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SYNTHESIS AND EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF PURIFIED AND UNPURIFIED MULTI-WALLED CARBON NANOTUBES

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Abstract

Multi-walled carbon nanotubes (MWCNTs) were purified and unpurified in this study to obtain hybrid materials with improved activity. The production stage comprised a chemical purification of the produced sample. Raman spectroscopy analyzed the structural composition of purified and unpurified samples. The disc diffusion assay, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill assessment analyzed antimicrobial activity. MWCNT performed well against the tested bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter aerogenes, Staphylococcus aureus,* and *Streptococcus sp. Staphylococcus epidermidis*). The disc diffusion assay revealed inhibition zone differences caused by purified and unpurified MWCNTs. MIC and MBC values of purified and unpurified MWCNTs were similar. The purified and unpurified nanotubes of *Staphylococcus epider*midis (ATCC 35985) exhibited inhibition zone diameters of approximately 8 mm and 9 mm, respectively. The microdilution method revealed a MIC of 1.23 mg/ml for the purified nanotube and 0.156 mg/ml for the unpurified nanotube against the same microorganism. The killing curve analysis demonstrated that unpurified carbon nanotubes were more effective against all tested microorganisms. MWCNTs represent a promising method for microbiology, but studies on the toxicity of these materials remain scarce.

Keywords: Anti-infective agents. Nanocomposite. Nanotechnology. Spectrum analysis. X-ray diffraction.

1. Introduction

Nanotechnology allows the creation, fabrication, and manipulation of nanometric structures and particles (Nasrollahzadeh et al. 2019). Among the advantages of nanostructures are different chemical, physical, and behavioral properties when in contact with the physiological systems of animals and humans. Thus, nanomaterials become more chemically reactive, granting them new properties (El-Kady et al. 2023).

Nanobiotechnology mainly explores carbon nanotubes, which may be single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). These materials may also be classified according to chirality: armchair, zigzag, or chiral (Yang et al. 2020). Carbon nanotubes' hazardous or beneficial effects on the biological environment are among the most explored research fields in biomedical nanoparticle applications (Negri et al. 2020).

The indiscriminate use of antimicrobial agents is a relevant public health problem that allows the development of resistant and multidrug-resistant strains and increases nosocomial infections (Ventola 2015). Hence, there is an increasing search for new and efficient therapeutic alternatives with minimal side effects (Miethke et al. 2023).

Some studies have attributed antimicrobial activity to carbon nanotubes, and others confirmed the antimicrobial potential of SWCNTs (Abo-Neima, Motaweh, and Elsehly, 2020; Foo et al. 2018). However, only a few have investigated the potentially toxic effects of MWCNTs on microorganisms and their relationship with the purification process. Therefore, this study developed, characterized, and evaluated the antimicrobial activity of purified and unpurified MWCNTs in microorganisms.

2. Material and Methods

MWCNT synthesis and purification

This study synthesized MWCNTs by chemical vapor deposition (CVD), described by Eletskii (1997), in a horizontal bed reactor. MWCNT growth used metal catalysts, iron (Fe), molybdenum (Mo), and magnesium oxide (MgO) as oxide support. The system comprising the metal catalysts (Fe and Mo) and oxide support (MgO) was produced through solution combustion synthesis (SCS), using MgO, iron(III) oxide (Fe2O3), and molybdenum trioxide (MoO3) precursors: magnesium nitrate hexahydrate (Mg(NO3)2·6H2O, Merck), iron(III) nitrate nonahydrate (Fe(NO3)3·9H2O, Merck), and ammonium heptamolybdate tetrahydrate ((NH4)6Mo7O24·4H2O, Synth). Ethylene (C2H4) was the carbon source for MWCNT growth. The standard synthesis time was 30 minutes, and the temperature was 900°C. Argon (Ar, AGA, 99.995%) was the gas carrier.

MWCNTs were thermally purified to remove the amorphous carbon. Then, they were taken to a quartz tube, packed into ceramic containers (the same as the synthesis), and connected to an air pump. The system was subjected to 500°C, establishing an airflow in the quartz tube for one hour and 30 minutes for purification. Next, natural cooling occurred, and the sample was chemically purified.

