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Modelling Microbial Inactivation in Water Treatment using Extracts from Stored *Moringa oleifera* Seeds

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Abstract
Proteins were isolated from Moringa oleifera (MO) seeds that had been stored in various forms, namely Winged seed in bottle (WS_{BO}), Winged seed in cellophane (WS_C), Seed pod in basket (SP_{BA}), Shelled seed in bottles (SS_{BO}), Shelled seed in basket (SS_{BA}) and Winged seed in basket (WS_{BA}). The isolated proteins were used as coagulants in the clarification of turbid water. Microbial analysis of the water before and after clarification showed that the protein extract from stored MO seed was effective in reducing the bacterial load of the water. The rate of bacterial removal from the purified water was monitored by plating out samples of the water taken at 10minutes intervals for the first 1hour and at 30minutes intervals for the next two hours as treatment progressed. At the end of the experimental period, Total Viable Count (TVC) removals were 96.93% for protein from WS_{BO}, 95.09% for WS_C, 92.73% for SP_{BA}, 92.73% for SS_{BO}, 96.67% for SS_{BA} and 94.76% for WS_{BA}. The TVC removals observed at the various intervals of time were fitted into Cerf (1977) inactivation kinetics model. These observed data effectively fitted into the model. Coefficients of determination ranged from 0.84 to 0.99. It was concluded that the inactivation kinetics of total viable bacterial count using Moringa oleifera seed extract as a disinfecting agent can be successfully predicted using an already existing Cerf (1977) model. However, the tailing effects observed, which suggested bacterial resistance, needs to be overcome to make the use of Moringa oleifera more effective in water treatment.

Keywords: *Moringa oleifera, coagulant, clarification, microbial, inactivation, kinetics*

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1. Introduction

A number of plant extracts including *Moringa oleifera* have been identified to have exhibited antimicrobial properties (Oluduro, 2012; Adetitun et al., 2013; Kalpana et al., 2013; Fouad et al., 2019). Gram positive and gram-negative bacteria have generally been susceptible to inactivation by plant extracts (Ajayi and Fadeyi 2015; Falowo et al., 2016; Amabye and Tadesse, 2016; Abdallah, 2016; Idris and Abubakar, 2016; Sanusi et al., 2017; Muhuha et al., 2018; Abdel-shafi et al., 2019; Nwonuma et al., 2019; Bancessi et al., 2020;). However, in spite of the number of researches undertaken to elucidate the antimicrobial properties of these plant extracts, work on the inactivation process is sparse. Kinetic models are useful for prediction of inactivation rate of microbes through the period of the reaction (Li et al., 2018). There is an exponential decrease with time of microbial population when exposed to any form of disinfection (Klotz et al., 2007).

Two hypotheses have been proposed to explain the disinfection process (Lambert and Johnston, 2001). These are the mechanistic and vitalistic hypotheses. The mechanistic hypothesis supported by the works of Chicks (1908) and others opined that the disinfection process operates in a manner similar to a chemical reaction, influenced by temperature in an orderly manner, making the Arrhenius equation applicable. This school of thought presented the inactivation process as a first order reaction (Klotz, 2007), although Chicks (1908) observed a departure from the simple law following resistance of some younger microbes within the population. The proponents of the vitalistic hypothesis, however are of the opinion that survivor response curves vary according to differences in resistance of the individual microbes (Klotz, 2007). Withell (1942) identified three

general types of time survivor response curves by bacteria to disinfectants, namely sigmoid, exponential and lag phase followed by exponential curves. On the other hand, Xiong et al. (1999), grouped the survival curves into four, namely linear curves, curves with a shoulder, biphasic curves (curves with a tailing) and sigmoidal curves (curves with a shoulder and a tailing). The linear survival process has been modelled by Chick (1908), curves with a shoulder (Buchanan et al. ,1993), biphasic curves (Cerf, 1977), curve with shoulder and tail (Whiting and Buchanan, 1992) and all response survivor curves model (Xiong, 1999). The aim of this paper is to establish that the inactivation kinetics of total viable bacterial count using *Moringa oleifera* as a disinfecting agent can be predicted using an already existing model.

2. Materials and methods

2.1 Collection of water sample

Turbid water samples were collected near a 35-metre-long bridge at Amansea, on the Enugu-Onitsha Express Road. The water was collected from the river side using a thoroughly cleansed plastic container. The container was further rinsed with the river water and then immersed until it was full. The cap was inserted while the container was still underwater. The water was transported to the laboratory immediately for determination of the microbial load. Ice packs were used to keep the water cool while being transported.

2.2 Collection of *Moringa oleifera* seeds

Moringa oleifera seeds were obtained from local growers located in Agulu, Anambra State, Nigeria. The seeds were thoroughly mixed together, and air-dried for fourteen days before being segregated for storage.

