Neotrop Entomol (2017) 46:685–693
DOI 10.1007/s13744-017-0501-7

PEST MANAGEMENT

Toxicity and Detoxification Mechanism of Black Pepper and Its Major Constituent in Controlling *Rhynchophorus ferrugineus* Olivier (Curculionidae: Coleoptera)

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Keywords

Cytochrome P450, glutathione S-transferase, nutritional indices, red palm weevil, toxicity

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Edited by Moisés J Zotti - UFPel

Received 17 March 2016 and accepted 20 February 2017
Published online: 21 March 2017

Abstract

The survival, feeding response, and detoxification mechanism of *Rhynchophorus ferrugineus* Olivier, a key pest responsible for destruction of date palm, was examined with different extracts of *Piper nigrum* and its major constituent (piperine) identified by GC-MS. In the present study, toxicity of different extracts of black pepper was evaluated by incorporating diffferent doses of extracts into the artificial diet of red palm weevil larvae. All extracts showed dose-dependent insecticidal activity to the tested eighth-instar red palm weevil larvae. Among all the extracts, maximum larvicidal activity was exhibited by chloroform (LD_{50} = 342.62 mg/l), followed by dichloromethane (LD_{50} = 357.78 mg/l), acetone (LD_{50} = 372.57 mg/l), and ethanol (LD_{50} = 408.88 mg/l). However, piperine, a major constituent of all black pepper extracts identified by GC-MS in the present work, was found to be the most potent treatment exhibiting the least LD_{50} (219.88 mg/l). In addition, nutritional indices evaluated by calculating the efficiency of the conversion of ingested food (ECI) and digested food (ECD) at the same dose (219.88 mg/l) showed that there was maximum reduction in the ECI (49.90%) and ECD (62.21%) index of larvae fed diets incorporated with piperine. Larvae that were fed diets incorporated with different black pepper extracts experienced increases in the expression of detoxification genes (*glutathione S-transferase* and *cytochrome P450*), and this upregulation in detoxification genes (*glutathione S-transferase*, *cytochrome P450* and *esterase*) was tremendously high in larvae fed diets incorporated with piperine. Results suggest that piperine is a promising bio-pesticide agent for the control of *R. ferrugineus* Olivier.

Introduction

Phoenix dactylifera L. is an important palm species that grows in arid, sub-tropical, and tropical regions (Khiyami and Alyamani 2008). This species not only tolerates harsh desert temperatures but is also resistant to drought and high levels of salinity. However, desert beauty (date palm) is not able to tolerate the invasion of the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera, Curculionidae), which results in serious threats to the date industry, especially in Gulf Cooperation Council (GCC) countries (Hussain *et al* 2013b).

Red palm weevil control is primarily based on the frequent application of synthetic pesticides (Hussain *et al* 2013a). However, environmental pollution, possible deleterious effects on non-target animals, applicator safety issues, concerns over human health, and the development of resistance

among red palm weevils to insecticides have forced scientists to search for and develop new and sustainable methods of biological control (Al-Ayedh *et al* 2016). The development of plant-based red palm weevil control technology appears to be an attractive alternative.

The use of plant extracts is known to have a wide array of effects due to the presence of several active compounds. These plant extracts are comprised of secondary metabolites. These metabolites are concentrated in bark, leaves, fruit, and seeds (Isman 2006). The incorporation of plant extracts in pest management strategies aimed to kill or repel pests has a long history, as these extracts have been used throughout the world because of their ability to produce chemicals (Isman 2006). These chemicals function as defense mechanisms that reduce feeding injury caused by pests. Numerous modes of action of these chemicals have been described, including (1) cuticle disruption, (2) reduced fecundity, (3) molting inhibition, (4) growth reduction, (5) respiratory inhibition, (6) anti-feeding activity, (7) repellent , and (8) toxic effects (Isman 2000, Tsao *et al* 2002, Akhtar and Isman 2004, Maia and Moore 2011, Erdogan *et al* 2012, Deletre *et al* 2013). In addition, botanical pesticides, unlike synthetic pesticides, contain complex mixtures of several compounds (Isman 2006). These diverse characteristics are advantageous over synthetic pesticides because they delay the development of resistance among pest populations (Feng and Isman 1995, Joseph *et al* 2012). Species of *Piper* are a good source of naturally occurring insecticides (Scott *et al* 2007b). For instance, piperine is a compound derived from *P. nigrum* and is known to have insecticidal properties against pests (Su 1977, Scott *et al* 2007b).

