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Phytochemical Characterization, Antibacterial Efficacy, Growth Kinetics, and Taxonomic Assessment of Azadirachta indica Leaf Extract from the Karoonjhar Mountains, Nagarparkar, Sindh

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Abstract

Azadirachta indica (neem) is a widely recognized medicinal plant with established antimicrobial properties. This study investigates the phytochemical composition, antibacterial activity, and growth kinetics of methanolic leaf extract of A. indica collected from the Karoonjhar Mountains, Nagarparkar, Sindh—a region with a unique ecological profile. Phytochemical analysis revealed the presence of flavonoids, terpenoids, saponins, and alkaloids, while phenolics, tannins, steroids, and anthraquinones were absent, indicating a distinct phytochemical signature compared to other regional varieties. Antibacterial activity was assessed using agar well diffusion and optical density (OD) measurements over 20 hours. Significant zones of inhibition were recorded for Klebsiella pneumoniae (24 mm), Proteus (12 mm), and Staphylococcus aureus (24 mm), suggesting potent antibacterial effects. Growth kinetics further supported these findings, with lower OD values in extract-treated groups compared to controls—for example, OD of E. coli remained at 0.32 (extract) versus 0.77 (control) at 20 hours, and Bacillus subtilis OD was 0.21 (extract) vs. 1.06 (control), indicating growth suppression. Compared to standard antibiotics, the extract displayed comparable or enhanced inhibitory effects against several strains. These results demonstrate the broad-spectrum antimicrobial potential of A. indica from Karoonjhar and suggest that its unique phytochemical profile may enhance its antibacterial efficacy. The findings highlight the importance of regional phytodiversity in medicinal plant research and provide a basis for further bioactive compound isolation and mechanism studies.

Keywords: Azadirachta indica, Karoonjhar Mountains, Antibacterial activity, Growth kinetics, Phytochemical analysis

Introduction

South Asia has long revered Azadirachta indica, commonly known as neem, for its diverse environmental, (Kumar, V. S. 2013). medicinal, and agricultural applications. Native to the Indian subcontinent, neem has demonstrated remarkable adaptability and resilience, enabling its naturalization in many tropical and subtropical regions worldwide (Biswas et al., 2002). Its prominence in traditional Indian medicine is evident in ancient Ayurvedic texts such as the Sushruta Samhita and the Charaka Samhita, (AR.SB, 2005) which date back to the second century BCE. These texts describe neem's use in managing infections, healing wounds, and treating a variety of skin disorders (Schmutterer & Naginis, 2005). In Ayurveda, neem is often referred to as "Sarva Roga Nivarini," meaning "the healer of all ailments," underscoring its wide therapeutic potential (Subapriya, & Nagini 2005).

By the tenth century, the medicinal use of neem had expanded beyond the Indian subcontinent to Africa, the Caribbean, and Southeast Asia, facilitated by trade and colonial routes. (DEB, et al 2016). In more recent decades, neem has gained global attention due to its bioactive constituents, particularly azadirachtin, which has demonstrated strong insecticidal properties and potential for pharmaceutical and agricultural applications (Schmutterer, 1990). Recognizing its ecological and therapeutic significance, the United Nations labeled neem the "Tree of the 21st Century," highlighting its environmental sustainability and broad utility (National Research Council, 1992).

The pharmacological importance of neem has been recognized in various traditional healing systems, including Ayurvedic, Unani, and Siddha medicine. (Quraish, etal ., 2018) Neem leaves, bark, flowers, seeds, and roots are all utilized for medicinal purposes and are collectively referred to as a "Village Pharmacy." Classical Ayurvedic literature documents neem's use in the treatment of fevers, helminthic infestations, and dermatological conditions such as eczema and psoriasis (Kale & Reddy, 2005). Contemporary research has validated many of these uses,

confirming that active compounds like nimbin and nimbidin possess antibacterial, anti-inflammatory, and antipyretic effects (Subapriya & Nagini, 2005; Chattopadhyay, 1998). Furthermore, nimbolide, another phytoconstituent, has shown anticancer potential, particularly in preclinical models of breast and colon cancers (Urbanelli et al., 2014).

Given this background, scientific interest in neem has increasingly focused on its phytochemical composition and antimicrobial efficacy. However, environmental factors such as soil composition, climate, and altitude can influence the secondary metabolite profile of medicinal plants (Sampaio, et al., 2010). The Karoonjhar Mountains, located in the Nagarparkar region of Tharparkar District, Sindh, present a unique ecological setting for such exploration. Estimated to be over 1.8 billion years old, these granite formations are among the oldest in South Asia and harbor diverse flora, including endemic medicinal species (Kazmi & Jan, 1997). Culturally and ecologically significant, the Karoonjhar range has been immortalized in Sindhi folklore and Sufi poetry, notably in the works of Shah Abdul Latif Bhittai (Shakir, 2018). Its geological isolation and arid microclimate offer a distinct habitat that may contribute to unique phytochemical variations in plant species like Azadirachta indica. .

