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Insecticidal activities of pellitorine isolated from *Zanthoxylum zanthoxyloides* roots against *Sitophilus oryzae* L. (Coleoptera: Curculionidae)

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Abstract

In the present study, the insecticidal activity of *Z. zanthoxyloides* was studied to isolate its bioactive compound against *S. oryzae*. Bioassay guided isolation led to the isolation of (2*E*, 4*E*)-*N*-(2-methylpropyl) deca-2,4-dienamide (pellitorine) as the main bioactive compound responsible for insecticidal activities against *S. oryzae*. The amount of isolated pellitorine from the 1g of methanol extract was 6.25 mg and its LC₅₀ against the adult *S. oryzae* was found to be 1.92 mg/mL (95% CL: 1.352 – 2.737 mg/mL; Slope ± SE: 3.60 ± 0.288; X²: 24.0). The LT₅₀ value ranges from 48.23 h (for the highest concentration of 10.0 mg/mL to > 5183.08 h for the least concentration of 0.5 mg/mL of pellitorine. This compound justifies *Z. zanthoxyloides* as an insecticidal plant for managing stored product pests especially *S. oryzae*, based on the results of present study.

Keywords: *Zanthoxylum zanthoxyloides*; (2*E*, 4*E*)-*N*-(2-methylpropyl) deca-2,4-dienamide; Pellitorine; *Sitophilus oryzae*; insecticidal activities

1. Introduction

Sitophilus oryzae L. (Coleoptera: Cucurlionidae) is one of the most serious post-harvest pests of stored products including rice and maize grains in tropical regions of the world. The adult females bore holes into the grains and lay their eggs inside them^[1]. The eggs hatch into larvae which feed within the grains, pupate and the adults emerge out through the feeding holes created in the grains^[1]. These activities lead to the reduction in both the nutritive and germination quality of the grains. Thus, both the adults and larvae are destructive to the stored rice grains.

A huge loss from both the damage caused by the pest and the cost involved in controlling them occurs annually and this is quite problematic to farmers. The control of *S. oryzae* like other stored product pests includes the use of insecticides, biological and physical control. The use of botanicals in recent times has been found to be useful^[2, 3]. These are plant products with purported insecticidal activities against insects as well as little or no mammalian toxicities. In most cases the leaves, stems or roots are blended into powder and are mixed with the stored produce at storage. The powder as well as methanol extract treatments from its various parts has been found to be useful in protecting stored products against insect attack^[4, 5].

Natural compounds present in these botanicals have been known to be responsible for the protection they offer against insect pests^[6-9]. Pellitorine is one of the many compounds known for fungicidal, larvicidal and insecticidal activities^[8]. Pellitorine was isolated from *Piper tuberculatum* as insecticidal compounds active against the velvet bean caterpillar^[10]. It has also been isolated from several *Zanthoxylum* species as a bioactive compound against several insect pests^[11, 12]. Insecticidal activities of pellitorine has been studied against mosquitoes^[13] and lepidopteran species^[14], however little has been reported on its insecticidal activities against stored product beetles especially the weevils.

Zanthoxylum zanthoxyloides Lam has also been found to show insecticidal activities against several insects^[15, 16]. It is widely grown in savanna forest habitats and sometimes in coastal thickets^[17] and it thrives well in dry and well drained soils. It is a shrub that can grow to a small tree of up to 1.25 m high and 0.13 m girth^[18]. Since the roots, stem and leaves powder has been used traditionally for pest management by farmers, it is important that the compound or compounds responsible for the insecticidal activities of *Z. zanthoxyloides* are elucidated to

determine the amounts required to achieve maximum control of these insects. In the present study, we used a bioassay guided approach to isolate and identify the bioactive compound in *Z. zanthoxyloides* roots that is responsible for insecticidal activities against *S. oryzae*.

2. Materials and methods

2.1 Insect rearing

The adult *S. oryzae* were cultured in brown rice under a temperature of 27 ± 2 °C, 70% relative humidity and 12 L: 12 D photo regime, in the Chemical ecology laboratory of Kochi University, faculty of Agriculture, Nankoku, Japan.

