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Full Length Research Paper

Phytotoxic, cytotoxic and insecticidal activities of *Calendula arvensis* L.

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Plants are the natural factories for the synthesis of variety of bioactive compounds. This diverse chemical setup of the plants speaks their important role as biomedicine. These biomolecules are often toxic to both plants and animals. The methanolic extract of *Calendula arvensis* was screened for its toxic potential against *Lemna minor, Artimia salina* (Brine shrimps) larvae and some important grains pests. It was observed that *C. arvensis* exhibited dose dependent toxicity towards *Lemna minor,* with low toxicity at 10µg/ml and 100µg/ml and moderate activity at 1000µg/ml. Moderate level of cytotoxicity was found LD_{50} value 9.23µg/ml against brine shrimp larvae. The insecticidal potential was also dose dependent, while different insects showed variable degree of susceptibility to the same treatment. *Callosobruchus analis* was the most susceptible pest with LD_{50} 0.51mg/ml, where *Trogoderma granarium* was the most resistive pest among the five tested insect with LD_{50} 90.50mg/ml.

Key words: *Calendula arvensis*, Phytotoxicity, Cytotoxicity, Insecticidal.

INTRODUCTION

Biologically active compounds with in plant extracts are often toxic to the larvae of *Artemia salina* (Brine shrimp). Brine shrimps lethality assay is a rapid, inexpensive, general bioassay, which has been developed for screening, fractionation and monitoring of physiologically active natural products (Kivack *et al.,* 2001, Carballo *et al.,* 2002). Cytotoxic effect of biomolecules on shrimp's larvae is correlated to the anticancer potential, because shrimps larval tissues respond in very similar manner as do the mammalians carcinoma (Mclaughlin, 1991; Solis, 1993). Members of the family Lemnaceae are suitable organisms to investigate physiological processes and effects of different biochemical substances. *Lemna* plants are miniature aquatic monocot consists of a central oval frond or mother frond with two attached daughter fronds and a filamentous root. Under normal conditions, the plants reproduce exponentially with budding of daughter fronds from pouches on the sides of the mother frond. Using the *Lemna* assay, it is observed that natural antitumour compounds can inhibit *Lemna* growth. In addition, it was also discovered that some substances stimulate frond proliferation, and the assay may be useful to detect new plant growth stimulants.

The commercial need for such natural, biodegradable, herbicides and plant growth stimulants may someday be filled with natural products detected by the simple and convenient *Leman* bioassay (Atta ur Rehman, 1991). Freedom from insect infestation and contamination has become an important consideration in storage of grain and to maintain high quality product (Coolins, 1998). Nearly one thousand species insect have been associated with store products throughout the world, of which the majority belong to Coleopteran (60%) and Lepidoptera (8-9%) (Champ, 1981). Pesticides, including residual grain protectants and fumigants are used extensively in grain industry. Resistance to one or more of these materials has occurred in most major pest species. This relentless development of resistance is a serious threat to the future use of this material and consequently, there is an urgent need to develop economically safer and sounder pest control techniques (Tabassum *et al*., 1997). Biological screening is an

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important step in evaluation medicinal plants activity (Nisar *et al.,* 2010a, b, 2011, Qayum *et al*., 2012, Zia-Ul-haq *et al.,* 2011). *C. officinalis* exhibits potential therapeutic properties in cases of cheilitis exfoliative (Roveroni-Favaretto *et al.,* 2009). The occurrence of acute dermatitis of grade 2 or higher was significantly lower (41% v 63%; P <.001) with the use of calendula than with trolamine (Pommier *et al*., 2004*). E. coli* growth inhibited by sage, catnip, and lavender, while bayberry actually facilitated bacterial growth. *Calendula* had no effect on the bacterial cultures. Growth of *B. cereus* was not affected by any of the applications (Errickson and Sedia 2005).

