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Anti-fungal efficacy of aqueous leaf extracts *Neem* (*Azadirachta indica*) in the treatment of tap water

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ABSTRACT

High-quality drinking water coming from treatment plants is susceptible to pollution and severe deterioration due to the drinking water delivery system prior to access to consumers' faucets. The results of this study confirmed that *Neem* leaves extract and chlorine at all concentrations had antifungal activity during tap water treatment. This study has provided excellence about the ability of *Neem* leaves extraction and chlorine as a disinfectant for *Rhodotorula mucilaginosa* and *Aspergillus spp*. in treating tap water. The most notable factors were the dosage, time, and agitation selected to evaluate their effects on reducing the development of fungal communities in drinking water using the central composite design (CCD) in the response surface methodology (RSM). The CCD was performed with a 2 complete central composite design with five different stage coordinate components. Because the *Neem* leaves can be processed locally and should also be encouraged for use in water treatment. This will eventually reduce the high costs and health risks associated with chemical water treatment. This technology is cheap, conventional, readily available, and suitable for rural areas, as nature's biological methods generate no treatable waste.

Key words : Neem *leaves (Azadirachta indica), Fungi, Aspergillus spp., Rhodotorula mucilaginosa, Drinking water, Tap water*

Introduction

Neem trees are evergreen and grow in tropical and subtropical regions around the world. *Neem* tree is known as *Azadirachta indica (A. indica*). The medicinal properties of every part of the *Neem* tree have been used for more than 4000 years (Sultana *et al.,* 2011). *Neem* produces large amounts of phytochemicals, including various parts such as leaves, seeds, roots, flowers, and oils, pharmacological activities, and various biological methods. *Neem* extract is a varied medicine due to its ability to act as a cancer therapy and prevention (Patel *et al.,* 2016). *Neem* trees can be used as a home remedy against various human ailments ranging from leprosy, oral care, and intestinal worms to ancient diseases (Kumar and Navaratnam, 2013; Pandey *et al.,* 2014; Reddy *et al.,* 2018). Besides, various pharmacological properties are attributed to different parts of the tree, such as antifungal, antiviral, antibacterial, anti-inflammatory, antifeedant pesticides, sterilant, antiscab, anti-allergenic, analgesic, and nematicidal (Ogbuewu *et al.,* 2011).

It has been revealed that aqueous, methanolic, and acetonic extracts of *Neem* leave inhibited the growth of *Escherichia coli, Micrococcus luteus,*

Enterobacter, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Streptcoccuspyogens, and *Staphylococcus aureus* when studied using agar well and disc diffusion methods (Lall *et al.,* 2013; Salam *et al.,* 2014; Tirumalasetty *et al.,* 2014; Vijayaram *et al.,* 2016). *Neem* trees are cultivated globally in piles of fields. Nutrition, vegetables, vitamins, calcium, phosphorus, vitamin C, carotene, and so on are part of the *Neem* leaves. It comprises amino acids such as glutamic acid, tyrosine, aspartic acid, alanine, praline, glutamine and cysteine, and various fatty acids (Suneeta *et al.,* 2020). Excellent anti-microbial interest in microorganisms has been researched and documented in leaf extracts of *Neem*, based on the overall availability of enormous amounts of bioactive compounds that can be useful for bacterial and fungal infections. The relationship of isolated components from the *Neem* tree leaf and their potential for antimicrobial effectiveness against *Staphylococcus aureus* (staph) bacteria and *Salmonella typhi* bacteria was investigated within the current research.

The pollution problem of drinking water sources with many forms of biological and chemical toxins is concerning in several countries worldwide. Therefore, the consistency of piped water is not always an opportunity for human use (Mirzabeygi *et al.,* 2016). Microbial pollution has been reported in rural as well as urban areas as one of the severe problems. This is due to pipe contamination, emissions from the infiltration of sewer pipes into drinking water sources, and so on (Daud *et al.,* 2017). Many reactions, contaminants, and biological agents, as well as encounters with pipe walls, will reach the pipeline from several outlets, causing a significant issue that can lead to outbreaks of complex diseases and low water safety (Miller *et al.,* 2016). However, these contacts at the interface of the pipe network wall are central to this degradation and are regulated mainly by dynamic and not well-understood microbial biofilms (Fish *et al.,* 2017).

There were fungi in the tap water in the Kuantan area in different concentration were *Aspergillus spp., Rhodotorula mucilaginosa, Penicillium citrinum, Cladosporium cladosporioides, Cerrena spp., Aspergillus aculeatus, Aspergillus flavus, Cryptococcus sp., Cladosporium perangustum, Purpureocillium lilacinum, and Candida catenulate* (Salah HO *et al.,* 2020). It is necessary to mitigate the pollution of fungal activity through the tap water system to keep our drinking water healthy. The goal is to preserve protected watercourses and provide recommendations on monitoring priority contaminants and contaminants. The EU is planning a revised watchlist of emerging toxins to supplement the Directives (Loos *et al.,* 2018). In addition to these preventive papers, in a memorandum, the organization of European waterworks recommended the introduction of a 0.1 ìg/L (0.1 ng/mL) threshold for fungal involvement in tap water to reduce toxicity. In the present study, we selected *Neem* leaves to evaluate the effectiveness of natural disinfection petri-dishes and pilot-scale fungus growth removal on synthetic tap water in university laboratories and compare them with chlorine disinfection. The characterization of antifungal extraction has been done from tree leaves before and after the use of FT-IR, SEM, and XRD.

Materials and Methods

Study Site

Kuantan is the state capital of Pahang, Malaysia. It is located at latitude 3°492N and longitude 103°202E. Moreover, the total area of Kuantan 324 km^2 and located near the estuary of the Kuantan River and faces the South China Sea (Zainutdin *et al.,* 2017). Kuantan is the 17th largest city in Malaysia, based on a population of around 427,000 and the largest city on the East Coast of Peninsular Malaysia (Kozaki *et al.,* 2016).

Sample Collection

Thirty tap water samples were obtained from public buildings and private homes in Kuantan (samples were coded 1 to 30). The samples were taken from a tap that collects water directly from the Semambu Water Treatment Plant, Kuantan is located at Latitude 3°52’8.41"N, and longitude 103°19’45.10"E. All the samples were analysed within 4 h.

Samples were taken from a tap at various distances (0 to 30 km) that receives direct water supply from the Semambu Water Treatment Plant in Kuantan. Within four hours of collection, all of the samples were analysed. The tap was washed with a clean cloth until the samples were taken to eliminate some soil. The tap was then switched on for three minutes at full flow. Before collecting, the sampling bottle was rinsed three times with tap water. In a 1500 mL polyethylene plastic container, tap water samples were obtained.

