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INSECTICIDAL ACTIVITY OF COMPOUNDS FROM *Ailanthus altissima* AGAINST *Spodoptera littoralis* LARVAE

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We determined the efficacy of *Ailanthus altissima* extracts, obtained using solvents of various polarity, in terms of acute and chronic toxicity, antifeedant efficacy and larval growth inhibition of *Spodoptera littoralis*. Different efficacy was found for individual fractions, in terms of both acute and chronic toxicity, as well as antifeedant efficacy and growth inhibition of *S. littoralis* larvae. Fractions 1 and 2, obtained at the beginning of the separation period, were the only ones to cause significant chronic toxicity accompanied by larval growth inhibition. A major proportion of tocopherol isomers were detected in these fractions. On the contrary, fractions 5 and 6 caused acute toxicity, associated with significant antifeedant efficacy and related strong growth inhibition of *S. littoralis* larvae. Five major quassinoids (Ailanthone, Chaparrinone, Glaucarubinone, 13(18)-Dehydroglaucarubinone and Shinjulactone H) were detected in fraction 5, which showed the greatest efficacy in terms of acute toxicity and feed intake inhibition of *S. littoralis*. Methanol extracts or active substances obtained from *A. altissima* leaves can be recommended for the development of new botanical insecticides targeted against some phytophagous larval species of butterfly pests.

Keywords: *Ailanthus altissima*, *Spodoptera littorallis*, botanical insecticides, tocopherol, quassinoids, toxicity, plant extracts, antifeedant

INTRODUCTION

The development of botanical insecticides is important given that the number of pest populations resistant to the active substances of synthetic insecticides has been continually rising (Ahmad *et al.*, 2009; Baldwin and Graves, 1991; Saleem *et al.*, 2008). The use of botanical insecticides is a plant protection alternative, generally considered safe for the environment and health (Isman, 2000; Pavela, 2007; Pavela *et al*., 2009; Rattan, 2010; Pavela, 2011b).

Ailanthus altissima Swingle, commonly known as the "tree of heaven", is used in traditional medicine in many parts of Asia, including China, Japan and Korea, to treat cold and gastric diseases and as an antitumor agent. Phytochemical investigation into the genus *Ailanthus* has afforded nearly 200 compounds (Albouchi *et al.*, 2013) with varying structural patterns, such as alkaloids, terpenoids, steroids and flavonoids, together with some other compounds. Previous phytochemical studies have demonstrated the presence of quassinoids (Kubota *et al*., 1996) as well as indole and b-carboline alkaloids in this plant (Ohmoto and Koike, 1984; Souleles and Waigh, 1984). Quassinoids are reported for their various biological activities such as antitumor, anti-malarial and anti-inflammatory effects (Kundu and Laskar, 2010).

Besides the possible uses mentioned above, the extracts could potentially be used in plant protection. In particular, extracts from *Ailanthus altissima* showed important nematocidal antifeedant, insecticidal and insect growth-regulating effects (Caboni *et al*., 2012; Kraus *et al*., 1994; Pavela, 2011a). It is therefore expected that extracts from *A. altissima* will be used in the development of new botanical pesticides.

In our paper, we determined the efficacy of extracts, obtained using solvents of various polarity, in terms of acute and chronic toxicity, antifeedant efficacy and larval growth inhibition of *Spodoptera littoralis* Boisd (*S. littoralis)*. The larvae of *S. littoralis* were chosen for the tests since they are an important polyphagous pest, widely distributed in Africa and Mediterranean Europe (Baldwin and Graves, 1991). Larvae of this pest can feed on 90 economically important plant species belonging to 40 families, and the rate of development has a strong nutritional component (Azab *et al*., 2001). Commonly, the control of this pest has largely been dependent on the use of neurotoxic insecticides, including chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids. However, the control achieved has not been successful because of the insect's high capacity to develop resistance to the majority of these compounds (Ahmad *et al.*, 2009; Elghar *et al*., 2005). The main objective of our study is to find which of the Ailanthus compounds are responsible for different insecticidal activities against *S. littoralis* larvae.

MATERIALS AND METHODS

***Plant material*:** *Ailanthus altissima* (Mill.) Swingle (Family: Simaroubaceae DC) – herbarium items are stored under registration number 09097 at the Crop Research Institute, Prague. The leaves of *Ailanthus altissima* were collected manually in August, 2012, from plants grown in the field of the Crop Research Institute. Impurities were removed from the collected materials, and the materials were dried in the dryer Stericell BMT – Medical Technology JSC, Czech Republic, at 40°C (48 hours).

***Preparation of plant fractions*:** The powdered leaves (50 g) were extracted using 100% pure methanol (1,000 ml). The extract was concentrated (after 48 h of maceration) under a reduced pressure of 22–26 mm Hg at 45°C, and the residue obtained was stored at 4°C. The concentrated crude *Ailanthus* extract was then resolved in 100% pure methanol. For purposes of purification and the separation of a large amount of extract, silica gel column chromatography was utilised. A silica gel (Merck *Silica gel* 60, 70-230 mesh ASTM) column (50 cm x 4 cm diameter) was prepared. A sample of crude *Ailanthus* extract was then loaded with a Pasteur pipette. The column with sample was then washed with dichloromethane : methanol mobile phase. Different fractions for bioassays were obtained with a step gradient (from 100:0 v/v up to 80:20 v/v). The obtained fractions were then evaporated to dryness and preserved in 4°C until the bioassays (Jegorov *et al.*, 2003; Zabka *et al.*, 2006).

GC/MS analysis:

***Chemicals, reagents, and glassware*:** Ethylacetate (EtOAc) and chloroform, used as solvent for the samples, were obtained from BioSolve BV. Derivatization agent BSTFA:TMS (99:1, v/v), dimethylformamide (DMF) and standard α (+) tocopherol were obtained from Sigma-Aldrich, Inc. Helium (purity 99.9999%) was obtained from Linde (CZ). The tocopherols mixture, Tocobiol, was purchased from BTSA Biotecnologías Aplicadas, S.L.