MATERIALS AND METHODS

Preparation of Extracts

Whole plants of *Calendula arvenisis* was collected from the campus of University of Peshawar, Pakistan and was identified by a Taxonomist Prof. Dr. Abdur Rashid, Department of Botany, University of Peshawar, Peshawar, Pakistan. The voucher specimen was deposited in the Herbarium Department of Botany, University of Peshawar, Pakistan. The plant was shade dried and was ground to 60 mesh. Fifty grams of sample were soaked in 250 ml methanol for 72 hours. Thereafter, plant extract was passed through Whatman filter paper No. 1823 for 3 times. It was evaporated in a rotatory evaporator at 40 ^{o}C to concentrate the extracts. These extracts were stored at 4 ^{o}C prior to use. The plant extract and the standard drug were dissolved in dimethylsulphoxide (DMSO) at the concentration of 10 mg/ml and 1 mg/ml for cytotoxic 30 mg/ml and 1 mg/ml for phytotoxic and 200 mg/ml and 1 mg/ml for insecticidal activities, respectively. The dissolved plant extract were diluted with distilled water up to the required concentrations (given in results).

Cytotoxicity

The materials and reagents used for cytotoxicity includes test sample *Artemia salina* (shrimps eggs), sea salt (38 g/L of D/W, pH 7.4), hatching tray with perforated partition, lamp to attract brine-shrimp larvae, micro pipette (5, 50,500µl), vials tray, 30 vials, organic solvents methanol and acetone. The cytotoxic activity of the crude extracts of the plants was carried out by following the method of Meyer *et al.* (1982).

Hatching

The hatching tray (a rectangular dish 22x32 cm) was halffilled with filtered brine solution and 50mg (eggs of brine shrimp were sprinkled in it). It was incubated at 37°C and after 24h brine shrimp hatched. The plant extracts were applied to see the cytotoxicity of these extracts.

Sample preparation

Test sample was dissolved (10mg) in 1ml of DMSO and from this solution 5, 50 and 500µl was transferred to vials (3vials/concentration). The concentrations were made as 10, 100 and 1000µg/ml respectively. After 2 days of hatching and maturation 10 larvae/vials were placed, using a Pasteur pipette. The volume was made 5 ml with seawater (38.5g of sea salt / 1000ml of distilled water). It was incubated at 25 - 27 °C for 24 hours under illumination. Other vials were supplemented with DMSO and etoposoid was used as reference cytotoxic drug which served as negative and positive controls, respectively. The data was analyzed with Probit Analysis program to determine LD_{50} values (Finney, 1971).

Phytotoxicity

Phytotoxic activity of the extracts was carried out against the *Lemna minor* following McLaughlin *et al*. (1991). The medium was prepared by mixing various constituents in distilled water (1000 ml) and the pH was adjusted (5.5-5.6) by adding KOH pellets. The medium was then autoclaved at 121 ^{o}C for 15 minutes. The extracts (30.0 mg) dissolved in methanol (1.0 ml) served as stock solution. 30 petri plates, three for each concentration, were inoculated with 1000, 100 and 10 µl of the stock solution to give the final concentration of 1000, 100 and 10µg/ml, respectively. The solvent was allowed to evaporate overnight under sterile conditions. To each plate, medium (20 ml) and plants (10), each containing a rosette of three fronds of *Lemna minor,* were added. Other plates supplement with solvent and reference growth inhibitor (Paraquate), served as a negative control. All plates were kept in the growth cabinet for seven days. The number of fronds per plates were counted and recorded on day seven.

Insecticidal activity

The materials and reagents used for insecticidal activity included test insects, volatile organic solvent (methanol), standard insecticide (Permethrin), Petri plates (9cm diameter), micropipette (1000µl), growth chamber, test sample, filter paper, glass vials, and brush. The insecticidal activity of the crude extract was carried out by impregnated filter paper method following Naqvi and Parveen, (1991).

Preparation of Test sample

The test sample is prepared by mixing 0.1, 1 and 10 mg test sample per 1 ml of methanol.

Rearing technique

The stored grain pests are reared in the laboratory under controlled conditions (temperature and humidity) in plastic bottles containing sterile breeding media. Insects of uniform age and size are used for the experiment.

Procedure

On the first day, filter paper was cut according to the size of Petri plate (9 cm or 90 mm) and was put in the plate. Then the whole sample of different concentrations was loaded over the

Table 1. Phytotoxic activity of *Calendula arvensis* against *Lemna minor*.