Sources of Fungi

The fungi cultures *Aspergillus spp*. and *Rhodotorula mucilaginosa* used in this work were obtained from the tap water in Kuantan Malaysia. The cultures of the fungi on Potato Dextrose Agar (P.D.A) were kept at 4°C until when required for use at Universiti Malaysia Pahang.

Preparation of Extracts

Fresh *Neem* leaves were washed thoroughly with tap water and distilled water. Then, it is left at 45°C in the oven for 24 hours until it removes the amount of moisture. After that, it is dried. The dried leaves were crushed into a coarse powder using a laboratory grinder. After drying, *Neem* leaf powder was collected, and some changes were made and stored at 4 °C (Paray *et al.,* 2018). The extract of the aquatic cake was prepared using 50 g *Neem* leaves and 250 ml of distilled water in a sterile conical flask and kept overnight in a hot air oven at 55 °C (Paray *et al.*, 2018). After incubation, the supernatant was filtered through No. 1 Whatman filter paper at room temperature. The supernatant was collected and frozen-dried at -80 °C for 2 days. The *Neem* powder was obtained with the help of a laboratory grinder and driven from 250 mm to get the appropriate particle size.

Characterization of active agent

In this study, the efficiency of plant extraction was tested in two parts and compared with the general chemical disinfection in the second part. In the first part, the growth diameter of the fungus was measured in PDA and expressed as percentage resistance. In the second part, the application of natural synthesis on synthetic water was done in the university laboratory and compared with chlorine. The characterization of the antifungal agent of Neem was done using FT-IR, SEM, X Ray Diffraction (XRD), and TGA to obtain their physiochemical properties.

The surface morphology of the active agent

To observe the morphological profile of biomass, it is essential to analyse the surface of the biomass before and after the extraction phase. Neem's surface morphology was measured using the scanning electron microscope (SEM) HITACHI/ TM3030 PLUS. SEM is a form of electron microscope that scans the surface of a sample with a directed beam of electrons to obtain photographs of the sample. The electrons communicate with the atoms in the sample, resulting in a variety of signals that provide details about the sample's surface topography and composition. A raster scan pattern is used to scan the electron beam, and the direction of the beam is coupled with the observed signal to generate an image. In addition, the dimension, shape, and number of pores in bio-aggregates and bio-composites can be inspected by image processing analysis (Senthil Kumar *et al.,* 2016; Kebede *et al.,* 2018).

The molecular structure of the active agent

The molecular structure of the antifungal agent of Neem was measured by using FTIR (Fourier transform infrared spectroscopy) is a method for determining the chemical bonding or molecular structure of organic or inorganic products. A molecule's vibrational continuum is a special physical property and a function of the molecule. As a consequence, when matching the emission from an unseen with previously reported reference spectra, the infrared spectrum may be used as a fingerprint for detection. In this case, the FT-IR studies were carried out to deduce the specific functional groups present in the sample and the nature of the sample's charge (Senthil Kumar *et al.,* 2016; Kebede *et al.,* 2018).

Determine the atomic and molecular structure of the active agent

To determine the atomic and molecular structure of a crystal of Neem was measured using XRay Diffraction (XRD). XRD can do the active agent's semi-crystalline nature due to the high composition of protein and oil determined the atoms in the crystal, chemical bonds, and crystallographic disorder (Kebede *et al.,* 2018).

Thermal properties of the active agent

The Neem leaves' thermal stability was determined using the thermogravimetric analyser (TGA Instruments Model Q 500 TGA with TA 5000 Controller). Monitoring the seeds' active agent structural degradation and leaves powder through temperature changes from 50 to 1000 °C (Kebede *et al.,* 2018).

Perform the study, the extract of the *Neem* leaves

39 g/l of potato dextrose agar (DifcoTM, Becton, USA) was prepared and autoclaved for 15 minutes at 121 °C. Without the inclusion of any plant extract as a control, Media PDA was poured into Petri-

dishes under aseptic conditions. Plant extracts and chlorine were combined with PDA at 45 °C at final concentrations of 0.1, 0.5, 1, 2, 3, 4, and 5 mg / ml and poured with some modifications into Petri dishes under aseptic conditions (Ndamane *et al.*, 2013).

A disk of a pure culture of the fungus (using sterile cork sack of 5 mm diameter) was placed on top of 5 days old culture extraction transferred to modified media with different concentrations of plant extract (Abd El-Ghany *et al.*, 2015). This comparison was made by placing a 5 mm diameter disc of a pure culture of the fungus transferred to the modified media with different chlorine concentrations as a general disinfectant. Each concentration was replicated three times. The Petri dish was cooked at 26±1 °C for 7 days. The growth diameter of the fungus was measured and expressed as a percentage barrier with some amount of change (Abd El-Ghany *et al.*, 2015).

Obtain the best condition of the disinfection of *Neem* leaves

The fungal count test was performed after treatment of the introduction of the *Neem* leaves solution into the water sample in order to achieve the best condition of disinfection on synthetic water at the university laboratory. A McFarland standard of 0.5 has been standardized according to the Clinical Laboratory Standards Institute (2002), which is roughly $1.0x10^6$ CFU/ml relative to regular saline. *Aspergillus spp.* and *Rhodotorula mucilaginosa* were harvested from 7 days old Potato dextrose agar (PDA) slant cultures by washing with 10 ml sterile normal saline containing 3% w/v 80 with the help of sterile glass beads to assist in dispersing the spores (Aboh *et al.*, 2014). After that, using a single-beam spectrophotometer (UV-1800) at 530 nm of suspension equal to McFarland standard 0.05, the spore suspension was normalized to 1.0×10^3 spores/ml. By spreading 100 µl on the Potato dextrose agar tray, all modified suspensions were quantified and then incubated at 26 °C for 1 day with some adjustments (Idris *et al.*, 2017).

The most widely used way to assess a coagulant and disinfection efficacy is a jar test since it is simple to administer. A typical jar tester was performed to investigate the elimination of target pollutants from both synthetic water samples by using the chosen biomass. To explore the elimination of fungi by using the selected biomass, several experiments were performed. In this study, the mixture and simultaneous dosing of more than one biomass procedure were also attempted to increase the medication's effectiveness. A jar test apparatus with six beakers was the equipment used in this research. 1 litre of synthetic water was filled into each container. Using the three stages of factorial design, the process requirements were calculated. The optimal concentration of factors was calculated, and the significant factors were demonstrated in the experimental area. The three essential considerations during the disinfection process are doses of 1, 3, and 5 mg/l, agitation of 100, 120, and 140 rpm, and time of 30, 60, and 90 minutes, with some changes (Idris *et al.*, 2017). At moderate, medium, and high stages, these variables have been studied. The three-factor factorial configuration using Design Expert Tools is 20 experimental runs with six centre points, as shown in Table 1.

