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ORIGINAL ARTICLE

Cardioactive properties of Solanaceae plant extracts and pure glycoalkaloids on *Zophobas atratus*

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Abstract

Glycoalkaloids, the biologically active secondary metabolites produced by Solanaceae plants, are natural defenses against animals, insects and fungi. In this paper, the effects of glycoalkaloids present in extracts of Solanaceae plants (potato, tomato and black nightshade) or pure commercial glycoalkaloids on the coleopteran *Zophobas atratus* F. were evaluated by *in vitro* and *in vivo* bioassays using heart experimental models. Each tested extract induced a dose-dependent cardioinhibitory effect. The perfusion of *Zophobas atratus* semi-isolated heart using the highest potato and tomato extract concentration (1 mmol/L) caused irreversible cardiac arrests, while extract from black nightshade produced fast but reversible arrests. Pure commercial glycoalkaloids caused similar but less evident effects compared with extracts. Our results showed that the bioactivity of tested compounds depended on their structure and suggested the existence of synergistic interactions when combinations of the main glycoalkaloids of potato and black nightshade were used for trials. Surprisingly, injection of tomato and potato extracts in 1-day old pupae of *Zophobas atratus* induced reversible positive chronotropic effects and decreased the duration of the both phases (anterograde and retrograde) of the heart contractile activity. Furthermore, these extracts affected the amplitude of the heart contractions.

Key words cardioinhibitory effect, glycoalkaloid, heart perfusion, Solanaceae, *Zophobas atratus*

Introduction

Glycoalkaloids (GAs), a class of nitrogen-containing steroidal glycosides, are naturally occurring secondary metabolites commonly found in the Solanaceae family, which includes many agricultural crops of economic importance (Milner *et al.*, 2011), such as potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), aubergine (*Solanum melongena* L.), sweet peppers (*Capsicum annuum* L.) and herbaceous plants as black nightshade (*Solanum nigrum* L.). GAs are contained in all parts of the plant but the highest concentrations are detected in the very active growing young tissues as flowers, sprouts, unripe berries, and young leaves (Friedman & McDonald, 1997; Friedman, 2006).

GAs contain a polar water-soluble sugar moiety with three or four monosaccharides attached to an aglyconic part formed by a hydrophobic 27-carbon skeleton of cholestane and by a basic portion (Milner *et al*., 2011). The glycosidic residue consists of different combinations of D-glucose, D-galactose, D-xylose and L-rhamnose (Fig. 1). All GA types can contain double bonds and hydroxyls (OH) in various positions. At least 90 structurally unique steroidal alkaloids have been identified in over 350 *Solanum* species (Friedman & McDonald, 1997). The main potato GAs contained in commercial cultivars are α-chaconine and α-solanine, high concentrations of α-tomatine and dehydrotomatine have been found in tomato plants, while black nightshade plants mainly contain solamargine and solasonine (Milner *et al*., 2011; Eldridge & Hockridge, 1983). The presence of couples of main GAs in Solanaceae plants has been principally attributed to plant evolution (Friedman, 2002).

These biologically active secondary metabolites seem to be associated with plant resistance to pests and pathogens and exhibit a concentration-dependent toxicity to a wide range of organisms, such as fungi, insects, and other animals. Parnell *et al.* (1984) observed that the level of 200 mg/kg fresh weight, generally accepted as the human safe limit for potatoes, only relates to acute and/or subacute effects and not to possible chronic effects. Korpan *et al*. (2004) in a review on potato GAs, underlined that some GAs have been indicated as toxic for humans, while others have been proved to exert antiviral and anticancer activity. To date, the maximum daily dose of GAs has not yet been determined, however an intake of 2–5 mg GAs/Kg body weight can be toxic to humans (Langkilde *et al.*, 2009) and for this reason, the development of modern biotechnology strategies to reduce GAs content in potatoes, resulting in their lower toxic potential, and confirming adequate level of disease resistance, is promoted. On the other hand, although we cannot neglect the problem of GA human toxicity, we believe that the proper use of GAs could represent a good alternative approach for pest management and consistently could reduce the use of synthetic pesticides in sustainable agriculture.

