APPLICATION OF RESPONSE SURFACE METHODOLOGY FOR OPTIMIZATION OF BIO-PESTICIDE PRODUCTION FROM NEEM SEED OIL

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Abstract: The cost and effect of inorganic chemical pesticides on the environment and living organism as well as growing resistance of inorganic chemical pesticides is a threat to their future application as pesticide on crops. This research focuses on optimization of bio-pesticide production from neem seed oil using response surface methodology. The neem seed oil was characterized to test it suitability for production of bio-pesticide and the use of response surface methodology involving central composite design to optimize the pesticide formulation parameters. The characterized oil was found with a specific gravity, saponification value, iodine value, pH value of 0.912, 180.55mg/g, 86.42 g/g and 5.36 respectively. The optimum response was found to be 64.53% weight reduction of pest after 3 days under the optimized conditions of 17.96ml of neem seed oil, 4.51ml of emulsifier and 5.42ml of water, the developed model was significant and is confirmed by the P-value of less than 0.0001 and the higher F value of 21.0196. Design Expert 7.0 software was used for the optimization of bio-pesticide production from neem seed oil, emulsifier and water as variables using response surface methodology.
Keywords: Neem Seed oil, Characterization, Bio-pesticide, Optimization, Army worm weight reduction

Introduction

Pesticides are chemical materials utilized to aid the production and yield in agricultural practices by preventing, repelling and destroying pests (Choudhary *et al*., 2017). Herbivorous insects have been said to be responsible for destroying one fifth of the world's total crop production annually. Insect pests are capable of evolving to biotypes that can adapt to new situations, for example, overcome the effect of toxic materials or bypass natural or artificial plant resistant, which further confounds the problem (Roush and Mckenzie, 1987). One of the serious challenges confronting mankind has always been sustainability of food. A major cornerstone in this challenge is the competition from insect pests. Particularly in the tropics and sub-tropics, where the climate provides a highly favorable environment for a wide range of insect pests, strong efforts are being required to suppress population densities of the different pests in order to achieve an adequate food supply.

Pesticides are predominantly classified into two base on their source of origin as synthetic (Chemical) pesticide and natural (bio-pesticide) (Devi and Maji, 2011). The utilization of synthetic pesticides has given undoubtedly results in increasing of crop production. However, synthetic pesticides that are used to control plant diseases are bringing irreparable harm and damage to our fragile environment. Environmental issues, detrimental health effects, insect resistance or marketing opportunities for organically produced food are well-known arguments against the use of synthetic pesticides (Devi and Maji, 2011). The increasing awareness and concern about the impact of agricultural practices on the environment and in food and fiber production is promoting the concept of sustainable agriculture; thus, raising the thrust for bio-pesticides over synthetic pesticides. The increasing incidence of pesticide resistance is also fueling the search for more environmentally and toxicologically safe and more selective and efficacious new bio-pesticides.

Neem is a member of the Mahogany family (Meliaceae) and it contains an active ingredient known as Azadirachtin, which is a proven natural anti-feedant, anti-ovipository, growth regulator and insect repellent (Khanam *et al.,* 2017). It is toxic to soft bodied insect larvae. Azadiracthin has proven effectiveness as a pesticide against many insect species by repelling or reducing their feeding (Usharani et al., 2019). Since 1963 Indian scientists extensively studied the chemistry of the active principles of Neem (Gebrehawaria, 2017). Following the discovery of Neem kernel as a locust feeding deterrent, its chemistry has grown considerably. Many compounds have been isolated and characterized. The important feature is that majority of them are chemically similar and biogenetically derivable from a Tetracyclicterpenes. These are also refered to liminoids bitter principles and occur in other botanical species as well. The unraveling of high complex structural features and biogenetic interrelationship represent classic piece of work on natural product chemistry.

From the practical side, these compounds also show a broad variety of biological activity (Gebrehawaria, 2017). Additionally, it is in use and have high curative in some rural and urban areas in the developing countries. For example, in the Northern part of Ethiopia (Tigray), specifically in central part, the Neem leave and seed were utilized to protect the crops from pests by steeping the crops to a 24 hr water solution of Neem leaves (Gebrehawaria, 2017).

