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Isolation and characterization of biofumigant from leaves of *Lantana camara* for control of stored grain insect pests

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ABSTRACT

Due to environmental concerns, health hazards to man and the evolution of resistance in insect pests, there have been constant efforts to discover newer insecticides both from natural sources and by chemical synthesis. Natural sources for novel molecules hold promise in view of their eco-friendly nature, selectivity and mammalian safety. We have isolated one natural bioactive molecule from the leaves of *Lantana camara* named Coumaran, based on various physical–chemical and spectroscopic techniques (IR, ^{1}H NMR, ^{13}C NMR and MS). Coumaran is highly toxic and very low concentration is needed for control of stored product insects. This molecule has potent grain protectant potential and caused significant reduction in F1 progeny of all the three species in the treated grain and the progeny was completely suppressed at 30 μg/l. The differences in germination between the control and treated grains were not significant. The lack of any adverse effect of Coumaran on the seed germination is highly desirable for a grain protectant, becoming a potential source of biofumigant for economical and environmentally friendly pest control strategies against stored grain pests during storage of grains or pulses.

1. Introduction

Insect infestation control of stored grain is primarily achieved by the use of synthetic gaseous insecticides such as methyl bromide and phosphine (Leelaja et al., 2007; Liu et al., 2010; Zettler and Arthur, 2000). In several countries including India, mixing of any synthetic insecticide with stored grain is not permitted (Rajashekar et al., 2012a). Due to environmental concerns and human health hazards, many insecticides have been replaced by modern insecticides (Subramanyam and Hagstrum, 1995). Further, due to the problem of resistance to insecticides, there is an urgent need for safer alternatives to conventional chemical insecticides particularly from natural sources, for the protection of grain against insect infestation. In view of all the aspects for control of stored grain insects, and these problems have highlighted the urgent need to develop newer eco-friendly safer and effective stored-product insecticide (Alexander, 1980). Essential oils and their individual constituents have been used as potential fumigants against stored grain insect pests (Rajashekar et al., 2012a; Rajendran and Sriranjini, 2008; Shaaya and Kostyukovsky, 2011).

Biofumigants have the advantage of providing novel modes of action against insects that can reduce the risk of cross-resistance as well as offering new leads for design of target-specific molecules (Liu et al., 2010; Rajashekar et al., 2012b). At present, there is no botanical insecticide to replace phosphine and methyl bromide for protection of stored grain from insect infestation. Biofumigants of plant origin often show selectivity to insect species, easily biodegradable, and high chance of acceptability and, therefore, are considered to be the best source of newer chemicals for development of new, ecofriendly, and safer insect control agents (Copping and Duke, 2007; Dayan et al., 2009; Isman, 2006; Khater, 2012; Ogendo et al., 2003; Yao et al., 2008).

Lantana camara (Verbenaceae), an erect shrub, grows widely throughout the tropical, sub tropical and temperate parts of World. Earlier work has shown that leaves of *L. camara* are source of insecticidal activity (Dua et al., 2010; Rajashekar et al., 2012c) and preliminary studies indicated that the leaves of *L. camara* possessed rich source of bioactive molecules (Khan et al., 2002). The leaves of *L. camara* seem to be promising choice as a source of a new biopesticide. In the present paper we report the isolation and characterization of biofumigant molecule from leaves of *L. camara* against the storage pests *viz.* rice weevil, *Sitophilus oryzae* L. (Coleoptera: Curculionidae), rust red flour beetle, *Tribolium castaneum* Herbst. (Coleoptera: Tenebrionidae) and adzuki bean weevil,

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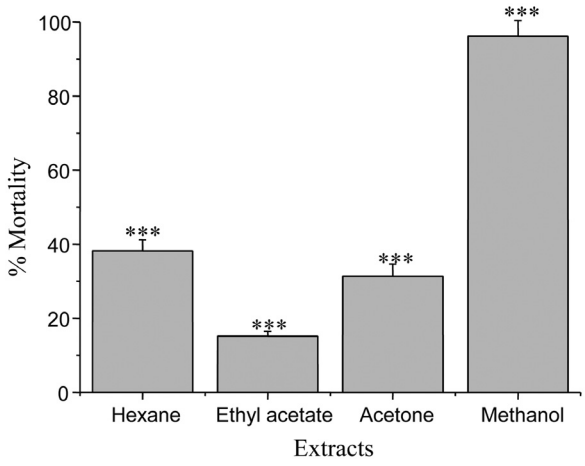

Fig. 1. Insecticidal activity of the solvent extracts of *L. camara* to *Sitophilus oryzae* in the fumigant bioassay. The extracts were applied at 500 μl/l ($n = 4$, error bars, s.e.m.) one-way ANOVA, $^{***}P < 0.001$.