Adverse effects of GAs on developmental biology have been described for several insects (Friedman & McDonald, 1997; Milner *et al*., 2011). However, the knowledge of GA activity on insect physiology is limited. Potato extract and pure GAs exhibited considerable acute and residual toxicity against adults of the red flour beetle *Tribolium castaneum* Herbst and the rice weevil *Sitophilus*

oryzae L. in a dose-dependent manner. The extract was more toxic than single pure GAs (Nenaah, 2011a). Moreover, potato extract and its major GAs displayed considerable contact toxic effects on khapra beetle *Trogoderma granarium* Everts when a topical application technique was used (Nenaah, 2011b). Potato GAs showed ovicidal effect and repellent activity against *Spodoptera exigua* (Hübner) moths (Adamski *et al*., 2009), while α-tomatine proved to have the highest mortality (95%) against the potato leafhopper *Empoasca fabae* Harris (Friedman & McDonald, 1997).

The composition of the carbohydrate side chain and the nature of the aglyconic moiety of GAs are crucial for defining their biological activities (Milner *et al*., 2011). The mechanism of toxicity induced by GAs is associated with their inhibitory activity of acetylcholinesterase and with their membrane-disruptive properties (Milner *et al*., 2011). The membrane lytic activity has been attributed to the bond with hydroxy sterols in cellular membrane and the extent of the loss of membrane integrity has been correlated with the concentration of these sterols (Milner *et al*., 2011). GAs also alter the membrane potential and cause a reduction in sodium active transport; α-tomatine exhibited the highest potency towards membrane depolarization (Blankemeyer *et al*., 1995; 1997).

The aim of present study was to evaluate the effects on the cardioactivity of model insect *Zophobas atratus* F. by potato, tomato and black nightshade plant extracts and their major GAs as single pure compounds at different concentrations. Moreover, we have examined the combined effects of *α*-chaconine with α-solanine, main GAs contained in potato extract, and solamargine with solasonine, main GAs contained in black nightshade extract, to evaluate the eventual synergistic interactions of GAs on insect cardioactivity.

Materials and methods

Insects

Zophobas atratus F. (Coleoptera: Tenebrionidae) adults (4-week old) and pupae (1-day old) were obtained from a colony maintained at the Department of Animal Physiology and Development, Adam Mickiewicz University, Poznań, Poland, according to the Quennedy procedure (Quennedy *et al*., 1995).

Chemicals

Pure solamargine (97.5%) and solasonine (98.3%) were purchased from Glycomix (United Kingdom), pure α-chaconine (≥ 95%) and α-solanine (≥ 95%) were obtained from LabService Analytica (Italy), while hydrate *α*-tomatine, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium chloride (NaCl), calcium chloride ($CaCl_2$) were supplied by Sigma-Aldrich (Germany). Commercial α-tomatine contained dehydrotomatine as impurity (*α*-tomatine : dehydrotomatine 10 : 1 w : w). Acetic acid, formic acid, acetonitrile, LC-MS grade methanol were obtained from Carlo Erba (Milan, Italy); potassium chloride (KCl) was purchased from Chempur (Germany), glucose was supplied by Polish chemicals (POCH) S.A, while proctolin was purchased from Bachem (Switzerland). Ultrapure water was produced using a QPAK 1 Purification Pack system from Millipore (Molsheim, France).

Plant material and sample extraction

Young and small leaves were harvested in May 2011 before fruit appearance from the same stem position (top) of potato plants (*Solanum tuberosum* L.) cultivar *Nicola* and tomato "cherry" (*Lycopersicon esculentum* Mill.) grown in a field of Castellana Grotte (40°53′15″ N–17°12′34″ E, Bari, Italy). Green unripe berries were harvested from autochthonous black nightshade (*Solanum nigrum* L.) plants in a greenhouse of Metaponto (40°23′37″ N–16°47′54″ E, Matera, Italy) in May 2011. The vegetable material was washed and stored at −20 °C to arrest maturation. The vegetable samples were lyophilized and ground to a fine powder using a laboratory mill; then, the same optimized procedure of extraction (Cataldi *et al*., 2005) was employed for each freeze-dried sample. Briefly, in each centrifuge tube 1.5 g of sample were placed in 20 mL of 1% acetic acid aqueous solution; slightly acidic pH values enhance GAs solubilization. To facilitate contact between plant tissue and extraction solvent, the suspension was stirred for about 2 hours and then centrifuged at 6000 r/m for 30 minutes. The obtained pellet was resuspended in 5 mL of 1% acetic acid, shaken, centrifuged and the two supernatants were mixed together. To remove solid particles, the extract was filtered by means of a single-use 0.22 mm nylon filter (Whatman, Maidstone, UK) and then was

injected into the LC/MS system. For the determination of extract bioactivity, the liquid phase was eliminated by means of lyophilization.

