Research Article

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Insecticidal activities of cinnamic acid esters isolated from *Ocimum gratissimum* L. and *Vitellaria paradoxa* Gaertn leaves against *Tribolium castaneum* Hebst (Coleoptera: Tenebrionidae)

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Abstract

BACKGROUND: Pest management using botanicals has been widely practiced in sub-Saharan Africa and other parts of the world in recent times. The natural compounds present in these botanicals are known to be responsible for the protection they offer against insect pests. Some of these compounds may act as single compounds to produce an effect or they may be synergistically effective. In the present study using a bioassay guided approach, two cinnamic acid derivatives, methyl cinnamate and sitosterol cinnamate, were isolated from the leaves of *Ocimum gratissimum* and *Vitellaria paradoxa*, respectively.

RESULTS: The two cinnamic acid derivatives were found to show higher levels of insecticidal, larvicidal and larval growth inhibition activities against *Tribolium castaneum*. The LC_{50} of methyl cinnamate was determined to be 26.92 mg mL^{-1} (95% CL: 1.18.66–38.84 mg mL^{-1}; slope ± SE: 2.84 ± 0.81) for the adult 8.31 mg mL^{-1} (95% CL: 2.39–28.83 mg mL^{-1}; slope ± SE: 0.66 ± 0.28) for the larvae while the LC_{50} of sitosterol cinnamate was determined to be 6.92 mg mL^{-1} (95% CL: 3.97–12.06 mg mL^{-1}; slope ± SE: 1.59 ± 0.12) the adult and 3.91 mg mL^{-1} (95% CL: 2.21–6.93 mg mL^{-1}; slope ± SE: 1.52 ± 0.13) for the larvae.

CONCLUSION: Generally, the susceptibility of adult *T. castaneum* to these cinnamic acid esters can be directly associated with the concentration as well as time of exposure to the compounds. The isolated compounds support the use of *O. gratissimum* and *V. paradoxa* as important botanicals for the management of storage pests.

Keywords: methyl cinnamate; β -sitosterol; sitosterol cinnamate; bioassay; larvicidal activity; bioactive compounds

1 INTRODUCTION

The rust red flour beetle *Tribolium castaneum* Hebst (Coleoptera: Tenebrionidae) is a secondary pest that attacks a wide range of stored products.[1] It is an unspecialized feeder on a wide range of durable commodities including cereal, grains and grain products, ground nuts, spices, dried fruits, peas, cocoa, coffee, animal products and feed. Infestation by this insect leads to a persistent and objectionable odor in the commodity due to the secretion of benzoquinones from a pair of abdominal defense glands from this insect.[1]

Considering the nature and extent of damage to the stored product, it is important that effective management practices are employed. An interruption in the life cycle can be one way to manage this pest.

Pest management using botanicals has been widely practiced in sub-Saharan Africa and other parts of the world in recent times.[2–6] Natural compounds present in these botanicals are known to be responsible for the protection they offer against insect pests.[7–11] Some of these compounds may act as single compounds to produce an effect or they may be synergistically effective. The bioactivities of compounds may differ by changing one or more functional groups attached to the compounds. By varying the level of saturation of the aliphatic carbon chain, the cardanols isolated from cashew nutshell liquid showed different levels of insecticidal activities against *Sitophilus oryzae* L.[7]

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Ocimum is a genus of aromatic annual and perennial herbs and shrubs in the family Lamiaceae native to the tropical and warm temperate regions of all six inhabited continents, with the greatest number of species in Africa.[12–15] *Ocimum* is known for its pesticide properties due to the diverse group of compounds in its essential oil.[16] *Ocimum gratissimum* is grown for the essential oil in its leaves and stems and is reported to contain bioactive constituents that are insecticidal and repellent.[17]

Indegenous to Africa, the shea tree, *Vitellaria paradoxa*, is known to contain several antioxidants.[18–21] Its fruits are edible and contain a lot of important fatty acids. Shea butter, which is the most economically important part of the tree, is an edible fat extracted from the seed kernel and is known to consist of an olein and a stearin fraction along with non-saponifiable (nonlipid) compounds.[22,23] The leaves have also been known to contain several useful antioxidants and are therefore used in folk medicine to treat certain illnesses. The methanol extract of *V. paradoxa* kernels was investigated for its constituents, and 15 oleanane-type triterpene acids and glycosides, among other compounds, were isolated, showing several bioactivities.[23]

Traditionally, the various parts of these plants are pounded into powder and used to treat stored products by local farmers, and this offers protection against storage pests.[5] In the present study using a bioassay guided approach, the insecticidal, larvicidal and larval growth inhibition activities of cinnamic acid derivatives isolated from the leaves of *O. gratissimum* and *V. paradoxa* against *T. castaneum* were studied in the laboratory.

2 MATERIALS AND METHOD

2.1 Insects

The initial stock of adult *T. castaneum* was from a laboratory stock obtained from Earth Chemicals Ltd, (Tokyo) Japan. The adult beetles were introduced into uninfested wheat flour (100 g) purchased from a licensed flour shop and were kept in 500 mL glass jars. The insects were allowed to oviposit in the flour. They were allowed for oviposition after which the adults were filtered out 15 days later. The adults that emerged were transferred into another 500 mL glass jar such that the F1 adults (which were used as the culturing stock for the experiments) were of uniform size and age. The set up was kept under a temperature of 32 ± 2 °C, 70% relative humidity and 12L:12D photo regime.

2.2 Plant materials

The leaves of *O. gratissimum* and *V. paradoxa* previously identified and confirmed by the Department of Botany, University of Ghana, Legon were harvested from the University Farms (5.6506° N, 0.1962° W), University of Ghana. The *O. gratissimum* used was about 6 months old whiles the *V. paradoxa* was about 5 years old. The leaves harvested were cut into pieces using a pair of laboratory scissors and dried in the laboratory under a temperature of 32 °C and were then grounded into fine powder using mortar and pestle. About 200 g of the leaf powder of each plant was soaked in 1.5 L of methanol (extra pure grade; purchased from Nalacai Tesque Inc., Japan) and 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 2.0 g equivalent (eq.) mL^{-1} and 1.0 g eq. mL^{-1} of the extract and were used for the bioassays.

2.3 Insecticidal bioassay

One-week-old, unsexed adult insects were each dipped in turns for 10 s into 10 mL each of the isolated extracts and then transferred into clean Petri dishes (210 mm in diameter) containing 10 g of wheat flour. The Petri dishes were covered with their lids and the set up was observed daily for insect mortalities and survivals until there were no changes in the mortality and survival of the insects. An insect was considered dead if it did not respond to probing of a blunt probe.[24] Methanol alone was used for negative control while deltamethrin applied at a rate of 0.5 ppm[25] was used for positive control. Six replicates were made, with each replicate consisting of ten adult insects.

