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Synergistic Activity of Multi-Walled Carbon Nanotubes Suspension against Clinical Isolates of Pathogenic Bacteria

Zuhair S. Alsehlawi [1],Wasna'a M. Abdulridha[2], Ahmed N. Abd[3], Nadir F. Habubi[4*]

[1]*Head of Department of Basic Science, College of Dentistry, Kufa University, Iraq.*
[2] *Department of Basic Science, College of Dentistry, Kufa University, Iraq.*
[3]*Physics Department, Science Faculty, Mustansiriyah University, Iraq.*
[4]*Physics Departement, College of Education, Mustansiriyah University, Iraq*

Abstract:

The current work was designed to study the *in vitro* antimicrobial activity of multi-walled carbon nanotubes suspension (MWCNTs) on growth of pathogenic bacteria isolated from clinical samples. A concentration of 0.0875mg/mL MWCNTs was prepared by dispersing in distilled water with homogenized by sonication. The MWCNTs were physically characterized by using X-ray diffraction (XRD), Fourier Transform-Infrared Spectroscopy (FT-IR), Scanning Electron Microscopy (SEM) and UV–Vis spectrophotometry. Three isolates of each *Staphylococcus aureus* and *Escherichia coli* were randomly recovered from different clinical samples by cultivation and isolation on bacteriological selective media. Antimicrobial activity of MWCNTs were assayed individually on bacteria and incorporated with cefotaxime and gentamicin by different methods included; disk diffusion, well diffusion and macro broth dilution. Structural and morphological study improved that MWCNTs has good dispersion in water, scanning electron microscopy image of multi-wall carbon nanotubes that deposited on substrate of silicon after process of sonication revealed that MWCNTs were segregated from the bundles to separately tubes with diameters ranging between (14.2 and 42.8) nm. Antimicrobial activity results recorded highest activity when using 100 µg/ml of MWCNTs by disk and wells diffusion methods. However, slight increasing in diameters (mean; ≤ 3 mm) of inhibition zones were showed when used Gentamicin/MWCNTs, 100 µg/ml combination. While, MWCNTs (100 µg/ml) which had high synergistic action on both bacterial species when combined with cefotaxime. Thus *E.coli* was more susceptible to MWCNTs activity, and the minimum concentration of MWCNTs was 125 µg/ml. In conclusion , MWCNTs had enhancement antibacterial activity against clinical isolates of Staphylococcus aureus and Escherichia coli.

Keywords: *Multi-Walled Carbon Nanotubes, Antimicrobial Activity, S. aureus, E.coli and MIC.*

INTRODUCTION:

Nanotechnology is an novel field that effects all aspects of human's life [1]. Nanoparticle (NP) is utilized in nanomedicine, they are paid so much attention in this field [2]. Carbon nanotubes (CNTs) has allotropy property, with a cylindrical nano tubes-structure [3], which clasified as single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) and these classes could be functionalized or nonfunctionalized so as to obtain more cohesion for diverse substrates [4]. In the last decade MWNTs were contributed as components in different composites that have medical properties [5]. The physicochemical properties including ultra-light weight, high tensile strength, metallic electronic properties, chemical and thermal stability of carbon nanotubes are highly eligible for using within the medical sectors and the addition of carbon nanotubes to recover the performance and the quality of products that used widely [6]. Microbial infections cause serious risk to human life with diseases of acute and chronic, that may cause of death [7]. Consequently, nanoparticles such as CNT plays a very important role in the microbial growth control. CNTs , among all nanoparticles, have unparalleled features like magnetic, chemical, optical and electronic properties, that is make CNT to apply in different applications such as, tissue engineering, biosensing, drug delivery and wound dressing, as well as carbon nanotubes have enhanced the strong antimicrobial activity [8]. Recently, epilson-polylysine covalent bonding with carbon nanotube promoted the antimicrobial activities with *S. aureus, E. coli* and *Pseudomonas aeruginosa* through, the immediate contact of CNT conglomerates cause cell damage and cell death [9,10]. Also it has been revealed that, the chemical functionalization or purification of carbon nanotube can convert the physiochemical properties which makes it disperse or fast to suspend in different solvents. However, the surface area and size of CNTs are significant parameters cause effect on their antibacterial activity, which is, increment the surface area of nanoparticles by decrement their size lead to enhance their activity for interaction with bacteria [11]. The increment resistance of the microorganisms towards antibiotics has been led to severe health problems in the current years and most infection-causing bacteria were resistant to at least one of the antibiotics that are usually used to remove the infection[12]. Moreover, this study aimed to evaluate the MWCNTs which can effectively inhibit of human pathogenic bacteria.

