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Development and Evaluation of Aztreonam-Functionalized Graphene Oxide Nanosheets for Enhanced Antibacterial Efficacy Against Multidrug-Resistant Bacteria

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Abstract

The present study aimed to develop a new nanocomposite by covalently functionalizing graphene oxide (GO) nanosheets with Aztreonam (AT), to target multidrug-resistant bacteria isolated from wound infections. The successful synthesis of the GO-AT nanocomposite was confirmed using advanced characterization techniques. Ultraviolet-Visible (UV-Vis) spectroscopy peaks were observed at 226.5nm and 217.1nm for GO and GO-AT, respectively. Fourier Transform Infrared (FTIR) spectroscopy exposed functional groups. Transmission Electron Microscopy (TEM) revealed morphological features, including GO sheets not exceeding 6nm and 12nm for GO-AT. Zeta potential indicated high colloidal stability (-33.7mV). The Raman spectroscopy characterization demonstrated an increased I_D/I_G ratio (0.90 to 1.14), indicating higher defect levels due to functionalization. Cytotoxicity assays conducted on selected cell lines showed an IC_{50} value of 1761.3363 μ g/ml, confirming a wide safety margin for potential biomedical applications. The antibacterial efficacy of the GO-AT was evaluated through minimum inhibitory concentration (MIC) testing, demonstrating synergistic antibacterial activity against resistant strains, as confirmed by statistical analysis ($p < 0.01$). The results highlight a significant synergistic antibacterial effect of Aztreonam-modified graphene oxide nanosheets against MDR isolates for both Gram-positive and Gram-negative bacteria.

Keywords: Graphene oxide, Aztreonam, Nanocomposite, Antibacterial Activity, Multidrug-Resistant Bacteria.

تطوير وتقييم صفائح أوكسيد الغرافين المعدلة بالأزترونام لتعزيز الفعالية المضادة للبكتيريا متعددة المقاومة للمضادات الحيوية

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الخلاصة

هدفت الدراسة الحالية الى تطوير مركب نانوي جديد عن طريق التفاعل التساهمي الوظيفي لصفائح أوكسيد الغرافين (GO) مع الأزترونام (AT)، لاستهداف البكتيريا المتعددة المقاومة للمضادات الحيوية والمعزولة من

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التهابات الجروح. تم تأكيد نجاح تحضير المركب النانوي GO-AT باستخدام تقنيات متقدمة للتوصيف. أظهر التحليل الطيفي بالأشعة فوق البنفسجية-المرئية (UV-Vis) قمم امتصاص عند 226.5 و 217.1 نانومتر لكل من GO و GO-AT على التوالي. أما التحليل الطيفي بالأشعة تحت الحمراء باستخدام تقنية فوريير (FTIR) ، فقد كشف عن وجود المجموعات الوظيفية. وأظهرت صور المجهر الإلكتروني النافذ (TEM) المورفولوجية، بما في ذلك صفائح GO و GO-AT بأبعاد التي لا يتجاوز طولها 6 و 12 نانومتر على التوالي . كما أظهر قياس الجهد زيتا ثباتاً غروباً عالياً (-33.7mV). وبالإضافة إلى ذلك، أظهر تحليل مطيافية رامان ارتفاعاً في نسبة I_D/I_G من 0.90 إلى 1.14، مما يشير إلى زيادة في العيوب الناتجة عن التعديل الوظيفي. تم إجراء اختبارات السمية الخلوية على خطوط خلوية مختارة، وأظهرت نتائجها أن قيمة IC_{50} بلغت 1761.3363 ميكروغرام/مل، مما يدل على أمان واسع لإمكانية الاستخدامات الطبية الحيوية. وتم تقييم الفعالية المضادة للبكتيريا لمركب GO-AT من خلال اختبارات التركيز المثبط الأدنى (MIC)، حيث أظهرت نشاطاً مضاداً للبكتيريا بشكل تآزري ضد السلالات المقاومة، وذلك كما تم تأكيده بالتحليل الإحصائي ($p < 0.01$). وتُبرز النتائج التأثير التآزري الكبير لأوكسيد الغرافين المعدل بالأزترينومام ضد العزلات متعددة المقاومة، سواء من البكتيريا موجبة الغرام أو سالبة الغرام..

1 Introduction

The proliferation of antibiotic resistance in bacterial pathogens is a significant global concern that poses a significant risk to public health, being associated with substantial morbidity and mortality rates. Multidrug-resistant patterns have resulted in infections in which the Gram-positive and Gram-negative bacteria are difficult to treat or are rendered untreatable with conventional antimicrobial agents [1]. Among the threatening pathogens causing this crisis are *Pseudomonas aeruginosa* and *Staphylococcus aureus* [2]. The bacterium *P. aeruginosa* is known to be aerobic, mobile, and Gram-negative [3]. *P. aeruginosa* is a pathogenic, opportunistic bacterium and one of the most common pathogens that cause nosocomial infections in patients undergoing chemotherapy, those with cystic fibrosis, or those with burns [4]. Due to the formation of biofilm, *P. aeruginosa* acquires drug resistance, making the bacteria resistant to conventional antibiotics [5]. *S. aureus* is a Gram-positive opportunistic bacterium that possesses virulence traits that have been developed in the environment [6], causing a wide range of diseases, from mild skin lesions to more severe and life-threatening conditions, such as septicemia [7].

Nanotechnology and science have provided a new concept in scientific materials, leading to promising advancements across all study domains [8]. The fabrication and creation of nanocarriers for the delivery of medications is one of the most interesting research areas [9]. Consequently, nano-platforms may be utilized in biosensing, bioimaging, antimicrobial agents, medication delivery, and other applications. Nano-platforms provide specific enhancement and efficacy on particular tissues or pathogens, which reduces the pathogenicity of pathogens [8]. Functional groups on graphene oxide (GO) facilitate its utilization for various domains, like drug /gene delivery systems [10]. GO exhibits antibacterial activity by damaging the bacterial cell membrane, enhancing the release of reactive oxygen species (ROS), and surrounding and separating these invasive bacteria from their nutrient supply and environment [11]. Yang Gao *et al.*, tested three graphene-loaded antibiotics against *Escherichia coli* and *S. aureus*. This system operates by generating ROS, which damages the cellular membrane and leads to cell lysis [12]. While the former studies primarily relied on physical adsorption or non-covalent interactions for loading different antibiotics onto GO, in this study, Aztreonam was covalently attached to the GO surface. This procedure leads to a more stable covalently bound association between the drug and the nanocarrier, thus leading to a more sustained release profile, greater antibacterial activity, and release control of the drug [13].

Consequently, the combination of antibiotics and carbon-based nanomaterials such as GO has been studied as a potential solution to increase the antibacterial spectrum and enhance antibacterial efficacy [8]. Aztreonam is a monobactam antibiotic that received approval in 1986 for the treatment of infections caused by aerobic Gram-negative bacteria; however, its clinical utility diminished alongside cephalosporins due to the emergence of extended-spectrum β -lactamases (ESBLs) and novel (serine) carbapenemases, which hydrolyze and inactivate Aztreonam and other β -lactam antibiotics [14]. New drug delivery systems, including one with graphene oxide-based nanocarriers, are being developed to have higher efficacy and specialized activity against resistant bacterial infections.

