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Synergistic Antibacterial Activity of Copper-doped Carbon Quantum Dots with *Azadirachta indica* (Neem) Leaf Extract on Multidrug-Resistant Bacteria

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Abstract

The emergence of multidrug-resistant bacteria poses a significant challenge to healthcare systems worldwide. Natural products, such as plant extracts, have garnered attention for their potential antibacterial properties. This study aimed to assess the synergistic antibacterial activity of Copper-doped Carbon Quantum Dots (Cu-CQDs) with ethanolic leaf extract of *Azadirac indica* (*A. indica*) (Neem) against multidrug-resistant bacteria. The study employed standard microbiological techniques to isolate bacteria from clinical samples and determine their antibiotic susceptibility profiles. The ethanolic leaf extract of *A. indica* was prepared and subjected to antibacterial susceptibility testing using disc diffusion and broth microdilution methods. Synergistic antibacterial activity of Cu-CQDs with *A. indica* leaf extract was demonstrated by using checkerboard assay. Results demonstrated varying degrees of susceptibility among the multidrug-resistant bacteria tested, indicating the potential of *A. indica* extract as an antibacterial agent. Further investigations are warranted to elucidate the mechanisms underlying its antibacterial activity and explore its potential therapeutic applications.

Keywords: *Azadirachta indica*, multidrug-resistant bacteria, antibacterialactivity, synergistic activity, clinical isolates.

Introduction

The emergence of multidrug-resistant bacterial infections poses a critical crisis in medicine, particularly for immunecompromised individuals, leading to a high mortality rate worldwide. The lack of novel antimicrobial treatments exacerbates the antimicrobial resistance (AMR) crisis, with India notably labelled the "AMR capital of the world" due to a surge in resistant bacteria alongside excessive antibiotic use [1,2]. Projections indicate staggering economic burdens and millions of annual fatalities by 2050 due to AMR [3]. The United Nations General Assembly has addressed this issue, emphasizing the urgent need for alternative therapeutic approaches [4]. Scientists are exploring nanotechnology for novel antibacterial compounds, particularly nanomaterials made of metal or metal oxide, while ensuring safety for clinical use [5]. Carbon-based nanomaterials have shown promising antimicrobial activity and biocompatibility. The inappropriate use of antibiotics contributes significantly to host antibiotic resistance, complicating the treatment of common infectious diseases and posing risks in both hospital and community settings [6,7]. Discovering effective alternatives to antibiotics is crucial across various medical contexts, including organ transplantation, surgery, and cancer treatment, to combat multidrug-resistant events.

Carbon quantum dots (CQDs) are a novel type of carbon-based nanomaterial characterized by unique properties such as photostability, environmental friendliness, water dispersibility, ease of synthesis, low cost, and low toxicity, as well as compatibility with biological systems [8]. These qualities make CQDs excellent candidates for various applications including optical chemical analysis [9], fingerprint imaging [10], biosensors [11], fluorescent labelling, and bioimaging [12]. Recent studies have highlighted the antibacterial properties of CQDs. Researchers have demonstrated that super-cationic CQDs exhibit potent antibacterial effects against multidrug-resistant bacteria, particularly in treating eye-related bacterial infections [13].Additionally, various surface-charged carbon dots have been shown to induce programmed bacterial death, with specific analysis of the apoptosis mechanisms in E. coli cells [14]. Furthermore, cationic carbon dots have been developed to selectively target Gram-positive bacteria through electrostatic interactions, with higher positive charges correlating to stronger antibacterial abilities [15]. Notably, CQDs with the highest positive charge have shown significant inhibition of bacterial growth by interacting with negatively charged components of bacterial cell walls. Thus, controlling the surface charge and synthesizing CQDs with adjustable positive charges are critical for enhancing their antibacterial efficacy.

Several researchers throughout the world have conducted antibacterial investigations on medicinal plants such as Betula pendula [16] and Ageratum houstonianum [17]. The World Health Organization states that medicinal plants are one of the excellent sources for the production of antimicrobial drugs [18]. Many studies have demonstrated the significant effect of plant bioactive chemicals against drug-sensitive and resistant microorganisms [19–21]. Secondary metabolites found in medicinal plants are abundant and have been utilized for centuries as an alternative form of treatment [22]. In developing countries, 60–80% of people use medicinal plants to heal ailments [23]. The secondary metabolites found in medicinal plants include alkaloids, flavonoids, tannins, and phenolic acids. These secondary metabolites provide numerous health benefits, including antibacterial, anti-carcinogenic, and anti-mutagenic properties [18].