Dose (µg/ml)	Number of fronds in test	Number of frond in control (-ve)	% inhibition	FI_{50}
10	52		17.46	1617.163
100	43	63	31.74	
1000	34		46.03	

Table 2. Cytotoxic activity of *Calendula arvensis.*

Extract Conc. (µg/ml)	Total Number of Larvae	Number of survivors	% inhibition	LD_{50}	95% CL		Least square line	X^2 (p)
					LCL	UCL		
10	30	15	40					0.02
100	30	7	60	9.23	0.43	48.50	Y= 4.37+0.65X	(0.89)
1000	30	3	67					

filter paper and these plates were left for 24 hours to evaporate the solvent completely. On the second day (after the evaporation of solvent) put 10 healthy and active insects of same size and age of each species in each plate (test and permethrin was used + ve and Methanol was used as – ve control, respectively) with the help of a clean brush. The plates were incubated at 27 ^{o}C for 24 hours. On the third day readings were noted and the percentage inhibition or percentage mortality with the help of the following formula was calculated:

$$\% \text{ Mortality} = 100 - \frac{Number\ of\ insects\ alive\ in\ test\ sample}{\text{Number of insects alive in control}} \times 100$$

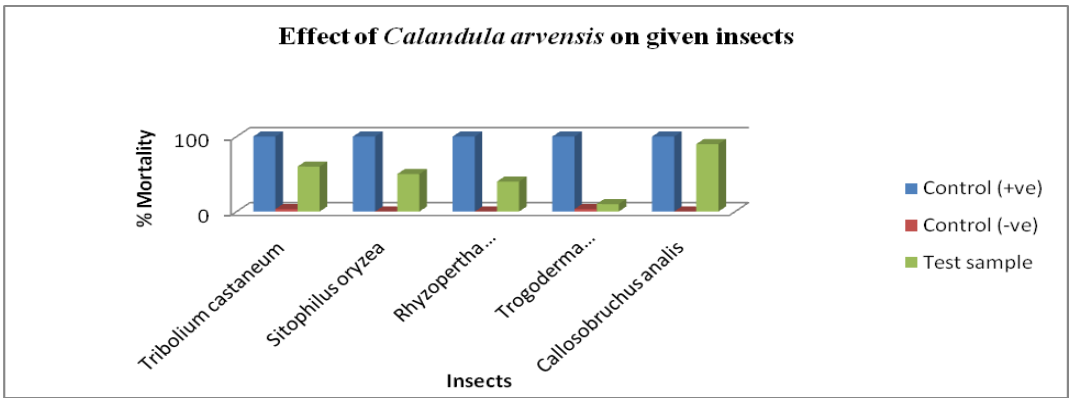
By means of Biostate 2009 Professional statistical package, data was also analyzed for Regression Line to determine LD_{50} values (Finney, 1971).

RESULTS AND DISCUSSION

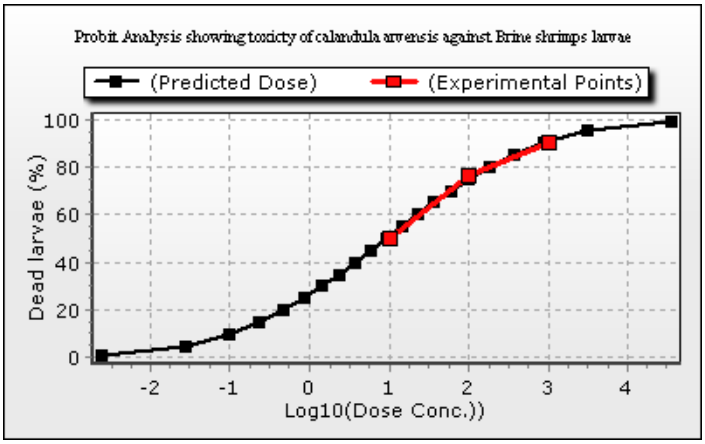
In the present study, *Lemna minor* bioassay was conducted to evaluate the toxic potential of the crude methanolic extract of *Calendula arvensis.* The phytotoxicity was observed to be dose dependent, as low phytotoxic activity (% inhibition≤40%) was observed at 10 and 100µg/ml concentration and moderate activity (% inhibition= 40-50%) at 1000µg/ml. FI_{50} (Concentration which causing 50% fronds proliferation inhibition) was high (1617.163µg/ml) because of low toxic effect towards *Limna minor* (Table 1). Hussain *et al*. (2010) reported the phytotoxicity of the *Rumix hastatus*, *R. dentatus*, *R. nepalensis*, *Rheum australe*, *Polygonum persicaria* and *P. plebejum* against *Lemna minor*. Moderate activity was shown by *R. nepalensis*, *R. austral* and *P. persicaria* at the concentration of 100 µg/ml. Ali *et al,* (2009) carried out the phytotoxic activity for the root extracts of *Euphorbia wallichii* obtained from chloroform, n-hexane, n-butanol and ethyl acetate. All of these exhibited a high degree of Phytotoxicity (60-100%) at high concentration (1000 µg/ ml) while at low concentration (10 µg /ml) they exhibited 30-80% Phytotoxicity. The findings of these workers are supporting our current results.