***Derivatization procedure*:** Samples were dissolved in DMF:EtOAC (1:9 v/v) and after addition of 100 □L BSTFA:TMS were heated at 70°C in the oven. Reagent agent was removed after 30 min. under a gentle nitrogen stream, and 1 □L of sample was injected into the GC/MS equipment (Kresinova *et al.*, 2012)

***GC/MS analysis*:** GC/MS analysis was performed with a Varian 450-GC (USA) instrument equipped with a Combi-Pal injector (CTC Analytics, Sweden) and a Varian 240 MS ion trap detector employing an electron impact ion source (-70 eV). 1 µL of the derivatized/non-derivatized sample solutions was injected into the GC-MS systems. The analyses were performed with a 30 m length × 0.25 mm i.d., 0.25 mm film thickness, DB-5MS column (Agilent, USA). The GC oven temperature program started at 60°C (hold 1 min.), then heated up to 150°C at 25°C min^{-1}, then to 200°C at 1°C min^{-1} and finally to 240°C at 15°C min^{-1} and held isothermally for 28 min. Helium was used as the carrier gas, with a constant flow rate of 1 mL/min. The injector was operated in the split/split less mode, with a split less time of 1 min. The injector temperature was 240°C. The source, ion trap and transfer line temperatures were 250, 220 and 280°C, respectively. Full scan spectra were acquired in the range from m/z 100 to 1000. Mass spectrometry workstation (version 6) software (Varian, USA) was used for data processing. Structures were suggested by comparing the mass spectra with data in the NIST 08 library and independently by interpreting the fragmentation pattern.

UHPLC-DAD-ToFMS analysis:

***Chemicals, reagents and glassware*:** Acetonitrile (ACN) and methanol, used as the chromatographic mobile phase, were LC/MS grade and were obtained from Biosolve (The Netherlands). Formic acid (98-100%) was from Merck (Germany); dichloromethane (CH_2Cl_2) was LC grade and was purchased from Chromservis (Czech Republic). Ultrapure water was prepared using the Milli-Q water purification system (18.2 MΩ; Millipore, USA).

***UHPLC-DAD-ToFMS analysis*:** The obtained extract was evaporated to dryness and reconstituted in 100 µL of methanol-CH_2Cl_2 (80:20, v/v). For ToFMS analysis, the sample was diluted 50 times.

UHPLC-DAD-ToFMS analyses were carried out on the Waters Acquity UPLC System (Waters, Prague, Czech Republic), consisting of the Acquity UPLC Solvent Manager, Acquity UPLC Sample Manager, Acquity UPLC Column Heater/Cooler, and Acquity UPLC Diode Array Detector (set at 200-600 nm). The Waters LCT Premier XE orthogonal accelerated time of flight mass spectrometer (Waters MS, Manchester, UK), with electrospray interface, was operating in both positive and negative ion mode, using the following parameters: cone voltage, 50 V; capillary voltage, +2500 V (positive), -2500 V (negative); ion source block temperature, 120°C; nitrogen desolvation gas temperature, 350°C; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h. Full scan spectra from m/z 100 to 1600 were acquired with a scan time of 0.1 s and 0.01 s inter scan delay. Instrument calibration was performed externally with a sodium formate solution, and mass accuracy was maintained by lock spray using Leucine Enkephalin as the reference compounds. Fragmentation using in-source, collision-induced dissociation was achieved by setting the Aperture I value to 70 V. Mass Lynx V4.1 software was used for data processing. Analyses were performed on an Acquity UPLC BEH C18 column (50 x 2.1 mm i.d.; 1.7 µm) with a mobile phase flow rate of 0.4 mL/min, column temperature of 25 °C, and injection volume of 1 µL. The mobile phase consisted of (A) formic acid-water (0.1:99.9, v/v), and (B) ACN using a gradient elution program (min/ %A): 0.0/ 95, 15.0/ 65, 27.0/ 0, followed by a 1.5 min wash step with 100% B and 1.5 min equilibration step.

For verification of the compounds' identity, the parameters set for the Elemental Composition editor were: CHNO algorithm; mass measurement error tolerance, 5 mDa; i-FIT (norm) error, 5. In-source fragmentation was employed for verification of the generated fragment ions, with a mass measurement error tolerance of 10 mDa, set in the Mass Fragment software. Verification of the compounds' identity was performed in both positive and negative ion mode, for both Elemental Composition prediction and identification of fragments.

Insects: *S. littoralis* Boisd. (Lepidoptera: Noctuidae): Bioassays were conducted using larvae of the tobacco cutworm, *S. littoralis*, obtained from an established laboratory colony (> 20 generations; out-crossed once). The larvae fed on an artificial insect diet (Stonefly Industries, Bryan, TX, USA); adults fed on a 10% honey solution and were able to oviposit on filter paper. The colonies were reared at 25 ± 1 °C and a 16:8 (L:D) photoperiod. This experiment was performed with pre-weighed, newly-moulted (0–6 h after ecdysis) 3rd instar larvae.

Bioassays:

***Toxicity*:**

***Acute toxicity*:** The acute toxicity, measured as mortality after 24 hours of exposure, was determined by topical application to early 3rd instar larvae of *S. littoralis*.

The stock solutions of fractions (or α-tocopherol) were dissolved in acetone as a carrier, and each larva received 1 µL of the solution per treatment, with acetone alone as the control treatment. A range of six doses (8, 15, 30, 50, 80 and 100 µg) for larvae were used to establish the lethal doses. Four replications of 20 larvae were tested per dose. The doses were applied to the dorsum of each larva's body using a repeating topical dispenser attached to 100 µl syringes. All treated larvae from each replicate were transferred to the relevant diet in plastic boxes measuring (10 × 10 × 7 cm), which were closed using perforated caps to make sure that the experiment was not affected by the fumigation effect of the acetone. The boxes were placed for 24 hours in a growth chamber (L16:D9, 25°C). Death was recorded when the larvae did not respond to prodding with forceps.

***Chronic toxicity*:** Chronic toxicity of fractions (or α-tocopherol), measured as mortality after 5 days, was determined by oral application to early 3rd instar larvae of *S. littorralis*.

The maximum dose of 200 µg/cm^2 of tomato leaves was applied with the aim of determining the chronic toxicity of the extracts. The fractions (or α-tocopherol) were dissolved in acetone (20 mg of the extract in 1 ml acetone) and the solution thus prepared was applied to the tomato leaf discs (surface area 2 cm^2) using an automatic pipette, at a dosage of 10 µL per cm^2 of the disc area. After the solvent evaporated, the tomato discs were inserted in Petri dishes (6 cm in diameter) with an agar bottom layer (height 3 mm) in order to maintain stable moisture. A prepared diet (tomato discs) was thus administered *ad libitum* to new larvae of *S. littoralis*, 3rd instar. Larval mortality was assessed 5 days after the experiment was established. The fractions which caused >50% mortality were chosen for determining lethal doses. Diets contaminated with fractions (or α-tocopherol) in 8 doses (200, 150, 100, 80, 50, 30, 15 and 10 µg/cm^2) were administered to *S. littoralis* larvae in order to determine lethal doses; the diet was prepared identically as described above.