This study aimed to synthesize and characterize GO covalently loaded with Aztreonam antibiotic (GO-AT), with a wide safety margin for potential biomedical applications, and to evaluate its synergistic antibacterial effect against MDR Gram-positive and negative bacteria.

2 Materials and methods

Graphene oxide synthesis

This work used the modified Hummer's method with simple changes [15]. 1 g of graphite (Sigma-Aldrich), 4 g of NaNO_3 (HI-MEDIA), and 6 g of KMnO_4 (Fluka) were added to 50 ml of cool concentrated H_2SO_4 (CDH) and stirred for six hours on a cold-water bath that contained ice. The mixture turned deep red-brown. Then, 50 ml of deionized (DI) water was added to the solution and heated to 90-98 °C. 250 ml of warm water was used to dilute the mixture. Then, 30ml of H_2O_2 (Intron) was added until the mixture changed to bright yellow. After that, the liquid was washed and purified with 10% hydrochloric acid (CDH). Decanting and centrifugal processes were repeated several times. The resultant was left at room temperature to dry.

Functionalization of GO and preparation of GO-AT

First, 0.5 g of GO was combined with 100 mM of N-Hydroxysulfosuccinimide sodium salt (NHS) and 100 mM of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) aqueous solutions (Sigma-Aldrich) to activate the GO molecule [16]. The mixture was stirred at room temperature for 24 hours. Then, the product was centrifuged at 4000 rpm for 5 min to disperse the product. The filtrate was decanted, and the precipitate was redispersed in ethanol and centrifuged to eliminate the remnant of the starting material. 0.5 g of Aztreonam (Alfa-Aesar) was added to the product and stirred for 12 h. This mixture was extracted using water and purified by decantation and centrifugation multiple times, and the resulting product was dried to room temperature.

P. aeruginosa and S. aureus clinical Isolates

Fifty-one isolates of *P. aeruginosa* and 44 isolates of *S. aureus* were randomly taken from infected wounds at Medical City Hospital in Baghdad. Culture media and the VITEK2 System at the hospital laboratory had previously identified these isolates. The identity of the isolates was reconfirmed in this study using culture on selective media, biochemical tests, and the VITEK 2 System, by the routine diagnostic procedures employed in hospitals.

Antibiotic susceptibility testing

The disc diffusion-based antibiotic susceptibility testing was performed with 51 and 44 clinical isolates of *P. aeruginosa* and *S. aureus* [17]. The clinical isolates were tested with the following antibiotics: Meropenem (10), Imipenem (10), Ceftazidime (30), Aztreonam (30), Ciprofloxacin (5), Tobramycin (10), Gentamicin (10), Piperacillin-tazobactam (100/10), Penicillin (10), Rifampin (5), Levofloxacin (5), Clindamycin (2), Linezolid (30), Azithromycin (15), Doxycycline (30) and Trimethoprim-sulfamethoxazole (1.25/ 23.75). An isolate occurring

or having multiple resistances to at least three different classes of antibiotics is described as an MDR [18]. A result of antibiotic susceptibility was defined as resistant, susceptible, or intermediate based on Clinical and Laboratory Standards Institute (CLSI, 2024).

Characterization of the prepared materials

The surface functional groups of GO and GO-AT were studied using Raman spectra and Fourier-transform infrared spectroscopy (FTIR), the UV–Vis spectra of GO and GO-AT to determine the optical properties, the zeta potential of the compounds was evaluated to check the stability, and Transmission Electron Microscopy (TEM) to assess the morphology [8, 19, 20].

Determination of antibacterial by the minimum inhibitory concentration (MIC) of GO-AT

Minimal inhibitory concentrations (MICs) for GO-AT, GO, and Aztreonam were determined for *P. aeruginosa* and *S. aureus* using the broth microdilution method in 96-well plates. To achieve 0.1 mL of each concentration in each well, GO-AT was diluted two-fold in Muller-Hinton broth. To achieve the final concentration of 0.5 McFarland, a standardized inoculum was prepared from a suspension and a diluted colony in normal saline. After that, another 1:20 dilution was performed when the yield reached 5×10^6 CFU/ml. In the end, every well received 0.01 ml of the suspension. The microtiter plate was incubated at 37 °C for 24 hours. On the following day of incubation, resazurin was added to each well and incubated for 2 hours. Then, the MIC was determined by changing the colour from blue to pink in the presence of live cells [18].

Cytotoxicity test of GO-AT nanomaterials

The toxic effects of the test material were evaluated using a 96-well plate MTT cell viability assay on normal Human Dermal Fibroblasts neonatal (HDFn). 10^4 cells per well were used to seed the cell lines. Cells were treated with the test substance after 24 hours or when a confluent monolayer was established. The cell proliferation rate was evaluated by assessing cell viability after 72 hours of treatment, during which the medium was removed. Subsequently, 28 µL of a methyl thiazolyl tetrazolium (MTT) solution (2 mg/mL) was added, and the mixture was incubated at 37 °C for 15 min while shaking. The leftover crystals in the wells were solubilized by removing the MTT solution and adding 130 µL of dimethyl sulfoxide (DMSO), followed by incubation at 37 °C for 15 min while shaking [21].

Statistical analysis

Statistical analysis was performed using SPSS software (version 27, IBM Corp., USA). The MIC data were analyzed using a paired t-test. Results were expressed as mean $\pm$ standard deviation (SD), and a p-value less than 0.01 was considered statistically significant. A 99% confidence interval (CI) was applied to evaluate the precision of the mean differences.

3 Result and Discussion

P. aeruginosa and S. aureus identification

All 95 isolates are cultured on cetrimide and mannitol salt agar (MSA). Cetrimide agar was utilized to detect *P. aeruginosa*. Whereas the medium transitions from pale or neutral to pale green, the green color of the colony easily identifies the *P. aeruginosa* colonies [22]. The *S. aureus* fermentation of mannitol changes the media to yellow, and the colony on MSA is translucent, yellow-pigmented, creamy, and smooth [23] (Figure 1).



Figure 1: (a) Colonies of *P. aeruginosa* on cetrимide agar (b) Colonies of *S. aureus* on mannitol salt agar

VITEK 2 system

VITEK 2 compact system (bioMérieux, France) was carried out as a confirmatory test for the identification of *P. aeruginosa* and *S. aureus* using GN cards (Cat. No. 21341, LOT 2413024503) and GP cards (Cat. No. 21342, LOT 2422975403).

Antibiotic Susceptibility Testing

Most bacterial isolates identified in wounds belong to the genus *P. aeruginosa*. The results show that most of the isolates had characteristics indicating they were multidrug-resistant; 100% of isolates were resistant to Ciprofloxacin, Aztreonam, Piperacillin/tazobactam, and Ceftazidime, while in Imipenem (52.9%), Levofloxacin (76.5%), Tobramycin (80.4%), and Meropenem (58.8%). Findings of this study were supported by Ijaz *et al.*, who found that *Pseudomonas* is one of the major bacteria causing hospital-acquired infection [24]. *S. aureus* was resistant to Penicillin (100%), Rifampin (72.7%), Gentamicin (84.1%), Levofloxacin (79.6%), Clindamycin (75%), Linezolid (63.6%), Azithromycin (77.3%), Doxycycline (56.8%), Trimethoprim-sulfamethoxazole (65.9%), and Ciprofloxacin (68.2%). The results of the present study align with a study conducted by Asif *et al.*, [23]. Antibiotic resistance is usually referred to as intrinsic, acquired, or adaptive. An organism's nature includes resistance genes, but it can gain more by acquiring them from other organisms or by beneficial mutations or effective resistance brought about by its environment [25].

