

***In vitro* evaluation of the effect of *Azadirachta indica* (Neem) leaf extract on *Escherichia coli* from gut source**

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Abstract

The human gut microbiota plays a crucial role in maintaining host health, and disruption of its balance can promote the growth of opportunistic bacteria such as *Escherichia coli*. Medicinal plants such as *Azadirachta indica* (Neem) have gained attention as potential sources of alternative antimicrobial agents. This study evaluated the *in vitro* effects of Neem leaf extract on a gut-derived *E. coli* isolate. Phytochemical characterization using UV–Vis spectrophotometry indicated the presence of diverse bioactive compounds. Antibacterial activity was assessed using optical density (OD₆₀₀), colony-forming unit (CFU) enumeration, biofilm formation, growth kinetics, and haemolysis assays. Neem extract exhibited concentration-dependent inhibitory effects, with 250 µg/mL of plant extract, bacterial growth reduced by approximately 23% and viable counts by ~52.8%, while 125 µg/mL reduced CFU (~45.6%). Growth kinetics analysis demonstrated delayed but sustained inhibition after 12 h of exposure. No significant effect on biofilm formation was observed, and haemolytic activity remained unchanged across treatments. The discrepancy between OD₆₀₀ and CFU highlights limitations of turbidity-based measurements when evaluating crude extracts. Overall, Neem extract demonstrated moderate inhibitory effects on bacterial viability, supporting its potential as a source of antimicrobial compounds targeting gut-associated bacteria.

Keywords: *Escherichia coli*, Plant extracts, Time-kill assay, Biofilm formation, Haemolysis

INTRODUCTION

The gut microbiota (GM) represents a complex and dynamic microbial ecosystem inhabiting the human gastrointestinal (GI) tract, with the colon alone harboring approximately 10¹⁴ microorganisms, predominantly bacteria (Dekaboruah *et al.* 2020). This community exists in a symbiotic relationship with the host, contributing to metabolic, immunological, and structural functions essential for maintaining intestinal homeostasis (Ursell *et al.*, 2014). Although earlier estimates suggested a bacterial-to-human cell ratio exceeding 10:1, more recent studies indicate a ratio closer to 1:1 (Sender *et al.*, 2016). The gut microbiome exhibits remarkable diversity, comprising thousands of species, many of which remain uncultivable due to strict anaerobic requirements and complex ecological interdependencies (Dethlefsen *et al.*, 2007). Microbial density and composition vary along the GI tract, with the colon representing the most densely populated

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region, containing up to 10^{12} cells per gram of intestinal content (Sears, 2005). The intestinal microbiota is dominated by anaerobic bacteria, primarily belonging to the phyla Bacillota, Bacteroidota, Pseudomonata, and Actinomycota (Khanna *et al.*, 2014). These microbial populations play critical roles in nutrient metabolism, maintenance of epithelial integrity, immune modulation, and protection against pathogenic invasion (Natividad *et al.*, 2013).

Disruption of a balanced ecosystem, termed dysbiosis, can arise from factors such as antibiotic exposure, dietary changes, and environmental stressors (Hao and Lee, 2004). Dysbiosis has been associated with increased susceptibility to opportunistic pathogens, including *Escherichia coli* and *Clostridium difficile*, as well as the development of metabolic and inflammatory disorders (Yoon *et al.*, 2014; Fukui, 2016). The ability of gut microbiota to resist pathogen colonization is largely mediated through competitive exclusion, nutrient utilization, and the production of antimicrobial compounds.

Medicinal plants have long been utilized in traditional healthcare systems such as Ayurveda, Unani, and Traditional Chinese Medicine for the treatment of gastrointestinal and infectious diseases (Petrovska, 2012). Among these, *Azadirachta indica* (Neem), a member of the Meliaceae family, is widely distributed across the Indian subcontinent, including Bangladesh (Bhadraa *et al.*, 2019). Neem is rich in bioactive phytochemicals, including alkaloids, flavonoids, triterpenoids, and phenolic compounds, which exhibit diverse pharmacological activities such as antimicrobial, anti-inflammatory, hypoglycemic, and hepatoprotective effects (Verkerk & Wright, 1993; Chaturvedi, 2011). Traditionally, Neem leaf extracts have been used for the treatment of skin infections, intestinal parasites, and microbial diseases, as well as for oral health management due to their antibacterial properties (Khine, 2013; Imran *et al.*, 2010). Key active constituents such as nimbin, nimbidin, and azadirachtin are known to contribute to its therapeutic efficacy (Mondall *et al.*, 2009). These compounds exhibit varying degrees of solubility and bioactivity, enabling their interaction with microbial systems (Pankaj *et al.*, 2011).

