International Journal of Life Sciences. Vol. 7 No. 3. 2018. Pp. 92-98

Full Length Research Paper

Optimization of Insecticidal Potency of *Piper guineense* Seed Extracts on *Sitophylus zeamais* using the Rotatable Central Composite Design (RCCD)

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Article history
Received: 12-04-2018
Revised: 15-04-2018
Accepted: 24-04-2018

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Abstract
For safe foods and healthy environment, plant-based alternatives to the synthetic chemical insecticides are urged. Piper guineense seed is among the plant materials which have shown promising insecticidal potency on many common agricultural pests and insects.- Piper guineense seed was obtained from the fresh fruit. Common solvents were deployed to obtain extracts of the plant materials. Acute toxicity of the extracts was tested on Sitophylus zeamais using the rotatable central composite design of the response surface methodology. Optimization analysis showed that 48 hours, 45%, 72 hours and acetone/diethyl ether of exposure, time, concentration, time of extraction, solvent respectively exhibited the lowest LD_{50} (highest insecticidal potency) on the insect model. Insecticidal potency of the extracts followed the trend: 2.99>3.03>3.97µl/g of the insect at 24 hours for acetone/DME, acetone and DME respectively. The method could be useful to the local grain farmers.

Keywords: *Piper guineense, extraction coefficient, Sitophylus zeamais, RCCD.*

Introduction

Food security is hampered by unsuccessful storage of farm produce. Excess maize at harvest needs to be released at controlled, safe and functional state at off seasons. Maize (*Zea mays* L) provides food and feed, raw materials for the manufacture of general chemicals, pharmaceutical industries and cultivation. Cultivation and processing of maize has strong potential for employment (Tchoumbougnang, 2009).

Synthetic insecticides like aldrin dust, DDT, methylbromide, ethylene oxide, phostoxin are costly, and require high technical skill for effective and safe application. The high persistence on the point of application causes high toxicity level on treated food materials and the environment (Jessup and Sloggott, 1993; Guedes *et al.*, 1997; Chowanski *et al.*, 2014). Most of them contain heavy metals like lead, halogens and chemical subsistent like cyanide which are naturally toxic especially when accumulated in sufficient concentrations (Chowanski, *et al.*, 2014). Research works on identification and development of safe, cheap, renewable and easy-to-use plant-based as an alternative to the chemical synthetic insecticides have been undertaken, for example, Shah *et al.*, (2008) insecticidal characteristics in *Syzygium aromaticum*, *Piper nigrum*, and *Cinnamomum zeylanicum* on pulsed beetle, Ojimelukwe and Adler, (1999) on potential of zimtaldehyde, 4-allyl-anisol, linalool, terpineol and other phytochemicals for the control of the confused flour beetle (*Tribolium confusum* reported adequate mortality of this insect to allow the determination of LD_{50} and LD_{95} values. Ojimelukwe and Okoronkwo, (1999) on effect of preservation with *Xylopia aethiopica* on the shelf-life of cowpea reported high viability of stored seeds. Ratanabhumma *et al*., (2010) on their work, field evaluation of efficacy of bioinsecticides against the diamondback moth on Chinese Kale in Chiang Mai reported sufficiently efficacy. Dhliwayo *et al.* (2005) demonstrated the traditional methods of preservation of cereals and legumes with smoke, wood ashes, products of spices and the hermetic environment, they reported effective insecticidal potency, though for a short time and at low scale of operation. Dhliwayo *et al.* (2005) and Tchoumbougnang, (2009) on their works, agreed that application of the materials was not fully standardized, unpredictable nor target specific.

In most of the reported studies, optimization of insecticidal potential of the materials was rarely used. Bondari, (1999); Udofia *et al*. (2008) on mixture experimental design of the response surface method to optimize insecticidal potency of extracts of *Piper guineense* seed, reported a positive correlation between concentration of the extracts, time of exposure of the test insects and type solvent and insect mortality. Application of rotatable central composite design of the response surface methodology is rarely reported elsewhere.
The work optimized insecticidal potency of *Piper guineense* seed extracts on *Sitophylus zeamais* under the conditions of concentration, exposure time, time of extraction and solvent using the rotatable central composite design (RCCD) of the response

surface methodology. The work seeks to find a save and renewable source of pesticide and insecticide of plant origin to replace the synthetic counterparts. Synthetic insecticides have long history of toxicity to man and the environment. RCCD optimizes insecticidal parameters of the test substance on the test insect.