MATERIALS AND METHODS

Preparation of Multi-Walled Carbon Nanotubes (MWCNTs) Suspension

MWCNTs powder (nanotubes with diameter 10–60 nm and length 10–15µm) provided by NanoTech Labs, Inc.USA. A concentration of 0.0875mg/mL MWCNTs was prepared by dispersing in distilled water without any surfactant, suspension was homogenized by using sonication (GT Sonic, Germany) for 6 hours. To make the dispersion of MWCNTs in distilled water fast and easy, the temperature of sonication water was reached to 40°C [13].

Physical Investigations of MWCNTs

The Structural, morphological and optical properties of MWCNTs were previously investigated by means of (CuKα) XRD-6000,Shimadzu X-ray diffractometer (Japan), Fourier Transformation Infrared spectroscopy, JEOL (JSM-5600) Scanning Electron Microscopy (Netherland), Angstrom AA 3000 atomic force microscopy and Cary 100 Conc plus UV–Vis spectrophotometer (Japan).

Isolation and Identification of Bacterial Isolates

During the period of study, three isolates of each *Staphylococcus aureus* and *Escherichia coli* were randomly recovered from different clinical samples after culturing on blood agar, manitol salt agar and MacConkey agar (Difco, USA). Plates were incubated for overnight at 37^{o}C. Traditional method used for identification of bacterial isolates, which includes culture characteristic on selective media, biochemical reactions and Gram-staining, relative to systematic Bacteriology Bergeys Manual [14] .

Antibiotic Susceptibility Testing

Disk diffusion method on Muller-Hinton agar medium (MH) (Oxoid, UK) was carried out to determine the susceptibility of *S. aureus* and *E. coli* isolates against standard antibiotic disks included; gentamicin (10µg), cefotaxime (30µg) and tetracycline (30µg) (Himedia, India). MacFarland microbial suspension was used as an inoculum approximately 1×10^{8} CFU/ml. The cultures were incubated at 37^{o}C for 18 hr under aerobic conditions according to Kirby-Baur [15]. zones of inhibition were calculated and interpreted as recommended by the National Committee for Clinical Laboratories Standard guidelines. In this study, *Escherichia coli* (ATCC 25922) was used as the reference strain for antimicrobial susceptibility testing [16].

Antimicrobial Activity Measurements of MWCNTs

The activity of MWCNTs against *Staphylococcus aureus* and *E. coli* isolates were evaluated alone and incorporated with standard antibiotics using the following methods:

a) Disk diffusion method

The stock solution of MWCNTs was diluted at the time of disc preparation to obtain the working solution with concentrations (25, 50, 100) µg/ml. A Whiteman filter paper 6 mm disks were impregnated completely in 20µl of each concentration. The standard antibiotic discs of, gentamicin (10µg) and cefotaxime (30µg) also absorbed 20µl of 100µg/ml MWCNTs. The loaded discs with MWCNTs were dried and placed on MH agar medium which seeded by 100 µl (1×10^{8} CFU/ml) of fresh culture of *Staphylococcus aureus* and *E. coli* by spreading method [17]. Each plate had three powers of MWCNTs treated discs, inhibition zones were calculated after one day of incubation at 37 ◦C. Tetracycline used as a positive control while solvent blank disk was used as negative control.

b) Wells diffusion method

The antibacterial activity was done by modified Kirby-Bauer disk diffusion method. A lawn of test organism culture (1×10^{8} CFU/ml) was prepared by spreading on MH agar plates. Plates were left standing for 15min, after that holes with (8mm) were punched into the bacterial seeded agar plates. The bottom of holes were sealed with one drop of molten agar to avoid nanomaterials leakage out side [18]. Using a micropipette, a combining equal volumes 100µl (1/1) of (100 µg/ml MWCNTs suspension with gentamicin 10 µg/ml and 100 µg/ml MWCNTs colloidal with cefotaxime 30µg/ml) were separately poured onto wells. After overnight incubation at 37°C, inhibition zones were calculated. Tetracycline used as a positive control while solvent blank disk was used as negative control.

