GMR
The Original

FUNGICIDAL EFFICACY AND VALIDATION OF NEEM (AZADIRACHTA INDICA) AND LEMON (CITRUS LIMON) BASED HERBAL SURFACE CLEANER AGAINST ASPERGILLUS NIGER: A NEUTRALIZATION AND LOG REDUCTION KINETICS STUDY

R K Vijayraj[1], Pavan R[2]

[1,2]Department of Microbiology, Sri Siddhartha Medical College and Hospital, Sri Siddhartha Academy of Higher Education, Agalakote, B H Road, Tumkur-572107, India. Email: vijayraj5071@gmail.com

ABSTRACT

With increasing concerns about antimicrobial resistance and the environmental impact of synthetic disinfectants, there is growing interest in plant-derived alternatives for surface sanitation. In this study, the fungicidal effectiveness of a newly developed herbal surface cleaner containing neem (*Azadirachta indica*) and lemon (*Citrus limon*) extracts was investigated against *Aspergillus niger* ATCC 16404. The formulation was evaluated using neutralization validation, appropriate positive and negative controls, and quantitative suspension testing. Concentrations ranging from 3% to 7% were assessed at contact times of 10, 20, 30, and 40 minutes, with all tests performed in triplicate. The neutralization study confirmed that the neutralizer effectively quenched the antimicrobial activity of the formulation without affecting fungal recovery, yielding inoculum counts between 1.40×10^7 and 1.52×10^7 CFU/mL. Positive control results demonstrated stable fungal populations, with initial counts ranging from 1.48×10^7 to 1.55×10^7 CFU/mL ($\log_{10}$ 7.17–7.19), confirming the reliability of the test system. The herbal cleaner exhibited strong antifungal activity across all tested concentrations. Complete elimination of *A. niger* was observed with the 3% and 7% formulations at every contact time examined, corresponding to a log reduction of 7.18. Although the 4% and 6% formulations showed slightly lower activity after 10 minutes of exposure (mean log reduction 6.88), complete fungal inactivation was achieved within 20 minutes. Statistical analysis using one-way ANOVA demonstrated significant differences among the tested concentrations and exposure times ($p < 0.05$), highlighting the influence of both factors on fungicidal performance. Overall, the results indicate that the neem–lemon herbal surface cleaner possesses potent antifungal properties and can serve as an effective, environmentally friendly alternative to conventional chemical disinfectants for routine surface hygiene applications.

KEYWORDS: Herbal surface cleaner, *Azadirachta indica*, *Citrus limon*, *Aspergillus niger*, fungicidal activity, log reduction kinetics, natural disinfectants, sustainable sanitation.

1. INTRODUCTION

1.1 Background and Rationale

The increasing incidence of healthcare associated infections (HAIs) and foodborne diseases continues to pose a major public health challenge worldwide according to [09]. These infections contribute substantially to patient morbidity, mortality, prolonged hospital stays, and rising healthcare costs. As a result, maintaining hygienic surfaces through effective disinfection remains a critical component of infection prevention programs. Although conventional chemical disinfectants are widely used and highly effective, concerns have been raised regarding their potential toxicity, environmental impact, and contribution to the emergence of antimicrobial resistance. At the same time, growing consumer awareness of sustainability and environmental safety has led to increased interest in natural and eco friendly cleaning products.

Among the many medicinal plants investigated for antimicrobial activity, neem (*Azadirachta indica*) and lemon (*Citrus limon*) have received considerable attention. Neem has long been used in traditional medicine and is known for its diverse biological activities, including antibacterial, antifungal, antiviral, and anti-inflammatory effects. These properties have been attributed to bioactive compounds such as azadirachtin, nimbidin, and quercetin, which are capable of disrupting microbial growth through multiple mechanisms. Similarly, lemon contains a range of antimicrobial constituents, including citric acid, flavonoids, and essential oils rich in limonene. These compounds can damage microbial cell structures and inhibit metabolic processes. Combining neem and lemon extracts in a single formulation may therefore provide enhanced antimicrobial activity through complementary or synergistic effects.

1.2 *Aspergillus niger*: A Fungal Pathogen of Concern

Aspergillus niger is a common environmental fungus that can be found in soil, air, water, food products, and indoor surfaces. Although it is generally regarded as an opportunistic pathogen, it can cause serious infections in immunocompromised individuals, including pulmonary aspergillosis and otomycosis. In addition to its clinical significance, *A. niger* is associated with food spoilage and mycotoxin production, creating concerns for both public health and food safety.

One of the reasons this organism is frequently used in disinfectant efficacy studies is its remarkable resistance to environmental stress. The fungal cell wall contains structural components such as chitin, glucans, and melanin, which provide protection against adverse conditions and antimicrobial agents. Furthermore, *A. niger* is capable of forming biofilms on a variety of surfaces, allowing it to survive and persist in healthcare settings, food processing environments, and domestic spaces. These characteristics make it a challenging test organism and an appropriate model for evaluating the performance of novel antifungal surface cleaners.