Materials and methods

Culturing of Sitophylus zeamais

The insect was cultured according to the method adopted by Asawalam and Emosairue, (2006). Adult *S. zeamais* stock used in the study was obtained from infested flint maize from Ikot Ekpene, Main market, Akwa Ibom State, Nigeria. The infested grain was incubated with whole, uninfected grains, in a porous earthen pot. The mouths of the pots were covered with muslin cloth to provide adequate ventilation for survival, growth, reproduction of the adult and progenies at room temperature (26.00±1.00^{o}C) and relative humidity of between 65%-70%. The freshly emerged progenies (light brown) were selected and used in the experiments. *Piper guineense* seed were obtained from Obo Annang market, in Essien Udim Local Government Area, Akwa Ibom State, and Southeast Nigeria and authenticated at the Botanical Unit of Department of Science Technology, Akwa Ibom State Polytechnic, Nigeria. 'Soft' local Maize was obtained from Ariam Market, Abia State, Nigeria.

Preparation of Piper guineense seed powder

Sun-dried *Piper guineense* seed was sorted of contaminants, washed and dried in the sun again to a moisture content of about 13%. The plant material was ground with an attrition mill (Model 112A Germany) to pass through a sieve pore of 200µm. The powdered plant material was stored in air-tight glass containers for analyze.

Solvent extraction of P. guineense seed powder for chromatographic analysis

The *Piper guneense* seed powder was extracted in acetone, 1:1 v/v mixture of acetone/dimethyl ether (DME) and DME. About 10g of the sample was weighed in an analytical balance (SCIENTECH 12A 120 USA) into a cold solvent extractor. The temperature of the electro-thermal heater was kept at boiling points of the solvents. Extraction was carried out according to the experimental design in Table 1. The extract-solvent mixtures were evaporated in a rotary evaporator of the extractor leaving the extracts of the plant material. The extracts were stored in glass vials and used for chromatographic analysis.

Experimental design and data analysis

The rotatable design was chosen to minimizes the number of experimental runs, cost and time (Box-Behnken 1960, Udofia *et al.* 2008). The method of response surface scales the conditions of an experiment which are optimal. Each level of the variables is combined for all the variables. The method is reliable, efficient, and provides accurate results with the minimum number of experimental level combination. The experimental design is shown in Table 1 and equation 1.

$$Y_i = \beta_o + + \sum_{i=1}^{n} \beta i x i + \sum_{i=1}^{n} \beta i i x i**2 + \sum_{i<j=2}^{n} \beta i x i + e \qquad \dots \qquad 1$$

Where Y_i = predicted response and β_o , = overall mean effect of the terms, β_I = linear effect, β_{ij} = interaction term, β_{ii} = quadratic term and , and e = random error.

Table 1: Coded and real levels of independent variables in RCCD

Independent variable						
	Unit	**-α**	**-1**	**0**	**1**	**+α**
Exposure time of insects (X_1)	Hr	15.81	24	36	48	58.18
Concentration (X_2)	(%)	3.63	10	30	50	63.63
Time of extraction (X_3)	Hr	-0.07	3	7.50	12	15.06
Solvent (X_4)	0,1,2	-0.69	0	1.0	2	2.68
Response (Y)						
Lethal dose (LD_{50})						

Solvent, 0 = acetone, 1 = dimethyl ether, 2 = 50:50 acetone/dimethyl ether mixture

Theory of lethal dose of insecticides and the probit analysis

According to Abbot (1925), the principle of the probit analysis is that, the percentage mortality with and without adjustment for natural mortality in treated and control runs calculated according to equations 2 and 3.