The nanotubes were immersed in a hydrochloric acid (HCl) and deionized water (3:1) solution to remove the oxide support and catalyst particles. Then, the suspension was stirred for one hour at 100°C and later solubilized and filtered on activated charcoal. Filtering occurred with a vacuum on a Buchner funnel associated with a Kitassato flask.

MWCNT characterization

Raman spectrometry (InVia Renishaw) characterized the MWCNTs to verify carbon nanotube quality and purification efficiency. The assays used a laser excitation line with a 514nm wavelength. The spectra had resolutions of 1 cm^{-1} and ranged from 100 to 3000 cm^{-1}. X-ray diffraction (XRD) verified the product and synthesis phases, determining the respective mean size of crystallites using a Bruker X-ray diffractometer, D2 PHASER model, equipped with a copper anode and operated at 30 kV and 10 mA. The data for determining crystalline phases were collected through angular-range scan $10° < 2\theta < 80°$ with steps of 0.05°, for one second each step. Scanning electron microscopy (SEM) (MEV-EDS Inspect F50, FEI, UK) analyzed MWCNT structure and morphology.

Microorganisms

The study used eight American Type Culture Collection strains and one clinical isolate. It also included four Gram-negative bacteria (*Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter aerogenes* (ATCC 13048), and *Escherichia coli* (ATCC 25922)), three Gram-positive bacteria (*Staphylococcus aureus* (ATCC 25923) *Streptococcus* sp. (Clinical isolate), and *Staphylococcus epidermidis* (ATCC 35985)), and two yeasts (*Candida albicans* (ATCC 14053) and *Candida krusei* (ATCC 6258)).

Culture conditions

Various bacterial and fungal strains were tested to evaluate the potential antimicrobial activity of MWCNTs. These microorganisms remained in a glycerol-containing culture medium and were stored at -80°C. The samples were thawed, inoculated into brain heart infusion (BHI) broth, and incubated for 24 hours. Subsequently, they were plated on nutrient agar and incubated at 37°C for 24 hours. Suspensions were then prepared from the resulting growth, and the turbidity was adjusted to 0.5 McFarland standard using a spectrophotometer (OD_{600} = 0.08 to 0.1).

Disc diffusion assay

Disc diffusion tested susceptibility in Mueller-Hinton agar (Wikler et al. 2009). The suspension with the microorganisms was seeded into a Petri dish with the agar. Then, the discs received 10 µL of purified and unpurified MWCNTs at 10 mg/mL and were placed on the dish, which was incubated at 37°C for 24 hours to measure inhibition zones. The assay occurred in triplicate in two independent experiments.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

This analysis used only microorganisms sensitive to the treatments tested in the disc diffusion assay. The microdilution method, performed on a 96-well plate, determined MIC - the lowest concentration for inhibiting microbial growth (CLSI 2015). Wells containing Mueller-Hinton broth received different concentrations of purified and unpurified MWCNTs (0.039 – 10 mg/ml). This assay occurred in triplicate, with saline as a growth control and an inoculum with only broth as a negative control. Then, the plate was incubated at 37°C for 24 hours. Next, the test revealed a substance signaling microbial growth (2,3,5 triphenyltetrazolium chloride). The MBC was determined by taking a 1µL aliquot from each well, plating it onto a BHI agar plate, and incubating it for 24 hours. After incubation, the colonies were identified. The assay occurred in triplicate in two independent experiments (Lopes 2019).

Time-kill curve

The killing test was against one Gram-positive and one Gram-negative strain, according to the CLSI standard protocol for comparing purified and unpurified MWCNTs (CLSI, 1999). This test determined the time MWCNTs take to eliminate microorganisms. The suspensions with 1.5 x 10^8 CFU/mL of *Escherichia coli* and *Staphylococcus aureus* were exposed to MBCs for each MWCNT in the BHI broth. A tube containing BHI with the suspension was the positive control, and another with only broth was the negative control. The tubes remained in the agitator at 37°C, and an aliquot was taken from all tubes and seeded onto nutrient agar after zero, six, 12, and 24 hours. After 24 hours of incubation at 37°C, the plates were examined for colonies and expressed in log colony-forming units per milliliter (CFU/mL). The assay occurred in triplicate in two independent experiments.