1.3 Standardized Antimicrobial Testing Protocols

The effectiveness of disinfectants must be demonstrated through standardized testing procedures that ensure accuracy, reliability, and reproducibility. Quantitative suspension testing is one of the most widely accepted methods for assessing antimicrobial activity and forms the basis of several international standards, United states pharmacopeia and related validation protocols. [7,8]

In this method, a standardized microbial suspension is exposed to the test product for predetermined contact times. Following exposure, any remaining antimicrobial activity is neutralized before viable microorganisms are recovered and enumerated. The neutralization step is particularly important because it prevents residual disinfectant from continuing to act during microbial recovery, which could lead to misleading results. Neutralizing systems commonly include substances such as polysorbate 80, lecithin, L-histidine, and glycine, either individually or in combination.

Antimicrobial performance is generally expressed as a log reduction value, which represents the decrease in viable microbial numbers after treatment. Higher log reductions (99.99% kill) indicate greater antimicrobial efficacy and provide a quantitative basis for comparing different formulations and conditions.

1.4 Previous Studies on the Antifungal Activity of Neem and Lemon

A growing body of literature supports the antifungal potential of both neem and lemon extracts. Several researchers have reported that neem exhibits strong inhibitory activity against a variety of fungal species, including *Aspergillus niger*. For example, researchers observed that neem leaf extract produced superior antifungal effects compared with other plant extracts tested against *A. niger* [2]. Similarly, some demonstrated complete inhibition of several fungal pathogens by *A. indica*, while *C. limon* showed excellent activity against *Fusarium* species [3].

More recently, interest has expanded from individual extracts to the development of plant-based antimicrobial formulations. It was reported promising antifungal activity of medicinal plant extracts against fungi isolated from daycare center surfaces [1]. Likewise, researchers demonstrated the effectiveness of a herbal denture cleanser containing plant extracts against *Candida albicans* [5], while a study showed that an herbal disinfectant spray could significantly reduce microbial contamination on smartphone surfaces [6].

Despite these encouraging findings, relatively few studies have examined the combined use of neem and lemon extracts in surface-cleaning formulations, particularly against *A. niger*. In addition, limited information is available regarding how concentration and contact time influence fungicidal performance. Addressing these gaps is important for establishing scientifically validated recommendations for practical applications.

1.5 Study Objectives

The present study was undertaken to assess the fungicidal efficacy of a neem–lemon herbal surface cleaner against *Aspergillus niger* ATCC 16404. Specifically, the objectives were:

1. To validate the effectiveness of the neutralizer system used during antimicrobial testing.
2. To evaluate the fungicidal activity of the herbal surface cleaner at concentrations of 3%, 4%, 5%, 6%, and 7%.
3. To investigate the influence of contact time (10, 20, 30, and 40 minutes) on fungal reduction.
4. To determine whether significant differences exist between test concentrations and exposure periods using statistical analysis.
5. To identify the most effective formulation parameters and provide data supporting the use of neem–lemon-based surface cleaners as sustainable alternatives to conventional disinfectants.

2. MATERIALS AND METHODS

2.1 Test Organism and Culture Conditions

Aspergillus niger ATCC 16404 was used as the test organism in this study. The culture was maintained on Sabouraud Dextrose Agar with chloramphenicol(SDAC) slants and stored at 4°C until required. Prior to each experimental run, fresh fungal cultures were prepared by inoculating SDAC plates and incubating them at 25 ± 1°C for 5–7 days to ensure adequate sporulation. To harvest the spores, a sterile saline solution containing Tween 80 is poured onto the surface of the culture, and the spores are gently dislodged using a sterile loop or bent glass rod, taking care not to scrape the underlying

agar and collect hyphal fragments. The resulting cloudy suspension is then aspirated and filtered through a plug of sterile cotton wool or several layers of gauze, a crucial purification step that removes mycelial debris and yields a suspension of primarily free spores. This filtrate is centrifuged at a moderate speed to pellet the spores, which are then washed and resuspended in fresh saline-Tween solution. The concentration of the spore suspension is determined either by a quick estimate using a hemocytometer to count all spores or, more accurately, by a viable count via serial dilution and spread plating, which provides the concentration in CFU/mL after incubation. The suspension is subsequently diluted to the desired challenge concentration, typically of 1.5 x 10^7 CFU/mL where the cell concentration is less than 100CFU. Finally, the standardized suspension is aliquoted into sterile vials and stored under refrigeration; however, fungal spores are generally less stable and the viable count should be confirmed on the day of use to ensure accuracy.

2.2 Preparation of the Herbal Surface Cleaner

The herbal surface cleaner evaluated in this investigation was formulated using aqueous extracts of neem (*Azadirachta indica*) leaves and lemon (*Citrus limon*) peel. To ensure consistency between batches, the extracts were standardized before formulation. Working solutions containing 3%, 4%, 5%, 6%, and 7% (v/v) of the herbal cleaner were prepared by diluting the stock formulation with sterile distilled water. Fresh dilutions were prepared on each day of testing to maintain product stability and effectiveness.