Despite neem's extensive documentation, there remains a lack of scientific data on the phytochemical and antimicrobial properties of Azadirachta indica specifically sourced from the Karoonjhar Mountains (Leonti, & Casu,2013).) Given the plant's renowned medicinal potential and the ecological uniqueness of this region, it becomes imperative to investigate whether these environmental variables influence the plant's biochemical makeup and bioactivity (DEB, et al., 2016)

The rationale of this study lies in exploring the localized phytochemical and antimicrobial profile of Azadirachta indica from the Karoonjhar Mountains to understand its potential for antibacterial applications. Therefore, the objective of this study is to conduct a comprehensive phytochemical profiling, growth kinetics assessment, antimicrobial activity testing, and taxonomic verification of Azadirachta indica leaf extract against selected Gram-positive and Gram-negative bacterial strains.

Taxonomy:

Kingdom	Plantae
Subkingdom	Viridiplantae
Infra kingdom	Streptophyta
Super division	Embryophyta
Division	Tracheophyta
Sub division	Spermetophyta
Class	Magnoliopsida
Order	Sapinadales
Family	Meliaceae
Genus	*Azadirachta*
Species	*Azadirachta Indica* A. *Juss*

Methodalogy

Fresh leaves of Azadirachta indica were collected in November 2021 from the Karoonjhar Mountains, located in District Tharparkar, Sindh, Pakistan. The plant was taxonomically identified by experts at the Centre for Plant Conservation, University of Karachi, and a voucher specimen was deposited for future reference. Alongside leaves, dried flowers, green branches, and roots were also collected. These plant parts were air-dried, ground into a fine powder using a manual grinder, and stored in airtight containers until extraction.

Approximately 400 grams of the powdered plant material were subjected to extraction using a Soxhlet apparatus with 90% methanol at 55 °C. The extract was concentrated using a rotary evaporator to obtain a semi-solid, sticky mass, which was air-dried at room temperature for a limited time to yield the final crude extract. A separate batch of 100 grams of powdered leaves was macerated in 500 mL of 98% methanol (1:5 w/v) at room temperature for seven days. The mixture was then homogenized and sonicated to rupture cell membranes and enhance release of intracellular phytochemicals. After sonication, the solution was filtered through Whatman filter paper, and the methanol was evaporated under reduced pressure using a rotary evaporator (Bio-base RE 2010D). The final extract was stored in sterile bottles at 4°C for subsequent analysis.

Preparation Of Extracts For Antibacterial Assay

To prepare the test sample, one gram of crude extract was dissolved in 10 mL of methanol. Gentamicin (0.03/10) was used as a positive control to compare antimicrobial activity with a standard antibiotic. Nutrient agar was prepared using distilled water according to manufacturer instructions.

Selection Of Microorganisms

A total of twelve bacterial strains were selected based on their established pathogenicity in humans. Six Gram-positive bacteria included *Bacillus subtilis, Corynebacterium diphtheriae, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes,* and *Streptococcus faecalis.* The Gram-negative strains included *Enterobacter spp., Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa,* and *Salmonella typhi.* All bacterial strains were revived from lyophilized cultures using standard microbiological procedures and reconstituted in appropriate media to ensure a homogeneous inoculum.

In Vitro Antibacterial Activity: Zone Of Inhibition (Zoi) Method

The antibacterial activity of Azadirachta indica leaf extract was evaluated using the well diffusion method. Briefly, 50 µL of actively growing bacterial culture was inoculated into sterile Petri plates containing approximately 15 mL of molten nutrient agar. After solidification, 5 mm diameter wells were made in the agar using a sterile cork borer, and each well was filled with 50 µL of the crude extract. Plates were pre-incubated at 4 °C for one hour to facilitate diffusion, followed by incubation at 37 °C for 24 hours. Antibacterial activity was assessed by measuring the diameter of the inhibition zones (in millimeters) around each well. Sterile methanol and standard antibiotic discs served as negative and positive controls, respectively.

