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Optimization of Media for the Production of Biomass and Pesticidal Metabolites by the Entomopathogenic Fungi *Nomuraea rileyi* (Farlow) Samson using Response Surface Methodology and Evaluation of their Biocontrol Potential against major Peanut Defoliator *Spodoptera litura* (Fab.) (Lepidoptera; Noctuidae)

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***Nomuraea rileyi* (Farlow) Samson is an important fungal biocontrol agent and extensively used as mycoinsecticide against economic important insect pests in various parts of the world. Success of biocontrol agent is primarily based on the production of adequate quantity of inoculam using suitable media.Supply of nutrients is an important factor which influences the biomass production.Statistical optimization strategy as response surface methodology (RSM) is adopted to maximize the biomass production through submerged and solid state fermentation. RSM can identify the various interactions among different parameters and it has been extensively applied for optimization of cultural medium conditions and other process parameters in bio processes In the present study, Central composition design (CCD) was used for optimizing the parameters. Temperature (X_1), pH (X_2), Maltose (X_3), Peptone (X_4) was chosen as independent variables. The maximum percentage of biomass yield is obtained at 3.87g/mL of maltose and peptone at 0.55g/mL,temperature 28.1ºC and pH 7.09.Metabolites were extracted from the culture filtrate of fungi grown in media and showed distinct effect on the life stages of *Spodoptera litura*. Lethal time 50(LT_{50}) and lethal concentration 50 (LC_{50}) was highly influenced by larval stages and concentration.**

Key words: *Nomuraea rileyi*, *Spodoptera litura*, Response surface methodology, Metabolites.

Insect pest management in agriculture is more important to protect the crop yield and increase the productivity. An average of 33% of crop loss occurs due to insect pests and the loss as been estimated around Rs.2000 billion annually [1].The adverse effects of chemical pesticides on the environment, health and social economic conditions of the community, farmers resort to self defeating practices such as the applications of more dosage of pesticides or increasing the frequency of applying the pesticides to minimize the crop loss. This has led to search for bio-intensive eco-friendly integrated pest management [2]. The IPM has laid special emphasis on the use of bio-agents in order to minimize the injudicious and indiscriminate use of chemical pesticides. Microbial pesticides are a promising alternative for the chemical pesticides which have opened up new vistas in insect pest management to enable the promotion of safe eco-friendly pest management. Due to biodegradable nature the microbial pesticides do not contaminate the aquatic systems

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or leaves any residue on crops. The microbial control includes the utilization of micro organisms in all aspects and their by products are used for the control of insect pest and plant diseases. These microbial agents do not interfere with other biotic systems as they are relatively host specific. The use of microbial pesticides has been limited to the generation of information born on the efficacy in micro plots than in the fields of farmers. The large scale use of microbial pesticides will provide a huge environmental impact on improving the sustainable agriculture and pest management [3,4].The rational for the deployment and development of microbial insecticides for pest management is for their environmental safety, specificity and biodegradability. These new generation microbial pesticides have been introduced for crop production and pest management and requires several steps to be addressed right from its isolation in pure culture to bioefficacy assays performed *in-vivo*, *ex-vivo*, *in-vitro* or in pilot scale trials under field conditions. Different microbial agents such as bacteria, fungi and baculoviruses are quite promising for pest control. Nowadays bacteria and fungi are gaining importance due to their amenability for mass multiplication in artificial media [4,5]. Environmental impact on microbial pesticides). Entomopathogenic fungi are currently attracting attention as potential biological control agents against coleopteran, lepidopteran and acridid pests [6.] .Fungal bio agents have been used for controlling pests all over the world such as *Beauveria bassiana, Metarhizium anisopliae, Nomureae rileyi, Verticillium lecanii* [7] .Entomopathogenic fungi are known to produce a wide range of metabolites known to affect the insects.The toxins, beauverucin produced by the fungi of the genus *Beauveria* were shown to be cyclodepsipeptides by Onafre *et al* [8] and destruxins of *Metarhizium* species were also subsequently found to be cyclodepsipeptide [9] . Apart from the fungal biomass and their conidia which were conventionally used as entomopathogenic biopesticdes to penetrate, grow and kill the insect pests, the toxins they produce (beauverucin/ destruxins) also hold good for the same purpose. The utility of such toxins for pest control has not yet been attempted in the Indian context. Preferred method of liquid fermentation to produce the fungus, conidia and the toxin as the case may be need to be cheaper to produce high yield over a reduced time scale for purposes of commercial production and marketing of the biopesticide. Both *B. bassiana* and *M.anisopliae* are extensively investigated for their use as biopesticides and formulation with appropriate carriers and adjuvants has resulted in the release of many commercial products for controlling pests in the farmer's field [10] .Although efficacy of the said fungal biopesticides in the form of active hyphae and conidial suspensions has been repeatedly demonstrated, and the efficacy equals that of conventional chemical pesticides without the residual effects characteristic of the chemicals, biopesticides are thought to take longer to kill the pests than the current insecticides [11]. In other words, there is a significant lag phase in the infection of the insect through germ tube penetration into the cuticle of the insect, multiplication of the hyphae and formation of conidiophores before the actual killing of the host takes place. This may be 3-14 days as already reported and may be crucial in avoiding considerable foliage and hence yield loss in severely infested groundnut crop. It is imperative that an alternative formulation with improved technological inputs is made so that it acts on the insect pest instantaneously, immobilizing it and possibly killing it without leaving time for it for further defoliation under field condition [12].