2.4 Larvicidal bioassay

Ten larvae (1 week old) were dipped in turns into each sample and then transferred into clean Petri dishes (210 mm in diameter) containing 10 g of wheat flour. These were incubated in a rearing chamber for a period of 6 weeks. The set up was closely monitored twice a week to see the various developmental stages from larvae to adult. To assess the effect of the samples on the different developmental stages of *T. castaneum*, insect mortality and growth activity were determined following a modified scale.[26] Using the modified scale, the number of dead larvae (Stage 1), the number of dead pupa (Stage 2), the number of adultoids (Stage 3, alive and dead), the number of adults with deformity (Stage 4) and finally the number of normal adults (Stage 5) were defined. Adultoids were defined as pupa having a characteristic appearance with the front part of the body like an adult and pigmented with widespread forewings and hind wings (if developed), while the abdomen has a typical appearance of pupa and is not pigmented.[26] An adult with deformity is a fully developed adult with some part of its body undeveloped. Features considered as a deformity include a broken or underdeveloped wing, leg or antenna. Methanol alone was used for the negative control while deltamethrin applied at a rate of 0.5 ppm[25] was used for positive control. Six replicates per treatment were made and methanol was used for the control.

2.5 Isolation and identification of bioactive compounds from methanol extracts of *O. gratissimum*

The methanol extract of the *O. gratissimum* leaves (yield: 4.48 g) was dissolved in distilled water (110 mL), and its components were separated by liquid–liquid partitioning using 80 mL each of hexane, diethyl ether and ethyl acetate (AcOEt) one after the other. The hexane layer (1.84 g) was chromatographed on a silica gel open column (55.2 g, 20 mm Ø × 351.4 mm). The column was eluted under gravity with 1104 mL each of hexane, 5% AcOEt in hexane, 30% AcOEt in hexane, 50% AcOEt in hexane, 70% AcOEt in hexane, AcOEt and methanol. The 5% AcOEt in hexane fraction (1.08 g) was submitted to normal phase silica gel HPLC (column: Cosmosil 5SL, 10 mm Ø × 250 mm) eluted with 3% AcOEt in hexane at a flow rate of 3 mL min^{-1} and a UV detector (254 nm). Four fractions [fraction (Fr.) 1 (t_R: 0.00–16.00 min), Fr. 2 (t_R: 16.01–19.99 min), Fr. 3 (t_R: 20.00–22.00 min) and Fr. 4 (t_R: 22.01–30.00 min)] from the HPLC analysis were collected and used for bioassay. Fr. 3 was designated compound **1** and was further collected and analyzed by GC–MS and ^1H and ^{13}C NMR for structural elucidation.

2.6 Isolation and identification of bioactive compounds from methanol extracts of *V. paradoxa*

The methanol extract of the *V. paradoxa* leaves (yield: 52.17 g) was dissolved in distilled H_2O (1.3 L) and its components were

separated by liquid–liquid partitioning using 885.5 mL each of hexane, diethyl ether and AcOEt. The hexane layer (4.17 g) was chromatographed on a silica gel open column (246 g, 30 mm Ø × 347.97 mm). The column was eluted under gravity with 2.5 L each of hexane, 5% AcOEt in hexane, 30% AcOEt in hexane, 50% AcOEt in hexane, 70% AcOEt in hexane, AcOEt and methanol. The 5% AcOEt in hexane fraction (2.08 g) was submitted to normal phase silica gel HPLC (column: Cosmosil 5SL, 10 mm Ø × 250 mm) eluted with 3% AcOEt in hexane at a flow rate of 3 mL min^{-1} and a UV detector (254 nm). Four fractions [fraction (Fr.) 1 (t_R: 0.00–13.74 min), Fr. 2 (t_R: 13.75–15.85 min), Fr. 3 (t_R: 15.86–17.72 min) and Fr. 4 (t_R: 17.73–30.00 min)] from the HPLC analysis were collected and used for bioassay. Fr. 2 was further divided to peak (Pk.) 2–1 (t_R: 13.75–14.91 min) and Pk. 2–2 (t_R: 14.92–15.85 min). The bioactive Pk. 2–1 was designated compound **2** and was further collected and analyzed by GC–MS and ^{1}H and ^{13}C NMR for structural elucidation.

2.7 Instruments

GC–MS data(EI positive) were recorded with GC 2010 Plus (Shimadzu) equipped with an HP-5 MS column (cross-linked 5% PH ME siloxane, 30 m × 0.32 mm × 0.25 µm film thickness). The carrier gas was helium at a flow rate of 11.7 mL min^{-1}, the split ratio was 1:10, and the detector and vaporizer temperature was held at 300 °C. The column temperature was initially at 150 °C for 6 min, raised to 300 °C at 5 °C min^{-1} and held for 10 min. The identification of the components was confirmed by comparing with the mass spectra of compounds documented by the National Institute of Standards and Technology 14 library. ^{1}H and ^{13}C NMR spectra were measured with a Jeol JNM-ECX500 using TMS as internal reference.

2.8 Chemicals

All the chemicals used were all purchased from Nalacai Tesque Inc., Japan. They were all extra pure grade. The ones used for HPLC were all HPLC grade and the ones used for the NMR were of NMR grade.

2.9 Median lethal dose (LC_{50}) and time (LT_{50}) of isolated compounds

Concentrations of 40, 20, 10 and, 5 mg mL^{-1} of compound **1** and 23.45, 11.73, 5.86, 2.93 and 1.47 mg mL^{-1} of compound **2** were prepared separately and was used for both insecticidal bioassay, and larvicidal and larval development inhibition bioassay. Adult and larval mortality data were analyzed using probit analysis (Finey, 1952)[27] and the lethal time value at 50% mortality (LT_{50}) for each concentration as well as the LC_{50} value of the isolated compounds were determined appropriately.

2.10 Data collection and analysis

Data on adult insect mortality and survival as well as larvae to adult development were collected and were analyzed 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 mean 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 ($y = \sin^{-1}\sqrt{x/100}\sqrt{x}/100$) transformation before analysis. Percentage mortality was calculated and control mortalities were corrected for using Abbott's formula: (% test mortality – % control mortality)/100 – control mortality × 100.[28] Mean (±SE) of untransformed data are reported.

3 RESULTS

3.1 Bioactivities of the methanol extracts of *O. gratissimum* and *V. paradoxa* leaves

The different concentrations of the crude extracts of the *O. gratissimum* and *V. paradoxa* leaves showed different levels of bioactivities against the adult and larvae of *T. castaneum*. The 2.0 g eq. mL^{-1} treatment of the *O. gratissimum* leaves showed a significantly ($P < 0.01$) higher percentage mortality than the 1.0 g eq. mL^{-1} (Table 1**).** Percentage mortalities of 80.0 ± 0.0% and 60.0 ± 0.0% were observed for the 2.0 and 1.0 g eq. mL^{-1} treatments, respectively, 48 h after exposure. For the *V. paradoxa*, the 2.0 g eq. mL^{-1} also showed a significantly ($P < 0.01$) higher percentage mortality than the 1.0 g eq. mL^{-1} (Table 1). After 96 h, the highest percentage mortality of 70.0 ± 6. 7% was recorded for the 2.0 g eq. mL^{-1} treatment while 50.0 ± 6. 7% was recorded for the 1.0 g eq. mL^{-1} treatment. The control, which consisted of methanol alone, recorded no insect mortality, whilst the deltamethrin recorded 100% insect mortality after 24 h. These results thus confirm the insecticidal potential of the crude extract of *O. gratissimum* and *V. paradoxa* leaves against *T. castaneum*.