Recent evidence suggests that the biological activity of phytochemicals may be mediated, at least in part, by their interactions with the gut microbiota, influencing both microbial composition and metabolic function (Shin *et al.*, 2006; Lampe, 2009). However, despite the extensive traditional use of Neem, there remains limited experimental evidence regarding its direct effects on gut-associated bacterial species, particularly under controlled in vitro conditions. Given the increasing interest in plant-derived antimicrobials and the need for alternatives to conventional antibiotics, it is essential to evaluate the antimicrobial potential of

traditional medicinal plants against gut-derived pathogens. Therefore, the present study aims to investigate the in vitro effects of *Azadirachta indica* leaf extract on *Escherichia coli* isolated from a gut source. This study seeks to provide insight into the potential role of Neem-derived phytochemicals in modulating gut-associated bacteria and to contribute to the development of plant-based antimicrobial strategies.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions: The research was conducted in the Pathology Laboratory, Department of Botany, Jahangirnagar University, Savar, Dhaka, Bangladesh, during the academic session 2021-2022. The bacterial strain *Escherichia coli* was obtained as a pure culture from the laboratory, and the isolate originated from a mammalian gut source and had been previously identified and preserved. For routine cultivation, the bacterium was grown in Nutrient Broth (NB), Nutrient Agar (NA), and Tryptic Soy Broth/Agar (TSB/TSA) under aseptic conditions. Stock cultures were maintained in TSB supplemented with 50% (v/v) glycerol at -20°C .

Preparation of Standardized Inoculum: A single colony from an overnight NA culture was inoculated into 10 mL NB and incubated at 37°C for 24 h. The bacterial suspension was adjusted to an optical density of $\text{OD}_{600} = 0.5$ to standardize the inoculum and to ensure equal seeding (10 μl from the prepared inoculum) prior to all experiments.

Preparation of Plant Extracts: Fresh leaves of *Azadirachta indica* were collected from the Botanical Garden of Jahangirnagar University and authenticated using standard taxonomic references. Leaves were washed, air-dried, and ground into a fine powder. Ethanolic extracts were prepared using a Soxhlet apparatus (Heidolph, Germany), followed by solvent removal using a rotary evaporator according to previously described methods (Shuvo et al., 2024). Aqueous extracts were prepared using a similar approach with distilled water. The extracts were subjected to preliminary phytochemical analysis using UV–Vis spectrophotometry (Analytik Jena, Specord) over a wavelength range of 190–790 nm to identify characteristic absorbance peaks.

Bacterial Growth Assay: The effect of *A. indica* extracts on bacterial growth was evaluated using a spectrophotometric assay. Overnight cultures of *E. coli* grown on NA were inoculated into TSB and exposed to plant extracts at concentrations of 125 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$. Bacterial growth was monitored at different time points by measuring optical density at 600 nm (OD_{600}). Untreated cultures and

antibiotic, Metronidazole-treated cultures served as negative and positive controls, respectively. For viable cell enumeration, serial dilutions (10^{-6} – 10^{-8}) were prepared, and 10 μ L aliquots were spread onto NA plates. Colony-forming units (CFU/mL) were calculated using the standard formula:

$$\text{CFU/mL} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume plated (mL)}}$$

Biofilm Formation Assay: Biofilm formation was assessed using a crystal violet staining method as described by Shil & Chichger (2021). Briefly, overnight cultures of *E. coli* were inoculated into fresh TSB with or without plant extracts (1:200 dilution) and incubated at 37 °C for 48 h under static conditions. Following incubation, biofilms formed on glass surfaces (5 cm) were stained with crystal violet, and excess stain was removed. The retained dye was solubilized with 3 ml of 30% acetic acid, and absorbance was measured at 450 nm. Biofilm biomass was normalized against untreated controls.

