BIOLOGICAL SCREENING OF *CLERODENDRON INERME* LEAF EXTRACTS FOR REPELLENCY AND TOXICITY POTENTIALS AGAINST STORED PRODUCT INSECTS

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ABSTRACT: Botanicals have been used traditionally as grain and legume protectants against stored product insects. The objective of this study was to evaluate the repellent and insecticidal activity of different solvent extracts of *Clerodendron inerme* (L.) leaf against the red flour beetle *Tribolium castaneum* (Herbst), the lesser grain borer *Rhyzopertha dominica* (F.), and the cowpea weevil *Callosobruchus chinensis* (L.). The area preference method and dose-mortality was used to determine repellency rate and insecticidal activity. The average repellency of 0.02µl, 0.04µl, 0.08µl of the methanol, petroleum ether and ethyl acetate extract concentrate per cm^2 of *C. inerme* leaves totally achieved class II, III, class IV and class V respectively at different intervals (2, 4, 8 hours) of time. The order of repellent activity was *Callosobruchus chinensis* < *Rhyzopertha dominica* < *Tribolium castaneum.* The concentrate of methanol- extract of *Clerodendron inerme* leaves was found to be more effective than the commercial product teflubenzuron (No: SZBB291XV) and also more effective than other solvent extracts. The order of repellent activity was *Callosobruchus chinensis* < *Rhyzopertha dominica* < *Tribolium castaneum.* The dose-mortality test of methanolic extracts of *C. inerme* leaf indicates that the LD_{50} (1.74 mg/cm^2) and LD_{99} (3.01 mg/cm^2) was very high in *C. chinensis* compared to the other two beetles. Our result indicates that the repellent activity was dose and extract dependent in different intervals of time and dose-mortality of the methanolic extract varied depending on the insect species.

KEY WORDS: Phytochemicals, Repellency, *Rhyzopertha dominica, Tribolium castaneum, Callosobruchus chinensis,* Teflubenzuron.

Insects are the major threat for stored grain products throughout the world due to the qualitative, quantitative, commercial and agronomic losses they cause. It is estimated that more than 20,000 species of field and storage pests destroy approximately one-third of the world's food production, valued annually at more than $ 100 billion among which the highest losses (43%) occurring in the developing world (Jacobson 1982, Ahmed & Grainge 1986). The quantitative and qualitative damage to stored grain and grain products from the insect pests may amount to 20-30% in the tropical zone and 5-10% in the temperate zone (Talukder, 2006). Although synthetic chemical pesticides have been commonly used to reduce losses, there is great risk of negative effects such as the development of insecticide-resistant strains, environmental pollution, serious public health hazards, and pesticide residues in food and increase of the cost of application. The continuous application and dependence on chemical pesticides have also resulted in potential toxicity hazards for non target organisms and users (Amanda et al., 2012; Silva et al., 2002; Regnault-Roger et al., 2004). The increase in the above problems and contamination of the biosphere associated with large-scale use of broad spectrum synthetic pesticides have led to the need for effective biodegradable pesticides with greater selectivity. This awareness has created a world-wide interest in the development of alternative strategies, including the discovery of new insecticides (Sharma & Meshram, 2006). Therefore, there is an urgent need for new alternative approaches to control stored product insects by eco-friendly organic sources that are easily available, affordable, less toxic to mammals and less detrimental to the environment.