Few studies have explored the potential of plant-based products as control agents for red palm weevils. The findings of Salama and Ismail have found that *Ambrosia maritima*, *Calotropis procera*, and *Curcuma longa* are highly toxic to red palm weevils (Salama and Ismail 2007). In another study, neem extract and insect growth regulator (flufenoxuron) were used to investigate their potential to control red palm weevils. Their results suggested that both neem and IGR at higher doses (500 mg/l) caused 22.2 and 14.3% adult mortality, respectively (El-Bokl *et al* 2010). Plant-based essential oils have also been used to investigate their potential to control red palm weevils (Shukla *et al* 2012). Their findings have shown that the essential oils from *Eupatorium adenophorum* flowers and *Artemisia nilagirica* aerial parts promote anti-feeding activity at a concentration of 1000 ppm. Recently, the insecticidal potential of phenylpropanoids, an important class of plant secondary metabolites against red palm weevils, was evaluated. Results showed that coumarin greatly disturbed the growth and detoxification mechanism of red palm weevils that ultimately leads to the death of *R. ferrugineus* Olivier larvae (AlJabr *et al* 2017). However, none of the study has investigated the insecticidal potential of *P. nigrum* extracts to control red palm weevils. Our study aimed to (1) compare the toxicity of *P. nigrum* extracts with different solvents to the main chemical component (piperine), (2) assess the impact of *P. nigrum* extracts and piperine on the larval growth and development of *R. ferrugineus* Olivier, and (3) evaluate the impact of extracts and piperine on the quantitative expression of detoxification genes from the gut of *R. ferrugineus* Olivier larvae. Determining how *P. nigrum* extracts and piperine affect the growth and detoxification mechanisms of *R. ferrugineus* Olivier will facilitate the development of inexpensive IPM strategies to control *R. ferrugineus* Olivier infestations in date palm plantations.

Material and Methods

Rearing of experimental insects

Rhynchophorus ferrugineus Olivier adults were collected in October–December 2014 from infested date palms (25°23′ 02.9″N 49°36′33.6″E). Mated females were shifted to pineapples for egg laying. Second instar larvae were reared in the laboratory with a 16-h light photoperiod at 30 ± 1°C, 75% ± 5% relative humidity (RH) on artificial diets as per the protocol standardized previously (Hussain *et al* 2015).

Extraction procedure

Piper nigrum seeds were ground to fine powder in an electric grinder and loaded into extraction thimbles. *Piper nigrum* powder was extracted using pure solvents (>99%) including acetone, chloroform, dichloromethane, or ethanol separately until the solvent ran clear in the soxhlet apparatus. Extracts were evaporated until they were dry under reduced pressure. The extracts were freeze dried until a powder was formed and were then stored at −20°C. The desired stock solution of each solvent *P. nigrum* extract was prepared with their respective solvent. Further dilutions of each extract were prepared with ddH_2O.

Identification of active compounds

Qualitative analysis of *P. nigrum* extracts was performed with a GCMS-QP 2010 Plus (Shimadzu). The capillary column used was RTx®-1 (30 m × 0.32 mm × 0.25 μm) operated with the following program: the initial oven temperature (60°C) was maintained for 60 s following injection, and then the temperature was raised to 180°C at a rate of 10°C/min and held for 1 min. Next, the oven temperature was raised to 280°C at 20°C/min and held for 15 min. Carrier gas, helium (99.99% purity), was used. Electron ionization (EI) was induced at 70 eV, and mass spectra were repetitively scanned between 35 and 335 atomic mass unit (amu). Identification of major

compound (e.g., piperine) was confirmed by comparing the retention time and mass spectra with pure standard purchased from Sigma-Aldrich.

Laboratory toxicological bioassays

Preliminary laboratory toxicity bioassays (data not shown) were performed to identify a range of each *P. nigrum* extract dose to be used for toxicological bioassays. Separate preparations of artificial diets were prepared using individual *P. nigrum* extract solutions. Based on preliminary laboratory toxicity bioassays, five doses (150, 300, 450, 600, and 750 mg/l) were used to evaluate the toxicity of acetone, chloroform, dichloromethane, and ethanol extract of *P. nigrum* to newly molted eighth-instar larvae. Different doses of piperine, the major constituent of each extract as depicted from GC-MS, were prepared by dissolving it in 1 ml of acetone with ddH_2O to obtain 100, 200, 300, 400, and 500 mg/l of piperine. A control treatment diet was prepared using ddH_2O along with similar volume (1 ml) of the respective solvent used to prepare the artificial diet for each extract. All of the treatments were incubated at 30 ± 1°C with 75% ± 5% relative humidity in an incubator (Sanyo). Each replicate consisted of 25 larvae. Five replicates for each treatment from separate generations were prepared on different dates. The study was repeated over time. Dose mortality response was recorded daily until 100% mortality was achieved. A larva was considered dead if no signs of movement were observed. Control mortality was adjusted using Abbott's formula (Abbott 1925). Corrected percent mortality data were angularly transformed. Corrected angularly transformed cumulative percent mortality data were analyzed by repeated measures ANOVA with Fisher's LSD test (Statistix 2003). Lethal dose to kill 50% (LD_{50}) of red palm weevil larvae and lethal time to kill 50% (LT_{50}) larvae were calculated using probit analysis using POLO software (Russell *et al* 1977).