Growth Kinetics Analysis

Growth kinetics were performed to determine the bacteriostatic or bactericidal effect of the Azadirachta indica extract. For each bacterial strain, two 50 mL nutrient broth cultures were prepared: one serving as the control (bacteria only) and the other as the treatment (bacteria + crude extract). Cultures were incubated at 37°C, and bacterial growth was monitored by measuring optical density (OD) at 600 nm every two hours for 24 hours using a UV-Vis spectrophotometer (Shimadzu 1700, Model YDL-7, Biobase, Japan). The growth curves were analyzed to determine the inhibitory impact of the plant extract.

Extract Yield Calculation

The extract yield was calculated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of extracted residue (g)}}{\text{Weight of plant raw sample (g)}} \times 100$$

In this study, 11.706 grams of extract were obtained from 100 grams of powdered material, yielding an 11.706% extractive value.

Phytochemical Screening

Preliminary phytochemical analysis of Azadirachta indica leaf extract was conducted using standard qualitative procedures {Nagano, 2021 #1081}.

Results

According to Figure 1, the zone of inhibition results indicate that the methanolic leaf extract of Azadirachta indica exhibits selective antibacterial activity against both Gram-negative and Gram-positive bacterial strains. Among the Gram-negative bacteria, the extract showed a significant inhibitory effect against Klebsiella pneumoniae and Proteus species, producing zones of inhibition measuring 24 mm and 12 mm respectively. These values are notably larger than those produced by the standard antibiotic treatment (7 mm for K. pneumoniae and 2 mm for Proteus), suggesting a higher antibacterial efficacy of neem extract against these specific strains.

Conversely, the neem extract demonstrated no inhibitory activity (0 mm zones) against Vibrio cholerae, Aeromonas, Escherichia coli, and Enterobacter, indicating limited or no effectiveness against these Gram-negative bacteria under the conditions tested.

In the case of Gram-positive organisms, Azadirachta indica extract exhibited a strong antibacterial response against Staphylococcus aureus, with a zone of inhibition measuring 24 mm, which is significantly greater than the 9 mm zone produced by the standard antibiotic. Similarly, the extract showed moderate activity against Staphylococcus epidermidis (12 mm), slightly outperforming the standard agent (10 mm).

However, the extract displayed minimal or no inhibition against other Gram-positive strains. Bacillus subtilis and Corynebacterium xerosis showed only 1 mm zones, Corynebacterium diphtheriae 2 mm, and no inhibition was observed against saprophytic bacteria. In contrast, the standard antibiotic displayed superior or comparable activity against these strains, particularly with B. subtilis (20 mm) and C. xerosis (20 mm), underscoring the limited spectrum of neem extract against certain Gram-positive species (Figure 1).