In recent years, studies have been focused on plant materials and their bio-active chemical constituents as a rich source of natural substances which can be used to develop ecologically safer methods for Insect control (Cloyd, 2004, Arnason et al., 1989). Insecticidal activities of many plants against insect pests have been well demonstrated (Jilani & Su 1983, Isman, 2000, Rajesh Kumar et al., 2008; Regnault-Roger et al., 2004; Karina Caballero-Gallardo et al., 2012). The deleterious effects of plant extracts or pure compounds on insects can be manifested in several manners including toxicity (Dubey et al., 2007; Park et al., 2003; Jouda et al., 2012; Tripathi et al., 2000a,b, 2001c,d), mortality (Jouda et al., 2012; Kim et al., 2011), repellent activity (Mc Donald et al., 1970; Xie et al., 1995; Fields et al., 2001; Mohan & Fields, 2002, Hou et al., 2004; Kumar et al., 2004; Tapondjou et al., 2005; Isman, 2006; Nerio et al., 2009; Amanda et al., 2010; Olivero-Verbel et al., 2010; Karina Caballero-Gallardo et al., 2012, Lu & Shi 2012), antifeedent, growth inhibitor, suppression of reproductive behavior and reduction of fertility and fecundity (Metcalf & Metcalf, 2002, Rajasekaran & Kumaraswami, 1985; Papachristos & Stamapoulos, 2002) on stored product insects. Natural plant products such as essential oils (Isman, 2000) mixtures of volatile terpenes and alkaloids, polyphenols, steroids and other components isolated mostly from plants (Liu & Su, 1999; Prajapati et al. 2001; Regnault-Roger et al., 2004, Arthur et al., 2011; Hamid et al. 2011) are being extensively studied as repellents and insecticidal compounds which are biodegradable. There is a considerable importance for phytochemicals because of their repellent effect, which is highly species - specific and generally advantageous being specific and having low mammalian toxicity (Malik & Naqvi, 1984). The repellent properties of Neem leaves on the insect pests of both stored products and field crops have also been extensively studied (Nakanishi, 1975; Radwanski, 1977).

Many authors also reported the grain protectant activity of some plant materials against insect pests due to their repellent properties (Malik & Naqvi, 1984, Quersi et al., 1988; Jalini et al. 1994; Bekele et al., 1996; Othira et al., 2009). A number of workers stated the gustatory repellent properties of the seed kernel extracts of neem against different insects (Pradhan et 1963, Jotwani & Sircar 1965). The *Clerodendron inerme* (L.) Gaertn belongs to (Verbenaceae) and its bio-efficacy on the public health flies like *Musca domestica* have been studied by Periera and Gurudutt, 1999 but there is no attempt made to study bio-efficacy on stored-product insects. Therefore the present work has been carried out to evaluate the repellent and insecticidal activity of *Clerodendron inerme* (L.) leaf extract on the lesser grain borer *Rhyzopertha dominica* (F.), the red flour beetle *Tribolium castaneum* (Herbst) and the cowpea weevil *Callosobruchus chinensis* (L.).

MATERIALS AND METHODS

Collection and Extraction of plant material

Fresh plant leaves of *Clerodendron inerme* belongs to (L.) Gaertn. (Verbenaceae) were collected during August-October 2012 from the ICMR regional centre, Belgaum campus (N15.88668; E74.523653) Karnataka, India and authenticated by Dr. Harsh V Hegde Scientist. Regional Medical Research Centre, Belgaum. Approximately 500 gms harvested leaves of *Clerodendron inerme* were washed, air-dried in shade for 2 days and lyophilized. The dried leaves were ground to powder using electric grinder. The resulting powder was passed through a 25-mesh sieve to obtain a fine powder. This powder was soaked with 1 liter of methanol, petroleum ether and ethyl acetate solvents separately and stirred for 30 minutes using a magnetic stirrer and then filtered through Whatman No. 1 filter paper (Rahman and Talukder 2006). After 2 days solvents from the pooled filtrated solution was concentrated to dryness using a rotary flash evaporator at 70°C. The yield of final crude extracts was 5.2 gms and they were preserved in sealed bottles in a refrigerator at 5°C until used for further insect repellent bioassays. Sample specimens of these plants were deposited in form of herbarium in the FPIC Department, under the following codes *C. inerme* Geartn0012.

