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Decaleside: a new class of natural insecticide targeting tarsal gustatory sites

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Abstract Natural sources for novel insecticide molecules hold promise in view of their eco-friendly nature, selectivity, and mammalian safety. Recent progress in understanding the biology of insect olfaction and taste offers new strategies for developing selective pest control agents. We have isolated two natural insecticidal molecules from edible roots of *Decalepis hamiltonii* named *Decalesides I* and *II*, which are novel trisaccharides, highly toxic to household insect pests and stored-product insects. We have experimentally shown that insecticidal activity requires contact with tarsi on the legs but is not toxic orally. The insecticidal activity of molecules is lost by hydrolysis, and various sugars modify toxic response, showing that the insecticidal activity is via gustatory sites on the tarsi. Selective toxicity to insects by virtue of their gustatory site of action and the mammalian safety of the new insecticides is inherent in their chemical structure with 1-4 or 1-1 α linkage that is easily hydrolyzed by digestive enzymes of mammals. *Decalesides* represent a new chemical class of natural insecticides with a unique mode of action targeting tarsal chemosensory/gustatory system of insects.

Keywords Natural insecticides · *Decalesides I* and *II* · Trisaccharides · Tarsi · Gustatory receptors · Digestive enzymes

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Introduction

In view of environmental concerns and human health hazards, many insecticides have been replaced by modern insecticides derived from natural molecules (Casida and Quistad 1998; Isman 2006; Nauen 2006; Copping and Duke 2007; Ishaaya et al. 2007; Dayan et al. 2009). Some of the earlier natural insecticides, such as, rotenone from the Derris root, one of the earlier plant-derived insecticides, were not acceptable because of their mammalian toxicity (Isman 2006). Azadirachtin from the well-known neem (*Azadirachta indica*) tree is an antifeedant and insect growth regulator but lacks contact toxicity, finds use mainly in integrated pest management (Ruscoe 1972; Schmutterer 1990; Morgan 2009). The widely used and successful synthetic pyrethroids were originally derived from flowers of *Tanacetum cinerariaefolium* (Casida et al. 1975; Casida 1980). Recently, diamide insecticides, originally derived from a natural molecule, have been introduced as a promising new class of insecticides targeting ryanodine receptor with high selectivity and mammalian safety (Nauen 2006; Lahm et al. 2009). Compounds with a new mode of action are needed to deal with the problem of resistance and selectivity (Nauen 2006; Copping and Duke 2007; Ishaaya et al. 2007; McCheeney et al. 2007). Recent progress in understanding the biology of insect olfaction and taste offers new strategies for developing selective pest control agents (der Goes et al. 2006; Fischler et al. 2007). Despite the large number of plants that show insecticidal activity, and

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the diversity of natural chemistry with inherent eco-friendly structures, newer classes of natural insecticides have eluded discovery (Isman 2006; Nauen 2006).

The tuberous roots of *Decalepis hamiltonii* (family, Asclepiadaceae), a woody climber found in the forests of peninsular India, are traditionally consumed as pickles and as a health drink for their alleged health benefits. Several bioactive molecules with health-promoting potential have been isolated from its roots in our laboratory (Harish et al. 2005; Srivastava et al. 2006; Srivastava et al. 2007). An initial clue for the roots as a potential source of bioinsecticides was from the observation that the tuberous roots are not infested by insects during storage and the powder had biopesticidal property (George et al. 1999; Rajashekar et al. 2010). In this paper, we report the discovery of a new class of natural insecticides based on the structure as well as their mode of action targeting the chemosensory/gustatory system.

Materials and methods

Insect culture

The stored-product insects, lesser grain borer (*Rhyzopertha dominica*) and rice weevil (*Sitophilus oryzae* L.), were reared on whole wheat, and the rust-red flour beetle (*Tribolium castaneum* Herbst.) on wheat flour with 5 % yeast; the pulse beetle (*Callosobruchus chinensis*) was reared on whole green gram as described elsewhere (Rajendran and Muralidharan 2006). The insect cultures are derived from the original collection since 1960 and maintained in the insect culture room under standard conditions (25±2 °C and 70 % relative humidity). Housefly (*Musca domestica*) larvae were reared in a mixture of sterilized bran, milk powder, and water, and the adults were allowed free access to water and thick paste of condensed milk and milk powder (Pavela 2008). The German cockroach (*Blatella germanica*) was reared in plastic tubes with harborages, containing broken wheat, biscuits, and water provided ad libitum (Favlde et al. 2006). The cockroaches and housefly were maintained at 23.6±2.5 °C, 70 % relative humidity, and a photoperiod of 12:12 (light:dark).

Isolation

Tuberous roots of *D. hamiltonii* (10 kg), procured from the local supplier, were washed, crushed with a roller, and the outer fleshy layer was separated from the inner woody core. The fleshy portions were cut into smaller pieces, dried at 40 °C, and finely powdered using a blender.

The root powder (100 g) was sequentially extracted with a series of solvents of increasing polarity, viz., hexane, ethyl acetate, acetone, and methanol, in a Soxhlet apparatus. The extract was concentrated in a flash evaporator (Heidolph, Laborota 4000) and the residue dissolved in a known volume of methanol (Harish et al. 2005). The aqueous extract was prepared by homogenizing the root powder (100 g) in warm water (50 °C), allowed to stand for 24 h, filtered through Whatman No. 1 paper, and the filtrate lyophilized (Srivastava et al. 2006).