2.3 Neutralizer Validation Study

Before evaluating fungicidal activity, a neutralization study was conducted to confirm that the selected neutralizer could effectively stop the action of the test formulation without affecting fungal viability. The study was performed in accordance with the principles outlined in Pharmacopeia [7,8].

The neutralizing solution consisted of polysorbate 80 (30 g/L), lecithin (9 g/L), L-histidine (1 g/L), and glycine (10 g/L) prepared in phosphate-buffered saline (PBS). Four experimental conditions were included:

- **Neutralization test:** disinfectant + neutralizer + fungal inoculum
- **Neutralizer toxicity control:** water + neutralizer + fungal inoculum
- **Positive control:** water + PBS + fungal inoculum
- **Negative control:** water + neutralizer without fungal inoculum

All tests were carried out in triplicate. The neutralizer was considered effective when the recovery of viable fungal cells was at least 50% of that obtained in the positive control, indicating that the disinfectant activity had been successfully neutralized.

2.4 Tube Dilution Assay

Inoculate 0.1mL containing 10^7 CFU of test organism (Vegetative microorganism) into the 10 mL of specified concentration of disinfectants. After completion of contact time, 1ml of disinfectant with challenged microorganism was transferred into 100ml of D/E Broth & allowed for some minutes for neutralization. In the case of spore-forming organisms inoculate 0.1mL containing 10^7 cfu into the 10 mL of the specified concentration of disinfectants. After the specified contact time, aseptically transfer 1 mL of the neutralized sample into a sterile empty petri plate. Pour approximately 15-20 mL of molten Sabouraud Dextrose Agar with Chloramphenicol Media (SDAC) supplemented with a neutralizer (0.5% tween 20), cooled to approximately 45°C, into the petri plate containing the sample. Gently swirl the plate in a motion to ensure even distribution of the sample and to mix it thoroughly with the agar. Allow the agar to solidify. For spore-forming organisms, follow the same procedure: after neutralization, transfer 1 mL of the neutralized sample into a sterile empty petri plate and pour with the appropriate agar as described above. Incubate all the plates at 20-25°C for NLT 3-5 days.

Positive Control:

Inoculate 0.1mL containing 10^7 cfu of test organism (Vegetative microorganism) into the 10 mL of 0.9% sterile normal saline. In case of spore forming organisms, inoculate 0.1mL containing 10^7 cfu into the 10 mL of 0.9% sterile normal saline. Perform serial dilution with 0.9% sterile Saline up to 10^{-7}. From each dilution (e.g., 10^{-5}, 10^{-6}, 10^{-7}), aseptically transfer 1 mL into a sterile empty petri plate in duplicate. Pour approximately 15-20 mL of molten Sabouraud Dextrose Agar with Chloramphenicol Media (SDAC) supplemented with a neutralizer (0.5% tween 20), cooled to approximately 45°C, into the petri plate. Gently swirl each plate in a "figure-8" motion to ensure even distribution. Allow the agar to solidify. Incubate all the plates at 20-25°C for NLT 3-5 days.

Negative Control:

Aseptically transfer 1 mL of the neutralized disinfectant (e.g., 1 mL of disinfectant added into 100ml of D/E Broth) into a sterile empty petri plate. Pour approximately 15-20 mL of molten Sabouraud Dextrose Agar with Chloramphenicol Media (SDAC) supplemented with a neutralizer (0.5% tween 20), cooled to approximately 45°C, into the petri plate. Gently swirl each plate in a "figure-8" motion to ensure even distribution. Allow the agar to solidify. Incubate all the plates at 20-25°C for NLT 3-5 days.

2.5 Data Analysis and Statistics

Fungicidal efficacy was expressed as log reduction in viable fungal counts. The log reduction value was calculated using the equation:

Log Reduction = $\log_{10}$ (Positive Control Count) − $\log_{10}$ (Surviving Count)

Statistical analysis was performed using SPSS software. Differences among concentrations and contact times were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. Statistical significance was accepted at a confidence level of 95% ($p < 0.05$).

Graphs and figures were generated using GraphPad Prism to facilitate visualization of fungicidal trends and treatment effects.

3. RESULTS

3.1 Neutralization Study

The neutralization study confirmed that the selected neutralizing system effectively inactivated the residual antimicrobial activity of the neem–lemon herbal surface cleaner. Recovery counts obtained from the neutralization test were comparable to those observed in the positive control, demonstrating that the neutralizer neither inhibited fungal growth nor interfered with the recovery of viable spores.

