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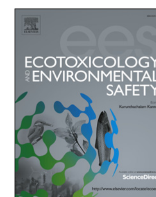

Insecticidal properties of *Solanum nigrum* and *Armoracia rusticana* extracts on reproduction and development of *Drosophila melanogaster*

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ABSTRACT

Plant-derived substances, because of high biological activity, arouse interest of many scientists. Thus, plant extracts and pure substances are intensively studied on various insects as potential insecticides. In such studies, *D. melanogaster* is one of the most important model organisms. In our studies, we analysed the contents of two plant extracts and tested the activity of their main components against fruit flies and compared observed effects to effects caused by crude extracts. Then, we assessed the development of the next, unexposed generation. The chemical analysis of extracts revealed the presence of numerous glycoalkaloids and glucosinolates in *Solanum nigrum* and *Armoracia rusticana* extracts. These extracts, as well as their main components, revealed lethal and sublethal effects, such as the altered developmental time of various life stages and malformations of imagoes. Interestingly, the results for the extracts and pure main compounds often varied. Some of the results were also observed in the unexposed generation. These results confirm that the tested plants produce a range of substances with potential insecticidal effects. The different effects of extracts and pure main components suggest the presence of minor compounds, which should be tested as insecticides.

1. Introduction

Biologically active agents, defined as biopesticides, generally have several advantages compared to conventional pesticides (Alyokhin et al., 2008). Chemical pesticides are responsible for the extensive pollution of the environment. They cause serious health hazards due to the presence of their residues in food; development of resistance in targeted insect pest populations; and decrease the biodiversity (Chowański et al., 2014). Biopesticides, in contrast, are inherently less toxic to humans and the environment, do not leave harmful residues and are typically more specific to target pests (Sporleder and Lacey, 2013). These compounds generally decompose rapidly, and some biopesticide compounds, such as semiochemicals, are used in small doses. To effectively use biopesticides, it is necessary to have good knowledge about managing the particular pests or pest complexes. Due to limited commercial use, biopesticides are often developed by research institutions rather than by the pesticide industry (Damalas and Koutroubas, 2018). While effective active ingredients have been discovered, biopesticide products might lack appropriate formulations for efficient field use. A broader set of perspectives in the design and launch of biopesticides would be helpful. Farmers often consider biopesticides as alternatives to chemical pesticides, in which the active ingredient is thought to be synthetic, having a similar mode of action to the chemical pesticide. However, biopesticides considerably differ in their modes of action from conventional chemical pesticides, and their modes of action are almost always specific. A key step is to estimate the correct dose, necessitating intensive research into the pest infestation pattern on plants (Alyokhin et al., 2008). A low or inadequate dosage would lead

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to a failure of protection, leading to the abandonment of the programme by the farmer in favour of other methods that yield immediate rewards, such as chemical pesticides. Therefore, efficiently using biopesticides requires specific user knowledge on the agent and the target pest for optimizing application time, field rates, and application intervals. Biopesticides vary, and therefore the application of the same environmental and consumer safety criteria to all of these compounds is impossible.

Secondary plant metabolites play an important role in plants. Basically, these organic compounds are produced to decrease herbivory. They show cytotoxic, antifungal, zoocidal (Bennett and Wallsgrove, 1994) and antimicrobial (Bednarek, 2012) activity. Amongst other compounds, glycoalkaloids [GAs] have drawn the attention of many entomologists, who focus on plant protection and study relations between plants and herbivorous insects (Friedman, 2015). The sublethal and lethal activities of both pure alkaloids and extracts obtained from plants have frequently been described (Chowański et al., 2016). These substances can alter metabolic pathways, cause ultrastructural and morphological malformations, alter the duration of larval, pupal and imaginal development, show deterrent activity and cause the death of insects (Ibanez et al., 2012). Consequently, these compounds can limit the feeding of insects (Nenaah, 2011b) and may lead to increased crop yield. Next, natural substances can replace or at least lower a number of synthetic insecticides present in the environment. The important advantage of plant-derived substances is that these compounds are recommended for organic farming, an intensively developing branch of agriculture. In such cases, plant-derived substances are relatively cheap and their extracts are easy to obtain, making these compounds available for crop protection with less environmental damage and for small-area farmers.

Solanaceae is a family of plants that contain many substances with potential to be used for plant protection. Moreover, many *Solanaceae* plants are harvested as popular, widely distributed crops. Therefore, the organs that are not harvested but contain active substances can be easily obtained in large quantities and used against pests. In a previous study (Ventrella et al., 2016), we described the effects of extracts and pure glycoalkaloids (GAs) obtained from two widely cultivated plants, tomato (*Lycopersicon esculentum* Mill.) and potato (*Solanum tuberosum* L.), on the development of fruit flies (*Drosophila (Sophophora) melanogaster*). We observed developmental abnormalities and malformations of the body: deformations of wings and abdomens, smaller black abdominal zone or overall smaller body size. To some extent, these changes were also observed in the next, unexposed generation. These findings supported the hypothesis that agricultural plants can be sources of substances used as pesticides or deterrents (Atanu et al., 2011).

Wild plants contain various alkaloids and other substances that have insecticidal potential. Therefore, we decided to examine the effects of extracts and pure alkaloids present in black nightshade (*S. nigrum*) berries against the model organism, *D. melanogaster*. This plant is widely dispersed across the globe and can be easily obtained from various areas and widely used, implying that detailed studies on the toxic activities of the extracts obtained from this plant, as well as its pure alkaloids, should be conducted in relation to various insect species.

Moreover, other plant families produce bioactive compounds too. *Brassicaceae* are amongst the most important plants, particularly from an agro-economic point of view. Horseradish (*Armoracia rusticana*) is commonly grown and used as a spice due to the properties of its roots. Similar to *S. nigrum*, horseradish grows in various regions around the world. This plant is also used in crop protection, mainly traditional, but there are scientific reports describing its effects on insects (Kinae et al., 2000; Tedeschi et al., 2011). For example, sinigrin is a glucosinolate (GLS) crucial for the flavour of horseradish and perhaps might play an important role in the insecticidal activity of horseradish extracts.

Notably, it is important to test not only the pure substances obtained from plants but also extracts, for which activity is occasionally underestimated. The explanation of the activity of single substances is crucial for basic studies, particularly when the research leads to new commercially used insecticides. Additionally, the amount of toxic substances in extracts may vary from season to season and may depend on environmental and genetic factors (Dreger et al., 2012; Srivastava and Srivastava, 2010). However, the substances present in extracts may act synergistically and reveal potent activity, as previously shown for various *Solanaceae* plant extracts on beetles (Ventrella et al., 2015). Such effects were also demonstrated for other biological phenomena, including the toxic effect of plant extracts and animal venoms (Lee and Bae, 2016; Nenaah, 2011a; Smith et al., 2001). Thus, these effects limit the amount of substances necessary to obtain the desired effect, due to a multiplied effect of mixtures. Therefore, the use of plant extracts may lead to further limitations of the use of synthetic insecticides.

The purpose of the present study was to assess the effect of extracts obtained from black nightshade unripe fruits (*S. nigrum*) and horseradish sprouts (*A. rusticana*) and their main pure metabolites, solamargine and sinigrin, respectively, on the development of *D. melanogaster*. Although this species is not an important crop pest, it is regarded as one of the most important model organisms for testing insecticide activity and the development of resistance. The well-described genome and the development and physiology of fruit flies suggested that pesticides, particularly new compounds, should be tested on *Drosophila* to assess activities and development of resistance in exposed insects at various levels (Daborn et al., 2012; Pontecorvo and Fantaccione, 2006). The effects of pesticides on *D. melanogaster* have frequently been described (Akmoutsou et al., 2011; Yun et al., 2017). We primarily focused on sublethal developmental effects, but lethality was also estimated. Two generations of insects were tested. The first generation was exposed to plant extracts and pure metabolites, whereas the next generation was maintained under control conditions to observe whether the exposure may affect filial generations of insects or not. Such a situation may indicate that although the lethality may be low, the effect on pests would be prolonged.