Azadirachta indica (*A. indica*) (Neem) is a fast-growing tree that belongs to the Meliaceae family. The United Nations has proclaimed neem the "Tree of the 21st Century". More than 300 phytochemicals have been identified to date, and they exhibit a wide range of chemical and structural complexity [24]. The active ingredient in Neem is azadirachtin. Other elements found in Neem include nimbidol, salannin, nimbidin, nimbanene, and ascorbic acid. Neem's active components are responsible for its medicinal qualities, which include anti-oxidant, antibacterial, antifungal, and anti-inflammatory action [25]. According to a study (Herrera Calderon et al., 2019) [26], the crude Neem extraction contains antibacterial components that can be utilized to treat ear and ocular infections. Furthermore, Shaila, Sumaiya, and Laisa (2016) [27] reported that azadirchtin possesses the capability to inhibit DNA topoisomerase II.

Multi drug-resistant bacteria represent a significant public health concern due to limited treatment options and the potential for severe infections. Traditional antibiotics are becoming increasingly ineffective against these pathogens, necessitating the exploration of alternative treatment strategies. Natural products, including plant extracts, have emerged as promising candidates for combating multidrug-resistant bacteria due to their diverse chemical compositions and potential antibacterial properties. *A. indica*, commonly known as Neem, is a plant with a long history of medicinal use in various traditional systems of medicine. Their leaves, in particular, are rich in bioactive compounds that exhibit antimicrobial properties. This study aims to evaluate the synergistic antibacterial activity of Cu-CQDs and *A. indica* leaf extract against MDR bacteria.

Materials and Methods

Collection of plant materials

The plant material utilized in this research comprised leaf of *A. indica*, sourced from the premises of Shree Guru Gobind Singh Tricentenary University in Gurugram, Haryana, India, during July 2023. The collected leaves underwent taxonomic identification and authentication processes.

Preparation of extract by Cold extraction method

The leaves were carefully washed multiple times with distilled water and then air-dried in the shade at room temperature for duration of 15 days. Subsequently, the dried leaves were crushed and utilized for extraction purposes. Approximately 50 grams of this powder were soaked in 200 mL of 95% ethanol and left to macerate for a period of seven days at room temperature, with occasional agitation. After the seven-day maceration period, the extract was filtered either through sterile Whatman No. 1 filter paper or transferred to sterile 500 mL conical flasks. Following filtration, sediments settled at the bottom, and the residual ethanol was removed using a rotary evaporator apparatus at 50°C under reduced pressure. The solvent was completely evaporated, resulting in the formation of a sticky green material, which was then stored in sterile airtight containers in a refrigerator at 4°C for subsequent use [28].

Bacterial cultures and growth conditions

Multidrug-resistant (MDR) clinical isolates including Methicillin-resistant *Staphylococcus aureus* (MRSA) (11 isolates), *Escherichia coli* (*E. coli*) (20 isolates), *Klebsiella oxytoca* (*K. oxytoca*) (10 isolates), *Klebsiella pneumoniae* (*K. pneumoniae*) (6 isolates), and *Pseudomonas aeruginosa* (*P. aeruginosa*) (9 isolates) were obtained from the Department of Medical Microbiology, SGT Medical College Hospital and Research Institute in Gurugram, India, along with their respective antibiotic resistance profiles. Standard strains such as *Staphylococcus aureus* (*S.aureus*) ATCC 25923, *Enterococcus casseliflavus* (*E. casseliflavus*) ATCC 700668, *E. coli* ATCC 25922, and *K. pneumoniae* ATCC 700603 was employed for quality control purposes. All test strains were cultured on Luria agar slants (Hi-Media Laboratories Pvt. Limited) and subcultured onto Luria broth for 24 hours before testing. These bacterial strains were utilized as test pathogens for antibacterial assays.