3.2 Bioassay guided isolation of compound 1

Compound **1** was isolated from the crude extracts of *O. gratissimum* leaves using a bioassay guided isolation. The various

Table 1. Percentage mortality of *T. castaneum* for the two concentrations of methanol extracts of *O. gratissimum* leaves and *V. paradoxa* leaves

Plant	Concentration (g. eq mL^{-1})	% Mortality†							
		0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
O. gratissimum	1.0	0 ± 0^a	60.0 ± 0.0^c	60.0 ± 0.0^c	60.0 ± 0.0^c	60.0 ± 0.0^c	60.0 ± 0.0^c	60.0 ± 0.0^c	60.0 ± 0.0^c
	2.0	0 ± 0^a	65.0 ± 0.5^c	80.0 ± 0.0^d	80.0 ± 0.0^c	80.0 ± 0.0^e	80.0 ± 0.0^e	80.0 ± 0.0^e	80.0 ± 0.0^e
V. paradoxa	1.0	0 ± 0^a	46.7 ± 8.9^b	46.7 ± 8.9^b	50.0 ± 6.7^b	50.0 ± 6.7^b	50.0 ± 6.7^b	50.0 ± 6.7^b	50.0 ± 6.7^b
	2.0	0 ± 0^a	50.0 ± 6.7^b	60.0 ± 0.0^c	60.0 ± 0.0^b	70.0 ± 6.7^e	70.0 ± 6.7^d	70.0 ± 6.7^d	70.0 ± 6.7^d
Control									
Methanol		0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a
Deltamethrin*		0 ± 0^a	100 ± 0^f	100 ± 0^f	100 ± 0^f	100 ± 0^f	100 ± 0^f	100 ± 0^f	100 ± 0^f

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different. Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA (for significant difference $P < 0.05$).

Table 2. Percentage mortality of *T. castaneum* for the four separated layers from liquid–liquid partitioning of the methanol extracts of *O. gratissimum* leaves

	% Mortality†							
Treatment (per 2 g eq. mL^{-1})	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Hexane	0 ± 0^{a}	80 ± 0^{c}	80 ± 0^{c}	80 ± 0^{d}	80 ± 0^{d}	80 ± 0^{d}	80 ± 0^{d}	80 ± 0^{d}
Ether	0 ± 0^{a}	0.0 ± 0^{a}	16.7 ± 6.7^{b}	16.7 ± 6.7^{b}	20.0 ± 6.7^{b}	23.3 ± 6.7^{b}	23.3 ± 6.7^{b}	23.3 ± 6.7^{b}
Ethyl acetate	0 ± 0^{a}	16.7 ± 4.4^{b}	23.3 ± 4.4^{b}	26.7 ± 4.4^{b}	30.0 ± 6.7^{b}	30.0 ± 6.7^{b}	30.0 ± 6.7^{b}	30.0 ± 6.7^{b}
Water	0 ± 0^{a}	23.3 ± 6.7^{b}	23.3 ± 6.7^{b}	40.0 ± 6.7^{c}	40.0 ± 6.7^{bc}	40.0 ± 6.7^{bc}	40.0 ± 6.7^{bc}	40.0 ± 6.7^{bc}
All	0 ± 0^{a}	76.7 ± 4.4^{c}	76.7 ± 4.4^{c}	76.7 ± 4.4^{d}	76.7 ± 4.4^{d}	76.7 ± 4.4^{d}	76.7 ± 4.4^{d}	76.7 ± 4.4^{d}
Control								
Methanol	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Dealtamethrin*	0 ± 0^{a}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.

Table 3. Percentage mortality of *T. castaneum* for the various fractions from the silica gel open column chromatography of the hexane layer of *O. gratissimum*

	% Mortality†							
Treatment (per 2 g eq. mL^{-1})	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
100% Hexane	0 ± 0^{a}	6.7 ± 4.4^{a}	10.0 ± 6.7^{b}	10.0 ± 6.7^{b}	10.0 ± 6.7^{b}	10.0 ± 6.7^{b}	10.0 ± 6.7^{b}	10.0 ± 6.7^{b}
5% AH	0 ± 0^{a}	73.3 ± 8.9^{d}	73.3 ± 8.9^{d}	73.3 ± 8.9^{d}	73.3 ± 8.9^{d}	73.3 ± 8.9^{d}	73.3 ± 8.9^{d}	73.3 ± 8.9^{d}
30% AH	0 ± 0^{a}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}
50% AH	0 ± 0^{a}	10.0 ± 0.0^{ab}	10.0 ± 0.0^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	20.0 ± 0.0^{c}
70% AH	0 ± 0^{a}	10.0 ± 4.4^{ab}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}
100% AcOEt	0 ± 0^{a}	10.0 ± 0.0^{ab}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}	15.0 ± 3.3^{b}
100% Methanol	0 ± 0^{a}	25.0 ± 3.3^{c}	25.0 ± 3.3^{c}	35.0 ± 3.3^{c}	35.0 ± 3.3^{c}	35.0 ± 3.3^{c}	35.0 ± 3.3^{c}	35.0 ± 3.3^{d}
All	0 ± 0^{a}	80.0 ± 0.0^{d}	80.0 ± 0.0^{d}	80.0 ± 0.0^{d}	80.0 ± 0.0^{d}	80.0 ± 0.0^{d}	80.0 ± 0.0^{d}	80.0 ± 0.0^{e}
Control								
Methanol	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Deltamethrin*	0 ± 0^{a}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA. ‡ AH: Ethyl acetate in hexane

separated components of the crude extracts of the *O. gratissimum* leaves showed different levels of bioactivities against the adult and larvae of *T. castaneum* (Table 2). The four different layers obtained from the liquid–liquid partitioning of the crude extract of *O. gratissimum* showed varying levels of insecticidal activities against the adult *T. castaneum*. The hexane layer yielded a significantly ($P < 0.01$) higher insecticidal activity of $80.0 \pm 0.0\%$ mortality, but this was not significantly different from the $76.7 \pm 4.4\%$ mortality recorded when all four layers were combined in equal amounts. The diethyl ether, AcOEt and water layers yielded $23.3 \pm 11.1\%$, $30.0 \pm 6.7\%$ and $40.0 \pm 6.7\%$ respectively (Table 2).

Seven fractions were obtained from the silica gel open column chromatography of the hexane layer. Of the seven fractions, the 5% AcOEt in hexane fraction yielded the significantly ($P < 0.01$) highest percentage mortality of $73.3 \pm 8.9\%$ than the other fractions 24 h after exposure (Table 3). The other fractions, on the other hand, yielded relatively much lower insect mortalities with significantly ($P > 0.01$) no difference between them. When all the fractions were combined in equal amounts, the percentage mortality of 80% was observed 24 h after exposure. This was not significantly different ($P > 0.01$) from the percentage mortality of $73.3 \pm 8.9\%$ observed for the 5% AcOEt in hexane fraction. Therefore, it can be inferred that the bioactivity of the crude extract is retained in the 5% AcOEt in hexane fraction.

The analysis of the 5% AcOEt in hexane fraction by HPLC showed several peaks that were grouped into four fractions designated fraction (Fr.) 1 (yield: 37.7 mg), Fr. 2 (yield: 5.7 mg), Fr. 3 (yield 37.3 mg) and Fr. 4 (yield 31.2 mg), as shown in Fig. 1.