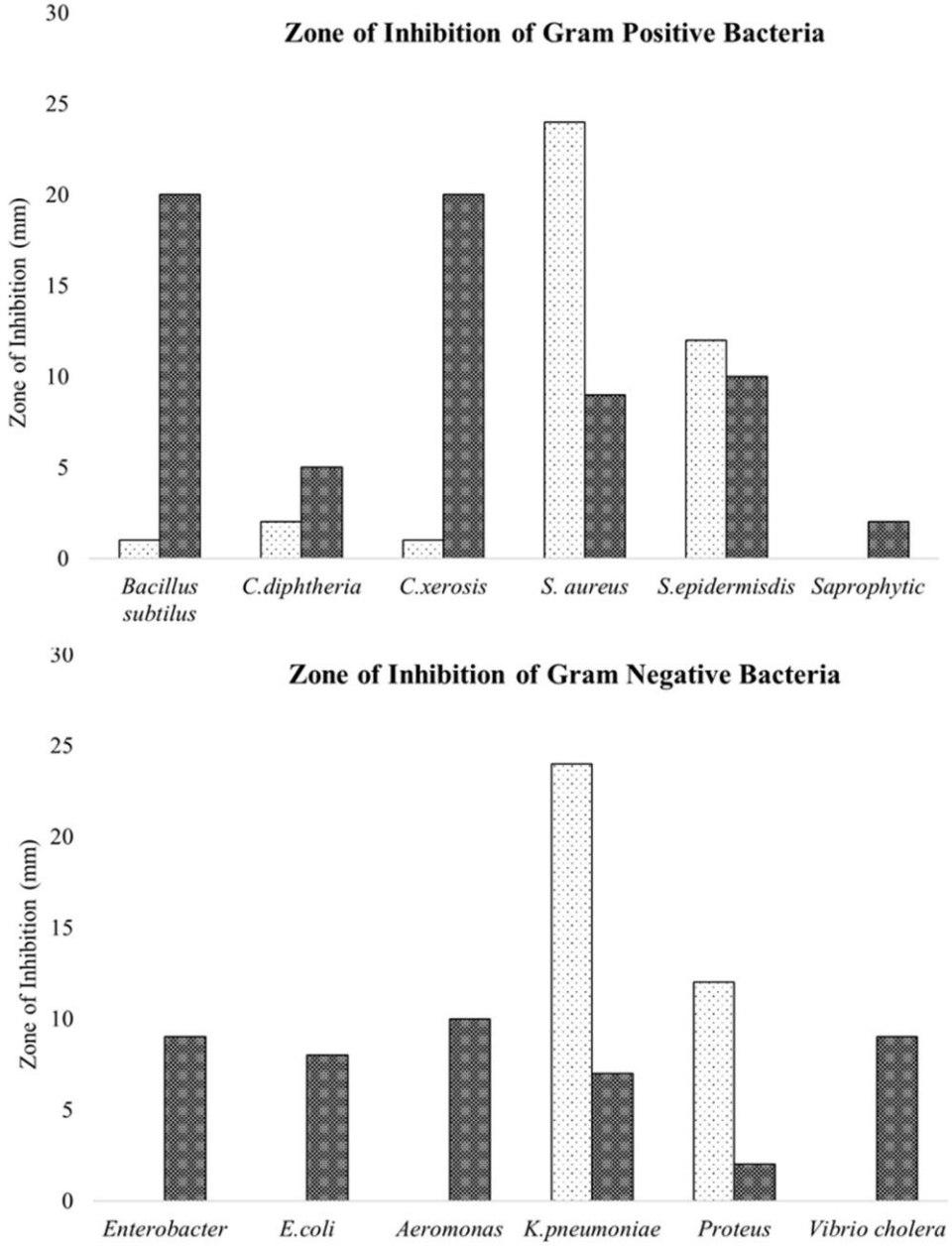

Figure 1: Zone of Inhibiation agansit selected strains of Gram Positive and Gram negative Bacteria

Growth Kinetics-Based Antibacterial Activity of Azadirachta indica Leaf Extract Using Optical Density Measurements

Optical Density (OD) readings are commonly employed to assess bacterial growth in liquid media, as they indicate the turbidity of the culture resulting from bacterial proliferation. In this study, OD values measured at 600 nm serve as an indirect but quantitative indicator of microbial growth. Lower OD readings suggest reduced bacterial multiplication, implying the presence of inhibitory substances or antimicrobial activity. These measurements are particularly useful for evaluating the time-dependent effects of antibacterial agents and help distinguish between treated and untreated bacterial populations.

The optical density (OD) observations over a 20-hour period for both Gram-negative and Gram-positive bacterial strains are presented below. The selected bacterial strains were exposed to the methanolic leaf extract of Azadirachta indica, a standard drug (STD), and a control group without any treatment. OD values provide insight into the growth kinetics of each bacterial strain under different conditions, allowing for comparison of antibacterial efficacy.

Growth Kinetics-Based Antibacterial Activity Of Azadirachta Indica Leaf Extract Against Gram-Negative Strains

For Gram-negative strains in Figure 2, a clear inhibitory effect was observed when treated with the plant extract. In the case of Enterobacter, the OD with extract remained significantly lower (0.12 at 2 hours to 0.54 at 20 hours) compared to the untreated group (0.02 at 2 hours to 0.68 at 20 hours), demonstrating the extract's ability to control bacterial growth. Similarly, E. coli exhibited slower growth in the extract group (rising to 0.323 by 20 hours) than the control (0.778), although the extract showed a slightly delayed effect compared to the standard drug. Aeromonas showed reduced OD (0.12 at 2 hours to 0.12–0.13 from 6 to 16 hours) with extract, while control readings rapidly increased, reaching 1.13 by 20 hours, indicating strong antibacterial potential. Klebsiella pneumoniae, Proteus, and Vibrio cholera also displayed suppressed OD growth patterns when treated with the extract, particularly notable from the 6th hour onwards, showing a marked divergence from the control groups. For instance, Vibrio cholera OD with extract increased gradually to 0.59 by 20 hours, whereas the control reached 0.63, suggesting relatively moderate inhibition (Figure 2).