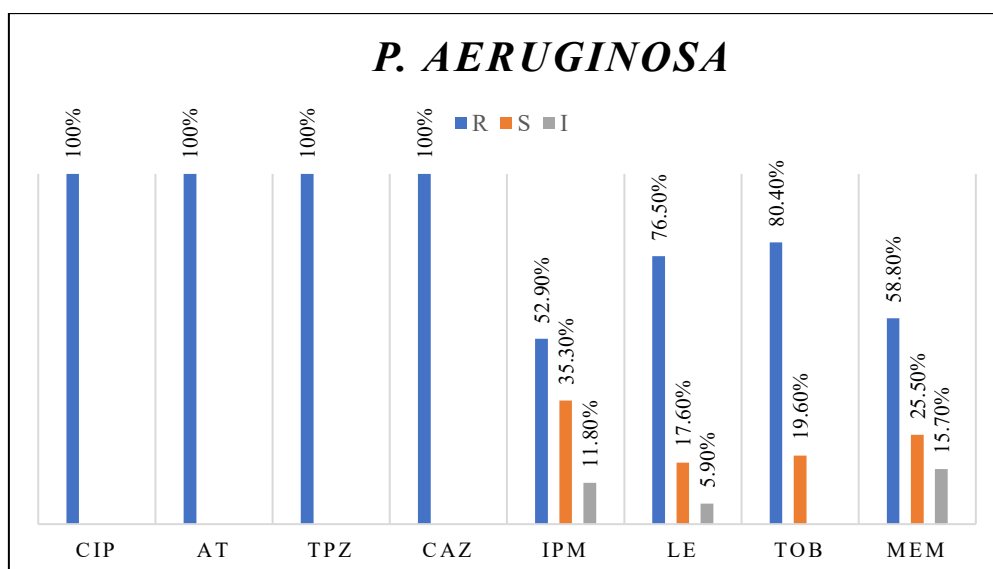


Figure 2: Antibiotics susceptibility against resistant *P. aeruginosa* (CIP= Ciprofloxacin, AT= Aztreonam, TPZ= Piperacillin/ Tazobactam, CAZ= Ceftazidime, IPM= Imipenem, LE= Levofloxacin, TOB= Tobramycin, and MEM= Meropenem).

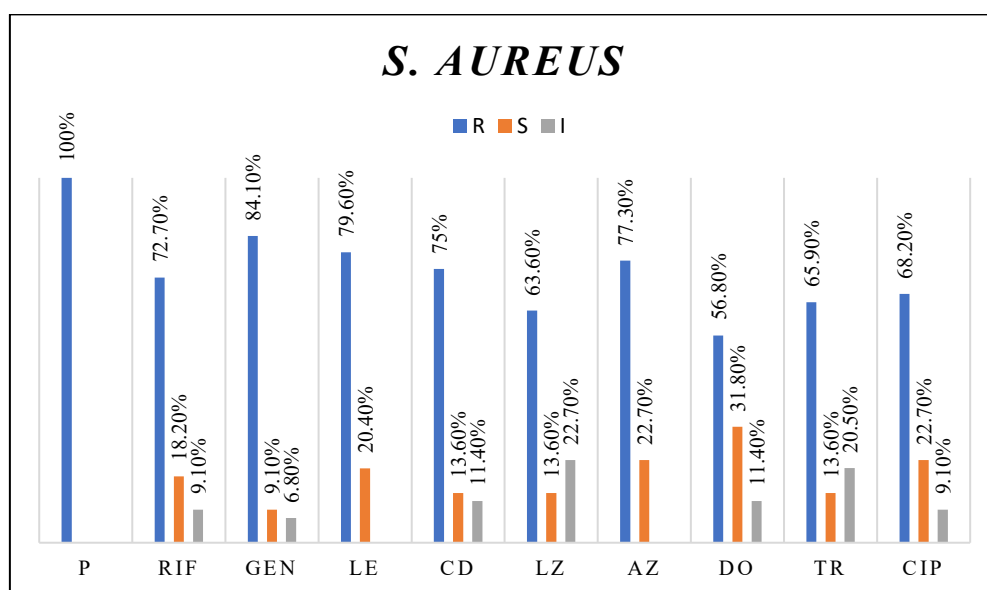


Figure 3: Antibiotics susceptibility against resistant *S. aureus* (P= Penicillin, RIF= Rifampin, GEN= Gentamicin, LE= Levofloxacin, CD= Clindamycin, LZ= Linezolid, AZ= Azithromycin, DO= Doxycycline, TR= Trimethoprim-sulfamethoxazole, and CIP= Ciprofloxacin).

Characterization of GO and GO-AT

Ultraviolet-Visible spectroscopy

UV - Vis spectroscopy of GO showed an intense absorption peak at 226.5 nm assigned to the $\pi \rightarrow \pi^*$ electronic transitions of the aromatic C=C bonds. A shoulder peak at about 300 nm was also apparent, which was assigned to $n \rightarrow \pi^*$ transitions involving oxygen-containing functional groups like carbonyls that were accepted by Trikkaliotis *et al.*, [26]. After Aztreonam loading (GO-AT), the maximum absorption peak shifted to 217.1 nm, or a blue shift was observed, and its shoulder substantially diminished. Therefore, the change in the spectrum shall mean an altered electronic environment in GO due to functionalization, implying that the chemical conjugation was successful through EDC/NHS [27]. In addition, a low-intensity

shoulder at ~ 300.8 nm in GO -AT indicates unreacted or newly formed $n \rightarrow \pi^*$ transitions of carbonyl groups in the antibiotic. This further shows partial retention or addition of oxygenated functional groups. Others have discussed comparable spectrum changes in GO-antimicrobial nanocomposites. Previously, Vancomycin has been loaded on the GO by Singh *et al.*, [20]. The study proves that a red shift was monitored at 225 and 279 nm, which was attributed to the drug conjugation effect on the π -electron of GO [20]. In this study, there was a red shift, whilst GO-AT observed a blue shift, indicating that the electrical property of Aztreonam has a different interaction with the GO matrix. Both outcomes show that the drug loading induces specific spectrum shifts, proving that optical modulations in GO reflect functionalization (Figure 4).