Antibacterial activity assay

The antibacterial efficacy of the ethanolic Neem leaf extract was assessed using the disc diffusion method. Each bacterial culture, with an inoculum containing 10^8 CFU/mL, was spread onto Mueller-Hinton agar plates using a sterile swab saturated with the bacterial

suspension. The extract was initially tested at a concentration of 100mg/mL, with three replicates conducted for each trial. Sterile discs (6mm diameter, Himedia, India) were impregnated with different volumes (20µL, 30µL, 40µL) of the *A. indica* leaf extract solution (100mg/mL) and placed on the agar plates. Positive controls, consisting of 30µg of Amikacin (AK), and negative controls, involving sterile water, were included for comparison. The plates were then aerobically incubated at 37°C overnight. Following incubation, the diameter of the inhibition zones was measured in millimeters. Data were presented as mean values with standard deviation calculated [29].

Minimum inhibitory concentration (MIC)

MIC of *A. indica* leaf extract was determined using broth microdilution method against the test strains. Initially, all bacterial strains were cultured until reaching the logarithmic growth phase in LB medium, and then diluted in the same medium to achieve a concentration of approximately 10^6 CFU/mL. Subsequently, 50 µL of Muller Hinton broth was dispensed into each well of microtiter plates. *A. indica* leaf extract (50 µL) was added to the first well of rows A to H, followed by two-fold serial dilutions. Bacterial culture (~10^6 CFU/mL) was then added to wells in columns 1 to 11, while column 12 contained only medium as a negative control in each plate. The plates were incubated for 16-18 hours at 37°C with agitation (180 rpm). After incubation, the MIC was determined as the lowest concentration of the test compound that completely inhibited the visible growth of the bacterial strains. [30].

Evaluation of the synergistic interaction between Cu-CQDs and *A. indica* leaf extract against clinical pathogens

After determining the MIC of individual *A. indica* leaf extract and Cu-CQDs, their synergistic effect was assessed using the microdilution checkerboard method. All experiments were conducted in triplicate. *A. indica* leaf extract was tested at MIC levels with prepared Cu-CQDs against selected ATCC strains and MDR clinical isolates. Each well of a microtiter plate received a total of 50 µL of MHB medium. One set of wells received 50 µL of Cu-CQDs, serially diluted along the y-axis, while another set received 50 µL of *A. indica* leaf extract, serially diluted along the x-axis. Subsequently, 100 µL of the clinical isolate, equivalent to a 0.5 McFarland suspension, was added to each well. The MICs of the *A. indica* leaf extract and Cu-CQDs in combination were determined after 24 hours of incubation at 37°C. The fractional inhibitory concentration index (FICI) was then calculated as follows: FICI = (FIC of antibiotic

A) + (FIC of antibiotic B). Based on the calculated FICI, the interaction between the compounds was categorized as follows: FICI ≤ 0.5 indicated a synergistic effect, $0.5 < FICI < 1$ indicated a partial synergistic effect, $FICI = 1$ indicated an additive effect, $2 \leq FICI < 4$ indicated indifference, and $FICI > 4$ indicated antagonism [31].

Characterization

Fourier transform infrared spectroscopy (FTIR) analysis was employed to characterize the *A. indica* leaf extract. The functional moieties from the plant extract responsible to stabilize these particles were identified using JASCO-FT-IR 410 spectrophotometer with a scan range of 4000 cm^{-1} to 600 cm^{-1}.

Results

This study aimed to assess the antibacterial efficacy of an ethanolic extract derived from *A. indica* (Neem) leaf against both ATCC bacterial strains and clinical isolates. Additionally, the potential synergistic antibacterial effect of Cu-CQDs in combination with *A. indica* leaf extract was examined against selected ATCC bacterial strains and clinical isolates. A total of 56 multidrug-resistant (MDR) bacterial clinical isolates were obtained from the Department of Medical Microbiology, SGT Medical College, Hospital and Research Institute in Gurugram. The antibacterial activity of the *A. indica* leaf extract was evaluated using a modified disk diffusion assay, with the diameter of inhibition zones around the charged disks measured for analysis (refer to Tables 1 and 2). Furthermore, the antibacterial activity of the *A. indica* leaf extract was quantitatively assessed against clinical isolates through MIC determination using the broth microdilution method (refer to Table 3). Confirmation of synergistic antibacterial activity of Cu-CQDs with *A. indica* leaf extract against selected ATCC strains and clinical isolates was achieved through the checkerboard assay (refer to Tables 4 and 5).