Response surface methodology (RSM) is an important statistical technique employed for multiple regression analysis by using quantitative experimental data obtained from properly designed experiments using central composite design (CCD) [13]. RSM can identify the various interactions among different parameters and it has been extensively applied for optimization of cultural medium conditions and other process parameters in bio processes. Optimization of the variables in a fermentation process can give information about the main effects of the variables and also the interaction between variables in varying level. The main advantage of using response surface methodology includes the reduction in number of experiments saving time, chemicals and labor and also its rapid and reliable prediction of responsmade it a lucrative option to explore [14]. In the present study, the optimization of media for the biomass and pesticidal metabolites production through

shake flask culture of entomopathogenic fungi *Nomuraea rileyi* (Farlow) Samson adopting response surface methodology (RSM) and evaluation of bio control potential of metabolites against major groundnut defoliator *Spodoptera litura* (Fab.)(Lepidoptera;Noctuidae) has been carried out.

MATERIALS AND METHODS

Soil sampling

N.rileyi was isolated from the soil sample obtained from groundnut field, Chengalpet,Kanchipuram district,Tamil Nadu and processed for the isolation of fungi [15]. Approximately 2 kg of soil was collected from four points a few meters apart by digging to a depth of 10-15 cm with a small spade. The soil samples were put in plastic bags and taken to the laboratory and stored at 25°C. For processing, the soil was thoroughly mixed and passed through a 0.4 mm mesh sieve to break or separate any coarse lumps of soil or litter. Before microbial analysis, soil aggregates were broken by hands, trays with soil were kept open until moisture was at equilibrium [16]. Soil texture pH electrical conductivity organic matter nitrate, phosphorous, potassium, calcium, magnesium sulphur, sodium, zinc, iron, copper were determined for all soil collected. These measurements were determined in national agro foundation at Taramani TamilNadu, India

Isolation of *N.rileyi*

N.rileyi SSK 7 strain was isolated from the processed soil sample by the modified method of Clark [15] using CTC (Chloramphenicol Thiobenzodazole Cyclohexamine) media. The organism was identified based on the morphological and cultural characteristics adopting standard methods and the pure culture was maintained on CTC agar slant.Fungal morphology was confirmed by lacto phenol blue staining [17].

Inocula preparation

Fungal inoculam was prepared from 7 days old SMYA slant culture by scrapping off with a sterilized glass rod. A homogenous conidial suspension was prepared in sterile distilled water by adding a few drops of the wetting agent Tween 80 (0.01%). The conidial concentration of the suspension was determined using an improved Neubauer haaemocytometer (Germany) and used as the source of inocula.

Experimental design and optimization studies

Central composition design (CCD) was used for optimizing the parameters. Temperature (X-$_{1}$), pH (X_2), Maltose (X_3), Peptone (X_4) was chosen as independent variables. Experimental analysis was carried out in 250mL Ermenlayer flasks. To 100mL of sterile double distilled water the maltose and peptone was added according and used as medium for the growth of fungi. The flasks were maintained under shaking condition at different temperatures and pH (Table 1,2).