2.3 Seed storage

The harvested *MO* seeds were air-dried for a period of two weeks and then stored at room temperature of about 30^{o}C in different forms and different containers for a period of 150days. Winged seeds were stored in covered baskets hereafter called winged seed in basket, winged seeds in corked glass bottles (winged seed in bottle) and winged seeds stored in cellophane bags (winged seed in cellophane). Some shelled seeds were stored in covered baskets (shelled seed in basket) and shelled seeds were stored in corked glass bottles (shelled seed in bottle). Seeds not removed from the pods were stored in covered baskets (seed pods in baskets). Winged seeds refer to seeds removed from the pod with the outer brown covering intact, which has some wing-like components. Shelled seeds are the white *Moringa oleifera* kernel.

2.4 Extraction of disinfectant

The method of Nwaiwu et al. (2012) was adopted. Good quality seeds of *Moringa oleifera* were selected. The seed kernels were ground to fine powder, using an ordinary food processor. The seed powder was defatted using a Soxhlet extractor. The remaining cake after oil extraction from *Moringa oleifera* seeds was blended with seven hundred millilitres of water for about 2-3minutes, using a domestic food blender. The sample was filtered through a muslin cloth. The filtrate was the crude aqueous extract.

2.5 Protein precipitation

The crude extract was further processed to obtain a precipitate of protein. Cold Acetone Precipitation was adopted. Chilling the acetone was for the purpose of preventing protein denaturing during the precipitation process. The filtrate from the aqueous extraction was centrifuged for 6minutes at 10,000rpm to remove all remaining particles. The supernatant was poured into a measuring cylinder and the volume noted. The measured supernatant was poured into a container that had a volume capacity of twice its volume. An equal volume of chilled acetone was poured into the supernatant. The mixture was immediately stirred with a spatula and centrifuged for 6minutes at 10,000rpm. The supernatant was poured out. 20ml of chilled acetone was added to the sediment to enable the sediment to be scraped off the walls of the centrifuge tubes using a long spatula. The added acetone was filtered off through a Whatman filter paper to obtain the precipitate. The precipitates were air-dried for 24hours to let off all residual acetone, ground to fine dry powder and transferred into a tightly corked glass bottle. The dry-powdered form served to further protect the precipitated protein from being denatured.

2.6 Disinfection studies

2.6.1 Determination of microbial load of the raw water

As soon as the water sample was transported from the river to the laboratory, the microbial load of the water was determined. Fifty microlitre (50µl) of the water was placed on Petri dishes plated out with nutrient agar and incubated for 24hours. Developed colonies were manually counted and converted to residual total viable counts (TVC) in colony forming units per millilitre (Cfu/ml) of the water using Equation (1)

$$Number\ of\ colony\ forming\ units = \frac{N}{V\ x\ D} \quad (1)$$

where N is the number of colonies counted, V is the volume plated out(ml) and D is dilution.

2.6.2 Determination of microorganism removal from the water

The turbid water samples were clarified by flocculation-coagulation method, using the *Moringa oleifera* seed protein extracts as the coagulant. The coagulation process also ensured the disinfection of the water by the protein extract (Nwaiwu, 2011). Fifty milligrams (50mg) of the dry extract were added to one litre of raw water from Ezu river. Using a magnetic stirrer, the mixture was rapidly stirred for 25-30minutes and slowly for 40 minutes, to effect flocculation and coagulation. After stirring, the microorganism removal from the water samples (disinfection) was monitored by taking samples of the supernatant as settlement of the colloids progressed. 25 millilitres of supernatants were pipetted into sterilized sample bottles every 10 minutes for 1hour and every 30minutes for the next 2hours. The microbial loads of the pipetted supernatants were then determined using the same procedure as in (a). The entire process was repeated using the extract from seeds stored in each of the various modes of storage.

2.7 Theory of modelling biphasic concave upward curves

Establishment of sterilization or disinfection cycles is simpler when a survival curve can be described by a mathematical model. Two fraction models for describing biphasic curves have been proposed by Cerf (1977). These models assume a mix of unidentical individual microorganisms in a population possessing intrinsic variable degrees of resistance to the disinfection which is permanent (Cerf, 1977). A biphasic concave upward curve without shoulder results when two homogeneous populations of dissimilar resistance microbes are mixed (Cerf, 1977). These models accord a separate constant inactivation rate to each sub-population (Xiong et al., 1999). The less-resistant microorganisms are represented by the first straight portion of the curve while the deaths of the more resistant ones are shown by the second part (Xiong et al., 1999). The independent and irreversible inactivation of the two groups of microorganisms follow a first order reaction. Other models include those of Whiting -Buchanan (1992) and Xiong et al. (1999).