Table 1. Experimental design using three levels of factorial design in response and the output of fungal count

Run	Dosage (mg/ml)	Time (minutes)	Agitation (rpm)
1	1	90	140
2	5	60	120
3	1	30	140
4	1	90	100
5	5	90	100
6	3	60	120
7	3	30	120
8	1	30	100
9	5	90	140
10	3	60	120
11	5	30	100
12	5	30	140
13	3	60	120
14	3	90	120
15	1	60	120
16	3	60	120
17	3	60	140
18	3	60	100
19	3	60	120
20	3	60	120

Results and Discussion

Characterization

The surface morphology of *Neem* leaves powder

The structure and surface character of the powder of

the *Neem* leaves were analyzed using SEM, as shown in Figure 1. As shown in Figure 1, the fatty substances at 100x and 500x are excluded from the SEM surface picture of the *Neem* leaves. After extracting the fatty substances from the *Neem* leaves seen in Figures 1(c) and 1(d), the molecular powder was homogeneous, and the surface area increased.

The molecular structure of the active agent

In analysing the existence of certain functional groups in a molecule, FTIR spectroscopy is a powerful instrument since each complex chemical bond reveals unique energy (Zhu *et al.*, 2012). The practical aspect of the surface spectra before and after for *Neem* leaves was illustrated in Figure 2. The disappearance or decrease of the peak value was illustrated in Figure 2 when comparing the fatty substances before and after elimination. For instance, the fatty substances from the *Neem* leaves were reduced near peaks in Figure 2. Due to the stretching of NH and OH stretch in the secondary amide group, the FTIR spectrum of *Neem* leaves exhibited a prominent peak at 3289 cm^{-1} and 3284 cm^{-1}. Due to the asymmetric and symmetric stretch of the C-H bond and the CH_2 methyl group, the band lies between 2921 cm^{-1} and 2848 cm^{-1} of the *Neem* leaves. *Neem* spectra are alternating bands in 2112 to 778 cm^{-1} regions of the number. Also, C=O stretching can be observed between the bands of 1726 to 1030 cm^{-1} of the *Neem* leaves.

Determined the atomic and molecular structure of the active agent

To gain further details about a *Neem* crystal's atomic and molecular composition, X-Ray Diffraction (XRD) research was performed. The XRD pattern of *Neem* Protein Leaves is shown in Figure 3. The XRD trend reveals a poorly resolved peak that suggests a

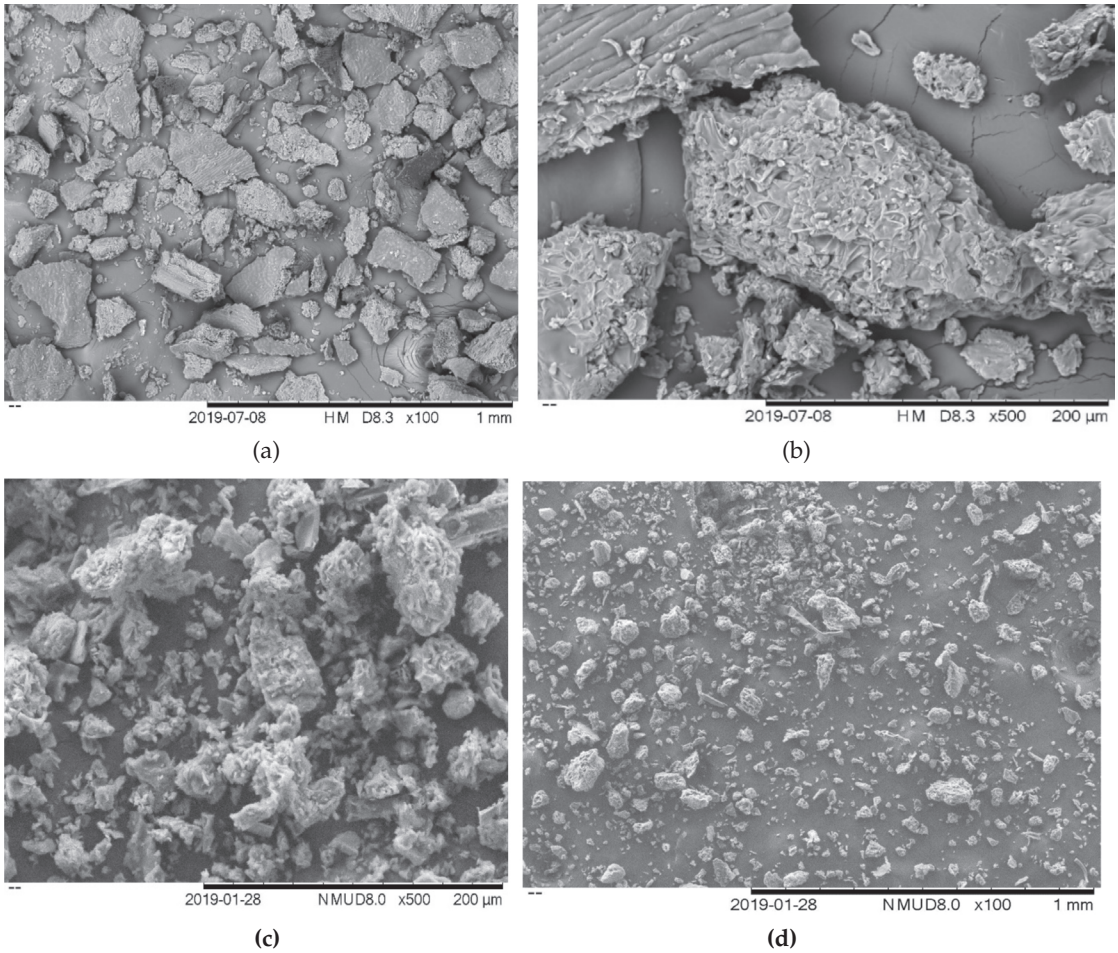


Fig. 1. SEM surface image of the *Neem* leaves before removing the fat at a magnification of (a) 100x, (b) 500x, and after removing fat at a magnification of (c) 100x, (d) 500x.

predominance of amorphous content due to the high quantity of proteins, fatty substances present (about 69%) in the composition of the material (Abdulkarim *et al.*, 2005; Kebede *et al.*, 2018). The XRD trend showed broadband about 2´ = 15° to 25° due to the high composition of proteins, fatty substances, except Figure 3 where 2 = 25° for the leaves powder, which is attributable to the predominance of the material's semi-crystalline nature. The presence of these peaks is presumably related to the diffraction of the constituent protein accompanying the other more amorphous or semi-crystalline elements.

Thermal properties of the active agent

The thermal gravity characteristics of decomposition stages are seen in TGA research and thermal stability, which is characterized as the mass loss of a material subjected to a constant heating rate at a specified period in a controlled setting. Figures 4(a) and 4(b) display the mass loss curve for the samples in Neem leaves, which shows a standard profile that suggests many phases of the decomposition process.