Haemolysis Assay: Haemolytic activity was evaluated using blood agar plates containing 7% sheep blood. *E. coli* cultures were treated with Neem extract at concentrations of 125 μ g/mL and 250 μ g/mL and incubated at 37 °C with shaking for 24 h. Standardized cultures (OD₆₀₀-adjusted) were streaked onto blood agar plates and incubated at 37 °C for 24 h. Haemolytic activity was assessed qualitatively based on the presence and extent of clear zones surrounding bacterial growth (Shil *et al.*, 2023).

Statistical Analysis: All experiments were conducted in three independent replicates. Data were recorded using Microsoft Excel and analyzed using GraphPad Prism (version 9.5.0). Statistical significance was determined using one-way ANOVA followed by uncorrected Dunn's test, with $p < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical Characterisation of *Azadirachta indica* Leaf Extract: The UV–Vis spectrophotometric profile of the ethanolic leaf extract of *Azadirachta indica* exhibited multiple absorbance peaks across the wavelength range of 190–790 nm, indicating the presence of diverse bioactive phytoconstituents (Fig. 1). These peaks are characteristic of phenolic compounds, terpenoids, and glycosides, suggesting that the extract contains chemically active components that may contribute to its antimicrobial properties.

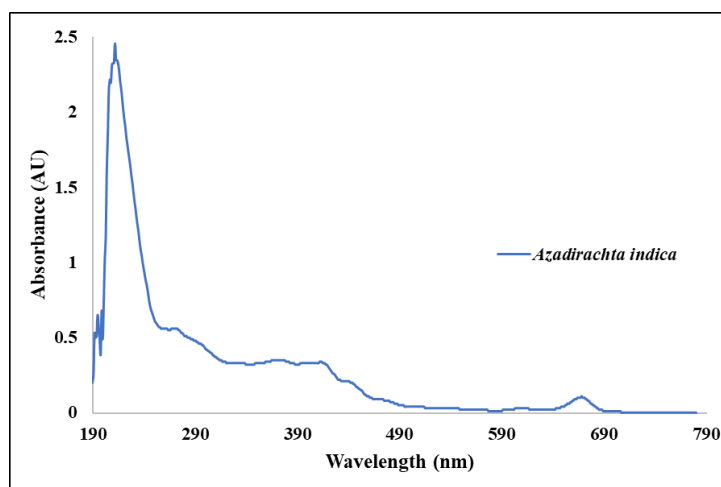


Fig. 1. Ultraviolet visualisation of the Neem leaf extracts in ethanol.

Crude ethanolic extract was prepared from dried grinded leaves using Soxhlet device and concentrated using rotary evaporator. Absorbance was measured using spectrophotometry (Analytik jena, Specord) at a range of 190 to 790 nm wavelengths against ethanol as vehicle.

Effect of Neem Leaf Extract on *Escherichia coli* Growth: The effect of Neem extract on *E. coli* growth was assessed by measuring optical density at 600 nm (OD₆₀₀). Exposure to 250 µg/mL Neem extract resulted in a reduction of bacterial growth by approximately 54% compared to the untreated control, whereas treatment with 125 µg/mL resulted in an approximate 59% decrease in growth. The antibiotic-treated group showed a substantial reduction in growth (~90%), confirming the effectiveness of the assay conditions (Figure 2). Statistical analysis indicated that the reduction in growth at 250 µg/mL and 125 µg/mL was not statistically significant.

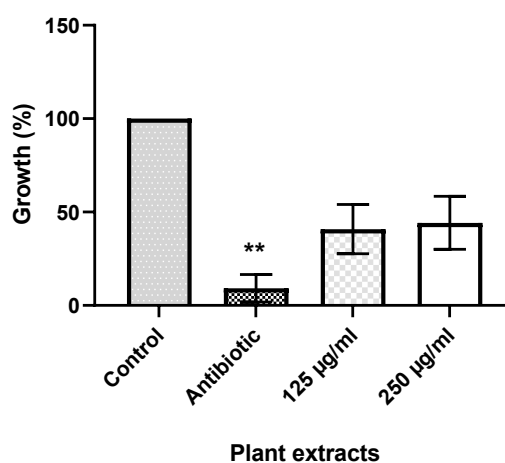


Fig. 2. Effect of Neem leaf extract on *E. coli* growth at different concentrations.

A single bacterial colony was inoculated into 5 ml liquid media and incubated for 24 hours at 37 °C. Growth was measured as absorbance at 600 nm using a spectrophotometer. Data are presented as the mean $\pm$ S.E.M., $n=3$.