Feeding performance bioassays

To evaluate the impact of different extracts and their major constituent (piperine) on the growth and development of *P. nigrum*, nutritional analyses were performed. The experiment was conducted using eighth-instar (newly molted) larvae. In this experiment, 25 larvae per treatment per replicate were fed individually on artificial diet supplemented with either piperine, acetone, chloroform, dichloromethane, or ethanol extract of *P. nigrum* at the dose of 219.88 mg/l based on the LD_{50} value of the most potent treatment (piperine) as determined from experiments on eighth-instar red palm weevil larvae. Five replicates for each treatment from separate generations were prepared on different dates. A control treatment diet was prepared using ddH_2O along with a similar volume of the respective solvent used to prepare the artificial diet for each extract. All of the experimental units were incubated at 30 ± 1°C with 75 ± 5% relative humidity in an incubator (Sanyo). The initial weight of the artificial diet offered to the larvae and the food remaining after 3 days were measured using an analytical balance. In addition, the frass produced over 3 days and the initial and final weights of the larvae were also measured. Insect, diet and frass weights were used to calculate nutritional indices. These indices were calculated by computing the efficacy of conversion of ingested food [ECI = 100 × dry weight gained by the larva/dry weight of food consumed by larva] and efficacy of conversion of digested food [ECD = weight gained by the larva/(food ingested by the larva − dry weight of frass excreted by larvae)] as described earlier by Hussain *et al* (2009). Another important nutritional index, approximate digestibility (AD), was calculated as (food ingested − frass weight)/food ingested × 100 (Hussain *et al* 2016). Significant differences between nutritional indices generated from larvae fed on different diets were determined using one-way ANOVA and Fisher's LSD test (SAS Institute 2000).

Quantitative expression of detoxification genes of red palm weevils by qRT-PCR

For total RNA isolation, eighth-instar red palm weevil larvae were fed on artificial diet incorporated with piperine, acetone, chloroform, dichloromethane, or ethanol extract of *P. nigrum* at the dose of 219.88 mg/l based on the LD_{50} value of the most potent treatment (piperine) as determined from experiments on eighth-instar red palm weevil larvae. After 72 h, larvae were dissected in saline and then transferred to a mortar for fine grinding in liquid nitrogen. Total RNA from the mid-gut of red palm weevil larvae was extracted using RNeasy® Universal Mini Kit (cat no. 73404; Qiagen). Total RNA for each treatment was separately reverse-transcribed as per the protocol of PrimeScript First Strand cDNA Kit (cat no. 6110A; TaKaRa, Clontech). The synthesized first-strand cDNA was used as a template to quantify target gene expression sequenced from Macrogen sequencing facility (Macrogen, South Korea) (Table 1) with specific primers synthesized from Macrogen Korea in CFX96 Touch™ (Bio-Rad) as per the manufacturer's protocol of the SYBR® Premix Ex Taq™ II kit (cat no. RR820W; TaKaRa Clontech). Three replicates were prepared using three larvae mid-guts. The results of each experimental unit were compared with those of the control by relative fold expression obtained by transforming the obtained results into absolute values using $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). The relative expression of each gene was set to 1 for the uninfected (control) treatment. Significant differences between gut samples extracted from different treatments were determined using one-way ANOVA and Fisher's LSD test (SAS Institute 2000).

Table 1 Primers used for quantitative PCR expression analysis of detoxification genes from red palm weevil larvae.

Target gene	Accession no.	Product size	Forward primer (5′–3′)	Reverse primer (5′–3′)
Cytochrome P450	KT748789	118 bp	TGGAGAAACACCCG CAAGAA	CGGCGATTTTGCCT ACCAAG
Glutathione S-transferase	KR902496	92 bp	ATAGCCAACCACCA CTGTCG	CGTTCCTCTTGCCG CTAGTT
Esterase	KT748822	70 bp	ACCTACAAGAATCC GACGCC	ACTCCGAAACTTTG GGCCAT
beta-Actin	KM438516	129 bp	AAAGGTTCCGTTGC CCTGAA	TGGCGTACAAGTCC TTCCTG