The insecticidal activities of the separated fractions are shown in Table 4. Fr. 3 showed significantly ($P < 0.01$) the highest activity against the adult *T. castaneum*. Percentage mortality of $70.0 \pm 0.0\%$ was observed 144 h after exposure. On the other hand, Fr. 4 showed the least bioactivity with $23.3 \pm 4.4\%$, but this is not significantly different from the response of $26.7 \pm 8.8\%$ mortality showed by Fr. 2. When all the fractions were combined in equal amounts, $86.7 \pm 11.1\%$ mortality was observed. Therefore, with the highest percentage mortality of $70.0 \pm 0.0\%$ among the fractions, Fr. 3, which contains only one peak, was considered to be the compound responsible for the bioactivity of the 5% AcOEt in hexane fraction and was therefore designated compound **1**.

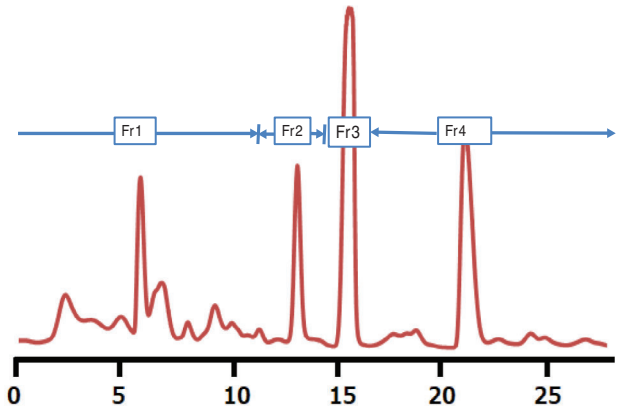

Figure 1. The HPLC chromatogram for the 5% AcOEt in the hexane fraction of *O. gratissimum* leaf extracts [Fr 1 (i_R: 0.00–13.00 min), Fr. 2 (t_R: 13.01–14.99 min), Fr. 3 (t_R: 15.00–16.99 min) and Fr. 4 (t_R: 17.00–30.00 min)].

3.3 Larvicidal and larval growth inhibition activities of compound 1

The larvicidal and larval growth inhibition activities of the separated components of *O. gratissimum* leaves and the isolated compound **1** are shown in Table 5. The methanol extract, hexane layer, 5% AcOEt in hexane fraction as well as the isolated compound **1** showed significantly ($P < 0.01$) higher larvicidal and larval growth inhibition activities than the control. There were 100 ± 0.0% larval mortalities in the methanol extract of *O. gratissimum* leaves, 5% AcOEt in hexane fraction and the isolated compound **1**. These were not significantly different from the 86.7 ± 7.8% larval mortality observed in the hexane layer, which also recorded 3.3 ± 0.4% adults with deformities. Consequently, no adults without deformities were recorded in all these treatments. The control consisting of methanol alone had 10.0 ± 6.7% larval mortalities, 6.7 ± 4.4% dead adultoids, 3.3 ± 0.4% adults without deformities and a significantly higher number of adults without deformities of 80.0 ± 7.3% emerging, whilst the deltamethrin treatment recorded 100% larval mortality with no adult emergence. These results indicate that isolated compound **1** exhibits high larvicidal and larval growth inhibition activities against *T. castaneum*, and thus cuts short the life cycle of the insect.

3.4 Bioassay guided isolation of compound 2

Compound **2** was isolated from the crude extracts of *V. paradoxa* leaves. The four different layers obtained from the liquid–liquid partitioning of the crude extract showed various levels of insecticidal activities against the adult *T. castaneum* (Table 6). The hexane layer showed a significantly ($P < 0.05$) higher insecticidal activity of 80.0 ± 5.0% mortality after 24 h of exposure. The diethyl

Table 4. Percentage mortality of *T. castaneum* for the fractions isolated from the 5% AcOEt in hexane fraction from *O. gratissimum* by HPLC

	% Mortality†							
Treatment (per 2 g eq. mL^{-1})	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Fraction 1	0 ± 0^{a}	20.0 ± 4.4^{b}	20.0 ± 4.4^{b}	20.0 ± 4.4^{b}	20.0 ± 4.4^{bc}	40.0 ± 4.4^{c}	40.0 ± 4.4^{c}	40.0 ± 4.4^{c}
Fraction 2	0 ± 0^{a}	6.7 ± 3.3^{a}	6.7 ± 3.3^{a}	6.7 ± 3.3^{a}	10.0 ± 3.3^{b}	16.7 ± 4.4^{b}	26.7 ± 4.4^{b}	26.7 ± 4.4^{b}
Fraction 3	0 ± 0^{a}	43.3 ± 4.4^{c}	43.3 ± 4.4^{c}	50.0 ± 0.0^{c}	50.0 ± 0.0^{d}	56.7 ± 4.4^{d}	70.0 ± 0.0^{d}	70.0 ± 0.0^{d}
Fraction 4	0 ± 0^{a}	16.7 ± 4.4^{b}	16.7 ± 4.4^{b}	16.7 ± 4.4^{b}	16.7 ± 4.4^{b}	20.0 ± 0.0^{b}	23.3 ± 4.4^{b}	23.3 ± 4.4^{b}
All fractions	0 ± 0^{a}	86.7 ± 11.1^{d}	86.7 ± 11.1^{d}	86.7 ± 11.1^{d}	86.7 ± 11.1^{a}	86.7 ± 11.1^{e}	86.7 ± 1.1^{e}	86.7 ± 11.1^{e}
Control								
Methanol	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Deltamethrin*	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.

Table 5. Effect of the separated and isolated components of *O. gratissimum* on larval to adult development of *T. castaneum*

	% Mortality†					
	Stages‡					
Treatment	1	2	3	4	5	6
Hexane layer	86.7 ± 17.8^{b}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	3.3 ± 0.4^{b}	0 ± 0^{a}
5% EtOAc in hexane	100.0 ± 0.0^{c}	0 ± 0^{a}	0 ± 0^{a}	$0 \pm 0^{a\,a}$	0 ± 0^{a}	0 ± 0^{a}
Compound 1	100.0 ± 0.0^{c}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Deltamethrin*	100.0 ± 0.0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.
‡Stage 1, dead larvae; stage 2, larvae transforming to pupae; stage 3, dead pupae; stage 4, adultoids (alive and dead); stage 5, deformed adults; stage 6, normal adults.

Table 6. Percentage mortality of *T. castaneum* for the four separated layers from liquid–liquid partitioning of the methanol extracts of *V. paradoxa leaves*

	% Mortality†							
Treatment (per 2 g eq. mL^{-1})	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Hexane	0 ± 0^{a}	80.0 ± 5.0^{f}	80.0 ± 5.0^{f}	80.0 ± 5.0^{f}	80.0 ± 5.0^{f}	80.0 ± 5.0^{f}	80.0 ± 5.0^{f}	80.0 ± 5.0^{f}
AcOEt	0 ± 0^{a}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}	10.0 ± 0.0^{b}
Ether	0 ± 0^{a}	53.3 ± 6.7^{d}	53.3 ± 6.7^{d}	53.3 ± 6.7^{d}	53.3 ± 6.7^{d}	53.3 ± 6.7^{d}	53.3 ± 6.7^{d}	53.3 ± 6.7^{d}
Water	0 ± 0^{a}	20.0 ± 10.0^{c}	20.0 ± 10.0^{c}	20.0 ± 10.0^{c}	20.0 ± 10.0^{c}	20.0 ± 10.0^{c}	20.0 ± 10.0^{c}	20.0 ± 10.0^{c}
All	0 ± 0^{a}	70.0 ± 0^{e}	70.0 ± 0^{e}	70.0 ± 0^{e}	70.0 ± 0^{e}	70.0 ± 0^{e}	70.0 ± 0^{e}	70.0 ± 0^{e}
Control								
Methanol	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Deltamethrin*	100 ± 0^{g}	100 ± 0^{g}	100 ± 0^{g}	100 ± 0^{g}	100 ± 0^{g}	100 ± 0^{g}	100 ± 0^{g}	100 ± 0^{g}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.