Viable Cell Count (CFU/mL) Analysis: To validate the spectrophotometric findings, viable bacterial counts were determined using the standard plating method, and the dilution factor was determined using the standard plating method (Figure 3). Colony enumeration from 10^{-7} diluted plates revealed that the untreated control (C1) exhibited a bacterial load of 1.25×10^{11} CFU/mL, whereas treatment with 125 μ g/mL (S1) and 250 μ g/mL (S2) Neem extract reduced the counts to 6.8×10^{10} CFU/mL and 5.9×10^{10} CFU/mL, respectively (Figure 4, Table 1). These correspond to reductions of approximately 45.6% and 52.8% relative to the control, demonstrating a concentration-dependent inhibitory effect of Neem extract on bacterial viability. Colony morphology remained consistent across all groups, with colonies appearing circular, opaque, flat, and moist with off-white pigmentation; however, colonies from treated groups were noticeably smaller in size. Although CFU values are reported as representative counts, they are interpreted alongside spectrophotometric data.

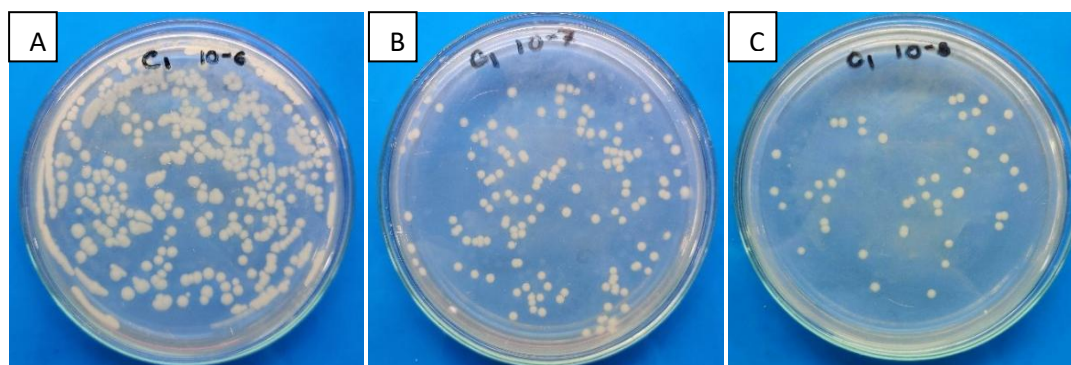


Fig. 3. *E. coli* growth (representative) after overnight incubation.

[A, B, and C indicate 10^{-6} , 10^{-7} , and 10^{-8} diluted plates, respectively.]