Results

Insecticidal activities

All of the extracts and the major constituent of black pepper (piperine) exhibited insecticidal activities against red palm weevil larvae. Overall, piperine-fed larvae had the lowest LD_{50} value (219.88 mg/l) as shown in Table 2. The mortality of red palm weevil larvae fed on piperine differed significantly after 3, 6, 9, and 12 days post-feeding time intervals ($F_{3,\ 64}$ = 1260.23, P < 0.0001), doses ($F_{4,\ 64}$ = 316.29, P < 0.0001), and their interaction ($F_{12,\ 64}$ = 38.71, P < 0.0001) (Fig 1). In addition, the most potent compound (piperine) showed 2.40 days to impart 50% larval mortality (LT_{50}) at 500 mg/l as shown in Table 3. Red palm weevil larvae fed on diets incorporated with ethanol extract of black pepper showed the lowest response, resulting in the highest LD_{50} value (408.88 mg/l) (Table 2), along with the highest LT_{50} values (11.60 days) as shown in Table 3. However, ethanol extract-fed larvae differed significantly at all of the studied time intervals ($F_{3,\ 64}$ = 1697.90, P < 0.0001), doses ($F_{4,\ 64}$ = 313.17, P < 0.0001), and their interaction ($F_{12,\ 64}$ = 21.55, P < 0.0001) (Fig 2). Treatment of chloroform extract incorporated diet was also toxic to the tested larvae, resulting in significant differences in mortality at different time intervals ($F_{3,64}$ = 2019.51, P < 0.0001), doses ($F_{4,64}$ = 253.57, P < 0.0001), and their interaction ($F_{12,64}$ = 39.60, P < 0.0001) (Fig 3). Mortality percentages of red palm weevil larvae fed black pepper dichloromethane extract showed significant differences at all of the studied time intervals ($F_{3,\ 64}$ = 2024.50, P < 0.0001), doses ($F_{4,\ 64}$ = 270.11, P < 0.0001), and their interaction ($F_{12,\ 64}$ = 39.91, P < 0.0001) (Fig 4). Red palm weevil mortality was also highly significantly different at all of the studied time intervals ($F_{3,\ 64}$ = 1728.39, P < 0.0001), doses ($F_{4,\ 64}$ = 309.63, P < 0.0001), and their interaction ($F_{12,\ 64}$ = 25.75, P < 0.0001) among larvae fed diets incorporated with black pepper acetone extract (Fig 5).

Growth-retarding activities

Nutritional indices of tested compounds greatly differed against red palm weevil larvae at a dose of 219.88 mg/l. The larvae fed diets incorporated with piperine showed the greatest reduction (49.90%) in ECI and remained different compared to all other diets incorporated with black pepper extracts ($F_{5,\ 24}$ = 364, P < 0.0001). However, the lowest reduction (5.37%) was observed in larvae fed diets incorporated with black pepper ethanol extract (Table 4). Similarly, ECD also differed ($F_{5,\ 24}$ = 348, P < 0.0001) between larvae fed different diets. The most potent compound (piperine) greatly reduced (62.21%) ECD (Table 4). However, black pepper ethanol extract caused little reduction (8.56%) in ECD compared to control larvae. Larvae fed diets incorporated with black pepper chloroform, dichloromethane, and acetone extracts generated 48.36, 39.27, and 22.72% reductions in ECD compared to control larvae, respectively (Table 4).

The feeding of artificial diet incorporated with different extracts of black pepper and piperine resulted in significant increase in AD of red palm weevil larvae ($F_{5,\ 24}$ = 182, P < 0.0001). The most potent piperine tremendously enhanced the AD (24.57%) of red palm weevil larvae compared

Table 2 Susceptibility of *Rhynchophorus ferrugineus* larvae to different products.

Treatment	LD_{50} (95% CL) (ppm)	χ^2	Slope ± SE
Chloroform extract of black pepper	342.62 (310.00–378.67)	5.76	2.33 ± 0.25
Dichloromethane extract of black pepper	357.78 (324.67–394.27)	6.06	2.92 ± 0.27
Acetone extract of black pepper	372.57 (337.09–411.78)	5.69	2.78 ± 0.27
Ethanol extract of black pepper	408.88 (371.43–450.10)	6.40	2.87 ± 0.28
Piperine	219.88 (199.90–241.85)	6.69	3.12 ± 0.28

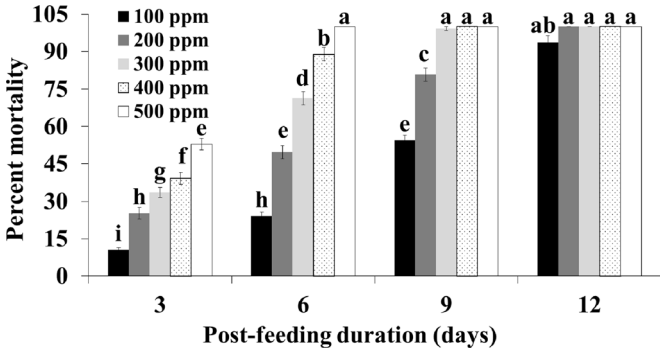

Fig 1 Average cumulative corrected percent mortality of *Rhynchophorus ferrugineus* Olivier larvae fed artificial diets incorporated with piperine. Bars (means ± SE) followed by different *letter(s)* are significantly different (Fisher's LSD test, α = 0.05).

with control larvae (Table 4). Chloroform and dichloromethane extract of black pepper increases 16.78 and 17.77% AD compared with the control, respectively. The least potent extract (ethanol) of black pepper negligibility (3.34%) enhance AD of red palm weevil larvae compared to control larvae (Table 4).