The methanolic extract of *Calendula arvensis* was examined for cytotoxicity while using Brine shrimps lethality assay. Shrimps larvae showed variable response towards different concentration of test sample. The experimental findings confirm moderate to significant cytotoxic effect of *calendula arvensis* with percent mortality of 40, 60 and 67% at 10, 100, and 1000µg/ml respectively with LD_{50} (9.23µg/ml). The best fitted line and Chi square values are also given (Table 2). The best fitted regression line of probit for cytotoxicity is shown (Graph 2). Ramachandran *et al.* (2011) reported significant cytotoxicity of *Agave cantula* against Shrimps larvae with LC50 15 and 12.5mg for aqueous and alcoholic extracts respectively. These findings are lines with our results. The significant lethality of brine shrimps larvae due to the methanolic extract of *Calendula arvensis* speaks about the presence of potent cytotoxic constituents which needs further investigation.

The plant extract showed variable effect in term of toxicity towards different insects' species. The test sample concentration was found to be correlated with the degree of toxicity of against the same test specie. Pest types differentially respond because of their variability in morpho-histolgy, physiology and genomics. Graph 1 showing the comparative mortality rate of five pest species to +ve and –ve controls along with the extract of C. arvensis. The extract exhibited low toxicity against *Trogoderma granarium,* moderate activity against *Rhyzopertha dominica* and *Sitophilus oryzea,* good activity against *Tribolium castaneum* and significant activity against *Callosobruchus analis.* The percent mortality, LC_{50} with 95% confidence levels, best fitted regression lines and Chi square values for insecticidal activity of *Calendula arvensis* are given (Table 3). Similarly the best fitted regression line of probit for insecticidal potential of *C. arvensis* at various doses



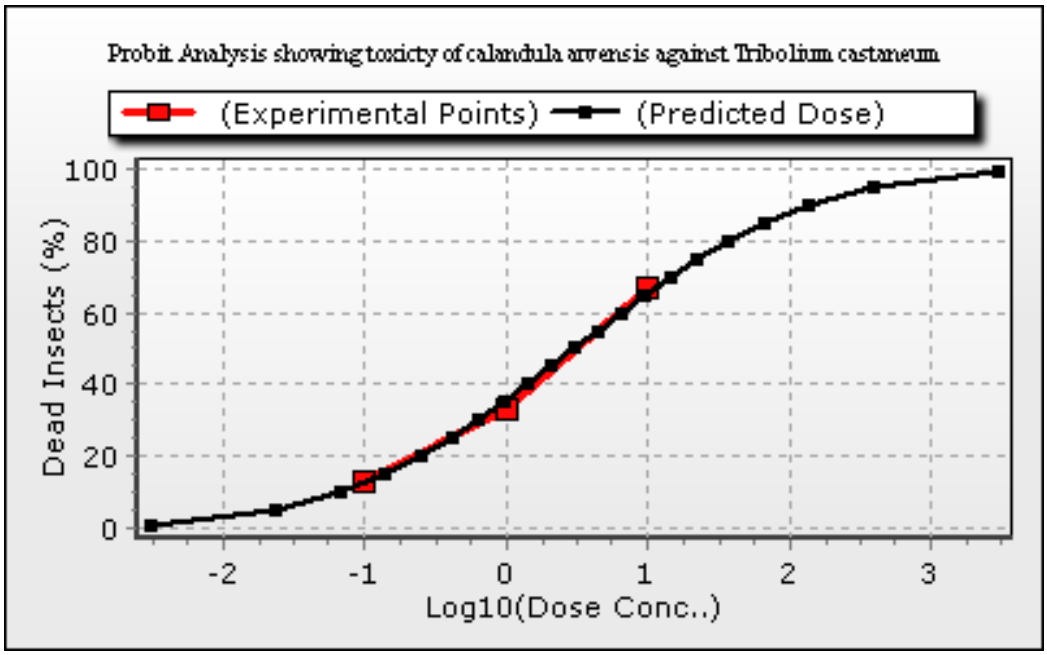
Graph 1. Showing the percent mortality rate of various pest species at +ve and –ve controls along with the *calendula arvensis* extract.