Table 1. Antibacterial activity of ethanolic *A. indica* leaf extract against standard ATCC strains

Zone of Inhibition Mean±SD(mm)				
Bacterial strains	**Concentration(100mg/mL)**			**Positive control**
	20µL	30µL	40µL	AK Disc 30µg
S. aureus (ATCC 25923)	9.34±0.58	11±1.0	14.67±0.58	17±1.0
E. casseliflavus	10.34±0.58	12.67±1.16	15.34±0.58	18±1.0

(ATCC 700327)				
E. coli (ATCC 25922)	9.67±1.16	12±1.0	15±1.0	16.34±0.58
K. pneumoniae (ATCC 700603)	11.34±0.58	13.34±0.58	16±1.0	17±1.0

Against standard ATCC bacterial strains, *A. indica* leaf extract demonstrated its highest antibacterial effectiveness on *K. pneumoniae* ATCC 700603, displaying a zone of inhibition measuring 16±1.0 mm at a concentration of 40µL;100mg/mL. This was closely followed by notable inhibition against *E. casseliflavus* ATCC 700327, exhibiting a zone of inhibition of 15.34±0.58 mm at the same concentration. Additionally, considerable inhibition was observed against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, with zone diameters of 15±1.0 mm and 14.67±0.58 mm respectively, at the same concentration. At lower concentrations, the antibacterial activity against standard strains *S. aureus* and *E. coli* appeared to be comparable.
A. indica leaf extract demonstrated notably high antibacterial effectiveness against both Gram-positive and Gram-negative isolates. Specifically, it exhibited significant activity against MRSA, with a zone of inhibition measuring 16.46±0.94 mm at a concentration of 40µL;100mg/mL, and against *E. coli*, with a zone of inhibition measuring 16.05±0.89 mm at the same concentration. Moderate antibacterial activity was observed against *K. pneumoniae* (zone of inhibition: 15.17±1.33 mm at 40µL;100mg/mL) and *P. aeruginosa* (zone of inhibition: 15.12±0.93 mm at 40µL;100mg/mL). Among all the test isolates, *K. oxytoca* exhibited the least susceptibility to the extract, with a zone of inhibition measuring 14.1±0.99 mm at 40µL;100mg/mL. Overall, the antibacterial activity of the *A. indica* leaf extract displayed a dose-dependent trend against most of the tested strains, with a statistically significant increase in activity observed with higher doses.

Table 2. Antibacterial activity of *A. indica* extract against clinical isolates

Zone of Inhibition Mean±SD(mm)				
Bacterial strains	**Concentration (100mg/mL)**			**Positive control**
	20µL	**30µL**	**40µL**	**AK Disc 30µg**
MRSA (11)	10.46±1.13	13.19±0.99	16.46±0.94	17.09±0.71
E. coli (20)	10.85±0.94	13.3±1.09	16.05±0.89	17.6±0.95
K. pneumoniae (6)	10.33±1.22	12.84±1.48	15.17±1.33	16.84±0.76
K. oxytoca (10)	9.5±1.27	11.6±1.18	14.1±0.99	16.1±0.88