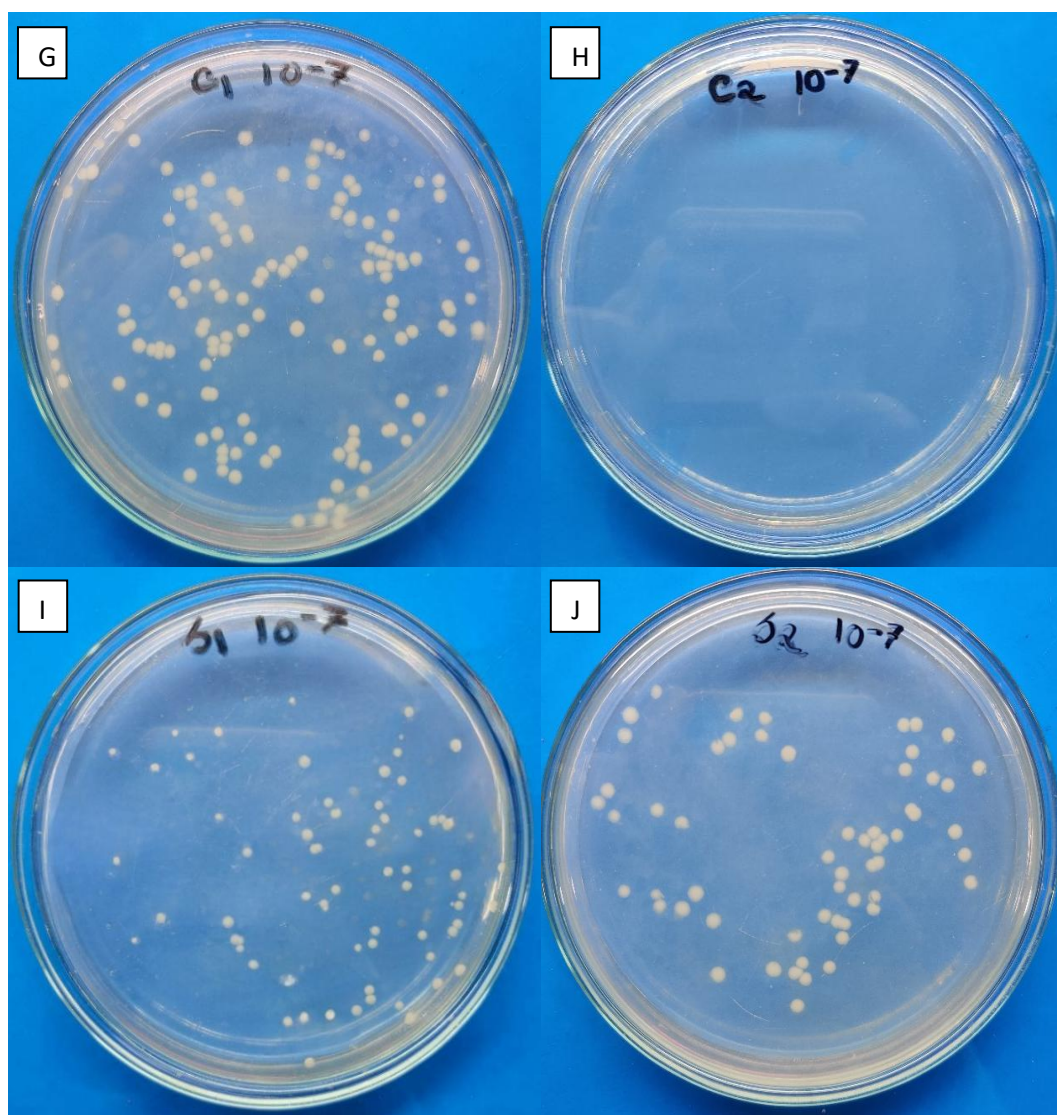


Fig. 4. *E. coli* growth (representative) after being exposed to the antibiotic and the plant extract.

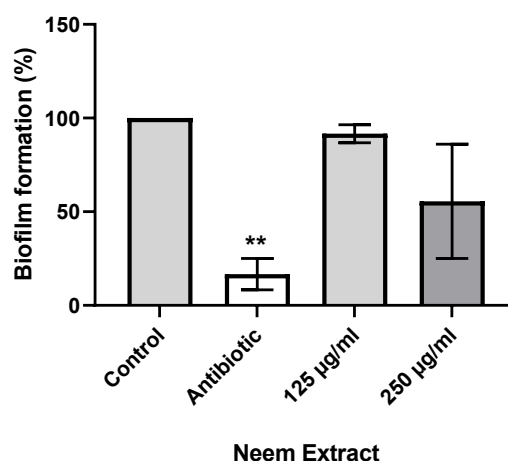
[G, H, I, and J indicate 10^{-7} diluted plates of control (C_1), negative control (C_2), 125 $\mu\text{g/mL}$ plant extract (S_1), and 250 $\mu\text{g/mL}$ plant extract (S_2), respectively.] After overnight incubation of a single colony in a liquid media at 37 °C, the *E. coli* culture was exposed to two concentrations of plant extract: 125 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$. Colonies were counted from 10^{-7} diluted plates.

Table 1. Colony counting for the diluted plates of the growth experiment.

Serial dilution was done up to 10^{-8} . Growth of three independent assays was measured using standard plating method and colonies were counted from 10^{-7} diluted plates.

Plates	No. of colonies (average of 3)	Dilution factor× no. of colonies	CFU/ml
Control (C ₁)	125	$10^7 \times 125$	125×10^9
125 µg/ml plant extract (S ₁)	68	$10^7 \times 68$	68×10^9
250 µg/ml plant extract (S ₂)	59	$10^7 \times 59$	59×10^9

Effect of Neem Leaf Extract on Biofilm Formation: The effect of Neem extract on *E. coli* biofilm formation was evaluated using the crystal violet assay, with absorbance measured at 450 nm and normalized to the untreated control. Treatment with 125 µg/mL Neem extract produced little change (91.7%) in biofilm formation relative to the untreated control, while exposure to 250 µg/mL resulted in a 44.4% reduction in biofilm biomass. Neither concentration of Neem extract caused a statistically significant change in biofilm formation compared with the control ($p > 0.05$). In contrast, treatment with the antibiotic metronidazole significantly reduced biofilm formation ($p < 0.01$) (Figure 5). These findings suggest that, although Neem extract exhibits antibacterial activity against planktonic *E. coli* growth, it does not significantly inhibit biofilm formation under the experimental conditions tested.