2.7.1 Cerf Model

According to Xiong et al. (1999), the above scenario can be expressed by the following differential equations which assist to propound the Cerf model.

$$\begin{cases} \frac{dN_1(t)}{dt} = -K_1 N_1(t) & t \geq 0 \\ N_1(0) = N_{01} & (N_{01} > 0; t = 0 \end{cases} \quad (2)$$

$$\begin{cases} \frac{dN_2(t)}{dt} = -K_2 N_2(t) & t \geq 0 \\ N_2(0) = N_{02} & (N_{02} > 0; t = 0 \end{cases} \quad (3)$$

$$\begin{cases} N(t) = N_1(t) + N_2(t) \\ N_0 = N_{01} + N_{02} \\ K_1 > K_2 \geq 0 \end{cases} \quad (4)$$
$$N_1(0) = N_{01}; N_2(0) = N_{02}$$

where $N_1(t)$ is the less resistant fraction with death rate K_1 at time t; $N_2(t)$ is the more resistant fraction with death rate K_2 at time t; K_1 is the death rate constant for $N_1(t)$ (/day); K_2 is the death rate constant for $N_2(t)$ (/day); N_t is the concentration of the whole population at time (t); N_0 is the concentration of the whole population at time t equal to zero and t is time (minutes)

Solving Equations (2) – (4) will yield

$$N_t = N_0(fe^{-k_1 t}) + (1-f)e^{-k_2 t} \quad (5)$$

2.7.2 *Whiting -Buchanan (1992) model*:

This model is expressed as follows:

$$log\frac{N_t}{N_0} = \log\{\frac{f(1+e^{-k_1 t_{lag}}}{1+e^{k_1(t-t_{lag})}} + \frac{(1-f)(1+e^{-k_2 t_{lag}}}{1+e^{k_2(t-t_{lag})}}\} \quad (6)$$

2.7.3 Xiong et al (1999) model

This is written as:

$$log\frac{N_t}{N_0} = log\{fe^{-k_1(t-t_{lag})} + (1-f)e^{-k_2(t-t_{lag})}\} \quad (7)$$

The Cerf (1977) model, Equation (5) was used to fit observed total viable bacterial count (TVC) data from the *Moringa oleifera* water disinfection process. The linear relationships between the observed data and the predicted values using the model were evaluated statistically with the use of coefficient of determination (R^2), Adjusted R^2, Standard error of estimate (€), Root mean square error (RMSE), Bias and percentage (%) differential between R^2 and Adjusted R^2. The adjustment for the degree of freedom in the derived linear models made to the coefficient of determination (R^2) to avoid an upward bias is represented by the adjusted R^2 (Murphy, 1973). The best overall prediction

accuracy of the inactivation models was determined using RMSE of the linear models, comparing predicted and observed values, Equation (8). Model over-prediction or under-prediction was evaluated using the Bias, Equation (9) (Nwaiwu et al, 2005)

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n} d_i^2} \quad (8)$$

$$d = \frac{1}{n}\sum_{i=1}^{n} d_i \quad (9)$$

where d_i is the difference between predicted and measured values while n is the number of the measured values of total viable counts. The percentage difference between R^2 and adjusted R^2 was used to quantify the disparity between the two occasioned by the adjusted. The purpose was to establish the model possessing the best coefficient of determination R^2 and least affected by the adjustment.

3. Results

3.1 Residual total viable counts (RTVC)

Table 1 shows values of residual total viable counts of samples taken at intervals of 10 minutes for the first one hour and 30 minutes for the next two hours. The Table also shows the percentage TVC removals at the end of the three hours.

Table 1: Residual total viable counts against settling time

Settling Time	Winged seed in bottle ($x10^5$)	Winged seed in cellophane bag ($x10^5$)	Pod seed in basket ($x10^5$)	Shelled seed in bottle ($x10^5$)	Shelled seed in basket ($x10^5$)	Winged seed in basket ($x10^5$)
0	3.26	3.26	1.1	1.1	2.1	2.1
10	1.14	1.98	0.98	0.9	0.94	0.98
20	0.72	1.76	0.36	0.5	0.6	0.93
30	0.52	0.96	0.32	0.44	0.30	0.38
40	0.4	0.62	0.3	0.42	0.25	0.36
50	0.34	0.58	0.28	0.36	0.20	0.32
60	0.2	0.52	0.24	0.28	0.18	0.28
90	0.19	0.34	0.18	0.12	0.10	0.15
120	0.15	0.34	0.1	0.08	0.08	0.14
150	0.12	0.21	0.08	0.08	0.07	0.11
180	0.1	0.16	0.08	0.08	0.07	0.11
%Final Removal	96.93	95.09	92.73	92.73	96.67	94.76

3.2 Log-inactivation curves

In Fig. 1 is shown the plot of log inactivation curves against time for *Moringa oleifera* treated water using extracts from the six storage modes namely winged seed in bottle, winged seed in cellophane bag, seed pod in basket, shelled seed in basket and winged seed in basket. The two segments of the biphasic - upward concave curve for the winged seed in bottle (for the less and more resistant microbes respectively) yielded inactivation rate constants of K_1= 0.0508 and K_2 = -0.0062. The respective coefficients of determination for the linear equations describing the respective segments were 0.844 and 0.972. The commencement of the tail section of the curve was observed after 60 minutes of disinfection until the end of the experiment at 180 mins. This yielded initial proportion of the less resistant fraction f = 0.939 (Table 2).