2. Materials and methods

2.1. Extractions and analytical features

The voucher specimens of both plants were deposited at Herbarium Lucanum (HLUC, Potenza, Italy) with the ID Code: 2320 and 9197 for *S. nigrum* and *A. rusticana*, respectively. Green unripe berries were harvested from autochthonous black nightshade (*S. nigrum* L.) plants (pooled from 7 different individuals). The extraction procedure is based on the method of Cataldi et al. (2005). The vegetable samples were lyophilized and ground to a fine powder by using a laboratory mill; then, 1.5 g of sample was placed in 20 mL of 1% acetic acid aqueous solution. To facilitate contact between the plant tissue and the extraction solvent, the suspension was stirred for approximately 2 h and then centrifuged at 6000 rpm for 30 min. The obtained pellet was suspended in 5 mL of 1% acetic acid, shaken, and centrifuged, and the two supernatants were subsequently mixed together. To remove solid particles, the extract was filtered by a single-use 0.22 µm nylon filter (Whatman, Maidstone, UK) and then injected into the LC/MS system. All chemical analysis were conducted at Department of Sciences, University of Basilicata by Prof. Sabino Bufo's team.

The sprouts were harvested from *A. rusticana* P. Gaertner, B. Meyer & Scherbius plants from a horseradish field established at the Institute of Plant Genetics-National Research Council, located in Policoro (MT). After harvest, the vegetable material was immediately washed and stored at −20 °C or frozen in liquid nitrogen and stored at −80 °C until extraction. The extraction was performed according to Agneta et al. (2012). Briefly, the sprout samples were lyophilized and homogenized into a fine powder by using a laboratory mill. Subsequently, polypropylene tubes containing 200 mg of dry material were placed in a

water bath and heated at 75 °C for 1 min. For GLS extraction, 2 mL of 70% methanol (75 °C) was added to the sample and briefly vortexed, incubated for 10 min in a water bath at 75 °C, mixed twice on a vortex mixer, and centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was collected and filtered through a 0.22 µm nylon filter (Whatman, Maidstone, UK). The extraction procedure was repeated again with 2 mL of 10% methanol, as previously described.

Plant tissue analyses were performed by using a Surveyor Autosampling LC system, interfaced to a LIT-FTICR mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) via electrospray source, equipped with a 20 W CO_2-laser (Synrad, Mukilteo, WA, USA; 10.6 µm) as described elsewhere (Bianco et al., 2012). The following source settings were used for the ionization of GAs: ESI needle voltage, + 4.5 kV; capillary voltage, + 35 V; temperature of the heated capillary, 300 °C; and sheath gas (N_2) flow rate of 80 arbitrary units (a.u.). The following source settings were used for the ionization of GLSs: ESI needle voltage, −4.6 kV; capillary voltage, −22 V; temperature of the heated capillary, 350 °C; and sheath gas (N_2) flow rate of 80 arbitrary units (a.u.). The LTQ and FTICR mass spectrometers were calibrated according to manufacturer's instructions using a solution of sodium dodecyl sulfate (*m/z* 265) and sodium taurocholate (*m/z* 514) (Handbook of instrument).

The chromatographic separation of GAs was performed at an ambient temperature on a Supelcosil LC-ABZ, amide-C_{16} HLPC column (5 m, 250 × 4.6 mm) with a guard column of the same material (Supelco Inc., Bellefonte, PA, USA) and a mobile phase consisting of 0.1% formic acid in water (solvent A) and methanol (solvent B). The following gradient at 0.8 mL/min was applied: 30–43% B in 0–8 min; 43–60% B in 8–20 min; and 60% B in 20–24 min. Prior to the next injection, the column was equilibrated for 6 min.

The chromatographic separation of GLSs was performed at an ambient temperature on a Discovery C_{18} column, 250 × 4.6 mm i.d., 5 mm particle size, equipped with a Discovery C_{18} 20 × 4 mm i.d. security guard cartridge (Supelco Inc., Bellefonte, PA, USA) and a mobile phase consisting of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The following gradient at 0.8 mL/min was applied: 10–24% B in 0–10 min; 24–60% B in 10–12 min; and 60–10% B in 12–15 min. Prior to the next injection, the column was equilibrated for 5 min. The injection volume was 20 µL, and the flow to the source was reduced to 200 µL/min by a post-column splitter. Mass spectrometric data were acquired in the positive ion mode while scanning *m/z* 50–1300 at a rate of 2 scans/s for GAs and in the negative ion mode while scanning *m/z* 50–1000 at a rate of 2 scans/s for GLSs (Agneta et al., 2012).

The data were acquired and processed by the Xcalibur software package (version 2.0 SR1; Thermo Fisher Scientific). The chromatographic raw data were imported, elaborated, and plotted by using SigmaPlot 10.0 software (Systat Software, Inc., London, UK).

2.2. Insects and exposure

The wild-type *D. [Sophophora] melanogaster* Meigen flies [Diptera: Drosophilidae] were obtained from a culture maintained at the Department of Genetics, Adam Mickiewicz University in Poznań, Poland. We used wild-type Oregon R strain of fruit flies. Control and exposed insects were maintained on modified sugar/yeast [SY] culture medium, as previously described (Ventrella et al., 2016). The tested substances [extracts or single, pure solamargine (Glycomix, United Kingdom) and sinigrin (Sigma-Aldrich, Steinheim, Germany)] were added to the culture medium as water solutions, which was poured in each vial to obtain the following final concentrations in the medium: 0.005, 0.05, 0.5, 5 and 50 µM. Stock solutions at concentration 1 mM of extracts, and pure solamargine and sinigrin were prepared by dissolving lyophilized extracts, and solamargine and sinigrin powder in 0.1% acetic acid water solution (slightly acidic pH values enhanced solubilisation of GAs), thus to control medium 0.1% acetic acid was added. The experiments carried out on *D. melanogaster* were performed at the Faculty of Biology, Adam Mickiewicz University.

7-day-old virgin females of wild type were collected and crossed to males for mating overnight. Next, three fertilized females were transferred to new ones with medium containing tested samples and maintained to lay eggs [approximately 100 eggs per vial]. Three vials in three repetitions were used per each sample and concentration. After 24 h, the imagoes were removed from the vials, and the development of the juveniles present in the vials, named as exposed [parental or P] generation, was observed. Number of third instar larvae, pupae and imagoes [males and females] were counted throughout the testing period (28 days). The adult flies were immediately removed from the vials. As previously described, the time when 50% of the population reached larval, pupal and imaginal developmental stage [T_{50}] was calculated (Ventrella et al., 2016). Additionally, the percentages of dead pupae and malformed imagoes were determined. Malformed insects were observed and described by the Stereo Lumar V12 microscope (Zeiss, Germany). On the basis of the number of organisms in control and exposed groups, a percentage of the final number of organisms [FNO] compared to the control was determined according to Eq. 1: FNO = 100 [T – C]/C; where: T — final number of organisms counted in tested sample-containing vials; C — final number of organisms counted in control vials. Next, three adult females and three adult males from each parent generation [P] were transferred to another vial containing the control medium. After mating overnight, the mated flies laid eggs and the first generation [F_1] completed their development. The same parameters were observed in that generation.

2.3. Statistics

The data analysis was performed by R 2.10.1 software. The results were expressed as the means ± SD of three replicates of each series of experiments. The data were analysed with analysis of variance, ANOVA, or Kruskal-Wallis test was used if non-normal distribution was noted. The differences between control and tested group were analysed with Tukey Post-hoc test. Values at $p < 0.05$ were regarded as significantly different from the control (Ventrella et al., 2016). To determine the correlations between the concentration of the extracts or pure alkaloids and measured factors [number of organisms, malformations, T_{50}], Pearson's correlation coefficient was calculated, and a significant correlation was shown when the value was higher than 0.7 or lower than -0.7.

3. Results

3.1. Glycoalkaloids and glucosinolates in S. nigrum and A. rusticana extracts

Total ion chromatogram (TIC) provides a good summary of the ions being separated/detected, but it works best when the analytes dominate the ion signals in any given retention time window or at least when there is a good signal-to-noise (S/N) ratio and a low-drifting baseline. The use of extracted ion chromatograms by LIT MS, generated with a tight mass-to-charge ratio window of ± 0.5 units around each selected protonated molecule, greatly reduces the signal complexity of the total ion current trace.