**Fig. 5. Effect of crude Neem leaf extract on the biofilm formation of the model bacteria, *E. coli*.**

The organism was exposed to 125 µg/ml and 250 µg/ml of crude Neem extract or antibiotic, or vehicle for 48 hours at 37 °C. Crystal Violet-Acetic Acid biofilm formation assay was performed, and absorbance at 450 nm of the retained Crystal Violet was measured as biofilm. Data were normalized with control and presented as percentage. Data were analysed using GraphPad Prism 9.3.1 and are presented as the mean $\pm$ S.E.M., $n = 3$.

Time-Kill (Growth Kinetics) Analysis: The time-dependent effect of Neem extract on *E. coli* growth was assessed over a 24-hour incubation period by measuring OD₆₀₀. At the 2-hour time point, all groups exhibited negligible growth. By 4 hours, the control group began to show an increase in absorbance, while Neem-treated samples recorded OD₆₀₀ values of approximately 0.12 and 0.18 for 125 µg/mL and 250 µg/mL, respectively. At 12 hours, the control group reached an OD₆₀₀ of 0.21, whereas both Neem-treated groups maintained significantly lower values (~0.05), indicating effective inhibition of bacterial growth. The control culture continued to grow, reaching 0.27 at 18 hours before declining slightly to 0.23 at 24 hours, consistent with entry into the stationary phase. In contrast, Neem-treated cultures maintained low absorbance values beyond 12 hours, comparable to those observed in the antibiotic-treated group, which showed complete inhibition of growth throughout the experiment (Figure 6).

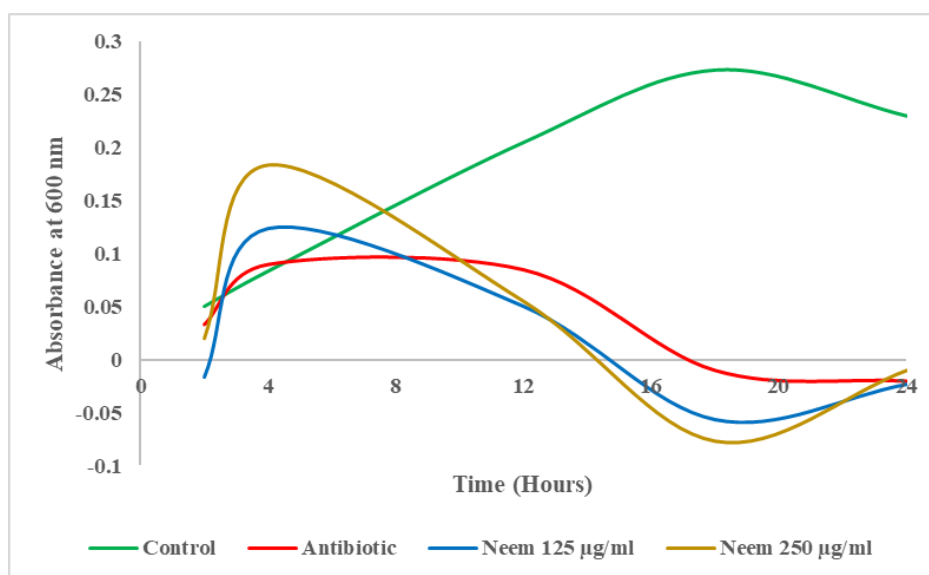


Fig. 6. Effect of Neem leaf extract on the viability of *E. coli*.

Time-kill study was conducted with or without the presence of plant extract, and growth was measured as absorbance at 600 nm using spectrophotometry after 2, 4, 8, 12, 18 and 24 hours of incubation. For each treatment, individual blank (treatment without bacteria) was maintained, and the data presented are derived after deducting the blank from the relevant bacteria culture. Data were analysed using Microsoft Excel and are presented as the mean, $n = 3$.