Table 7. Percentage mortality of *T. castaneum* to the various fractions from the silica gel open column chromatography of the hexane layer of *V. paradoxa*

	% Mortality†							
Treatment (per 2 g eq. mL^{-1})	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
100% Hexane	0 ± 0^{a}	1.7 ± 2.8^{a}	15.0 ± 67^{b}	15.0 ± 67^{b}	15.0 ± 67^{b}	15.0 ± 67^{b}	15.0 ± 67^{b}	15.0 ± 67^{b}
5% AH	0 ± 0^{a}	66.7 ± 10.0^{d}	71.7 ± 12.2^{d}	73.3 ± 11.1^{e}	73.3 ± 11.1^{e}	73.3 ± 11.1^{e}	73.3 ± 11.1^{e}	73.3 ± 11.1^{e}
30% AH	0 ± 0^{a}	16.7 ± 2.3^{b}	16.7 ± 2.3^{b}	23.3 ± 2.3^{c}	25.0 ± 2.3^{c}	26.7 ± 2.3^{c}	30.0 ± 2.3^{c}	30.0 ± 2.3^{c}
50% AH	0 ± 0^{a}	11.7 ± 3.3^{b}	11.7 ± 3.3^{b}	11.7 ± 3.3^{b}	11.7 ± 3.3^{b}	11.7 ± 3.3^{b}	11.7 ± 3.3^{b}	11.7 ± 3.3^{b}
70% AH	0 ± 0^{a}	16.7 ± 3.3^{b}	20.0 ± 6.7^{b}	23.3 ± 4.4^{c}	23.3 ± 4.4^{c}	25.0 ± 5.0^{c}	33.3 ± 4.4^{c}	33.3 ± 4.4^{c}
AcEOt	0 ± 0^{a}	8.3 ± 3.3^{a}	8.3 ± 3.3^{a}	8.3 ± 3.3^{a}	8.3 ± 3.3^{a}	8.3 ± 3.3^{a}	8.3 ± 3.3^{a}	8.3 ± 3.3^{a}
MeOH	0 ± 0^{a}	33.3 ± 4.4^{c}	40.0 ± 10.0^{c}	43.3 ± 8.9^{d}	48.3 ± 2.8^{d}	48.3 ± 2.8^{d}	48.3 ± 2.8^{d}	48.3 ± 2.8^{d}
All	0 ± 0^{a}	80.0 ± 10.0^{e}	83.3 ± 6.7^{e}	83.3 ± 6.7^{e}	88.3 ± 2.8^{f}	90.0 ± 3.3^{f}	96.7 ± 4.4^{f}	96.7 ± 4.4^{f}
Control								
Methanol	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Deltamethrin*	0 ± 0^{a}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}	100 ± 0^{f}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA. ‡ AH: Ethyl acetate in hexane

ether, AcOEt and water layers yielded mortalities of 53.3 ± 6.7%, 10.0 ± 0.0% and 20.0 ± 10.0%, respectively. However, when all the four layers were combined in equal amounts a percentage mortality of 70.0 ± 0.0% was recorded.

The 5% AcOEt in hexane fraction from the silica gel open column chromatography of the hexane layer yielded the significantly ($P < 0.05$) highest percentage mortality of 73.0 ± 11.1% than the other fractions after 72 h of exposure (Table 7). The other fractions yielded relatively much lower insect mortalities with no significant difference ($P > 0.05$) between them. When all the fractions were combined in equal amounts, a percentage mortality of 96.7 ± 4.4% was observed 144 h after exposure. From these results, it can be inferred that the bioactivity of the crude extract is retained in the 5% AcOEt in hexane fraction.

The analysis of the 5% AcOEt in hexane fraction by HPLC showed several peaks that were grouped into four fractions designated fraction (Fr.) 1, Fr. 2, Fr. 3 and Fr. 4, as shown in Fig. 2(A). The amount of each fraction yielded is as follows: Fr. 1 (yield: 22.7 mg), Fr. 2 (yield: 17.4 mg), Fr. 3 (yield 7.2 mg) and Fr. 4 (yield 4.1 mg).

From the bioassay results, Fr. 2 showed significantly ($P < 0.05$) the highest activity against the adult *T. castaneum* (Table 8). A percentage mortality of 68.3 ± 2.8% was observed 168 h after exposure. On the other hand, Fr. 3 and Fr. 4 showed the least bioactivity with 16.0 ± 0.0% mortalities each. However, this is not significantly different from the response of 17.5 ± 7.5% mortality showed by Fr. 1. When all the fractions were combined in equal amounts, 80.0 ± 0.0% mortality was observed.

Fr. 2 was further separated into two peaks, namely, peak (Pk.) 2-1(yield: 13.3 mg) and Pk. 2-2 (yield: 6.5 mg) as shown by the chromatogram in Fig. 2(B). The bioassay results of the two peaks separated from Fr. 2 are shown in Table 9. Pk. 2-1 showed a significantly ($P < 0.05$) higher insecticidal activity than Pk. 2-2. Pk. 2-1 showed 64.0 ± 3.0% mortality, which is not significantly different ($P > 0.05$) from the 68.3 ± 2.8% mortality recorded by Fr. 2. It can therefore be inferred that Pk. 2-1 (compound **2**) is the compound in the 5% AcOEt in hexane fraction responsible for the insecticidal activities of *V. paradoxa* leaves against adult *T. castaneum*.

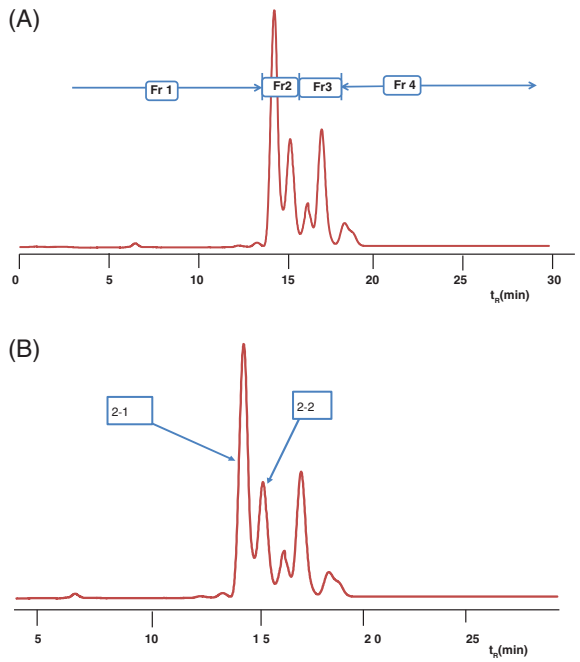

Figure 2. (A) HPLC chromatogram for the 5% AcOEt in hexane fraction [Fr. 1 (t_R: 0.00–13.74 min), Fr. 2 (t_R: 13.75–15.85 min), Fr. 3 (t_R: 15.86–17.72 min) and Fr. 4 (t_R: 17.73–30.00 min)]. (B) HPLC chromatogram for the 5% AcOEt in hexane fraction showing Pk. 2–1 (t_R: 13.75–14.91 min) and Pk. 2–2 (t_R: 14.92–15.85 min).