As presented in Table 1, the chromatographic peaks of GAs in a sample extract of black nightshade unripe berries were identified (malonylated derivatives are not reported). All GAs found in unripe berries gave good signals corresponding to the protonated precursor ion, $[M+H]^+$. In addition, the structures of the GAs were confirmed by extensive sequential MS analysis. Several common product ions were observed, which were generated by losses of the sugar moiety or aglycone fragmentation in the B- or E-ring, which can provide information on the accurate mass of aglycone and the primary sequence and branching of the oligosaccharide chains. The multistage CID made it feasible to understand the fragmentation pathways, thereby

Table 1
GAs identified in extract of black nightshade unripe berries by LC-ESI-LIT MS: peak number (N°), retention time (t_R), common name, mass/charge ratio (*m/z*) of precursor ion $[M+H]^+$ and characteristic fragments observed in MS/MS and MS^3 spectra.

N°	t_R (min)	GA Common Name	$[M+H]^+$ (*m/z*)	Characteristic Fragments	
1	5.6	Solanidadienol Chacotriose	866.4	*m/z* 412.4	$[Solanidadienol+H]^+$
					$[M\text{-}Chacotriose+H]^+$
				m/z 720.4	$[M\text{-}Rha+H]^+$
				m/z 848.4	$[M\text{-}H_2O+H]^+$
2	4.4	Solanidenetriol Solatriose	916.3	*m/z* 410.5	$[M\text{-}Solatriose\text{-}2\ H_2O+H]^+$
					$[Solanidenetriol\text{-}2\ H_2O+H]^+$
				m/z 718.3	$[M\text{-}Glc\text{-}2\ H_2O+H]^+$
				m/z 862.4	$[M\text{-}3\ H_2O+H]^+$
				m/z 880.4	$[M\text{-}2\ H_2O+H]^+$
				m/z 898.3	$[M\text{-}H_2O+H]^+$
3	7.3	Spirosolentriol Chacotriose	916.3	*m/z* 408.3	$[M\text{-}Chacotriose\text{-}3\ H_2O+H]^+$
				m/z 426.1	$[M\text{-}Chacotriose\text{-}2\ H_2O+H]^+$
				m/z 444.2	$[M\text{-}Chacotriose\text{-}H_2O+H]^+$
					$[Spirosolentriol\text{-}H_2O+H]^+$
				m/z 752.4	$[M\text{-}Rha\text{-}H_2O+H]^+$
				m/z 880.4	$[M\text{-}2\ H_2O+H]^+$
				m/z 898.5	$[M\text{-}H_2O+H]^+$
4	5.5	Solanidenetriol Chacotriose	900.3	*m/z* 410.2	$[M\text{-}Chacotriose\text{-}2\ H_2O+H]^+$
					$[Solanidenetriol\text{-}2\ H_2O+H]^+$
				m/z 718.4	$[M\text{-}Rha\text{-}2\ H_2O+H]^+$
				m/z 846.4	$[M\text{-}3\ H_2O+H]^+$
				m/z 864.5	$[M\text{-}2\ H_2O+H]^+$
				m/z 882.4	$[M\text{-}H_2O+H]^+$
5	8.5	Spirosolenol Solatriose	900.3	*m/z* 394.4	$[Spirosolenol\text{-}2\ H_2O+H]^+$
					$[M\text{-}Solatriose\text{-}2\ H_2O+H]^+$
				m/z 702.4	$[M\text{-}Glc\text{-}2\ H_2O+H]^+$
				m/z 718.5	$[M\text{-}Rha\text{-}2\ H_2O+H]^+$
				m/z 736.3	$[M\text{-}Rha\text{-}H_2O+H]^+$
				m/z 864.5	$[M\text{-}2\ H_2O+H]^+$
				m/z 882.5	$[M\text{-}H_2O+H]^+$
6	9.2	α-Chaconine	852.5	*m/z* 398.5	$[Solanidine+H]^+$
					$[M\text{-}Chacotriose+H]^+$
				m/z 706.5	$[M\text{-}Rha+H]^+$
7	11.4	Spirosolenol Chacotriose	884.4	*m/z* 394.2	$[M\text{-}Chacotriose\text{-}2\ H_2O+H]^+$
				m/z 412.3	$[Spirosolenol\text{-}H_2O+H]^+$ $[M\text{-}Chacotriose\text{-}H_2O+H]^+$
				m/z 702.4	$[M\text{-}Rha\text{-}2\ H_2O+H]^+$
				m/z 848.5	$[M\text{-}2\ H_2O+H]^+$
				m/z 866.4	$[M\text{-}H_2O+H]^+$
8	13.6	Solasonine	884.4	*m/z* 378.4	$[Solasodine\text{-}2\ H_2O+H]^+$
				m/z 396.5	$[Solasodine\text{-}H_2O+H]^+$
				m/z 414.2	$Solasodine+H]^+$ $[M\text{-}Solatriose+H]^+$
				m/z 704.4	$[M\text{-}Glc\text{-}H_2O+H]^+$
				m/z 720.5	$[M\text{-}Rha\text{-}H_2O+H]^+$
				m/z 866.4	$[M\text{-}H_2O+H]^+$
9	9.0	α-Solanine	868.5	*m/z* 380.5	$[Solanidine\text{-}H_2O+H]^+$
				m/z 398.5	$[Solanidine+H]^+$ $[M\text{-}Solatriose+H]^+$
				m/z 560.5	$[M\text{-}Rha\text{-}Glc+H]^+$
				m/z 706.5	$[M\text{-}Glc+H]^+$
				m/z 722.5	$[M\text{-}Rha+H]^+$
10	14.1	Solamargine	868.4	*m/z* 378.3	$[Solasodine\text{-}2\ H_2O+H]^+$
				m/z 396.4	$[Solasodine\text{-}H_2O+H]^+$
				m/z 414.2	$[Solasodine+H]^+$ $[M\text{-}Chacotriose+H]^+$
				m/z 557.8	$[M\text{-}2\ Rha\text{-}H_2O+H]^+$
				m/z 704.4	$[M\text{-}Rha\text{-}H_2O+H]^+$
				m/z 850.4	$[M\text{-}H_2O+H]^+$

substantially improving the identification of unknown compounds.

The LC/ESI-FTICR TIC analysis of the black nightshade berries extract confirmed the presence of two main glycoalkaloids, solamargine and solasonine, and showed the presence of a third compound not yet reported in the literature for *S. nigrum*, malonyl-solamargine (Fig. 1). In addition to the main GAs, several minor GAs were displayed in the chromatograms through the narrow window extracted ion chromatograms (XICs) of each compound (± 1 mDa) from the complex matrix of berry pulp (data not shown) and tentatively identified. Among all types of GAs found in berries extracts, only solasonine and solamargine were identified with standard compounds. The identification of the other compounds was based on chromatographic behaviour, accurate mass measurements, fragmentation analyses, and comparison with the data from the literature.

Altogether 10 different GAs were tentatively identified in *S. nigrum* (Fig. 2A): solanidadienol chacotriose, solanidenetriol solatriose, spirosolentriol chacotriose, solanidenetriol chacotriose, spirosolenol solatriose, α-chaconine, spirosolenol chacotriose, solasonine, α-solanine and solamargine. We cannot exclude the presence of other minor GAs that were not revealed by the instrument. In fact, during the synthesis of GAs in plants, the glycosylation of aglycone is achieved in a subsequent phase after the synthesis of aglycone and depends on the availability of the different sugars (Milner et al., 2011). Even if mass spectrometry cannot accurately distinguish structural-isomers, spirosolenol chacotriose and spirosolenol solatriose likely contain the aglycone 12-β-hydroxysolasodine. In fact, the presence of this aglycone