Adjusted % mortality = x 100 … (2)

Percentage mortality = x 100 …..(3)

The most adopted method for the determination of LD_{50} is the probit analysis (Finney, 1952; Steel and Torrie, 1980). Probit analysis is a specialized regression model of binomial response variables to determine dose-response relationship according to equation 4.

$$P = \frac{1}{\sqrt{(2\pi)}} \int_{-8}^{Y-5} e^{-\frac{1}{2}u^2} du \quad \dots (4)$$

The standard method makes use of analysis of maximum and minimum working probits.

$$Y_{max} = Y + \frac{Q}{Z}$$

$$Y_{min} = Y - \frac{Q}{Z}$$

and the range $\frac{1}{Z}$, where $Z = \frac{1}{\sqrt{(2\pi)}} e^{-0.5(Y-5)2}$

Acute toxicity of P. guineense of extracts on S. zeamais

zPlacement of experimental units on acute toxicity randomized with the ballot method (Steel and Torrie, 1980; Scharf *et al.,* 2006). Allocation of experimental variables was done according to the experimental design in Table 1. Quantities of 0.1, 0.2, 0.3, 0.4, 0.5 ml of extract from *P. guineense* seed extracts were further diluted in 10ml of 95% ethanol to give 0.1, 0.2, 0.3, 0.4, and 0.5µl/ml solution of the compound. Ash-free, 9cm diameter, Whatman filter papers were impregnated with graded concentrations of its solution, and placed on the bottom of glass Petri dishes and left opened for 12 hours to allow for complete evaporation of the ethanol solvent.

Twenty-two (22) to thirty-nine (39) test insects (WHO, 1952) were introduced into the Petri dishes, the lids replaced. Mortality of the insects for each experimental run was examined at 24, 48 and 72 hours' intervals. The weevils were presumed dead if they remained immobile and did not respond to six probing with a blunt peaked object and knocked down if neither death or alive.

The equation given as follows was used to calculate the percentage mortality of the insects since death of control experiments for all the experimental units was uniform (Abbot, 1925; Finney, 1952)

$$\text{Mortality (\%)} = \frac{\text{Number of dead insects}}{\text{Total number of insects used}} \times 100 \quad \dots \quad (5)$$

$$Y_i = \beta o + kx + e \quad \dots \quad (6)$$

$$Y_i = \beta o + k\frac{\text{antilog of Concentration (x)}}{100} + e$$

Where Y_i = expected **L**ethal **D**ose level at a particular concentration of the test substance, β_o = intercept, probit value when concentration is zero, k = slope of the probit-regression plot, x = equivalent of concentration of the test substance (antilogarithm of concentration ÷ 100), e = experimental error. The data analysis of the probit values was carried out with DesignExpert Version 7 (Statease, USA) using backward elimination regression method with alpha to exit at 0.1000 to reduce model 1.

Plots of probit values versus logarithm of concentration x 100 of the extracts were made. Probit regression analysis was fitted to the data obtained. The regression line provided a lead to the relationship between the probit values and the antilogarithm of the concentrations. The regression equation provided the model for the determination of LD_{50} values.

Result and discussion

Table 2 presents the 2^{nd} degree polynomial fitted to the data of probit values (not shown here) using the response surface analsis and parameters of the general linear model (GLM). The table shows that the linear (X_1, X_2, X_4), cross product (X_1X_4, X_2X_4) and quadratic (X_2^2) terms of the variable were significant showing near adequate quadratic fit ($p<0.05$, $R^2 = 0.6991$) for the predictive model. Since the coefficent of estimates for X_2 is higher than all others, it can be concluded that the variable is the most important influence on insecticidal potency of the extract and others following the following trend in Table 2. Variable X_3 was not significant on the insecticidal potency of the plant product, hence it was removed from the model, but the plots showed that they could be significant in combination with other variables, the coefficient of variable X_4 was not significant, indications are that interactions with X_1 and X_2 may produce synergistic effects for insect control. In general, positive interaction coefficients indicate synergism if higher responses are better and negative coefficients indicate antagonism. The inverse is true if lower responses are favored. The observation is elucidated by equation 1 and Fig. 3 to 5..