Callosobruchus chinensis Fab. (Coleoptera: Bruchidae). The results of the present study would be useful in promoting research aiming at the development of new biofumigant for pest control.

2. Materials and methods

2.1. Insect culture

Cultures of *S. oryzae* were maintained on whole wheat (*Triticum aestivum*) whereas, *T. castaneum* were reared on the whole wheat flour with 2% yeast powder. *C. chinensis* was reared on whole green gram (*Vigna radiata*). Adults of *S. oryzae* (3–5 d) and other species (2–3 d) were used in the experiments. Experiments were carried out in the laboratory and insect cultures were maintained at 27 ± 2 °C and 70 ± 5% r.h. (Leelaja et al., 2007; Senthilkumar et al., 2012).

2.2. Isolation

The healthy mature leaves of *L. camara* were collected from Bangalore and its surrounding region, India. The leaves were cut into small pieces, dried at 40 °C and powdered. One hundred gram of leaf powder was sequentially extracted with a series of solvents of increasing polarity *viz*., hexane, ethyl acetate, acetone and methanol in a Soxhlet apparatus. The solvent was evaporated *in vacuo* and the residue was dissolved in a known volume of methanol. This solution was screened for insecticidal activity through for fumigant toxicity (Fig. 1).

The methanolic extract (0.7 g) was subjected to column chromatography using a glass column (length; 50 cm, diameter: 3 cm) packed with silica gel (60–120 mesh) and eluted with hexane followed by a stepwise gradient of ethyl acetate and methanol (100:0, 75:25, 50:50, 25:75, 0:100 in this ratio). Ten fractions of 300 ml each were collected, concentrated under reduced pressure, and assayed for insecticide activity. Fractions showing insecticide activity were pooled into active fractions.

The active fraction was further fractionated on a silica gel column (length; 50 cm, diameter: 3 cm) eluted with a stepwise gradient of chloroform, ethyl acetate and methanol. The subfractions were further subjected to fractionation on a silica gel column as mentioned above (Supplementary Scheme 1). The purity of the bioactive compound was checked by RP-HPLC (Rajashekar et al., 2012b).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2013.09.006.

2.3. Fourier transformer infrared (FTIR) spectrometry

The FTIR spectrum was recorded using a Nicolet 5700 (Thermo electron, Madison, WI, USA) spectrometer at room temperature. The bioactive compound was dissolved in chloroform and scanned in the range of 4000–400 cm^{-1}.

2.4. ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX 500 NMR instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C at room temperature. A region from 0 to 12 ppm for ^{1}H and 0–200 ppm for ^{13}C was employed. Signals were referred to the internal standard tetramethylsilane. About 10 mg of the sample dissolved in 0.5 ml of $CDCl_3$ were used for recording the spectra.

2.5. Gas chromatography–mass spectrometry

The purified compound of the *L. camara* leaves was analyzed on a gas chromatograph (HP6890; Agilent Technologies UK Ltd., South Queensferry, West Lothian EH30 9TG) directly linked to a HP5973 mass selective detector (Agilent Technologies) operated in electron impact mode (source temperature 230 °C; transfer line 250 °C). The column used was HP-5MS, 5% phenylmethylsiloxane capillary column (30 m × 250 μm i.d. × 0.25 μm), 350 °C max (Agilent 19091S-433) and the carrier gas was helium of 99.999% purity (1 ml min^{-1}). The column temperature was held at 70 °C for 2 min, raised to 240 °C at a ramp of 4 °C/min, held for 10 min; raised to 270 °C at a ramp of 6 °C/min, held for 12 min, again raised to 290 °C at ramp of 8 °C per min held for 14 min, and finally raised to 310 °C at ramp of 10 °C per min and held for16 min. Mass spectra were compared with those in the Wiley 275 L mass spectral library (Rajashekar et al., 2012c).