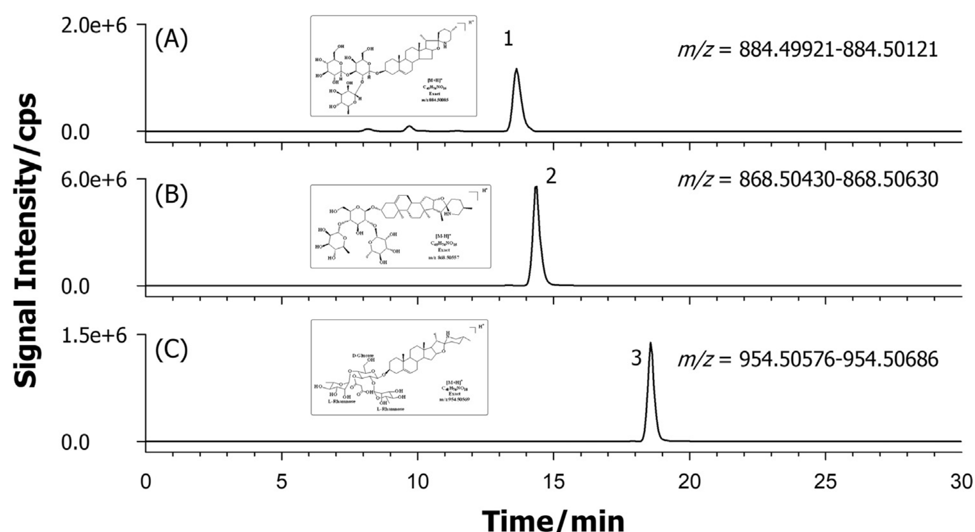

Fig. 1. LC/ESI-FTICR TIC chromatogram acquired in positive mode of a black nightshade berries extract. Mass spectra of three main peaks, corresponding to solasonine (found at *m/z* 884.50079), solamargine (found at *m/z* 868.50476), and malonyl-solamargine (found at *m/z* 954.50580) are reported in the insets.

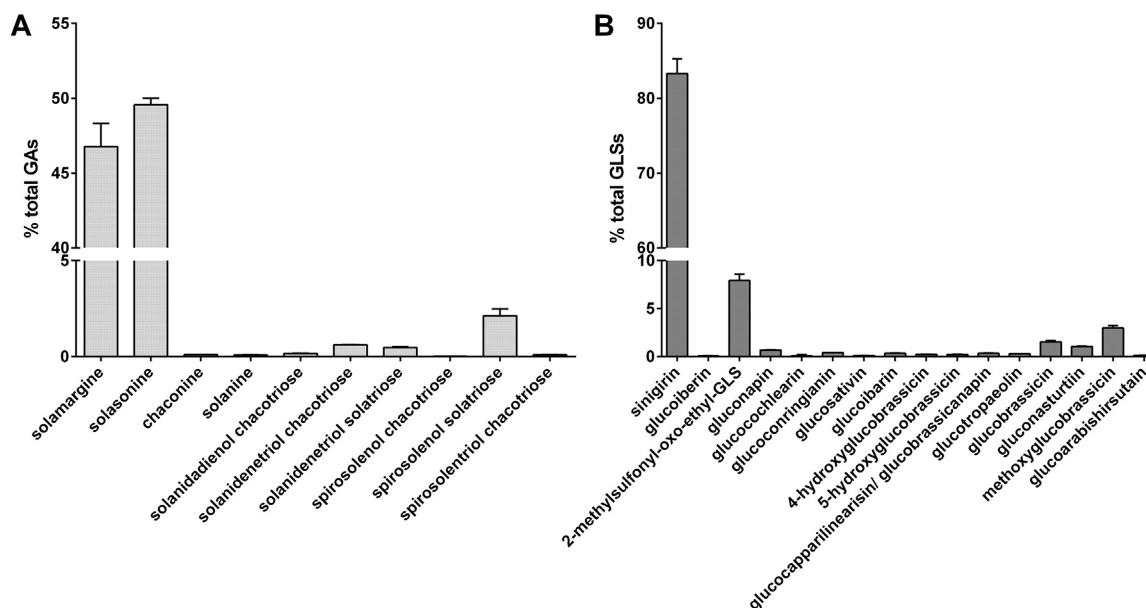

Fig. 2. The composition of glycoalkaloids in *S. nigrum* extract (A) and glucosinolates in *A. rusticana* extract (B). The results are presented as a percentage of the total amount of determined compound ± SD, $n = 3$.

in black nightshade has previously been shown (Pelletier, 1998).

Atanu et al. (2011) shown that absolute amount of GAs per leaf increased during leaf development, whereas the concentration of single substances declined. The small unripe fruits of *S. nigrum* had a high concentration of solasodine and solasodine-containing GAs [solamargine and solasonine], but the concentration and the absolute amount per fruit decreased with fruit maturation (Atanu et al., 2011). The analysed unripe berries contained a similar quantity of the two main GAs: solamargine: 138.4 ± 11.0 mg/100 g dry weight and solasonine: 149.3 ± 11.3 mg/100 g dry weight. The other GAs, which constitute less than 4% of dry weight include solanidadienol chacotriose, solanidenetriolsolatriose, spirosolentriol chacotriose, solanidenetriol chacotriose, spirosolenol solatriose, spirosolenol chacotriose, α-solanine, and α-chaconine.

The horseradish sprout extract analysis was performed with LC/ESI-FTICR TIC in negative ion mode. The GLS were identified as intact deprotonated molecules, $[M-H]-$, by using high-resolution LC–ESI–FTICR MS. Overall, 17 GLS were tentatively identified and reported previously (Agneta et al., 2012).

In sprouts of *A. rusticana*, seventeen different GLSs were found (Fig. 2B). The following substances were identified (Agneta et al., 2012): six major GLSs found in previous studies: sinigrin, which contributes to more than 4/5 of the total GLSs content, and other GLSs previously reported in the literature: glucobrassicin, gluconasturtin, gluconapin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin, two aliphatic GLSs having a side chain with 4 carbon atoms: 1-methylpropyl-GLS [glucocochlearin] and 2-methylpropyl-GLS [glucoconringianin]; three olefinic GLSs: 2-propenyl-GLS [sinigrin] as the main component of the extract, 3-methyl-3-butenyl [glucocapparilinearisin] or pentenyl-GLS [glucobrassicanapin], 3-butenyl-GLS