Four replications of 20 larvae were tested per dose. All larvae from each replicate were transferred into plastic boxes (15 × 15 × 7 cm). The boxes were placed for 5 days in a growth chamber (L16:D8, 25°C). Death was recorded when the larvae did not respond to prodding with forceps.

***Effect on larval growth*:** Diet (leaf discs) containing fractions (or α-tocopherol) at a dose of 25 µg/cm^2 was administered to *S. littoralis* larvae in order to determine the efficiency of the extract on larval growth. The tomato discs were prepared identically as described above. Newly emerged 3rd instar were weighed and placed individually into Petri dishes (6 cm in diameter). A contaminated diet was given to the larvae *ad libitum* for 5 days. Subsequently, the larvae were weighed, and the growth inhibition index was calculated based on the determined weight increments according to the formula: GI (%) = 100-[(T/C)*100)], where C and T are weight increments of the larvae that consumed the control and contaminated diets, respectively (Pavela, 2011a).

Twenty new larvae of the 3rd instar were always tested for every dose. The experiment was placed in the growth room (L16:D8, 25°C). The experiment was replicated 3 times.

***Antifeedant activity*:** The no-choice test was chosen to determine antifeedant activity, since its design most closely approximates a practical application (Pavela, 2010). *S. littoralis* larvae were left with no food before the experiment, always for 5 hours. The experiment itself was done in Petri dishes (9 cm in diameter). Damp filter paper was laid on the bottom of the dishes, and 4 discs, 1.5 cm in diameter and prepared using cork borer from tomatoes leaves, were always placed on the filter paper.

An adequate amount of stock solution of fractions (or α-tocopherol) was dissolved in acetone to obtain a 10.0, 5.0, 1.0, 0.5, 0.25, 0.12, 0.06 and 0.03 % (w/v) solution. 20 µl of the solution was uniformly applied to every disc using an automatic dosing device, corresponding to doses of approximately 1000, 500, 100, 50, 25, 12.5, 6.2 and 3.1 µg/cm^2. Discs to which only the solvent had been applied were used as the control. After application, the leaf discs were left at rest for approximately 10 minutes to allow the solvent to evaporate. Afterwards, 2 starved larvae of *S. littoralis* were inserted into the centre of every dish. The entire experiment was done in 15 repetitions. The experiment was terminated when the control larvae had consumed approximately 90% of the leaf discs (about 10

hours, and 25°C). The area of the leaf discs consumed by larvae was then assessed and compared with control discs, using a screener software program (unpublished), to determine antifeedant activity.

From test data, the following could be calculated: feeding deterrence index:

(FDI) = 100*[(C.T)/(C + T)], where C and T are the control and treated leaf consumed by the insect (Pavela, 2010).

***Statistical analysis*:** *Acute and chronic toxicity*: Experimental tests demonstrated that more than 20% of the controlled mortality was discharged and repeated. When the controlled mortality reached 1-20%, the observed mortality was corrected using Abbott's formula.(Abbott, 1987) Probit analysis of dose-mortality data was conducted to estimate the LD_{50} and LD_{90} values and associated 95% confidence limits for each treatment (Finney, 1971).

Doses causing 50% (ED_{50}) and 90% (ED_{90}) FDI, including corresponding values within a 95% confidence limit (CI_{95}), were estimated using probit analysis applied to the FDI values found.(Finney, 1971) Data with numbers and percentages were subjected to the HOVTEST = LEVENE option of SAS to account for homogeneity of variance and normality. In the case of non-homogeneity, percent values were transformed using the arcsine–square-root (arcsine√) transformation, and insect count values were transformed by the square root (√) transformation before running an ANOVA (Gomez and Gomez, 1984). Treatment differences in the GI were determined by Tukey's test. Differences between means were considered significant at $P \leq 0.05$ (SAS, 2000).

RESULTS

***Toxicity*:** The effect of the obtained fractions on larval mortality of *S. littoralis* is shown in Table 1. A significant difference ($F=96.53$; $P<0.01$ and $F=82.61$; $P<0.01$ for acute and chronic toxicity, respectively) was found in the efficacy of individual fractions.

After topical application of the highest tested dose of 100 µg per larva, only fractions 5 and 6 caused mortality higher than 50%, while fraction 5 showed significantly higher efficacy (LD_{50} = 35 µg/larvae) compared to fraction 6 (LD_{50} = 71 µg/larvae). When presented to the larvae in their feed, the greatest effect was shown by fractions 1 and 2, which caused mortality higher than 50% after application of the highest dose of 200 µg/cm^2. A comparison of the lethal doses showed that fraction 2 was significantly more efficient (LD_{50} = 42 µg/cm^2) compared to fraction 1 (LD_{50} = 75 µg/cm^2).

***Effect on larval growth and antifeedant activity*:** Table 2 presents efficient doses that caused contaminated feed intake inhibition in the larvae by 50% and 90% compared to untreated feed. All fractions caused feed intake inhibition; however, significant differences ($F=41.16$; $P<0.01$; Table 2) were found among the fractions. Fraction 5 showed the greatest efficacy, with the ED_{50} estimated at 2 µg/cm^2 and ED_{90} at 26 µg/cm^2, and thus significantly less compared to the other fractions obtained. All fractions also inhibited larval growth in the larvae that consumed feed contaminated with the fractions at a dose of 25 µg/cm^2. The greatest inhibition of larval growth was observed for fractions 2, 5 and 6, where the larvae of *S. littoralis* grew less by more than 90% compared to the control.

Based on an evaluation of biological efficacy, fractions 2 and 5 were chosen as those providing the greatest effect. Qualitative and quantitative analysis of major substances was performed for these fractions. Results of the analyses are shown in Table 3.

***Identification of major substances in the most efficient fractions*:** Fractions 1, 2 and 5 were subjected to chemical analysis in order to determine the qualitative content of major substances. These fractions were chosen based on a comparison of their biological efficacy. Fractions 1 and 2 were chosen as the only fractions that caused significant chronic toxicity, while fraction 5 exhibited the greatest effect on acute toxicity and feeding deterrent activity, compared to the other fractions.

Analysis of fractions 1 and 2: Analytes were detected with GC-MS techniques without/with trimethylsilylation with BSTFA. Identification of analytes was based on comparing the EI spectra with the NIST library, and also on the basis of commercially available standards.