Experimental Design

Experimental design was carried out using Design expert software (Stat Ease, 9.0 trial version). This software was used towards the construction of a quadratic model. Four independent variables such as temperature, pH, Maltose and peptone were studied. Three levels such as -1 , 0 , +1 was analyzed for each independent variable and the variable factors in coded and actual values are given in table 1 . The second order polynomial function was also used for regression analysis to study the interaction effect as follows

$$Y_i = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4; \quad ...(1)$$

Where Y_i is the predicted response ; X_1, X_2, X_3, X_4 the independent variables ; b_o , the offset term ; b_1, b_2, b_3, b_4, the coefficients of linear effects ; b_{11}, b_{22}, b_{33}, b_{44}, the coefficients of squared effects ; and b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34}, coefficients of interaction terms. The regression equation contains four linear terms (,,,), four quadratic terms (, , ,), and six cross interaction terms (, , , ,) plus 1 block term.

Extraction of pesticidal metabolites

Pesticidal metabolites were isolated and purified according to the method [18] with minor modification. A litre of Sabouraud maltose yeast extract broth was prepared in 2 litre conical flasks and inoculated with 10 ml of fungal spore suspension (1.0 x 108 spores/ml) and incubated at 28°C on a rotatory shaker at 150 rpm for seven days. After the incubation period, the broth was filtered through sterile cheese cloth and the

collected filtrate was extracted with ethyl acetate and concentrated in a rota evaporator, concentrated extract thus obtained was stored in screw cap vial and used for further studies.

Screening of pesticidal activity against *Spodoptera litura*

The metabolites thus obtained was reconstituted in dimethyl sulphoxide (DMSO) at the concentrations of 1.0, 0.1, 0.01 and 0.001 mg/ml and the pesticidal activity was carried out by determination of development period lethal time 50 (LT50) [19], lethal concentration 50 LC50 [20].

Laboratory bioassay on *Spodoptera litura*

The 2nd and 3rd instars larvae of S.litura selected for bioassay studies. 20 larvae in each instars (2nd and 3rd) of S.l and H.R were sprayed with respective concentration using ULV (Ultra Low Volume) sprayer. The treated larvae were introduced into the plastic container (34mm X 21mm) provided with moist cotton swap covered with tissue paper at the bottom of the container to provide humidity. The containers were covered with meshed lid to provide aeration to the larvae for control category another 20 larvae of each instars treated with distilled water only. The containers were incubated at room temperature 28 ± 0.5 C in a incubator (Remi BOD incubator, Mumbai, India). Daily observation on larval mortality was recorded

The LT_{50} of the dose of fungi to kill the different larval instars was assessed in hours followed Blever and Hostetter [19]

$$LT_{50} = a + e\ c - b\ /d$$

Where a = the no of hours from the initiation of the test until the reading made just before the 50% value was recorded; b= the total number of larvae dead at the reading just before 50% value was recorded; c = 50% of the total number tested; d = the no of larvae dying in 24 hrs period during which the 50% mortality was reached and e = the number of hours between mortality counts. The dose mortality data were subjected to profit analysis for LC_{50} [20].

Pupa

About 300 gram of finely sieved soil was taken in 500 ml capacity bottle and autoclaved at 15 psi pressure for 30 minutes. After the sterilization this soil was transferred in to a clean surface sterilized 500 ml capacity plastic container (65 x 32 mm) and the soil moisture was maintained by adding 5 ml of sterilze-distilled water. Different concentrations of metabolites was added separately. Pupa of *S. litura* was placed individually. Each treatment was replicated 10 times. Another set was maintained by adding only distilled water as control. Observations on the pupal mortality and adult emergence were recorded.

RESULTS AND DISCUSSION

Fungi isolation

N.rileyi was isolated from the groundnut field soil adopting culture dependent method and the isolated fungi was identified based on the cultural characteristics on the CTC media which revealed brilliant green aerial mycelia and the microscopic examination of fungal spore by lactophenol cotton blue showed spherical conidia. Soil physico-chemical parameters highly influenced the natural occurrence of *Nomurea rileyi*. *Nomurea rileyi* isolated from the respective soil sample reveals high organic matter, available nitrogen and phosphorous(Table 4).This may favour the viability of the fungal spore and thus improved the natural occurrence of *Nomurea rileyi*

Regression analysis

Regression analysis was carried out considering full quadratic model equation (1) on the responses to evaluate the adequacy of fit. The determination of coefficients (R^2 values) for the model equation (1) were R^2= 0.9931, adjusted R^2=0.9866, and predicted R^2=0.9635 for the responses (Table 3). The adjusted R^2 and predicted R^2 values indicate the experimental values posses