2.2 Preparation of plant materials and roots extract.

Z. zanthoxyloides roots were harvested from the University Farms, University of Ghana, Legon in March 2014. They were cut into pieces and dried in the laboratory under a temperature of 32 °C and were then blended into fine powder. About 200.40 g of the root powder was soaked in 1 L of methanol was kept for 48 h after which the solvent was evaporated to obtain the crude extract. The concentrations of the crude extract were adjusted with methanol at 0.5 g equivalent (eq.)/mL 1.0 g eq. /mL and 2.0 g eq. /mL of the extract and were used for bioassay.

2.3 Insecticidal bioactivity bioassay method

Ten adult insects were each dipped in turns into each sample and then transferred into clean Petri dishes containing brown rice. Six replicates were made and these were observed daily for insect mortalities and survivals until there were no changes in mortalities and survivals. An insect was considered dead if it did not respond to probing of a blunt probe [19].

2.4 Isolation of bioactive components

The methanol extract (6.0 g) of the *Z. zanthoxyloides* roots was dissolved in H₂O (150 mL), and then extracted twice with diethyl ether (110 mL) and ethyl acetate (AcOEt) (110mL). The diethyl ether layer (1.06 g) was chromatographed on Silica gel open column (31.8 g, 15 mm Ø x 350 mm). The column was eluted with 600 mL each of hexane, 5% AcOEt in hexane, 30% AcOEt in hexane, 70% AcOEt in hexane, AcOEt and methanol. The 30% AcOEt in hexane fraction (0.30 g) was submitted to normal phase Silica gel HPLC

(Column: Cosmosil 5SL, Nacalai Tesque Inc.: Kyoto, Japan; 10 mm Ø x 250 mm) eluted with 23 % AcOEt in hexane at a flow rate of 3mL/min and UV detector (254 nm).

Five fractions {fraction (Fr.) 1 (t_R : 0.00 min – 14.00 min), Fr. 2 (t_R : 14.01 min – 16.80 min), Fr. 3 (t_R : 16.81 min – 18.80 min), Fr. 4 (t_R : 18.81 min – 22.00) and Fr. 5 (t_R : 22.01 min – 35.00 min)} from the HPLC analysis were collected and used for bioassay. The Fr. 3 (t_R = 16.81 min) was further collected and analysed by LC-MS and ¹H and ¹³C- NMR analysis.

2.5 Median lethal dose (LC₅₀) and time (LT₅₀) of isolated compound

The LC₅₀ and LT₅₀ of the isolated compound was determined using 10 mg/mL, 6 mg/mL, 3 mg/mL, 1 mg/mL and 0.5 mg/mL of the isolated compound for bioassay. The set up was monitored after every 24 h for insect mortality. Insect mortality data was analyzed using probit analysis and the LT₅₀ value for each concentration as well as the LC₅₀ value were determined.

2.6 Instrument

LC-MS data (EI-positive) were recorded with Shimadzu LCMS-2010 (UV: 254 nm, column temperature 32 °C, Solvent 100% MeOH, Flow rate 0.2 mL/min). ¹H and ¹³C- NMR spectra were measured with Jeol JNM-ECX500, TMS as internal standard.

2.7 Data collection and analysis

Data collected were analysed using GenStat Statistical Package 9.2 (9th Edition, VSNi, London, UK) and SPSS Statistics (IBM, USA). Analysis of variance was run at 95% confidence level and men separation was done using Tukey's HSD. Data involving counts were transformed using square root ($y = \sqrt{x}$) transformation while those involving percentages were transformed using arcsine ($y = \sin^{-1}\sqrt{x / 100}$) transformation before analysis. Mean (\pm SE) of untransformed data are reported.

3. Results

The three concentrations prepared from the crude extracts of the *Z. zanthoxyloides* roots showed significantly ($F_{pr} < 0.01$) high insecticidal activities against adult *S. oryzae* from the control (Table 1).