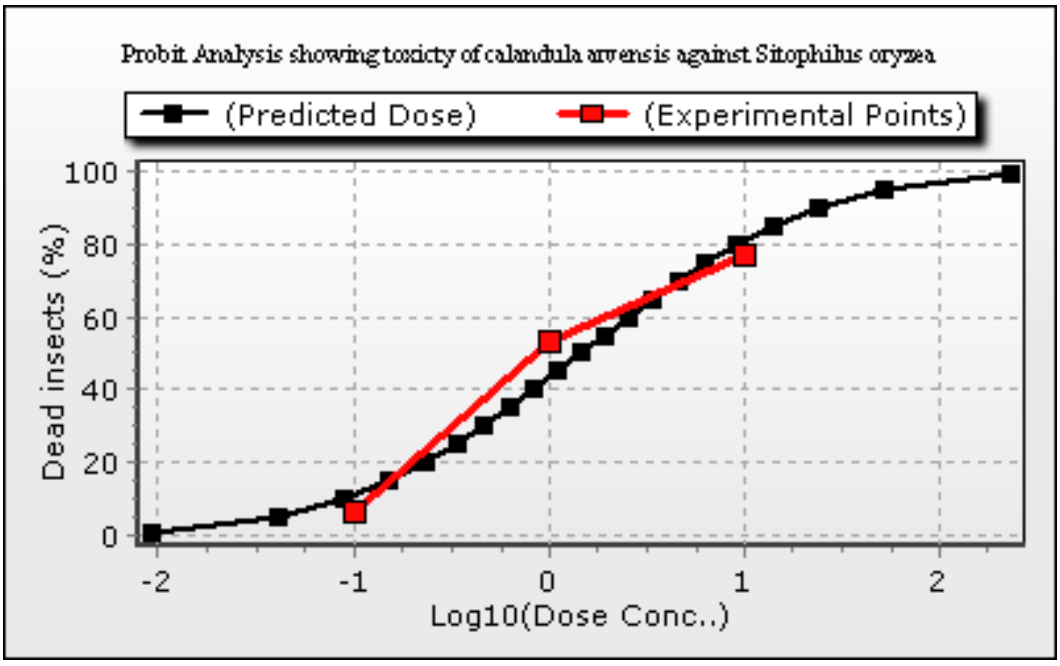
Graph 2. Showing the experimental response, expected response and regression line of Brine shrimps larvae using different concentration of *calendula arvensis*.

Table 3. LC50, 95% confidence limits, Least Square line and χ2- values for the calendula arvensis against different insect species.

Insect	Concentration (mg/ml)	Dead insect (Total)	LC50	95% confidence level		Least Square line	χ^2 (p)
				LCL	UCL		
Tribolium castaneum	0.1	4 (30)	3.035	1.31	11.07	Y= 4.62+0.78X	0.05 (0.81)
	1	10 (30)					
	10	20 (30)					
Sitophilus oryzea	0.1	2 (30)	1.45	0.75	3.01	Y= 4.83+1.06X	1.29 (0.26)
	1	16 (30)					
	10	23(30)					
Rhyzopertha dominica	0.1	1 (30)	17.25	6.53	209.51	Y= 3.88+0.91X	0.45 (0.50)
	1	3 (30)					
	10	13 (30)					
Trogoderma granarium	0.1	0 (30)	90.50	17.55	14792992.80	Y= 3.35+0.84X	0.20 (0.65)
	1	2 (30)					
	10	6 (30)					
Callosobruchus analis	0.1	7 (30)	0.51	0.18	1.18	Y= 5.23+0.80X	0.97 (0.32)
	1	21 (30)					
	10	24 (30)					