The lyophilized aqueous extract (16.08 g) was re-extracted thrice with methanol in a total volume of 300 ml and concentrated under reduced pressure. The residue was subjected to fractionation by silica gel (60–120 mesh) column chromatography using a glass column (length 50 cm, diameter 3 cm) and eluted with chloroform followed by stepwise gradient of chloroform, ethyl acetate, and methanol. Ten fractions of 300 ml each were collected, concentrated, and assayed for insecticidal activity by contact bioassay.

The fractions showing insecticidal activity were pooled and further fractionated on a silica gel column (length 50 cm, diameter 3 cm) and eluted with a stepwise gradient of ethyl acetate, acetone, and methanol. The active subfraction was further fractionated on a LH-20 column (length 50 cm, diameter 1 cm) using methanol as the eluant (Fig. 1) (Srivastava et al. 2007). The purity of the isolated compound was checked by reverse phase high-performance liquid chromatography (RP-HPLC) on a C_{18} column with

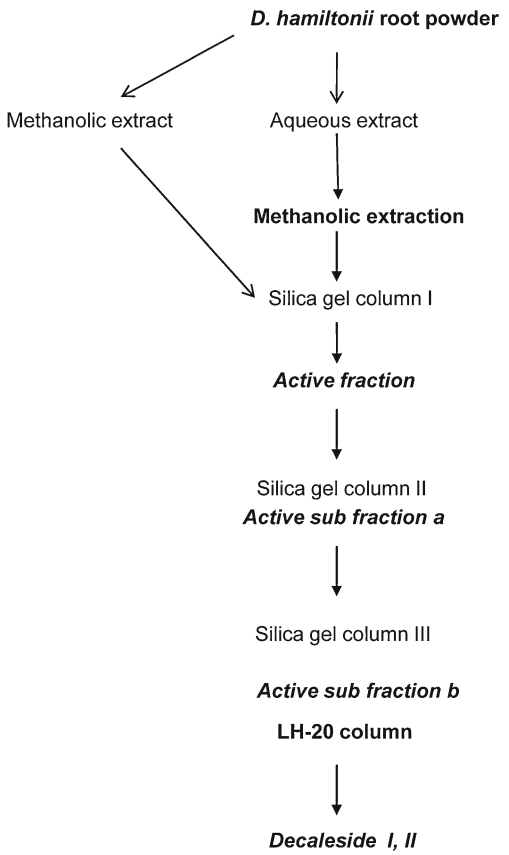


Fig. 1 Scheme for the isolation of the *Decalesides I* and *II* from the methanolic and aqueous extracts of the roots of *D. hamiltonii*

methanol:water with 0.1 % trifluoroacetic acid (TFA) (1:1) as the mobile phase. The purified compound (purity > 99 %) from the aqueous extract was designated *Decaleside I* (Supplementary Fig. 2a, b).

The methanolic extract (15.7 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 chloroform followed by a stepwise gradient of ethyl acetate and methanol. Ten fractions of 300 ml each were collected, concentrated under reduced pressure, and assayed for insecticidal activity. Fractions showing the activity were pooled and subjected to the second round of chromatography on a silica gel column (length 50 cm, diameter 3 cm) and eluted with a stepwise gradient of chloroform, ethyl acetate, and methanol. The active subfractions were pooled and further fractionated on a silica gel column (length 32 cm, diameter 2 cm) and eluted with a stepwise gradient of ethyl acetate, acetone, and methanol. The active super fractions were subjected to a final round of purification on a LH-20 column (length 50 cm, diameter 1 cm) and eluted with methanol (Fig. 1). The purity of the bioactive compound was checked by RP-HPLC on a C_{18} column with methanol:water with 0.1 % TFA (1:1) as the mobile phase. The purified compound (purity > 99 %) from the methanolic extract was designated *Decaleside II* (Supplementary Fig. 3a, b).

Reverse phase high-performance liquid chromatography

Reverse phase HPLC was performed using a Shimadzu's LC-8A system equipped with a Rheodyne 7725i injection valve fitted with a 20 μl sample loop and a C_{18} column (5 μm particle size, 25 cm×0.4 cm i.d.; Supelco, USA). Pooled subfractions were eluted with an isocratic solvent mixture comprising 0.1 % TFA in water:methanol (50:50) with a flow rate 0.5 ml/min and monitored with an ultraviolet (UV) detector at 220 nm.

Infrared spectrometry

IR spectra were recorded with a PerkinElmer FT-IR spectrophotometer (Spectrum 2000) at 400–4,000 cm^{-1}.

Liquid chromatography–mass spectrometry

The liquid chromatography (LC) system consisted of a Hitachi L-6000 pump (Hitachi, Tokyo, Japan), a Rheodyne Model 7125 injector with a 25 μl loop and a 4.6 i.d. 325 mm Devosil C30 UG-5 column (Nomura Chemical, Seto, Japan). LC was performed using a solution containing 1 % acetonitrile, 20 mM ammonium heptafluorobutyrate, and 10 mM ammonium formate (pH 4.0) as the mobile phase at a flow rate of 0.4 ml/min at 15 °C. The column was connected to ion interface of the mass spectrometer through a fused silica capillary without splitting. Liquid chromatography–mass spectrometry (LC–MS) spectra were recorded on a triple quadrupole TSQ 700 mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an API-ES source with an ICIS II data system in the positive ion mode. API-ES was affected by a spray voltage of 14.8 kV and the heated capillary temperature was maintained at 250 °C. Nitrogen served as the sheath gas at an operating pressure of 60 psi and as the auxiliary gas at a flow rate of 3.0 ml/min. The dwell time was set at 500 ms/Da.