Fraction 1: The major peak, with molecular weight of 430.6 and major mass fragments of 165.2 and 205.2 *m/z* (relative height 95 and 17%, respectively), was identified as α-tocopherol (Fig. 6). The structure was supported by analysis with the (+)-α-tocopherol standard, which showed the same retention behaviour and MS spectra.

Fraction 2: The presence of γ-tocopherol in fraction 2, with molecular weight of 416.5 and major mass fragment of 191.2 and 151.3 *m/z* (relative height 89 and 32%, respectively), was confirmed by analysis with the tocobiol commercial standard, which includes both β- and γ-tocopherol. These two have the same molecular weight, but on the basis of EI fragmentation γ-tocopherol (Fig. 6) was identified (80% compliance with the NIST library of γ-tocopherol, compared to 0.9% consistent with β-tocopherol). The structure was also supported by analyzing the derivatized sample, where the major peak was at the molecular weight 488.7 and fragment of 223.3 (24%) *m/z*. This data corresponds with the trimethylsilyl derivate of γ-tocopherol, and was also found in the tocobiol mixture with the same retention time and identical MS spectra.

Analysis of the tocobiol mixture confirmed the presence of two identical methyl esters of fatty acids in F2 (retention time 35.3 and 35.6 min).

Table 1. Acute and chronic toxicity of *A. altissima* fractions against larvae of *Spodoptera littoralis*.

Fraction	Toxicity					
	Acute			Chronic		
	100 µg/larvae* (% ± S.E.)	LD_{50} (CI_{95})	Chi.***	200 µg/cm²* (% ± S.E.)	LD_{50} (CI_{95})	Chi.***
1	33.3 ± 3.6^{c}	> 100		83.3 ± 9.2^{e}	75 (63-89)	1.707
2	6.7 ± 3.2^{b}	> 100		93.3 ± 8.1^{e}	42 (28-59)	0.708
3	6.9 ± 1.3^{b}	> 100		43.3 ± 4.0^{d}	> 200	
4	30.1 ± 7.1^{c}	> 100		33.4 ± 1.6^{c}	> 200	
5	81.7 ± 5.4^{d}	35 (28-46)	0.023	36.7 ± 1.8^{cd}	> 200	
6	68.5 ± 7.2^{cd}	71 (65-82)	1.838	10.5 ± 2.7^{b}	> 200	
7	38.9 ± 5.7^{c}	> 100		33.6 ± 2.5^{c}	> 200	
8	3.3 ± 0.8^{b}	> 100		16.7 ± 4.1^{bc}	> 200	
9	0.0 ± 0.0^{a}	> 100		0.0 ± 0.0^{a}	> 200	
α-tocopherol	28.3 ± 3.6^{c}	> 100		72.5 ± 7.3^{e}	77 (55-97)	0.467
P; F-value**	0.0001; 96.53			0.0001; 82.61		

* Average mortality in % obtained after application; the uppermost limit (100 µg/larvae or 200 µg/cm²) for ascertaining the activity of fractions of *A. altissima* on acute or chronic toxicity. Mean values followed by same letters in a column are statistically not significant by Tukey's HSD at p<0.05. **ANOVA parameters. CI_{95} denotes confidence intervals, compound activity is considered significantly different when the 95 % CI fail to overlap, *** Chi = Chi-square value, significant at P < 0.05 level.

Table 2. ffective doses (µg/cm²) causing 50% (ED_{50}) and 90% (ED_{90}) feeding deterrence and growth inhibition of *S. littoralis* larvae relative to the control.

Fraction	Feeding deterrent activity			GI***
	ED_{50} (CI_{95})	ED_{90} (CI_{95})	Chi*	(% ± S.E.)
1	201 (182-227)	412 (359-452)	4.017	43.5±8.2^{b}
2	171 (151-196)	420 (337-486)	1.585	96.9±1.8^{c}
3	119 (102-138)	378 (301-410)	0.576	56.6±2.9^{b}
4	300 (269-355)	1058 (920-1102)	0.028	13.4±5.9^{a}
5	2 (2-3)	26 (18-31)	1.811	92.2±2.5^{c}
6	22 (18-27)	163 (119-187)	6.459	98.9±0.7^{c}
7	221 (185-268)	1170 (938-1243)	4.334	85.9±1.6^{c}
8	176 (159-196)	358 (302-475)	0.107	51.7±4.1^{b}
9	175 (154-202)	462 (361-498)	2.333	51.8±0.6^{b}
α-tocopherol	222 (178-253)	429 (398-483)	2.333	76.2±7.8^{bc}
P; F-value**				0.0001; 41.16

CI_{95}: 95% confidence intervals are shown in parenthesis. *Chi-square value, significant at P < 0.05 level. **ANOVA parameters. ***Average growth inhibition in % obtained after application dose 25 µg/cm² of fractions from *A. altissima*.

Analysis of fraction 5: The identity of the five major compounds (Compounds A-E) presented in fraction 5 was evaluated according to the obtained accurate masses, in both positive and negative ion mode, using the Elemental Composition editor and Mass Fragment software (after in-source collision). Three major compounds were then characterized as follows:

Compound A was characterized by MS spectra of m/z 377.1589 (positive ion mode; ES+) and 375.1438 (ES−), which corresponds with Elemental Composition of $C_{20}H_{24}O_7$ with obtained mass error of Δ = −2.9 ppm (ES+), Δ = −1.6 ppm (ES−) and i-FIT (norm) error of 0.2 (ES+) and 0.0 (ES−). MS spectrum is showed in Fig. 1.

Compound B was characterized by MS spectra of m/z 379.1742 (ES+) and 377.1609 (ES−), which corresponds with Elemental Composition of $C_{20}H_{26}O_7$ with obtained mass error of Δ = −4.0 ppm (ES+), Δ = 2.4 ppm (ES−) and i-FIT (norm) error of 0.1 (ES+) and 0.0 (ES−). MS spectrum is showed in Fig. 2.