Table 1: Percentage survival of *S. oryzae* to the different concentrations of methanol extracts of *Z. zanthoxyloides* roots.

Treatment (g eq./mL)	% Survival							
	0 h	24 h	48 h	72 h	96h	120 h	144 h	168 h
2 g eq. /mL	100.0 \pm 0.0 ^a	50.0 \pm 5.7 ^a	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a	0.0 \pm 0.0 ^a
1 g eq. /mL	100.0 \pm 0.0 ^a	65.0 \pm 7.1 ^a	3.3 \pm 2.9 ^a	0.0 \pm 0.0 ^a				
0.5 g eq. /mL	100.0 \pm 0.0 ^a	81.7 \pm 2.4 ^b	38.3 \pm 1.9 ^b	26.7 \pm 5.7 ^b	19.2 \pm 3.6 ^b	18.3 \pm 3.8 ^b	15.8 \pm 4.0 ^b	14.2 \pm 4.8 ^b
Control	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^c						

Values with different small letters within the columns are significantly different ($F_{pr} < 0.01$). based on Tukey's HSD test following ANOVA.

* Each value is expressed as mean \pm S. E (n=6 x 10).

The 2.0 g eq. /mL and the 1.0 g eq. /mL concentrations both yielded a percentage survival of 0.0 \pm 0.0% after the 168 h while the 0.5 g eq. /mL yielded a percentage survival of 14.2 \pm 4.8%. There were no mortalities recorded in the control

which consisted of only methanol.

The results of the insecticidal activities of the three layers obtained from the liquid-liquid partitioning (2.0 g eq./mL) are shown in Table 2.

Table 2: Percentage survival of *S. oryzae* to the four separated layers from liquid-liquid partitioning of the methanol extracts of *Z. zanthoxyloides* roots.

Treatment (Per 2g eq./mL)	*% Survival							
	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Diethyl Ether Layer	100.0 ± 0.0 ^a	66.7 ± 4.4 ^a	8.0 ± 5.4 ^a	0.0 ± 0.0 ^a				
Ethyl Acetate Layer	100.0 ± 0.0 ^a	100.0 ± 0.0 ^b						
Water Layer	100.0 ± 0.0 ^a	100.0 ± 0.0 ^b						
All layers	100.0 ± 0.0 ^a	48.3 ± 9.4 ^c	3.3 ± 1.4 ^a	0.0 ± 0.0 ^a				
Control	100.0 ± 0.0 ^a	100.0 ± 0.0 ^b						

Values with different small letters within the columns are significantly different ($F_{pr} < 0.01$). Based on Tukey's HSD test following ANOVA. * Each value is expressed as mean ± S. E (n=6 x 10).

Of the three layers, the diethyl ether layer significantly ($F_{pr} < 0.01$) showed the highest insecticidal activity of $0.0 \pm 0.0\%$ insect survival after 72 hours while the water and AcOEt layers as well as the control on the other hand showed no insect mortalities. From these results, the activities of the original extract are considered to be concentrated in the

diethyl ether layer.

The six fractions obtained from the silica gel open column chromatography of the active diethyl ether layer yielded various insecticidal activities against the adult *S. oryzae* (Table 3).

Table 3: Percentage survival of *S. oryzae* to the various fractions from the Silica-gel open column chromatography of the diethyl ether layer of *Z. zanthoxyloides* roots.