Quantification of detoxification genes by qRT-PCR

The toxicity of different treatments induced different levels of detoxification genes such as *cytochrome P450*, *glutathione S-transferase*, and *esterase*. The expression of detoxification genes ($F_{2,\ 20}$ = 283.63, P < 0.0001), upon exposure to different treatments ($F_{4,\ 20}$ = 671.32, P < 0.0001) and their interaction ($F_{8,\ 20}$ = 53.58, P < 0.0001), showed significant differences. *Cytochrome P450* was highly expressed compared to *esterase* (Fig 6). Overall, red palm weevil larvae fed on diets incorporated with piperine greatly induced the expression of *cytochrome P450*. However, the lowest fold expression of *cytochrome P450*, *glutathione S-transferase*, and *esterase* was induced by the least toxic treatment of black pepper ethanol extract (Fig 6).

Piperine percentage in different extracts of black pepper

The GC-MS analysis of the crude extracts of black pepper revealed marked differences in the proportion of piperine. Overall, the highest proportion of piperine was detected from the chloroform extract of black pepper (74.15%). On the other

Table 3 Dose mortality response (LT_{50} values) of *Rhynchophorus ferrugineus* larvae to different products.

Treatments	Dose (mg/l)	LT_{50} (days)	*F* value	*P* value
Piperine	100	8.20 ± 0.19a	136	<0.001
	200	5.17 ± 0.31b		
	300	3.72 ± 0.21c		
	400	3.11 ± 0.09d		
	500	2.40 ± 0.12e		
Chloroform extract of black pepper	150	8.57 ± 0.32a	145	<0.001
	300	5.56 ± 0.30b		
	450	3.45 ± 0.13c		
	600	2.83 ± 0.10 cd		
	750	2.42 ± 0.06d		
Dichloromethane extract of black pepper	150	8.75 ± 0.22a	129	<0.001
	300	5.89 ± 0.30b		
	450	3.51 ± 0.28c		
	600	2.91 ± 0.14 cd		
	750	2.35 ± 0.18d		
Acetone extract of black pepper	150	9.26 ± 0.41a	128	<0.001
	300	5.99 ± 0.19b		
	450	3.82 ± 0.25c		
	600	3.11 ± 0.17 cd		
	750	2.47 ± 0.09d		
Ethanol extract of black pepper	150	11.60 ± 0.36a	113	<0.001
	300	7.61 ± 0.50b		
	450	5.01 ± 0.34c		
	600	3.60 ± 0.14d		
	750	3.28 ± 0.15d		

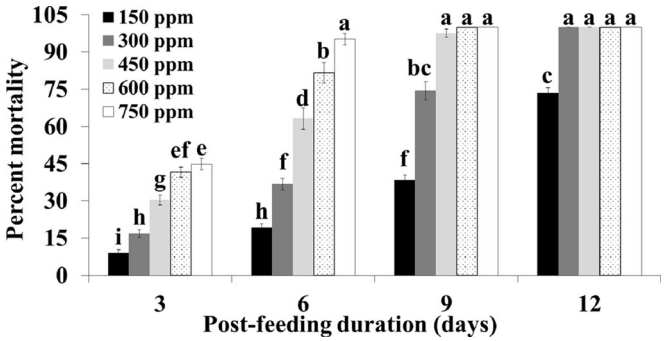

Fig 2 Average cumulative corrected percent mortality of *Rhynchophorus ferrugineus* Olivier larvae fed artificial diets incorporated with ethanol extract. Bars (means ± SE) followed by different *letter(s)* are significantly different (Fisher's LSD test, α = 0.05).

hand, the lowest piperine proportion (14.07%) was detected from the least toxic ethanol extract of black pepper (Fig 7).

Discussion

Laboratory dietary bioassays of black pepper extracts and its major constituent (piperine) had various biological effects on the red palm weevil, *R. ferrugineus* Olivier. All of the tested treatments showed insecticidal activities against red palm weevil larvae from moderate to high degrees. The difference in toxicity among all of the extracts probably stemmed from the piperine content. The toxicity of the treatments was measured by their ability to disrupt larval growth and development, induce larval weight loss, enhance detoxification gene expression levels, and increase mortality.