Nowadays, because of environmental, health, resource utilization, conservation as well as specific target effect on a particular pest have been the main reasons for considering organic, or bio-pesticide production instead of synthetic ones that is largely encouraged by different countries. According to WHO report of 2008, twenty five percent of the world production of inorganic chemical pesticide is applied in developing countries which lead to 99% death rate as result of pesticides. Up to 20,000 people die because of inorganic chemical pesticide poisoning in the third World countries each year (Choudhary *et al.*, 2017; WHO, 2008) In a nutshell, the major driving forces that drew the attention of researchers on the need for bio-pesticide to inorganic chemical pesticide are: quick decomposition, effectiveness in smaller quantities, environmental friendly and often cheaper to chemical pesticides (Devi and Maji, 2011; National Research Council, 1992). Neem based pesticides has less prone to pest resistance.

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables and also can help in investigating the interactive effect of process variables and in building a mathematical model that accurately describes the overall process. The conventional linear optimization involves changing a parameter and fixing other variant parameters constant which is more commonly referred as one-variable-at-a-time. RSM uses several variables by operating the regression statistical analysis on the independent variables to obtain the optimum overall factor surface. Through the representation of the surface model, responses on the basis of the combined variable can be determined. This investigation focuses on optimization of bio-pesticide production from neem seed oil using a full-factorial central composite design (CCD) method of RSM statistical analysis with a probability of significance 5% (α = 0.05).

Materials and Methods

Source of Neem Seed and Preparation

Matured seeds containing kernels at a light-yellow color stage were collected from Neem trees in the immediate environment of ModibboAdama University, Yola. After collecting the sample, it was packed in a polyethylene bag, washed to remove dirts, sun dried and stored at room temperature in an open air before use (Naniwadekar and Jadhav, 2012).

The neem seeds were further processed for moisture content. In this case, the seeds were hulled and put in an oven at 45 ^{o}C for four days, such that the moisture content was determined every 24 hours from equation 1 (Gebrehawaria, 2017).

$$\text{Moisture content (\%)} = \frac{W_1 - W_2}{W_1} \times 100\% \quad (1)$$

Where: W1 - initial weight of sample, (g); W2 – final weight of sample, (g).

The dried sample was then milled by an attrition miller **(**Retsch GmbH, 5657 HAAN**)** and made ready for Ash content analysis before extraction. Ash content of an organic sample was determined by igniting the oven dried sample obtained from the moisture content determination performed in a muffle furnace at 750 ^{o}C. The substance remaining after ignition is the ash. It is expressed as a percentage of the mass of the oven-dried sample (equation 2). The organic matter was evaluated based on equation 3 (Gebrehawaria, 2017).

$$\text{Ash conent (\%)} = \frac{Weight\ of\ Ash\ (g)}{Weight\ oven\ dried\ sample\ (g)}(100) \quad (2)$$

$$\text{Organic Matter(\%)} = 100 - \text{Ash content(\%)} \quad (3)$$

Neem Seeds Oil Extraction Method

Dried grounded seeds were sorted based on particulate sizes from 0.5mm to 1mm. The ground powder and hexane were placed into a batch soxhlet extractor for the purpose of extraction. The powder sample was placed in the timble while hexane was placed in the bottom flask which was heated until the extraction was completed. The samples (oil-hexane solution from the solid fraction) obtained were further heated and evaporated to get solvent free oils which were further analyzed for oil content, saponification value, iodine value, specific gravity and infrared spectroscopy.

The oil content was determined after extraction, filtration and stripping of solvent utilizing equation 4.

$$Oil\ content\ (\%) = \frac{weight\ of\ oil\ extracted}{weight\ of\ sample\ seed\ used}(100) \quad (4)$$

Physical and Chemical Characterization of Neem Seed Oil

The specific gravity was determined by specific gravity bottle known as Pycnometer using IS: 1460-2000 method based on the Archimedes principle of displacement. This was carried out at room temperature and pressure. Specific gravity is the measurement of relative density of sample with respective to water according to equation 5.

