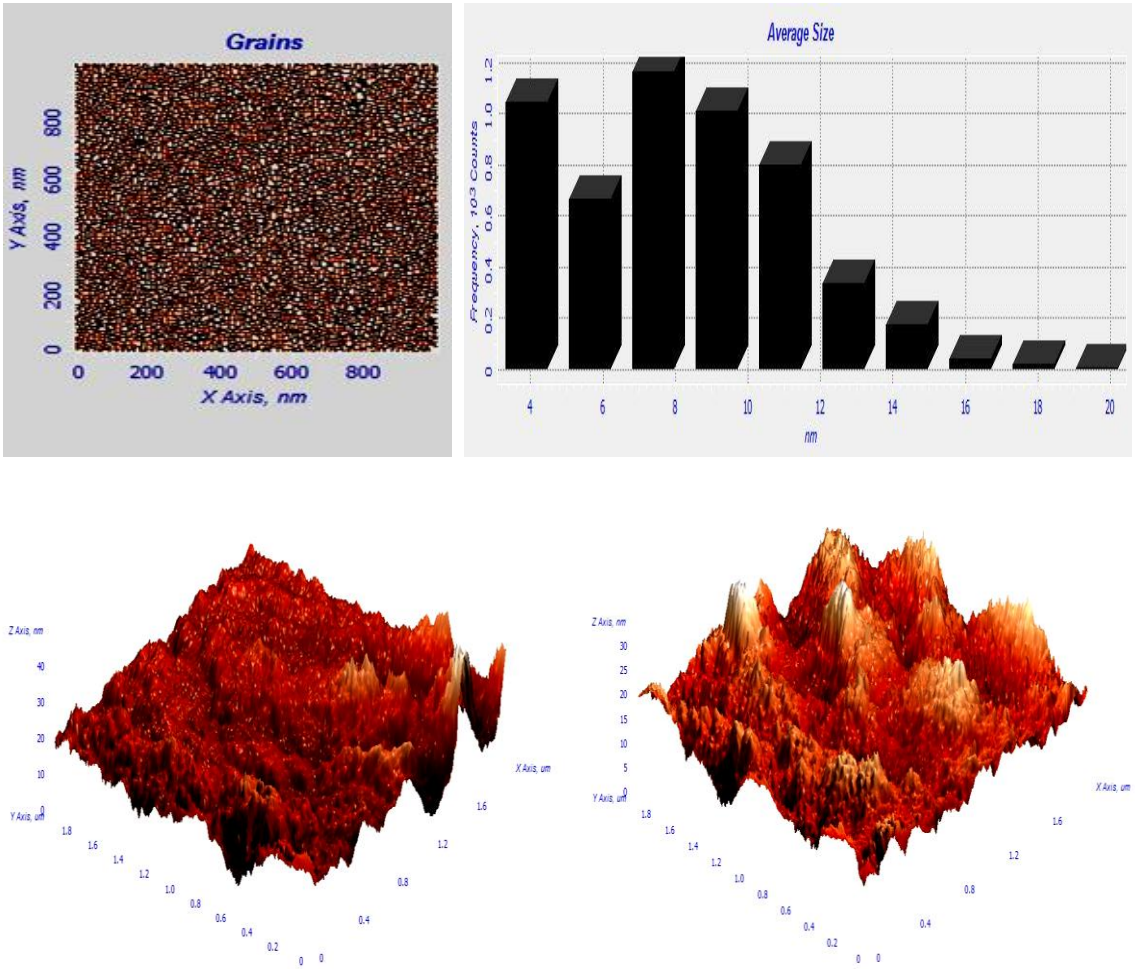



**Figure 1: AFM analysis of xylitol carbon dots**

X-Rays are can be used efficiently for the determination of positions/symmetry of the atoms in the unit cell, unit cell type, size, crystalline mixture and nano-scale assembly of

nano-structures [14]. X-ray diffraction pattern of sample, shown in Figure 2 indicated that sample showed very poor crystallinity and its polymorph can't be identified.