2.6. Insecticidal activity

The purified compound (Coumaran) was further evaluated as grain protectants on wheat and green gram. From the above cultures, adults (1–2 weeks old) were taken for preparation of mixed-age cultures. About 300 adults of test insects (*T. castaneum*, *S. oryzae* and *C. chinensis*) were allowed to breed in a 2 l glass jar with 1 kg of respective culture medium. The insect culture flask (jar) were kept under the rearing temperature conditions i.e. 25 ± 1 °C for other insects. After one week, the adults were shifted from the cultures jars. A series of cultures of the respective species were thus maintained continuously. Insect cultures in six successive weeks (containing 5–6, 4–5, 3–4, 2–3, 1–2, and 0–1 week old insects) were pooled such that the pooled populations contained all developmental stages of the particular species. Rearing media containing mixed-age cultures of individual species were weighed separately in 50 g aliquots into cloth bags (20 cm × 14 cm size) Cloth bags containing mixed-age cultures of individual species were placed individually in 850 ml screw cap glass bottles that served as the fumigation chambers. The impregnated filter paper was then attached to the under surface of the screw cap of a glass bottle (850 ml) (Hansen et al., 2012; Obeng-Ofori et al., 1998; Rajashekar et al., 2012c)

2.7. Fumigation

The mixed age insect cultures were exposed to a range of doses of purified compound (Coumaran) (5–30 μl/l) for 24 h and 7 d at

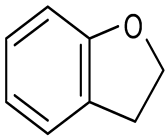

Fig. 2. Molecular structure of Coumaran (2, 3, dihydrobenzofuran).

27 ± 2° C. For each species, there were 4 replications per dose with an equal number of untreated control replications. At the end of exposure, the test insect bags were taken out of the desiccators (glass bottles). The contents of the bags were transferred to individual bottles (12 cm × 5 cm size) and kept at the rearing temperature and humidity conditions for 8 weeks. The insects, which emerged from wheat (*S. oryzae*) and green gram (*C. chinensis*) or survived as adults (*T. castaneum*) in their respective media were checked at weekly intervals for 8 weeks. Similarly, counts were made in untreated control batches every week. Percent kill was determined by taking the survival/emergence in total controls as 100% (Athanassiou et al., 2009; Rajashekar et al., 2012c).

After counting mortality at 7 d of the treated grain the insects (dead and live) were removed and the grain kept under the same experimental conditions (for 28 d) until the emergence of F1 progeny. Based on the life cycle of the insect species, the counting period of F1 progeny was monitored so as to avoid an overlap of generations for each species. At weekly intervals, the F1 progeny produced were recorded for 8 consecutive weeks. Percentage reduction in adult emergence of F1 progeny or inhibition rate (%IR) was calculated as

$$\mathrm{IR}(\%) = \frac{(C_n - T_n) \times 100}{C_n}$$

where C_n is the number of newly emerged insects in the untreated jar and T_n is the number of insects in the treated jar (Rajashekar et al., 2010; Tapondjou et al., 2002).

2.8. Seed germination

Wheat and green gram seeds were treated with Coumaran at 10 and 30 μg/l and germination tests were done at 24 h and 7 d of exposure treatment. Fifty seeds from each treatment were randomly selected from each group and soaked in distilled water for about 30 min, and kept on filter paper (Whatman No. 1) in a petri dish, moistened daily with distilled water and allowed to germinate at room temperature (25 ± 2 °C). After 5 d, germinated seeds were counted and percentage germination was calculated (Nikpay, 2007).

2.9. Statistical analysis

LC_{50} were determined by Probit analysis (Finney, 1971). The data were analyzed using one-way ANOVA ($P < 0.05$) by Duncans Multiple range test using Statplus 2007 software and Statistical software, 1999.