^{1}H and ^{13}C nuclear magnetic resonance

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX 500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz for ^{13}C). Proton and carbon 90° pulse widths were 12.5 and 10.5 μs, respectively. About 40 mg of the sample dissolved in DMSO-d_6 was used for recording the spectra at 25 °C. Chemical shift values were expressed in parts per million relative to the internal standard tetramethysilane. Two dimensional heteronuclear multiple quantum coherence transfer spectra (2D-HMQCT) were recorded in magnitude mode with sinusoidal shaped Z-gradient of strengths 25.7, 15.42, and 20.56 G/cm with a gradient recovery delay of 100 μs to defocus unwanted coherences. The t_1 was incremented in 256 steps. The spectra were processed using unshifted and $\pi/4$-shifted since bell window function in F1 and F2 dimensions, respectively.

Insecticidal activity

The insecticidal activity of the extracts/fractions and the compounds were tested on *S. oryzae* adults by the contact bioassay method (Obeng-Ofori et al. 1998; Rajashekar et al. 2010). One milliliter of the extract containing 17.5 mg residue was applied onto Whatman No. 1 (9 cm) filter paper and placed in a glass petri dish and the solvent was allowed to evaporate for 10 min prior to the release of 20 adults of *S. oryzae* into each dish. The control filter paper discs were treated with the solvent only. Each treatment consisted of four replicates. Insect mortality for each extract/fraction was recorded after 24-h exposure, percent mortality determined (Abbott 1925), and results are shown in Supplementary Fig. 1.

The insecticidal activity of the isolated compounds was tested by the filter paper method of contact bioassay on several insect species: housefly (*M. domestica*), German cockroach (*B. germanica*), and stored-product insects (rice weevil, *S. oryzae*; lesser grain borer, *R. dominica*; pulse beetle, *C. chinensis*; and rust-red flour beetle, *T. castaneum*). Twenty insects for each treatment were used for all the

species except in the case of cockroach, where ten individuals per plate were used. The dosages ranged from 0.004 to 0.272 mg/cm^2, and the effective dosages were chosen based on trial experiments. Four replicates were used for each dosage. LC_{50} (24 h exposure) were determined from the dose–response data using probit regression analysis (Finney 1971).

Insect toxicity by oral route was tested on cockroaches by administering 1 mg/insect in a 25–50 μl of aqueous solution applied to the mouth using a microsyringe. Contact toxicity by topical application was tested on the cockroaches by applying 1 mg/insect in 25–50 μl aqueous solution on the abdomen of cockroaches. After 24 h, mortality, if any, was noted.

Comparison of insect toxicity with chemical insecticides

In order to compare the insect toxicity of *Decaleside* with that of chemical insecticides, LC_{50} values were determined for endosulfan (organochlorine), monocrotophos (organophosphate), and deltamethrin (pyrethroid) using the contact bioassay procedure as described earlier. The timecourse of toxicity was studied separately by measuring the knockdown effect, which precedes mortality (0–60 min) at LC_{50} concentration using the contact bioassay procedure. For each time point, ten cockroaches were used. The knockdown effect on insects was evaluated by observing cessation of movement but being alive to touch as distinct from dead insects. The number of insects knocked down at various time points (0–60 min) was counted and percentage mortality determined.

Effect of hydrolysis of the compounds on insecticidal activity

Acid hydrolysis

Forty-four milligrams of *Decaleside I* or *Decaleside II* was refluxed with 20 ml of 2 N methanolic sulfuric acid for 6 h in a water bath. Methanol was removed under vacuum and 20 ml distilled water added when a solid separated out and then neutralized with barium carbonate and filtered. The filtrate was concentrated under vacuum and the residue extracted with alcohol (Aguilar et al. 2002). The filtrate was subjected to paper chromatography and HPLC and the presence of individual sugars (psicose, altrose, galactose/gulose) were identified from R_f values and retention times, respectively. Alcohol concentrate of the hydrolysate was subjected to insecticidal activity by using the filter paper bioassay method.

Enzymatic hydrolysis

A mixture of 1.5 ml of 33 % *Decaleside II* in 0.1 M sodium phosphate buffer (pH 6.5) containing 0.5 ml of 15 U of β-galactosidase or α-glucosidase (Sigma-Aldrich Fine Chemicals) was incubated at 37 °C for 6 and 12 h, and the reaction was stopped by heating the mixture in a boiling water bath for 10 min, cooled, and subjected to paper chromatography (Toba et al., 1980). The hydrolysate was checked for insecticidal activity by the contact bioassay.

Effect of sugars on insecticidal activity

Whatman No. 1 (diameter 9 cm) filter paper discs were treated with *Decaleside II* at LC_{50} concentration (0.07 mg/cm^2) with or without sugars at equimolar (1:1) concentration and the insects were released and mortality recorded after 24-h exposure. Effect of sugars on the knockdown effect of *Decaleside II* was studied by recording the number of insects knocked down at 0–60 min.

Mode of action

The effect of surgical ablation of the tarsal part of the insect leg on the toxicity of *Decaleside II* was investigated on German cockroaches. The tarsal segments of all six legs were surgically ablated by using fine scissors and released to bioassay plates containing filter paper treated with *Decaleside II* at LC_{50} concentration (0.07 mg/cm^2). Four replicates of ten insects each were used per group. The control group consisted of intact cockroaches. Mortality was recorded after 24 h exposure.

Similarly, the effect of molten wax application on the lower part of the legs (tarsi) of the insects on the contact toxicity of *Decaleside II* was determined by contact bioassay. Molten paraffin wax (m.p. 56–58 °C) was applied on to the tarsi of cockroach, allowed to cool and solidify, and then released to the bioassay dishes containing the paper applied with LC_{50} concentration of *Decaleside II* (0.07 mg/cm^2). Mortality was compared with untreated insects.