Higher mortality rates were observed when *R. ferrugineus* Olivier larvae were fed artificial diets incorporated with black pepper chloroform extract. These exposed larvae could not tolerate this extract and failed to grow. In addition, these larvae were exceptionally sluggish and all of the larvae died

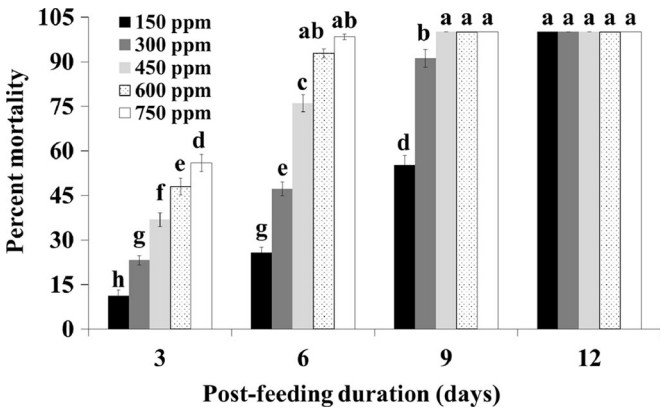

Fig 3 Average cumulative corrected percent mortality of *Rhynchophorus ferrugineus* Olivier larvae fed artificial diets incorporated with chloroform extract. Bars (means ± SE) followed by different *letter(s)* are significantly different (Fisher's LSD test, α = 0.05).

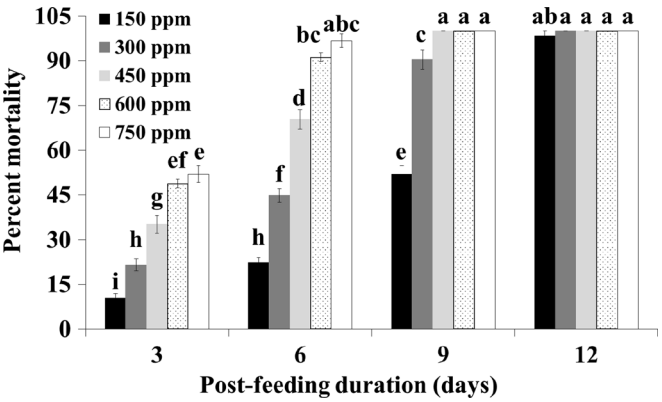

Fig 4 Average cumulative corrected percent mortality of *Rhynchophorus ferrugineus* Olivier larvae fed artificial diets incorporated with dichloromethane extract. Bars (means ± SE) followed by different *letter(s)* are significantly different (Fisher's LSD test, α = 0.05).

within 6 days at 500 mg/l. The present black pepper chloroform extract shows promise in its ability to control *R. ferrugineus* Olivier larvae. The efficacy of this extract is equivalent to the efficacy observed by Scott *et al* (2007a), who examined the effect of this extract on common home and garden insect pests. They found that *P. nigrum* extract showed the best control efficacy against the eastern tent caterpillar, *Malacosoma americanum* Fabricius (Lasiocampidae: Lepidoptera). Khani *et al* (2012) reported acute toxicity (LD_{50} = 12.52 µl/ml) of *P. nigrum* extract against the rice moth, *Corcyra cephalonica* Stainton (Pyralidae: Lepidoptera). In addition, previous research has found that crude hexane extract of *P. nigrum* performed better among other black pepper extracts, having a 1.806 mg/g LD_{50} against *Spodoptera litura* Fabricius (Noctuidae: Lepidoptera) (Fan *et al* 2011). However, the acute toxicity levels of crude hexane extract of *P. nigrum* against *S. litura* Fabricius appear to be much higher compared to the *P. nigrum* extracts evaluated in the current study. Even the least potent ethanol extract of *P. nigrum* incorporated into the diet in the current

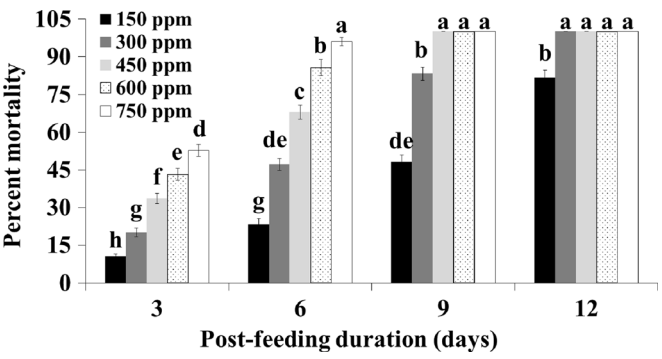

Fig 5 Average cumulative corrected percent mortality of *Rhynchophorus ferrugineus* Olivier larvae fed artificial diets incorporated with acetone extract. Bars (means ± SE) followed by different *letter(s)* are significantly different (Fisher's LSD test, α = 0.05).