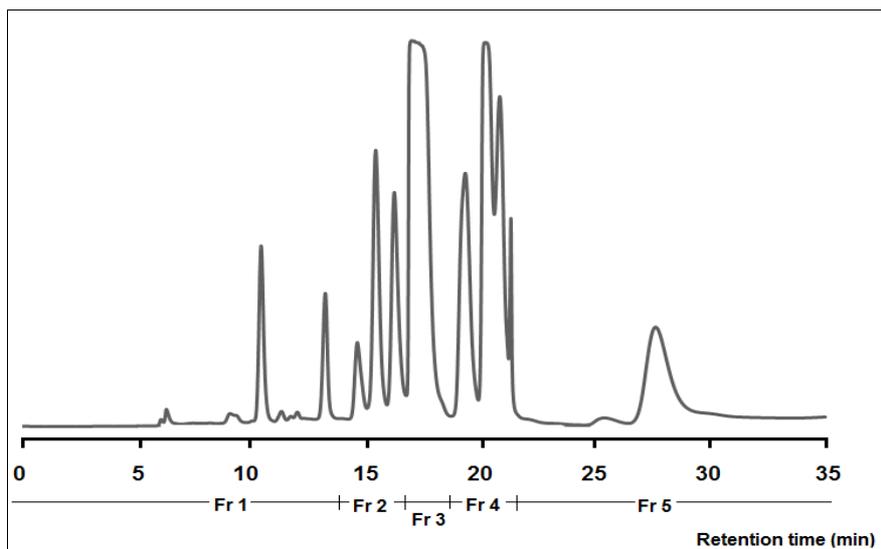
Treatment (Per 2g eq./mL)	*% Survival							
	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
100% Hexane	100.0 ± 0.0 ^a							
5% AcOEt in hexane	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	96.0 ± 2.2 ^a	96.7 ± 2.2 ^a				
30 % AcOEt in hexane	100.0 ± 0.0 ^a	75.0 ± 4.29 ^b	16.7 ± 6.5 ^b	16.7 ± 6.5 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b
70 % AcOEt in hexane	100.0 ± 0.0 ^a							
100 % AcOEt	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a
100 % MeOH	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a
All fractions	100.0 ± 0.0 ^a	35.0 ± 2.7 ^b	13.3 ± 7.8 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b
Control	100.0 ± 0.0 ^a							

Values with different small letters within the columns are significantly different ($F_{pr} < 0.01$). based on Tukey's HSD test following ANOVA.

* Each value is expressed as mean ± S. E (n=6 x 10).

The 30% AcOEt in hexane fraction showed a significantly ($F_{pr} < 0.01$) higher insecticidal activity from the other fractions. It yielded percentage survival of $0.0 \pm 0.0\%$ after 96 h while the 5% AcOEt in hexane, 100% AcOEt, methanol fractions on the other hand yielded $96.7 \pm 2.2\%$ survival each after 96 h. There were no insect mortalities in the 70% AcOEt

in hexane fraction, 100% hexane fraction and the control. When the 30% AcOEt in hexane fraction was submitted to HPLC analysis several peaks were observed at different retention times. These peaks were grouped into five fractions (Fig. 1) which showed significantly different ($F_{pr} < 0.01$) insecticidal activities against the adult *S. oryzae* (Table 4).

**Fig 1:** HPLC profile for the 30% AcOEt in hexane fraction [(Fr.) 1 (tr: 0.00 min – 14.00 min), Fr. 2 (tr: 14.01 min – 16.80 min), Fr. 3 (tr: 16.81 min – 18.80 min), Fr. 4 (tr: 18.81 min – 22.00) and Fr. 5 (tr: 22.01 min – 35.00 min)].

After 120 h, percentage survival of $10 \pm 0.0\%$ was observed for Fr. 3, while $96.7 \pm 2.2\%$ survival was observed for Fr. 4 and Fr. 5 and $96.7 \pm 2.2\%$ was observed for Fr. 1. No insect mortalities were observed for Fr. 2 and the control. When all these fractions were combined in equal volumes (1.0 mL), the

insecticidal activity increased further to give $0 \pm 0.0\%$ insect survival after the period of study. Fr. 3 which consists of only one peak was designated compound 1 is the compound responsible for the insecticidal activity in *Z. zanthoxyloides* against *S. oryzae* from the bioassay results

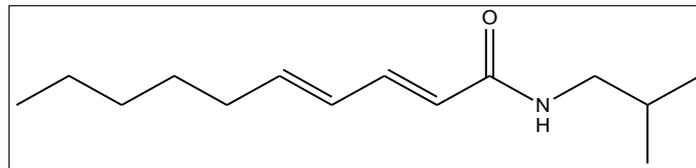
Table 4: Percentage survival of *S. oryzae* to the HPLC fractions isolated from the 30% AcOEt in hexane fraction.