Toxicity by direct application on the tarsi

In order to test the direct action of *Decaleside* on the chemosensilla of the tarsi, experiments were done by applying *Decaleside II* at 1 mg/leg to the forelegs of an insect (cockroach) in 25–50 μl of aqueous solution by using a

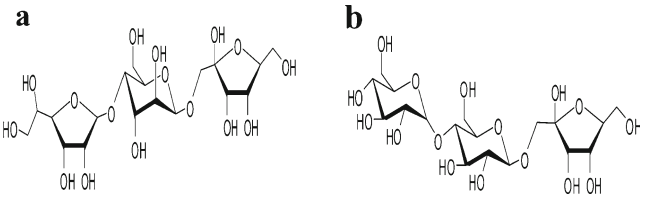


Fig. 2 Molecular structures: **a** *Decaleside I* (α-D-Gulosefuranose-(1-4)–β-D-altrosepyranosyl-(1-1)–α-D-psicosefuranose); **b** *Decaleside II* (β-D-Galactopyranose-(1-4)–α-D-altrosepyranosyl-(1-1)–α-D-psicosefuranose)

Table 1 Insecticidal activity of *Decaleside I* against adults of household and stored-product insects in 24-h contact bioassays

Values in parenthesis represent fiducial limits

Bold is the mean value to distinguish from the values in parenthesis

Insect species	LC_{50} (mg/cm^2)	LC_{99} (mg/cm^2)	Slope ± SE
B. germanica	**0.077** (0.067–0.08)	**0.152** (0.143–0.164)	0.395±0.076
M. domestica	**0.033** (0.029–0.037)	**0.066** (0.052–0.074)	0.611±0.141
C. chinensis	**0.042** (0.032–0.053)	**0.083** (0.072–0.091)	0.734±0.076
T. castaneum	**0.115** (0.102–0.127)	**0.227** (0.217–0.234)	0.371±0.030
S. oryzae	**0.044** (0.031–0.059)	**0.087** (0.072–0.095)	0.729±0.081
R. dominica	**0.042** (0.038–0.046)	**0.083** (0.071–0.092)	0.825±0.115

microsyringe. Control insects were treated with water only. Similarly, application of *Decaleside II* (1 mg/leg) on tarsi treated with molten wax was done and mortality determined.

Mammalian toxicity

Mammalian toxicity of *Decalesides* was evaluated by administering single oral doses (400–2,400 mg/kg bw in aqueous solution) by gavage to adult Swiss mice (four animals per group) and the control group received distilled water only. The animals were observed for symptoms or mortality for 1 week post-treatment.

Statistical analysis

LC_{50} were determined by probit analysis (Finney 1971). The data were analyzed using one-way ANOVA ($p<0.05$) by Duncan's multiple range test using the softwares Statplus 2007 and Statistica 1999.

Results

Isolation and identification

Using a bioassay-directed procedure, we isolated two novel bioactive molecules from the roots of *D. hamiltonii*. The two compounds characterized were novel trisaccharides named *Decaleside I* (α-D-Gulosefuranose-(1-4)–β-D-altrosepyranosyl-(1-1)–α-D-psicosefuranose) and *Decaleside II* (β-D-Galactopyranosyl-(1-4)–α-D-altrosepyranosyl-(1-1)–α-D-psicosefuranose) (Fig. 2). The IR spectrum of both *Decalesides I* and *II* showed intensive transmittance in the range of *v* 3,100–3,600 cm^{-1} indicating the presence of several hydroxyl groups. The carbon NMR showed the presence of 16 signals for 18 carbons (two carbon signals overlapped) of *Decaleside I* and 17 signals for 18 carbons of *Decaleside II* (one carbon signal overlapped) in the range δ 60.7–104.5 ppm, indicating these could be trisaccharides (Breitmaier and Voelter 1990; Fraser-Reid et al. 2008). Three anomeric carbons at δ 92.1, 98.3, and 104.5 ppm (*Decaleside I*) and 91.8, 99.2, and 104.2 ppm (*Decaleside II*) indicated the presence of three sugar units. The carbon signals at δ 60.9, 104.5, 72.2, 71.9, 82.9, and 62.2 ppm (*Decaleside I*) and δ 66.9, 104.2, 71.1, 71.4, 82.5, and 62.4 ppm corresponds to C-1, C-2, C-3, C-4, C-5, and C-6 of Psicose units, respectively. The up field shift of C-1 carbon (δ 60.9 for *Decaleside I* and 66.9 for *Decaleside II*) indicated the linkage of the second sugar unit at that position. The carbon signals at δ 92.1, 70.2, 70.2, 73.4, 73.5, and 60.9 ppm (*Decaleside I*) and δ 91.8, 71.6, 69.6, 68.7, 69.0, and 60.7 ppm corresponds to C-1^1, 2^1,3^1, 4^1, 5^1, and 6^1 of the second sugar unit viz., altrose, respectively. The up field shift of C^1-4 carbon (δ 73.4 for *Decaleside I* and δ 68.7 for *Decaleside II*) indicated the linkage of the third sugar unit at that position. In both compounds, the first and second sugar units are the same, but differ in isomeric forms and also differ in the third sugar unit. The carbon signal at δ 98.3, 73.8, 72.7, 70.2, 81.6, 72.8, and 62.7 ppm corresponds to C-1^{11}, 2^{11}, 3^{11}, 4^{11}, 5^{11}, and 6^{11}, respectively, of the third sugar unit of *Decaleside I* viz., D-gulosefuranose. In *Decaleside II*, the carbon signals at δ 99.2, 74.4, 73.0, 77.2, 70.5, and 62.4 ppm corresponds to C-1^{11}, C-2^{11}, C-3^{11}, C-4^{11}, C-5^{11}, and C-6^{11}, respectively, of the third sugar unit viz., D-galactose. The proton NMR signals confirmed the same. Three anomeric proton signals were observed at δ 5.25, 5.09, and 3.63 ppm