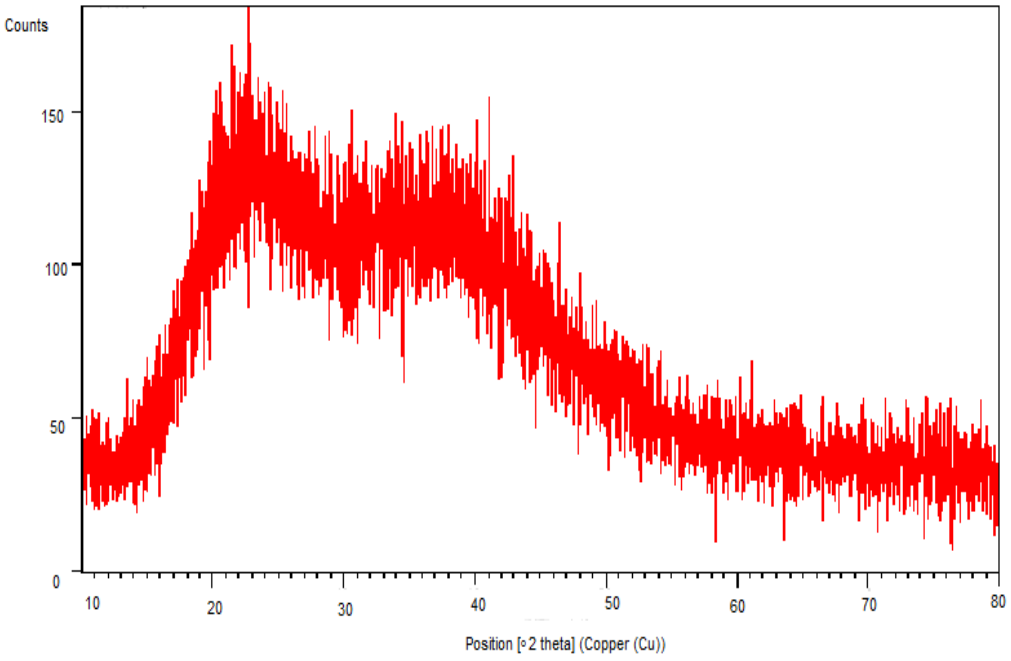



**Figure 2: X-ray diffraction pattern of xylitol carbon dots**

Exposure to heat may affect the physical and chemical nature of compound therefore the C-dots was analyzed through FTIR to determine the chemical bonds and functional groups. In FTIR analysis, functional groups and bonds are represented in form of peak. FTIR analysis of sample indicated that even after microwave treatment -OH functional group was remaining conserved (Figure 3). Both the xylitol samples including test and standard (Alfa Aesar) showed broad and intense peaks around 3386.01 $cm^{-1}$ of H-bonded hydroxyl groups (-OH) stretching absorption and the weak band at 2911.45 $cm^{-1}$ and 2940.95 $cm^{-1}$ due to intra-molecular hydrogen bonded -OH. Strong absorption band at 1642.75 $cm^{-1}$ and peaks ranging from 1460-1200 $cm^{-1}$ represented the ester carbonyl (C=O) group and the variable angle vibrations of C-H, respectively. Peak at 1405.24 $cm^{-1}$ can be attributed to C-H stretching vibration [15-16]. Each polysaccharide has a particular band in 1200-1000 $cm^{-1}$ region, which is dominated by glycosidic bond C-O and sugar ring stretching vibrations [17]. Tri and bi substituted C-H bending showed peaks at 877.64 $cm^{-1}$ and 756.40 $cm^{-1}$. Peak at 661.81 $cm^{-1}$ can be attributed to –OH that is out of plane.