Broth dilution method

Bacterial minimum inhibitory concentration (MIC) for MWCNTs was determined by the method of broth-dilution [19]. Within a set of tubes, 5 ml of MH broth medium (Oxoid, UK), was amended with two fold dilutions of MWCNTs (2000-62.5) µg/ml were prepared separately. Each set was inoculated aseptically with 100µl of bacterial colloidal (1×10^{8} CFU/ml). The inoculated tubes were incubated at 37°C for one day. Viable bacterial colonies were detected by the naked eye determining the MIC which is the minimum concentration which cause inhibit in bacterial growth. Control was also carried out in the presence of standard antibiotics.

RESULTS

Morphological and structural analysis of MWCNTs

Ultrasound technique represents a very effective technology for decreasing the particle size and dispersing of nanotubes in water. Figure 1 (a and b) shows the suspensions MWCNTs before and after sonication process, respectively. This process produced a black and homogenized suspension denoted to very good dispersion of MWCNTs in distilled water after 6 hrs. of sonication at 40 ^{0}C, the suspension may contain more separated MWCNTs and a less number of aggregates. The physical investigations of MWCNTs revealed that, the typical XRD patterns of the MWCNTs layer were deposited on a silicon surface and left at room temperature to dried for X-ray diffraction (Figure 2). The peaks located at 25.9◦and 78.1◦ are catalogued to G (002) and G (110) diffractions of graphite, respectively. As well as, a cross-sectional SEM image of multi-walled carbon naotubes dropped on a silicon surface by drop casting method after sonication process revealed that MWCNTs were separated from the aggregates to form individually nanotubes with radii ranging between 14.2 and 42.8 nm. While, SEM images of MWCNTs pristine (powder) demonstrated an agglomeration, while MWCNTs suspension dropped by drop casting method exhibited sphere-shaped and a little rounded with diameters ranged from few nanometers to few micrometers because of the effect of aggregation.

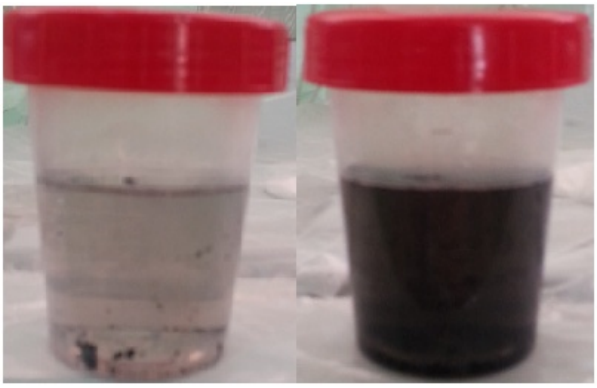

Figure 1: Multi-walled carbon nanotubes suspension. (A) before sonication process and (B) after 6 hours of sonication

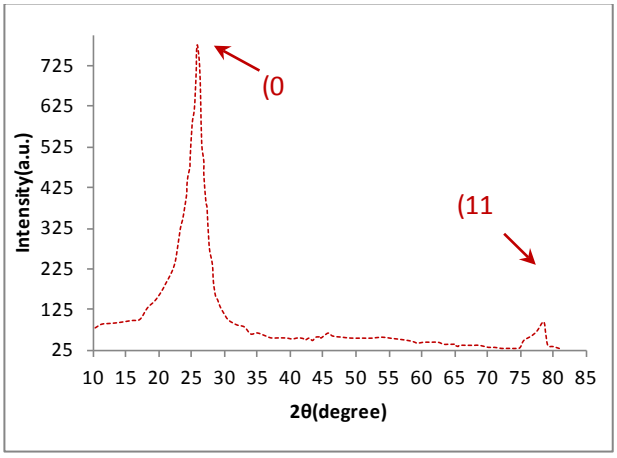

Figure 2: X-ray diffraction patterns of multi-walled carbon nanotubes layer deposited on silicon substrate