Table 4 Nutritional indices of *Rhynchophorus ferrugineus* larvae after feeding on different products.

Treatments	AD	ECI (%)	ECD (%)
Piperine	70.72a	9.37 ± 0.14f	13.26 ± 0.28f
Chloroform extract of black pepper	64.09b	11.60 ± 0.27e	18.13 ± 0.58e
Dichloromethane extract of black pepper	64.86b	13.81 ± 0.26d	21.32 ± 0.59d
Acetone extract of black pepper	58.76c	15.94 ± 0.13c	27.12 ± 0.25c
Ethanol extract of black pepper	55.18d	17.71 ± 0.08b	32.09 ± 0.26b
Control	53.34e	18.71 ± 0.14a	35.10 ± 0.51a

ECI and ECD indicate efficacy of conversion of ingested food and efficacy of conversion of digested food, respectively. Means ± SE values within each column by different letter(s) are significantly different (Fisher's LSD test, α = 0.05)

study exhibited a much lower LD_{50} (408.88 mg/l) compared to the results of Fan *et al* (2011). Current toxicological studies of red palm weevils suggest that all of the tested extracts of *P. nigrum* are toxic. However, their toxicity to the weevils varies with the solvent. The difference in toxicity may stem from the chemical composition of extracts. GC-MS analyses of crude extract revealed marked differences in the proportion of piperine (Fig 7). *P. nigrum* extract with the least toxicity showed a relatively low percentage of piperine and vice versa. Toxicological bioassays, in which piperine was incorporated into the artificial diet, showed acute toxicity, resulting in the lowest LD_{50} (219.88 mg/l) against red palm weevil larvae. Therefore, our findings suggest that high larvicidal activity of *P. nigrum* extracts are apparently attributed to higher percentages of the main constituent piperine. In the past, the insecticidal activity of piperine has been well documented in other insect pests (Scott *et al* 2003). However, the present work, for the first time, highlights the potential of piperine to be used as a botanical insecticide against red palm weevils.

Piperine caused significant reductions in the growth and development of *R. ferrugineus* Olivier eighth-instar larvae, when it incorporated into the diet at a dose of 219.88 mg/l.

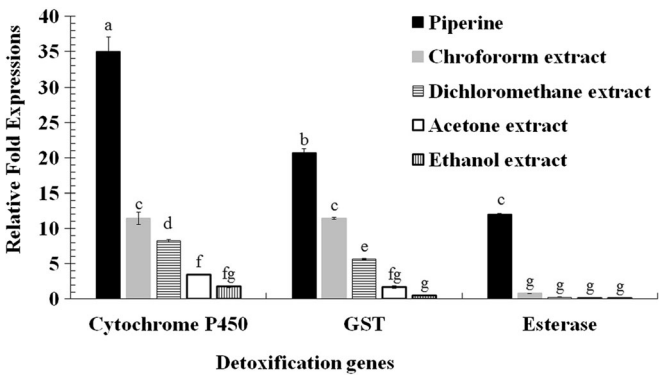

Fig 6 Effect of black pepper extracts and piperine on the expression of detoxifying genes of red palm weevils. Newly molted eighth-instar larvae were fed for 72 h on artificial diets incorporated with piperine, chloroform, dichloromethane, ethanol, or acetone extract of black pepper. Bars (means ± SE) followed by different *letter(s)* are significantly different (Fisher's LSD test, α = 0.05).

Eighth-instar larvae fed on piperine-containing diets showed the lowest efficacy of conversion of digested and ingested diet, which meant that less food was available for growth because most of the energy was metabolized for the degradation of toxin. This finding was confirmed in the present work by comparing ECI and ECD, which were higher for larvae fed on diets incorporated with black pepper ethanol extract. Silva *et al* (2009) observed similar nutritional indices in *Anagasta kuehniella* Zeller larvae when they studied the insecticidal potential of *Croton urucurana* extracts by incorporating them in their artificial diet. The chloroform extract (LD_{50} = 342.62 mg/l), dichloromethane extract (LD_{50} = 357.78 mg/l), and acetone extract (LD_{50} = 372.57 mg/l) of *P. nigrum* incorporated into the artificial diet *al*so interfered with nutritional indices resulting in 37.98, 26.17, and 14.82% reductions in ECI and 48.36, 39.27, and 22.72% reductions in ECD indices compared to control larvae, respectively. Similar growth inhibition patterns of ECI and ECD in *R. ferrugineus* Olivier larvae were observed upon exposure to conidial suspension (1×10^7 conidia/ml) of *Beauveria bassiana* (Hussain *et al* 2015). The decrease in ECI and ECD values compared to the control larvae reported before, and confirmed in the present work, revealed that most of the energy from ingested food is being used to perform physiological activities to combat the toxin, which means that less food is being utilized for larval growth.