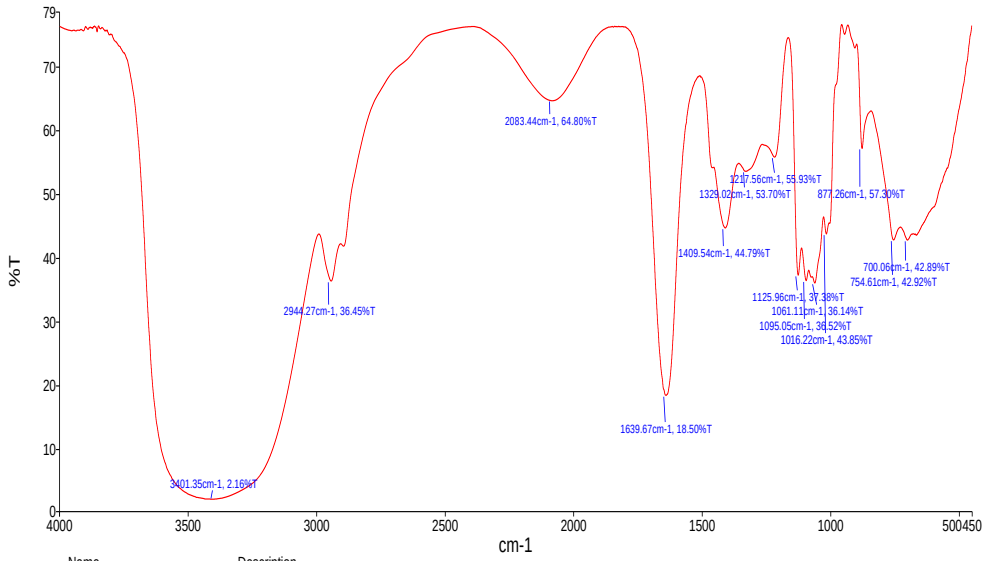



**Figure 3: FTIR analysis of xylitol carbon dots**

FTIR analysis confirmed that -OH (alcohol) was the main functional group followed by the fractions of other functional group formed due to side reactions with HCl and ethylene diamine which evidently suggested the successful passivation.

Carbon dots may have distinct optical property; therefore carbon dots were analyzed under Gel doc system and spectro-photo fluorometer. The sweet CD was pale yellow under visible light and bright blue under UV light. The spectroscopic study revealed that the maximum fluorescence emission intensity was between 400-450 nm when excitation wavelength was 340 nm (Figure 4).

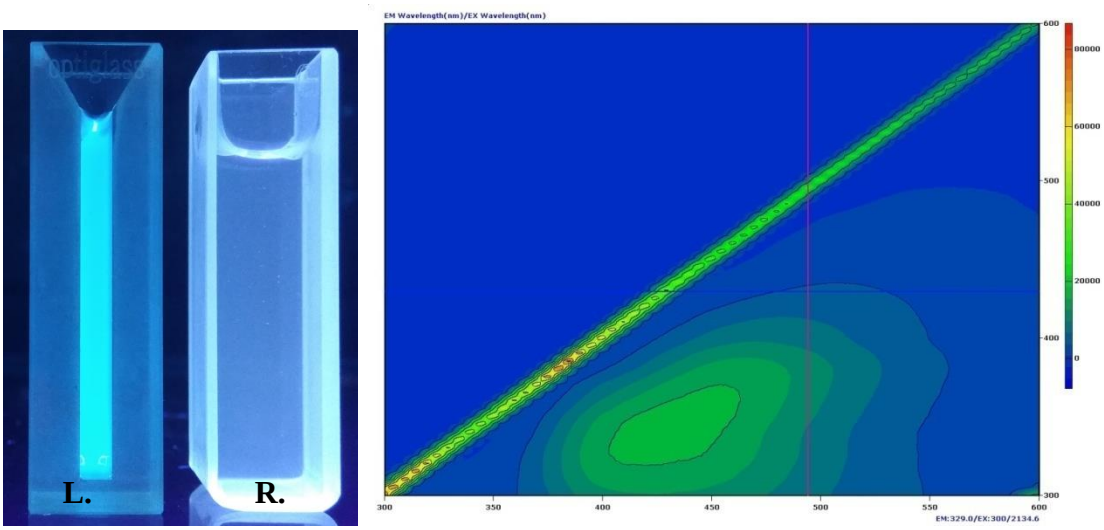


**Figure 4: Analysis of fluorescence property of carbon dots showing fluorescence** A: control (R) & carbon dots (L) and B. Spectro-photo-fluorometric analysis of C-dots

Carbon dots showing fluorescence under UV light can possibly be used for imaging of specific cells or proteins. Synthesized CDs were further dissolved dimethyl sulfoxide (DMSO) to investigate its anti-microbial potential against selected pathogens.