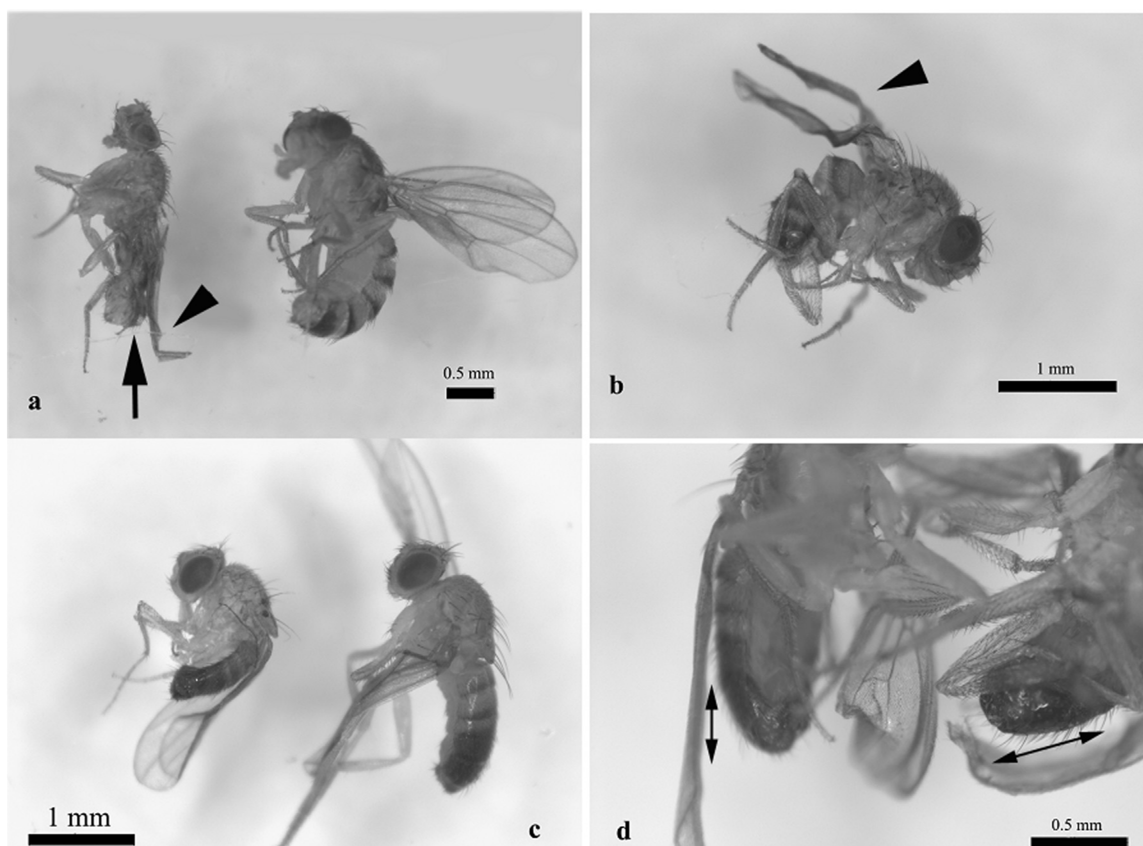

Fig. 3. Examples of the most prominent and most frequent malformations of *D. melanogaster* imagoes. a) Malformed abdomen and wings (left) compared to the control insect (right); b) deformed wings of a male insect with a curved body shape; c) smaller overall size of the body and curved body shape of the male (left) compared to the normal size of the control male (right); d) smaller dark abdominal zone of an exposed male (left) compared to that of a control insect (right). $n = 100$ per extract per concentration.

[gluconapin]; three aromatic GLSs: benzyl-GLS [glucotropaeolin], 2-phenylethyl-GLS [gluconasturtiin] and 2(R)-hydroxy-2-phenylethyl glucosinolate [glucobarbarin]; four indolic GLSs: 4-hydroxy-3-indolylmethyl-GLS [4-hydroxyglucobrassicin]; 5-hydroxy-3-indolylmethyl-GLS [5-hydroxyglucobrassicin]; 3-indolylmethyl-GLS [glucobrassicin]; 4-methoxy-3-indolylmethyl-GLS [4-methoxyglucobrassicin]; five GLSs having one sulfur atom in their side chain: 3-methylsulfinylpropyl-GLS [glucoiberin]; 2-methylsulfonyl-oxo-ethyl-GLS; [R]-7-[methylsulfinyl]heptyl-GLS [glucoibarin]; 4-mercaptybutyl-GLS [glucosativin] and 7-[methylthio]heptyl-GLS [glucoarabishirsutain]. Comparing the GLS classes, the aliphatic GLSs were predominant due to the significant contribution of sinigrin, which accounts for more than 83.2% of the total GLS (Agneta et al., 2012).

3.2. Effect of solamargine, sinigrin, S. nigrum and A. rusticana extract on D. melanogaster

All tested substances altered the development of fruit flies. In some cases, we observed lethal effects, but the most significant effects were sublethal, showing altered developmental time and malformations of imagoes. Overall, the body of the exposed insects was smaller and curved. The most prominent malformations, detected by a stereomicroscope, were found in the abdomen, which was shrunken and lost its turgidity, and in the wings, which were smaller and folded. Interestingly, the dark area of the abdomen, which is characteristic for males, was often reduced (Fig. 3). For a majority of the effects, we did not observe a dose-dependent intensity of changes [Tables 2–3].

Although *S. nigrum* extract and its main component, solamargine, did not lead to the high mortality of the insects of the exposed generation, these compounds significantly shortened the duration of the larval (*S. nigrum* extract: F = 79,0696, $p < 0.0001$; solamargine: F = 62.1096, $p < 0.0001$), pupal (*S. nigrum* extract: F = 100.0627, $p < 0.0001$; solamargine: F = 8.9432, $p < 0.0001$) and imaginal stages (*S. nigrum* extract: F = 185.7068, $p < 0.0001$; solamargine: F = 81.2936, $p < 0.0001$). However, there was a high correlation between extract/solamargine concentration and pupal mortality. Pure GA caused malformations more frequently than the extract, but the T_{50} factor was more altered by the extract than by solamargine [Table 2]. For the imagoes, this difference varied between 14% and 31%.

In the non-exposed generation, the T_{50} values were still significantly lower for insects exposed to the extract than for those exposed to the pure GAs in each stadium (larvae: *S. nigrum* extract vs solamargin t = 7.204, $p < 0.0001$; *A. rusticana* extract vs sinigrin t = 6.488, p = 0,0002; pupae: *S. nigrum* versus solamargin t = 3.599, p = 0.0070; *A. rusticana* versus sinigrin t = 4.645, p = 0,0017; imagoes: *S. nigrum* versus solamargin t = 5.884, p = 0.0004; *A. rusticana* versus sinigrin t = 3.725, p = 0,0058). This parameter was significantly lower than that in the control. For imagoes exposed to solamargine, the T_{50} values were insignificantly higher than those for insects exposed to the extracts. Additionally, the mortality and malformations did not significantly differ from those for the control [Table 3].

The tested substances caused significant lethal toxicity to fruit fly pupae exposed to two concentrations of sinigrin, i.e., 0.5 and 50 µM, where mortality reached ca. 25% of the exposed population (post-hoc test: 0.5 µM diff = -25.93, p = 0.0001; 50 µM diff = -24.22, p = 0.0002) [Table 2]. The final number of organisms was also significantly lower for groups exposed to sinigrin (post-hoc test: 0.005 µM diff = -12.00, p = 0.0003; 5 µM diff = -9.33, p = 0.0077; 50 µM diff = -8.33, p = 0.0229). In addition to the lethal effects, both the extract and its main components shortened the development of all

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Table 2
Effect of the pure substances (solamargine and sinigrin) and extracts of *S. nigrum* and *A. rusticana* on the parental [exposed] generation of *D. melanogaster*.