Compound C was characterized by MS spectra of m/z 495.2229 (ES+) and 493.2066 (ES−), which corresponds with Elemental Composition of $C_{25}H_{34}O_{10}$ with obtained mass error of Δ = −0.2 ppm (ES+), Δ = −1.6 ppm (ES−) and

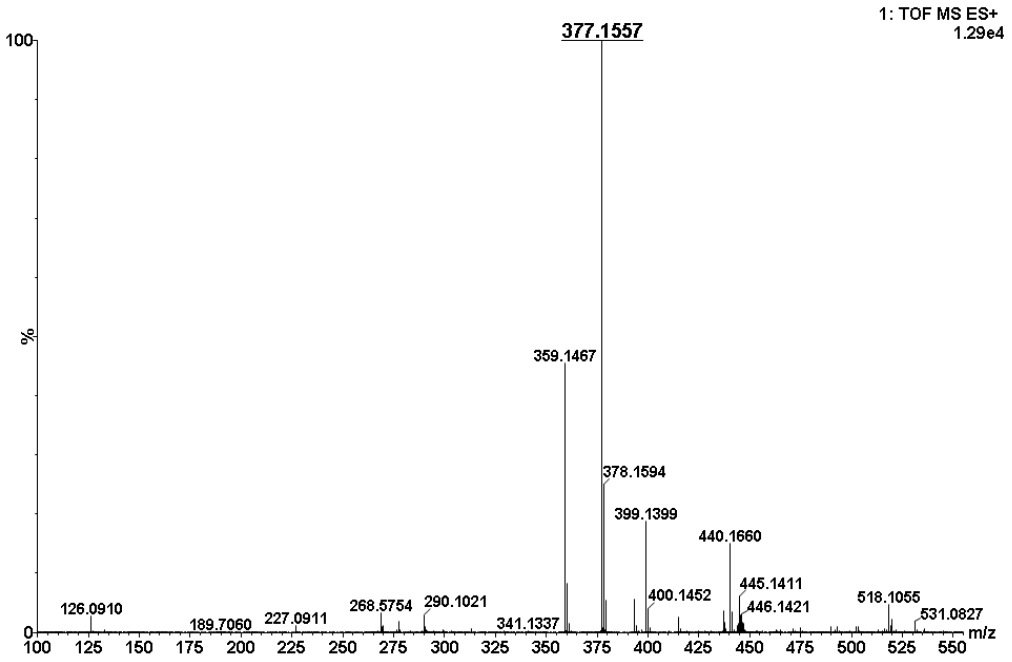

Figure 1. MS spectrum of Ailanthone, tR=3.71 min.

For chromatographic and MS conditions, see chapter 2.3.2. in Experimental section.

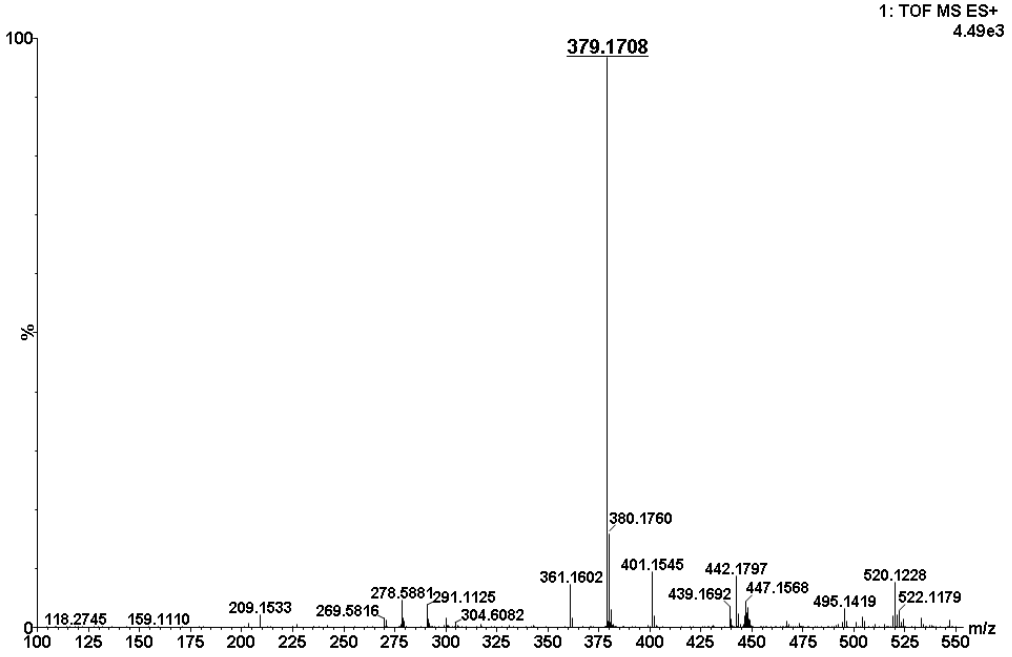

Figure 2. MS spectrum of Chaparinone, tR=3.50 min

For chromatographic and MS conditions, see chapter 2.3.2. in Experimental section.

i-FIT (norm) error of 0.0 (ES+) and 0.0 (ES−). MS spectrum is showed in Figure 3.

Compound D was characterized by MS spectra of m/z 493.2070 (ES+) and 491.1917 (ES−), which corresponds with Elemental Composition of $C_{25}H_{32}O_{10}$ with obtained mass error of $\Delta = -0.8$ ppm (ES+), $\Delta = 0.0$ ppm (ES−) and i-FIT (norm) error of 0.8 (ES+) and 0.2 (ES−). MS spectrum is showed in Fig. 4.

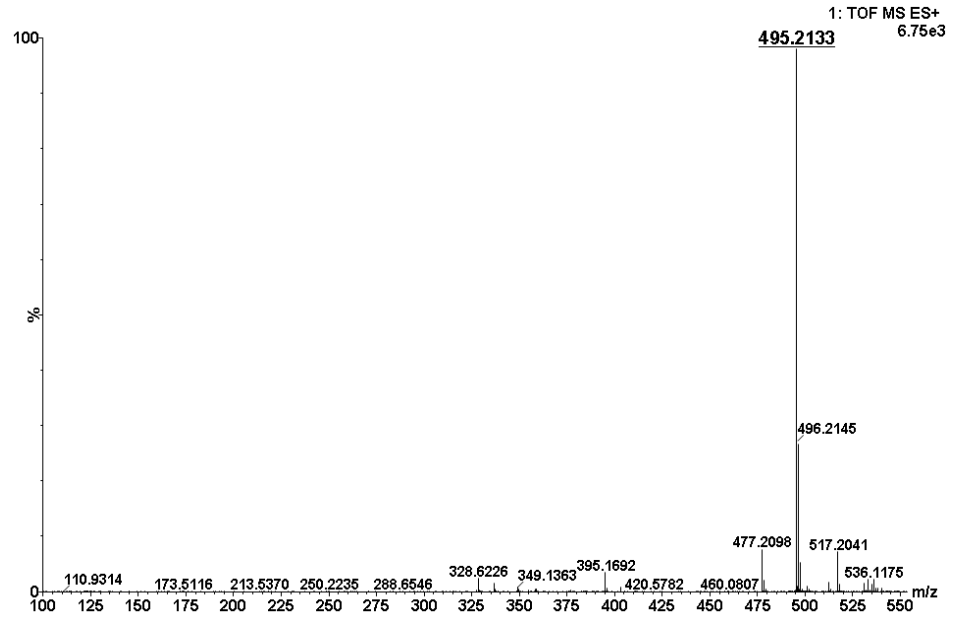

Figure 3. MS spectrum of Glaucarubinone, tR=7.05min

For chromatographic and MS conditions, see chapter 2.3.2. in Experimental section.