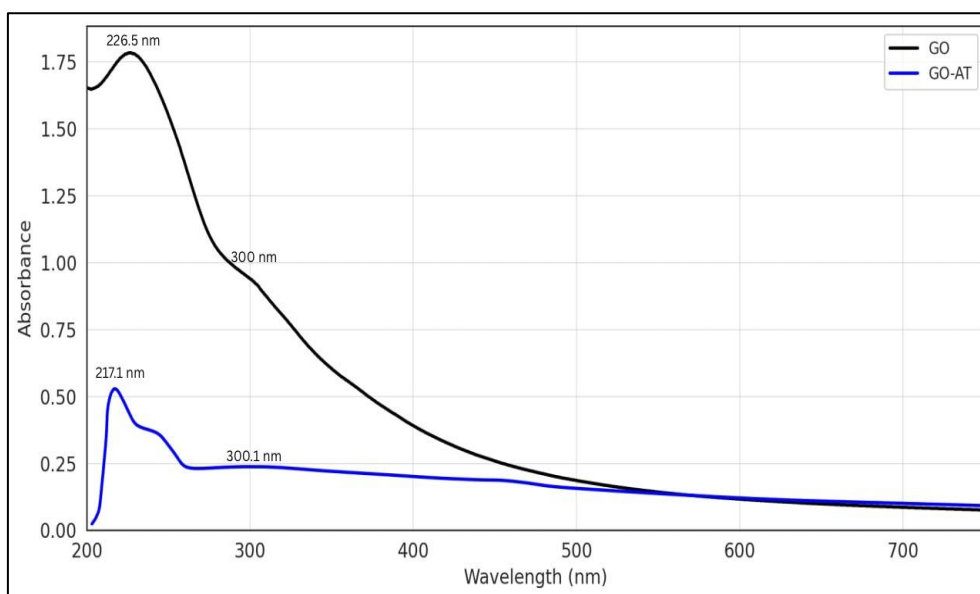


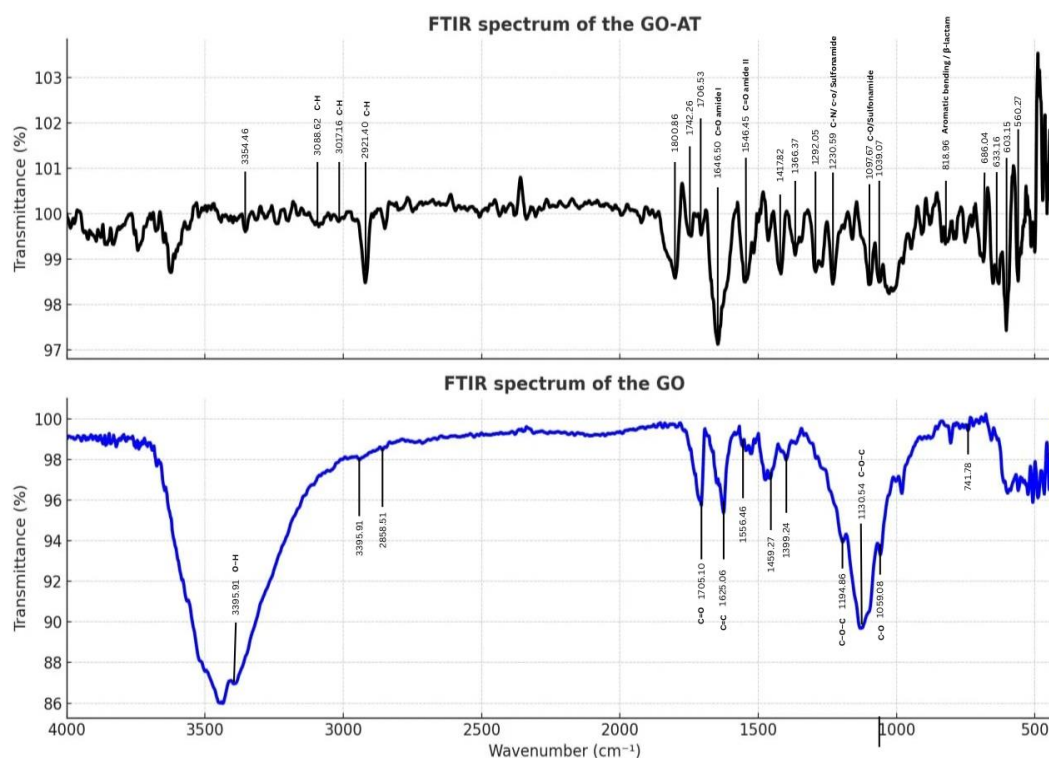
Figure 4 : UV-Vis Spectrum of graphene oxide (black) and graphene oxide-Aztreonam (blue).

Fourier-Transform Infrared Spectroscopy

The results of FTIR of GO in Table 1 showed the characteristic bands for the functional groups of the GO molecule [8]. In the case of GO-AT composite, the spectrum showed a band at 3088.62 cm^{-1} and 3017.16 cm^{-1} , which were attributed to the stretching vibrations of aromatic C-H of the Aztreonam molecule, whilst the band at 2921.40 cm^{-1} was assigned to the stretching vibrations of aliphatic C-H. The formation of amide bonds by coupling reactions with EDC/NHS is indicated by successful records on the bands at 1646.50 cm^{-1} and 1546.45 cm^{-1} , as previously proved in the literature [16]. The vibrations associated with aromatic compounds and amines appeared at 1417.82 , 1366.37 , and 1292.05 cm^{-1} . Other bands at 1230.59 to 1039.07 cm^{-1} (including prominent bands at 1230.59 , 1097.67 , and 1039.07 cm^{-1}) represent the C-N, C-O, and sulfonamide groups present in the aztreonam structure. The presence of new absorptions at 818.96 , 686.04 , 633.16 , 603.15 , 560.27 , and 425.92 cm^{-1} in the fingerprint region means something changed in the structure and vibrations after functionalization [28] (Figure 5). These findings can be correlated with the earlier studies [8] on FTIR analysis of β -lactam antibiotics loaded on graphene oxide nanocarriers, whereby the compounds had the same shift and manifestation of new peaks as in our study.

Table 1: FTIR major bands of GO and GO-AT nanoparticle.

FTIR Peak (cm ⁻¹)	Assigned Functional Group	Compound Class
3395.91	O–H stretching	Alcohol
1705.10	C=O stretching	Carboxylic Acid
1625.06	C=C stretching	Aromatic Ring
1194.86, 1130.54	C–O–C stretching	Epoxy
1059.08	C–O stretching	Alcohol
1646.50	Amide I (C=O stretching)	Amide bond (EDC/NHS coupling)
1546.45	Amide II (N–H bending + C–N stretching)	Amide bond (EDC/NHS coupling)
3088.62, 3017.16	Aromatic C–H stretching	Aromatic (from Aztreonam)
2921.40	Aliphatic C–H stretching	Alkane (from Aztreonam)
1230.59, 1097.67	C–N, C–O stretching	Sulfonamide / β -lactam structure
818.96	Fingerprint of substituted ring	Structural change (GO-AT)

**Figure 5:** Major bands of FTIR represent the functional groups of nanoparticles graphene oxide (blue) and Graphene oxide-Aztreonam (black).

Transmission Electron Microscopy

TEM measurement was conducted to investigate GO and GO-AT morphology. The TEM image of GO exhibited typical characteristics of well-exfoliated nanosheets, as it is displayed as ultrathin, transparent layers with a wrinkled morphology, as accepted by Nasreen *et al.*, [29]. After conjugation with Aztreonam, GO-AT showed a noticeable morphological change in TEM pictures. Moreover, dispersed electron-dense characteristics were seen along the nanosheets, indicating the potential presence of aztreonam molecules affixed to the GO surface (Figure 6). Similar structural features have been reported by Memar *et al.*, small dots aggregated on Mrp-GO were obtained based on TEM images, indicating the loading of meropenem on GO sheets

[19]. Li *et al.*, reported that such small particles may improve antibacterial effects because of the greater chance of interaction with bacteria than free GO sheets [30].