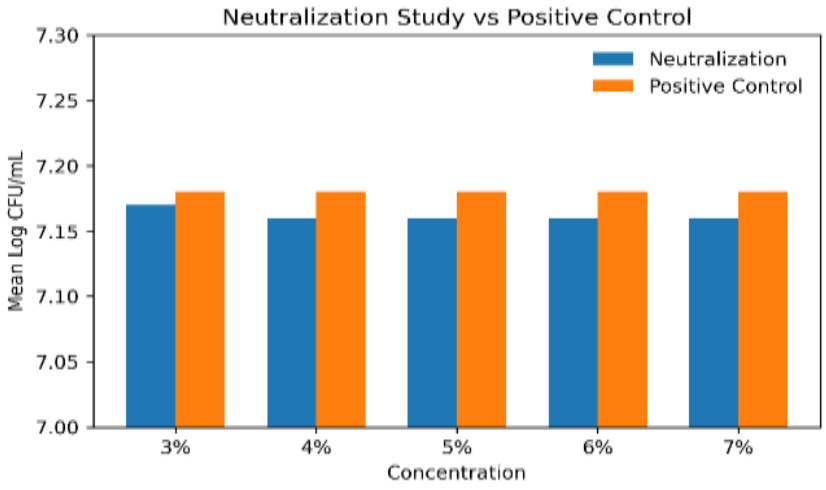
Across all tested concentrations, recovered fungal counts ranged from 1.40×10^7 to 1.52×10^7 CFU/mL, which exceeded the minimum acceptance criterion of 50% recovery relative to the positive control. The neutralizer toxicity control also showed satisfactory recovery, indicating that the neutralizing agents themselves were non-toxic to *A. niger* under the experimental conditions.

These findings validated the suitability of the neutralizer system for subsequent fungicidal efficacy testing and confirmed that any reduction in fungal counts observed during the study could be attributed to the activity of the herbal surface cleaner rather than residual disinfectant effects. Results of the neutralization study are presented in **Table 1**.

Table 1: Neutralization Study and Positive Control Results for A. niger

Parameter	Range (cfu/mL)	Log Range
Neutralization Study	1.40×10^7 - 1.52×10^7	7.1461 - 7.1818
Positive Control	1.48×10^7 - 1.55×10^7	7.1703 - 7.1903

Figure 1: Comparison of Neutralization Study and Positive Control Results



Neutralization Study Summary:

- **Neutralization validity:** Valid growth observed across all concentrations and trials
- **Positive control range:** 1.48-1.55 × 10^7 cfu/mL (Log 7.17-7.19)
- **Negative control:** No growth observed

- **Test control growth:** Recovered in neutralization study

3.2 Tube Dilution Test Results
The tube dilution test results are presented in Tables 2-6.

Table 2: Tube Dilution Test Results - Neem & Lemon 3%

Contact Time	Trial 1	Trial 2	Trial 3	Mean ± SD
10 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
20 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
30 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
40 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101

Table 3: Tube Dilution Test Results - Neem & Lemon 4%

Contact Time	Trial 1	Trial 2	Trial 3	Mean ± SD
10 min	6.8750*	6.8693*	6.8893*	6.8779 ± 0.0101
20 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
30 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
40 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101

*Indicates incomplete log reduction

Table 4: Tube Dilution Test Results - Neem & Lemon 5%

Contact Time	Trial 1	Trial 2	Trial 3	Mean ± SD
10 min	7.1760	7.1703	6.8893*	7.0785 ± 0.1664
20 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
30 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
40 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101

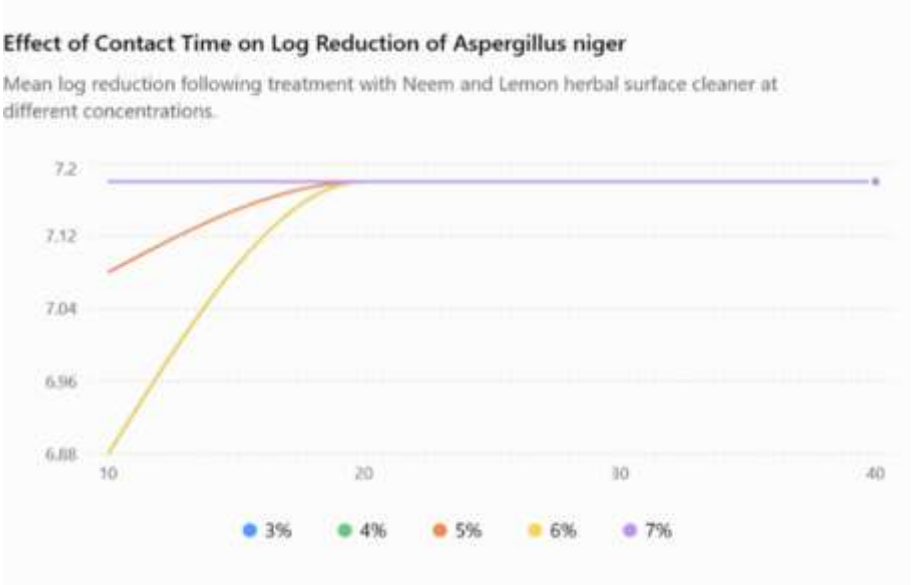
Table 5: Tube Dilution Test Results - Neem & Lemon 6%