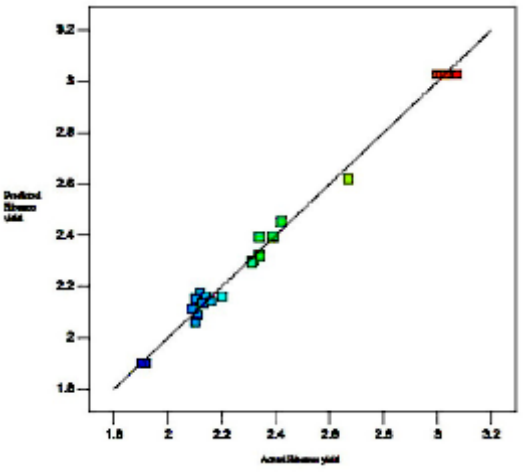

Fig. 1. Graphical comparison between actual and predicted percentage of Biomass yield.

a good agreement with the predicted values (Figure 1). Therefore, the model is found to appreciable and adequate in representing the response and the yield of biomass will be useful for further analysis. The regression equation relating to the coded levels of the variables developed as follows

$$Biomass\ yield = 3.03 + 0.013X_1 + 0.039X_2 + 0.083X_3 + 0.12X_4 + 0.026X_1X_2 + 0.16X_1X_3 + 0.005X_1X_4 - 0.037X_2X_3 + 0.005X_2X_4 - 0.25X_3X_4 - 0.24X_1^2 - 0.20X_2^2 - 0.49X_3^2 - 1.01X_4^2$$

… (2)

The above regression equation (2) shows an optimal increase in biomass yield with respect to temperature, pH, maltose and peptone. But further decrease in temperature, pH, maltose and peptone was observed in square term of regression equation (Figure 1)

ANOVA of responses

The statistical significance of equation (2) was evaluated by perfuming the test such as F-test and ANOVA using the design expert software 9.0. The model was found to be statistically valid with a lower probability value (P_{model}<0.0001) and

Table 1. Coded levels for independent factors used in experimental design

Factor	Coded levels		
	-1	0	+1
Temperature(X_1),ºC25	30	35	
pH (X_2)	5.5	6.5	7.5
Maltose (X_3), g/100mL	3	4	5
Peptone (X_4),g/100mL0.	25	0.5	0.75

Table 2. Experimental designs used in RSM studies by using four independent variables showing observed and predicted values of Biomass yield

Run	Temp (X_1)	pH(X_2)	Maltose(X_3) (g/100mL)	Peptone(X_4) (g/100mL)	Response (R_1)	Predicted value	Residual value
1	30	6.5	4	0.25	1.90	1.90	0.00
2	30	6.5	3	0.5	2.42	2.45	-0.03
3	30	6.5	5	0.5	2.67	2.62	0.05
4	30	6.5	4	0.5	3.03	3.03	0.00
5	25	5.5	4.5	0.625	2.14	2.16	-0.02
6	30	6.5	4	0.5	3.01	3.03	-0.02
7	25	5.5	4.5	0.375	2.12	2.18	-0.06
8	35	5.5	3.5	0.375	1.92	1.90	0.02
9	35	5.5	4.5	0.375	2.32	2.30	0.02
10	30	6.5	4	0.5	3.02	3.03	-0.01
11	30	6.5	4	0.5	3.03	3.03	0.00
12	35	7.5	3.5	0.375	2.10	2.06	0.04
13	35	5.5	3.5	0.625	2.12	2.14	-0.02
14	30	6.5	4	0.75	2.16	2.14	0.02
15	25	7.5	3.5	0.375	2.10	2.15	-0.05
16	25	5.5	3.5	0.625	2.34	2.32	0.02
17	25	7.5	4.5	0.625	2.10	2.15	-0.05
18	30	6.5	4	0.5	3.07	3.03	0.04
19	40	6.5	4	0.5	2.09	2.11	-0.02
20	30	8.5	4	0.5	2.31	2.29	0.02
21	30	4.5	4	0.5	2.13	2.13	0.00
22	30	6.5	4	0.5	3.00	3.03	-0.03
23	35	7.5	4.5	0.375	2.34	2.39	-0.05
24	35	7.5	4.5	0.625	2.39	2.40	-0.01
25	25	7.5	4.5	0.375	2.20	2.16	0.04
26	35	7.5	3.5	0.625	2.34	2.32	0.02
27	20	6.5	4	0.5	2.10	2.06	0.04
28	35	5.5	4.5	0.625	2.31	2.30	0.01
29	25	5.5	3.5	0.375	2.11	2.09	0.02
30	25	7.5	3.5	0.625	2.39	2.39	0.00