Rearing of test insects

The lesser grain borer *R. dominica,* the red flour beetle *T. castaneum* and the cowpea weevil *C. chinensis* were reared for the present study. A small population of these insects was obtained from the entomology laboratory stock, Food Protectants and Infestation Control Department, CSIR-CFTRI, India. The rearing of above insect was done on whole wheat, wheat flour and cow pea seeds (food media) inside a growth chamber at 27 ± 2°C, L : D 12:12 and with 70 ± 5% RH (Rahman & Talukder, 2006).

100 numbers of 1-2 days old adult insects were placed in a glass jar (1.5-L) containing 500 gms of food media in glass containers covered by muslin cloth. Maximum of 7 days were allowed for mating and oviposition. Then the parent stocks were removed and food media containing eggs were incubated in a temperature/humidity controlled cabinet (27 ± 2° C and RH 70 ± 5%) in darkness to obtain same aged insects (Rahman & Talukder, 2006). Thus subsequent progenies of the insects were used for assays.

Repellency bioassay

Repellency assay was carried using paper strip method described by Mc Donald et al., 1970, Tapondjou et al., 2005; Nerio et al., 2009; Olivero-Verbel et al., 2010; Karina Caballero-Gallardo et al., 2011; Lu & Shi, 2012). Test solutions were prepared by dissolving different volumes of the plant leaf extract with acetone (0.02µl, 0.04µl, 0.08µl of the extracts per cm^2). Each Whatman filter paper (18 cm in diameter) was cut into two halves to fit into glass petri dish (18 cm in diameter) and each volume of plant extract was applied to a half filter paper as uniform as achievable using a pipette. The other half was treated only with acetone and it served as control. The plant-extract treated and acetone treated halves were air-dried to evaporate the solvent completely for 10 minutes. Each treated strip was attached lengthwise, edge to edge with cellophane tape. A filter paper was placed on the bottom of the glass petri-dish. Twenty five unsexed adult insects of *T. castaneum* (6 days old) *R. dominica* (6 days old) and *C. cinensis* (3 days old) were released at the center of each filter paper disc, and then petri dishes were covered and placed in darkness at 27 ± 2°C with relative humidity of 70 ± 5%. The numbers of *T. castaneum* specimens on treated and untreated portions of the experimental paper halves were counted for each dish after 2, 4, 8 hours exposure. Percentage repellency (PR) for a given treatment time was obtained using the formula: $PR = [(Nc-Nt)/(Nc+Nt)] \times 100$, where Nc= the number of insects in the untreated (control) and Nt = treated areas, respectively (Karina Caballero-Gallardo et al. 2012). Positive values expressed repellency and negative values attractancy (Karina Caballero-Gallardo et al. 2012). Along with this standard repellent Teflubenzuron from Sigma-Aldrich batch no: SZBB291XV was used as positive control (Saeideh Loni et al. 2011), utilizing the same experimental conditions as the extracts. The averages were then assessed to different class using the following scale. Percent repulsion >0.01 to<0.1= class 0; 0.1-20 = class I; 20.1-40 = class II; 40.1 to 60 = class III; 60.1to 80=class IV; 80.1-100= class V (Mc Govern et al. 1977). Five replicates were performed for each test concentrations of plant leaf extract and also for positive control.

For dose-mortality calculation, surface film assay method was used. The stock solution of *Clerodendron inerme* leaf extract was prepared at 100 mg/ml concentration level. This stock was serially diluted with acetone to give a concentration of 0.2, 0.4, 0.8, 1.0, 1.4 mg/cm^2. Then 1ml of each of the solutions was poured onto the Whatman No. 1 filter circle in each of the peri-plate (6 cm diameter) and allowed for about 10 minutes in a hood for the solvent to evaporate. 30 unsexed insects (6 days old *T. castaneum,* 6 day old *R. dominica* and 3 days old *C. chinensis* (due to less life span) were released separately into each petri-plate. Dead insects were counted after 24 hours of exposure. The mortality (%) was calculated using the Abbott's formula (Abbott 1925) in treated filter papers in comparison with the control ones.