Table 2 Insecticidal activity of *Decaleside II* against adults of household and stored-product insects in 24-h contact bioassays

Values in parenthesis represent fiducial limits

Bold is the mean value to distinguish from the values in parenthesis

Insect species	LC_{50} (mg/cm^2)	LC_{99} (mg/cm^2)	Slope ± SE
B. germanica	**0.07** (0.060–0.078)	**0.138** (0.129–0.147)	0.911±0.195
M. domestica	**0.023** (0.013–0.03)	**0.041** (0.033–0.049)	0.688±0.194
C. chinensis	**0.026** (0.02–0.028)	**0.051** (0.042–0.061)	1.381±0.171
T. castaneum	**0.093** (0.086–0.100)	**0.184** (0.173–0.195)	0.384±0.036
S. oryzae	**0.032** (0.03–0.036)	**0.063** (0.052–0.074)	1.638±0.128
R. dominica	**0.02** (0.018–0.021)	**0.039** (0.031–0.048)	2.062±0.231

Table 3 Comparison of insecticidal activity of *Decalesides I* and *II* with the chemical insecticides

Insecticides	LC_{50} (mg/cm^2)		
	Musca domestica	*Blatella germanica*	*Sitophilus oryzae*
Decaleside I	**0.033** (0.029–0.037)	**0.077** (0.067–0.088)	**0.044** (0.031–0.059)
Decaleside II	**0.023** (0.013–0.030)	**0.070** (0.06–0.078)	**0.032** (0.03–0.036)
Endosulfan	**0.036** (0.029–0.044)	**0.069** (0.053–0.076)	**0.031** (0.029–0.034)
Monocrotophos	**0.03** (0.027–0.039)	**0.112** (0.105–0.121)	**0.027** (0.023–0.032)
Deltamethrin	**0.029** (0.021–0.032)	**0.098** (0.088–0.107)	**0.02** (0.017–0.024)

Values in parenthesis represent fiducial limits

Bold is the mean value to distinguish from the values in parenthesis

for *Decaleside I* (Supplementary Table S1) and δ 5.19, 4.65, and 3.48 ppm for *Decaleside II* (Supplementary Table S2). The compounds *Decaleside I* and *II* were found to be novel trisaccharides (Liptak et al. 1990; Lutteke et al. 2006; Fraser-Reid et al. 2008) with insecticidal properties.

Insecticidal activity

Decalesides I and *II* showed potent insecticidal activity by contact bioassay against several insect species viz., housefly (Dipetera), cockroach (Orthoptera), and the stored-product insects (Coleoptera) (Tables 1 and 2). The contact toxicity (mortality) as well as the rapidity of action (knockdown) of the compounds was comparable to that of synthetic insecticides (Table 3, Fig. 3a and b).

Mode of action

Insect toxicity of *Decaleside II* was abolished by tarsal ablation as well as wax treatment (Fig. 4a, b). Direct application of *Decaleside II* to the tarsi of the legs was effective in killing the cockroaches, whereas wax application protected against the toxic action (Fig. 4c).

Effect of sugars on insecticidal activity

Experiments wherein cockroaches were exposed to *Decaleside*-treated filter paper with or without various sugars showed that sugars, but not amino acids, protected against toxicity (Fig. 5a and b, Fig. 6a). Among the sugars, maltotriose, a trisaccharide, was most effective in rescuing the toxicity of *Decaleside* (Fig. 6b) and the effect was dose-dependent (Fig. 5c).

Effect of hydrolysis of the compounds on the insecticidal activity

When *Decaleside* was subjected to chemical and enzymatic hydrolyses, its insecticidal activity was lost as evident from the results of contact bioassay (Fig. 6c).