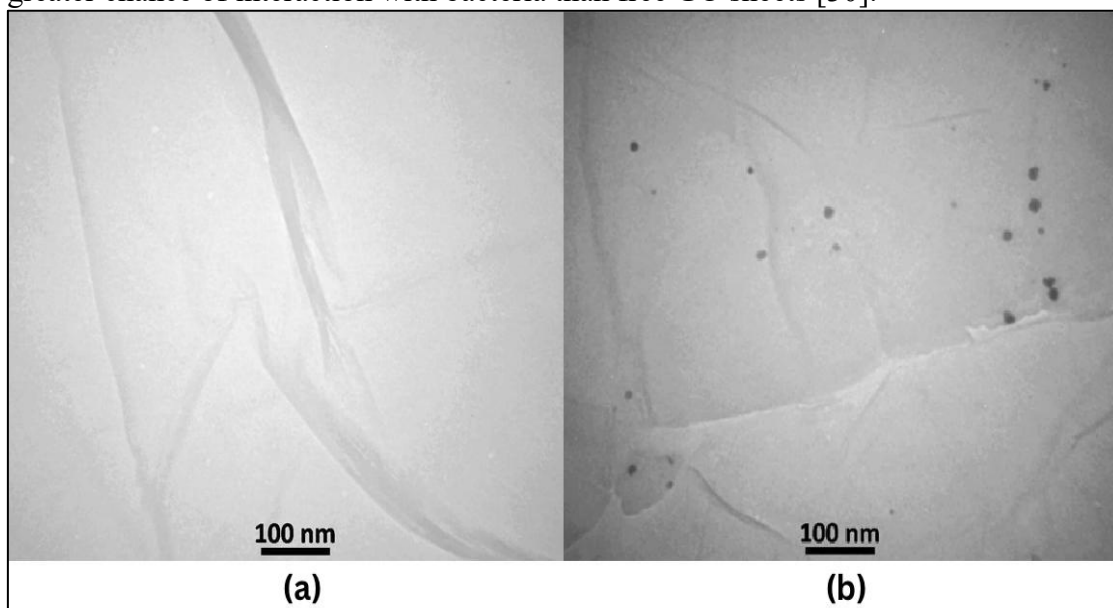


Figure 6: TEM of the (a) Graphene oxide and (b) Graphene oxide-Aztreonam.

Zeta potential

The zeta potential of the synthesized GO was determined to be -33.7mV , depicting a high negative surface charge and a high colloidal stability. This large value indicates that numerous negatively charged groups, including oxygen-containing functional groups (e.g., $-\text{COOH}$, $-\text{OH}$) on the GO surface, contribute to dispersion and avoid aggregation. This is consistent with the findings of Gilsanz-Muñoz *et al.*, who reported that a zeta potential of GO has been established at $-35.4 \pm 2.1\text{ mV}$, validating the high electric repulsion caused by the prevalence of oxygen functional groups (e.g., carboxyl and hydroxyl) [31]. Conversely, the zeta potential of GO-AT significantly decreased to -2.76mV . This significant decrease can be explained by the covalent functionalization of aztreonam molecules on the GO surface that possibly masked or neutralized the most negatively charged functional groups (Figure 7). The same was seen in a study by Xiong *et al.*, in which the zeta potential of GO was higher following the EDC/NHS activation and protein conjugation [32], which further reaffirms the finding that covalent functionalization reduced the surface charge by modifying the GO interface.

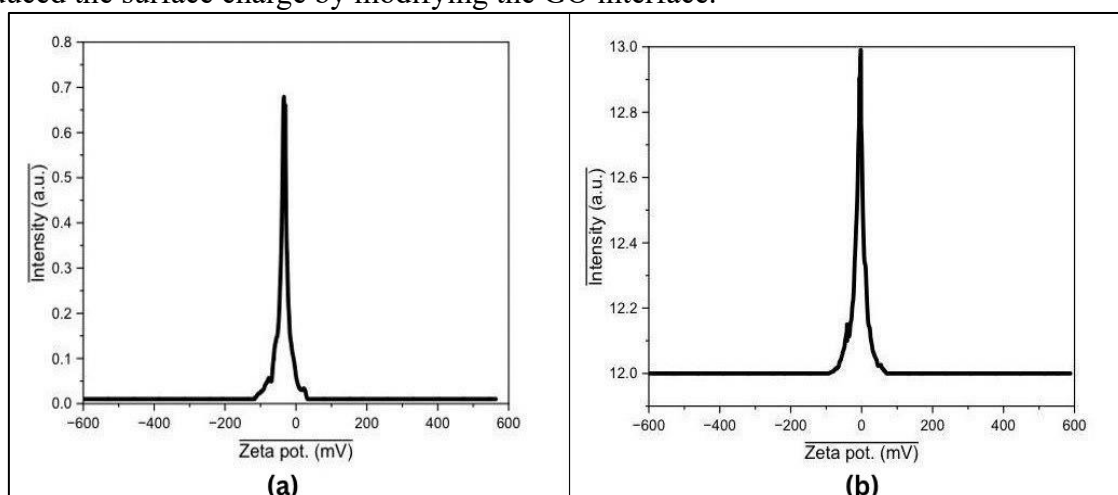


Figure 7: Zeta-potential measurement of (a) Graphene oxide and (b) Graphene oxide-Aztreonam.

Raman Spectra

The Raman spectra of both GO and GO-AT employed two typical peaks at the D and G bands. In GO, the D-band was noted at 1352.50 cm^{-1} , and the G-band was observed at 1582.75 cm^{-1} , and the intensity ratio (I_D/I_G) is approximately 0.90. These bands are assigned to the occurrence of structural defects (D-band) and graphitic sp^2 domains (G-band), respectively, and the ratio reflects a middling degree of disorder, which results in oxidation. The D-band appeared at 1344.67 cm^{-1} and the G-band at 1607.54 cm^{-1} with an increased (I_D/I_G) ratio of 1.14 in the case of GO-AT. This increment indicates the occurrence of special structural defects as a result of aztreonam functionalization [33]. The shift in peak positions and increase in disorder level strongly demonstrate the successful loading of the drug on the GO surface and the partial breaking up of the sp^2 -hybridized carbon-based structure of GO (Figure 8). Amiri-Zirtol *et al.*, noticed the minor shifts of G-and D-bands and the increase in I_D/I_G of GO functionalized with amino acids, which supports the link between new sp^3 hybrid sites and Raman defect signals [34].