Concentration [μM]	Larvae	Pupae		Imagoes			
	T_{50} ± SD [days]	Mortality ± SD [%]	T_{50} ± SD [days]	Malformations ± SD [%]	N° final organisms	FNO	T_{50} ± SD [days]
Insects exposed to *S. nigrum extract*							
Control	13.2 ± 0.8^{a}	$1.5 \pm 2.6^{a,b}$	15.4 ± 0.3^{a}	0.0 ± 0.0^{a}	52.3 ± 9.6^{a}	0	21.2 ± 1.0^{a}
0.005	$8.6 \pm 0.3^{b,d}$	0.0 ± 0.0^{a}	9.5 ± 0.0^{b}	0.0 ± 0.0^{a}	$36.0 \pm 7.8^{b,d}$	− 31.2	13.8 ± 0.0^{b}
0.05	$9.4 \pm 0.3^{b,c,d}$	0.0 ± 0.0^{a}	10.7 ± 0.3^{e}	0.0 ± 0.0^{a}	$39.0 \pm 9.0^{b,d}$	− 25.5	13.8 ± 0.0^{b}
0.5	$8.8 \pm 1.0^{b,c}$	$4.2 \pm 7.2^{a,b}$	$9.8 \pm 1.2^{b,e,f}$	3.6 ± 3.1^{a}	$23.7 \pm 8.3^{b,c}$	− 54.8	$13.6 \pm 0.8^{a,b}$
5	$8.8 \pm 0.5^{b,c,}$	0.0 ± 0.0^{a}	$9.6 \pm 0.3^{b,c,f}$	$21.9 \pm 33.1^{a,b}$	19.7 ± 12.5^{c}	− 62.4	$13.5 \pm 0.6^{a,b}$
50	$9.7 \pm 0.0^{c,d}$	5.8 ± 2.9^{b}	$10.9 \pm 1.0^{d,e}$	36.9 ± 44.1^{b}	$27.0 \pm 6.0^{b,c,d}$	− 48.4	15.0 ± 0.8^{c}
Correlation coefficient	nc	0.74	nc	0.88	nc	nc	nc
Insects exposed to solamargine							
Control	15.2 ± 0.3^{a}	0.0 ± 0.0^{a}	19.8 ± 1.7^{a}	0.0 ± 0.0^{a}	21.3 ± 3.5^{a}	0	24.2 ± 1.7^{a}
0.005	12.2 ± 1.1^{b}	5.0 ± 8.7^{a}	16.9 ± 3.3^{b}	$22.5 \pm 21.5^{a,b}$	11.3 ± 5.5^{b}	− 46.9	21.1 ± 0.2^{b}
0.05	$12.0 \pm 0.5^{b,c,d}$	2.9 ± 5.0^{a}	16.5 ± 0.3^{b}	37.3 ± 13.3^{b}	$18.7 \pm 4.9^{a,b}$	− 12.5	20.4 ± 0.6^{b}
0.5	10.0 ± 0.3^{d}	3.8 ± 3.6^{a}	15.0 ± 0.9^{b}	$17.4 \pm 13.9^{a,b}$	$16.0 \pm 6.1^{a,b}$	− 25	16.3 ± 0.3^{c}
5	$12.5 \pm 0.9^{b,c}$	0.0 ± 0.0^{a}	16.4 ± 0.7^{b}	$18.9 \pm 20.1^{a,b}$	$19.3 \pm 0.6^{a,b}$	− 9.4	20.7 ± 0.7^{b}
50	10.7 ± 0.6^{d}	12.7 ± 2.5^{b}	16.2 ± 0.6^{b}	33.4 ± 20.6^{b}	$14.7 \pm 10.0^{a,b}$	− 31.2	20.9 ± 0.6^{b}
Correlation coefficient	nc	0.876	nc	nc	nc	nc	nc
Insects exposed to *A. rusticana* extract							
Control	13.2 ± 0.8^{a}	1.5 ± 0.3^{a}	15.4 ± 0.3^{a}	0.0 ± 0.0^{a}	52.3 ± 9.6^{a}	0	21.2 ± 1.0^{a}
0.005	8.8 ± 1.3^{b}	6.7 ± 7.7^{a}	$10.9 \pm 1.6^{b,c,d}$	35.6 ± 48.0^{b}	28.3 ± 9.5^{b}	− 45.9	14.4 ± 1.8^{b}
0.05	9.5 ± 1.2^{b}	10.5 ± 16.2^{a}	$11.3 \pm 1.0^{b,c}$	$14.9 \pm 9.1^{a,b}$	30.0 ± 12.5^{b}	− 42.7	14.1 ± 1.2^{b}
0.5	8.9 ± 0.3^{b}	0.7 ± 1.2^{a}	$10.2 \pm 0.9^{b,c,d}$	1.8 ± 3.1^{a}	33.0 ± 18.3^{b}	− 36.9	13.5 ± 0.0^{b}
5	8.6 ± 0.3^{b}	2.6 ± 4.4^{a}	$9.6 \pm 0.9^{b,d}$	93.5 ± 5.8^{c}	20.7 ± 15.9^{b}	− 61.1	13.5 ± 0.3^{b}
50	9.5 ± 0.3^{b}	2.6 ± 4.4^{a}	$10.2 \pm 0.3^{b,c,d}$	82.0 ± 7.7^{c}	26.3 ± 15.0^{b}	− 49.7	13.6 ± 0.0^{b}
Correlation coefficient	nc	nc	nc	nc	ns	ns	ns
Insects exposed to sinigrin							
Control	15.2 ± 0.3^{a}	0.0 ± 0.0^{a}	19.8 ± 1.7^{a}	0.0 ± 0.0^{a}	21.3 ± 3.5^{a}	0	24.2 ± 1.7^{a}
0.005	12.5 ± 1.3^{b}	0.0 ± 0.0^{a}	$18.0 \pm 1.0^{b,c,d,e}$	0.0 ± 0.0^{a}	9.3 ± 4.0^{b}	− 56.3	20.1 ± 0.2^{b}
0.05	$11.0 \pm 1.0^{b,c}$	25.9 ± 23.1^{b}	$18.5 \pm 0.5^{a,c,d,e}$	$17.5 \pm 12.6^{a,b}$	$16.7 \pm 6.1^{a,b}$	− 21.8	20.7 ± 0.7^{b}
0.5	10.1 ± 0.1^{c}	8.2 ± 8.3^{a}	$16.1 \pm 0.9^{b,e}$	31.8 ± 15.3^{b}	$16.0 \pm 6.6^{a,b}$	− 25.0	16.7 ± 0.3^{c}
5	$11.2 \pm 2.4^{b,c}$	0.0 ± 0.0^{a}	$16.8 \pm 0.7^{b,c,e}$	23.6 ± 12.0^{b}	12.0 ± 3.7^{b}	− 43.7	18.6 ± 0.6^{d}
50	9.4 ± 0.1^{c}	24.2 ± 7.3^{b}	14.9 ± 0.8^{b}	31.6 ± 22.0^{b}	13.0 ± 7.2^{b}	− 39.1	16.6 ± 0.3^{c}
Correlation coefficient	nc	nc	− 0.710	nc	nc	nc	nc

Data are presented as the means ± SD; $n = 3 \times 3$ replicates; statistically significant differences between groups are indicated by letters (the same letter means lack of significant differences); $p \leq 0.05$; positive values of FNO show that the number of organisms was higher in the tested groups than in the control, and negative values indicate that the number of individuals was higher in the control than in the exposed groups; nc – no correlation higher than 0.7 or lower than -0.7 was found. T_{50} = the time when 50% of the population reached larval, pupal and imaginal developmental stage, respectively.

stages of fruit flies significantly. The difference was, with only one exception, higher for the extract than that for sinigrin. For example, the mean T_{50} values for the lowest concentration of sinigrin were approximately 15, 20% and 27% higher (i.e., closer to control) than those for the extract for larvae, pupae and imagoes, respectively. However, for larvae, the differences were not always clearly in favour of the extracts. Pupal and imaginal intermoult were consistently shorter for insects exposed to extracts than for those exposed to pure compound (pupae: *S. nigrum* extract vs solamargin $t = 6.514$, $p = 0.0002$; *A. rusticana* extract vs sinigrin $t = 4.564$ $p = 0{,}0018$; imagoes: *S. nigrum* versus solamargin $t = 4.151$, $p = 0.0032$; *A. rusticana* versus sinigrin $t = 2.487$, $p = 0{,}0377$). The number of malformed imagoes was significantly higher for sinigrin when compared to control but not in all tested concentration (post-hoc test: 0.05 μM diff = 37.25, $p = 0.0003$; 50 μM diff = 33.41, $p = 0.0013$). Similarly, in insects exposed to the extract but significant changes were observed only for the highest applied concentration (post-hoc test: 50 μM diff = 36.86, $p = 0.0130$) [Table 2].

In the next, not-exposed generation, we did not observe significant mortality, neither within pupae nor imagoes. However, the larval (*S. nigrum* extract: $F = 27{,}1960$, $p < 0.0001$; solamargine: $F = 17{,}3826$, $p < 0.0001$; *A. rusticana* extract: $F = 70{,}8378$, $p < 0.0001$; sinigrin: $F = 7{,}2248$, $p < 0.0001$), pupal (*S. nigrum* extract: $F = 110{,}2790$, $p < 0.0001$; solamargine: $F = 14{,}8804$, $p < 0.0001$; *A. rusticana* extract: $F = 37{,}7707$, $p < 0.0001$; sinigrin: $F = 10{,}6210$, $p < 0.0001$) and imaginal (*S. nigrum* extract: $F = 119{,}2314$, $p < 0.0001$; solamargine: $F = 20{,}3219$, $p < 0.0001$; *A. rusticana* extract: $F = 94{,}5671$, $p < 0.0001$; sinigrin: $F = 18{,}6322$, $p < 0.0001$) development was faster for fruit flies exposed to the extracts solamargine and sinigrin, similar to the observations in the first generation. Similar to the results for *S. nigrum* and its main glycoalkaloid, the activity of pure glucosinolate was lower than of the extract. Both substances did not cause significant malforming activity, except for the highest concentration of the extract, where we observed malformations in approximately half of the insects [Table 3].