The coefficients of determination for the winged seed stored in cellophane bag were 0.972 for both segments of the inactivation curve. Tailing began after 40 minutes from the onset of disinfection. The seed pod in basket had linear inactivation coefficients of K_1= 0.0347 and K_2= 0.0107. The two linear models describing the curve had coefficient of determination of R^2= 0.933 and 0.947 respectively and tailing set in after 40 minutes into the disinfection process. Tailing was observed to begin after 30 minutes of disinfection inception for the shelled seed in bottle (K_1=0.032, K_2= 0.013, R_1^2= 0.936; R_2^2=0.8722); shelled seed in basket (K_1=0.065, K_2=0.010, R_1^2=0.987, R_2^2= 0.8977) and winged seed stored in basket (K_1=0.054, K_2=0.009, R_1^2=0.913, R_2^2=0.912).

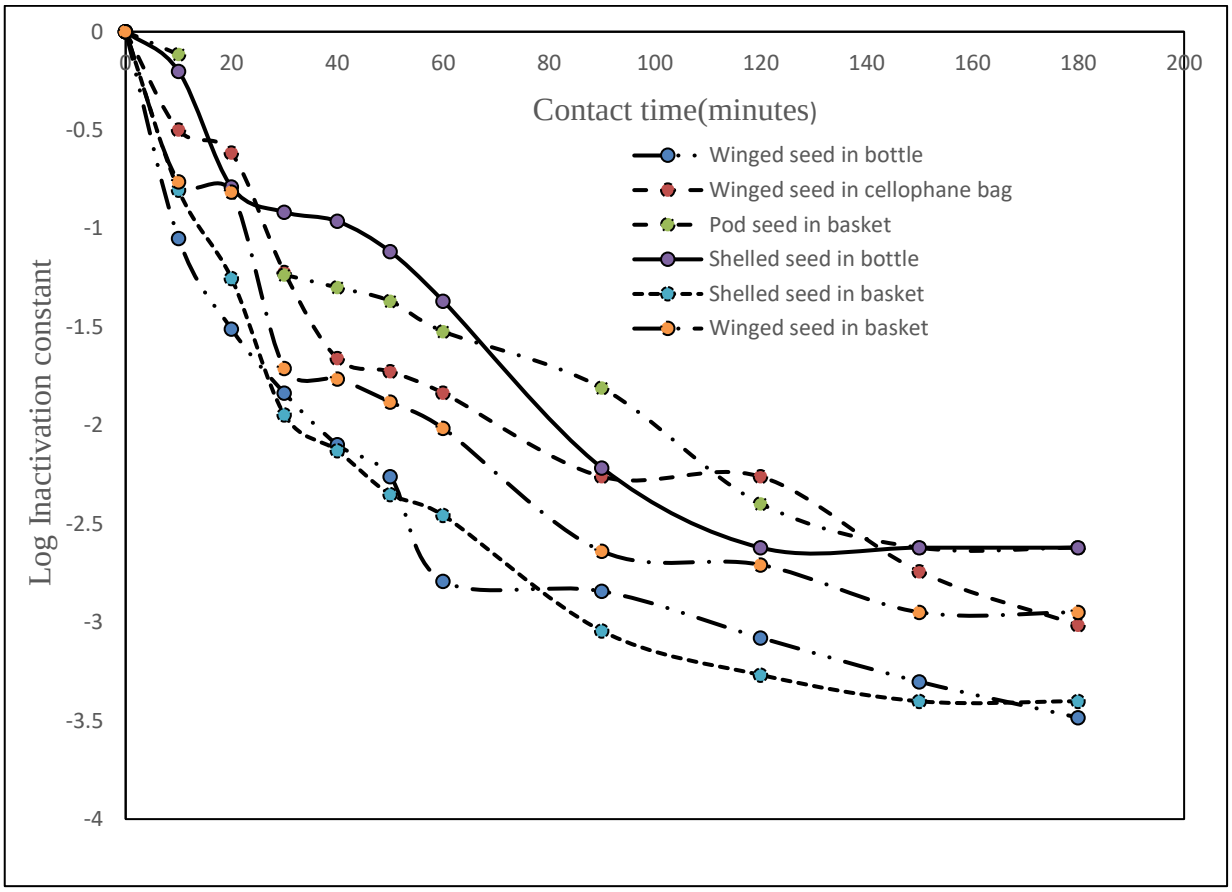

Fig. 1: Log inactivation constant versus contact time

Table 2: Microbial inactivation model kinetic parameters

Storage Mode	Regression Inactivation Relationships (log inactivation constant (y) versus contact time (x))	Coefficient of Determination ($R^{2)}$	Remarks
Winged seed in bottle	a) Less resistant fraction Linear: $y = -0.0508x$ Poly: $y = 0.0006x^2 - 0.0817x$ b) More resistant fraction Linear:$y = -0.0062x - 2.3614$ Poly:$y = -2E-05x^2 - 0.00148x - 2.6102$	0.8436 0.9568 0.9716 0.9843	Tailing was observed after 60 minutes of disinfection f = 0.939
Winged seed in cellophane	a) Less resistant fraction Linear: $y = -0.0401\,x$ Poly: $y = -0.0002x^2 - 0.0337x$ b) More resistant fraction Linear: $y = -0.0096x - 1.2699$ Poly:$y = -5E\,06x^2 - 0.0085x - 1.3163$	0.9718 0.9763 0.9727 0.973	Tailing began after 40minutes of disinfection f = 0.8098

Seed pod in basket	a) Less resistant fraction Linear: $y = 0.0347x$ Poly: $y = -9E-05x2 - 0.0315x$ b) More resistant fraction Linear $y = -0.0107x - 0.8939$ Poly: $y = 5E-05x2 - 0.0212x - 0.4509$	0.9327 0.9334 0.947 0.9739	Tailing began after 40mins with the exclusion of the out lier f = 0.727