Effect of Neem Leaf Extract on Haemolytic Activity: The haemolytic activity of *E. coli* following exposure to Neem extract was assessed using blood agar plates (Figure 7). No observable changes in haemolytic pattern were detected in treated groups compared to the control, indicating that Neem extract did not alter the haemolytic properties of the bacterium under the experimental conditions.



Fig. 7. Effect of *Azadirachta indica* leaf extract on haemolytic activity of *Escherichia coli*.

Representative blood agar plates showing haemolytic patterns of *E. coli* following treatment with Neem extract at 125 µg/mL and 250 µg/mL, compared to untreated control. Plates were incubated at 37°C for 24 h. No visible haemolytic zones were observed in any treatment group, indicating the absence of haemolytic activity under the tested conditions.

This study aimed to evaluate the *in vitro* effects of *Azadirachta indica* leaf extract on a gut-derived *Escherichia coli* isolate, focusing on bacterial growth, viability, biofilm formation, and haemolytic activity. The findings demonstrate that Neem extract exhibits concentration-dependent antibacterial activity, although its effects vary across different bacterial physiological responses and assay systems.

Phytochemical profiling of the extract revealed the presence of phenolics, terpenoids, and glycosides, which are widely associated with antimicrobial activity (Karpagasundari and Kulothungan, 2014; Al-Jadidi *et al.*, 2015). These compounds are known to interfere with bacterial cell membranes, enzyme systems, and nucleic acid synthesis, thereby contributing to growth inhibition (Pommier *et al.*, 2010; Elmasri *et al.*, 2017). The observed antibacterial activity at higher concentration (250 µg/mL) is therefore consistent with the established bioactivity of Neem-derived phytochemicals. However, while such spectral fingerprints are useful for qualitative characterization, they do not provide compositional specificity or identify active antimicrobial compounds (Wylie and Merrell, 2022). This limitation is widely acknowledged in phytochemical research, where extract composition varies significantly depending on the solvent system and plant part.

The growth assay revealed that both 250 µg/mL and 125 µg/mL decreased *E. coli* growth, although the reduction was not dose dependent. However, CFU analysis demonstrated a reduction in viable cells at both concentrations. This discrepancy likely arises from optical interference caused by the intrinsic absorbance or particulate nature of the crude extract, a limitation previously reported in spectrophotometric assays (Taylor *et al.*, 2001). More broadly, turbidity-based measurements such as OD₆₀₀ quantify light scattering rather than true absorbance, and therefore include contributions from non-viable cells, debris, and suspended particulates (Mira *et al.*, 2022). The CFU data

therefore provide more reliable evidence of antibacterial activity, confirming that Neem extract exerts a net inhibitory effect on bacterial viability, which is enhanced at higher concentrations. The observed reductions indicate partial inhibition rather than strong bactericidal activity. According to established antimicrobial susceptibility frameworks, such effects require confirmation through standardized minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) testing, which form the basis for clinical interpretation of antibacterial potency (CLSI, 2024; EUCAST, 2025). As these parameters are absent, the current findings should be interpreted as indicative of moderate suppressive activity under the tested conditions.

The antibacterial effect observed in this study aligns with previous reports demonstrating Neem's efficacy against Gram-negative bacteria, including *E. coli* (Abd El-Moez *et al.*, 2014; Rathod *et al.*, 2012). Neem phytochemicals such as flavonoids and triterpenoids have been shown to disrupt membrane integrity, inhibit DNA gyrase, and interfere with quorum-sensing pathways (Ramalhete *et al.*, 2011; Ouyang *et al.*, 2016). When compared with existing literature, the observed antibacterial effects align with reports of Neem extracts exhibiting activity against *E. coli*, although typically at higher (100 and 112.5 mg/mL) concentrations (Ali *et al.*, 2021). For example, variations in antimicrobial efficacy have been attributed to differences in extraction solvent, phytochemical composition, and bacterial strain variability (Yehia, 2016; Koonan & Budida, 2011; Hemdan *et al.*, 2023). The relatively lower concentrations used in the present study may reflect differences in extract potency, bacterial strain susceptibility, or experimental conditions, and should be interpreted cautiously pending further standardization.