The iodine value is the degree of unsaturation of fat or oil. It is defined as the amount in grams of iodine absorbed by 100g of oil and fat performed according to the AOCS Cd 1-25 method. The iodine value was determined according to equation 6.

$$\text{Specific gravity} = \frac{\text{density of sample}}{\text{density of water}} \quad (5)$$

$$\text{Iodine value} = \frac{12.69\ (B - S)N}{W} \quad (6)$$

Where B and S represent the volume in ml of standard sodium thiosulfate required for blank and sample, respectively, N stands for the normality of standard sodium thiosulfate solution and W stands for weight of neem seed oil sample.

Saponification value (SV) is the number in milligrams of KOH (potassium hydroxide) required to completely hydrolyze 1g of oil to glycerol and soap. The saponification value of the oil was determined by means of standard titration method (AOCS official method Cd 3-25) utilizing equation 7.

$$SV = \frac{56.1\ N\ (V_b - V_s)}{W} \quad (7)$$

Where, V_b is the volume of HCI used for blank, V_s is the volume of HCI used for sample, N is the nomality of the HCI, and W is the weight of the test sample

The structural functional group of the oil was assessed using the Fourier Transform Infrared (FTIR) Spectrophotometer (Bulk Scientific Model M530 Infrared Spectrophotometer, Thailand). The spectrum was recorded over the range of 4000 – 500 cm^{-1}

Pesticide Formulation

A water-soluble bio-pesticide were formulated using Neem Seed Oil, mixed with an emulsifier (liquid soap) to help the oil stick to the leaf on application (Mondal and Chakraborty, 2016) and water in varying ratios to obtain the optimum mixing ratio that is effective on the pest (Naniwadekar and Jadhav, 2012).

Optimization of Bio-pesticide Formulation

The experiment design was carried out using Design Expert 7.0. The RSM ascertain the number of experiments to be assayed for the optimization and a full-factorial central composite design (CCD) was adapted involving the three independent variables comprising of neem seed oil, emulsifier and water with reaction temperature of 35°C as presented in Table 1. The range of values chosen for each independent parameter was based on information from literature (Naniwadekar and Jadhav, 2012).

Table 1: Pesticide Formulation Variables and their Levels in RSM Design

Variables	Symbols	-1	0	+1
Neem seed oil (ml)	A	12	15	18
Emulsifier (ml)	B	3	5	7
Water (ml)	C	5	7	9

Numerical optimization of formulation variables

The formulation variables and response for the bio-pesticide were optimized numerically using the software to predict their optimum values. The variables were set at their individual ranges of lower and upper limit (Table 1). The response (% weight reduction) was set to maximize, solutions was clicked and optimum conditions were generated for the variables. The optimum conditions generated by the software were experimented in the laboratory to confirm the predicted % weght reduction.

Bioassay

Bean and corn leaves were sprayed with the formulated pesticide and army worms were used as hosts to determine their daily % weight reduction.

pH value

The pH value of the extract and formulation were measured by dissolving 500 mg of sample in 10 ml of deionized water then the electrode was inserted into the solution to be measured and the value was read directly at room temperature and 1atm.

Total weight lost (TWL)

The total weight lost or % weight reduction was measured to determine the effectiveness of the formulated pesticide and is given by equation 8.

$$TWL\ (\%) = \frac{intial\ weight\ of\ host\ before\ dosage - final\ weight\ of\ host\ after\ dosage}{intial\ weight\ of\ host\ before\ dosage} \times 100 \qquad (8)$$

Results and Discussion

Characterization of Neem Seed and Extracted Oil

Properties of the Neem Seeds that were used for Neem Seed Oil production were evaluated as shown in Table 2.

Table 2: Properties of NeemSeeds used for Oil Extraction.