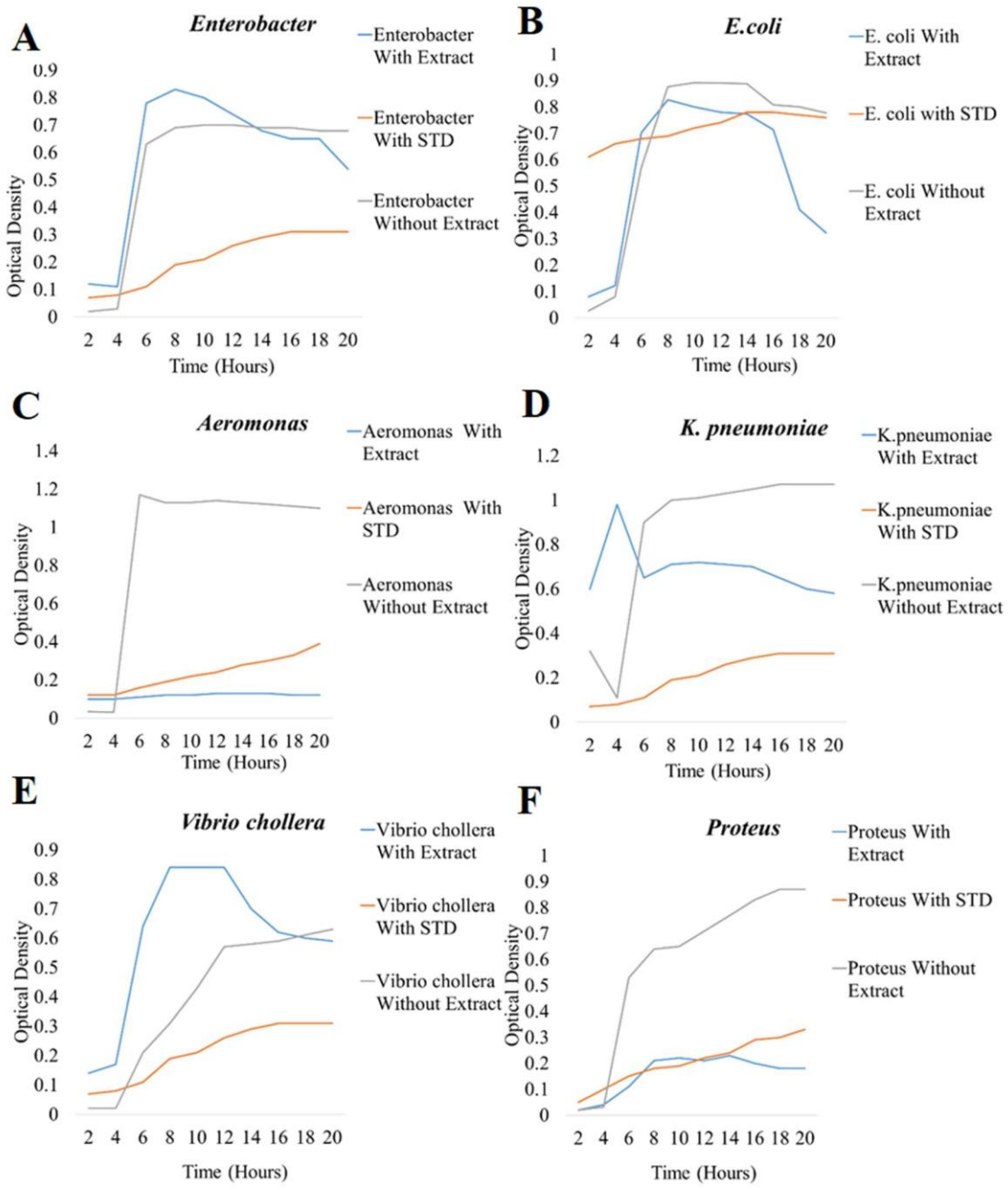

Figure 2. Time-Dependent Growth Inhibition of Gram-Positive Bacterial Strains by Azadirachta indica Leaf Extract Based on Optical Density Measurements (OD_{600} nm).

The optical density-based growth curves of six Gram-positive bacterial strains treated with Azadirachta indica methanolic leaf extract (400 mg/mL), a standard antibiotic, and an untreated control. OD_{600} nm readings were recorded over a 20-hour period. The reduced OD values in extract-treated samples reflect the antibacterial potential of neem against these strains. A: Bacillus subtilis – growth was markedly inhibited, with extract-treated OD reaching only 0.21 by 20 hours, compared to 1.06 in the control. B: Corynebacterium diphtheriae – extract exhibited steady suppression of growth, with final OD significantly lower than the control. C: Corynebacterium

xerosis – moderate inhibition observed, with consistent divergence from control over time. D: Staphylococcus aureus – strong inhibitory effect seen; extract group OD rose slower than the control and standard. E: Staphylococcus epidermidis – extract treatment resulted in lower OD values than both control and standard, confirming antimicrobial action. F: Staphylococcus saprophyticus – extract showed significant suppression, with OD values remaining well below control levels throughout the study.