4. Discussion

The present study examines the effect of whole GAs profile extract of black nightshade unripe berries. Whereas, the direct effect of solamargine and solasonine, main components of the extract, as well as the presence of α-solanine and α-chaconine in leaves and berries are well known (Eldridge and Hockridge, 1983; Ikeda et al., 2000). Solanine was found in most parts of *S. nigrum*, but the highest levels were found in unripe berries and in the leaves of ripe plants (Atanu et al., 2011). The results showed that the concentration of the main GAs in black nightshade unripe berries, solamargine and solasonine, were similar. These two phytochemicals likely interact (additive or synergistic effect) to improve the defensive capacity of the plant. Solamargine and solasonine were also the two major components of eastern black nightshade *Solanum ptycanthum*, but in the berries of this plant, a solasonine/solamargine ratio equal to 1.5 was found (Eldridge and Hockridge, 1983). The accurate detection of GAs and their derivatives is of great interest because these compounds play a major role in disease resistance in *Solanaceae* plants and may be biologically active in animals and humans (Friedman, 2006). Thus, the occurrence and function of minor

Table 3
Effect of the pure substances (solamargine and sinigrin) and extracts of *S. nigrum* and *A. rusticana* on the filial [non-exposed] generation of *D. melanogaster*.

Concentration [μM]	Larvae	Pupae		Imagoes			
	T_{50} ± SD [days]	Mortality ± SD [%]	T_{50} ± SD [days]	Malformations ± SD [%]	N° final organisms	FNO	T_{50} ± SD [days]
Insects exposed to *S. nigrum* extract							
Control	12.5 ± 1.0^{a}	0.0 ± 0.0^{a}	15.1 ± 0.6^{a}	0.0 ± 0.0^{a}	18.0 ± 3.9^{a}	0	20.6 ± 1.5^{a}
0.005	7.0 ± 0.6^{b}	0.0 ± 0.0^{a}	8.0 ± 0.8^{b}	16.0 ± 9.6^{b,c}	30.0 ± 13.2^{b,c}	66.7	12.7 ± 0.6^{b,d}
0.05	7.1 ± 0.0^{b}	0.0 ± 0.0^{a}	10.4 ± 0.5^{b,c}	2.8 ± 4.8^{a,c}	19.0 ± 7.0^{a,c}	5.6	14.0 ± 0.5^{c,d}
0.5	6.8 ± 0.6^{b}	0.0 ± 0.0^{a}	7.7 ± 0.3^{b}	15.3 ± 15.6^{b,c}	14.0 ± 1.7^{a}	− 22.2	12.0 ± 0.3^{b}
5	6.8 ± 0.6^{b}	0.0 ± 0.0^{a}	8.5 ± 1.4^{b}	20.3 ± 17.6^{b}	22.0 ± 8.7^{a,c}	22.2	12.7 ± 0.8^{b,d}
50	7.8 ± 2.8^{b}	7.8 ± 13.6^{b}	9.6 ± 0.6^{c}	20.5 ± 12.4^{b}	23.0 ± 9.5^{a,c}	27.8	13.6 ± 1.0^{c,d}
Correlation coefficient	nc	nc	nc	nc	Nc	nc	nc
Insects exposed to solamargine							
Control	15.3 ± 2.3^{a}	0.0 ± 0.0^{a}	18.0 ± 4.2^{a}	0.0 ± 0.0^{a}	17.7 ± 8.0^{a}	0	24.5 ± 3.3^{a}
0.005	12.5 ± 0.0^{b,c}	16.8 ± 22.9^{b}	13.6 ± 1.0^{b}	4.7 ± 5.1^{a}	21.3 ± 10.0^{a}	20.8	19.4 ± 0.6^{b,c,d}
0.05	12.4 ± 0.5^{b,c}	2.7 ± 2.9^{a}	13.3 ± 0.3^{b}	10.7 ± 9.5^{b}	23. 7 ± 16.9^{a}	34	19.0 ± 0.3^{b,c,d}
0.5	16.0 ± 1.0^{a}	0.0 ± 0.0^{a}	13.5 ± 0.0^{b}	3.3 ± 5.8^{a}	11.0 ± 3.0^{a}	− 37,7	17.8 ± 1.5^{b,d}
5	14.8 ± 0.3^{a,c}	0.0 ± 0.0^{a}	18.5 ± 1.2^{a}	8.7 ± 5.2^{b}	52.0 ± 4.0^{b}	194.3	20.9 ± 0.6^{b,c}
50	13.8 ± 0.5^{c}	0.0 ± 0.0^{a}	14.6 ± 0.5^{b}	2.4 ± 4.2^{a}	8.0 ± 5.0^{a}	− 54.7	19.4 ± 0.8^{b,c,d}
Correlation coefficient	nc	nc	nc	nc	Nc	nc	nc
Insects exposed to *A. rusticana* extract							
Control	12.5 ± 1.0^{a}	0.0 ± 0.0^{a}	15.0 ± 0.6^{a}	0.0 ± 0.0^{a}	18.0 ± 3.9^{a}	0	20.6 ± 1.5^{a}
0.005	7.5 ± 1.0^{b,c}	0.0 ± 0.0^{a}	7.8 ± 1.2^{b}	2.5 ± 2.1^{a}	17.0 ± 10.0^{a}	− 5.6	12.2 ± 1.0^{b}
0.05	8.0 ± 0.6^{c}	0.0 ± 0.0^{a}	10.2 ± 1.2^{c}	0.0 ± 0.0^{a}	16.0 ± 6.0^{a}	− 11.1	14.6 ± 0.8^{b,c}
0.5	6.8 ± 0.6^{b}	0.0 ± 0.0^{a}	7.8 ± 1.8^{b}	22.2 ± 38.5^{a}	15.0 ± 4.0^{a}	− 16.7	11.9 ± 1.4^{b}
5	7.3 ± 0.5^{b,c}	0.0 ± 0.0^{a}	8.3 ± 1.8^{b,c}	34.1 ± 38.0^{a}	14.3 ± 7.1^{a}	− 20.4	12.6 ± 0.6^{b,c}
50	6.6 ± 0.6^{b}	0.0 ± 0.0^{a}	7.8 ± 1.4^{b}	45.6 ± 25.5^{b}	15.7 ± 7.2^{a}	− 12.9	12.0 ± 0.6^{b}
Correlation coefficient	nc	nc	nc	nc	Nc	nc	Nc
Insects exposed to sinigrin							
Control	15.3 ± 2.3^{a}	0.0 ± 0.0^{a}	18.0 ± 4.2^{a}	0.0 ± 0.0^{a}	17.7 ± 8.0^{a,b,c}	0	24.5 ± 3.3^{a}
0.005	11.5 ± 0.0^{b}	3.0 ± 6.1^{a,b}	12.5 ± 0.0^{b}	10.8 ± 10.1^{a}	8.0 ± 0.0^{a,b,c}	− 54.7	18.1 ± 1.2^{b,c,d}
0.05	11.5 ± 0.0^{b}	9.2 ± 16.0^{a,b}	12.5 ± 0.0^{b}	16.7 ± 28.9^{a}	6.3 ± 4.2^{a,b,c}	− 64	16.5 ± 0.0^{b,c,d}
0.5	12.0 ± 2.1^{b}	12.2 ± 12.0^{b}	13.7 ± 1.7^{b}	10.3 ± 13.1^{a}	17.7 ± 9.4^{a,b,c}	0	20.2 ± 2.2^{b,d}
5	11.8 ± 1.5^{b}	0.0 ± 0.0^{a}	12.9 ± 1.2^{b}	26.9 ± 22.3^{b}	25.3 ± 15.3^{a}	43.4	18.9 ± 2.4^{b,c,d}
50	10.5 ± 2.9^{b}	2.6 ± 4.4^{a,b}	14.4 ± 0.6^{b}	13.3 ± 11.6^{a}	24.3 ± 12.0^{a,c}	37.7	17.6 ± 0.6^{b,c,d}
Correlation coefficient	nc	nc	nc	nc	Nc	nc	Nc

Data are presented as the means ± SD; $n = 3 \times 3$ replicates; statistically significant differences between groups are indicated by letters (the same letter means lack of significant differences); $p \leq 0.05$; positive values of FNO show that the number of organisms was higher in the tested groups than in the control, and negative values indicate that the number of individuals was higher in the control than in the exposed groups; nc – no correlation higher than 0.7 or lower than − 0.7 was found. T_{50} = the time when 50% of the population reached larval, pupal and imaginal developmental stage, respectively.

glycoalkaloids in the extracts of *S. nigrum* berries was systematically investigated. Rich fragmentation patterns were produced during the ionization of GAs and malonyl-GAs, which provided sufficient resolution for a priori structure elucidation. Typical product ions were observed, generated by losses of the sugar moiety or aglycone fragmentation in the B- or E-ring, which can provide information on the accurate mass of aglycone and the primary sequence and branching of the oligosaccharide chains (Lelario et al., 2015).