According to the lethal table (not shown here), acetone extract of *Piper guineense* seed on *S. zeamais* at 24 hours of exposure showed LD_{50} of 3.99µl/g of the test insect. The model did not appear to be significant (p=0.0795), and appeared predictable, R^2=0.8081. The model at 48 hours of exposure was significant, $p<<0.05$, and R^2=0.8077 and LD_{50} of 3.00µl/g of the test insect. The model for 72 hours exposure showed a negative slope which is difficult to explain statistically. Model of probit of mortality against concentration of acetone/dimethyl ether extract of *Piper guineense* seed on *S. zeamais* at 24 hours of exposure appeared to be significant (p=0.0581) and predictable (R^2=0.8118). The model produced LD_{50} of 3.03µl/g of the test insect. The model for 72 hours exposure did not show an explainable relationship between the variables. The model of probit of mortality against concentration of dimethyl ether extract of

Piper guineense seed was not significant, p=0.5213 and R^2=0.7705 (Sha, 2011), but with an LD_{50} of 2.99µl/g of the test insect. Similarly, the model for 48 hours of exposure was not significant, p= 0.1095, and reliable, R^2=0.8903 with an LD_{50} of 4.15µl/g of the test insect. The model at 72 hours of exposure followed the same trend and the models above. The trend of insecticidal potency of the plant product extracts was as follows: acetone/DME (48hr) at 2.76µl/g>DME at (24hr) 2.99µl/g>acetone at (48hr) 3.00µl/g>acetone at (24 hr)>DME at (24 hrs) 4.15µl/g.

Table 2. Shows the estimate of effect of the vatiables LD_{50} of *P. guineense* seed extracts

Effect of term	Coefficient of estimates	Standard error	p-value
Intercept	3.77	0.19	-
X_1	0.86	0.29	*0.0111*
X_2	1.14	0.29	*0.0017*
X_4	0.22	0.19	*0.2657*
X_1X_4	0.86	0.38	*0.0396*
X_2X_4	0.87	0.38	*0.0425*
X_2^2	-0.46	0.18	*0.0236*
R^2 = 0.6991			
Adj. R^2 = 0.5701			
Mean = 20.14			
Model = 0.0044			

Model = significance of the model

In this work, it was observed that the percentage insect mortality was a factor of concentration of the test substance and time of exposure of insects to the test substance (Fig). Sharma *et al.* (2004) reported that sex, age and source of the insect model may also play some role. It was also observed that among the extracts, the acetone/dimethyl ether extract exhibited the highest insecticidal potency, with the lowest value of LD_{50} indicating the lowest concentration of the test substance to cause 50% death on the test insects (Koona and Koona, 2006). This result was in agreement with that reported by Koona and Koona (2006), on testing fractionated extracts of ethnobotanical, *Pachypodanthium staudtii,* (Annonaceae) for Bruchid insect control (Cloleoptera: Bruchidae). This result might be attributed to increased extraction coefficient of the solvents in combination than when used singly. Extraction coefficient of solvent enhances higher extraction of desirable solutes from the target material.

According to Table 2**,** it appears that the potency of crude extracts of the plant product was more persistent at the point of application, beyond 72 hours, but the plots show that the insecticidal potency decreased after 48 hours. The depletion of the insects at this point may make accurate extrapolation of insecticidal potency difficult calling for higher population of insects. Lethal dose (LD_{50}) = $3.77+0.86\,X_1+1.14X_2+0.22X_4+0.87X_1X_4+0.86X_2X_4-0.46X_2^2$ (1)
Insecticides may be applied as fumigants, repellents, attractants, insect growth regulators, or pheromones to achieve the desired effect (Bolboaca and Jantschi 2005).