Statistical analysis

The mean number and standard deviation of insects on the treated and untreated area of the filter paper was calculated for average repellency rate. The Duncan's Multiple Range Test was used to compare percentages of mean repellency at different intervals of time. Probit analysis for dose-mortality was performed according to Finney (1947) and Busvine (1971) to find out the LD_{50} and LD_{99} values using STATS plus software.

RESULTS

The results of repellency rate assays are presented in Table 1. The different solvent extracts of *C. inerme* leaf and teflubenzuron (standard repellent) were tested to determine their repellency against *T. castaneum*, *R. dominica* and *C. chinensis*. The methanol extract exhibited strong repellent effect to the red flour bettle, *T. castaneum* and *R. dominica* at all the doses showing the repellency of class IV and class V in different dose and intervals of time. However, there is slight less effectiveness in case of *C. chinensis* at lowest dose (0.020µl/cm^2). All the concentrations of petroleum ether extracts were found to be moderately repellent to *T. castaneum* and *C. chinensis* showing (only class III) but in case of *R. dominica* repellency rate goes to class III at 0.020 µl/cm^2 and class IV at 0.080 µl/cm^2 concentrations respectively. The repellency rate of ethyl acetate showed least effective result to *C. chinensis* depicting class 0 and class 1 with negative results in lower concentrations and class II in higher doses of extract compared to *T. castaneum* and *R. dominica.* The standard repellent teflubenzuron was less effective with class III (58.1 ± 5.8 for *T. castaneum* and 51.6 ± 6.8; 59.2 ± 4.4; 63.4 ± 5.6 at 0.020 µl/cm^2, 0.040 µl/cm^2 and 0.080 µl/cm^2 respectively for *R. dominica*) and II, but not even class IV in any of three stored-product beetles. The results showed a dose, different extract concentration and time dependent repellent effects of *C. inerme* against *T. castaneum, R. dominica* and *C. chinensis* respectively.

The methanolic extract of *Clerodendron inerme* leaf offered dose-mortality action against *T. castaneum*; *R. dominica* and *C. chinensis* adults and the results were found to be promising as presented in Table 2. The LD_{50} and LD_{99} values for methanol leaf extract were 0.65 mg/cm^2 and 1.50 mg/cm^2 for *R. dominica* which is lower compared to *T. castaneum* (LD_{50}=1.28 and LD_{99}=2.20 mg/cm^{-2}). At the same time the LD_{50} and LD_{99} doses against *C. chinensis* reached LD_{50} (1.74

mg/cm^2) and LD$_{99}$ (3.01mg/cm^2) respectively. The methanolic extract of *C. inerme* significantly affected survival of all three stored product beetles according to Chi-Square values depicted in Table 2, Figure 1a, 1b, and 1c along with R^2 values.

DISCUSSION

Currently, botanicals constitute 1% of world insecticide market, despite the knowledge that plants constitute a rich source of bioactive chemicals and provide alternatives to regular insect control agents (Kim et al. 2003). Several species from the various plant families have been tested for their insecticidal potency (Abida et al., 2010; Belmain et al., 2001; Khanam et al. 2008; Ogendo et al., 2004). The present work revealed the effective repellant activity of three solvent extracts of *C. inerme* leaf, along with a standard repellant teflubenzuron and insecticidal activity of methanolic plant extract on three stored product insects. Significant repellant activity against *T. castaneum, R. dominica* and *C. chinensis* adults was observed with crude methanol extract from *C. inerme* leaf, followed by petroleum ether and ethyl acetate as given in Table 1.