**Antimicrobial potential of purified xylitol and carbon dots:** Xylitol has already been reported to have antimicrobial potential against many known pathogens. With the possibility of enhanced drug delivery and efficacy, its C-dots and conjugates with conventional antimicrobials were evaluated against selected human pathogens. Tetracycline and ketoconazole were used as positive control against bacteria and fungi respectively while DMSO was used as negative control.

Carbon dots were loaded with antimicrobials (Tetracycline and Ketoconazole) separately and washed with distilled water to remove unbound/free antimicrobials. Drug binding efficiency of C-dots was determined by UV-VIS spectrum. UV-VIS spectrum indicated that C-dots have maximum absorption at 328 nm, ketoconazole showed maximum absorption at 293 nm while tetracycline has three peaks at 216 nm, 376 nm, 348 nm and 361 nm. In comparison to this, differential peaks at 323 and 380 nm were recorded in C-dot conjugates of ketoconazole and tetracycline respectively. Drug loading efficiencies of C-dot conjugates were 89.16% in case of tetracycline was while 86.23 % in case of ketoconazole (Figure 5).

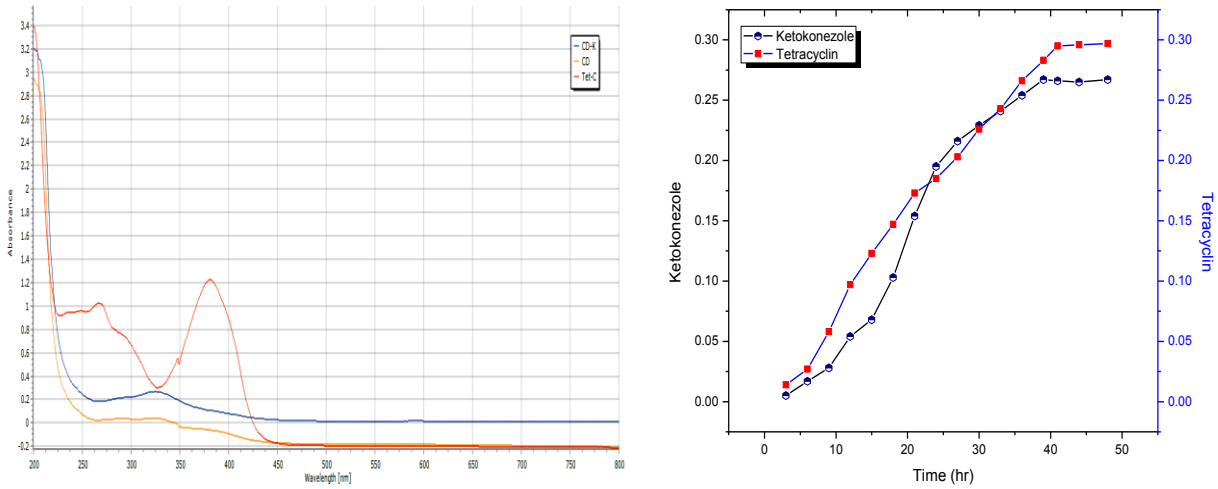



**Figure 5: Purification and drug loading efficiency of C-dots** A: UV-Vis spectrum and B: antimicrobial release profile of xylitol, carbon dots, carbon dot and antimicrobial conjugates

The results pertaining to antimicrobial activity of xylitol, C-dots, antimicrobials conjugates revealed interesting facts about efficiency of xylitol and other combinations. Both xylitol and C-dots were effective only against *E. coli S. pyogenes C. albicans* and *Cryptococcus* and no activity was recorded against other four pathogens. The efficiency of C-dots (18.52±0.15) was much higher than xylitol (18.52 mm against *E. coli*) and even than the positive control (11.41 mm) while least effective in case of *Candida* (Table 1).