3.5 Larvicidal and larval growth inhibition activities of compound 2

The larvicidal and larval growth inhibition activities of the separated components of *V. paradoxa* leaves and the isolated compound **2** are shown in Table 10. The hexane layer, 5% AcOEt in hexane fraction as well as the isolated compound showed significantly higher larvicidal activities than the control. There were 96.3 ± 3.3% larval mortalities and 3.3 ± 4.4% adult without deformity emerging for the hexane layer. The 5% AcOEt in hexane fraction and the isolated compound **2** showed no significant difference in terms of larval mortality. There was 76.7 ± 8.9% larval mortality recorded for both treatments with 3.3 ± 4.4% deformed adults and 20.0 ± 6.6% undeformed adults emerging for the 3% AcOEt in hexane fraction. However, the isolated compound **2** had no adult emergence, with 16.6 ± 8.9% of the larvae developing into adultoids which were found dead. The control consisting of methanol alone had 13.3 ± 3.3% larval mortalities and the significantly higher number of adults without deformities of 96.7 ± 4.4% emerging whereas the deltamethrin treatment recorded 100% larval mortality and no adult emergence. These results indicate that the isolated compound **2** exhibits a high larvicidal activity against *T. castaneum*, thereby reducing the level of adult emergence.

3.6 Identification of isolated compounds

*3.6.1 Compound **1***

The ^{1}H NMR data in $CDCl_3$ (500 MHz) of isolated bioactive compound **1** showed proton signals at δ (ppm): 7.67 (1H, d, J = 15.06 Hz), 7.37 (2H, m), 7.36 (3H, m), 6.44 (1H, d, J = 15.06 Hz)

Table 8. Percentage mortality of *T. castaneum* for the HPLC fractions isolated from the 5% AcOEt in hexane fraction from *V. paradoxa*

	% Mortality†							
Treatment (per 2 g eq. mL^{-1})	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Fraction 1	0 ± 0^a	7.5 ± 3.8^a	7.5 ± 3.8^a	7.5 ± 3.8^a	15.0 ± 5.0^b	15.0 ± 5.0^b	17.5 ± 7.5^b	17.5 ± 7.5^b
Fraction 2	0 ± 0^a	25.0 ± 8.3^b	33.3 ± 5.6^b	33.3 ± 5.6^b	43.3 ± 5.6^c	51.7 ± 2.8^c	60.0 ± 0.0^c	68.3 ± 2.8^c
Fraction 3	0 ± 0^a	2.0 ± 0.0^a	2.0 ± 0.0^a	4.0 ± 0.0^a	8.0 ± 0.0^a	10.0 ± 0.0^a	14.0 ± 0.0^b	16.0 ± 0.0^b
Fraction 4	0 ± 0^a	2.0 ± 0.0^a	2.0 ± 0.0^a	4.0 ± 0.0^a	8.0 ± 0.0^a	10.0 ± 0.0^a	14.0 ± 0.0^b	16.0 ± 0.0^b
All fractions	0 ± 0^a	63.3 ± 4.4^c	63.3 ± 4.4^c	70.0 ± 0.0^c	70.0 ± 0.0^d	76.7 ± 4.4^d	76.7 ± 4.4^d	80.0 ± 0.0^d
Control								
Methanol	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a
Deltamethrin*	0 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.

Table 9. Percentage mortality of *T. castaneum* for HPLC fraction 2 isolated from the 5% AcOEt in hexane fraction from *V. paradoxa*

	% Mortality†							
Treatment (per 2 g eq. mL^{-1})	0 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Fraction 2	0 ± 0^a	25.0 ± 8.3^b	33.3 ± 5.6^b	33.3 ± 5.6^b	43.3 ± 5.6^b	51.7 ± 2.8^b	60.0 ± 0.0^b	68.3 ± 2.8^b
Peak 2-1	0 ± 0^a	30 ± 0^b	40 ± 0^b	40 ± 0^b	40 ± 0^b	50 ± 0^b	64 ± 3.0^b	64 ± 3.0^b
Peak 2-2	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a
Control								
Methanol	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a
Deltamethrin*	0 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a	100 ± 0^a

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.

Table 10. Effect of the separated and isolated components of *V. paradoxa* on larval to adult development of *T. castaneum*

	% Mortality†					
	Stages‡					
Treatment	1	2	3	4	5	6
Hexane layer	96.7 ± 3.3^{c}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	3.3 ± 4.4^{a}
5% AcOEt in hexane	76.7 ± 8.9^{b}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	3.3 ± 4.4^{a}	20.0 ± 6.6^{b}
Compound **2**	76.7 ± 8.9^{b}	0 ± 0^{a}	0 ± 0^{a}	16.6 ± 8.9^{b}	0 ± 0^{a}	0 ± 0^{a}
Control						
Methanol	3.3 ± 3.3^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	96.7 ± 4.4^{c}
Deltamethrin*	100 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.
‡Stage 1, dead larvae; stage 2, larvae transforming to pupae; stage 3, dead pupae; stage 4, adultoids (alive and dead); stage 5, deformed adults; stage 6, normal adults.

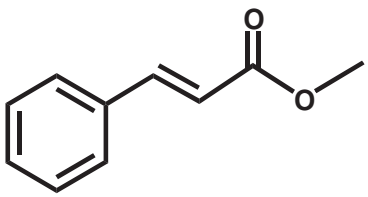

Figure 3. The structure of compound **1** (methyl cinnamate**)** isolated from *O. gratissimum* leaves.

and 3.72 (3H, s). The ^{13}C NMR data in $CDCl_3$ (125 MHz) indicated carbon signals at δ (ppm): 167.42, 144.85, 134.30, 130.27, 128.18, 128.04, 117.71, and 51.70.

The analysis of the NMR spectra and GC–MS (EI positive mode) data showed that compound **1** has a molecular weight of 162, with an aromatic (C_6H_5) ring, one methoxy (-O-CH_3) group, a carbonyl carbon (C=O) and two methine (CH) groups. By arranging the spectra information and comparing with literature,[29] compound **1** was identified to be methyl(2*E*)-3-phehylprop-2-enoate, also known as methyl cinnamate (Fig. 3).