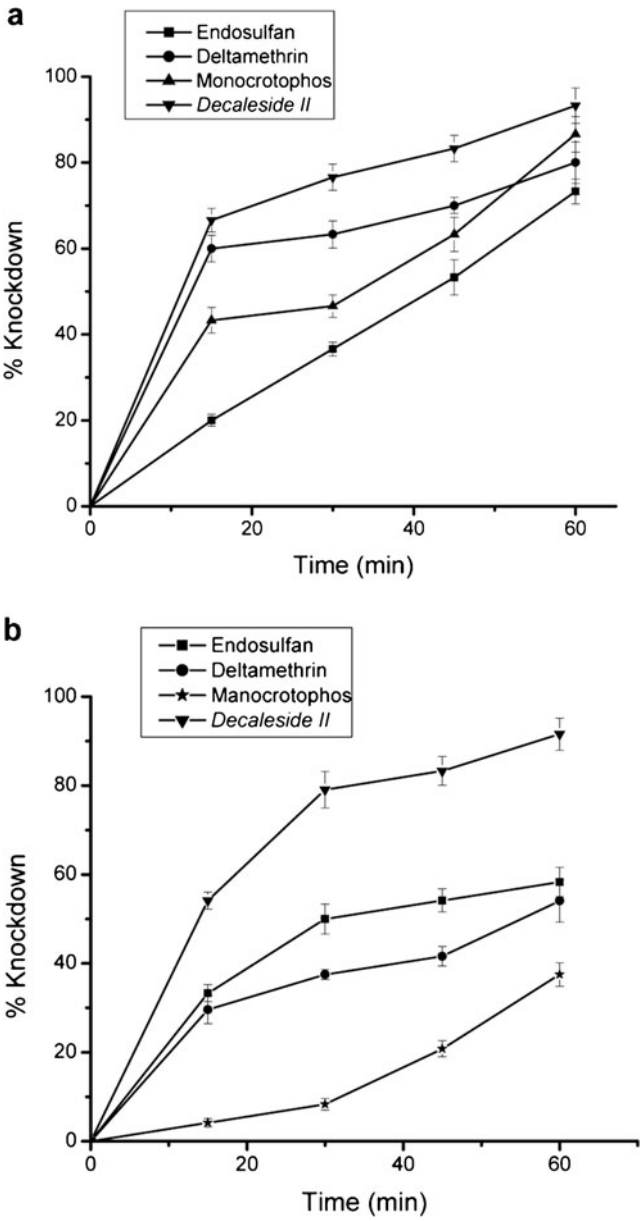
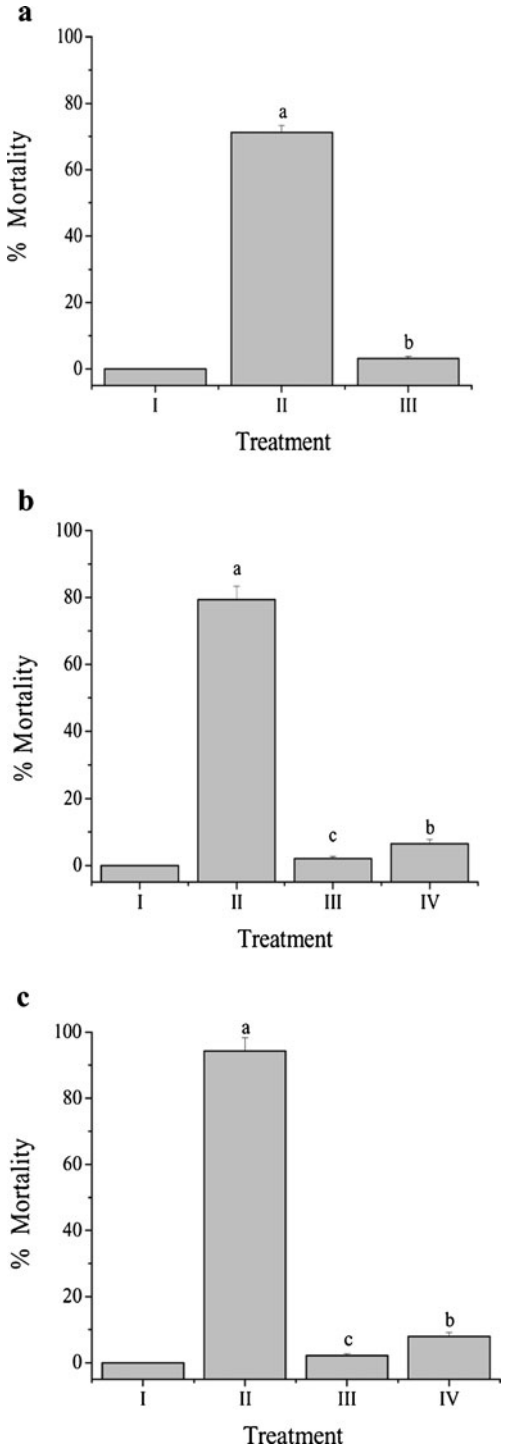


Fig. 3 Comparison of the insecticidal action (timecourse) of *Decaleside II* with that of chemical insecticides in **a** *Musca domestica* (housefly) and **b** *Blatella germanica* exposure at LC_{50} by contact bioassay (n=4, *error bars*, SEM)



◀ **Fig. 4** Experimental demonstration of the tarsi-mediated contact toxicity of *Decaleside II* in the German cockroach. **a** Effect of tarsal ablation on the insecticidal activity of *Decaleside II* in German cockroach by contact bioassay. *I* Intact control (solvent), *II* intact control + *Decaleside II*, *III* Tars *II* ablated + *Decaleside II* (n=4, error bars, SEM), one-way ANOVA, P<0.001. **b** Effect of wax application on tarsi, on the toxicity of *Decaleside II* in German cockroach, by contact bioassay. *I* Intact control (solvent), *II* intact control + *Decaleside II*, *III* wax treated (solvent control), *IV* wax treated + *Decaleside II* (n=4, *error bars*, SEM), one-way ANOVA, P<0.001. **c** Effect of wax application on the tarsi, on the toxicity of *Decaleside II* in the German cockroach, by topical application on the leg. *I* Control (solvent only), *II* untreated + *Decaleside II* (1 mg/insect), *III* wax treated on tarsi (+solvent), *IV* with wax-treated tarsi + *Decaleside II* (1 mg/insect) (n=4, *error bars*, SEM), one-way ANOVA, P<0.001

Mammalian toxicity

Decalesides I and *II* showed no acute mammalian toxicity in mice up to 2,400 mg/kg bw (data not shown). Since availability of the purified compound was limited, subacute studies were not done.