S/N	Properties	Values
1	Weight of Neem seed (g)	500
2	Sample size (mm)	0.5-1
3	Moisture content (%)	18.6
4	Mass of oil extracted (g)	212
5	Ash content of neem seed (%)	3.9
6	Organic matter (%)	96.1
7	% Oil content	42.4

Table 2 indicated that the seed has a moisture content of 18.6 % of its initial weight and an ash content of 3.9 %, which implies the seed has a good bio-active oil extract with less non-organic compounds present. In addition, the high content of Organic matters of 96.1 % shows the oil is biodegradable and environmentally sustainable (Gebrehawaria, 2017). The mass of oil obtained was 212 g which was 42.4 % of initial weight of neem seed.

The results of physical and chemical characterization of the neem seed oil extracted are presented in Table 3.

Table 3 Physical and Chemical Characterization of Neem Seed Oil

S/N	Property	Values
1	Specific gravity	0.912
2	Saponification value (mg/g)	180.55
3	Iodine value (g/g)	86.42
4	Colour	Yellow
5	P^H	5.36
6	Taste	Bitter
7	Odour	Irritating

Table 3 shows characteristics of the extracted oil. The specific gravity of the oil indicates that it will stay for a considerable period after the application as pesticide and the saponification value indicates that it can sufficiently be used for soap production. The iodine value from Table 3 is 86.42 g/g which is low. The low value shows that the oil can be classified as non-drying oil (Ochi et al., 2020).

Figure 1 depicts FTIR spectrum of the neem oil expressing the functional group present which is responsible for the biological activities, such as pesticidal activity and antifeedant etc. From the spectrum, peak at 3463.32 cm^{-1} corresponds to the stretching vibration of O-H bonds of water of the unsaturated fatty acid in the oil. Peaks at 2920.50 cm^{-1} and 2857.85 cm^{-1} attribute to the stretching mode of assymetrical and symetrical vibration of aliphatic C-H in CH_2 and CH_3 terminal groups. The peak appearing at 1641.49 cm^{-1} attributes to C=O bending vibraton of the carbonyl groups (Ochi et al., 2020). Peaks that appeared at 1454.97 cm^{-1}, 1155.18 cm^{-1} and 724.07 cm^{-1} can be attributed to C-H bending, C-O stretching and CH_3 stretching vibrations in the oil, respectively (Tulashie et al., 2021).

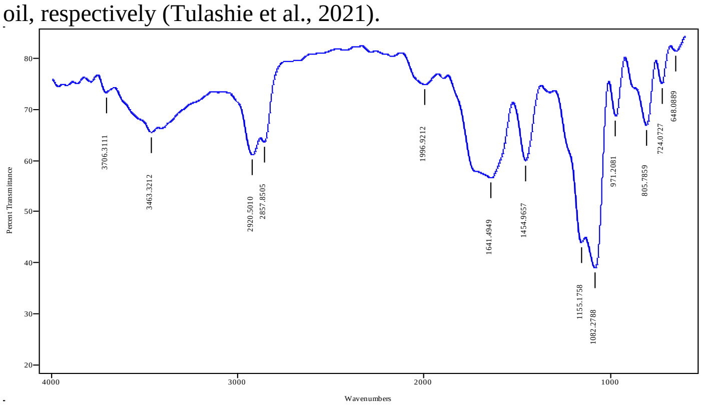


Figure 1: FTIR spectrum of neem seed oil recorded by Infrared Spectrophotometer M530

Optimization of Bio-pesticide Production by RSM-CCD

The total number of experimental runs performed were 20, with 8 factorial point runs, 6 axial point runs and 6 center point runs as replicate which helps to reduce experimental error (Rashid et al., 2011) as shown in Table 4.

Table 4: Experimental Design for Production of Pesticide from Neem Seed Oil.