P. aeruginosa (9)	10.34±1.33	13.34±1.12	15.12±0.93	17.12±1.27

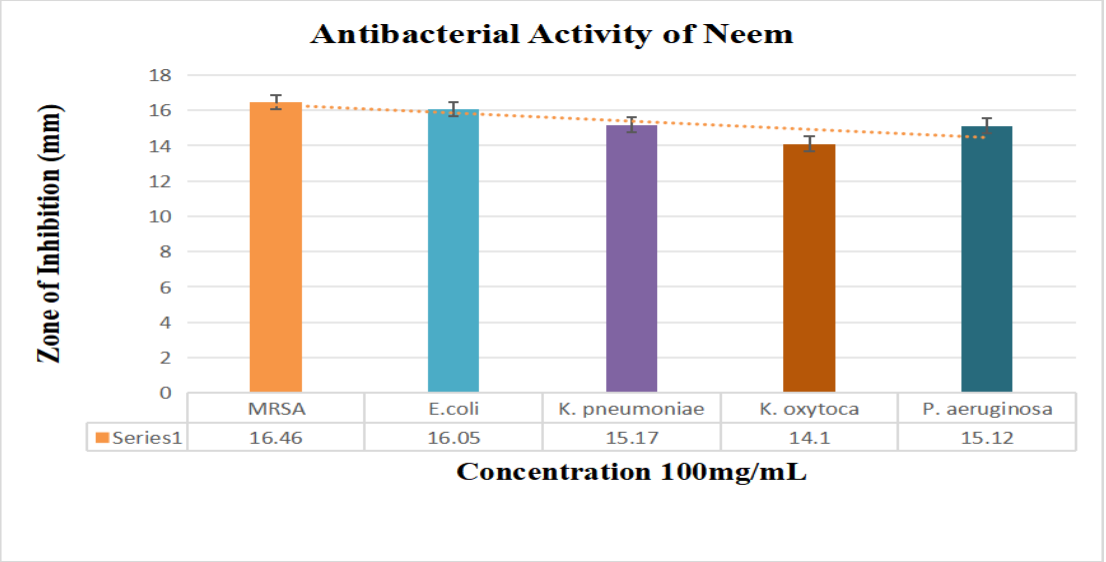

Figure 1. Antibacterial activity of *A. indica* extract against clinical isolates

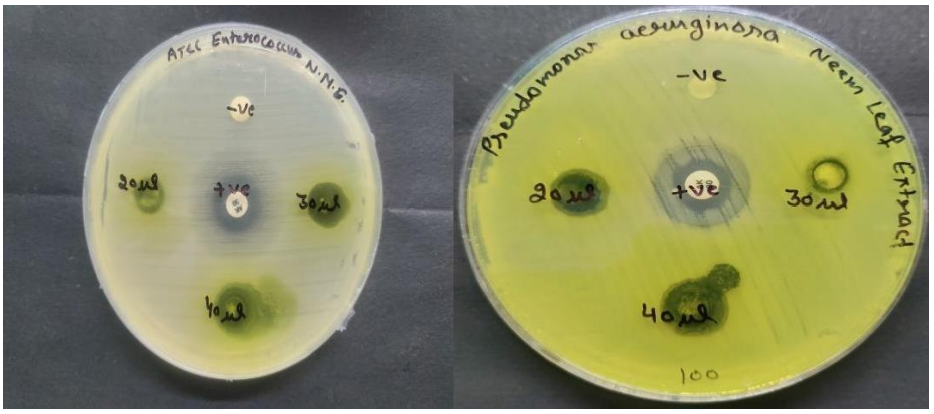

Figure 2. Showing zone of inhibition (ZOI) of tested samples against tested bacterial stains.

In this investigation, the clinical isolates exhibited the lowest MIC values against MRSA, with *K. pneumonia*, *K. oxytoca*, and *E. coli* following in order of decreasing susceptibility. Conversely, the highest MIC value of the extract was recorded against *P. aeruginosa*, as outlined in Table 4.

Table 3. Average minimum inhibitory concentration (MIC) of *A. indica* leaf extract against clinicalisolates.

Minimum inhibitory concentration (MIC)	
Bacterial strains	**Concentration (µg/mL)**
MRSA (11)	442.19
E. coli (20)	896
K. pneumoniae (6)	853.54
K. oxytoca (10)	870.4
P. aeruginosa (9)	910.23

The study showed that the checkerboard method demonstrated a synergistic interplay between Cu-CQDs and *A. indica* leaf extract. This combination displayed a synergistic impact against all ATCC strains, with the exception of ATCC 700603 *K. pneumoniae* as detailed in Table 4.

Table 4. Synergistic outcomes from the combination of Cu-CQDs with *A. indica* leaf extract on ATCC strains.

ATCC strains	**MIC (Cu-CQDs) µg/mL**	**MIC (*A. indica*) µg/mL**	**FIC_a (µg/ml)**	**FIC_b (µg/ml)**	**FICI (µg/ml)**	**EFFECT**
S. aureus ATCC 29213	16	512	0.125	0.25	0.375	Synergy
E.casseliflavus ATCC 700668	16	512	0.00195	0.25	0.25	Synergy
E. coli ATCC 25922	16	1024	0.125	0.125	0.25	Synergy
K.pneumoniae ATCC 700603	16	1024	0.5	0.5	1	Additive

In the checkerboard method, a synergistic interaction was observed between Cu-CQDs and *A. indica* leaf extract. This combination demonstrated a synergistic effect against all clinical isolates listed in Table 5.