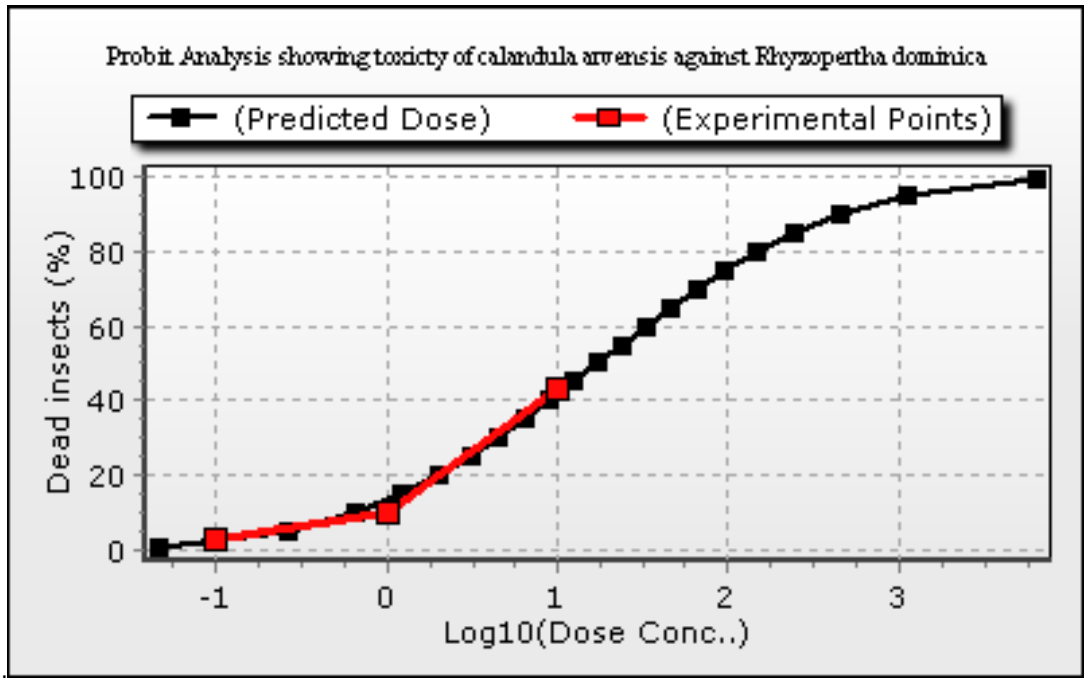


Graph 3A. Showing the experimental response, expected response and regression line of the effect of different concentration of *calendula arvensis* against *Tribolium castaneum.*