Shelled seed in bottle	a) Less resistant fraction Linear: $y = 0.0323x$ Poly: $y = -7E-05x^2 - 0.0341x$ b) More resistant fraction Linear : $y = -0.0133x - 0.6042$ Poly: $y = 0.0001x^2 - 0.0373x + 0.298$	0.936 0.9363 0.8722 0.9726	Tailing was taken to begin at 30minutes into the disifection process f = 0.6
Shelled seed in basket	a) Less resistant fraction Linear: $y = 0.0653x$ Poly: $y = 0.0004x^2 - 0.0745x$ b) More resistant fraction Linear: $y = -0.0102x - 1.8272$ Poly: $y = 9E-05x2 - 0.0282x - 1.1521$	0.9871 0.9905 0.8977 0.9958	Tailing was taken to begin at 30mins of disinfection f = 0.857
Winged seed stored in basket	a) Less resistant fraction Linear: $y = -0.0537x$ Poly: $y = 0.0001x^2 - 0.0504x$ b) More resistant fraction Linear: $y = -0.0093x - 1.4898$ Poly: $y = 6E-05x^2 - 0.0223x - 1.001$	0.9129 0.9135 0.9116 0.9751	Tailing was taken to begin at 30mins of disinfection f = 0.819

3.3 Data fitting into established model

The Cerf Model addresses the tail or tailing of biphasic or upward concave curves and so was used to predict microbial inactivation by extracts of stored *Moringa oleifera* seed. This decision is as a result of the shape obtained from the plot of log inactivation constant against reaction time as shown in Fig. 1. The plots of the observed and predicted values for all the extracts from the storage modes using the Cerf model are shown in Fig. 2 – 7.

The coefficient of determination (R^2) for the plots of Cerf predicted versus observed values for the extracts are respectively 0.9122, 0.971, 0.9464, 0.9641, 0.9801 and 0.9581 (Table 3). The percentage difference between R^2 and adjusted R^2 for all the predicted observed value equations were 1.1%, 0.33%, 0.45%, 0.43% 0.23% and 0.4801% (Table 3).

A one-way analysis of variance performed on the observed and Cerf model predicted values produced the following values. Winged seed in bottle ($0.683>p>0.05$); Winged seed in cellophane ($0.847>p>0.05$), seedpod in basket ($0.951>p>0.05$), shelled seed in bottle ($0.853>p>0.05$), shelled seed in basket ($0.8044>p>0.05$) and winged seed in basket ($0.8111>p>0.05$).