Table 5. Synergistic effects of the combination of Cu-CQDs with *A. indica* leaf extract on clinical isolates

Bacterial Isolate	**MIC (Cu-CQDs) µg/mL**	**MIC (*A. indica*) µg/mL**	**FIC_a (µg/ml)**	**FIC_b (µg/ml)**	**FICI (µg/ml)**	**EFFECT**
MRSA	16	512	0.00329	0.25	0.25	Synergy
E. coli	16	512	0.00195	0.0625	0.065	Synergy
K.pneumoniae	16	1024	0.00195	0.125	0.126	Synergy
K. oxytoca	16	1024	0.00195	0.125	0.126	Synergy
P. aeruginosa	16	1024	0.00195	0.125	0.126	Synergy

Fourier transform infrared spectroscopy (FTIR)

The FT-IR spectra (Figure 3) of the *A. indica* leaf extract revealed stretching vibrations of C-H and C-OH at 2920 cm^{-1} and 1242 cm^{-1}, respectively. These peaks indicate the presence of flavonoids and polyphenols [32]. Additionally, a strong stretching band observed around 3330 cm^{-1} suggests the presence of N-H stretching and bending vibrations of the amine group NH_2 and OH, likely due to the overlapping stretching vibrations attributed to water and phenolic groups within the *A. indica* leaf extract molecules. In aromatic ring C=C stretching vibrations was observed at 1607 cm^{-1}. The carbonyl stretching vibration of carboxylic acid and C-H in CH_3 peaks were observed at 1728 and 1372 cm^{-1} [33]. The terpenoids in the Neem extract exhibit C-O-C- linkage at 1159 cm^{-1} [34]. The presence of these linkages confirms the presence of phytochemicals constituents.

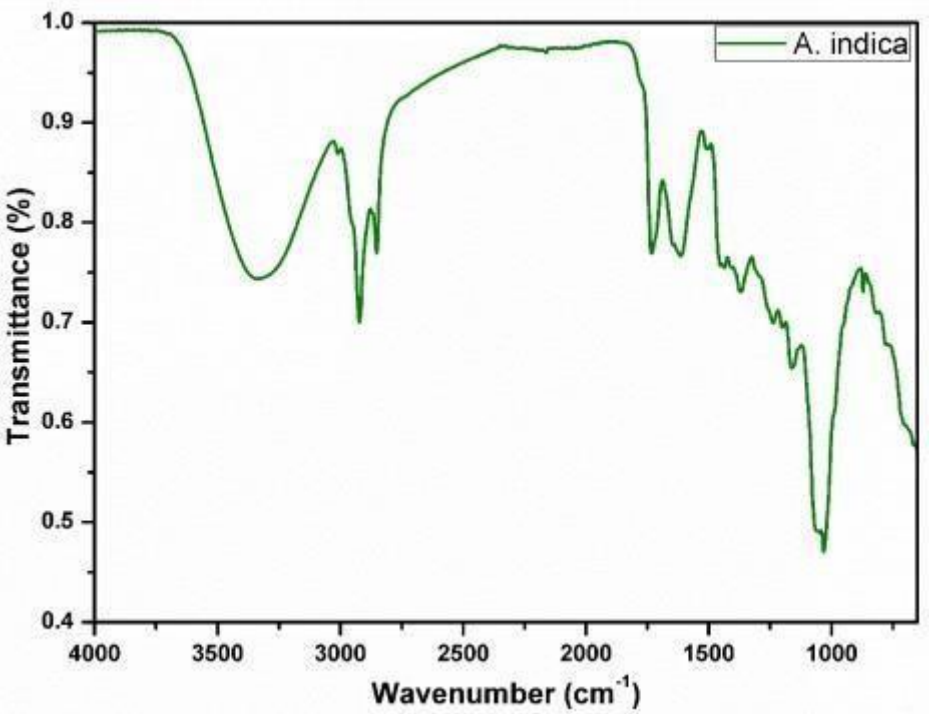

Figure 3. FTIR analysis of *A. indica* extract

Initial findings suggest that the ethanolic leaf extract of *A. indica* demonstrates diverse levels of antibacterial efficacy against MDR bacteria. Results from disc diffusion assays, as well as MIC obtained through broth microdilution method, indicate promising effectiveness of the extract against the targeted pathogens. Nonetheless, the degree of susceptibility appears to differ across various bacterial species and strains.