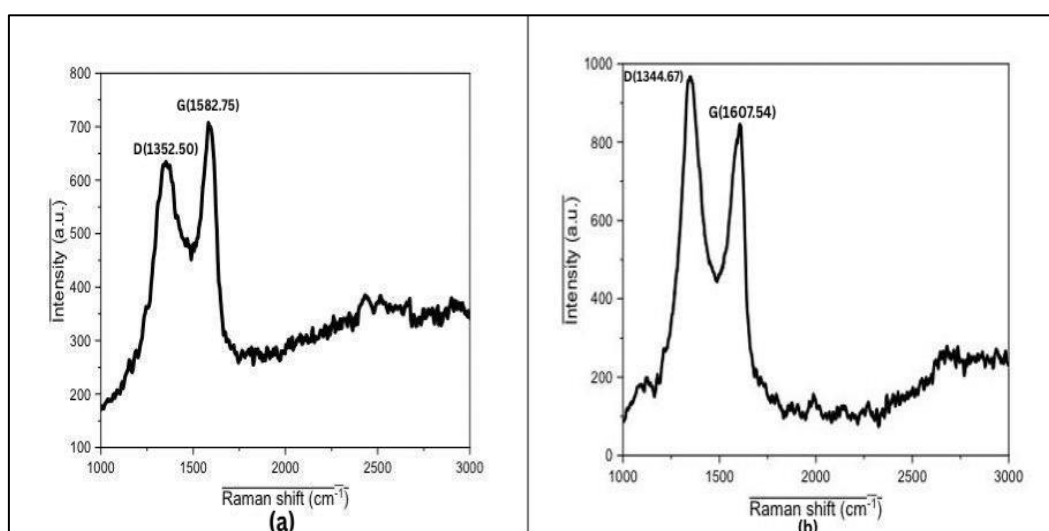


Figure 8: Raman spectra presenting shifts in the region of the D and G for (a) Graphene oxide and (b) Graphene oxide-Aztreonam.

Antibacterial activity analysis of GO-AT

The MIC of chemically modified graphene oxide-loaded Aztreonam (GO-AT) was evaluated against four MDR bacterial isolates, including two *S. aureus* and two *P. aeruginosa*. A two-fold serial dilution was carried out, starting with $1000\text{ }\mu\text{g/mL}$ and finishing at $1.953125\text{ }\mu\text{g/mL}$. The antibiotic Aztreonam is covalently added to the GO by using EDC/NHS coupling, which enhances its potential for delivering the drug.

The results revealed that GO exhibited only a moderate antibacterial effect, as the minimum inhibitory concentration (MIC) for all tested *S. aureus* and *P. aeruginosa* isolates was $500\text{ }\mu\text{g/mL}$. This suggests that its activity may be attributed to oxidative stress and disruption of bacterial membranes. For *P. aeruginosa*, the resistance to Aztreonam was $7.8125\text{ }\mu\text{g/mL}$ when treated with GO-AT. While the Aztreonam alone, the MIC was at the fourth dilution ($125\text{ }\mu\text{g/mL}$). The MIC of the first isolate of *S. aureus*, which was tested with GO-AT, shows a complete inhibition at all concentrations, indicating the highest inhibitory effect even at the lowest concentration. The MIC for the second isolate was $15.625\text{ }\mu\text{g/mL}$. Treatment with Aztreonam alone did not inhibit the growth of both *S. aureus*, which aligned with inefficiency against Gram-positive. According to the current research, Aztreonam loaded onto functionalized graphene oxide significantly increased its ability to fight *P. aeruginosa* and *S.*

aureus infections, as proven by lower MIC values (Figure 9). The paired t-test analysis revealed a significant decrease in the MIC values after treatment with GO-AT. The MIC before treatment was 562.5 ± 72.17 , as presented in Table 2, while after treatment it decreased markedly to 9.76 ± 3.90 . The mean difference between the two groups was 552.7, with a t-value of 14.84 (df = 3) and a highly significant p-value ($p = 0.001$, 99% CI: 335.18–770.28), as shown in Table 3. This significant drop in MIC is an indication of GO-AT's high efficiency as an antimicrobial agent. The very low p-value ($p < 0.01$) confirms that the above difference is statistically significant and not due to random variation. This most recent discovery implies that the functionalization of graphene oxide was successful in expanding the antibacterial spectrum of Aztreonam, enabling aztreonam-graphene oxide (GO-AT) to exhibit activity against the Gram-positive bacteria, making GO-AT an interesting nanocomposite to address the common problem of MDR pathogens. According to Memar *et al.*, who studied meropenem-loaded GO, similar MIC reductions were observed for carbapenem-resistant Gram-negative bacteria [19]. Besides, the better antibacterial effect toward *S. aureus* found here agrees with Mohammadi Tabar *et al.*, who discovered that modified graphene oxide enhanced the effects of Penicillin and Oxacillin against MRSA [8]. These findings show that GO nanocarriers may improve the effectiveness of regular antibiotics against dangerous bacteria.

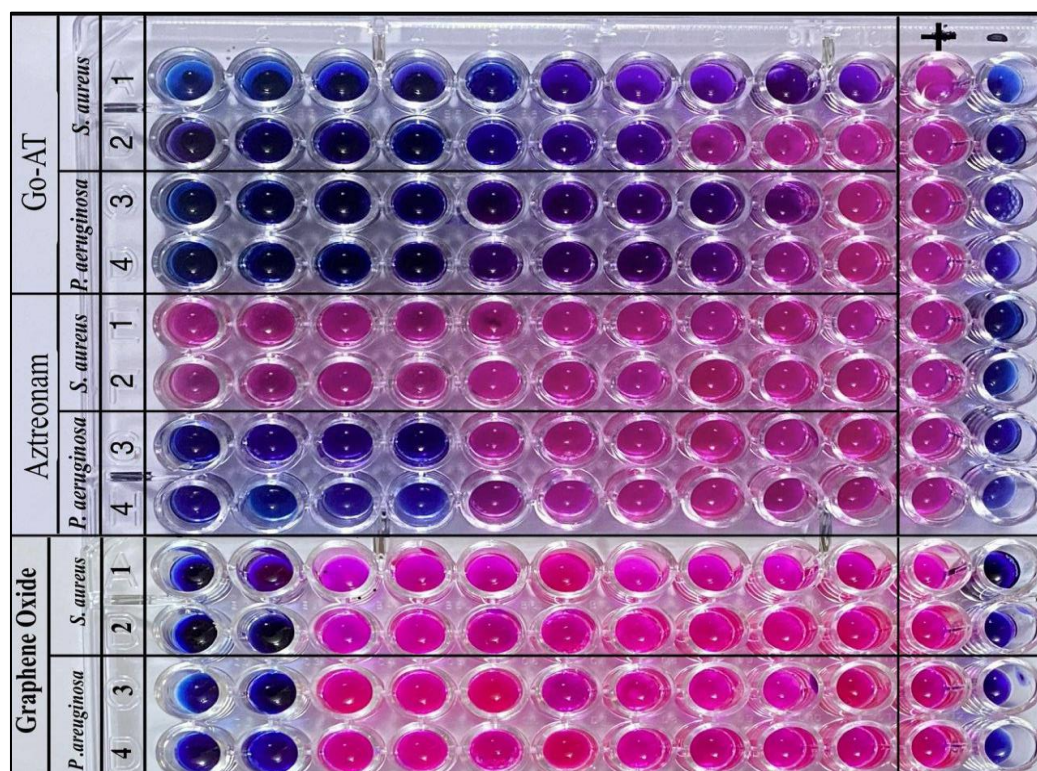


Figure 9: MIC of both GO-AT, Aztreonam, and GO (Numbers 1 and 2 refer to two isolates of *S. aureus* that were treated with GO-AT, AT, and GO, and numbers 3 and 4 refer to two isolates *P. aeruginosa* that were also treated with GO-AT, AT, and GO).