The careful scrutiny of Table1 indicates that repellent activity of the *C. inerme* plant methanolic extract varied depending on the insect species with different solvent system in intervals of time. The order of repellent activity was *C. chinensis* < *R. dominica* < *T. castaneum*. The concentrate of methanol-extract of *C. inerme* leaves was found to be more effective than other solvent extract (ethyl acetate, petroleum ether) of the same plant according to the repellency class (Mc Govern et al., 1977) in all the doses and in various intervals of time. For this purpose methanolic extract was selected to determine the dose mortality test for all the three stored-product insects. Further results showed that adults of *T. castaneum* were more susceptible to the plant methanolic extract and higher dose is required to achieve higher repellency rate. The consideration of the time exposure effect of methanolic extract dose is directly proportional to the time taken for the repellency activity in all three beetles. This is in contrast to the other solvent extracts (petroleum ether and ethyl acetate) and also for teflubenzuron standard repellent (Saeideh Loni et al., 2011), where the repellent activity is uneven in time and dose.

Further, our findings suggest that there may be different compounds in different solvent extracts possessing different bioactivities. Previous works by (Talukder & Howse, 1993) on repellant effect of different solvent extracts of Pitraj seed on *T. castaneum* showed that the acetone extract exhibited 88 and 93% repellency at 0.5% and 1% concentrations respectively to the beetles. The result is in agreement with Jilani & Su (1983) who reported that petroleum ether extract of neem leaf acted as repellent to *T. castaneum*. The garlic extracts were shown to be repellent to stored product insects by Ho & Ma (1995). Interestingly, repellent properties of the methanolic extract were better than those registered for the standard repellent teflubenzuron, which showed modest repellency rate with class II and III, but not even class IV in any of the three stored-product insects which is the contrast to work of (Saeideh Loni et al., 2011). These findings receive support from the result of Karina Caballero-Gallardo et al., 2012) who reported natural oils from *Cymbopogon martinii*, *Cymbopogon flexuosus* and *Lippia origanoides* were more effective as repellents than the commercial product IR3535 and Nerio et al., 2009 showing activity of repellency from seven aromatic plants. According to Table 2 and figure 1a, 1b, 1c the dose-mortality test of methanolic extracts of *C. inerme* leaf suggest that LD$_{50}$ and LD$_{99}$ was very high in case of *C. chinensis* and low in *R. dominica* compared to *T. castaneum*. This reflects *C. chinensis* is less sensitive to insecticidal activity of methanolic extract, which is also similar in the order of repellent behavior exhibited by the above three stored product insects. Based on the present findings, the plant extracts examined pose potential in controlling and chasing stored product pests. This study provides an interesting opportunity to develop bio-insecticides and repellent formulations based on the extracts from lesser known plants. Along with this our findings confirms the results of Karina Caballero-Gallardo et al., 2012. Since plant-derived pesticides are biodegradable and safer to higher animals, they offer a viable alternative to synthetic agrochemicals. Although this study has verified the scientific principle for use of *C. inerme* in controlling stored product insects, further research on mechanism of action of bioactive compounds extracted from *C. inerme* is necessary.

CONCLUSION

Evaluation of repellent activity by area preference method can be used for preliminary screening of plant products for their insecticidal activity on stored product insects. This will help to save time in identifying the insecticidal activity of plant products. Regarding the side effects of synthetic pesticides, the study suggests that these plant extracts which are eco-friendly, play an important role in protection of storage commodities. Therefore, these extracts may be potential candidates for their use in the formulation of commercial repellents and toxic agents that could be effective control options, in the management of stored product insects which are responsible for huge loss of food commodities in the world.

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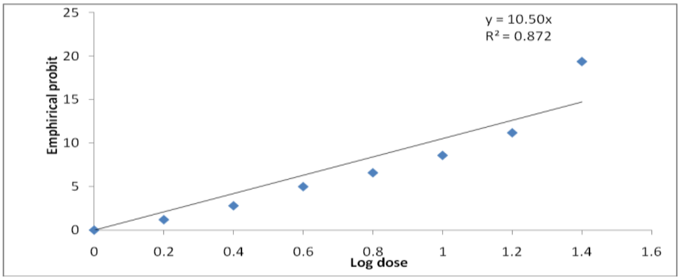

Figure 1a. Effect of dose mortality of *Clerodendron inerme* on *T. castaneum* adults.