3. Results

MWCNT characterization

The Raman spectrometry of purified and unpurified MWCNTs verified production success, and Figure 1 illustrates the results. This analysis revealed typical features of MWCNT structural properties. The D band, observed around 1350 cm^{-1}, is associated with disorders and defects within the carbon nanotube structure. The G band, located near 1580 cm^{-1}, corresponds to the tangential stretching mode of carbon-carbon bonds in the graphitic plane and represents the degree of graphitization. Additionally, the G' band, found around 2700 cm^{-1}, is a second-order overtone of the D band and provides further insight into the electronic properties and overall quality of MWCNTs. The intensity ratios of these bands helped assess the

purity and structural integrity of the MWCNT samples, confirming their suitability for subsequent applications.

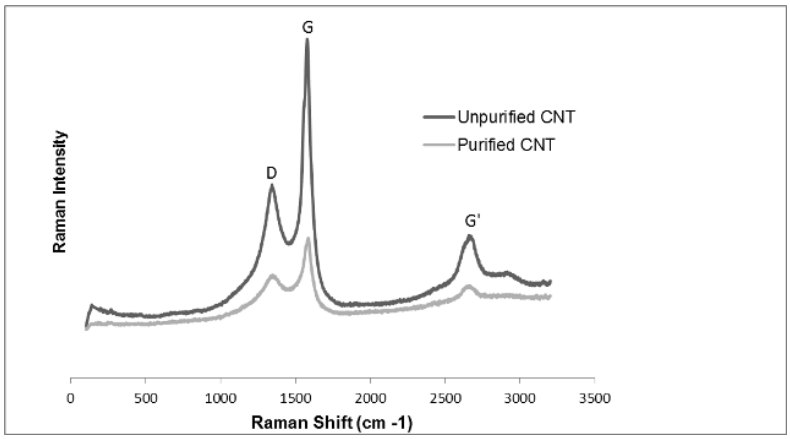

Figure 1. MWCNT characterization by Raman spectroscopy with D, G, and G' bands of purified and unpurified MWCNTs. Laser wavelength: 514 nm.

XRD investigated the structure of the synthesized MWCNTs and verified purification effectiveness. Figure 2 shows the diffractograms of the catalyst (a), unpurified carbon nanotubes (b), and purified carbon nanotubes (c). The representative morphology (SEM image) of MWCNTs was preserved after purification (Figure 3).

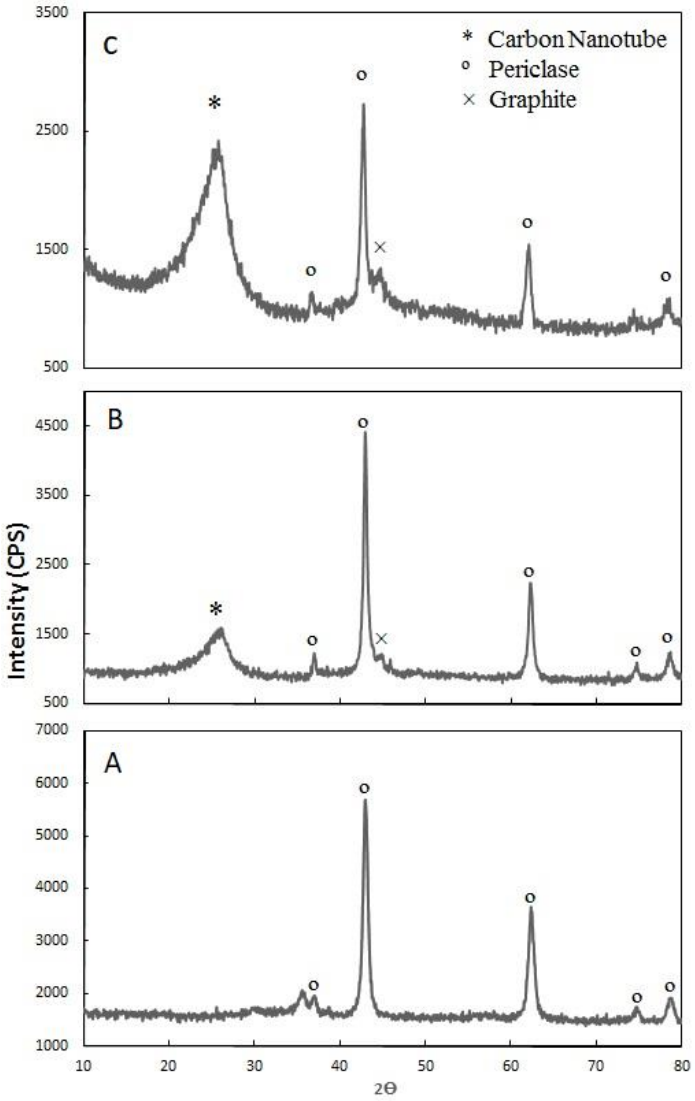

Figure 2. A - Diffractogram of the catalyst; B – Diffractogram of unpurified carbon nanotubes; C – Diffractogram of purified carbon nanotubes.