Treatment (Per 2g eq./mL)	% Survival							
	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Fraction 1	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	98.3 ± 2.4 ^a	96.7 ± 4.8 ^a	96.7 ± 4.8 ^a	96.7 ± 4.8 ^a	96.7 ± 2.2 ^a
Fraction 2	100.0 ± 0.0 ^a							
Fraction 3	100.0 ± 0.0 ^a	86.7 ± 3.8 ^b	56.7 ± 9.5 ^b	36.7 ± 9.5 ^b	20.0 ± 11.4 ^b	10.0 ± 0.0 ^b	10.0 ± 0.0 ^b	10.0 ± 0.0 ^b
Fraction 4	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a
Fraction 5	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a	96.7 ± 2.2 ^a
All fractions	100.0 ± 0.0 ^a	31.7 ± 4.8 ^c	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c
Control	100.0 ± 0.0 ^a							

Values with different small letters within the columns are significantly different (*Fpr.* < 0.01). based on Tukey's HSD test following ANOVA. * Each value is expressed as mean ± S. E (n=6 x 10).

The analysis of the NMR spectra and LC-MS (EI positive mode) data showed that compound 1 has a molecular weight is 223, with 3 methyl (CH₃) groups, 5 methylene (CH₂) groups, 5 methine (CH) groups, a carbonyl carbon (C=O) and

an amido (NH) group. By arranging the spectra information, compound 1 was identified to be (2*E*, 4*E*)-*N*-(2-methylpropyl)deca-2,4-dienamide. This compound is also known as pellitorine (Fig. 2).

**Fig 2:** The structure of isolated (2*E*, 4*E*)-*N*-(2-methylpropyl)deca-2,4-dienamide (pellitorine).

The amount of pellitorine isolated from the 1 g methanol extract was 6.25 mg and the lethal concentration required to cause 50% mortality in adult *S. oryzae* (LC₅₀) was determined to be 1.92 mg/mL (95% CL: 1.352 – 2.737 mg/mL; Slope ± SE: 3.60 ± 0.288; X²: 24.0). The time needed for pellitorine to cause 50% mortality in adult *S. oryzae* ranged from 48.23 h

(95% CL: 36.93 – 62.98) for the highest concentration of 10.0 mg/mL to > 5183.08 h for 0.5 mg/mL of pellitorine (Table 5). Generally, LT₅₀ values decreased with increase in pellitorine concentration and thus susceptibility of adult *S. oryzae* can be directly associated with pellitorine concentration as well as time of exposure.

Table 5: LT₅₀ of isolated pellitorine against *S. oryzae* at various concentrations.

Concentration (mg/mL)	LT50 a h (95% CLb)	Slope ± SE	cX2 (df)	R2
10.0	48.23 (36.93-62.98)	3.21 ± 0.06	56.0(49)	0.910
6.0	51.72 (40.03 – 66.83)	3.09 ± 0.06	48.0(42)	0.978
3.0	54.85 (41.35 – 72.75)	2.74 ± 0.06	48.0(42)	0.930
1.0	5183.08(1510.19 – 17788.63)	0.72 ± 0.27	16.0(14)	0.615
0.5	> 5183.08			

^a LT₅₀ units were applied for different concentrations at 27 ± 2 °C and 70% relative humidity

^b 95% lower and upper confidence limits are shown in parenthesis (n=6 x 10)

^c X² (df): Chi-square values (degrees of freedom)

4. Discussion

Zanthoxylum zanthoxyloides has been found to be an effective botanical in managing insect pests. Udo ^[16] reported that, the bark and root of *Z. zanthoxyloides* provided up to 100% protection of grains against infestation by *S. zeamais*, *T. castaneum* and *C. maculatus*. Application rates ranging from 0.125 g to 3 g per 20 g seed, root bark powder of *Z. zanthoxyloides* was used as an effective oviposition suppressant against the cowpea seed bruchid *C. maculatus* ^[17]. Several compounds have been isolated from *Z. zanthoxyloides*. The stem bark and root has been found to contain aromatic compounds such as benzophenanthridine, furoquinoline, aporphine alkaloids and several aliphatic amides ^[20]. However, in the study, pellitorine has been found to be the most active compound in the roots of *Z. zanthoxyloides* responsible for insecticidal activity against *S. oryzae*.