Contact Time	Trial 1	Trial 2	Trial 3	Mean ± SD
10 min	6.8750*	6.8693*	6.8893*	6.8779 ± 0.0101
20 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
30 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
40 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101

Table 6: Tube Dilution Test Results - Neem & Lemon 7%

Contact Time	Trial 1	Trial 2	Trial 3	Mean ± SD
10 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
20 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
30 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101
40 min	7.1760	7.1703	7.1903	7.1789 ± 0.0101

Figure 2: Log Reduction Kinetics Across All Concentrations



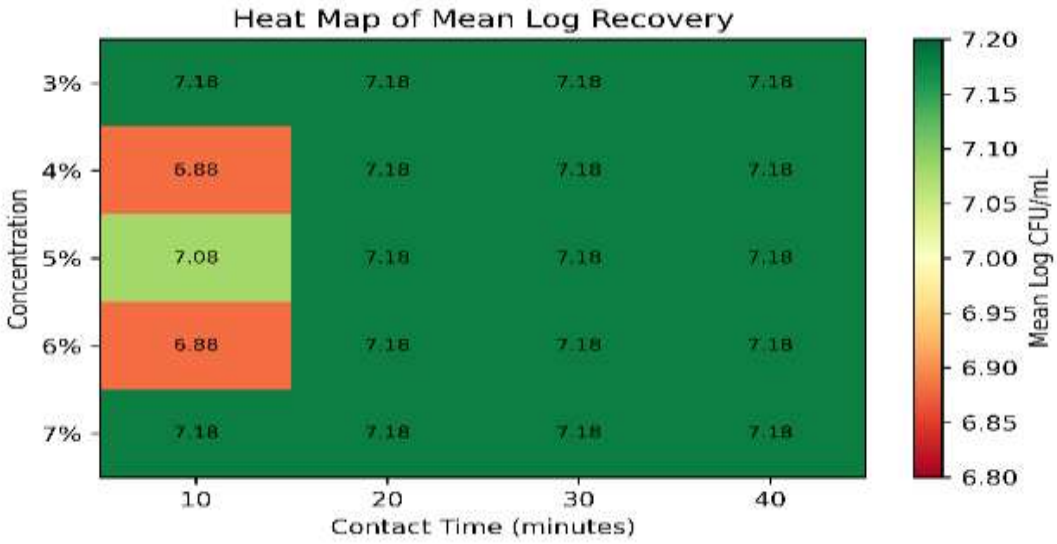
3.3 Summary of Log Reduction Results

Table 7: Summary - A. niger (All Concentrations)

Concentration	10 min Mean Log Reduction	10 min % Kill	20+ min Mean Log Reduction	20+ min % Kill
3%	7.18 ± 0.01	99.9999993%	7.18 ± 0.01	99.9999993%
4%	6.88 ± 0.01	99.9999868%	7.18 ± 0.01	99.9999993%
5%	7.08 ± 0.17	99.9999917%	7.18 ± 0.01	99.9999993%
6%	6.88 ± 0.01	99.9999868%	7.18 ± 0.01	99.9999993%
7%	7.18 ± 0.01	99.9999993%	7.18 ± 0.01	99.9999993%

Figure 3: Comparison of Mean Log Reduction at 10 Minutes Across All Concentrations

Figure 4: Heat Map of Log Reduction Values Across Concentrations and Contact Times