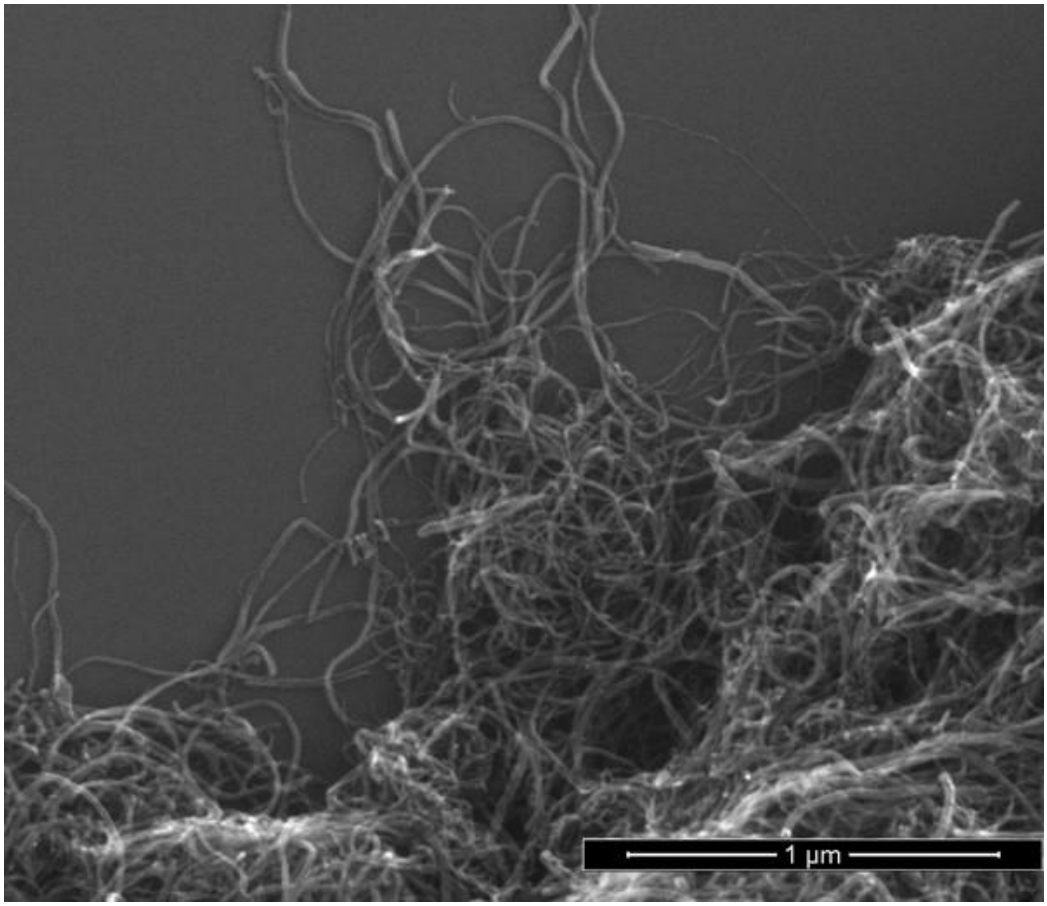

Figure. 3. MWCNT morphology by scanning electron microscopy.

Disc diffusion assay

Inhibition zones were measured after incubation. Disc diffusion showed that unpurified nanotubes formed larger inhibition zones than purified ones. Table 1 presents the values as mean ± standard deviation.

Table 1. Inhibition zones (mm) of the susceptibility test with MWCNTs.