Run	Type	A:Neem seed oil (ml)	B:Emulsifier (ml)	C:Water (ml)	Wt. reduction (%)
1	Center	15	5	7	60
2	Fact	18	3	5	64
3	Center	15	5	7	58.33
4	Fact	12	3	5	40
5	Fact	18	3	9	50
6	Axial	15	3	7	55
7	Axial	15	7	7	53.33
8	Fact	18	7	9	58.75
9	Fact	18	7	5	57.14
10	Center	15	5	7	60
11	Fact	12	3	9	36
12	Fact	12	7	9	27.5
13	Axial	18	5	7	63.33
14	Center	15	5	7	60
15	Center	15	5	7	61.4
16	Center	15	5	7	60
17	Axial	15	5	9	56.67
18	Axial	12	5	7	37.1
19	Fact	12	7	5	42
20	Axial	15	5	5	61.67

In this study, three variables were used in formulation of the pesticide, namely: neem seed oil (A), emulsifier (B) and water (C) and the response is the % weight reduction of army worm (selected pest) which was calculated for each formulation.

The results of the sequential model sum of squares for the experimental design and the model summary statistics are Presented in Tables 5 and 6, respectively.

Table 5: Sequential Model Sum of Squares.

Source	Sum of squares	Df	Mean square	F Value	P-value Prob> F	
Mean vs Total	56415.57	1	56415.5664			
Linear vs Mean	1356.431	3	452.14383	9.042778373	0.0010	
2FI vs Linear	16.72954	3	5.5765125	0.092552779	0.9628	
Quadratic vs 2FI	675.0116	3	225.003876	20.78221288	0.0001	Suggested
Cubic vs Quadratic	98.82546	4	24.706365	15.69976339	0.0025	Aliased
Residual	9.442065	6	1.57367754			
Total	58572.01	20	2928.60033			

Table 6: Model Summary Statistics

Source	Std. deviation	R-Squared	Adjusted R-Squared	Predicted R-Squared	Press	
Linear	7.071106	0.629014198	0.55945436	0.335832322	1432.237868	
2FI	7.762232	0.63677214	0.46912851	-1.63032065	5672.129136	
Quadratic	3.290403	0.949793402	0.90460746	0.015212093	2123.636212	Suggested
Cubic	1.254463	0.995621457	0.98613462	-1.683843201	5787.547316	Aliased

From the Tables 5 and 6, the software suggested the quadratic model as the best model for this design from the response of the experimental data.

Table 7: Analysis of variance (ANOVA) for response surface quadratic model.

Source	Sum of Squares	Df	Mean Square	F Value	P-value Prob> F	Degree of significance
Model	2048.173	9	227.574739	21.01966762	< 0.0001	significant
A-Neem seed oil	1223.678	1	1223.67844	113.0235902	< 0.0001	
B-Emulsifier	3.94384	1	3.94384	0.364268047	0.5596	
C-Water	128.8092	1	128.80921	11.89730806	0.0062	
AB	8.799013	1	8.7990125	0.812710227	0.3885	
AC	4.666512	1	4.6665125	0.431016825	0.5263	
BC	3.264012	1	3.2640125	0.301476596	0.5950	
A^2	200.8395	1	200.839546	18.55030357	0.0015	
B^2	58.08655	1	58.086546	5.365094095	0.0430	
C^2	0.460227	1	0.46022727	0.04250834	0.8408	
Residual	108.2675	10	10.8267525			
Lack of Fit	103.5308	5	20.706155	21.85692199	0.0021	
Pure Error	4.73675	5	0.94735			
Cor Total	2156.44	19				
Std. Dev.	3.290403		R^2	0.949793402		
Mean	53.111		Adjusted R^2	0.904607464		
C.V. %	6.195333		Predicted R^2	0.015212093		
PRESS	2123.636		Adeq. Precision	14.26228059		

Table 7 depicts the Analysis of variance (ANOVA) used to evaluate fitness, statistical importance and adequacy of quadratic model and also to investigate the effect of operational variables on the output response (% weight reduction) and also establishing the significance of each operating variable. From the results obtained from the analysis of variance (ANOVA) the model fitting was analyzed with the help of several statistical criteria. The p-value (probability of error value) represents the significance of the model and F-value represents the most influencing factor in a study.