the lack of fit value is found to be not significant (P= 0.0680), which also indicates the equation is adequate for the prediction of percentage of biomass yield under all conditions. The low coefficient of variation (CV= 1.78%), suggesting that the model is reliable and precise (Table 3).

Interactive effects of variables

Response surface methodology is specially used to evaluate the effect of process variables on biomass yield optimization. To evaluate and investigate the interaction effect of two factors on the percentage biomass yield, 3D

Table 3. Analysis of variance (ANOVA) results for response surface model developed for biomass yield optimization

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob >F	
Model	3.838	14	0.274	154.046	0.000	significant
X_1-Temperature	0.004	1	0.004	2.398	0.142	
X_2-pH	0.037	1	0.037	20.690	0.000	
X_3-Maltose	0.042	1	0.042	23.416	0.000	
X_4-Peptone	0.086	1	0.086	48.554	0.000	
X_1X_2	0.011	1	0.011	6.196	**0.025**	
X_1X_3	0.099	1	0.099	55.762	**0.000**	
X_1X_4	0.000	1	0.000	0.056	0.816	
X_2X_3	0.006	1	0.006	3.161	0.096	
X_2X_4	0.000	1	0.000	0.056	0.816	
X_3X_4	0.063	1	0.063	35.123	**0.000**	
X_1^2	1.517	1	1.517	852.755	0.000	
X_2^2	1.141	1	1.141	641.212	0.000	
X_3^2	0.413	1	0.413	232.096	0.000	
X_4^2	1.734	1	1.734	974.655	0.000	
Residual	0.027	15	0.002	-	-	
Lack of Fit	0.024	10	0.002	4.050	0.068	not
Pure Error	0.003	5	0.001	-	-	significant
Cor Total	3.864	29		-	-	
R^2	0.9931	-	-	-	-	
Adjusted R^2	0.9866	-	-	-	-	
Predicted R^2	0.9635	-	-	-	-	

Table 4. Physico chemical parameters of soil samples collected from Chengalpet groundnut field

S.no	Parameters	
1	pH	7.95
2	Electrical conductivity(ms/cm)	0.600
3	Organic matter (%)	2.33
4	Nitrate nitrogen (ppm)	24.9
5	Available phosphorous(ppm)	237.7
6	Potassium exchangeable k(ppm)	93
7	Calcium exchangeable (ppm)	1932
8	Magnesium exchangeable (ppm)	511
9	Sulphur available s as so4 ((ppm)	49.3
10	Sodium exchangeable Na((ppm)	302
11	Zinc available Zn (ppm)	2.15
12	Manganese available Mn (ppm)	4.72
13	Iron available Fe (ppm)	1.36
14	Copper available	1.84

Table 5. *Mortality of Spodoptera litura larval instars treated metabolites*

S.No	Concentration (mg/ml)	Mortality (%) 2nd instar	3rd instar
1	0.001	50.0	30.0
2	0.01	75.0	50.0
3	0.1	80.0	60.0
4	1.0	100.0	65.0
5	control	0.0	0.0

surface plots have used and analyzed. In a four parameter study, 3D surface plots play interactive role by varying any two parameters and keeping the other two variables as constant. Here in Figure 2(1), shows the interaction effect between temperature (X_1) and pH (X_2) towards biomass yield and the maltose and peptone are kept fixed. The 3D surface shows maximum percentage biomass yield, at the temperature around 28.1ºC and pH around 7.09. Further increase in temperature and pH will decrease the yield of biomass. In the case Figure 2(2), the pH and peptone was kept constant respectively. The maximum percentage of biomass yield is observed at 28.9ºC and maltose around 4.36 g/mL. Figure 2(3) represents the graph between maltose and peptone and keeping the temperature and pH constant. The maximum percentage of biomass yield is obtained at 3.87g/mL of maltose and peptone at 0.55g/mL.