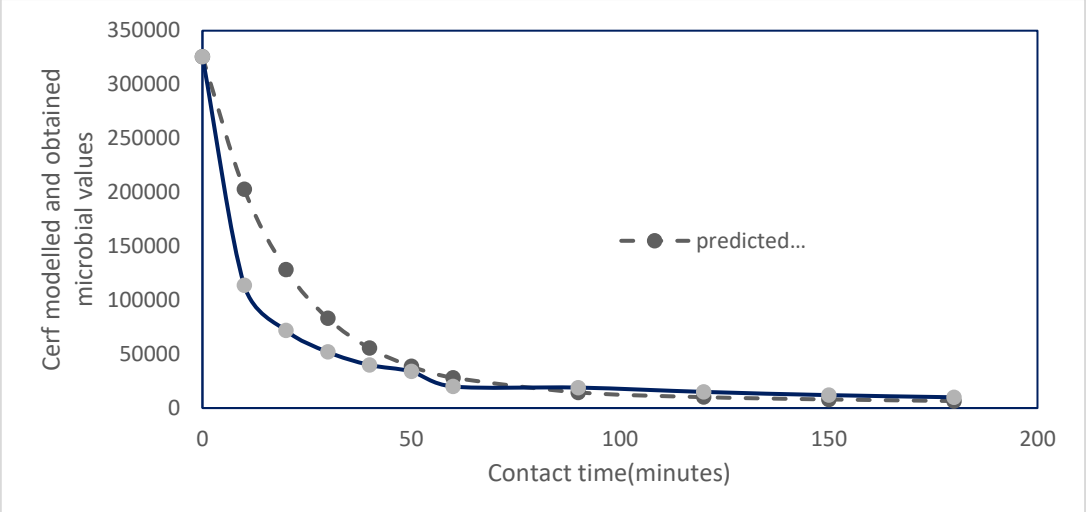

Fig. 2: Cerf modelled and observed microbial values versus contact time (winged seed in bottle)

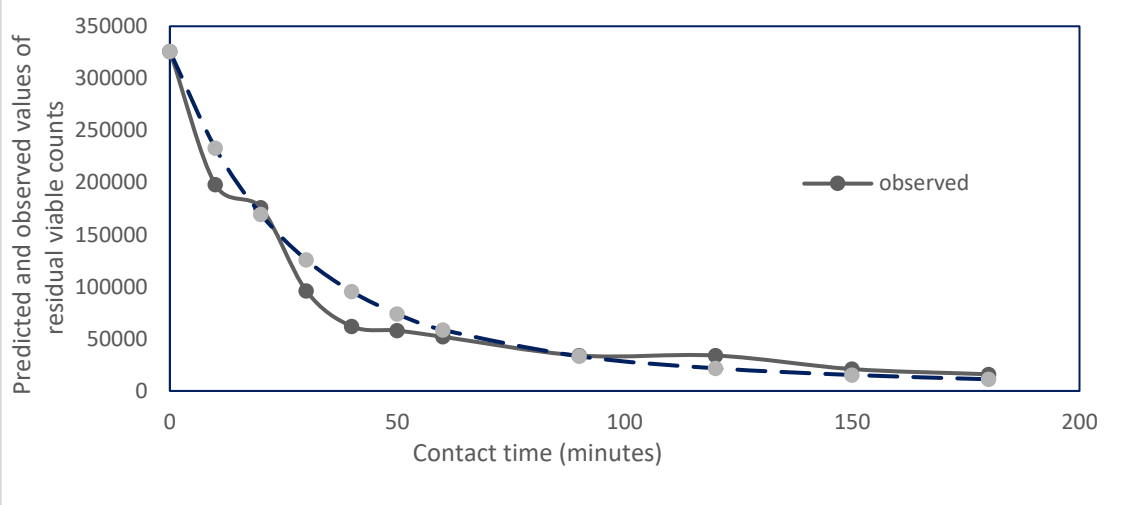

Fig. 3: Predicted and observed residual viable count against time (winged seed in cellophane)

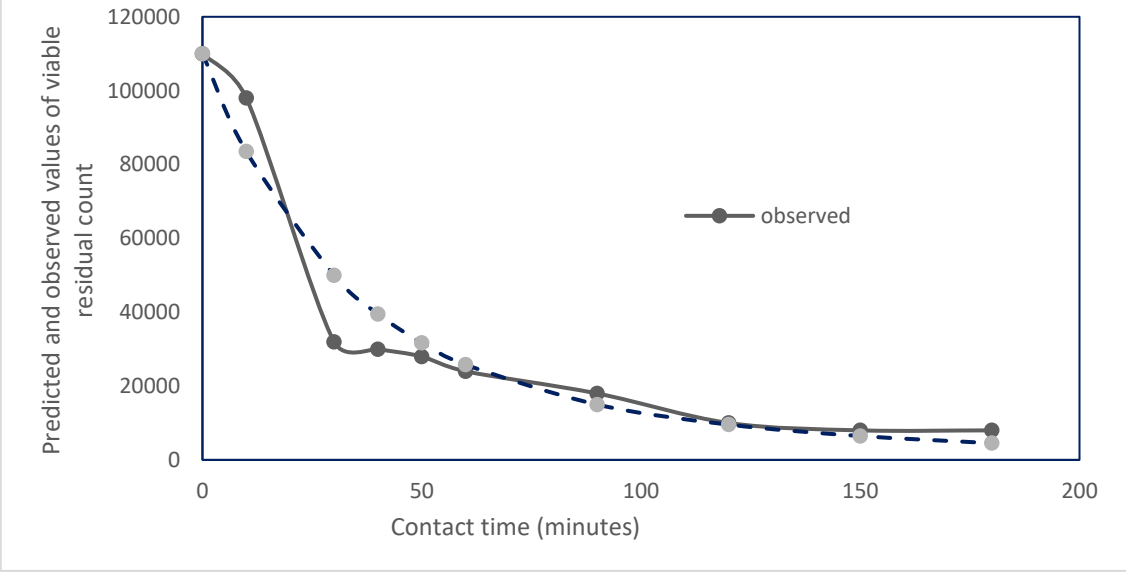

Fig. 4: Predicted and observed values of viable residual count against time (seed pod in basket)