Table 7 shows that P-values higher than 0.05 indicates insignificance and P-values less than 0.05 indicates significance of these parameters on the % weight reduction. Therefore, the results from the table indicated that the linear parameters A (neem seed oil) and C (water) and the quadratic parameters A^2 and B^2 have significant effect on the % weight reduction and AB, AC and BC were insignificant. A (neem seed oil) was established to be the most significant variables for the pesticide formulation to give a higher % weight reduction with a P-value of < 0.0001 and F-value of 113.024. F value normally shows the strength of the effect of each parameter to response (Lee et al., 2011). Thus, the high F value expresses stronger influence of variables on response (Boey et al., 2013). The variable C (water) appeared to be the second most influential variable with F-value of 11.897 and low significant effect P-value of 0.0062. Also, the model was significant and is confirmed by the P-value of less than 0.0001 and the higher F value of 21.0196.

The coefficient of regression R^2 which shows relationships between the predicted and actual % weight reduction is calculated as 0.949793402. In addition, the adjusted R^2 value of 0.904607464 was in close agreement with R^2 value which shows the model has related variable. The other important parameter for evaluating the model is C.V. which has to be less than 10%. The C.V. value of the fitting model is 6.195%, so this model and the experimental results are reliable and as well suitable for the predicted % weight reduction produced from the formulated pesticide (Kefas et al., 2018).

Regression coefficients and significance of response quadratic model are presented in Table 8.

Table 8: Regression coefficients and significance of response quadratic model

Factor	Coefficient Estimate	Degree of freedom	Standard Error	95% CI Low	95% CI High	VIF
Intercept	59.47736	1	1.13116104	56.95697978	61.99774749	
A-Neem seed oil	11.062	1	1.04051682	8.743584055	13.38041594	1
B-Emulsifier	-0.628	1	1.04051682	-2.946415945	1.690415945	1
C-Water	-3.589	1	1.04051682	-5.907415945	-1.27058406	1
AB	1.04875	1	1.16333317	-1.543317826	3.640817826	1
AC	0.76375	1	1.16333317	-1.828317826	3.355817826	1
BC	0.63875	1	1.16333317	-1.953317826	3.230817826	1
A^2	-8.54591	1	1.98418772	-12.96695483	-4.12486335	1.81818182
B^2	-4.59591	1	1.98418772	-9.01695483	-0.17486335	1.81818182
C^2	0.409091	1	1.98418772	-4.01195483	4.830136649	1.81818182

The % weight reduction was analyzed based on the response factor and the three independent variables. The % weight reduction regression equation in terms of the coded factor is as expressed in Equation 9.

$$\% \text{ weight reduction} = 59.47736 + 11.062A - 0.628B - 3.589C + 1.04875AB + 0.76375AC + 0.63875BC - 8.54591A^2 - 4.59591B^2 + 0.409091C^2 \quad (9)$$

Figure 2 shows a plot of predicted versus actual % weight reduction

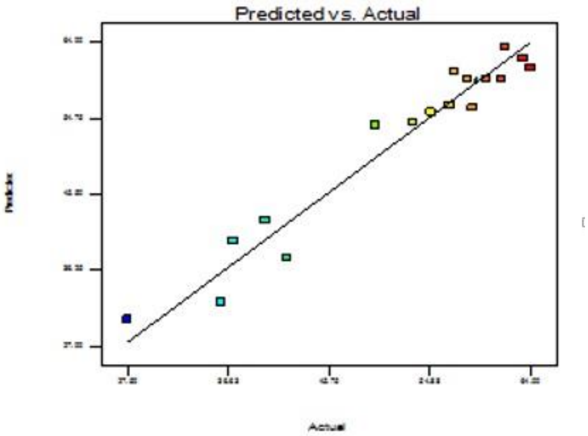
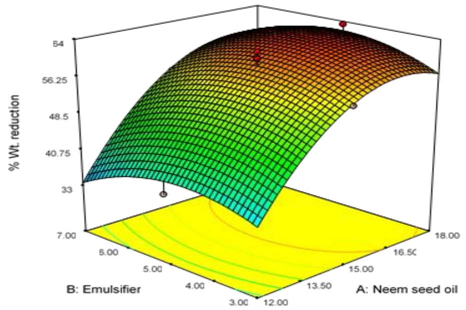