Table 2: Descriptive statistics of MIC values before and after treatment with GO-AT. Data are expressed as Mean $\pm$ SD (Standard Deviation) and SEM (Standard Error of Mean).

	Mean	SD	SEM
Before	562.5000	72.16878	36.08439
After	9.7650	3.90667	1.95333

Table 3: Paired t-test analysis of MIC values before and after treatment with GO-AT, showing mean difference, SD, SEM, t-value, degrees of freedom (df), significance level, and 99% confidence interval (CI).

	Paired Differences					t	df	Sig. (2 tailed)
	Mean	SD	SEM	99% Confidence Interval of the Difference				
				Lower	Upper			
Before - After	552.73500	74.49309	37.24654	335.18131	770.28869	14.840	3	.001

Cytotoxicity

MTT was used to determine the cytotoxicity of GO-AT on the cell line Human Dermal Fibroblasts neonatal (HDFn). This test was designed to measure the vitality of normal cell lines (1×10^4 cells/mL) using a variety of GO-AT concentrations. The cytotoxicity assay of the GO-AT showed that at the highest concentration (1000 $\mu\text{g/mL}$), the cell viability is $89.30 \pm 0.56\%$, indicating very mild cytotoxicity on the tested cells. As the concentration reduced, the viability increased gradually to more than 99 percent at reduced doses. This trend shows that GO-AT shows excellent biocompatibility at submaximal concentrations with negligible toxicity. In comparison to the values of MIC, GO-AT exhibited a strong antibacterial activity at much lower concentrations than those causing cytotoxic effects. In the case of *S. aureus*, the first clinical isolate inhibition was recorded entirely at all concentrations tested.

In contrast, the MIC of the second clinical isolate exhibited a result of 15.625 $\mu\text{g/mL}$. Moreover, both *P. aeruginosa* isolates exhibited an inhibitory effect on growth at a 7.8125 $\mu\text{g/mL}$ concentration treated by GO-AT. This information proves that GO-AT has high bactericidal levels at very low concentrations compared to the cytotoxic dosage ($\text{IC}_{50} \approx 1761.3363 \mu\text{g/mL}$), making it a highly safe drug. This promotes its perspective as a non-toxic and efficacious nanocarrier of antimicrobial treatment, especially towards drug-resistant pathogens. These results are consistent with those of Memar *et al.*, revealing that concentrations of Mrp-GO (1–128 $\mu\text{g/mL}$) exhibited no toxic effects on the cell line [19]. Also, this is consistent with the non-toxicity or mild toxicity of GO as reported in the scientific sources using GO in Caco-2 cells [35].

4 Conclusion

The current research was able to demonstrate covalently bound Aztreonam onto the nanosheets of graphene oxide to present a unique new nanocomposite (GO-AT) that exhibited better antibacterial activity as well as high biocompatibility. GO-AT structural integrity and functionalization were achieved since comprehensive physicochemical characterizations proved the success of the process. Antimicrobial assays *in vitro* demonstrated significant decreases in the MIC values compared to graphene oxide and free Aztreonam, especially against multidrug-resistant *P. aeruginosa*, as confirmed by statistical analysis ($p < 0.01$). Another interesting finding is that GO-AT has much greater activity against *S. aureus*, signifying a much wider antibacterial spectrum against Gram-positive bacteria. Furthermore, the cytotoxicity test conducted on Human Dermal Fibroblasts neonatal (HDFn) cells in terms of bactericidal concentrations indicated a high cell viability, and the composite is safe to utilize in terms of biological applications. These results indicate the synergistic property of GO-based nanocarriers to break the bacterial resistance mechanisms and warrant the future design of GO-AT as a possible candidate to address MDR bacterial infections in the clinical arena.

Ethical Approval

The investigation was duly permitted and approved by the Human Ethics Committee, College of Science, Baghdad University (Ref.: CSEC/0225/0017).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

- [1] M. Frieri, K. Kumar, and A. Boutin, "Antibiotic resistance," *Journal of infection and public health*, vol. 10, no. 4, pp. 369-378, 2017.
- [2] A. Rashid, M. Akram, O. T. Kayode, and A. Kayode, "Clinical features and epidemiological patterns of infections by multidrug resistance *Staphylococcus aureus* and *Pseudomonas aeruginosa* in patients with burns," *Biomedical Journal of Scientific & Technical Research*, vol. 25, no. 4, pp. 19272-19278, 2020.
- [3] E. F. Hussein, "Pseudomonas aeruginosa represents a main cause of hospital-acquired infections (HAI) and multidrug resistance (MDR)," in *Pseudomonas aeruginosa-New Perspectives and Applications*: IntechOpen, 2022.
- [4] I. Jurado-Martín, M. Sainz-Mejías, and S. McClean, "Pseudomonas aeruginosa: an audacious pathogen with an adaptable arsenal of virulence factors," *International journal of molecular sciences*, vol. 22, no. 6, p. 3128, 2021.
- [5] A. Haidar, A. Muazzam, A. Nadeem, R. Atique, H. A. Saeed, A. Naveed, J. Sharif, A. Perveen, H. R. Fatima, A. Samad, "Biofilm formation and antibiotic resistance in *Pseudomonas aeruginosa*," *The Microbe*, vol. 3, p. 100078, 2024.
- [6] N. A. Rasheed and N. R. Hussein, "Staphylococcus aureus: an overview of discovery, characteristics, epidemiology, virulence factors and antimicrobial sensitivity," *European Journal of Molecular & Clinical Medicine*, vol. 8, no. 3, pp. 1160-1183, 2021.
- [7] W. O. Fayisa and N. F. Tuli, "Review on *Staphylococcus aureus*," *International Journal of Nursing Care and Research*, vol. 1, pp. 1-8, 2023.
- [8] M. Mohammadi Tabar, M. Khaleghi, E. Bidram, A. Zarepour, and A. Zarrabi, "Penicillin and oxacillin loaded on pegylated-graphene oxide to enhance the activity of the antibiotics against methicillin-resistant *Staphylococcus aureus*," *Pharmaceutics*, vol. 14, no. 10, p. 2049, 2022.
- [9] V. Chandrakala, V. Aruna, and G. Angajala, "Review on metal nanoparticles as nanocarriers: Current challenges and perspectives in drug delivery systems," *Emergent Materials*, vol. 5, no. 6, pp. 1593-1615, 2022.
- [10] N. Karki, H. Tiwari, C. Tewari, A. Rana, N. Pandey, S. Basak, and N. G. Sahoo, "Functionalized graphene oxide as a vehicle for targeted drug delivery and bioimaging applications," *Journal of Materials Chemistry B*, vol. 8, no. 36, pp. 8116-8148, 2020.
- [11] I. M. Ng and S. Shamsi, "Graphene oxide (GO): a promising nanomaterial against infectious diseases caused by multidrug-resistant bacteria," *International journal of molecular sciences*, vol. 23, no. 16, p. 9096, 2022.
- [12] Y. Gao, J. Wu, X. Ren, X. Tan, T. Hayat, A. Alsaedi, C. Cheng, and C. Chen, "Impact of graphene oxide on the antibacterial activity of antibiotics against bacteria," *Environmental Science: Nano*, vol. 4, no. 5, pp. 1016-1024, 2017.
- [13] N. Osman, N. Devnarain, C. A. Omolo, V. Fasiku, Y. Jaglal, and T. Govender, "Surface modification of nano-drug delivery systems for enhancing antibiotic delivery and activity," *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, vol. 14, no. 1, p. e1758, 2022.
- [14] K. Bush, "Past, present, and future perspectives on aztreonam and avibactam," *Expert Review of Anti-infective Therapy*, vol. 23, no. 5, pp. 277-290, 2025.
- [15] V. Gupta, N. Sharma, U. Singh, M. Arif, and A. Singh, "Higher oxidation level in graphene oxide," *Optik*, vol. 143, pp. 115-124, 2017.
- [16] N. N. S. Aye, P. Maraming, P. Tipayawat, S. Daduang, A. Techasen, W. Jamnongkan, P. Sithithaworn, and J. Daduang, "Functionalized graphene oxide-antibody conjugate-based electrochemical immunosensors to detect *Opisthorchis viverrini* antigen in urine," *Materials Advances*, vol. 5, no. 10, pp. 4491-4503, 2024.
- [17] S. Milleville, L. Rouabah, S. Bernabeu, A. S. Henriksen, H. D. Swardt, I. Rezzoug, L. Dortet, and C. Emeraud, "Performance of disc diffusion and four commercially available MIC tests to