3.6.2 *Compound* ***2***

The ^{1}H NMR data in $CDCl_3$ (500 MHz) of the isolated bioactive compound **2** showed peaks at δ (ppm): 7.82 (d, 1H, $J = 16.0$ Hz), 7.57 (d, 1H, $J = 16.0$ Hz), 7.38 (dd, 3H, $J = 4.8$ Hz, 1.8 Hz), 6.45 (d, 1H, $J = 16.0$ Hz), 5.33 (d, 1H, $J = 16.0$ Hz), 3.52 (m, 1H), 2.27 (m, 2H), 2.0 (m, 2H), 1.80 (m, 3H), 1.50 (m, 6H), 1.20 (m, 4H), 1.00 (s, 3H), 0.92 (m, 4H), 0.80 (m, 9H), 0.67 (s, 3H). The ^{13}C NMR data in $CDCl_3$ (125 MHz) indicated carbon signals at δ (ppm): 14.1, 15.7, 16.8, 16.9, 17.5, 18.2, 21.4, 22.7, 23.2, 23.4, 23.7, 26.6, 28.0, 28.1, 28.7, 31.2, 31.6, 32.8, 33.7, 36.8, 37.9, 38.4, 39.6, 40.0, 41.5, 42.0, 55.2, 59.0, 81.0, 118.2, 124.3, 128.8, 130.1, 134.5, 139.6, 144.3 and 166.8.

Analysis of the ^{1}H and ^{13}C NMR data, GC–MS (EI positive mode) spectra and comparison with NMR data for standard compounds and literature[29,30] identified compound **2** as 3-phenylacrylic acid 17-(4-ethyl-1,5-dimethyl-hexyl)-10,13-dimethyl 2,3,4,7,8,9, 10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl ester, also known as sitosterol cinnamate, and its structure is shown in Fig. 4(A). This compound is an ester of cinamic acid (Fig. 4(B)) and β-sitosterol (Fig. 4(C)).

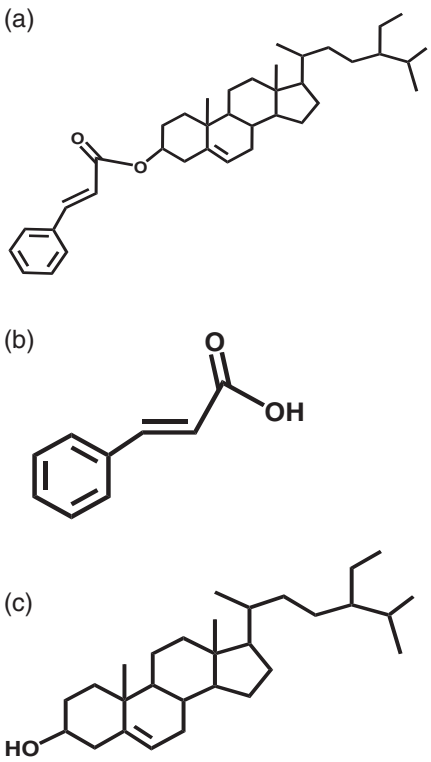

Figure 4. Structure of compound **2**: (A) 3-phenylacrylic acid 17-(4-ethyl-1,5-dimethyl-hexyl)-10,13-dimethyl- 2,3,4,7,8,9,10,11,12,13,14, 15,16,17-tetradecahydro-1*H*-cyclopenta[α]phenanthren-3-yl ester, (B) cinnamic acid and (C) β-sitosterol.

3.7 LC_{50} and LT_{50} of methyl cinnamate

The amount of methyl cinnamate isolated from the 1 g methanol extract of the *O. gratissimum* leaves was 9.43 mg. The effect of the five concentrations of isolated methyl cinnamate on adult and larval mortalities is shown in Table 11. Generally, as shown in the table, the mortalities of the adults and larvae of *T. castaneum* increased with increasing methyl cinnamate concentration. The median lethal concentration (LC_{50}) of methyl cinnamate was determined to be 26.92 mg mL^{-1} (95% CL:1.18.66–38.84 mg mL^{-1}; slope ± SE: 2.84 ± 0.81) for the adult and 8.31 mg mL^{-1} (95% CL:2.39–28.83 mg mL^{-1}; slope ± SE: 0.66 ± 0.28) for the larvae

Table 11. Effect of different amounts (mg mL^{-1}) of methyl cinnamate on the mortalities of adult and larvae of *T. castaneum*

Concentration (mg mL^{-1})	% Mortality†	
	Adults	Larvae
80.0	100.0 ± 0.0^{e}	83.3 ± 3.3^{e}
40.0	66.7 ± 3.3^{d}	56.7 ± 8.3^{d}
20.0	40.0 ± 5.0^{c}	53.3 ± 16.7^{c}
10.0	10.0 ± 0.0^{b}	53.3 ± 11.7^{c}
5.0	10.0 ± 0.0^{b}	50.0 ± 5.0^{b}
Methanol	0 ± 0^{a}	0 ± 0^{a}
Deltamethrin*	100.0 ± 0.0^{e}	100.0 ± 0.0^{e}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates after 168 h. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.

Table 12. LT_{50} of isolated methyl cinnamate against adult *T. castaneum* at various concentrations

Concentration (mg mL^{-1})	LT_{50} h (95% CL†)*	Slope ± SE
80.0	<18.33 (2.82–119.12)	
40.0	18.33 (2.82–119.12)	0.40 ± 0.15
20.0	590.07 (131.76–2642.54)	0.51 ± 0.33
10.0	4544.71 (1213.78–17016.59)	0.87 ± 0.29
5.0	>4544.71 (1213.78–17016.59)	

*LT_{50} units were applied for different concentrations at 27 ± 2 °C and 70% relative humidity.
†95% lower and upper confidence limits are shown in parenthesis ($n = 6 \times 10$).

Table 13. Effect of different amounts of sitosterol cinnamate on the mortalities of adult and larvae of *T. castaneum*

Concentration (mg mL^{-1})	% Mortality†	
	Adults	Larvae
23.45	75.0 ± 2.5^{f}	100.0 ± 0.0^{f}
11.73	70.0 ± 1.0^{e}	76.7 ± 8.9^{e}
5.86	40.0 ± 5.0^{d}	56.7 ± 11.1^{d}
2.93	36.7 ± 8.3^{c}	50.0 ± 13.3^{c}
1.47	10.0 ± 5.0^{b}	23.3 ± 11.1^{b}
Control	0 ± 0^{a}	0 ± 0^{a}
Deltamethrin*	100.0 ± 0.0^{e}	100.0 ± 0.0^{e}

*Deltamethrin was applied at the rate of 0.5 ppm.
†Each value is expressed as mean ± SE of six replicates after 168 h. Values with different small letters within the same row are significantly different ($P < 0.05$). Those with the same small letters have no significant difference based on Tukey's HSD test following ANOVA.

Table 14. LT_{50} of isolated sitosterol cinnamate against adult *T. castaneum* at various concentrations

Concentration (mg mL^{-1})	LT_{50} h (95% CL†)*	Slope ± SE
23.45	2.18 (0.30–15.65)	0.40 ± 0.44
11.73	12.60 (2.92–54.46)	0.51 ± 0.32
5.86	482.23 (134.68–1726.71)	0.60 ± 0.28
2.93	4173.28 (318.55–54 673.3)	0.30 ± 0.57
1.47	>4173.28	

*LT_{50} units were applied for different concentrations at 27 ± 2 °C and 70% relative humidity.
†95% lower and upper confidence limits are shown in parenthesis ($n = 6 \times 10$).