LC/ESI-MS analysis was carried out in positive mode using a LCQ Classic quadrupole ion trap mass spectrometer QITMS (ThermoFinnigan, San Jose, CA, USA). The column was a Supelcosil LC-ABZ, amide-C_{16} (5 μm, 250 × 4.6 mm) with a guard column of the same material (Supelco Inc., Bellefonte, PA, USA), which proved to be optimal for GAs chromatographic detection for its high resolution and short analysis time (Cataldi *et al*., 2005).

All extract concentrations have been referred to those of their major GA, which were determined by using opportune purchasable GA pure standard. Potato extract contained α-chaconine and α-solanine in a ratio 1.58:1, together with other minor GAs, especially dehydrochaconine and solanidadienol chacotriose. Extract of tomato leaves displayed the presence of three GAs sharing the same glycosidic group lycotetraose, α-tomatine, dehydrotomatine and filotomatine. Extract of black nightshade unripe berries contained similar concentrations of its main components, solamargine and solasonine, together with α-chaconine, α-solanine and other minor GAs.

Samples preparation

For *in vitro* bioassay, extracts of potato leaves, tomato leaves and black nightshade unripe berries were lyophilized and re-dissolved in saline A (274 mmol/L NaCl, 19 mmol/L KCl, 9 mmol/L $CaCl_2$, 5 mmol/L of glucose and 5 mmol/L of HEPES), pH 7.0. Stock solutions of extracts were obtained at concentration 1 mM referred to their main component metabolite (α-chaconine, α-tomatine and solamargine, respectively). Stock solutions of pure GAs (α-chaconine, α-solanine, α-tomatine, solamargine and solasonine) were prepared by dissolving them in saline A with 0.1% of acetic acid at a concentration of 1 mmol/L. The assayed solutions were prepared by dilution of the stock solutions in saline A to the desired concentrations, which varied in the ranges 10^{-3}–5×10^{-1} mmol/L for black nightshade extract and 10^{-3}–1 mmol/L for the other samples. Binary mixtures were obtained by mixing the selected GA stock solutions to appropriate ratios.

For *in vivo* bioassay, lyophilized potato and tomato extracts were dissolved in saline B (274 mmol/L of NaCl, 19 mmol/L of KCl, 9 mmol/L of $CaCl_2$) to prepare stock solutions 1 mmol/L of their main component metabolite and they were stored at −20 °C.

In vitro heart bioassay

The microdensitometric method was used to measure the effect of samples on semi-isolated heart of the adult beetle (Rosiński & Gäde, 1988). This method measures the intensity of light passing through the insect myocardium and allows to determine two type of parameters of the heart contractile activity: chronotropic (change in frequency) and inotropic (change in amplitude) parameters. In brief, 4 weeks old *Z. atratus* adults were immobilized by anesthetizing them in water for 5–10 minutes, they were decapitated and the abdomen was removed. The ventral body wall of the abdomen was cut away. The fat body, digestive system, and Malpighian tubules were removed from the abdomen. The final preparation consisted of the dorsal vessel (the heart), wing muscles, body wall muscles, and the dorsal cuticle. The heart preparations with regular heartbeat were selected and superfused in saline A (Marciniak *et al*., 2008). The superfusion chamber with the heart preparation was installed on the microdensitometer MD-100 (Carl Zeiss, Jena). An open perfusion system having an injection port 70 mm above the superfusion chamber was used. The flow rate of the fresh saline A was 140 μL/min and the solution in excess was removed from superfusion chamber using chromatographic paper (Whatman No. 3, UK). All tested samples were applied at the injection port with a Hamilton syringe (10 μL). Many applications of samples could be sequentially assayed in a single preparation. The open system was designed to enable the addition of the samples avoiding changes in pressure. After 15 min of initial stabilization, the activity of the isolated heart was recorded for 30 sec. Then, the sample was injected and the heart activity was recorded for further 1.5 min. This procedure was repeated with 5 min intervals for each tested sample. The used solvents (saline A, 0.1% of CH_3COOH in saline A) were also injected to check their probable effect on heart.

A computer software prepared in the Department of Animal Physiology and Development (Larwa) was used to record and analyse the cardiomyogram. The activities of samples have been presented as percentage change in the control frequency of the heart contractions after sample injection (ΔFreq):

(Frequency after the injection – Frequency before the injection) × 100/ Frequency before the injection

Each experiment was replicated six times. The heart responses were considered to be significant when the average changes in contractile activity were higher than 10%. The neuropeptide proctolin (10^{-4} mmol/L) was used as internal standard.