- determine mecillinam susceptibility on carbapenemase-producing Enterobacterales," *Journal of Clinical Microbiology*, vol. 63, no. 5, pp. e01473-24, 2025, doi: 10.1128/jcm.01473-24.
- [18] R. A. F. A. Fattah, T. A. H. Mohamed, and M. S. Elsayed, "Effect of chitosan nanoparticles on quorum sensing-controlled virulence factors and expression of LasI and RhII genes among *Pseudomonas aeruginosa* clinical isolates," *AIMS microbiology*, vol. 7, no. 4, p. 415, 2021.
- [19] M. Y. Memar, Y. R. Saadat, S. M. Dizaj, M. Yekani, S. M. Hejazian, B. Niknafs, S. Z. Vahed, and S. Sharifi, "Cytotoxicity and biocompatibility of Meropenem-loaded graphene oxide and its antibacterial effects against carbapenem-resistant Gram-negative bacteria," *OpenNano*, vol. 12, p. 100155, 2023.
- [20] V. Singh, V. Kumar, S. Kashyap, A. V. Singh, V. Kishore, M. Sitti, P. S. Saxena, and A. Srivastava, "Graphene oxide synergistically enhances antibiotic efficacy in vancomycin-resistant *Staphylococcus aureus*," *ACS applied bio materials*, vol. 2, no. 3, pp. 1148-1157, 2019.
- [21] B. H. Adil, A. M. Al-Shammari, and H. H. Murbat, "Breast cancer treatment using cold atmospheric plasma generated by the FE-DBD scheme," *Clinical Plasma Medicine*, vol. 19, p. 100103, 2020.
- [22] S. S. Al-Bayati, S. D. Al-Ahmer, A.-M. M. Shami, and A. H. Al-Azawi, "Isolation and identification of *Pseudomonas aeruginosa* from clinical samples," *Biochem Cellular Archives*, vol. 21, no. 2, pp. 3931-3935, 2021.
- [23] A. Asif, M. Asghar, H. U. Khan, I. Haq, and S. Luqman, "Antibiotic susceptibility pattern of clinical isolates of methicillin resistant *Staphylococcus aureus* in Peshawar, Pakistan," *Annals of the Romanian Society for Cell Biology*, vol. 25, no. 6, pp. 20116-31, 2021.
- [24] M. Ijaz, A. B. Siddique, M. H. Rasool, and M. Shafique, "Frequency of multi drug resistant *Pseudomonas aeruginosa* in different wound types of hospitalized patients," *Pakistan journal of pharmaceutical sciences*, vol. 32, 2019.
- [25] R. F. Langendonk, D. R. Neill, and J. L. Fothergill, "The building blocks of antimicrobial resistance in *Pseudomonas aeruginosa*: implications for current resistance-breaking therapies," *Frontiers in Cellular and Infection Microbiology*, vol. 11, p. 665759, 2021.
- [26] D. G. Trikkalitis, A. K. Christoforidis, A. C. Mitropoulos, and G. Z. Kyzas, "Graphene oxide synthesis, properties and characterization techniques: a comprehensive review," *ChemEngineering*, vol. 5, no. 3, p. 64, 2021.
- [27] M. S. H. Akash, K. Rehman, M. S. H. Akash, and K. Rehman, "Ultraviolet-visible (UV-VIS) spectroscopy," *Essentials of pharmaceutical analysis*, pp. 29-56, 2020.
- [28] N. Abidi, *FTIR Microspectroscopy*. Springer, 2021.
- [29] F. Nasreen, A. Anwar, M. Ahmad, A. Majeed, A. Afzal, and T. Hussain, "A FACILE IMPROVED OXIDATION METHOD FOR ECOLOGICAL PRODUCTION OF GRAPHENE OXIDE," *Digest Journal of Nanomaterials & Biostructures (DJNB)*, vol. 16, no. 1, 2021.
- [30] Y. Li, D. Yang, and J. Cui, "Graphene oxide loaded with copper oxide nanoparticles as an antibacterial agent against *Pseudomonas syringae* pv. tomato," *Rsc Advances*, vol. 7, no. 62, pp. 38853-38860, 2017.
- [31] M. F. Gilsanz-Muñoz, M. Martínez-Martínez, J. Pérez-Piñero, M. Roldán, M. P. Arce, R. Blasco, L. R.-S. Román, F. Esperón-Fajardo, A. Cerpa-Naranjo, and B. Martín-Maldonado, "Assessing the Antimicrobial Efficacy of Graphene Oxide and Its PEGylated Derivative Against *Staphylococcus aureus*," *Sci*, vol. 6, no. 4, p. 66, 2024.
- [32] K. Xiong, Q. Fan, T. Wu, H. Shi, L. Chen, and M. Yan, "Enhanced bovine serum albumin absorption on the N-hydroxysuccinimide activated graphene oxide and its corresponding cell affinity," *Materials Science and Engineering: C*, vol. 81, pp. 386-392, 2017.
- [33] J. Moon, M. Li, A. J. Ramirez-Cuesta, and Z. Wu, "Raman spectroscopy," in *Springer handbook of advanced catalyst characterization*: Springer, pp. 75-110, 2023.
- [34] L. Amiri-Zirtol, H. Emtiazi, S. N. Abootalebi, and A. Gholami, "Dual acid-base catalysis with biologically modified graphene oxide: a sustainable route to polyhydroquinolines with antimicrobial properties," *Scientific Reports*, vol. 15, no. 1, p. 10194, 2025.
- [35] J. Domenech, A. Hernández, E. Demir, R. Marcos, and C. Cortés, "Interactions of graphene oxide and graphene nanoplatelets with the in vitro Caco-2/HT29 model of intestinal barrier," *Scientific reports*, vol. 10, no. 1, p. 2793, 2020.