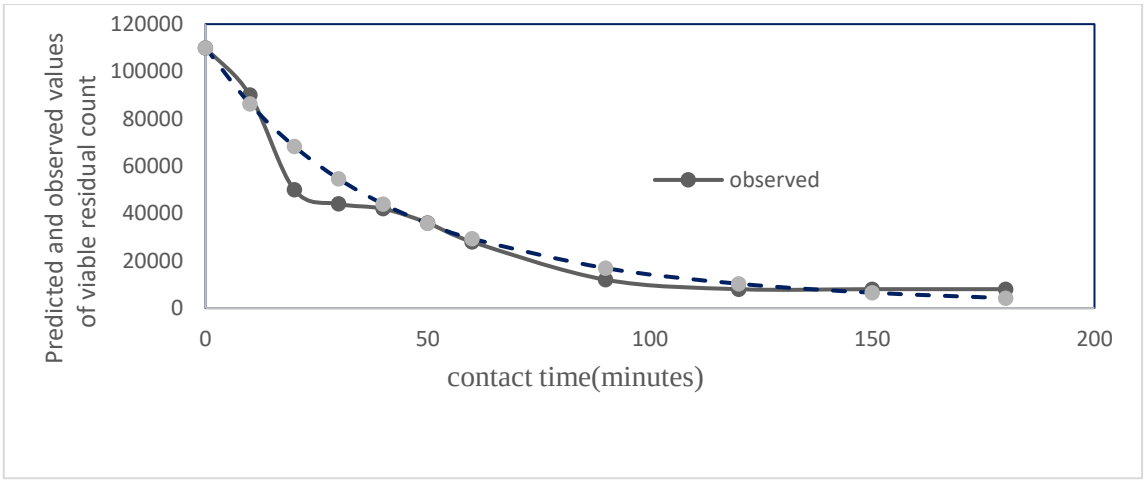

Fig. 5: Predicted and observed viable residual count versus time (shelled seed in bottle)

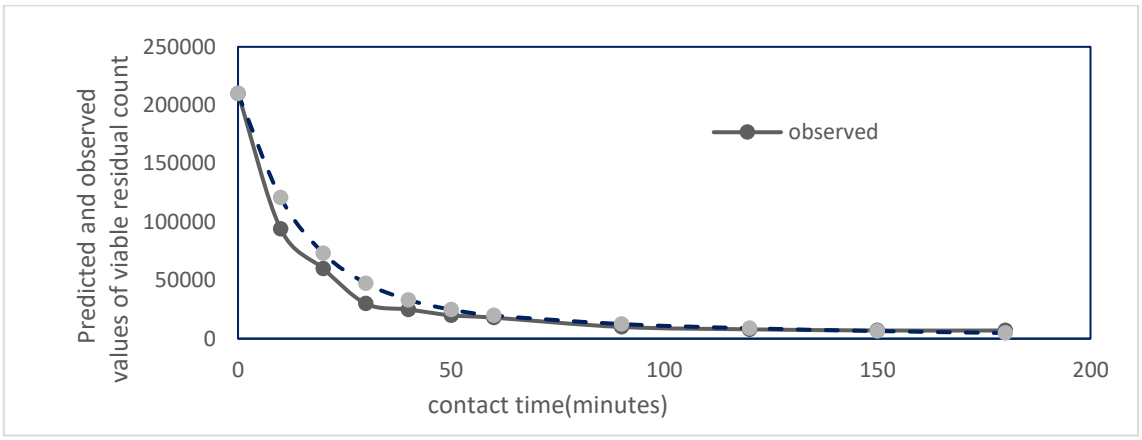

Fig. 6: Predicted and observed viable residual count versus time (shelled seed in basket)