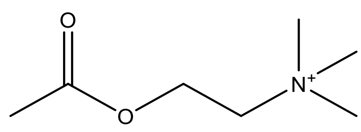

Fig 1: Molecular formula of acetylcholine molecule

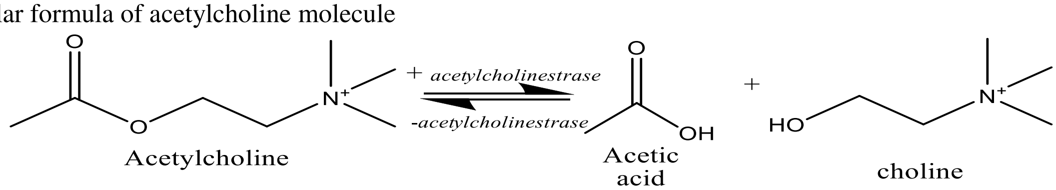

Fig 2: Degradation of acetylcholine molecule

According to Ratanabhumma *et al.* (2010), fumigants are low molecular weight molecules which are applied as gases, or in a form which vaporizes to form gas. Therefore, they can enter respiratory system of the insects and establish their inhibitive effects on *acetylcholinesterase* molecule. According to Fig. 2, acetylcholinesterase is a neurotransmitter which regulates the synthesis of acetyl choline in insects, alteration of the acetylcholinastrase creates excess of acetyl and choline in the system producing a state of over-firing, a situation which may lead to a knock down effect or death of the insect, depending on the insect, humidity, age of insect and temperature of the experimental unit (Ojimelukwe, 2008). The mechanism may also be attributed to electron transfer ability of the active molecules to acetylcholinastrase (Chowanski, 2014), partial charge transfer leads to knock down effect and probably development of resistance in the insects to future application of same chemical.

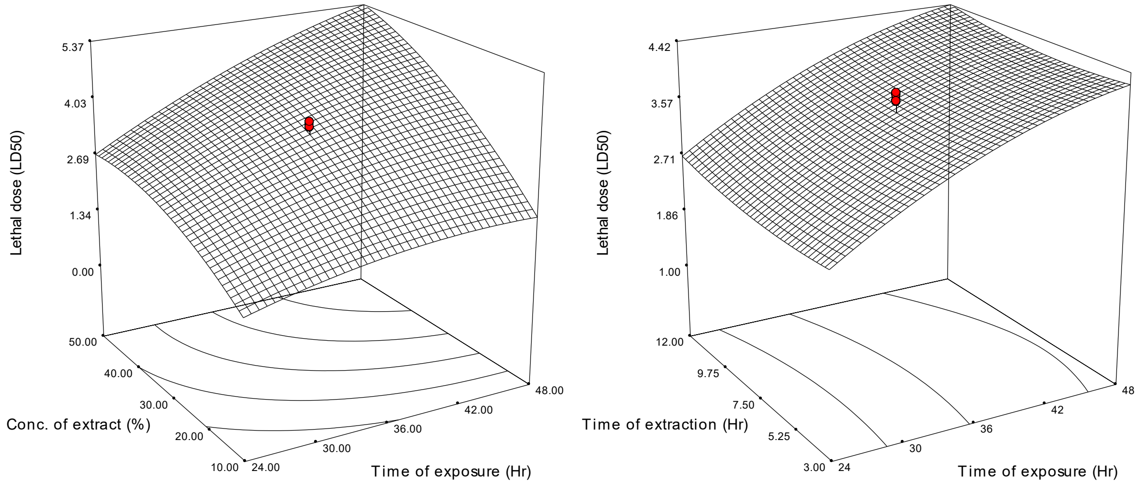

Fig 3: Response surface plot for LD_{50} versus concentration of extract, extraction time and exposure time.