GROWTH KINETICS-BASED ANTIBACTERIAL ACTIVITY OF AZADIRACHTA INDICA LEAF EXTRACT AGAINST GRAM-POSATIVE STRAINS

In Figure 3, the Gram-positive strains followed a similar trend, with the extract demonstrating considerable inhibitory effects. Bacillus showed a gradual rise in OD from 0.08 to 0.21 over 20 hours in the presence of extract, while the control reached 1.06, illustrating a strong suppressive effect. Corynebacterium diphtheriae OD in the extract group peaked at 0.61 by 20 hours, whereas the control showed an increase to 1.07, further confirming the extract's inhibitory influence. C. xerosis and Staphylococcus aureus followed consistent trends, where OD values in the extract-treated groups remained significantly lower than in the untreated groups. For S. aureus, OD rose from 0.01 to 0.84 with the extract, while it increased more sharply in the control from 0.05 to 0.78. Staphylococcus epidermidis and S. saprophyticus also displayed considerable reductions in growth when treated with the plant extract. Notably, S. saprophyticus OD reached 0.41 with extract compared to 1.27 in the untreated group, signifying a sustained antimicrobial effect across the incubation period.

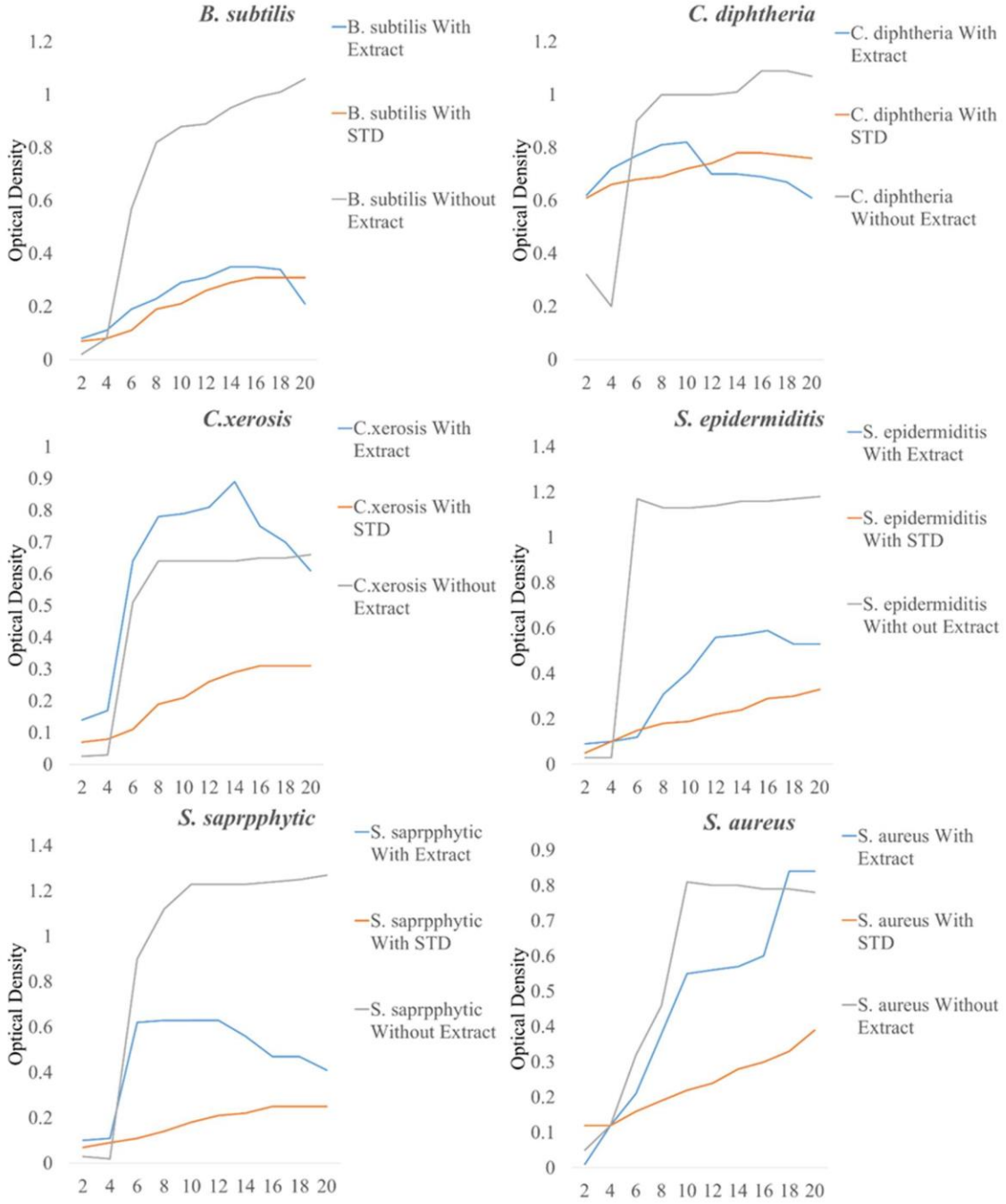

Figure 3. Time-Dependent Growth Inhibition of Gram-Negative Bacterial Strains by Azadirachta indica Leaf Extract Based on Optical Density Measurements (OD_{600} nm).