Table 6. Effect of metabolites on LT 50 of *Spodoptera litura*

S.No	Concentration (mg/ml)	LT 50 2nd instar	LT 50 3rd instar
1	1.0	1.61	2.17
2	0.1	2.13	3.07
3	0.01	3.17	3.57
4	0.001	4.21	4.51
5	Control	5.27	6.27

Biocontrol potential

To produce the pesticidal metabolites ,the fungi was grown in SMYB media aerobically at 28.9 ºC.After the incubation period, the media was filtered and the collected filtrate was extracted with the double the volume of ethyl acetate and concentrated.Both the tested instars of *Spodoptera litura* susceptible to all the tested concentration as dose dependent manner (Table 5,6).Maximum mortality was reported in high concentration in the both tested instars.The LT_{50} increased as the larvae grow older as well as the increase in the concentration. As the instars advanced a increased in time was recorded.The result of LC_{50} value determination through probity analysis was presented in table 7,8.Among the various estimate of regression based probity analysis, the chi-square test of the bioassay showed homogeneity of the test population which is a reflection of a good fit of the observed and expected response. From the table 6,7 it is very clear that the LC_{50} values of different larval instar of *Spodoptera litura* in

Table 7. Toxicity of tested samples against *Spodoptera litura* 2nd instar larvae

LC_{50} (mg)	95% Confidence limit Lower	95% Confidence limit Upper	LC_{90} (mg)	95% Confidence limit Lower	95% Confidence limit Upper	Chi square value
71.01	31.12	184.59	4523.77	1128.90	63898.54	0.036*
23699.88	2273.39	25468.00	5344184.00	64944.65	10053370.00	0.810*
15343.20	2214.94	57868423168.00	703534.31	21087.68	761807901.29	0.563*
2149.05	451.77	132843.00	845946.50	29095.87	3181850.82	0.658*

LC_{50} and LC_{90} values are expressed as percentage (n=24).
* Ç2 values are significant at P *d"* 0.05 levels.

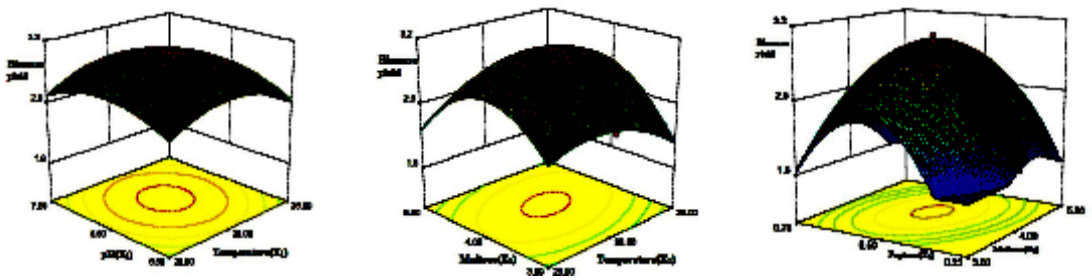

Fig. 2. (1) Interaction effect of temperature(X_1) and pH(X_2), (2) Interaction effect of temperature(X_1) and maltose (X_3), (3) Interaction effect of maltose (X_3) and peptone (X_4) on biomass yield

Table 8. Toxicity of tested samples against Spodoptera litura 3rd instar larvae

LC_{50}	95% Confidence limit		LC_{90}	95% Confidence limit		Chi square
(mg)	Lower	Upper	(mg)	Lower	Upper	value
172.21	72.04	578.47	13473.80	2590.73	397025.40	0.004*
49726.13	-	-	2758214.50	-	-	0.238*
-	-	-	-	-	-	-
5026.23	575.64	261078.10	13528330.00	90845.60	1045829.00	0.464*

LC50 and LC90 values are expressed as percentage (n=24).
* ?2 values are significant at P ? 0.05 levels.

response to the metabolites shown an increased trend in the LC_{50} value, when the age of larva advanced concentration. The medium lethal concentration of 2nd and 3rd instar of *Spodoptera litura* was 4525.77mg and 13473.80mg in 3rd instar.. The optimization of medium components by response surface methodology for biomass production through solid state fermentation of entomopathogenic fungus *Beauveria bassiana* has been studied [22,23].The present study might suggests the possible utilization of RSM strategy for the biomass production of mycoinsecticides and the pesticidal metabolites production which would used to control economic important insect pests.

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