**Table 1: Antimicrobial activity (mm) of xylitol, its carbon dots and conjugates**

| **Pathogens** | **Xylitol** | **Carbon dots** | **Am-C dots** | **Antimicrobial*** |
|---|---|---|---|---|
| *Staphylococcus aureus* | - | - | 0.42±0.15 | 18.16±0.33 |
| *Escherichia coli* | 13.27±0.33 | 18.52±0.15 | 14.68±0.33 | 11.41±0.67 |
| *Klebsiella pneumonia* | - | - | 12.47±0.67 | 12.47±0.33 |
| *Streptococcus pyogenes* | 8.41±0.67 | 11.41±0.33 | 18.64±0.67 | 15.63±1.15 |
| *Listeria monocytogenes* | - | - | 15.47±1.15 | 13.49±0.33 |
| *Salmonella typhi* | - | - | 14.76±0.33 | 12.16±0.67 |
| *Candida albicans* | 4.52±0.33 | 7.63±0.67 | 13.25±1.17 | 11.23±0.67 |

| | | | | |
|---|---|---|---|---|
| *Cryptococcus neoformans* | 6.73±0.67 | 8.92±0.33 | 12.49±0.67 | 10.52±1.15 |

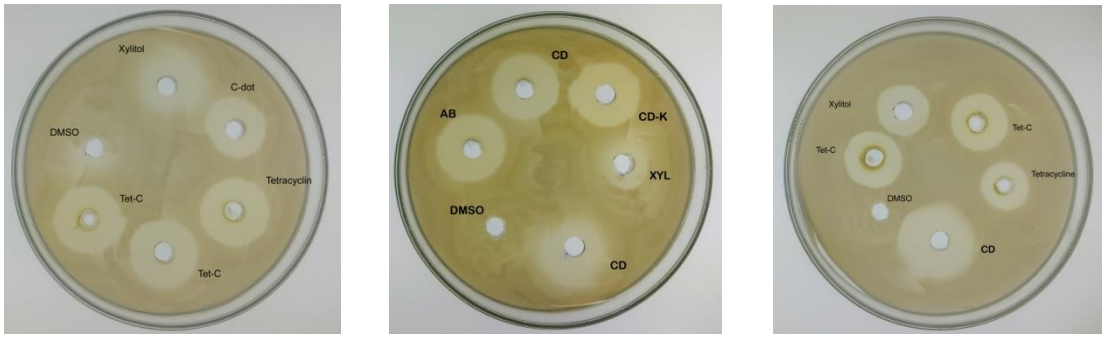
***** *Ketoconazole for fungi and tetracycline for bacteria*

The positive control ketoconazole and tetracycline were effective against all 8 certified microbial pathogens. The Am-C dots conjugates were not only quite effective against all pathogens but the efficacy was much higher in all cases in comparison to positive controls. Interestingly growth of *S. aureus*, *Listeria, Klebsiella, S. typhi* was not inhibited by xylitol and C-dots but in case of Am-C dots it was not only effective but also higher for *S. aureus* (20.42 mm) followed by for *S. pyogens* (18.64 mm) and almost similar for all other. This tremendous increase in antimicrobial activity of conjugates suggested it's possible in combination with conventional drugs.

The results on MIC w.r.t. four pathogens (inhibited by pure xylitol) showed that Am-C dots conjugates were more effective and even very small concentration of conjugates were sufficient to inhibit microbial growth in comparison to antimicrobials. As shown in table 2 and figure 6, only 0.01 mg/ml of conjugates was sufficient against *S. pyogenes* but 0.16 mg/ml xylitol and 0.08 mg/ml antimicrobial was required. Similarly only 0.04 mg/ml conjugates were sufficient to inhibit the growth of *C. albicans* and *Cryptococcus* while much higher concentration of xylitol (1.25 mg/ml) and antimicrobials (0.16 mg/ml) were required for the same purpose.

**Table 2: MIC analysis of xylitol, its carbon dots and conjugates**

| **Pathogens** | **mg/ml** | | | |
|---|---|---|---|---|
| | **Xylitol** | **Carbon dots** | **Am-C dots** | **Antimicrobial** |
| *Escherichia coli* | 0.65 | 0.65 | 0.16 | 0.32 |
| *Streptococcus pyogenes* | 0.16 | 0.08 | 0.01 | 0.08 |
| *Candida albicans* | 1.25 | 1.25 | 0.04 | 0.16 |
| *Cryptococcus neoformans* | 1.25 | 0.08 | 0.04 | 0.16 |