Pellitorine has been isolated from several *Zanthoxylum species* such as *Z. macrophylla*, *Z. gillettii*, *Z. actifolium* and *Z. petiolare* ^[21]. It has been found to exhibit potent ovicidal action against the potato beetle, *Leptinotarsa decemlineata* even at lower concentration ^[12].

For an insecticide to act at its target site, it must enter the insect through one or more absorption routes, including the cuticle, orally through the feeding on treated food, or inhalation through the spiracles ^[22]. When the insecticide enters the body of the insect, the active ingredient then distributes throughout the body to reach the target sites ^[22]. Considering the bioassay method used in this study, pellitorine can be considered to have been absorbed through the cuticle of *S. oryzae*. Perumalsamy *et al.* ^[23] reported that pellitorine is effective against *Culex pipiens pallens* larvae which has been found to have high levels of resistance to acetylcholinesterase (AChE) inhibitors such as chlorpyrifos, fenitrothion, and fenthion as well as axonic nerve poisons such as α – cypermethrin and deltamethrin. Their results thus mean that, pellitorine may not share a common mode of action as these categories of insecticides. However, this was not considered extensively in the present study.

Park *et al.* ^[13] isolated piperine, pellitorine, guineensine, piperidine and retrofractamide A from *Piper nigrum* fruit. Of the five compounds isolated, the isobutylamides; pellitorine, guineensine, piperidine and retrofractamide A were found to have larvicidal activities against three mosquito species ^[12].

This results indicate that the isobutylamine moiety appeared to be essential for insecticidal activities. This observation was similar with the findings of Miyakado *et al.* [24] who observed that by changing the amide moiety from isobutylamides of dihydropiperidine to other branched or cyclic aliphatic amines, the insecticidal activities was decreased by one-third or one-fourth compared to that of the parent dihydropiperidine. Additionally, a difference in insecticidal activities between these isobutylamides with and without a methylenedioxyphenyl moiety against *Plutella xylostella* has also been observed [14]. Without a methylenedioxyphenyl moiety, pellitorine was the least toxic and thus it can be concluded that the methylenedioxyphenyl moiety improved the insecticidal activity of isobutylamides by stabilizing the chemical structure [13].

The unsaturated isobutylamides are neurotoxic and they impair or block voltage dependent sodium channels on nerve axons [25]. Being neurotoxic, these amides show both knockdown and lethal action against pyrethroid susceptible and resistant insects. The neurotoxic activities may be caused by the amide functionality in these isobutylamides [26]. This can therefore be responsible for the observed insecticidal activities of pellitorine against *S. oryzae* in the present study.

The LC₅₀ value of pellitorine against *S. oryzae* was found to be 1.92 mg/mL in the present study. Comparably, this value is quite higher than LC₅₀ value of the standard insecticide, deltamethrin (2.8 EC) which was found to be 0.66 ppm (95% CL: 0.35 – 1.11, Slope: 1.13) against *S. oryzae* [27]. Pellitorine like other insecticides can be quite hazardous when proper care is not taken during its application. However, its use as flavoring agent in food makes it less harmful to humans [26].

5. Conclusion

The results of the present study has shown that (2*E*, 4*E*)-*N*-(2-methylpropyl) deca-2,4-dienamide (pellitorine) is the major compound in the root extract of *Z. zanthoxyloides* responsible for insecticidal activities against *S. oryzae*. Considering the continual search for renewable and biodegradable ways to manage stored product pests, we believe this compound justifies *Z. zanthoxyloides* roots as an insecticidal plant for managing stored product pests especially *S. oryzae*.

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