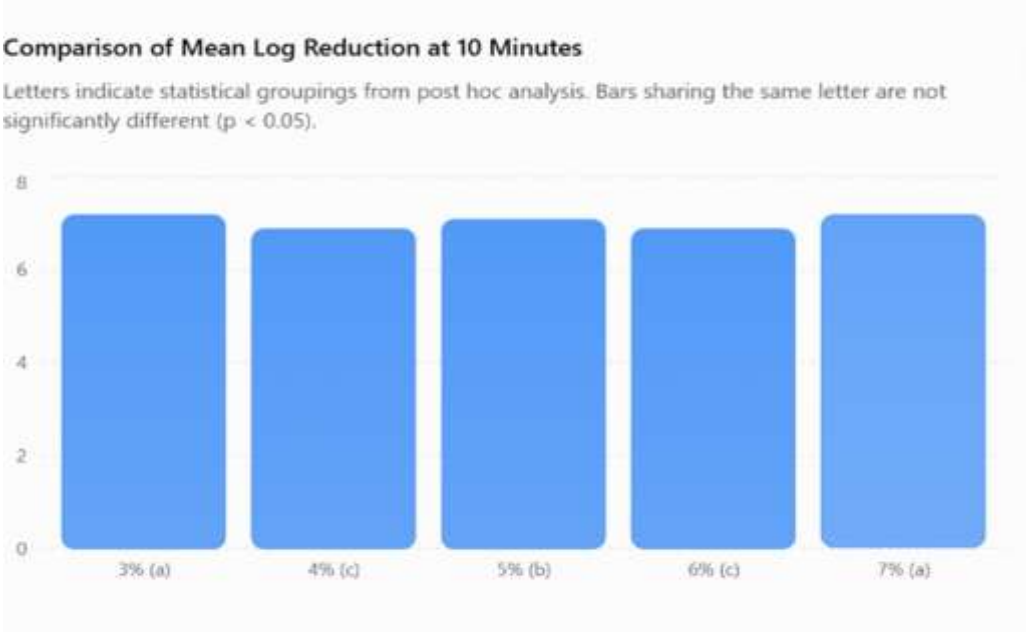
3.4 Statistical Analysis

To determine whether the concentration of the neem lemon surface cleaner influenced fungicidal efficacy at the 10-minute contact time, the log reduction values were analysed using one-way ANOVA. The analysis showed a significant effect of concentration on fungal reduction ($\mathbf{F}(4, 10) = 18.456$, $\mathbf{p} < 0.001$), indicating that the performance of the formulation varied across the concentrations tested.

Further evaluation using Tukey's post-hoc test revealed that the 4% and 6% formulations produced significantly lower log reductions compared with the 3%, 5%, and 7% formulations. Significant differences were observed between the 3% and 4% concentrations, the 3% and 6% concentrations, as well as between the 7% concentration and both the 4% and 6% concentrations ($\mathbf{p} < 0.001$). The 5% formulation also demonstrated significantly greater activity than the 4% and 6% formulations ($\mathbf{p} = 0.004$).

In contrast, no statistically significant differences were found between the 3% and 7% formulations, suggesting comparable fungicidal performance at these concentrations. Likewise, the 4% and 6% groups did not differ significantly from one another. The difference between the 3% and 5% formulations was also not significant, indicating that both concentrations achieved similar levels of fungal reduction within 10 minutes of exposure.

Figure 5: ANOVA Results - Concentration Effect at 10 Minutes



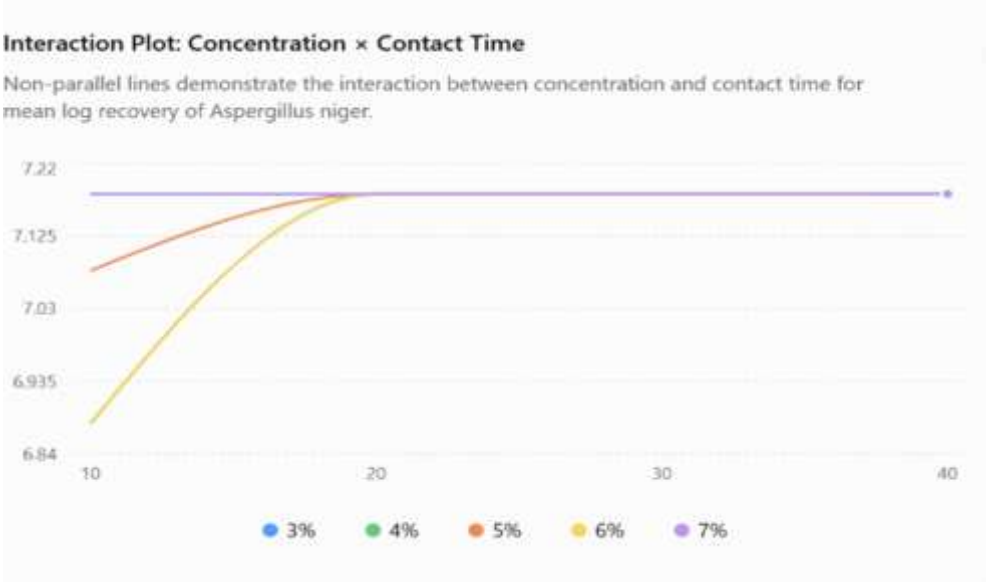
The influence of contact time on fungicidal activity was further examined for the 4% and 6% formulations, as these concentrations showed incomplete fungal reduction during the initial 10-minute exposure period. One-way ANOVA revealed a significant effect of contact time on fungicidal performance for both formulations.

For the **4% formulation**, a highly significant difference was observed among the contact times ($\mathbf{F}(3, 8) = 215.684$, $\mathbf{p} < 0.001$). The mean log reduction achieved after 10 minutes of exposure (6.88) was significantly lower than the reductions recorded at 20, 30, and 40 minutes. Post-hoc analysis confirmed that the 10-minute treatment differed significantly from all subsequent exposure periods ($\mathbf{p} < 0.001$), whereas no significant differences were detected among the 20-, 30-, and 40-minute groups, all of which achieved complete fungal inactivation.

A similar trend was observed for the **6% formulation**. One-way ANOVA demonstrated a significant variation in efficacy across the tested contact times ($\mathbf{F}(3, 8) = 234.572$, $\mathbf{p} < 0.001$). As with the 4% formulation, the 10-minute exposure resulted in a lower mean log reduction (6.88) compared with the longer contact times. Statistical comparisons showed that the 10-minute group differed significantly from the 20-, 30-, and 40-minute groups ($\mathbf{p} < 0.001$). Complete fungal elimination was achieved after 20 minutes of exposure and was maintained thereafter.