3. Results and discussion

Using a bioassay-driven procedure, we have isolated bioactive molecule from the leaves of *L. camara* (Fig. 2), which were toxic to a wide range of insects (Table 1), and named as Coumaran, water soluble. The isolated compounds were characterized by various physico-chemical and spectroscopic techniques like IR, ^{1}H NMR, ^{13}C NMR, MS, and elemental analysis. IR spectrum of isolated compound reveals the presence of aromatic stretching at 2857–2893 cm^{-1}. The ^{1}H NMR studies suggest that the aromatic protons were resonated at 6.90–7.30 cm^{-1} as multiplets and

Table 1
Insecticidal activity of Coumaran against adults of stored-product insects by fumigant toxicity.

Insects	LC_{25}	LC_{50} (μl/l)[a,b]	LC_{90}	Regression equation R^2
S. oryzae	0.225	**0.45** (0.39–0.50)	0.81	0.9962
C. chinensis	0.19	**0.38** (0.33–0.45)	0.69	0.8991
T. castaneum	0.27	**0.54** (0.47–0.63)	0.98	0.8892

Significant bold values indicated for the LC50 values are highlighting.
[a] LC_{50} and LC_{90} = μg/l.
[b] Values in parenthesis represent confidence limits by probit analysis (Finney, 1971), $n = 4$.

2-CH_2 benzofuron protons were signalled as triplets at 4.61–4.65 and 2.35–2.30 cm^{-1}. ^{13}C NMR studies revealed that all the respective aromatic carbons appeared at C4 (127.93), C5 (120.33), C6 (126.91), C7 (did not appear), C8 (127.3) and the benzofuron 2-CH_2 carbons were signalled at C2 (70.99), C3 (29.74). Thus, from the spectral studies the isolated bio-active molecule was confirmed as Coumaran (Fig. 2) (detailed characterization is given below).

2,3-Dihydrobenzofuran: colourless liquid; yield 0.2%; b.p 70; IR (neat) ν_{max} (cm^{-1}): 2857–2893 (Ar-H); ^{1}H NMR (400 MHz,$CDCl_3$) δ (ppm): 6.90–7.30 (m, 4H, Ar-H), 4.61–4.65 (t, 2H, O-CH_2), 2.35–2.30 (t, 2H, C-CH_2, $J = 8.75$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm C2 (70.99), C3 (29.74), C4 (127.93), C5 (120.33), C6 (126.91), C7 (did not appear), C8 (127.3); mass (m/z%): M^+ 120.2 Anal. Calcd.; C_8H_8O: C, 79.97; H, 6.71; O, 13.32%; found: C, 79.95; H, 6.74; O, 13.31%.

The organic extracts and its bioactive molecules from root powder of *Decalepis hamiltonii* showed potential to be used as grain protectant against grain insect pests (Rajashekar et al., 2010, 2012b). Azadirachtin from the well-known neem (*Azadirachta indica*) tree, is an antifeedant and insect growth regulator, but not commercially successful due to lack of fumigant toxicity, however finds use mainly in integrated pest management of field crop pests (Morgan, 2009; Schmutterer, 1990). Rotenone, from the Derris root, one of the earlier plant-derived insecticides, was not acceptable because of its high mammalian toxicity (Isman, 2006; Yi et al., 2012). Currently, widely used and successful synthetic pyrethroids were originally derived from the flowers of *Chrysanthamum cinerariaefolium* (Breese, 2006; Casida, 1980). Despite the large number of plants that show insecticidal activity, and the diversity of natural chemistry with inherent eco-friendly structures, newer classes of natural insecticides have eluded discovery (Lahm et al., 2009; Rajashekar et al., 2012b).

Volatile compounds of many plant extracts composed of many bioactive molecules, which exhibit fumigant/contact activity. The extracts of *L. camara* were used to protect grain against almond moth (Gotyal et al., 2010). Recently, in our laboratory, it was shown that methanol extract of *L. camara* can be used for the management of stored grain insect pests (Rajashekar et al., 2012c). Our preliminary screening showed that the methanol extract was most fumigant toxic against *S. oryzae*, followed by ethyl acetate, hexane and acetone (Fig. 1). There were no significant differences in the

Table 2
Comparison of insecticidal activity of the compound from *L. camara* with the chemical fumigants.