Microorganism	Unpurified MWCNT	Purified MWCNT
Klebsiella pneumoniae (ATCC 700603)	9 ± 1	8 ± 1
Pseudomonas aeruginosa (ATCC 27853)	9 ± 1	8 ± 1
Enterobacter aerogenes (ATCC 13048)	8 ± 1	-
Staphylococcus aureus (ATCC 25923)	10 ± 2	9 ± 1
Streptococcus sp. (CI)	8 ± 2	7 ± 2
Staphylococcus epidermidis (ATCC 35985)	9 ± 1	8 ± 2
Escherichia coli (ATCC 25922)	9 ± 1	7 ± 1
Candida albicans (ATCC 14053)	10 ± 1	8 ± 1
Candida krusei (ATCC 6258)	9 ± 2	7 ± 1

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Unpurified nanotubes inhibited some microorganisms at lower concentrations than purified ones. Table 2 describes these findings.

Time-kill curve

Colony counting showed that unpurified nanotubes reduced viable microorganisms more effectively. Unpurified nanotubes affected *E. coli* after 12 hours, while *S. aureus* experienced the effect after six hours of treatment. Figure 4 exhibits these results as mean ± standard deviation.

Table 2. Minimum inhibitory and minimum bactericidal concentrations (mg/ml).

Microorganism	Purified MWCNT		Unpurified MWCNT	
	MIC	MBC	MIC	MBC
Klebsiella pneumoniae (ATCC 700603)	1.23	5	1.23	5
Pseudomonas aeruginosa (ATCC 27853)	1.23	5	1.23	5
Enterobacter aerogenes (ATCC 13048)	1.23	5	0.625	5
Staphylococcus aureus (ATCC 25923)	1.23	5	0.625	5
Streptococcus sp. (CI)	0.5	1.23	0.5	1.23
Staphylococcus epidermidis (ATCC 35985)	1.23	5	0.156	5
Escherichia coli (ATCC 25922)	1.23	5	1.23	5
Candida albicans (ATCC 14053)	1.23	5	1.23	5
Candida krusei (ATCC 6258)	1.23	5	1.23	5

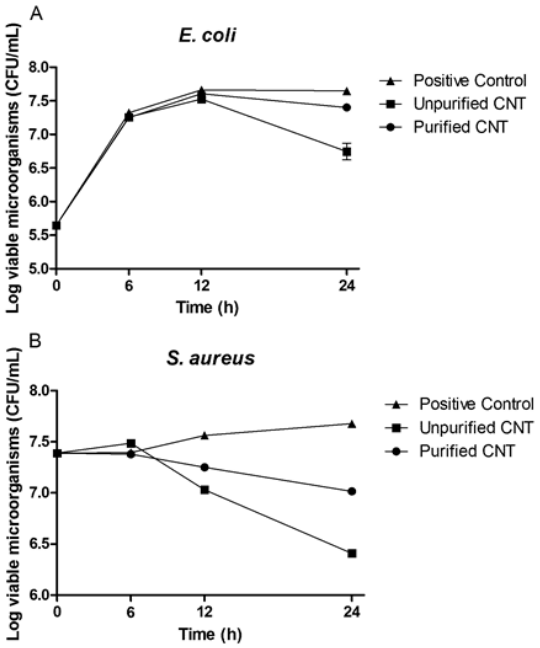

Figure 4. Time-kill curves of purified and unpurified MWCNTs against *E. coli* and *S. aureus*. The data was expressed as mean ± standard deviation.

4. Discussion

Even with perfect carbon nanotube (CNT) production methods, a purification process is required after the synthesis to remove impurities, such as amorphous carbon and oxides used during synthesis (Aghaei et al. 2023).

The Raman spectrum of multi-walled carbon nanotubes (MWCNTs) showed characteristic bands: the D band (D-disorder) at around 1360 cm^{-1} and the G band (G-graphite) at about 2670 cm^{-1}. A quality parameter emerged from the D and G band intensity ratio (ID/IG), indicating sp^2 carbon structures versus carbon impurities rich in sp^3 hybridization, such as amorphous carbon, polyhedral carbon shells, and carbon nanofibers (Machado et al. 2012). Lower ID/IG values indicate fewer impurities in MWCNT structures. The ID/IG in this study were 0.74 and 0.71 for unpurified and purified MWCNTs, respectively.

These findings suggest that the growth route produces high-quality MWCNTs, and the lower ID/IG ratio reflects purification efficiency (Machado et al. 2012).