In vivo heart bioassay

The *in vivo* heart bioassay was conducted on 1-day old *Z. atratus* pupae by non-invasive optocardiographic technique of Sláma and Rosiński (2005). Alternating visible red light (1-5 kHz) was emitted by some common light emitting diodes. The light beam was directed to the dorsal pericardial region of the pupa by means of a thin outgoing optic fibre. An associated incoming optic fibre collected the reflected pulse light (modulated by movements of the heart and other organs) and delivered it to a phototransistor at the opposite end of the fibre. The electronic responses, produced from the modulated pulse light by means of a transducer (CMY-17 Philips, USA), were filtered to be insensitive to incident light, then amplified and recorded. The tested extracts (4 μL) were injected, using a Hamilton syringe to a dorsolateral site between the second and third abdominal segments toward the head, avoiding injuries. The sample was injected and after 15 min of stabilization the heart rhythm was recorded for 24 hours. The recordings for each pupa started at the same time of the day. The heartbeat frequency after injection of sample was compared to the control rhythm (injection of physiological saline B). Each experiment was replicated five times.

Study of synergism

In this bioassay, combinations of binary mixtures (α-chaconine + α-solanine) and (solamargine + solasonine) were prepared at a total concentration of 1 mmol/L. The coactivity coefficients (CA) of

the mixtures were calculated according to the modified formula of Alonso-Amelot and Calcagno (2000):

$$CA = (X+Y)_{exp}/(X+Y)_{theor}, \quad \text{where:}$$

$(X+Y)_{exp}$= Sum of experimental (observed) cardioinhibitory effect of the two compounds (X and Y)

$(X+Y)_{theor}$= Sum of theoretic (expected) cardioinhibitory effect of the two compounds (X and Y)

Statistical analysis

The data analysis was performed through R 2.10.1 software. Results were expressed as mean ± SD of six replicates (*in vitro* assay) and five replicates (*in vivo* assay). The mean differences between treatments and control were statistically analyzed using Student *t*-test and analysis of variance (ANOVA) and individual pairwise comparisons were made by means of Tukey test.

Results

Effects of plant extracts and pure GAs on the adult Z. atratus *heartbeat in vitro*

The adult *Z. atratus* cardiac rhythm evaluated *in vitro* remained regular during superfusion with saline A and showed on average 35 ± 5 beats/min. The application of physiological saline A and 0.1% of CH_3COOH in saline A caused no significant change in heart activity (0.11 ± 1.67 and −0.54 ± 0.94, respectively). Saline A alone was used as control for all samples. Proctolin increased the heartbeat frequency (ΔFreq 41.56 ± 9.67) and decreased the amplitude of contraction.

Solanaceae plants' extracts and pure GAs were applied at a concentration of 1 mmol/L, except for black nightshade extract. In this latter case, the obtained extract at concentration of 1 mmol/L was not limpid and therefore not suitable for this assay, so a concentration of 0.5 mmol/L was used. Their application caused fast changes in the contractile activity of the heart (Fig. 2). Extracts of potato and tomato leaves and pure α-tomatine exerted a strong cardioinhibitory effect (Fig. 2) and caused the irreversible arrest of almost all the assayed hearts (Fig. 3C). Black nightshade extract produced a reversible decrease in the heart contractile activity (negative chronotropic effect) and stopped the hearts for a time of 46 ± 12 sec.

α-Chaconine, solamargine and solasonine used as pure substances showed a minor effect on heart contractions and caused reversible arrests of some hearts that lasted on average 23 ± 14, 26 ± 13 and 30 ± 16 sec, respectively (Fig. 3B).

A comparison of the dose-response curves (Fig. 4) indicated that potato and tomato extracts were the most effective samples. They were active in the range of concentration of 10^{-3}–1 mmol/L and 10^{-2}–1 mmol/L, respectively, while black nightshade extract affected the heart rhythm only at the highest considered concentrations (≥ 0.1 mmol/L). Significant correlations existed between the concentration of the extracts and the change in the heartbeat frequency ($r_{PEARSON}$ values were on average −0.98 for three tested extracts).

Interestingly pure GAs caused evident effects on *Z. atratus* heart only at concentrations ≥ 0.75 mmol/L, while α-solanine showed no activity also at the highest examined concentration 1 mmol/L (Fig. 3A). The results suggested that the activity of the potato and black nightshade extracts was higher than activity of their components individually taken.