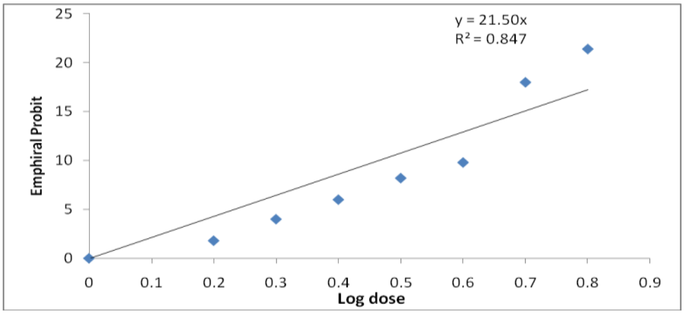

Figure 1b. Effect of dose mortality of *Clerodendron inerme* on *R. dominica* adults.

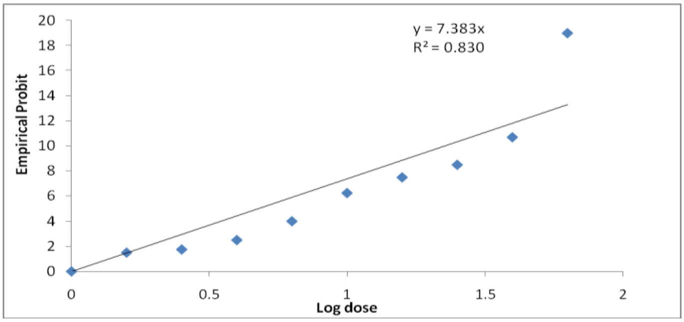

Figure 1c. Effect of dose mortality of *Clerodendron inerme* on *C. chinensis* adults.

Table 1. Percentage and rate of repellency of *Clerodendron inerme* leaf extracts on three stored product insects.