(Table 12). The time needed for methyl cinnamate to cause 50% mortality (LT_{50}) in adult *T. castaneum* ranged from 18.33 h (95% CL: 2.82–119.12) for the highest concentration of 40.0 mg mL^{-1} to >4544.71 h for 5.0 mg mL^{-1} of methyl cinnamate (Table 12). Generally, LT_{50} values decreased with increasing methyl cinnamate concentration and thus the susceptibility of adult *T. castaneum* can be directly associated with methyl cinnamate concentration as well as time of exposure.

3.8 LC_{50} and LT_{50} of isolated sitosterol cinnamate

The amount of sitosterol cinnamate isolated from the 1 g methanol extract of *V. paradoxa* leaves was 6.65 mg. The effect of the five different concentrations of the isolated sitosterol cinnamate on adult and larval mortalities is shown in Table 13. Generally, as shown, the mortalities of adult and larvae increased with increasing sitosterol cinnamate concentration. The median lethal concentration (LC_{50}) of sitosterol cinnamate was determined to be 6.92 mg mL^{-1} (95% CL: 3.97–12.06 mg mL^{-1}; slope ± SE: 1.59 ± 0.12) against the adult and 3.91 mg mL^{-1} (95% CL: 2.21–6.93 mg mL^{-1}; slope ± SE: 1.52 ± 0.13) against the larvae. The time needed for the compound to cause 50% mortality (LT_{50}) in adult *T. castaneum* ranged from 2.18 h (95% CL: 0.30–15.65) for the highest concentration of 23.45 mg mL^{-1} to >4175.28 h for 1.47 mg mL^{-1} of sitosterol cinnamate (Table 14). Generally, LT_{50} values decreased with increasing sitosterol cinnamate concentration and thus the susceptibility of adult *T. castaneum* is also directly associated with sitosterol cinnamate concentration as well as time of exposure.

4 DISCUSSION

The insecticidal activities of the crude extracts of plants has been widely studied. The ethanoic extract of *O. gratissimum* leaves exhibited a relatively higher insecticidal activity against adult mean mortality count of adult *Periplaneta americana* (L.) than its raw powdered form.[31] The chloroform extracts of *O. gratissimum* leaves were found to be larvicidal, pupicidal and adulticidal against *Culex quinquefasciatus* Say.[32] *Ocimum gratissimum* exhibited 100% mortality at 300 mg L^{-1} concentration within 24 h exposure against larvae of *Aedes aegypti* L.[33,34] Its constituents showed a strong dose and exposure time dependent repellence against some coleopteran pests, including *T. castaneum*.[35] The crude extract of *Vitellaria paradoxa* has been found to be active against bacteria, fungi and other disease pathogens.[36,37] Important essential oils have been isolated from the leaves of *V. paradoxa* and have been found to be responsible for the observed bioactivities. However, this work reports the first time a bioactive compound against *T. castaneum* has been isolated from its crude extracts.

Cinnamic acid esters and their derivatives are widely distributed in plants including cereals, legumes, oilseeds, fruits, vegetables, tea and coffee.[38] Cinnamic acid itself and its derivative cinnamaldehyde have been found to show several bioactivities.[39–41] Due to their common occurrence in plants and low toxicity for humans, animals and the environment,[42] they have been proved to possess diverse pharmacological activities and bioactivities such as antimicrobial,[43] anti-bacterial, anti-cancer,[44]

anti-inflammatory[45] etc. However, not much work on the insecticidal activities of the cinnamic acid esters has been reported.

In this study, methyl cinnamate (compound **1**) and sitosterol cinnamate (compound **2**) are the two cinnamic acid derivatives isolated from *O. gratissimum* and *V. paradoxa*, respectively. These have been found to show insecticidal and larvicidal and larval development inhibition activities against *T. castaneum* with the response almost similar to the insecticidal activities of deltamethrin, which was used as the positive control in this study.

Methyl cinnamate is the methyl ester of cinnamic acid and is a white or transparent solid with a strong aromatic odor. It is found naturally in a variety of plants, including fruits like strawberry and some culinary spices such as Sichuan pepper and some species of *Ocimum*.[46] Methyl cinnamate showed significant larvicidal activity against the third stage larvae of *Aedes aegypti* L. than linalool also isolated from *Ocimum* species, with LC_{50} values of 138.0 and 275.2 $\mu g\,mL^{-1}$, respectively. The repellent, insecticidal and larvicidal activities of methyl cinnamate and its derivatives were also reported in previous studies.[47–49]

Sitosterol cinnamate is an ester of cinnamic acid and β-sitosterol. In most plants sitosterol occurs as a sterol ester by the esterification of the hydroxyl group at carbon 3 with fatty/organic acids or carbohydrates.[50] The component compounds of the isolated ester (compound **2**) have separately been isolated from plants to show various levels of bioactivities. As a defense mechanism, most of the sterols produced by plants have anti-insect activities. β-sitosterol was isolated and identified as the main antifungal and antibacterial active compound from methanol extracts of *Senecio lyratus*.[51] It was also found to play a synergistic role in the deterrent activity of *Allophylus edulis* (A.St.-Hil).[52]

The activities of cinnamic acid esters are greatly influenced by varying both the phenyl and alkyl substituents that adjoin the phenol group.[53] With the –OH group replaced by a methoxy ($-OCH_3$) group in the case of compound **1** and β-sitosterol in the case of compound **2**, the insecticidal and larvicidal activities of the two compounds were enhanced as shown in this study. In the case of compound **2**, the structure–activity relationship was not considered extensively in this work to establish whether or not the two components act synergistically. Therefore, it is not clear which portion of compound **2** could be responsible for the activities observed. However, the two components are known to show various levels of bioactivities.[43,52] Therefore, since the isolated compound was found to be as an ester of the two components (sitosterol and cinnamic acid) in *V. paradoxa*, it can be established that the reported bioactivity is as a result of the cinnamic acid–β-sitosterol interaction. This is the first time the compound has been isolated in this form to be responsible for insecticidal activities against a stored product beetle.

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.[54] When the insecticide enters the body of the insect, the active ingredient then disperses throughout the body to reach the target sites. In the bioassay method used in this study, all the isolated compounds can be considered to have been absorbed through the cuticle of the insects studied. Essential oils affect the larval stage of an insect by the physical flooding of the tracheal system, by chemical toxicity, by interference with surface forces or by combined actions.[55] The larvae, in an attempt to eliminate the toxic substances, partially or totally eliminates its gut content as the first defense mechanism.[56–58] Thus, the compounds isolated in this study for showing larvicidal activities may have acted in any of the mechanisms described above, thereby preventing the larvae from developing to the next stage and consequently preventing the emergence adult without deformities. It is recommended that a further investigation is carried out to clearly determine the mode of action of cinnamic acid esters as insecticides.

5 CONCLUSION

This study has shown that the two cinnamic acid esters methyl cinnamate and sitosterol cinnamate, isolated from *O. gratissimum* and *V. paradoxa*, respectively, are responsible for the insecticidal, larvicidal and larval growth inhibition activities of these plants. Given the rapid volatilization and low persistence of natural products in the environment, it is unlikely that they will be used in field crops, but this property is conducive to using them to control stored product pests in controlled conditions. Several studies have confirmed the safety of these cinnamic acid esters by evaluating acute toxicity, skin irritation and genotoxicity, and therefore they can be used safely for stored product protection. However, it is important that care is taken during application and use of these compounds or the powdered form of the plants for stored product protection.

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