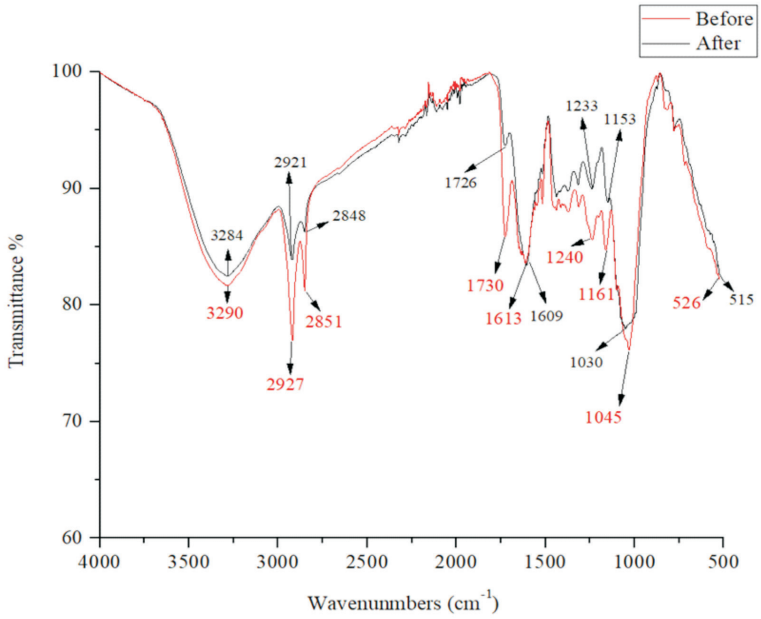


Fig. 2. FT-IR spectrum of *Neem* leaves before and after remove the fatty substances

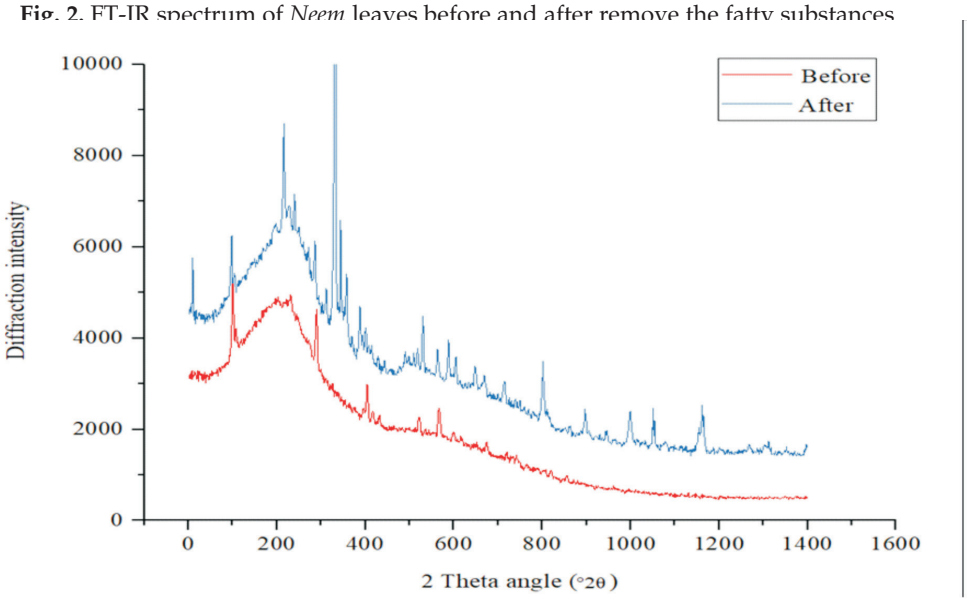


Fig. 3. XRD of *Neem* leaves before and after remove the fatty substances

Since the intermediates produced are a mixture of many elements, the thermogravimetric curve of the Neem verifies the heterogeneity of the samples. The sum of water loss from seeds measured by this methodology was 9.3%, which was close to the value of Neem leaves. The loss of mass was detected in the second level, ranging from 128 °C to 268 °C, with the loss of mass due to the degradation of organic matter and proteins found in the leaves.

The temperature ranges in the third stage, 268 to 541 °C, with a significant part of the decomposition in leaves and seeds components. Attributed to fatty acids, for instance, oleic acid has a boiling point of 360 °C in the elements in seeds and leaves. 14.6% was observed for the total residue due to the inorganic oxides and ash content at 950 °C.

Performance of the activities of the extract of the *Neem* leaves

The investigation of the antifungal activity of *Neem* was done through laboratory work. The standard two fungi existing in the tap water samples have selected are *Aspergillus sp.* (73%) and *Rhodotorula mucilaginosa* (63%) in the Kuantan area of Malaysia (Salah HO *et al.*, 2020). The plant extraction was added to the media (Potato dextrose agar) and determined the efficiency to inhibit the specific pure fungal growth. The fungal growth was measured and expressed as percentage inhibition in this part of the study. Table 2 showed that fungal mycelia

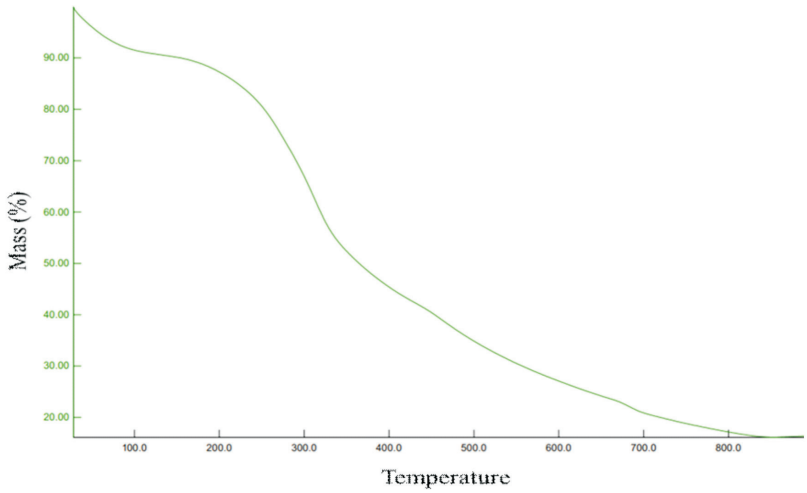


Fig. 4(a). Thermal properties curve of Neem leaves before remove the fatty substances