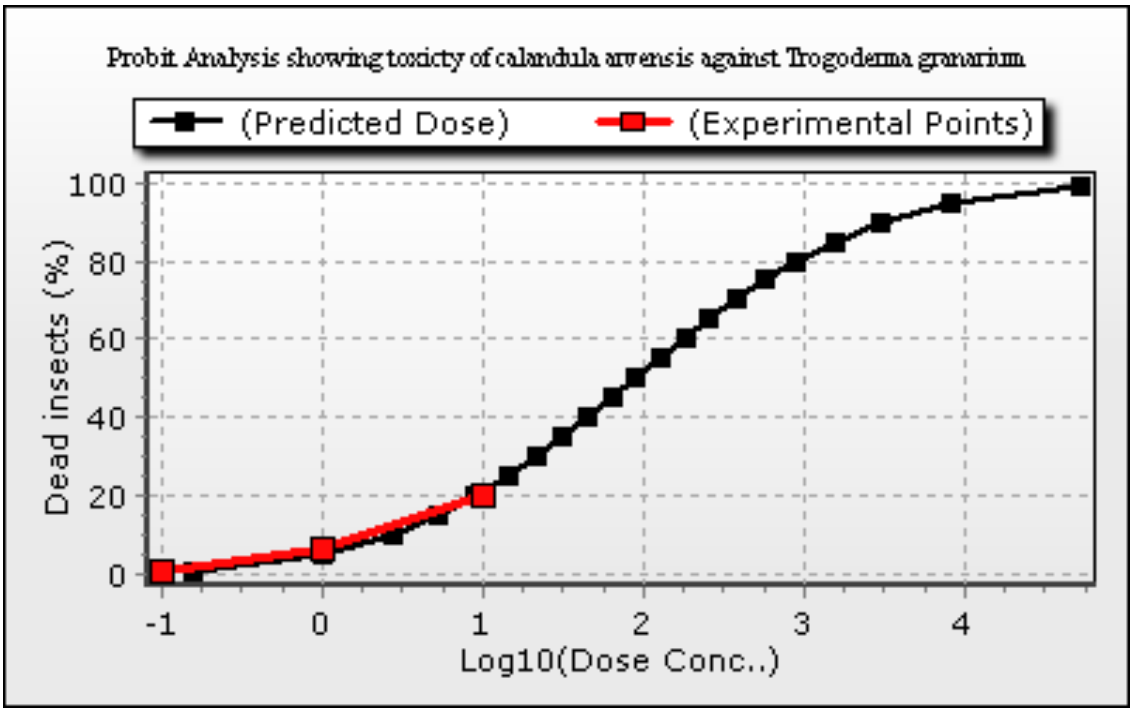


Graph3B. Showing the experimental response, expected response and regression line of the effect of different concentration of *calendula arvensis* against *Sitophilus oryzea.*

against different pest species are shown (Graph 3A, 3B, 3C, 3D and 3E). Srivastava and Gupta (2007) reported the effect of different formulations viz., aqueous suspension, aqueous extract and ether extracts of 10, 5, 2.5 and 1% concentrations of various parts (root, stem, leaf, fruit) of *Solanum surratense* (family: Solanaceae) on egg laying by the pulse beetle *Callosobruchus chinensis* Linn. The current results are also agreed with

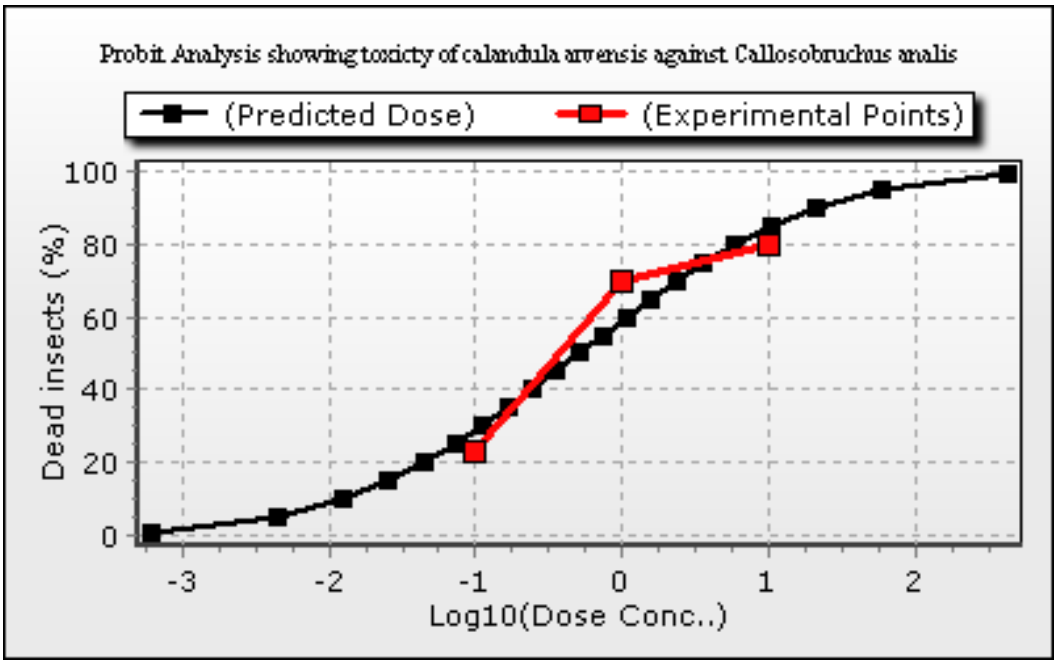


Graph 3C. Showing the experimental response, expected response and regression line of the effect of different concentration of *calendula arvensis* against *Rhyzopertha dominica*



Graph 3D. Showing the experimental response, expected response and regression line of the effect of different concentration of *calendula arvensis* against *Trogoderma granarium.*

Khalequzzaman and Sultan (2006) who reported the insecticidal activity of the seed of *Annona squamosa*



Graph 3E. Showing the experimental response, expected response and regression line of the effect of different concentration of *calendula arvensis* against *Callosobruchus analis.*

against *Tribolium castaneum.*

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