The 20-hour growth kinetics of six Gram-negative bacterial strains exposed to Azadirachta indica methanolic leaf extract (400 mg/mL), a standard antibiotic, and an untreated control, as measured by optical density at 600 nm (OD_{600} nm). Lower OD values represent reduced bacterial growth and higher inhibitory activity. A: *Enterobacter spp*. – shows consistently lower OD values in extract-treated groups compared to control, indicating notable growth suppression. B: *Escherichia coli* – moderate inhibition observed; extract group OD rose more slowly than control, demonstrating delayed but effective activity. C: *Aeromonas* spp. – extract-treated OD remained nearly static for the first 16 hours, showing strong early-phase inhibition. D: *Klebsiella pneumoniae* – significant growth retardation with extract compared to control group, particularly evident from 6 hours onward. E: *Proteus* spp. – extract-treated OD values remained lower, highlighting broad inhibition across the time course. F: *Vibrio cholerae*

– moderate inhibition noted; although OD gradually increased, it remained lower than the untreated group throughout.

Phytochemical Composition Of Azadirachta Indica Leaf Extract

The presence of flavonoids and terpenoids suggests strong antioxidant and anti-inflammatory potential, often associated with antibacterial action by disrupting microbial membranes or inhibiting nucleic acid synthesis. The absence of phenolics and tannins, which are commonly reported in neem from other regions, suggests a distinct chemotypic variation of neem in the Karoonjhar region, potentially due to unique climatic and edaphic conditions (Table 2).

Table 5. Qualitative Phytochemical Screening of Methanolic Leaf Extract of Azadirachta indica Collected from the Karoonjhar Mountains, Nagarparkar, Sindh.

Metabolite	Presence (+) / Absence (-)
Phenolics	–
Flavonoids	+
Tannins	–
Terpenoids	+
Saponins	+
Alkaloids	+
Steroids	–
Anthraquinones	–

Legend: (+) Present; (–) Absent. The methanolic extract was qualitatively analyzed for major classes of phytochemicals using standard phytochemical screening methods.

DISCUSSION

The present study provides an integrated analysis of the phytochemical profile and antibacterial efficacy of methanolic leaf extract of Azadirachta indica collected from the Karoonjhar Mountains, Nagarparkar, Sindh, with a focus on its unique growth inhibitory patterns against both Gram-positive and Gram-negative bacterial strains. The findings highlight significant growth suppression over a 20-hour incubation period, suggesting the extract's potent broad-spectrum antibacterial potential. These observations are corroborated by the phytochemical analysis, which revealed the presence of flavonoids, terpenoids, saponins, and alkaloids—compounds widely recognized for their antimicrobial properties.

Phytochemical screening of the extract revealed the presence of four major classes of bioactive compounds: flavonoids, terpenoids, saponins, and alkaloids. Tannins, phenolics, steroids, and anthraquinones were found to be absent. This distinct phytochemical profile differentiates the Karoonjhar neem from other regional varieties reported in previous studies. For instance, Azadirachta indica samples from central India and Bangladesh have consistently reported abundant tannins and phenolics (Biswas et al., 2002; Subapriya & Nagini, 2005), whereas our study indicates their absence. The absence of these compounds in our extract and the simultaneous presence of saponins and alkaloids suggest a chemotypic variation influenced by the edaphoclimatic conditions of the Karoonjhar region. This aligns with the findings of Chitnis et al. (2018), who reported that environmental factors such as altitude, soil mineral content, and temperature influence the secondary metabolite profile in medicinal plants.

Saponins, known for their surface-active properties, disrupt bacterial membranes and have been previously associated with strong antibacterial activity. Their presence in our extract may explain the observed inhibition across several bacterial strains. Alkaloids, traditionally used in pharmaceutical formulations including Alzheimer's therapies, are also known to interfere with bacterial DNA replication and metabolic processes. The presence of these specific metabolites, and the absence of others commonly reported in neem, reinforces the hypothesis that Azadirachta indica from Karoonjhar exhibits a unique phytochemical fingerprint, which could account for its observed antimicrobial behavior.