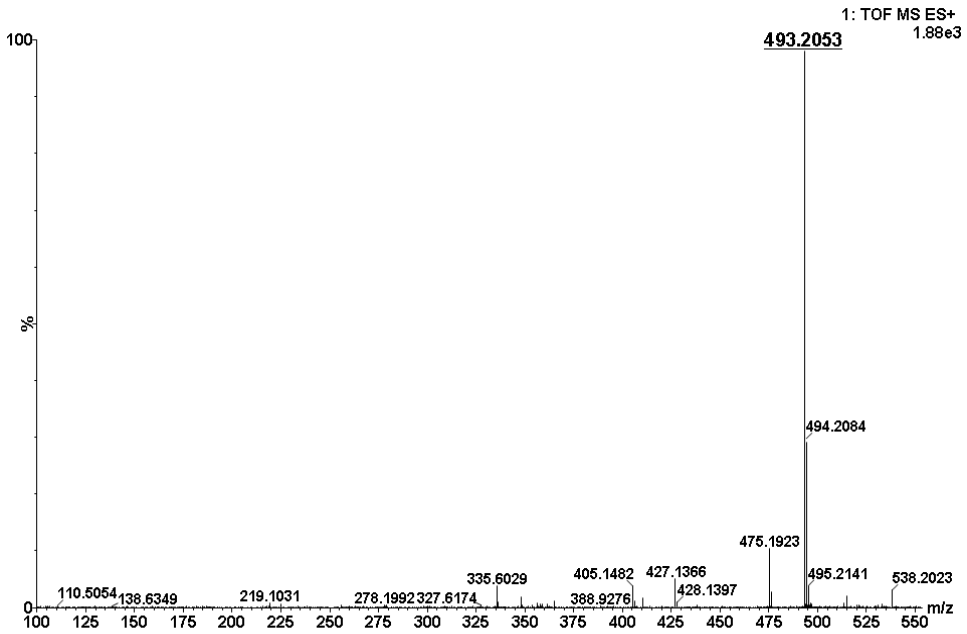

Figure 4. MS spectrum of 13(18)-Dehydroglaucarubinone, tR=7.76min

For chromatographic and MS conditions, see chapter 2.3.2. in Experimental section.

Compound E was characterized by MS spectra of m/z 365.1962 (ES+) and 363.1808 (ES−), which corresponds with Elemental Composition of $C_{20}H_{28}O_6$ with obtained mass error of $\Delta = -0.5$ ppm (ES+), $\Delta = 0.0$ ppm (ES−) and i-FIT (norm) error of 0.0 (ES+) and 0.5 (ES−). MS spectrum is showed in Fig. 5.

In general, for a ToFMS having a mass resolution of about 10,000 FWHM and external calibration, accurate mass measurement is secured (Williams *et al*., 2006). The available applications of Mass Lynx software, namely the Elemental Composition editor and Mass Fragment software, were employed for verification of the compounds' identity.

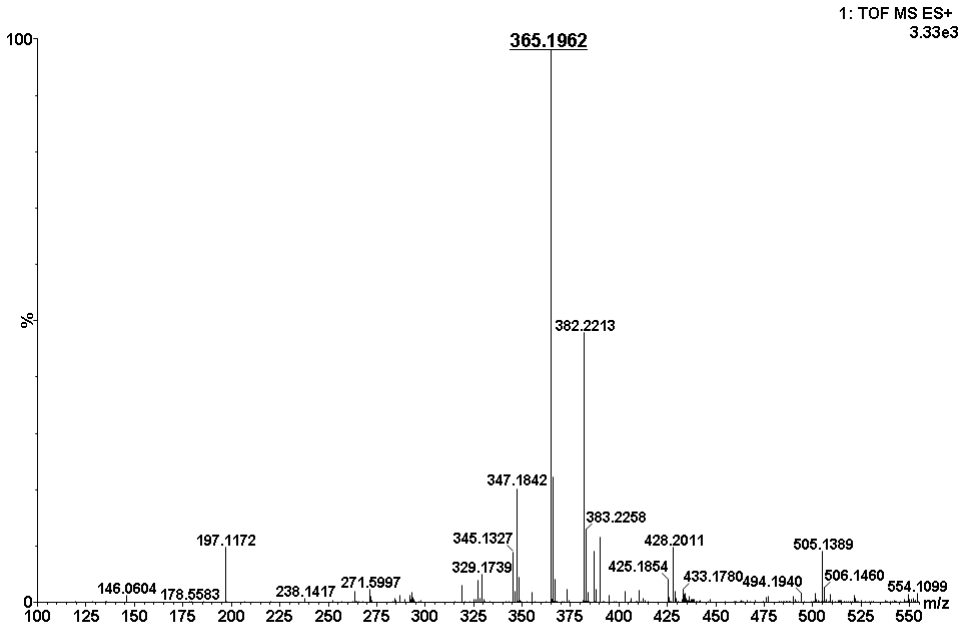

Figure 5. MS spectrum of Shinjulactone H, tR=5.07min

For chromatographic and MS conditions, see chapter 2.3.2. in Experimental section.

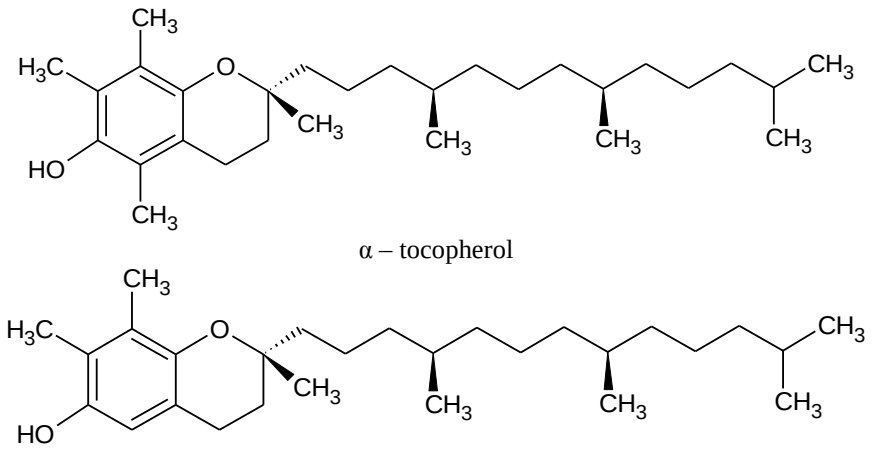

Figure 6. Structures of tocopherols detected in fractions 1 and 2.