Insecticides (fumigants)	LC_{50} (μg/l)		
	S. oryzae	*T. castaneum*	*C. chinensis*
Coumaran	0.45 (0.39–0.52)	0.54 (0.48–0.61)	0.38 (0.33–0.44)
Allyl acetate	0.92 (0.85–0.98)	1.43 (1.33–1.52)	0.81 (0.72–0.90)
Ethyl formate	1.07 (0.92–1.19)	1.08 (0.91–1.21)	0.72 (0.63–0.81)
Carbonyl sulfide	1.20 (1.09–1.32)	0.98 (0.87–1.08)	0.78 (0.67–0.89)

[a] Values in parenthesis represent confidence limits by probit analysis (Finney, 1971), $n = 4$.

Table 3
Mortality (%) of mixed-age cultures of stored-product insects exposed for 24 h and 7 d to purified compound (Coumaran) of *L. camara*.

Dosage μg/l	% Mortality (mean ± SE)					
	S. oryzae		*T. casatneum*		*C. chinensis*	
	24 h	7 d	24 h	7 d	24 h	7 d
5	10.5 ± 0.7[a]	15.8 ± 1.6[a]	12.0 ± 1.1[a]	20.3 ± 1.6[a]	5.6 ± 1.5[a]	22.3 ± 1.8[a]
10	25.6 ± 1.4[b]	30.6 ± 1.5[b]	29.3 ± 1.4[b]	38.85 ± 1.0[b]	36.47 ± 1[b]	44.6 ± 0.9[b]
15	42.5 ± 1.7[c]	48.1 ± 1.7[c]	50.8 ± 1.4[c]	62.9 ± 0.87[c]	66.2 ± 0.9[c]	73.5 ± 1.6[c]
20	61.9 ± 1.7[d]	66.3 ± 1.9[d]	71.4 ± 1.7[d]	79.5 ± 1.06[d]	80.0 ± 1.5[d]	86.5 ± 0.9[d]
25	81.9 ± 1.5[e]	87.2 ± 2.3[e]	89.5 ± 1.0[e]	94.3 ± 1.6[e]	94.1 ± 0.9[e]	100[e]
30	94.6 ± 1.01[f]	100[f]	98.5 ± 2.0[f]	100[f]	100[f]	

Values followed by different letters within the vertical columns are significantly different ($P < 0.05$) by Duncan's multiple range test ($n = 4$).

purified compound from leaves of *L. camara* of different geographical zones of India. Fumigant toxicity of the purified compound (Coumaran) from leaves of *L. camara* to adults of *S. oryzae*, *C. chinensis* and *T. castaneum* is shown in Table 1. Based on LC_{50} values and their respective significance level e.g. $P > 95\%$, the Coumaran was found to be significantly less effective to *T. castaneum* compared to *S. oryzae* and *C. chinensis* adults and this molecule showed potent fumigant toxicity. The LC_{50} value for all the three species was variable and species-specific. Antifeedant activity of Coumaran isolated from the *Cyperus* spp. was demonstrated against *Spodoptera litura* Fab. in laboratory assays (Morimoto et al., 1999a). Similarly, an 2, 3 dihydrobenzofuran derivative, 7, acetyl-4, 6-dimethoxy-2-isopropneyl-2,3-dihydrobenzofuran was most active antifeedant against *S. litura* (Morimoto et al., 1999b). The results clearly demonstrated that the molecule showed potent fumigant activity against stored grain insect pest and results indicated that the insecticidal mode of action of the compounds may be largely attributed to fumigant action. The fumigant toxicity of purified compound (Coumaran) of *L. camara* was more potent than the chemical fumigants for all the three species and, it was significantly different (Table 2).