Discussion

Decalesides I and *II* were toxic to a wide range of insects (Tables 1 and 2). The two trisaccharides differed only in one sugar in their structure (Fig. 2) and both of them exhibited insecticidal activity, the latter being slightly more potent (Tables 1 and 2). Since the compounds were toxic by contact exposure [but not by topical application on the abdomen or oral administration], we investigated if the toxic action required contact with the insect legs (Fig. 4a, b, c). Gustatory receptors in insects located in the labial palps (mouth) and legs are used in the detection of food and nonfood chemicals including sugars and other inedible (toxic) plant compounds (Amerein and Thorne 2005; Hallem et al. 2006). Since *Decaleside I* and *II* are trisaccharides, we reasoned whether sugar receptors located in the sensilla of the tarsi on the legs are involved in the insecticidal action. In order to test this hypothesis, we surgically ablated the lower part of the legs (tarsi) of insects (housefly and cockroaches) and exposed them to *Decaleside*-treated surface in the contact bioassay. The surgical ablation of tarsi did not cause mortality of the insects nor a drastic change in their movement in the bioassay. Further, a less invasive method, such as, masking the sensilla in the tarsi by application of molten wax, was done to see if it blocks the insecticidal effect. In both of these cases, toxicity was abolished by tarsal ablation as well as wax treatment (Fig. 4a, b). Direct application of *Decaleside II* to the tarsi of the first pair of legs was effective in killing the cockroaches, whereas wax application protected against the toxic action (Fig. 4c). This compelling evidence demonstrated the requirement of the exposure by contact of tarsi to *Decaleside II* for the toxic action and therefore, implicates the gustatory (sugar) receptors in the insecticidal action.

Further, to test the possible involvement of gustatory (sugar) receptors, we performed simple experiments in which we studied the effect of sugars (mono-, di-, and trisaccharides) on the toxicity of *Decaleside II*. Experiments in which cockroaches were exposed to *Decaleside*-treated paper with or

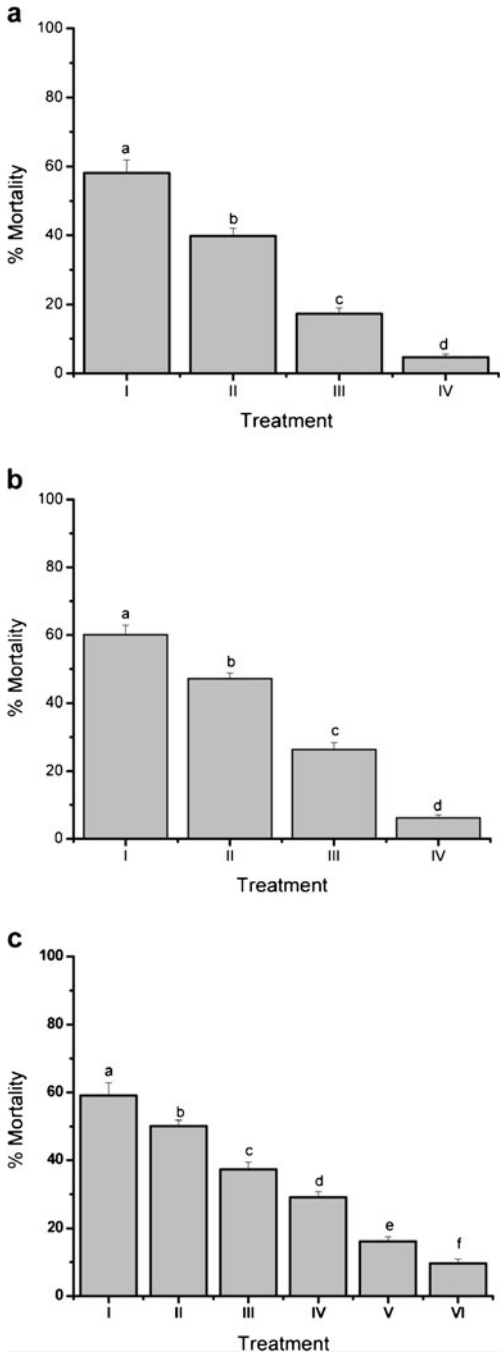


Fig. 5 Effect of sugars on the insecticidal activity of *Decaleside II* in German cockroach exposure at LC_{50} (0.07 mg/cm^2) by contact bioassay. **a** Monosaccharide (glucose). *I Decaleside II*, *II Decaleside II* + glucose (1:1), *III Decaleside II* + glucose (1:2), *IV Decaleside II* + glucose (1:3). Ratio in molar concentration (n=4, *error bars*, SEM), one-way ANOVA, P<0.001. **b** Disaccharide (trehalose). *I Decaleside II*, *II Decaleside II* + trehalose (1:1), *III Decaleside II* + trehalose (1:2), *IV Decaleside II* + trehalose (1:3). Ratio in molar concentration (n=4, *error bars*, SEM), one-way ANOVA, P<0.001. **c** Trisaccharide (maltotriose). *I Decaleside II*, *II Decaleside II* + maltotriose (1:0.1), *III Decaleside II* + maltotriose (1:0.25), *IV Decaleside II* + maltotriose (1:0.5), *V Decaleside II* + maltotriose (1:0.75), *VI Decaleside II* + maltotriose (1:1). Ratio in molar concentration (n=4, *error bars*, SEM), one-way ANOVA, P<0.001