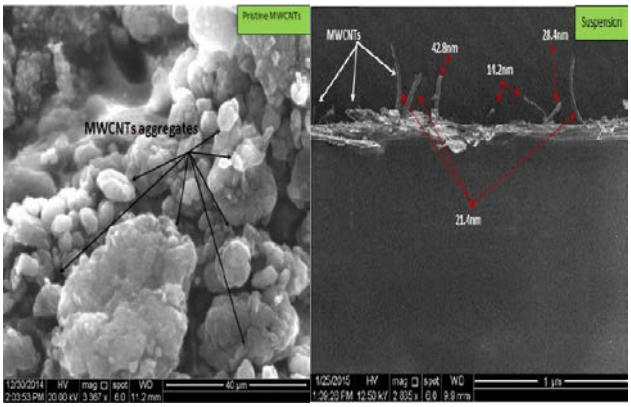

Figure 3: Scanning electron microscope images of top view of powder MWCNTs (left) and cross-section of MWCNTs layer dropped on silicon (right).

(Figure 4). Subsequently, the UV–Vis spectrum of multi-walled carbon nanotubes suspension exhibited a maximum absorption peak at 260 nm and the FT-IR spectra of MWCNTs suspension ranged between (500 and 4000)cm^{-1} as shown in Figures (4,5) .

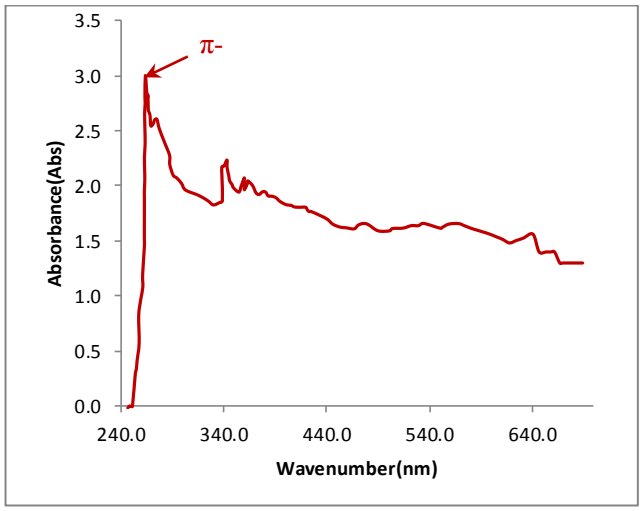

Figure 4: UV–Vis spectrum of suspension multi-walled carbon nanotubes.

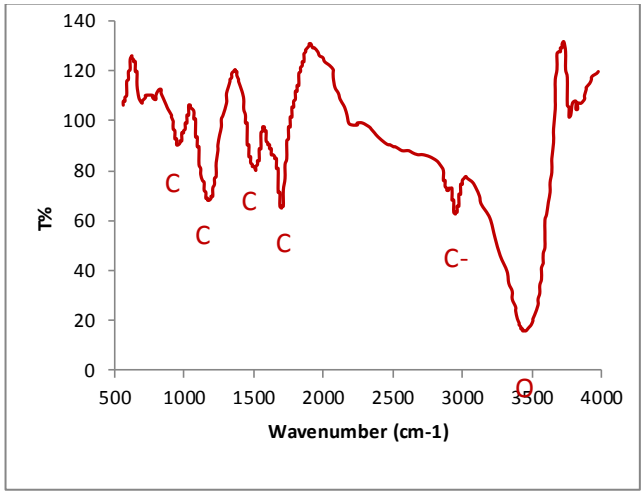

Figure 5: Fourier transformation infrared spectra of suspension MWCNTs.