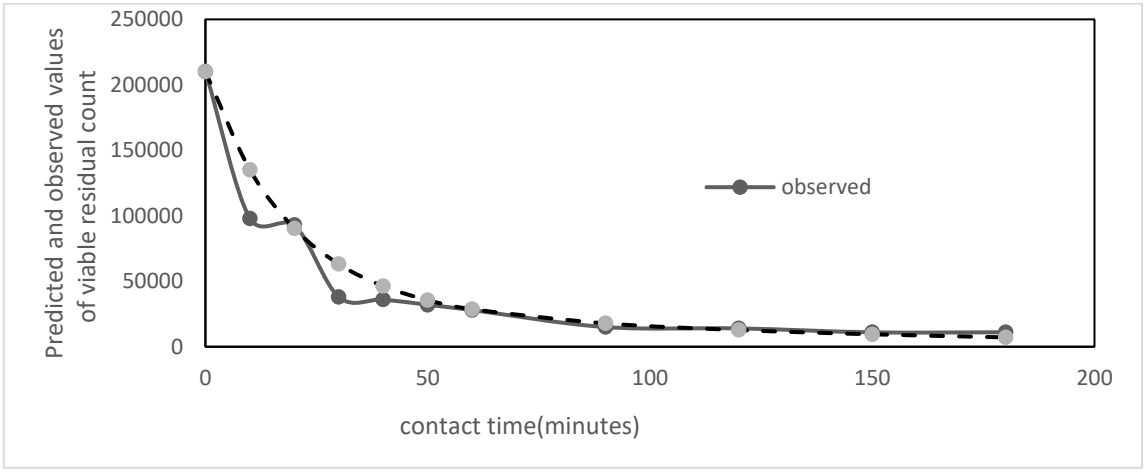

Fig. 7: Predicted and observed viable residual count versus time (winged seed in basket)

Table 3: Statistical comparison of observed and Cerf (1977) model predicted data

S/N	Storage Status/ Model	R^2	Adjusted R^2	Standard Error	RMSE	Bias	Percentage difference between R^2 and Adjusted R^2
1	Winged seed in bottle	0.9122	0.902	*	*	*	1.1%
2	Winged seed in cellophane	0.971	0.9678	*	*	*	0.33
3	Seed pod in basket	0.9464	0.9397	*	*	*	0.45%
4	Shelled seed in bottle	0.9641	0.96	*	*	*	0.43%
5	Shelled seed in basket	0.9801	0.9779	*	*	*	0.23%
6	Winged seed in basket	0.9581	0.9535	*	*	*	0.4801%

4. Discussion

4.1 Log-inactivation curve

Fig. 1 shows the biphasic – upward concave curves for the log inactivation of *Moringa oleifera* disinfection. These curves are reported with the nomenclature suggested by Pflug and Schidt (1968) and Board and Carr (1976) for biphasic and concave upward curves respectively. The upward concave shape of the curves may be a result of the free energy of the reversible reaction state of sensitive to resistant bacteria possessing a negative value as opined by Komemushi and Teni (1967). Tails are observed in all the curves plotted. Cerf (1977) aforesaid that the tailing phenomenon is a normal feature bound to the mechanisms of resistance and inactivation during disinfection and that survival curves are typically sigmoidal or at least concave upward. The resistance which midwifes the tailing phenomenon is thought to be brought about by natural random mutation (Long and Vester, 2008). In trying to explain the tailing effect, Cerf (1977) and Tan et al. (2017) postulated that the individual microbes possessing the average degree of resistance were in majority while maximum or minimum degree of resistance were possessed by the minority of the population. In conjunction with the above, two groups of microbes present in the population are responsible for the upward concave shape of the curves.

The two segments of the biphasic curves possessing two inactivation rate constants K_1 and K_2 (Table 1) show that two microbial groups are in the population in agreement with Cerf (1977). It is also seen that the rate constants K_1 are higher than K_2. K_1 and K_2 represent the rates of reaction of the sensitive and resistant groups respectively. The higher K_1 values show that this group of microbes are more susceptible to the disinfection unlike the resistant group with lower K_2 values. The tailing effect did not commence at the same time in each disinfection run. However, the average of the percentage of the population including the susceptible group was 79.2% while the resistant group comprised an average of 21.8%. The high coefficient of determination for the microbial decay models for the two groups ranged between 0.844 to 0.996. This shows a high correlation between the log inactivation constant and contact time. The higher reaction rates also mean that the rate of kill of the susceptible group proceeded faster.

4.2 Data fitting into Cerf (1977) model

The observed data for disinfection using six *Moringa oleifera* sample extracts were fitted into Cerf (1977) inactivation model as shown Figures 2-7. Linear regression equations were established to compare the observed data and the predicted values using Microsoft Excel for windows (version 10) The goodness of fit of the linear models were assessed between the observed and predicted values using the Root Mean Square Error (RMSE), coefficient of determination (R^2), Bias and percentage difference between R^2 and adjusted R^2. The Cerf (1977) model effectively fitted the observed data. The model did not consistently fit the observed survival curves best. In terms of coefficient of determination R^2, the Cerf model performed best for extracts from winged seed stored

in cellophane and shelled seed stored in basket with values of R^2=0.971 and R^2=0.980 respectively. The significance of the R^2 values for the model and extracts is exhibited by the low values of the percentage difference between R^2 and Adjusted R^2 values ranging between 0.23% and 1.2%. The RMSE for the models are all less than 0.5 and therefore considered not very significant (Xiong et al., 1999). This also goes for the Bias. This established model will therefore be useful in estimating the kinetic parameters governing water disinfection using natural coagulants (Li et al., 2018) such as *Moringa oleifera*.

5. Conclusion

The inactivation kinetics of total viable bacterial count using *Moringa oleifera* as a disinfecting agent can be successfully predicted using an already existing model. However, the tailing effect suggesting bacterial resistance needs to be overcome to make the use of *Moringa oleifera* more effective in water treatment.

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