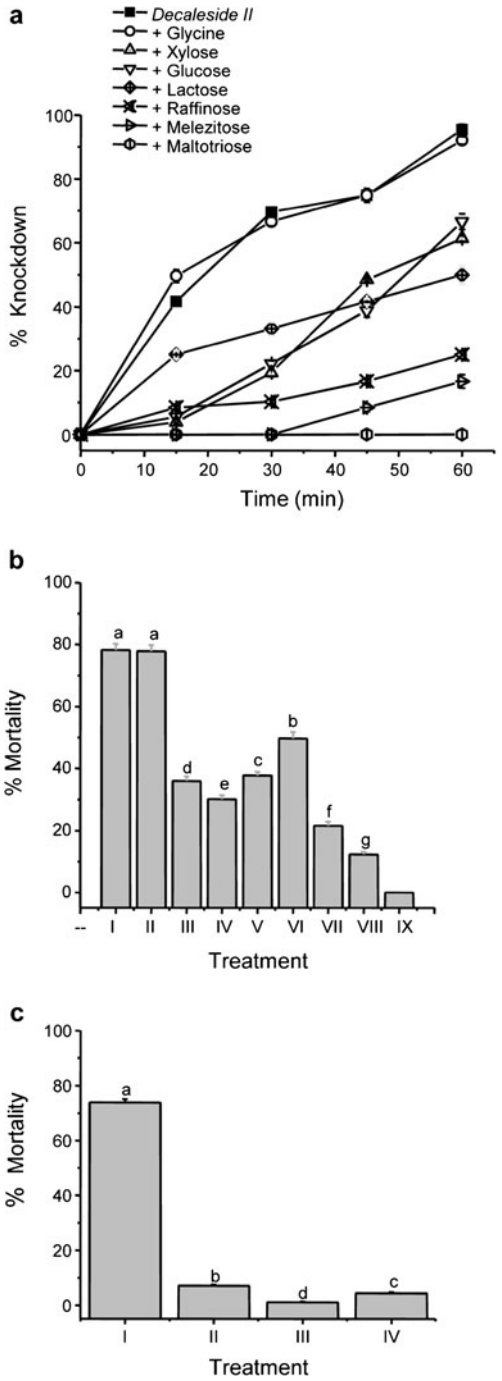


Fig. 6 Effect of sugars on the insecticidal activity of *Decaleside II* in the German cockroach. **a** Knockdown at equimolar (LC_{50}) concentration of *Decaleside II* + sugars (n=4, *error bars*, SEM). **b** Mortality at equimolar concentration: *I Decaleside II*, *II Decaleside II* + glycine, *III Decaleside II* + glucose, *IV Decaleside II* + xylose, *V Decaleside II* + lactose, *VI Decaleside II* + trehalose, *VII Decaleside II* + raffinose, *VII Decaleside II* + melezitose, *IX Decaleside II* + maltotriose. (n=4, *error bars*, SEM), one-way ANOVA, P<0.001. **c** Effect of hydrolysis of *Decaleside II* on the insecticidal activity. *I* Control (without hydrolysis), *II* acid hydrolysis, *III* enzymatic hydrolysis by β-galactosidase, *IV* enzymatic hydrolysis by α-glucosidase. (n=4, *error bars*, SEM), one-way ANOVA, P<0.001

without various sugars showed that sugars, but not amino acids, protected against the toxicity (Fig. 5a and b, Fig. 6a). These experiments clearly demonstrated the involvement of the gustatory (sugar) receptors located in the tarsi for the insecticidal action of *Decaleside*. Since *Decalesides I* and *II* are natural trisaccharides, they are detected by the gustatory receptors in the tarsi and the insecticidal action requires contact with the tarsi as shown experimentally. Abolition of toxicity of *Decaleside II* by hydrolysis indicates that only the intact trisaccharide molecules exhibit insecticidal activity (Fig. 6c). However, other natural trisaccharides, such as, raffinose, melezitose, maltotriose, and other sugars, were not toxic to insects (Fig. 6b) but protected against the toxic action of *Decaleside II*, indicating the common site of action possibly involving the gustatory receptors. This also shows that the specificity of the sugar sequence in the structure of *Decalesides I* and *II* is required for the insecticidal activity and that the latter containing galactose was somewhat more potent. Lack of oral toxicity of *Decalesides* to insects could be attributed to the enzymatic hydrolysis of the trisaccharides by the salivary enzymes and also the types of gustatory receptors in the insect mouth parts (Amerein and Thorne 2005, Hallem et al, 2006). This is the first time that such a novel biological activity for natural oligosaccharides has been shown (Breitmaier and Voelter 1990; Liptak et al. 1990; Lutteke et al. 2006; Fraser-Reid et al. 2008). *Decalesides I* and *II* could be considered a new class of natural insecticides targeting the insect gustatory receptors, reported for the first time (Breitmaier and Voelter 1990; Liptak et al. 1990; Lutteke et al. 2006; der Goes et al. 2006; Fischler et al. 2007). In insects, the axons of the gustatory receptor neurons from the chemosensilla directly report to the thoracic–abdominal and subesophageal ganglion as in *Drosophila* (Inoshita and Tanimura 2006). Therefore, the possible mode of action of *Decaleside* on the chemosensilla is likely to involve the blockade of nerve impulse or the amplification of the effect on neurons that could lead to the knockdown effect. However, further studies are needed to establish the precise molecular pathway involved in the toxic action. At the moment, we can only speculate that the mode of action of *Decalesides* could be mediated by interference with the signal transduction in neurons possibly interfering with the ionic movements across the nerve membrane. Therefore, there is great scope to unravel the mechanisms, particularly the neuronal responses involved in the interaction of the oligosaccharide compounds, such as, *Decalesides I* and *II*, with the sugar receptors of insects that lead to the toxic outcome. *Decaleside* could also serve, perhaps, as a chemical probe to investigate the newer dimensions of the tarsal sugar receptors in the biology of insects.

The unique site and mode of action of the *Decalesides* contribute to their high selectivity. *Decalesides I* and *II* were not toxic to up to 2,400 mg/kg bw to mice. The lack of mammalian toxicity is attributed to the 1,4 or 1,1 α linkage of the sugars which are easily hydrolyzed by mammalian digestive enzymes, such as, glucosidases. The possible application of *Decalesides* as insecticides in the field or as baits may have practical limitations. However, the insect selectivity and mammalian safety of *Decalesides* or similar molecules makes them highly suitable for use as novel grain or seed protectants of natural origin.

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