The Elemental Composition editor uses a CHNO algorithm, which provides standard functionalities such as mass measurement error (compared to the predicted mass), double bond equivalence, isotope predictive filtering (i-FIT), and an isotope modelling application for comparison of the theoretical and measured isotope pattern. The i-FIT value provides an exact numerical comparison of the theoretical and measured isotopic pattern of a compound. Mass Fragment software assigns structures to observed fragment ions, if in-source fragmentation is employed, by applying novel algorithms to known precursor structures. This approach is based on systematic bond disconnection of the precursor structure (Carrasco-Pancorbo *et al*., 2008; Peters *et al*., 2009).

The obtained molecular formulas were compared with previously published data dealing with the isolation of natural compounds from the same plant species (*Ailanthus* spp.) (Beutler *et al*., 2009; Jaziri *et al*., 1987; Joshi *et al*., 2003; Lin *et al*., 1995; Rahman *et al*., 1997). The identity of the substances was also confirmed by Mass Fragment software after in-source fragmentation, and by corresponding DAD UV/VIS-spectra. As presumed, the

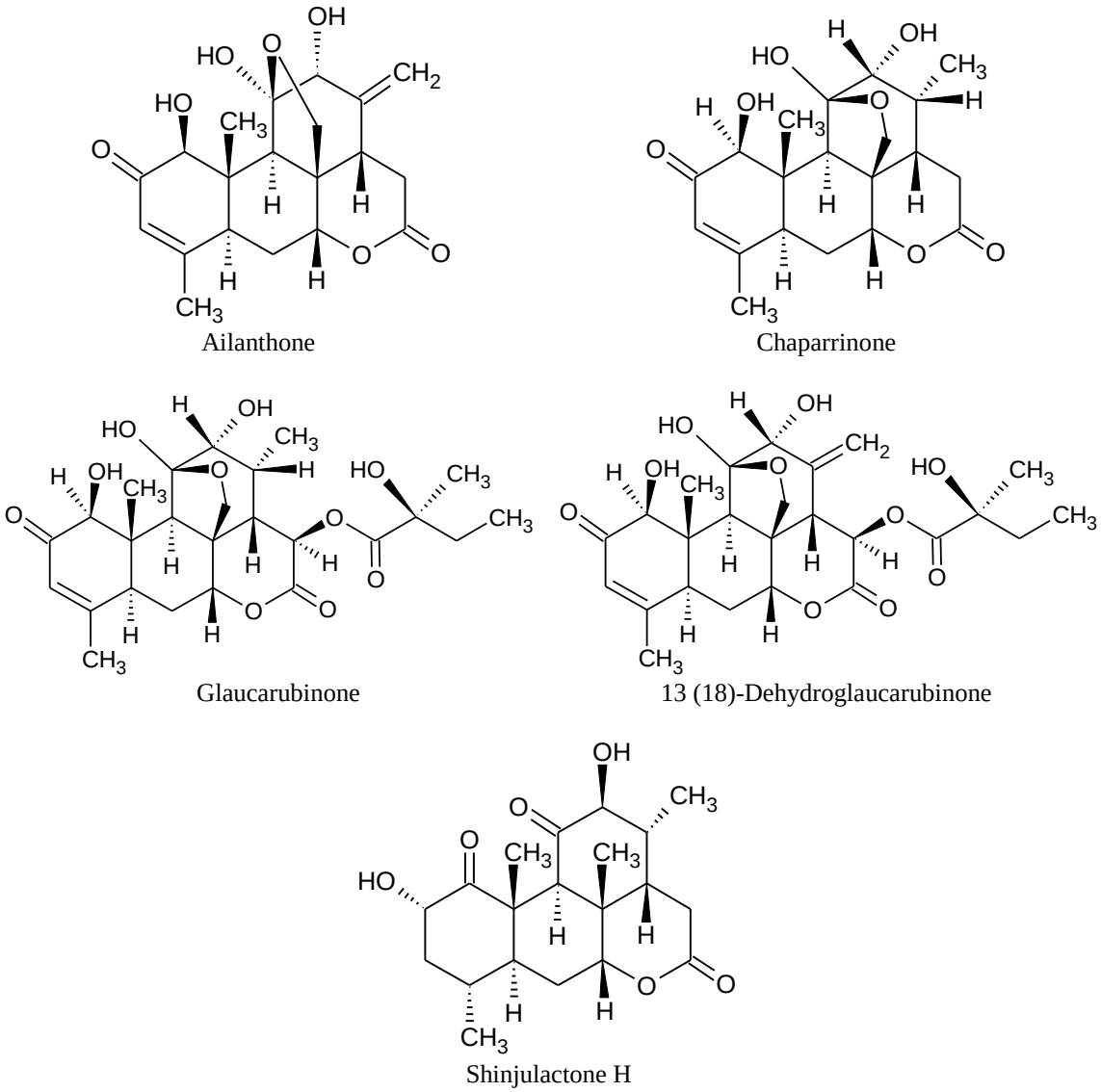

Figure 7. Structures of quassinoids detected in fraction 5

three compounds were characterized as quassinoids (Fig. 7): Compound A – Ailanthone (molecular formula $C_{20}H_{24}O_7$); Compound B – Chaparrinone ($C_{20}H_{26}O_7$); Compound C – Glaucarubinone ($C_{25}H_{34}O_{10}$); Compound D – 13(18)-Dehydroglaucarubinone ($C_{25}H_{32}O_{10}$); and Compound E – Shinjulactone H ($C_{20}H_{28}O_6$).

***Verification of biological efficacy of α-tocopherol*:** The efficacy of α-tocopherol on toxicity is shown in Table 1. It was found that while the acute toxicity of α-tocopherol was low (23.3% after application of a dose of 100 µg per larva), chronic toxicity corresponded to the efficacy of fractions 1 and 2, and LD_{50} was estimated at 77 µg/cm^2. Feed intake and larval growth inhibition is shown in Table 2. It was found that α-tocopherol significantly inhibited larval growth following a dose of 25 µg/cm^2 (GI = 76%). Feeding deterrent activity was estimated on the same level as fractions 1 and 2 (ED_{50} = 222 µg/cm^2).