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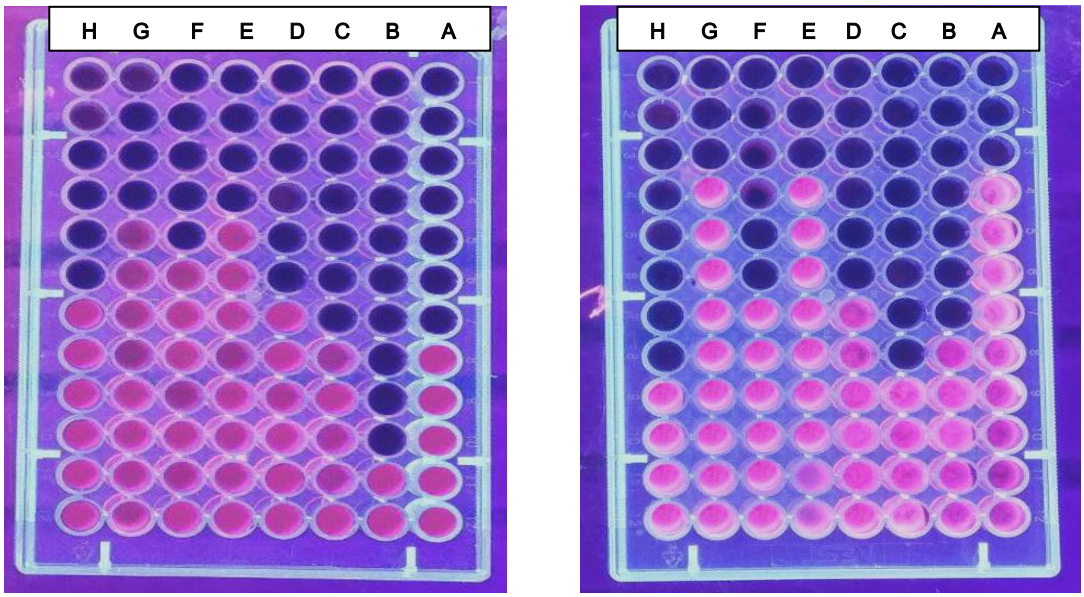


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**Figure 6: Antimicrobial activity of xylitol carbon dots against pathogenic microbes** A: *S. pyogenes*; B: *Candida albicans;* C: *E. coli;* D & E MIC analysis against the selected pathogens (D: MIC Plate 1: column 1-4: *E. coli*; 1: Tet-C, 2: xylitol, 3: tetracycline, 4: Carbon dots; column 5-8: *S. pyogenes;* 5: xylitol, 6: carbon dots, 7: Tet-c, 8: tetracycline; E : MIC Plate 2: column 1-4: *Candida albicans*; 1: Cd-K, 2: xylitol, 3: carbon dots, 4: Ketoconazole; column 5-8: *Cryptococcus neoformans;* 5: Ketoconazole, 6: Cd-K, 7: Carbon dots, 8: xylitol)

**DISCUSSION**

Xylitol is an important constituent of many healthcare products including toothpaste, mouthwash and anticariogenic products [18-19]. It prevents acute otitis infection caused by *Streptococcus pneumoniae* mediated by fructose phospho-transferase system [20].

*Burkholderia cepacia* complex (BCC) is one of the most prominent causes of pre-operative infection during lungs transplant. Out of 216 cystic fibrosis patients, 22 got pre-operative BCC infection and 9 patients died within the first year [18]. Xylitol and dextrans was evaluated for preventing *Burkholderia cepacia* complex infection and found that presence of dextran (40 kDa) decreased the bound organisms by 80%-99% while xylitol inhibited the binding of bacterial pathogen by 67%-85% [21].

de Sousa *et al.,* assessed antimicrobial and anti-adherent potential of xylitol against two *Pseudomonas aeruginosa* strains (ATCC 9027 and clinical) and found that xylitol didn't have any bactericidal activity against these strains, however it suppress microbial growth by inhibit the bacterial adherence [24]. Similar results were also warranted by Radmerikhi *et al.,* while evaluating the effect of xylitol on *Streptococcus mutans* and *Lactobacillus acidophilus* growth [19]. Different concentrations of xylitol were mixed with bacterial solutions and incubated at 37°C for 48 hr.