Insect species	Solvent used	Concentration of extract µlcm^{-2}	*Average repellency (%) after treatment (hours)			Mean	Repellency rate (class)
			2	4	8		
T. castaneum	Methanol	0.020	60.7 ± 2.6^{a}	71.3 ± 3.5^{b}	75.4 ± 3.1^{c}	69.1	IV
		0.040	75.4 ± 2.9^{a}	79.3 ± 5.2^{b}	85.6 ± 4.5^{c}	80.1	V
		0.080	90.3 ± 3.8^{a}	93.2 ± 3.4^{b}	95.3 ± 2.8^{c}	92.9	V
	Petroleum ether	0.020	41.4 ± 1.4^{a}	42.3 ± 5.2^{ab}	40.7 ± 3.2^{a}	41.4	III
		0.040	48.1 ± 4.1^{a}	45.6 ± 2.8^{b}	43.4 ± 1.6^{c}	45.7	III
		0.080	49.6 ± 4.4^{a}	50.2 ± 3.1^{b}	53.1 ± 2.1^{c}	50.8	III
	Ethyl acetate	0.020	24.1 ± 1.7^{a}	26.6 ± 2.9^{b}	23.4 ± 2.6^{c}	24.7	II
		0.040	46.7 ± 4.1^{a}	39.7 ± 4.4^{b}	40.1 ± 2.3^{c}	42.1	III
		0.080	48.5 ± 2.1^{a}	46.1 ± 6.5^{a}	41.2 ± 4.8^{c}	45.2	III
	Teflubenzuron	0.020	40.5 ± 6.7^{a}	32.7 ± 5.8^{b}	21.4 ± 4.3^{c}	31.5	II
		0.040	50.3 ± 4.2^{a}	41.1 ± 6.5^{b}	30.3 ± 5.2^{c}	40.5	II
		0.080	58.1 ± 5.8^{a}	60.8 ± 3.4^{b}	34.2 ± 4.7^{c}	51.1	III
R. dominica	Methanol	0.020	60.1 ± 6.2^{a}	63.4 ± 5.8^{b}	70.3 ± 4.4^{c}	64.6	IV
		0.040	69.3 ± 9.4^{a}	79.3 ± 7.1^{b}	81.2 ± 5.1^{c}	76.6	IV
		0.080	81.7 ± 6.4^{a}	89.2 ± 4.6^{b}	90.4 ± 3.9^{c}	87.1	V
	Petroleum ether	0.020	43.4 ± 8.1^{a}	40.1 ± 7.1^{b}	39.7 ± 6.2^{cb}	41.2	III
		0.040	53.2 ± 4.3^{a}	50.3 ± 6.3^{b}	40.5 ± 5.4^{c}	48.0	III
		0.080	79.1 ± 6.7^{a}	74.4 ± 7.1^{b}	69.2 ± 6.7^{c}	74.2	IV
	Ethyl acetate	0.020	20.1 ± 7.1^{a}	29.3 ± 3.4^{b}	30.6 ± 6.3^{cb}	26.6	II
		0.040	35.4 ± 8.6^{a}	41.5 ± 4.7^{b}	39.3 ± 4.6^{c}	38.7	II
		0.080	50.6 ± 6.4^{a}	49.8 ± 4.1^{ab}	38.2 ± 5.1^{c}	46.2	III
	Teflubenzuron	0.020	51.6 ± 6.8^{a}	44.1 ± 5.2^{b}	40.8 ± 3.7^{c}	45.5	III
		0.040	59.2 ± 4.4^{a}	64.1 ± 6.8^{b}	34.4 ± 5.4^{c}	52.5	III
		0.080	63.4 ± 5.6^{a}	54.2 ± 6.5^{b}	42.1 ± 6.9^{c}	53.2	III
C. chinensis	Methanol	0.020	65.3 ± 8.2^{a}	50.4 ± 6.2^{b}	58.4 ± 6.1^{c}	58.0	III
		0.040	61.7 ± 6.4^{a}	62.3 ± 5.7^{ab}	70.2 ± 5.3^{c}	64.7	IV
		0.080	65.6 ± 7.3^{a}	70.3 ± 7.1^{bc}	71.2 ± 3.2^{c}	69.1	IV
	Petroleum ether	0.020	41.2 ± 6.4^{a}	39.4 ± 4.1^{ab}	40.3 ± 4.6^{c}	40.3	III
		0.040	49.6 ± 7.1^{a}	41.2 ± 3.6^{b}	39.4 ± 4.1^{c}	43.3	III
		0.080	51.9 ± 6.4^{a}	50.1 ± 8.1^{ab}	40.7 ± 7.4^{c}	47.5	III
	Ethyl acetate	0.020	20.3 ± 7.2^{a}	-7	-12	00	00
		0.040	31.5 ± 7.1^{a}	-11	-12	18	I
		0.080	49.5 ± 3.2^{a}	31.8 ± 4.3^{b}	28.5 ± 3.9^{c}	36.6	II
	Teflubenzuron	0.020	30.1 ± 4.3^{a}	20.1 ± 4.2^{b}	10.2 ± 2.7^{c}	20.1	II
		0.040	41.2 ± 2.1^{a}	40.1 ± 5.4^{ab}	39.1 ± 5.2^{cb}	40.1	III
		0.080	50.6 ± 3.5^{a}	48.1 ± 3.2^{b}	46.2 ± 6.4^{c}	48.3	III

*values are averages of five replicates and mean ± SD; Means within same rows followed by same letter aren't significantly different according to DMRT ($P<0.05$)

Table 2. Dose-mortality effects of *Clerodendron inerme* methanol extracts against stored product insects.

	Exposure (h)	LD$_{50}$value (mg/cm^{2})	LD$_{99}$value (mg/cm^{2})	χ^{2} value (df)
T. castaneum	24	1.28	2.20	15.36 (4)
R. dominica	24	0.65	1.50	13.7 (4)
C. Chinensis	24	1.74	3.01	30.01 (4)