The most potent compound, piperine, incorporated into the artificial diet of red palm weevil larvae tremendously increased the AD values compared to control treatment.

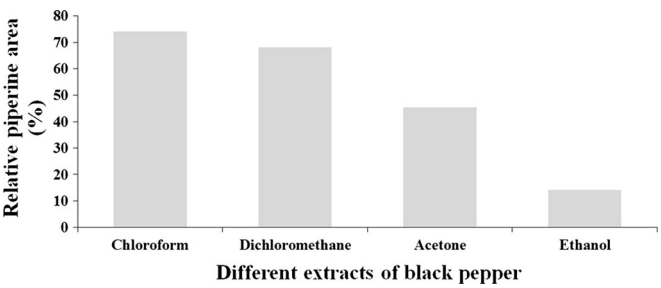

Fig 7 Percentage piperine composition detected by GC-MS in different extracts of black pepper.

The increase in AD values in piperine-fed larvae might reveal the fact that these larvae demand more energy for host defense. This energy shortfall could only be acquired through the use of intrinsic abilities to increase the AD of the limited foodstuff. In the past, similar enhanced AD response to fungal stress (Hussain *et al* 2015, 2016) and labramin (Martinez *et al* 2012) was obtained against *R. ferrugineus* Olivier and *A. kuehniella*, respectively.

Concerning the physiological impact, black pepper extract caused a tremendous increase in the expression of detoxification genes. This increase was even more pronounced in larvae fed diets incorporated with piperine. Enhanced expression of detoxification genes, including *glutathione S-transferase*, *cytochrome P450*, and *esterase*, is well documented as a defense mechanism against deleterious plant metabolites. The present laboratory experiments show that among the detoxification genes, *cytochrome P450* was greatly expressed in the mid-gut as a detoxification defense. We have noted approximately 35-fold higher expression of *cytochrome P450* from the eighth-instar red palm weevil larvae fed on diets incorporated with piperine. Similar enhanced activity (45-fold) of *cytochrome P450* was reported in variegated cut-worm fed peppermint leaves (Yu *et al* 1979). Furthermore, each extract of black pepper induced different levels of *cytochrome P450* expression. However, all of the extracts could only induce up to <12-fold expression in red palm weevil larvae. Our results are consistent with the findings of Rashid *et al* (2013), who demonstrated that sub-lethal concentrations of cantharidin in the artificial diet of *Helicoverpa armigera* Hübner enhanced *cytochrome P450* gene expression.

In the present study, all of the black pepper extracts were not able to induce *esterase* expression of eighth-instar red palm weevil larvae. These results are consistent with those reported by Liu *et al* (2008), who reported that fraxinellone-treated food was not able to induce the activity of *esterase* in *Ostrinia furnacalis* Guenee. In contrast, the most potent piperine-fed artificial diet greatly enhanced the expression of *esterase*, resulting in approximately a 15-fold increase in expression that coincides with the findings of Khosravi *et al* (2011), who reported that the crude extract of *Artemisia annua* Linnaeus significantly enhanced *esterase* activity of *Glyphodes pyloalis* Walker larvae.

Glutathione S-transferase is a gene with several functions but is primarily involved in detoxification. Data presented in the current study demonstrate that different extracts of *P. nigrum* significantly enhanced *GST* expression, and this expression was more prominent in piperine-fed larvae of red palm weevils. The findings of Zibaee and Bandani (2010) also showed high *GST* levels in *Eurygaster integriceps* Puton treated with *A. annua* Linnaeus. However, high levels of energy consumption in *Cydia pomonella* Linnaeus and *G. pyloalis* Walker were observed during detoxification that lead to a reduction in growth (Boivin *et al* 2001, Khosravi et al 2011). Similarly, our results do not reveal that there was any inhibition in the expression of detoxification genes, suggesting that they were involved in the detoxification mechanism of *R. ferrugineus* Olivier larvae.

Conclusion

In summary, incorporation of piperine in *R. ferrugineus* Olivier larval diet significantly inhibited the feeding performance signifying its utility as eco-friendly bio-pesticide against red palm weevils. The alterations in the expression of genes provide important insights for the first time about the detoxification mechanism of red palm weevils. Overall, detailed toxicological, growth inhibitory, and physiological responses at the molecular level in the current study demonstrate the efficacy of using environmentally friendly commercial development of piperine to control red palm weevil infestations.

Acknowledgments Deanship of King Faisal University (150083) of the Kingdom of Saudi Arabia provided the funds to carry out the whole experimentation.

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