Antimicrobial properties MWCNTs

In order to evaluate MWCNTs activity as an individual antibacterial agent, we tested it against *S. aureus* and *E.coli* at different concentrations included 25,50 and 100 µg/ml using disk and wells diffusion method. Results showed that the MWCNTs was recorded highest inhibition zone (9-10mm) at 100 µg/ml using both techniques. To compare MWCNTs activity with antibiotics that used in medical treatment for tested organisms, the antibacterial susceptibility of (gentamicin, cefotaxime and tetracycline) were analyzed on agar solid media. Results explain, the zone of inhibition of these antibiotics were in sensitive limits (CLSI, 2010). Moreover, cefotaxime had very high activity than gentamicin against both *E.coli* and *S. aureus* in addition to their sensitivity to tetracycline as positive control antibiotic (Table 1). In this manuscript, the synergistic effect of MWCNTs in combined with antibiotics were detected as well. However, Table (1) revealed that disk and wells diffusion methods showed no significant increasing in diameters (mean; ≤ 3 mm) of inhibition zones when used Gentamicin/(MWCNTs, 100 µg/ml) combination in compare with using antibiotic alone. While, MWCNTs (100 µg/ml) which seems more effective and had more synergistic effect on bacterial growth through combined with cefotaxime (the increment of inhibition zones was ≤ 7 mm for *S. aureus* and ≤ 14 for *E.coli*) in compare with using antibiotic alone. Furthermore, results exhibited that *E.coli* was more susceptible to Cefotaxime/(MWCNTs, 100 µg/ml) combination than other (Figure 6). In same manner, the minimum concentration of MWCNTs showed growth inhibition was determined against all selected bacteria (Table 2). The MICs values of MWCNTs were 500 and 125 µg/ml against *S. aureus* and *E.coli*, respectively. It clears that there is a strong antibacterial activity associated with MWCNTs, as compared to cefotaxime as positive control.

Table 1: The diameter of inhibition zones of antimicrobial agents using disk and well diffusion methods

Antimicrobial agents	Zones of Inhibition (mm)* using disk diffusion method		Zones of Inhibition (mm)* using wells diffusion method	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
MWCNTs 25 µg/ml 50 µg/ml 100 µg/ml	 0 0 9	 0 9 10	 0 7 9	 0 8 9
Gentamicin (10 µg)	26	25	15	21
Gentamicin + MWCNTs (100 µg/ml)	25	27	18	24
Cefotaxime (30 µg)	33	21	30	21
Cefotaxime / MWCNTs (100 µg/ml)	40	32	38	35
Tetracycline	23	22	23	20

Values are mean three replicates. MWCNTs: multi-walled carbon nanotubes

Table 2: The values of minimum inhibitory concentration of multi-walled carbon nanotubes using two fold broth dilution methods

Organism	MIC (µg/ml) of MWCNTs	MIC (µg/ml) of cefotaxime (Positive control)
S. aureus	500	64
E. coli	125	128

Values are mean three replicates. MWCNTs: multi-walled carbon nanotubes. MIC: minimum inhibitory concentration

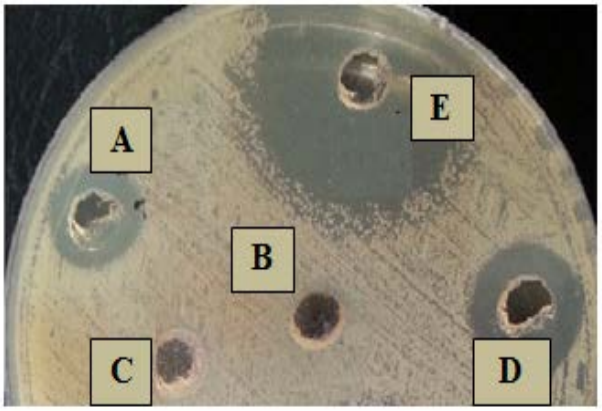

Figure 6: Overnight growth of *E.coli* on Muller Hinton agar medium at 37^{0}C exhibited the inhibition zones around wells filled by 100 µl of antimicrobial agents; A: multi-walled carbon nanotubes MWCNTs 100µg/ml alone, B: MWCNTs 50 µg/ml alone, C: MWCNTs 25 µg/ml alone, D: Cefotaxime (30 µg/ml) alone , E: Cefotaxime (30 µg/ml) + MWCNTs (100 µg/ml