Applications of xylitol is wide spread however, size reduction of any product specifically food and drug results in improved efficacy. Fluorescent carbon dots (FCDs), synthesized from natural carbon sources are gaining huge attention as new class of nano-materials are superior to traditional and conventional nano materials due to their solubility in water, bio-compatibility non-toxicity, applications in bio-imaging, drug delivery, cancer therapy and gene delivery [23-24]. Therefore carbon dots (8.87 nm) of purified xylitol were prepared and evaluated along with conventional antimicrobial compounds against selected human pathogens. Xylitol exhibited antimicrobial potential against *S. pyogenes, E. coli*, *C. albicans* and *C. neofomans* in itself which further enhanced when used in form of carbon dots. Moreover the presence of carbon dots increased the efficacy of conventional antimicrobial compounds against other pathogens as well.

Kim *et al.,* have prepared xylitol C-dots by microwave surface passivation method when characterized by TEM, AFM, photoluminescence lifetime measurement, cytotoxicity and cell imaging revealed that carbon dots were of 4.65 nm diameter showing intense peak at

365 nm in emission spectrum recorded with respect to excitation wavelength of 320-310 nm [25]. Thakur et al., have prepared carbon dots of gum arabic (GA) by microwave assisted synthesis and use the as molecular vehicle to ferry ciprofloxacin hydrochloride. During study, it was revealed that release of antibiotic from conjugates in extremely regulated manner under physiological conditions. Bare C-dots were successfully used for microbial imaging of the simplest eukaryotic model *Saccharomyces cerevisiae* (yeast). Moreover the conjugates showed comparatively higher antimicrobial activity against both model gram positive and gram negative microorganisms [26].

The researchers in the past have reported anticariogenic applications of xylitol besides a low calorie sweetener for diabetes but the present research have added new dimension to the applicability against wide spectrum of pathogenic microbes which needs to be explored further and if successful this will definitely increased the potential and market share of xylitol globally. Results have suggested the possible applications of xylitol in various industries but the present research efforts have proposed a new dimension to its applicability against wide spectrum of microbial pathogens which needs to be explored further and if successful this will definitely increased the potential and market share of xylitol globally. The fluorescence Am-C conjugate paves efficient nano-carriers with high antimicrobial activity but also not only a way for bioimaging.

C-dots can be a promising and efficient nano-carrier for drug delivery due to their excellent biocompatibility and distinct optical properties. Antibiotics can easily be anchored to C-dots without any chemical modification or activation with higher loading capacity which suggested it as an ideal vehicle for drugs and other therapeutics. The conjugate has been proved as potent and effective antimicrobials against both bacteria and fungi. Release of potential antibiotics like tetracycline and ketoconazole in regulated manner and fluorescence nature of C-dots increased their importance in pharmaceuticals and healthcare.

## MATERIALS

For xylitol production, sugarcane bagasse extract was fermented by *Pseudomonas gessardii* VXlt-16 (GenBank Accession Number MG770460) which was further used for carbon dots preparation. 1500 ml sugarcane bagasse extract, prepared by acidified steam blast (0.4N HCl) was fermented in 2l fermenter (New Brunswick Scientific, USA) for upto 52 hr and xylitol recovered by using activated charcoal [27] and Ion-exchange resins (DEAE-Sepharose and SP-Sepharose) [28-29] followed by concentration of fermentation broth and freeze drying.

**Preparation of xylitol carbon dots:** Xylitol carbon dots (CDs) were prepared by 'Microwave Based Surface Passivation Technique' using household microwave (700 W) following the method given by Kim et al [25]. Concept of passivation is important to protect the material from thermal and chemical damage for which ethylene diamine (EDA) was used.

For CDs preparation 152.15 mg of xylitol dissolved in 10 ml of distilled water to which 10µl of HCl and EDA (1mM each) were added and mixed by vigorous stirring for 2 min. Mixture was heated in microwave oven for 2-5 minutes.

**Purification of carbon dots:** Crude sample of C-dots was dissolved in distilled water, filtered from syringe filter (0.45 µm) and dialyzed against distilled water using dialysis membrane (MWCO of 500 – 1000D) for 24 hr.

**Characterization of purified xylitol carbon dots:** Xylitol carbon dots were characterized by Atomic Force Microscopy (AFM), X-Ray Diffraction (XRD), Fourier-Transform Infrared Spectroscope (FT-IR), spectrofluoro-photometry. Slide was prepared, dried overnight and scanned by 650 nm (NTGRA; USIC HPU Shimla) for analysis of surface topography.