The X-ray diffractogram in Figure 3a shows peaks around 37.1°, 42.9°, 62.3°, 74.9°, and 78.9°, indicating that the catalyst comprises the periclase phase of magnesium oxide (PDF 075-1526), corresponding to crystal planes (111), (002), (202), (113), and (222), respectively (Balamurugan, Ashna, Parthiban 2014). The diffractograms of unpurified and purified MWCNTs (Figure 2B and 2C, respectively) showed a high peak at around 26.1° and a lower one at around 44.1°, corresponding to (002) and (100) reflections of the crystallographic structure representing the graphitic structure of MWCNTs (PDF 41-1487) (Grassi et al. 2012). The persistence of periclase peaks after purification implies the process was not entirely efficient, although the increased intensity of peaks at 26.1° and 44.1° indicates the method removed some impurities.

The disc diffusion assay showed minimal differences between inhibition zones caused by purified and unpurified MWCNTs. This outcome might be due to the challenging MWCNT diffusion into the culture medium, with a significant amount remaining on the disc due to Van der Waals interactions and the large surface area, inevitably causing self-aggregation (Park et al. 2002).

The minimum inhibitory concentration (MIC) showed that a lower concentration of 1.375 mg/mL of purified and unpurified MWCNTs inhibited microorganisms without eliminating them, indicating a bacteriostatic effect. The minimum bactericidal concentration (MBC) required 5 mg/mL of purified and unpurified MWCNTs to kill the bacteria. Understanding the interactions between microorganisms and MWCNTs is of significant scientific interest (Maksimova 2019).

The time-kill curve indicated that unpurified CNTs were more effective against all tested microorganisms. This effect might be due to residual iron oxide from the catalyst, whose antimicrobial activity relates to the oxidative stress from reactive oxygen species (ROS) production (Alfei et al. 2024). The ROS may damage proteins and microbial DNA, controlling several cellular processes that trigger cell death (Juan et al. 2021). Current antimicrobial drugs induce ROS formation in bacterial cells (Qi et al. 2023).

Smaller nanoparticles may enhance the bactericidal effect of different compounds. The functionalization of CNTs with nanoparticles, such as silver, enhances their antimicrobial efficacy. For example, silver-functionalized CNTs have shown strong bactericidal properties by interacting with bacterial cells more efficiently due to the increased surface area and the unique properties of silver nanoparticles. Studies have demonstrated that these hybrid structures disrupt microbial cell membranes, leading to agglutination and subsequent cell death, particularly in Escherichia coli and Staphylococcus aureus (Dove et al. 2023).

CNTs may also be a promising alternative to conventional antimicrobials for treating infections from multidrug-resistant microorganisms due to physical properties that affect bacterial action (Li et al. 2023). Iron nanoparticles have inactivated E. coli by penetrating the cell membrane and inducing ROS production, which disrupts the bacterial plasma membrane. This effect is likely due to the residual oxide in the catalyst (Hetta et al. 2023)

Moreover, the significance of CNTs as a promising tool for combating bacteria lies in their unique physicochemical properties, including a large surface area, high mechanical strength, and the ability to generate ROS. These characteristics enable CNTs to disrupt bacterial cell membranes, leading to cell death. The antimicrobial activity of CNTs has been effective against diverse bacterial strains, including multidrug-resistant (MDR) pathogens, which pose a significant challenge in clinical settings (Alavi and Rai 2019). Additionally, the ability of CNTs to be functionalized with various chemical groups allows for targeted interactions with bacterial cells, enhancing their antimicrobial efficacy (Maksimova 2019). This adaptability makes CNTs a versatile platform for developing novel antimicrobial agents. Thus, integrating CNTs into antimicrobial strategies significantly advances the fight against bacterial infections, offering new avenues for treatment and prevention in healthcare and environmental applications.

5. Conclusions

Multi-walled carbon nanotubes showed improved antimicrobial activity in Gram-positive bacteria. These findings show possibilities for further studies and carbon nanotube applications in microbiology.

However, few studies have analyzed the toxicity of carbon nanotubes in biological systems. Still, the antimicrobial activity of multi-walled carbon nanotubes is an emerging research area in nanotechnology and nanoscience. Multi-walled carbon nanotube applications in human medicine require further studies to explore the real action mechanisms of these materials and reveal potential adverse effects.

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