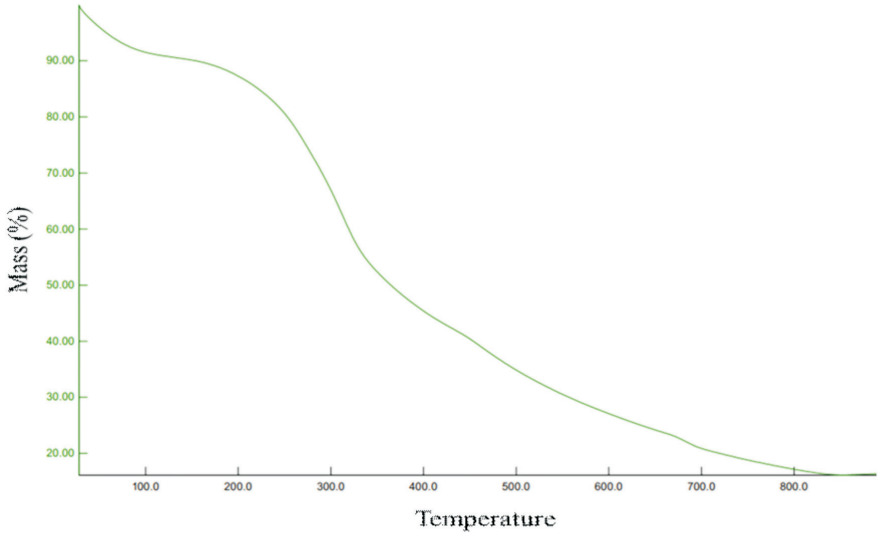


Fig. 4(b). Thermal properties curve of Neem leaves after remove the fatty substances

growth gradually decreased with an increase in the concentration of *Neem* leaves extract and chlorine on *Rhodotorula mucilaginosa (RM)* and *Aspergillus spp. (A).* The disparity between the quantity of *Neem* and chlorine applied and the market for *Neem* and chlorine is residual. Beyond the breakpoint, most water treatment facilities can add more. Small water treatment plants also only apply a fraction of the volume needed (compared to ammonium ions) and end up not disinfecting their water sources properly.

Unlike other products of these side reactions, ammonia and amines in tap water can combine with chlorine to yield chloramines with some biocidal activity. These chloramines will be oxidized once chlorination continues to the breakpoint, i.e. to a free-chlorine residual, allowing further added chlorine to be absorbed until a specific amount of free chlorine is reached. A microorganism is inactivated by chlorine by destroying the cell membrane. The chlorine will penetrate the cell and interact with cell respiration and DNA activity until the cell membrane is damaged (two processes that are necessary for cell survival). The results of this experiment confirmed that *Neem* leaves extract and chlorine at all concentrations had antifungal activity against this investigation. *Neem* leaves and chlorine at a concentration of 0.1 mg/ml inhibited the growth of *Rhodotorula mucilaginosa* by 13.51% and 35.43%, respectively. In contrast, percentage inhibition was 61.72% and 100% at the concentration of 5 mg/ml for *Neem* leaves and chlorine, respectively. It has been so successful that freedom from waterborne disease epidemics is now virtually taken for granted. As described in Drinking Water and Health (National Academy of Sciences, 1977), "chlorination is the standard of disinfection against which others are compared."

The inhibitory effect of *Neem* leaves extract and chlorine is shown in Table 2 at seven different concentrations of 0.1, 0.5, 1, 2, 3, 4, and 5 mg/ml on *Aspergillus spp. Neem* leaves and chlorine at a concentration of 0.1 mg/mL inhibited the growth of *Aspergillus spp.* 11.13% and 32.63%, respectively, while percentage inhibition at 53.46% and 100% at a 5 mg/ml concentration for Neem leaves and chlorine, respectively. *Aspergillus species* resistance to the plant extract was apparent, possibly due to the particular composition and growth of the fungal. At different percentage mean zones of inhibition, all the extracts display antifungal behaviours. Various Neem plant product extracts have demonstrated the inhibition against fungal growth. The extracts have been effective against fungal isolates. More efficacy than the positive control (Ketoconazole) against *Aspergillus spp.* was demonstrated by the mean percentage inhibition zones of all Neem plant part growth extracts. The toxic fungi potential of these plant extracts on fungi of *Rhodotorula mucilaginosa* and *Aspergillus spp.* recommends their use as an alternative to commercial/synthetic fungicides for treating tap water.

Obtaining the best condition of the disinfection Neem leaves

RSM is an essential means of achieving an experiment's optimum process settings. Compared to the conventional approach, this statistical methodology can provide data on optimal settings concerning the vicinity of the predicted response to achieve suitable responses in creating an approximate relationship between the dependent and independent factors and assess the degree of significance of the independent factors. It is then possible to create a set of appropriate functional relationships between a response of interest and deceptive variables. An experiments model is a required step to get the responses of interest calculated accurately and consistently (Mukhopadhyay and Khuri, 2012). In this analysis, as a linear function or first-order model was unfit for relationships between the studied responses and independent variables, a second-order

Table 2. Percentage inhibition (%) of *Neem* and chlorine on *Rhodotorula mucilaginosa (RM)* and *Aspergillus spp. (A).*

Concentration (mg/ml)	Neem (RM)	Chlorine (RM)	Neem (A)	Chlorine (A)
0.1	13.51±1.07%	35.43±0.08%	11.13±0.85%	32.63±1.92%
0.5	25.55±1.25%	50.18±2.58%	20.79±0.26%	66.56±1.38%
1	45.65±3.64%	100±0.00%	32.37±1.46%	100±0.00%
2	52.86±2.87%	100±0.00%	37.03±1.58%	100±0.00%
3	55.65 ±2.41%	100±0.00%	44.77±2.76%	100±0.00%
4	58.60±3.12%	100±0.00%	50.84±1.62%	100±0.00%
5	61.72±2.29%	100±0.00%	53.46±1.32%	100±0.00%

model was used to estimate the answers in the area close to optimum. Table 3 summarises the experimental outcomes of designated studies containing all studied responses.

Optimization of the Significant Variables using CCD after the Significant

For further evaluation of their effect on removing the growth of fungal communities in drinking water using CCD in RSM, dosage, time, and agitation were chosen as the most relevant factors. The CCD was performed at five different levels (relatively strong, high, basal, low, relatively low) coded (+ a, +1, 0, -1, -a) (Table 3) with a 2 complete factor central composite design of combination factors. Table 4 displays the spectrum and levels of the extraction parameters, where X1 represents the dosage, X2 represents the time, and X3 represents the agitation. A total of 20 experiments were conducted for each extract and fungal according to the complete factorial of 2 CCD to maximize all the essential variables, while CFU was taken as dependent responses. To prevent bias, the experimental run was done according to the standard order. Equation 1 was accompanied by a second-order polynomial model equation to predict the ideal point by changing the experimental outcome.

$$Y = b_o + \Sigma b_i X_i + \Sigma b_{ii} X^2_i + \Sigma b_{ij} X_i X_j \quad ..(1)$$

Where, Y is the response variable, b is the model's regression coefficient, X is the independent variables' encoded levels. The established second-order model was evaluated by evaluating the values of regression coefficients, ANOVA, F- and P-values. The level of fit of the regression model was represented by the coefficient of determination (R^2) and correlation (R). The mathematical Design-Expert program was developed for the CCD experiment (Stat-Ease Inc., Minneapolis, MN 55413, USA, version 7.1.6).