DISCUSSION

In this study, the major problem of the practical application of MWCNTs is to avoid using of organic solvents for preparation of carbon nanotubes suspension, Basically, the organic solvents already have powerful antimicrobial activity against bacteria may cause growth inhibition and give false positive results [20]. Moreover, Ultrasound technique was represent a very effective way for dispersing of nanotubes in distilled water, it is overcome the bonding forces between the nanotubes,. Hence, the characteristics of the Crystallinity, structure, and crystallite size of MWCNTs at room temperature were improved that MWCNTs has good dispersion in water. However, suspension of MWCNTs have high absorption peak (~3) due to higher exposed surface area of MWCNTs with sufficient sonication force, in addition to the disentanglement and dispersion, they correspond with more apparent spectral property like the presence of the peak of π-plasmon [21]. The FT-IR spectrum exhibited important peaks at 932 and 2936cm^{-1} represent C-H symmetrical mode [22]. Misra *et al*. detected the peak at 1490 cm^{-1} as unique to MWCNTs [23]. The bond around 1582 cm^{-1} can be refered to graphite structure in MWCNTs [24]. while C=C dilation vibration bond appears at 1668.43cm^{-1} inspire with CNTs consistency [25]. The 3444.87cm^{-1} wide transmission peak coincide to OH groups, this finding denotes an effective preface of the OH groups on the side walls of the multi-walled carbon nanotubes that make them simply dispersed in polar solvents like water [26].

Antibacterial activity results noticed that MWCNTs had poor activity when it used individually against both *S. aureus* and *E. coli* isolates and the activity MWCNTs appear to increase with highest concentration (~10 mm at 100 µg/ml). However, this findings correspond with [27]. they improved that the antimicrobial activities of SWCNTs against both Gram-positive and Gram-negative bacteria related to their surface groups of -OH and –COOH, while MWCNTs with the same surface groups did not exhibit any significant antimicrobial effect. Consequently, MWCNTs acted as excellent antibacterial agents against both *S. aureus* and *E. coli* when combined with antibiotics especially cefotaxime/(MWCNTs,100µg/ml) combination. In addition to the effect of oxidative stress as a primary antimicrobial activity of these nanostructures, cefotaxime is a β-lactams antibiotic that interfere directly during the final stages of cross-linking of peptidoglycan polymers in the wall of bacteria [28]. This mechanism leads to blockage and inhibition of bacteria when precursors of the cell wall is accumulate and the in activated wall is unable to resist osmotic pressure [28,29]. Results have been pointed that no significant increasing in diameters (mean; ≤ 3 mm) of inhibition zones when used Gentamicin/(MWCNTs, 100 µg/ml) combination. Indeed, CNTs may haven't direct bacterial effect but, have been used as vehicles to deliver other macromolecules such as gentamicin that is not able to pass easily through the cellular membrane by themselves into cells [30]. Thus, in this report, MICs values of MWCNTs have shown the best antibacterial behavior against *E. coli* compared with *S. aureus*. Furthermore, the interactions between MWCNTs and bacterial cells play a significant role in their antimicrobial mechanism [31]. Similar studies have been proposed that MWCNTs cause damage in cell wall of bacteria and then release of their DNA content [32]. As well as, the straight contact between MWCNTs and the cells which in turn lead to cell death [8]. In general, MWCNTs showed significant antimicrobial activities towards *E. coli* than *S. aureus*. However, our results are supported by Le *et al* [6]. who reported that the susceptibility of *E. coli* to CNTs was dependent on the initial density of cells in the treatment, with cells at the higher density being more resistant. Virtually, the cell wall of *E. coli* consist from thin layers of peptidoglycan below the outer membrane makes it more sensitive for damaging than *S. aureus*, which are having multi layers cell wall. This mechanism may explain the lower resistance of *E. coli* to MWCNTs compared to *S. aureus* [33].

CONCLUSIONS

From the results, our expectation this is a first inferred that the MWCNTs a good synergistically antimicrobial material and suitable for various antimicrobial and pharmaceutical applications. Moreover, this study suggests that; it can be used of MWCNTs in medical instrumentation, antibiotics disinfection and detergents.

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