***Atomic Force Microscopic*** analysis was done to visualization the surface properties of synthesized carbon dots by atomic force scanning probe microscope (NTEGRA NT-MDT) with 10 nm probe. Scanning was done in semi contact mode using high resonant-frequency (241 kHz) pyramidal cantilevers with silicon probes having force constants of 41 N/m.

Crystal nature of sample was determined by scanning the thin film of dried purified xylitol crystals and ***X-Ray Diffraction*** pattern was recorded by CuKα radiation source, using a voltage of 40 kV, and a scan rate of 10 $^{o}$min−1 from 2θ range of 10–80$^{o}$ using X-ray diffraction instrument (Rigaku MiniFlex 600; Central Instrumentation Facility 'CIF'-Jiwaji University, Gwalior).

Surface passivation may affect the surface as well as chemical structure which were determined by ***FT-IR spectrophotometer*** (Perkin-Elmer) at Central Instrumentation Facility (CIF) Jiwaji University, Gwalior. Liquid solution containing carbon dots were analyzed for transmission spectrum from 450 to 4000 $cm^{-1}$.

To determine the fluorescence property of carbon dots, sample and control were observed under UV light in ***gel doc system***. After confirmation of fluorescent nature, carbon dots were further analyzed for fluorescence properties by ***spectro-fluorophotometer*** (Shimadzu RF-6000) at CIF Jiwaji University Gwalior. Purified carbon dots were used for fluorescence analysis. In first analysis 300-900 nm of excitation wavelength was used and emission spectrum was recorded in continuous mode. In second analysis emission spectrum was recorded from 200-900 nm with respect to excitation wavelength of 340 nm. In both the cases, scan speed was 6000 nm/min.

**Loading of antibiotics on xylitol carbon dots (AM-C-dots):** Anti-microbial-carbon dots conjugates (AM-C) were prepared by using 0.5 ml (1 mM) anti-microbial solution mixed with 9.5 ml (95 mg/ml) C-dots by continuous stirring for 3 h. Conjugate formation was studied by analyzing their UV-VIS Spectrum (200–800 nm) w.r.t pure C-dots and anti-microbial solutions. Drug loading efficiency (DLE) of C-dots was determined by following equation [26]:

$$Drug\ loading\ efficiency = \frac{Theoretical\ amount\ of\ drug\ loaded - free\ drug}{Theoretical\ amount\ of\ drug\ loaded} 100$$

**Antibiotic release studies:** Unbounded/free antimicrobials from AM-C conjugates were removed by dialyzing it against 1% phosphate buffer saline (PBS, pH 7.2) in dialysis bag at 37 $^{o}$C [26]. During dialysis sampling was done at regular interval of time and recording absorbance at 348 nm for tetracycline and 293 nm for ketoconazole to determined the released AM. After each sampling, the phosphate buffer was replaced with fresh and pre-warmed buffer.

**Analysis of antimicrobial potential of xylitol:** The antimicrobial potential of xylitol, carbon dots and conjugates were evaluated against the known pathogens including *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Streptococcus pyogenes, Listeria monocytogenes, Salmonella typhi, Candida albicans* and *Cryptococcus neoformans* including cariogenic microbes, procured from Department of Microbiology, Indira Gandhi Medical College (IGMC) Shimla India. Anti-microbial potential of microbial pathogens was determined by well-diffusion method. Various concentrations of xylitol, carbon dots and conjugates were evaluated and compared to Di-methyl Sulfoxide (DMSO) as –ve control and antibiotic (antibacterial, antifungal; 5 mg/ml) as +ve control.

**Determination of Minimum Inhibitory Concentration (MIC):** '*Micro Broth Dilution Assay Method*' was used to determine minimum inhibitory concentration of xylitol, carbon dots and CD-AM conjugates using Resazurin dye (300 mg/ 40 ml sterile distilled water). Resazurin is non-fluorescent oxidation-reduction indicator, used to evaluate cell growth in various cytotoxicity assays [30]. It becomes pink and fluorescent when reduced to resorufin within viable cells.

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**CONFLICT OF INTEREST**

Not applicable

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