Further, the Coumaran was highly toxic to *S. oryzae*, *T. castaneum* and *C. chinensis*, recorded 70–88% mortality at dose of 20–25 μg/l in 24 h exposure, whereas 90–100% mortality was recorded at dose of 30 mg/l in 7 d exposure (Table 3), respectively. *T. castaneum* was, however, tolerant to the fumigant such that 93% mortality of the insects was achieved only at the higher dose of 25 μg/l in 7 d exposures. Generally, an extended exposure period of 7 d increased mortality in all three species (Table 3). Results of grain protection showed that Coumaran caused significant reduction in F1 progeny of all the three species in the treated grain and the progeny was completely suppressed at 30 μg/l (Table 4) In present study, for 84–88% population extinction of *T. castaneum* and *S. oryzae* the target concentration time (CT) product was high 25 μg/l in 7 d exposures. Coumaran was also recognized as one of the potential fumigant against the model insect, *Drosophila melanogaster* (Scharf et al., 2006). This may be due to the exceptionally high tolerance of the eggs of *T. castaneum* and *S. oryzae*. There is

Table 4
Grain protection potential of Coumaran: adult emergence in F1 progeny of stored product insects from treated grain.

Dosage (μg/l)	% Reduction in F1 progeny		
	S. oryzae	*T. castaneum*	*C. chinensis*
5	12.9 ± 1.4[a]	10.2 ± 4.04[a]	15.9 ± 1.7[a]
10	24.1 ± 1.4[b]	38.12 ± 1.9[b]	27.4 ± 1.7[b]
15	53.3 ± 1.5[c]	67.6 ± 1.4[c]	59.4 ± 0.6[c]
20	77.3 ± 0.21[d]	75.3 ± 0.6[d]	84.3 ± 1.4[d]
25	85.05 ± 0.4[e]	88.5 ± 0.4[e]	100[e]
30	100[f]	100[f]	

Values followed by different letters within the vertical columns are significantly different ($P < 0.05$) by Duncan's multiple range test ($n = 4$).

Table 5
Effect of Coumaran on seed germination.

Dosage (μg/l)	% of germination			
	48 h exposure		7 d exposure	
	Wheat	Green gram	Wheat	Green gram
5	95.86 ± 1.4	97.02 ± 1.7	94.12 ± 2.2	96.01 ± 0.6
30	95.04 ± 1.2	96.48 ± 1.2	93.6 ± 0.8	95.8 ± 2.4
Control	96.4 ± 1.2	97.8 ± 0.8	94.8 ± 1.6	96.4 ± 1.4

Values followed by different letters within the vertical columns are not significantly different ($P < 0.05$) by Duncan's multiple range test ($n = 4$).

no previous report about the toxicity of Coumaran from leaves of *L. camara* to stored grain insect pests. Our results clearly demonstrated that *C. chinensis* was relatively susceptible probably due to less tolerance of their eggs and comparable to mortality achieved with essential oils of *Artemisia mongolica* and *Artemisia capillaris* which also possess strong fumigant activity against *S. zeamais* adults with LC_{50} values of 7.35 and 5.31 mg/l, respectively (Liu et al., 2010). Liu and Ho (1999) reported that currently used grain fumigant methyl bromide and phosphine have fumigant toxicity (24 h) against *S. zeamais* adults with LC_{50} values 0.67 and 0.006 mg/l, respectively. Whereas the Coumaran was 10 times more toxic to the rice weevil compared with the commercial fumigant, methyl bromide. However, considering the currently used fumigants are synthetic insecticides, fumigant activity of the Coumaran is quite promising and showing the potential to be developed as possible natural fumigants for the control of stored product insects.

Further, our study showed that percentage germination of green gram and wheat seeds in 5 and 30 μg/l treatments at different exposure period ranged from 93.6 to 95% and 96.4 to 97.8% respectively, compared to respective controls of 94–97% in the control groups (Table 5). The differences in germination between the control and treated grains were not significant. The lack of any adverse effect of Coumaran on the seed germination is highly desirable for a grain protectant, which act by killing various life stages of stored grain insect pests and total suppression of emergence of progeny in treated grain.

The finding of the present study indicated that the biofumigant molecule Coumaran, isolated from leaves of *L. camara* is toxic to *S. oryzae*, *C. chinensis* and *T. castaneum*, and the lack of any adverse effect on the seed germination, showing promise of Coumaran becoming a potential source of biofumigant for economical and environmentally friendly pest control strategies against stored grain pests during storage of grains or pulses.

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