Discussion

The findings of this study strongly indicate that the ethanolic extract derived from *A. indica* leaf extract demonstrates notably potent antibacterial activity, not only against standard ATCC strains but also against a range of clinical isolates. These results align with several recent studies that have reported similar findings (35,36,37,38,39,40,41).

In a study conducted by Mehta et al., the antibacterial activity of ethanolic Neem leaf extract was evaluated using the disc diffusion method. Additionally, MIC values of the extract were determined via broth dilution against various bacterial strains including *S. aureus*, *Enterococcus* spp., *E. coli, K. pneumoniae, Citrobacter* spp., *Proteus mirabilis, P. aeruginosa*, and *Acinetobacter* spp. Among the tested strains, *E. coli* showed the highest susceptibility to

the extract, followed by *S. aureus*, *Enterococcus* spp., *K. pneumoniae*, *P. mirabilis*, and *P. aeruginosa*. *Acinetobacter* spp. exhibited the least susceptibility among all isolates [35].

Muhammad et al. conducted a study where they highlighted the notable antimicrobial efficacy of ethanolic Neem extract against *E. coli* and *K. pneumoniae*. However, the study revealed that *E. coli* strain exhibited the lowest susceptibility to the ethanolic extract [36].

In Chibuzo's study, Neem leaf extracts underwent phytochemical screening, and their antimicrobial efficacy against *S. aureus* and *E. coli* strains was assessed using the agar well diffusion method. Similar to our investigation, the researchers employed ethanol, methanol, and aqueous extracts of Neem leaves. These extracts displayed significant antimicrobial activity against the tested strains, with the ethanol extract showing the highest efficacy compared to the methanol and aqueous extracts against *S. aureus*. Additionally, Maleki et al., discovered that the methanol extract of Neem leaves exhibited the most potent inhibitory effect compared to ethanol and ethyl acetate extracts against both standard and clinical isolates of *P. aeruginosa* [38].

Ohalete et al., conducted a study to evaluate the antimicrobial effectiveness of ethanol extracts obtained from Neem leaf and bark using the agar well diffusion assay against various pathogenic bacterial strains. The findings revealed that the Neem leaf extract exhibited superior antimicrobial activity compared to the bark extract, particularly against *Salmonella* spp., followed by *Staphylococcus* spp [39].

In Bory et al., research, the antimicrobial efficacy of petroleum ether and methanol extracts derived from Neem leaves was examined against various bacterial strains. It was observed that the petroleum ether extract of Neem leaves displayed the most potent antimicrobial activity compared to the methanol extract, particularly against MRSA, followed by *S. aureus* [40].

In Benisheikh et al., study, extracts of Neem leaves were prepared using Soxhlet extraction with chloroform, methanol, ethyl acetate, and hexane. These extracts demonstrated significant antimicrobial activity against all tested strains. The efficacy of the extracts varied depending on the solvent used, with the chloroform extract exhibiting the highest activity, followed by the methanol extract, against *Streptococcus mutans, E. coli, S. aureus, P. aeruginosa,* and *Candida albicans*. However, weaker activity was observed against *Aspergillus niger, Aspergillus flavus,* and *Streptococcus pyogenes*. [41].

The results of this research underscore the promise of *A. indica* leaf extract as a potential

reservoir of antibacterial substances against bacteria resistant to multiple drugs. The detected antibacterial effects might stem from the existence of active compounds like nimbin, nimbidin, and azadirachtin, known for their antimicrobial qualities. Further investigations are necessary to clarify how *A. indica* extract exerts its antibacterial effects and refine its formulation for potential clinical applications. Moreover, it is essential to conduct in vivo studies to assess its safety and effectiveness in animal models before progressing to human clinical trials.

Conclusion

The ethanolic leaf extract of *A. indica* demonstrates promising antibacterial activity against multidrug-resistant bacteria isolated from clinical specimens. These findings underscore the potential of natural products as alternative therapeutic agents in the fight against antimicrobial resistance. Further research is necessary to explore the clinical implications of *A. indica* extract and its integration into current treatment protocols for multidrug-resistant bacterial infections.

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