The time-dependent growth kinetics further support the antibacterial activity of *A. indica* extract, demonstrating a delayed yet sustained inhibitory effect on *E. coli* growth (Fig. 6). While early time points showed minimal differences between treated and control groups, a pronounced divergence became evident after 12 hours, with Neem-treated cultures maintaining consistently lower OD₆₀₀ values compared to the untreated control. This pattern suggests that the extract does not exert an immediate bactericidal effect but instead produces a time-dependent inhibitory response, potentially reflecting gradual cellular damage or delayed interference with metabolic processes. Such delayed kinetics are characteristic of phytochemical mixtures that act through multi-target mechanisms, including membrane disruption, enzyme inhibition, and oxidative stress induction (Bhone Myint Kyaw *et al.*, 2012; Panda *et al.*, 2011). However, as this analysis is based on optical density measurements, the interpretation should be approached cautiously due to potential extract-related interference, as observed in the planktonic growth assay. Therefore, confirmation using time-resolved CFU-based kill kinetics would be necessary to accurately distinguish between bacteriostatic and bactericidal effects.

Despite its inhibitory effect on planktonic growth, Neem extract did not significantly reduce biofilm formation under the tested conditions. This contrasts with several studies reporting antibiofilm activity of Neem against *E. coli* and other bacterial species (Namasivayam & Roy, 2013; Bhattacharyya *et al.*, 2016). Although a numerical reduction in biofilm biomass was observed at 250 µg/mL, the effect was not statistically

significant because of the considerable variability among replicates. The lack of a significant effect observed here may be attributed to insufficient concentrations of bioactive compounds, strain-specific differences in susceptibility, or methodological variability associated with crystal violet-based biofilm quantification (Hancock *et al.*, 2011; Reisner *et al.*, 2006). These findings highlight the complexity of biofilm biology and emphasize the need for complementary approaches, such as microscopic visualization or biomass-independent methods, to validate biofilm inhibition.

Time-dependent growth analysis further supported the antibacterial potential of Neem extract, demonstrating delayed but sustained inhibition of bacterial growth, particularly after 12 hours of exposure. This delayed effect suggests that phytochemicals may require time to penetrate bacterial cells or accumulate to inhibitory concentrations, consistent with multi-target mechanisms of plant-derived compounds (Bhone Myint Kyaw *et al.*, 2012). Similar time-dependent bactericidal effects have been reported for other plant extracts, where maximum killing occurs after prolonged exposure (Panda *et al.*, 2011). However, as this assay relied on optical density measurements, the interpretation should again consider potential interference and would benefit from validation using time-resolved CFU measurements.

The haemolysis assay showed that Neem extract did not alter the haemolytic phenotype of the tested *E. coli* strain. Non-pathogenic gut-derived strain likely lacking or not expressing key haemolytic determinants such as *hly* and *clyA* (Mirsepasi-Lauridsen *et al.*, 2016). This finding is consistent with previous reports indicating that Neem extracts can exhibit antibacterial activity without inducing haemolysis, suggesting a degree of biological safety (Blum *et al.*, 2019). However, further studies using pathogenic strains and quantitative haemolysis assays are necessary to confirm this observation.

Overall, this study demonstrates that *A. indica* extract exerts moderate, concentration-dependent antibacterial effects against a gut-derived *E. coli* isolate, primarily affecting bacterial viability rather than biofilm formation or haemolytic activity. Although both tested concentrations reduced bacterial growth, a greater inhibitory effect was observed only at 250 µg/mL in the OD₆₀₀ assay, while viable count analysis further confirmed the antibacterial activity of the extract. The observed discrepancy between spectrophotometric and viable count data highlights an important methodological consideration when evaluating crude plant extracts and underscores the need for multi-assay validation. The reduction in viable bacterial counts observed in the plate count assay further supports the antibacterial potential of Neem extract and suggests that it may serve as a source of bioactive antimicrobial compounds.

Although this study is limited to a single bacterial strain, crude extract, and partial reliance on optical measurements, it still presents important evidence supporting the antibacterial activity of *A. indica* against gut-associated bacteria. The findings strengthen the growing evidence supporting plant-based antimicrobials and underscore the value of integrating traditional medicinal practices with modern microbiological research. Further investigations should concentrate on identifying and characterizing specific bioactive

compounds, evaluating their efficacy against a broader range of gut-associated and clinically important bacterial strains, and employing standardized antimicrobial susceptibility assays to better understand the therapeutic potential of Neem-derived compounds.

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