Table 3. Ranges and levels of factors tested in CCD for eliminating the growth of the fungi

Factors	Unit	levels				
		-2	-1	0	+1	+2
(X1) Dosage	mg/ml	1	5	-1	1	3
(X2) Time	min	30	90	-1	1	60
(X3) Agitation	rpm	100	140	-1	1	120

Table 4. Experimental design and results of the CCD for *Neem* leaves on *Rhodotorula mucilaginosa (RM)* and *Aspergillus spp. (A).*

Run	Dosage (mg/ml)	Time (minutes)	Agitation (rpm)	CFU/ml (RM)	CFU/ml (A)
1	1	90	140	39	52
2	5	60	120	29	37
3	1	30	140	40	53
4	1	90	100	37	49
5	5	90	100	29	33
6	3	60	120	28	36
7	3	30	120	35	42
8	1	30	100	42	48
9	5	90	140	27	36
10	3	60	120	29	37
11	5	30	100	37	43
12	5	30	140	33	42
13	3	60	120	32	36
14	3	90	120	30	35
15	1	60	120	41	47
16	3	60	120	31	38
17	3	60	140	27	36
18	3	60	100	35	41
19	3	60	120	29	37
20	3	60	120	31	34

Optimization of eliminating the growth of the fungi by RSM

Further research studies on their effects following the statistical method will enhance the process of removing the growth of the fungi. RSM is an analytical modelling methodology in which the problems associated with the response of interest influenced by multiple variables are studied (Swamy and Muthukumarappan, 2017).

Optimization of process condition

To analyse the mutual interactions of the most critical variables, dosage (A), time (B), and agitation (C), the CCD was used to assess the exact optimal values of the CFU factors. Twenty experiments were conducted, including six centre replicates, following the conditions indicated in Table 4. All the experiments in the CCD were performed randomly to minimize the effect of unexplained variation in the observed responses to reduce methodological errors. The input and output variables were fitted to the second-order equation and evaluated in terms of the accuracy of the model fit. The model's fitness has been verified by F-, P- values, determination coefficient (R^2), and correlation coefficient (R). The ANOVA results of the response surface quadratic models for CFUs in Table 5 summarise both parameters.

The F-value of the model is the answer, CFU of the *Rhodotorula mucilaginosa* and *Aspergillus spp*, based on ANOVA results. The findings of ANOVA with *Neem* leaves, the F-value of the responses describing the model, CFU of the *Rhodotorula mucilaginosa,* and *Aspergillus spp*. Both models were fundamental, suggesting a very low likelihood value of 0.0004 and < 0.0001, respectively. Furthermore, the value of R^2 for *Neem* leaves was 0.9115 and 0.9405, indicating a more significant linkage between the actual and expected CFU values. The modified coefficients of determination (adj R^2) of the UFC values for *Rhodotorula mucilaginosa* and *Aspergillus spp.* with *Neem* leaves, modified determination coefficients (adj R^2) for UFC of the *Rhodotorula mucilaginosa* and *Aspergillus spp*. with *Neem* leaves with high values of 0.8319 and 0.8870 respectively were also observed, suggesting that the models produced were essential and ideal for use in this experiment. For *Neem* leaves, the expected R^2 value for *Rhodotorula mucilaginosa* and *Aspergillus spp.* of 0.5002 and 0.5319 were considered to be in proper alignment with the adjusted values for coefficient calculation.

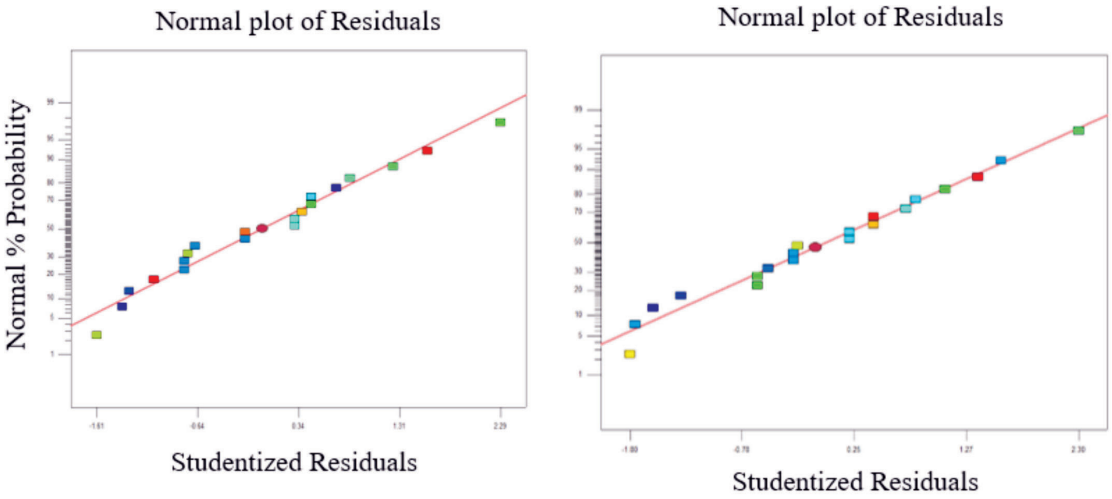
Notably, the negligible lack of fit (P > 0.050) was found to ensure a good fitness model for the experimental results for both regression equations. The acute effects of the extraction dosage (A) and time (B) were found to have the most impressive influence on the percentage of CFU. The most important factors were the second-order effect of extraction agitation (C) and two-level correlations between extraction dose and agitation (AC), dosage, and time (AB). In addition, the second effect of extraction dosage (A2) and extraction time (B2) and two-level extraction time interactions were observed to be the secondary effect of the CFU percentage.

Polynomial regression modelling was carried out based on the RSM effects on the corresponding coded values of the three different variables in this experiment, and the outcome was tested. The second-order equations in terms of coded factors for CFU of the *Rhodotorula mucilaginosa* and *Aspergillus spp*. with *Neem* leaves were Equations 2 and 3 respectively derived from the data regression analysis. The solvent extraction dosage (A) had the most significant effect on CFU, followed by extraction time (B) and agitation (C), according to all the equations. Similarly, the solvent extraction dosage (A) also has the most enormous effect on the percentage of CFU. These findings are consistent with the outcome of ANOVA in Table 5. An ANOVA table is typically used to summarise the test performed to substantiate a successful model.