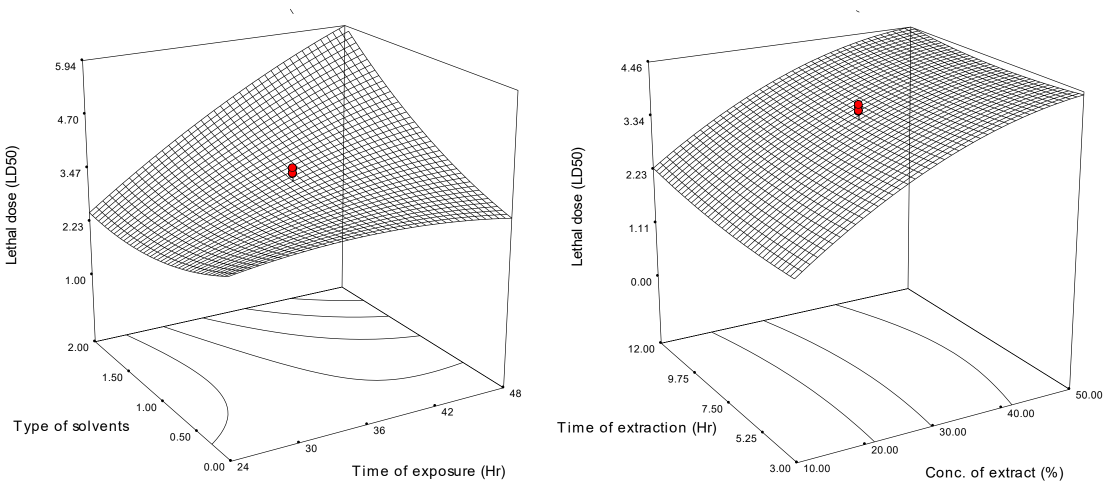

Fig 4: Response surface plot for LD_{50} versus solvent, exposure time and concentration of extracts.

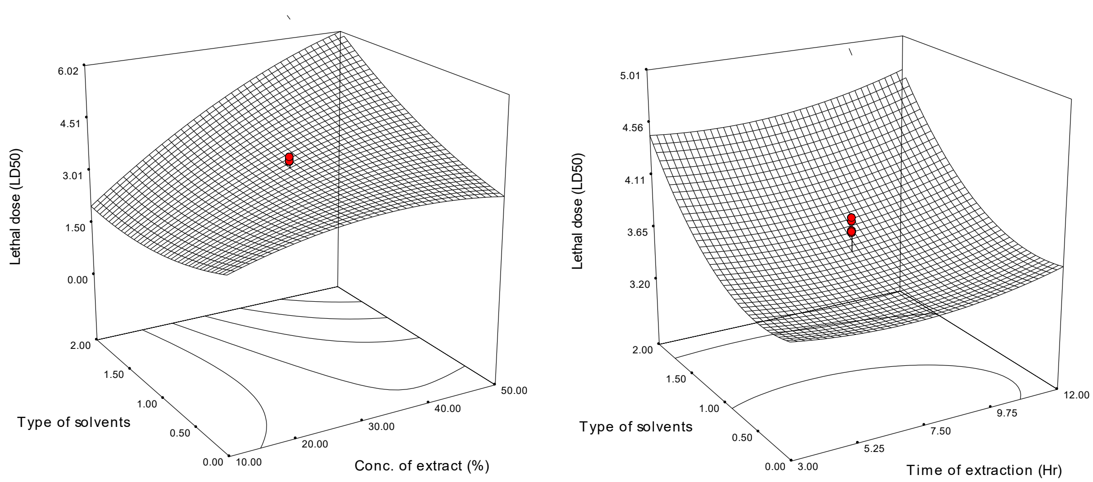

Fig 5: Response surface plot for LD_{50} versus solvent, concentration of extract and time of extraction.

Optimization analysis showed that 24 hours of exposure of the insect model to the test material, 10μl of the test martial, 3.0 hours of extraction time, and solvent between acetone and dimethyl ether could create the LD_{50} of μl/g of the model insect at 100% desirability level.

Conclusions and recommendations

This study thus demonstrates that cold and hot ethanol and acetone extracts of *Piper guineense* seed were relatively rich in crude fat, crude protein, carbohydrate and ash. The crude fat shows good industrial potency. Insecticidal potency of chemical components isolated from acetone, acetone/dimethyl ether and dimethyl ether extracts were comparable with that of the raw extracts of the plant materials, but the extracts were more persistent at the point of application. Insecticidal potency of the model molecule is proposed to arise from the chemical descriptors; insecticidal potency of other molecules may therefore be determined or enhanced using the same method. These hypotheses are the subject of ongoing investigations.

Declaration of interest

There is no actual or potential conflict of interest including financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence the work.

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