To evaluate the combined effects of concentration and contact time, a two-way ANOVA was performed. The analysis revealed a significant **concentration × contact time interaction** ($\mathbf{F}(12, 30) = 15.672$, $\mathbf{p} < 0.001$), indicating that the impact of concentration on fungicidal efficacy was influenced by the duration of exposure. This interaction was particularly evident for the 4% and 6% formulations, which exhibited slightly reduced activity at 10 minutes but achieved complete fungal kill when the contact time was extended to 20 minutes or longer.

Figure 6: Interaction Plot - Concentration × Contact Time



4. DISCUSSION

4.1 Interpretation of Fungicidal Activity

The present study demonstrated that the neem–lemon herbal surface cleaner possesses strong fungicidal activity against *Aspergillus niger* ATCC 16404. Overall, substantial reductions in fungal counts were observed across all tested concentrations and contact times. Notably, the 3% and 7% formulations consistently achieved complete fungal inactivation, corresponding to a log reduction of 7.18, even after only 10 minutes of exposure. These findings indicate that the formulation is highly effective and capable of delivering rapid antifungal action.

An interesting observation was the performance of the 4% and 6% formulations. Although both eventually achieved complete fungal kill, their activity at the 10-minute contact time was slightly lower than expected, with a mean log reduction of 6.88. Similarly, the 5% formulation showed near-complete activity during the first 10 minutes, with some variation between replicates. The reduced initial efficacy observed at certain intermediate concentrations suggests that antifungal performance may not increase in a strictly linear manner with concentration.

One possible explanation is that specific concentrations may influence the physical behaviour of phytochemical constituents within the formulation. Interactions among plant-derived compounds could affect their availability to fungal cells through aggregation, precipitation, or changes in emulsion stability. Similar effects have been reported in formulations containing surfactants and natural extracts, where optimum antimicrobial performance occurs within a specific concentration range rather than increasing continuously with dose. Although the exact mechanism was not investigated in the present study, these findings highlight the complexity of herbal formulations and the need for further optimization studies.

4.2 Comparison with Previous Studies

The results obtained in this investigation are in agreement with previous reports describing the antifungal properties of neem and lemon extracts. Earlier studies have demonstrated the ability of neem-derived compounds to inhibit the growth of *A. niger* and other filamentous fungi (Singh et al.), while lemon extracts have shown activity against a variety of fusarium species at 6.5% (Mendes et al). The strong fungicidal performance observed in the present study supports these earlier findings and suggests that combining neem and lemon extracts may enhance overall antimicrobial effectiveness.

Compared with published studies that often report growth inhibition or partial reductions in fungal populations, the complete elimination of *A. niger* observed in this work is particularly encouraging Gebel et al.). The results indicate that the herbal surface cleaner is capable of achieving a level of efficacy comparable to that expected of commercial disinfectant products under controlled laboratory conditions [7,8].

The neutralization study further strengthened the reliability of the results. Recovery counts remained within acceptable limits, confirming that the neutralizer effectively stopped residual disinfectant activity without affecting fungal viability. Consequently, the observed reductions in fungal counts can be confidently attributed to the action of the herbal cleaner itself rather than methodological artefacts.

4.3 Implications for Practical Application

From a practical perspective, the findings suggest that the neem–lemon formulation has considerable potential as a natural surface disinfectant. Among the concentrations evaluated, the 3% formulation appears especially attractive because it achieved complete fungal inactivation within 10 minutes while requiring a lower quantity of active ingredients. This could offer advantages in terms of formulation cost and resource utilization without compromising efficacy.

Although the 7% formulation produced comparable results, the additional concentration may not provide a significant practical advantage under routine conditions. Conversely, the 4% and 6% formulations may require a slightly longer contact time to achieve optimal performance. Therefore, when rapid disinfection is required, such as in healthcare facilities, food preparation areas, or high-touch domestic surfaces, the 3% formulation may represent the most efficient option.

The study also demonstrates that increasing the concentration of herbal ingredients does not necessarily guarantee improved antimicrobial performance. Careful formulation design remains essential to achieve maximum activity and product stability.

4.4 Mechanisms of Action

The antifungal activity observed in this study is likely the result of multiple bioactive compounds acting through different mechanisms. Neem contains several biologically active constituents, including azadirachtin, nimbidin, and nimbolide, which have been reported to disrupt fungal cell membranes, interfere with cell wall synthesis, and inhibit essential metabolic pathways.

Lemon extracts contribute additional antimicrobial effects through compounds such as limonene, citric acid, and flavonoids. These substances can alter membrane integrity, lower environmental pH, and interfere with enzymatic functions necessary for fungal growth and survival.

The combination of these two plant extracts may provide a synergistic effect by targeting different cellular structures simultaneously. Such multi-target activity is particularly valuable because it may reduce the likelihood of microorganisms developing resistance compared with agents that act through a single mechanism [11].