CFU of *Rhodotorula mucilaginosa with Neem* = + 60.84 - 4.81 D - 0.37 T - 0.034 S - 0.016 DT - 0.018 DS + 1.25 TS + 0.97 D2 + 1.56 T2 – 2.27 S2 .. (2)

CFU of *Aspergillus spp.* with *Neem* = + 108 – 6.15 D - 0.22 T - 0.84 S - 0.033 DT - 0.018 DS + 4.16 TS + 1.25 D2 + 1.66 T2 + 3.75 S2 .. (3)

The analysis of the standard probability plot of the residual for both responses to the CFU of the *Rhodotorula mucilaginosa* and *Aspergillus sp.* with *Neem* leaves is shown in Figure 4. Both plots closely reflect a straight-line distribution of residuals. In the diagram, the straight-line suggests that errors are spread uniformly and endorse the adequacy of the least square fit. A random scatter seen in Figure 5 greatly ensures that the suggested models are sufficient and free from any desecration of the presumption of freedom or constant variance.



(a) (b)

Fig. 5. Normal plot of Residuals of *Neem* leaves for (a) *Rhodotorula mucilaginosa* and (b) *Aspergillus sp.*

Interaction of Variables during Optimization by RSM

Figures 7 and 8 display the consequences of the CFU over the most critical input variables, which are the extraction dosage (A), time (B), and agitation (C) in the interaction forms. Each of the figures represents (a) two-dimensional (interaction plots) and (b) three-dimensional (response surface plot) graph that displays the equation of the regression model finding the optimal values of the input variables within the process's chosen ranges to optimize responses. As shown by their P-values, the predictor tested in CCD has the most critical effect on CFU (P-values less than 0.050 suggest that model words are significant), as shown in Table 5. A remarkable influence on CFU was the relationship between extraction dosage and time (AB). The interactions of main factors during RSM pectin extraction optimization are detailed below:

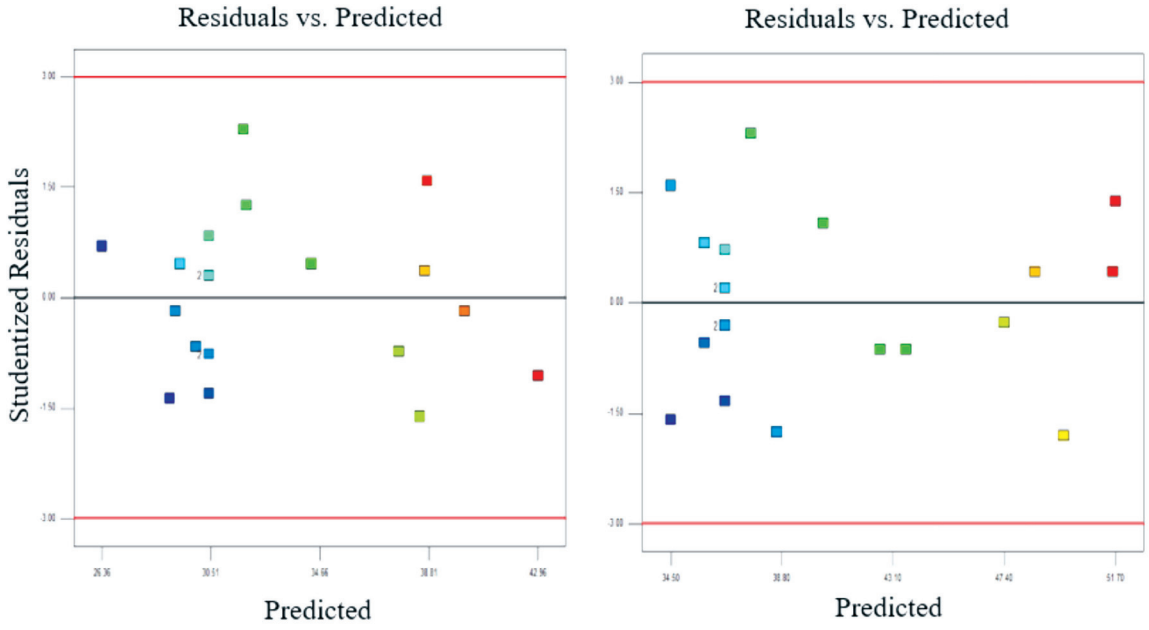
Table 5. ANOVA for the quadratic model adjusted to CFU of the *Rhodotorula mucilaginosa* and *Aspergillus spp.* with *Neem* leaves

Source	Sum of squares		Degree of freedom		Mean square		F-value		Prob > F	
	CFU/RM*	CFU/A*	CFU/RM*	CFU/A*	CFU/RM*	CFU/A*	CFU/RM*	CFU/A*	CFU/RM*	CFU/A*
Model	409.22	679.80	9	9	45.47	75.53	11.45	17.57	0.0004[a]	< 0.0001
A	193.60	336.40	1	1	193.60	336.40	48.73	78.23	< 0.0001	< 0.0001
B	62.50	52.90	1	1	62.50	52.90	15.73	12.30	0.0027	0.0057
C	19.60	2.50	1	1	19.60	2.50	4.93	0.58	0.0506	0.4634
AB	8.00	32.00	1	1	8.00	32.00	2.01	7.44	0.1863	0.0213
AC	4.50	4.50	1	1	4.50	4.50	1.13	1.05	0.3122	0.3304
BC	4.50	0.50	1	1	4.50	0.50	1.13	0.12	0.3122	0.7402
A^2	42.02	68.75	1	1	42.02	68.75	10.58	15.99	0.0087	0.0025
B^2	5.46	6.19	1	1	5.46	6.19	1.37	1.44	0.2682	0.2580
C^2	0.023	6.19	1	1	0.023	6.19	5.721	1.44	0.9412	0.2580
Residual	39.73	43.00	10	10	3.97	4.30				
Lack of Fit	27.73	33.67	5	5	5.55	6.73	2.31	3.61	0.1897[b]	0.0927[b]
Pure Error	12.00	9.33	5	5	2.40	1.87				
Cor Total	448.95	722.80	19	19						
R^2	0.9115	0.9405								
R	0.9645	0.9851	Prob > F less than 0.050 indicate model terms are significant							
Adj R^2	0.8319	0.8870	[a] model is significant, [b] lack of fit is not significant							
Pred R^2	0.5002	0.5319	CFU/RM *= UFC of the Rhodotorula mucilaginosa. CFU/ A* = UFC of the Aspergillus sp.							

When extraction agitation (C) was sustained at 120 rpm on *Rhodotorula mucilaginosa*, the interaction effect of the *Neem* leaves extraction dosage and time (AB) CFU was shown in Figure 7. Interaction and reaction surface plot analysis revealed that the CFU steadily decreased from 41 CFU at 1 mg/ml to 29 CFU at 5 mg/ml with the extraction dosage (A) while the time was sustained at 60 min, as shown in Figure 7 (a, b). However, *Neem*'s interaction effect leaves extraction dosage and time (AB) CFU on *Aspergillus sp.* when extraction agitation (C) was sustained at 120 rpm. In Figure 8, it is shown that interaction and reaction surface plot analysis revealed that the CFU steadily decreased from 47 CFU at 1 mg/ml to 37 CFU at 5 mg/ml extraction dose (A) while the time was constant at 60 min.