The presence of the solamargine malonyl derivative (solamargine: 121.5 ± 9.0 mg/100 g dry weight) comparable to those of solamargine and solasonine in *S. nigrum* berries is of interest. Since the occurrence of a malonyl ester on the glucosyl moiety causes a negative charge and could facilitate the efficient transport of these compounds into vacuoles by anionic transporters, the malonylation of secondary metabolites is considered an important step in plant defence processes. Generally, malonyl derivatives are not detected; in fact, the demalonylation of the thermally labile malonyl glycosides during processing or sample preparation may explain the low or non-existent content reported in berries and berry products.

Little is known about the GLSs composition in horseradish. The present results confirmed the presence of six major GLSs found in previous studies: sinigrin, gluconasturtin, glucobrassicin, gluconapin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin (Li and Kushad, 2004; Redovniković et al., 2008). Moreover, other identified compounds showed *m/z* values of precursor ions, and mass spectra and retention times similar to those reported by several authors for GLSs present in different species of *Brassicaceae* (Cataldi et al., 2005; Clarke, 2010).

The results of studies on horseradish are consistent with those reported by Redovniković et al. (2008). In fact, sinigrin was quantitatively dominant in sprouts and constituted more than 80% of total GLSs (Fig. 2B). The concentration of sinigrin in horseradish extract was calculated as 29.2 ± 1.1 mM. GLSs were found in the roots, seeds, leaves and stems of plants, and young tissues contained the highest amounts of these compounds (Blažević and Mastelić, 2009). Additionally, several studies showed changes in the total and individual GLSs content as a function of tissue age (Brown et al., 2003).

The biological activity of both extracts and their main components was similar, as observed for *S. tuberosum* and L. *esculentum*. All exposed populations showed shorter times of development, and some insects revealed morphological malformations (Ventrella et al., 2016). Moreover, another plant-derived substance, azadirachtin, caused sublethal effects that led to population-level effects, as this compound decreased mating success and reduced the number of offspring (Oulhaci et al., 2017). Furthermore, other insect larvae exposed to plant extracts showed growth disruption and a range of structural deformities (Kabir et al., 2013). In contrast, prolonged development was reported for the Mediterranean fruit fly *Ceratitis capitata* fed the aqueous extracts of *Cestrum paraqui* (*Solanaceae*) (Zapata et al., 2006). This difference may be due to species-specific factors in both the insects tested and the plants used, or due to variations in the ranges of extract concentrations. However, the other results were similar: the number of pupae and adults as well as reproduction was decreased. These data suggest that the stress caused by alkaloids significantly affects insects. Two different developmental strategies are possible. The first strategy is to speed up development so that the insects can reach imaginal stages earlier and

migrate [i.e., fly away] from the area where the stressor is present (Ventrella et al., 2016). The second strategy is to increase the developmental time, since energy expenses are concentrated on detoxification, rather than development (Mareggiani et al., 2002; Zapata et al., 2006). Next, the size of insects also plays an important role in detoxification, and smaller individuals typically do not have large energy supplies; hence, they are regarded as less resistant to stress (Harrison et al., 2013). Since fruit fly larvae are rather small and not protected by a thick, highly impervious cuticle, thus, the toxicity of stress factors may increase during a long exposure. Next, these larvae are not mobile and feed in one place. Therefore, these insects cannot migrate from the exposed environment. Hence, we propose that fast development may be the most successful strategy for fighting stress. However, the cost of this strategy may be high, as imagoes may be smaller or carry various malformations (Ventrella et al., 2016). We propose that the observed malformations may not only show effects directly from the activity of the stressor but also indirectly from improper larval or pupal development. The nature of these two effects is not clear. Pure alkaloids caused more frequent malformations than extracts. However, development was much faster within populations exposed to both tested extracts than that amongst insects exposed to pure substances. This finding suggests the synergistic action of various substances present in plants. Such activity may limit the production of particular substances present in plant organs and simultaneously lead to high toxicity. Therefore, not only pure alkaloids but also plant extracts seem to have a high potential for environmental protection.

Interestingly, not only the exposed generation but also the filial generation exposed to *S. nigrum* extracts showed faster development of larvae, pupae and imagoes compared to the control. In case of pure solamargine, such effect was only observed in adults. Similar to the effect in the exposed generation, the extracts caused more spectacular/striking changes than the pure main alkaloid, again suggesting that minor glycoalkaloids may play crucial roles in the toxic action of plant-derived extracts. These compounds can either increase the activity of the main toxic substance, or they can be responsible for some specific effects. Consequently, these compounds act synergistically with other substances present in plant organs. The shorter developmental time of F_1 insects may result from the selective pressure within the P generation. Since the final number of organisms was smaller, it is likely that the insects that showed longer development times died more frequently due to longer exposure to toxic substances, while insects with shorter development times survived. This feature [shorter developmental time] was more frequently observed in the next generation.

The toxic activity of *S. nigrum* extracts and solamargine alone was shown, but the effects varied among species. For example, solamargine and solasonine were inactive against *Manduca sexta* but inhibited the larval growth of *Tribolium castaneum* (Weissenberg et al., 1998). Solamargine negatively affected the reproduction of *Macrosiphum euphorbiae* (Günter et al., 2000) and altered the heart activity of *Zophobas atratus* (Ventrella et al., 2015). Interestingly, the lethal toxicity of *S. nigrum* extracts against mosquitos (Rawani et al., 2013) and beetles (Gokce et al., 2007) has previously been reported. The range of observed toxic effects, as well as the higher lethal toxicity of the extracts, suggests that not only the main alkaloids but also other substances may affect herbivores and synergistically or additively increase the toxicity of plant organs.

The insecticidal activity of sinigrin has also been reported. This compound showed lethal effects, affecting oviposition, larval weight, pupation and larval development (Ahuja et al., 2011). These results, which are consistent with the present data, show an insecticidal potential of glucosinolates at lethal and sublethal levels. The results may be significant, particularly for those insects that do not feed on *Brassicaceae* plants under natural conditions. For those pests, glucosinolates may be new toxic substances. Therefore, these insects will not easily reveal resistance. These findings were true for *D. melanogaster*. We observed a range of toxic effects. The second [non-exposed] generation also showed faster larval and pupal growth rates. These effects were more prominent for extracts than for pure sinigrin, similar to the effects observed in the first (exposed) generation, suggesting that amongst other substances present at lower concentrations, there may be some interesting chemicals toxic to insects. However, neither the extract nor the pure substance, except at the highest concentration of the extract, showed significant malforming effects in the second generation. This finding suggests that the substances are likely not genotoxic, at least at lower, not extreme concentrations.

As pesticides in general, biopesticides should be approved and registered in most countries before they can be used, sold, or supplied. Since 2009, new rules have been introduced, tightening the requirements for the chemical compounds used as pesticides (Czaja et al., 2015). As biopesticides pose fewer risks than conventional pesticides, authorities generally require less data for their registration. For example, the Environmental Protection Agency (EPA) in the USA often registers new biopesticides in less than a year, compared with an average of more than 3 years for conventional pesticides. However, in some cases, it is difficult to determine whether a product meets the criteria for classification as a biopesticide, and the decision by local agencies might vary, depending on the regulations in each country. Moreover, there might be specific requirements pertinent to the different categories of biopesticides. Although the lethal effect of natural substances may be weaker than that of synthetic insecticides, the natural substances have some advantages. The sublethal activity may seriously imbalanced population stability, making larvae, pupae and imagoes more susceptible to diseases, predators or unfavourable climate conditions. The exposed population may reproduce with lower intensity, which will decrease the number of insects in the environment. Notably, the repellent and antifeedant activity of some alkaloids (Adamski et al., 2016; Chopa et al., 2009; Nenaah, 2011a), particularly stored products, can also effectively protect crops. As observed in that present study and previous studies, the toxicity of extracts may be higher than that of pure substances. Such results suggest that extracts may be interesting, economically justifiable alternatives not only to synthetic insecticides but also for single, pure plant-derived substances.

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