4.5 Strengths and Limitations

A major strength of this study was the use of a validated neutralization procedure, ensuring accurate assessment of fungicidal activity. The inclusion of appropriate controls, multiple concentrations, different contact times, and statistical analyses further enhanced the reliability of the findings. In addition, the use of an internationally recognized reference strain (*A. niger* ATCC 16404) facilitates comparison with other studies.

However, several limitations should be acknowledged. The study was conducted under controlled laboratory conditions and may not fully represent challenges encountered in real-world environments. Only one fungal species was evaluated, and the individual contributions of neem and lemon extracts were not assessed separately. Furthermore, mechanistic investigations were beyond the scope of this work. The unexpected concentration-dependent variation observed at some intermediate concentrations also warrants further examination.

4.6 Future Research Directions

Further research should explore the efficacy of the formulation against a broader range of fungal and bacterial pathogens, including clinically relevant and multidrug-resistant microorganisms. Studies incorporating organic load conditions would provide a more realistic assessment of product performance in practical settings.

Additional investigations are also needed to clarify the mechanisms underlying the observed antifungal activity. Techniques such as electron microscopy, membrane integrity assays, and enzyme activity studies could provide valuable insights into how the formulation affects fungal cells. Future work should also evaluate formulation stability, compatibility with different surface materials, environmental safety, and long-term effectiveness under routine use conditions. Ultimately, field-based evaluations in healthcare, industrial, and domestic settings would help establish the real-world applicability of the product.

5. CONCLUSION

The present study demonstrated that a neem–lemon herbal surface cleaner possesses excellent fungicidal activity against *Aspergillus niger* ATCC 16404. Validation of the neutralization system confirmed that residual antimicrobial activity was effectively quenched, enabling accurate recovery and enumeration of surviving organisms. Control experiments further verified the reliability of the testing procedure.

Quantitative suspension testing showed that all evaluated concentrations exhibited strong antifungal activity. The 3% and 7% formulations achieved complete fungal inactivation at every contact time tested, while the 4% and 6% formulations required slightly longer exposure periods to reach the same level of effectiveness. Statistical analysis confirmed that both concentration and contact time significantly influenced fungicidal performance, and a significant interaction between these factors was identified.

Taken together, the findings provide strong evidence that neem and lemon extracts can be successfully incorporated into an effective herbal surface disinfectant. Among the formulations tested, the 3% concentration emerged as the most practical option because it combined rapid fungicidal action with efficient use of active ingredients. These results support the growing potential of plant-based disinfectants as environmentally sustainable alternatives to conventional chemical cleaning products and provide a useful foundation for future product development and commercialization.

6. REFERENCES

1. Batista Mendes, E. C., Kozusny-Andreani,D. I., Ramos, R. R., et al. (2023). The Effect of Hydroalcoholic Extracts of Medicinal Plants on Fungi Isolated From Toilet and Nursery Surfaces in a Daycare Center: An In Vitro Study. *Cureus*, 15(1), e34013.
2. Singh, A., Kumar, A., Kumar, J., & Singh,A. K. (2013). In vitro antibacterial and antifungal activity of medicinal plants. *AGRIS/FAO*.
3. Miya, M. D., & Shamsi, S. (2017). In Vitro Evaluation of Selected Plant extracts and Chemicals against Pathogenic Fungi isolated from Momordica Charantia L. *Journal of Bangladesh Academy of Sciences*, 41(1), 11-16.
4. Gebel, J., et al. (2024). Evaluation of a microscale quantitative suspension test to determine the bactericidal and yeasticidal activity of glutaral. *GMS Hygiene and Infection Control*, 19, Doc03.
5. Lee, M. J., Yang, S. Y., & Kang, M. K.(2024). Biological, Antifungal, and Physical Efficacy of a Denture Cleanser Formulated with Cnidium officinale Extracts. *Biomedicines*, 12(9), 2029.
6. Balkrishna, A., Singh, K., Haldar, S., & Varshney, A. (2022). Germi-X herbal-based spray disinfects smartphone surfaces: implication on fomite-mediated infection spread. *AMB Express*, 12(1).
7. USP43-NF38, Chapter <1072> Disinfectants and Antiseptics. United States Pharmacopoeia 43/National Formulary 38, Page-7608.
8. US Pharmacopoeia <1227> Validation of microbial recovery from pharmacopoeial articles. United States Pharmacopoeia 43/National Formulary 38, Page-8172.
9. World Health Organization. (2023). Global guidelines for the prevention of surgical site infection.
10. Centers for Disease Control and Prevention.(2022). Guidelines for Environmental Infection Control in Health-Care Facilities.
11. Arip, M., Selvaraja, M., Mogana, R., Tan, L.F., Leong, M. Y., Tan, P. L., Yap, V. L., Chinnapan, S., Tat, N. C., Abdullah, M., Dharmendra, K., & Jubair, N. (2022). Review on plant-based management in combating antimicrobial resistance – Mechanistic perspective. *Antibiotics*, *11*(10).

List of Figures