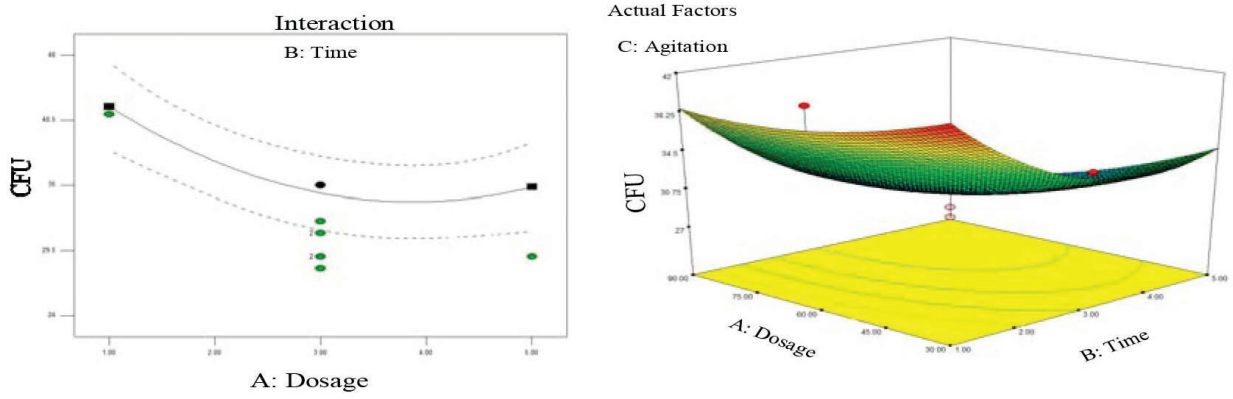
Conclusion

Microbial pollution has been described as a significant concern in both rural and urban areas. Until reaching the faucets of consumers, high-quality drinking water from treatment plants is subjected to contamination and severe deterioration by the



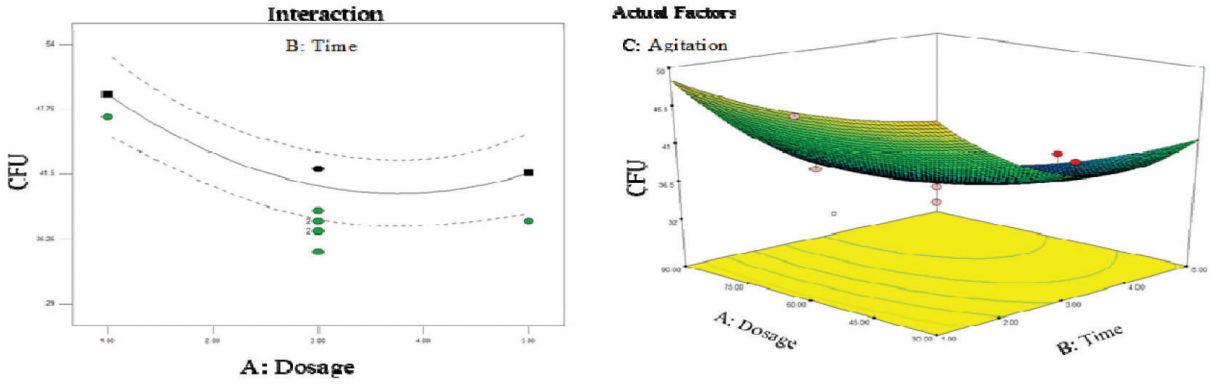
(a) (b)

Fig. 6. Residuals agents Predicted response plot of *Neem* leaves for (a) *Rhodotorula mucilaginosa* and (b) *Aspergillus sp.*



(a) (b)

Fig. 7. Interaction (a) & response surface plot (b) showing the effect of reaction dosage & time on CFU for *Neem* on *Rhodotorula mucilaginosa*



(a) (b)

Fig. 8. Interaction (a) and response surface plot (b) showing the effect of reaction dosage and time on CFU for *Neem* on *Aspergillus sp*.

drinking water delivery grid. Many reactions, chemicals, and biological agents and contacts with pipe walls can reach the pipeline from various sources, posing a big problem that could contribute to disease outbreaks and poor water quality. pH determination, temperature, heavy metal, total hardness, turbidity, sulphate, colour, chlorine, Escherichia coli, total coliform count, and fungal have all been included in this analysis as new data on the quality of drinking water in the Kuantan area. The samples were taken from the same resource in various parts of the study site. In Kuantan, Pahang, Malaysia, the tap water standard is deemed safe for domestic use. A study was conducted to see whether Neem leaves may function as antifungals in tap water. Water samples from public buildings and private homes in Kuantan were obtained and analysed. The samples were taken from a tap that collects direct water from Kuantan's Semambu Water Treatment Plant. Total coliform, Escherichia coli, pH, total hardness, sulphate, and identified heavy metals were all checked using Malaysian and WHO drinking water quality criteria. In most tap water tests, other consistency criteria such as colour, turbidity, and chloride were within reasonable limits, according to Malaysia's Food Act 1983 and the World Health Organisation.

The extraction and characterize of the Neem leaves were done in two steps. The extraction of the Neem leaves' active agent starts by wash, dry, and crush the biomaterials to make a powder. The traditional method (cold press) removed the oil and fatty substances without adding any chemicals. The aqueous cake extract was prepared using Neem leaves to powder and distilled water in a sterile conical flask and kept at 55°C overnight. In the end, the supernatant was collected and freeze-drying. The characterization of the antifungal extract from Neem before and after using FT-IR, SEM, TGA, and XRD to know the molecular structure, the surface morphology, the thermal gravity, and the thermal stability of the active agent of Neem leaves. Factors were identified from prior studies. The most significant factors are dosage, time, and agitation, which were selected for further evaluation of their impact on eliminating the fungi communities' growth in the drinking water using CCD in RSM. The CCD used a two-part complete factorial central composite architecture with five degrees of combination factors. Given the ease with which Neem leaves can be developed locally, their use in water treatment should be encouraged. This would inevitably reduce the high costs and health risks associated with commonly using chemical water treatments. The solutions in question are cost-effective, conventional, and simple to adopt, making them suitable for rural areas. Since the process is biological, no non-treatable wastes are generated.

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Declaration of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper. All listed authors are qualified for authorship as authors and mutually agree to submit it for consideration. Each author has participated and contributed sufficiently to take responsibility for every portion of the content.

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