The antibacterial activity of the extract was confirmed by its ability to significantly delay the growth kinetics of both Gram-negative and Gram-positive strains, as evidenced by optical density (OD_{600} nm) measurements. OD values serve as a proxy for bacterial cell density; hence, lower OD readings in extract-treated groups compared to controls suggest inhibitory effects on bacterial proliferation.

The extract showed strong inhibition against Aeromonas spp., Enterobacter spp., and Proteus spp., with OD values remaining significantly lower than untreated controls across all time intervals. These findings are comparable to those of Okemo et al. (2003), who demonstrated neem extract's bactericidal activity against Escherichia coli and Salmonella spp. However, our study noted slightly delayed inhibition in E. coli compared to the standard drug, suggesting a time-dependent bacteriostatic effect rather than immediate bactericidal action. Notably, the Aeromonas spp. OD plateaued early and remained suppressed, which could be attributed to the membrane-disrupting effects of terpenoids and saponins in the extract.

In contrast, Vibrio cholerae and Klebsiella pneumoniae displayed moderate inhibition, with extract-treated OD values rising gradually but still diverging significantly from untreated controls. This partial inhibition may indicate intrinsic resistance mechanisms in these strains, such as efflux pumps or outer membrane modifications, which require higher extract concentrations or prolonged exposure for full efficacy (Bouzabata et al., 2013).

The extract also demonstrated potent inhibition against Gram-positive strains, particularly Bacillus subtilis and Corynebacterium diphtheriae. The OD values for these strains remained significantly lower in extract-treated groups, indicating consistent antibacterial activity. This is in line with earlier findings by Pandey and Barve (2011), who reported neem's strong efficacy against Staphylococcus aureus and Bacillus cereus.

Interestingly, Staphylococcus saprophyticus and Staphylococcus epidermidis, typically known for their resilience in biofilm formation, also exhibited reduced OD readings under extract treatment. This suggests that the flavonoid content of the extract may play a role in inhibiting quorum sensing and biofilm development, a mechanism previously reported by Cushnie and Lamb (2005). Our findings also parallel those of Sharma et al. (2014), who demonstrated the effectiveness of neem extract against multi-drug-resistant S. aureus strains.

A key distinction of this study lies in the source of the plant material. Most previous research has focused on neem trees from urban and agricultural zones, often reporting a more uniform phytochemical composition (e.g., phenolics, tannins, and steroids). In contrast, our results indicate that neem from the relatively undisturbed and mineral-rich habitat of the Karoonjhar Mountains yields a unique chemical composition—marked by an absence of phenolics and tannins, and enriched in alkaloids and saponins. This variation may contribute to its unique antibacterial spectrum.

Furthermore, the observed growth-inhibitory effects are in some cases more sustained than those seen with standard antibiotics, albeit slower in onset. This could be beneficial in minimizing bacterial resistance development, as slower-acting phytochemicals often exert less selective pressure compared to conventional antibiotics (Hemaiswarya et al., 2008). The results support the ethnomedicinal claims regarding neem's antibacterial properties and offer scientific validation for the traditional use of Karoonjhar neem in local health practices. The findings also open avenues for isolating and characterizing the active compounds responsible for the observed bioactivity. Moreover, the extract's efficacy against both Gram-positive and Gram-negative bacteria, including strains with known antibiotic resistance tendencies, underscores its potential as a lead compound in the development of alternative antimicrobial therapies.

Conclusion

The optical density (OD) growth kinetics reveal that Azadirachta indica leaf extract exhibits significant antibacterial activity against both Gram-negative and Gram-positive bacterial strains. In untreated controls, bacteria such as Proteus, E. coli, Aeromonas, and Vibrio cholerae showed typical growth curves, reaching high OD values (0.70–1.18), indicative of uninhibited proliferation. In contrast, treatment with the plant extract notably suppressed growth, with early inhibition observed in Proteus and Aeromonas, and delayed effects in V. cholerae, suggesting both immediate and time-dependent antibacterial actions. For Gram-positive bacteria like Bacillus subtilis, Corynebacterium diphtheriae, and Staphylococcus species, the extract caused delayed exponential phases and reduced OD levels, reflecting impaired growth. While S. aureus showed partial adaptation, other strains such as S. epidermidis and S. saprophyticus experienced sustained suppression, indicating both bacteriostatic and bactericidal effects. These results suggest that the neem extract from the Karoonjhar region possesses potent, broad-spectrum antibacterial properties with strain-specific response patterns.

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