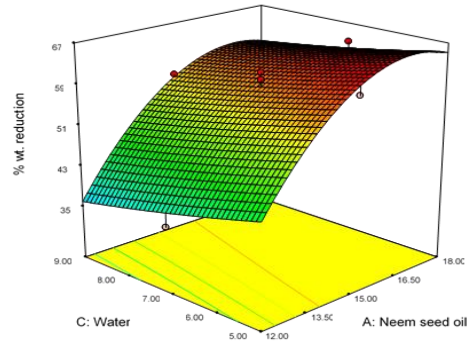
Figure 2: A plot of predicted versus actual % weight reduction

Actual % weight reduction compared to predicted % weight reduction graph is demonstrated in Figure 2. It can be clearly seen that an acceptable correlation was obtained between predicted and actual data of the pesticide formulation; hence, the errors of the distributed points can be said to be small (Lokman et al., 2015). In addition, the line of (y = x) mentioned a better fitting of the model with experimental results.

(a)



(b)



(C)

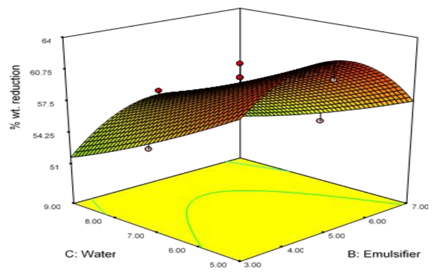


Figure 3: (a), (b) and (c) are response surface combined effect plots of the pesticide formulation variables

The interaction of variables and their effects on response is normally studied with the help of three dimension graphs. Figure 3 demonstrate the 3D response surface plots for % weight reduction of selected pests depending on neem seed oil and emulsifier (Figure 3a), neem seed oil and water (Figure 3b), emulsifier and water (Figure 3c). As expected, Figure 3a and 3b exhibit that the % weight reduction is increased when the content of neem seed oil increases. Therefore, neem seed oil is the most important factors that impact the % weight reduction. Figure 3c shows that the % weight reduction reduced when higher content of water is used in the mixture relative to the emulsifier used. It also shows that the best content for emulsifier is at 5ml and that further increase in the emulsifier content decreases the % weight reduction.

Percentage (%) weight reduction at numerically optimized conditions

The RSM was used to numerically optimize the bio-pesticide formulation variable. The software predicted 64.31 % weight reduction of army worm would be achieved at 17.96 ml neemoil, 4.51 ml emulsifier and 5.42 ml of water formulation conditions. However, the validation performed at the generated optimized conditions provided 64.53 % weight reduction of the army worm which is 0.22 % higher than the predicted response. The generated formulation conditions used in the validation above are in agreement with the ratio of the process parameters (15ml neem oil: 5ml liquid detergent: 7ml water) used in a research work performed for the development of pesticide to control white flaky insects on Rose plant by Naniwadekar and Jadhav (2012).

Conclusions

The following conclusions can be drawn from the results of this research:

1. Neem seed oil is effective for production of bio-pesticide for control of army worms which mostly affect bean, maize and guinea corn leaves on the farm.
2. The bio-pesticide from neem seed oil is non-toxic, biodegradable, environmentally friendly and relatively cheap.
3. Optimum formulation conditions generated by the RSM of 17.96 ml of Neem seed oil, 4.51 ml of emulsifier and 5.42 ml of water achieved a percent weight reduction of 64.53 % for the army worm.
4. The Design Expert 7.0 optimization software using response surface methodology and central composite design for the formulation of bio-pesticide from neem seed oil, emulsifier and water was successful.

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