DISCUSSION

In our work, we have explained the biological efficacy of a methanol extract from *A. altissima* leaves, which was divided into nine fractions using silica gel column chromatography. A different efficacy was found among individual fractions, in terms of both acute and chronic toxicity, as well as growth inhibition of *S littoralis* larvae. We thus successfully explained the mechanism of action of some substances contained in the extract. As previously found, methanol extracts obtained from the leaves of *Ailanthus altissima* provide significant insecticidal effects

(Kraus *et al*., 1994; Kundu and Laskar, 2010; Pavela, 2011a). However, it has not been explained until now which of the substances contained in the leaves are responsible for the insecticidal efficacy, and what the mechanism is of their action on the phytophagous larvae of butterflies.

In this work, we found a different mechanism of action between fractions obtained at the beginning of the separation (1 and 2) and fractions obtained in the middle of the separation period (5). While fractions 1 and 2 were the only ones to cause significant chronic toxicity accompanied by growth inhibition, fractions 5, by contrast, caused acute toxicity, associated with significant antifeedant efficacy and a related strong inhibition of *S. littoralis* larval growth. The content of major substances was determined for these most efficient fractions. A major proportion of two tocopherol isomers was determined in the fractions causing chronic toxicity. As far as we know, this is the first paper that describes the insecticidal efficacy of vitamin E on insect mortality. For this reason, we used identical biological tests to verify the efficacy of pure α-tocopherol. As clearly indicated by the results in Tables 1 and 2, the tests confirmed a similarity of effect between α-tocopherol and fractions 1 and 2.

The role of vitamin E in insects has not been quite explained; however, the work of other authors indicates that vitamin E plays an important role in biochemical actions related to the fertility of insects and their fat reserves. (Canavoso *et al*., 2001) mentioned possible associations between vitamin E and increased fecundity of moths and beetles. Moreover, absence of tocopherols in an artificial diet appeared to be responsible for a failure to produce viable sperm in the cricket *Acheta domestica* (Dadd, 1985; McFarlane, 1983). Other reports suggest that Vitamin E could be essential for the growth and reproduction of certain dipteran parasites(House, 1966), and even for the reproduction of honey-bees (Shekiladze, 1971). The actual presence and accumulation of α-tocopherol was found in *Drosophila*, (Parker and McCormick, 2005) and tocopheryl acetates were recently identified as a component of exocrine secretions in the squash beetle (Attygalle *et al*., 1996). All this information indicates that the mechanism of action of tocopherols on mortality of the phytophagous larvae of *S. littoralis* was associated with metabolic and/or hormonal disorders, caused by a gradual overdose of vitamin E provided in the feed (Pavela, 2010). However, further research is needed to confirm or disprove this presumption, and additional research is also needed to explore the different efficacies of individual tocopherol isomers, given that, as indicated by our tests, a significant difference was found between fraction 1, with a major proportion of α-tocopherol, and fraction 2, with a major content of γ-tocopherol.

In addition, vitamin E is an important antioxidant, which is also important in animal feed; it can therefore be assumed that when relatively high or repeated application doses are used, any potential residues may have no negative effects on human health and non-target organisms (Brigelius-Flohe and Traber, 1999).

Five major quassinoids (Ailanthone, Chaparrinone, Glaucarubinone, 13(18)-Dehydroglaucarubinone and Shinjulactone H) were detected in fraction 5, making this the fraction that showed the greatest efficacy on acute toxicity and feed intake inhibition of *S. littoralis*. Insecticidal and antifeedant activities of quassinoids have been known and studied before. For example, four semi-synthetic and fourteen quassinoids were tested for their antifeedant and insecticidal activity against 3rd instar larvae of the diamondback moth (*Plutella xylostella*) (Daido *et al*., 1995). The results of this work indicate that methoxyl groups at C_2 and/or C_{12}, or a methylenedioxy group between C_{11} and C_{12}, were required for both activities. The very weak activity suggests that the conjugated double bond system from C_{11} to C_{16} did not contribute to the antifeedant and insecticidal activities (Daido *et al*., 1995).

(Lidert *et al*., 1987) reported the effect of forty-six natural and semi-synthetic quassinoids on feeding of the tobacco budworm *(Heliothis virescens).* Their activity is compared to that of the well-known antifeedant azadirachtin from *Azadirachta indica* Juss. Four quassinoids, indaquassin C**,** samaderins C, B and A, isolated from the seeds and bark of *Samadera indica*, were tested for insect antifeedance against the tobaco cutworm, *Spodoptera litura* (Govindachari *et al*., 2001). Indaquassin C was the most effective antifeedant. Bioassay-directed fractionation of a methanol extract of the stem bark of *Ailanthus excelsa* led to the isolation and identification of the anti-feedant constituent excelsin. The leaf disc method of bioassay showed the potency of excelsin to prevent feeding to be 75.94%, at 1000 ppm concentration, against *Spilosoma oblique* (Kundu and Laskar, 2010).

Until now, the insecticidal efficacy of quassinoids was believed to be based on strong antifeedant efficacy, which has been associated with strong inhibition of contaminated feed intake, starvation and subsequent inhibition of larval growth resulting in death (Curcino Vieira and Braz-Felho, 2006; Daido *et al*., 1995). Efficacy on acute toxicity after contact application was also found in our tests. Thus the mechanism of action of some quassinoids seems to be probably associated with an effect on the nervous system of the insect. However, further research will be needed to demonstrate the exact mechanism of action of quassinoids from *A. altissima* on insects. In addition, mixtures of several active substances may exhibit a synergistic effect, similarly as in other secondary plant metabolites (Hummelbrunner and Isman, 2001; Pavela, 2008). In addition, more research will be needed, focused not only on the insecticidal efficacy of potential botanical insecticides containing quassinoids like a. i., but also on any phytotoxicity of these potential products, given that herbicidal effects have been found in some

quassinoids (for example, Ailanthone, detected by us) (Pedersini *et al*., 2011).

Our tests indicate that the insecticidal efficacy of vitamin E isomers may play an important role in the development of new, environmentally safe botanical insecticides. Similarly, plant extracts containing quassinoids can be recommended for the development of botanical insecticides. Based on our results, methanol extracts or active substances obtained from *A. altissima* leaves can be recommended for the development of new botanical insecticides targeted against some phytophagous larval species of butterfly pests.

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