Synergistic activity of major potato and black nightshade GAs in vitro

In order to assess the eventual synergistic effect of main potato GAs (α-chaconine and α-solanine) and black nightshade GAs (solamargine and solasonine), binary combinations of these phytochemicals were assayed on *Z. atratus* hearts (Fig. 5A,B). Results revealed considerable synergistic interactions between GAs in almost all tested combinations, since the negative cardiotropic effect of binary mixtures of two GAs was more intense than sum of effect of each individually assayed compound (Table 1). The binary mixtures' effect was evident even when the contractions frequency was not changed by respective tested single compounds. The biological potency depended on the ratio of the employed GAs mixture. The highest coactivity coefficients were noted for combinations 1 : 1 and 1.6 : 1 of both assayed mixtures (Table 1). On the contrary, the mixture having combination solamargine : solasonine equal to 1 : 1.6 (0.385 mmol/L of solamargine and 0.615 mmol/L of solasonine) inexplicably caused no evident effect on heartbeat frequency and no synergistic interaction between GAs was observed (Fig. 5B).

In vivo effects of potato and tomato extracts on the pupae heartbeat

A constant pattern of heartbeat in *Z. atratus* pupae was manifested by regular alternations of fast forward orientated (anterograde) and slow backward orientated (retrograde) intervals of cardiac pulsations, together with more or less prolonged periods of diastasis (cardiac rest). The figure 6A shows an example of typical heartbeat recording.

The frequency in control insects (exposed to physiological saline B) showed on average 28 beats/min in anterograde phase and 11 beats/min in retrograde phase. The injection of tomato and potato extracts (containing a concentration 1 mmol/L of α-tomatine and α-chaconine, respectively) induced a positive chronotropic effect in both phases of the heart contractile activity (Fig. 7B,C) in comparison to injection of saline B, which caused no effect (Fig. 7A). This effect was reversible and finished ten hours after the injection. Moreover, reversible changes in duration of both phases (anterograde and retrograde) in the heartbeat were observed after the application of tomato and potato extracts (Fig. 6B,C). Both extracts decreased the duration of two phases in the first 8 hours after injections (Fig. 8B,C). Until 18th hour after the injection of tomato extract, the retrograde phase duration was shorter than the control one (Fig. 8A). Furthermore, the application of the two extracts decreased the duration of periods of diastasis and this effect was more evident for potato extract (Fig. 8B,C).

The Figures 8 A,B,C show as the amplitude of heartbeat contractions varied with time. In the anterograde phase the maximum amplitude was evident 6-8 hours after the injection of saline B, while it was almost constant in retrograde phase (Fig. 9A). The tomato extract caused a decrease of the heartbeat amplitude of retrograde phase in the 8th hour after the application (Fig. 9B). Instead, after the injection of potato extract, the amplitude of contractions was almost constant in the anterograde phase (Fig. 9C). The lack of clear statistically significant differences between treatments and control (injection of saline B) was due to large fluctuations of the pupal heartbeat rhythm.

Discussion

Effect of plant extracts and pure GA on the adult Z. atratus *heartbeat*

Tested plant extracts had a complex chemical composition with two main GAs and other similar compounds at minor concentrations. It is possible to assume that the major activity of the potato and black nightshade extracts, with respect to pure GAs, should result from the sum of single effects of each compound. Moreover, a synergistic effect between the principal GAs could also exist and this hypothesis has been proved by our study of synergic effect of binary GAs combinations.

A preliminary study of the cardiotropic properties of potato *Désirée* leaves extract on several beetle species, *Zophobas atratus* F., *Tenebrio molitor* L. and *Leptinotarsa decemlineata* Say, have been performed previously (Marciniak *et al*., 2010a). The extract decreased the frequency of the adult *Z. atratus* heart contractions, caused fast but reversible cardiac arrests and decreased the amplitude and the duration of both retrograde and anterograde phases of the contractile activity of *Z. atratus* pupae. No effect was evident against the other two beetle species. Lack of inhibitory action of the potato *Désirée* extract on *L. decemlineata* has been explained to be due to the evolutionary adaptation of this beetle to potato GAs, while results of other works are expected to assess the effective resistance of *T. molitor* to GA extracts. The tested extracts in our assays have showed a stronger activity on adult and pupae of *Z. atratus*. In fact, potato *Nicola* and tomato cherry extracts caused irreversible cardiac arrests at the highest concentrations.

The GAs bioactivity depended on the structure of tested compounds, i.e. it varied with the type of aglycone and glycosidic unit. The tomato GA α-tomatine showed a major inhibitory effect because it caused an irreversible arrest of the heart activity. The black nightshade GAs, which share the same aglycone (solasodine), were more active than potato GAs having the solanidine group. α-Chaconine and solamargine possess the same glycosidic group (chacotriose) and they decreased the heartbeat frequency more than α-solanine and solasonine, which share the solatriose group (Milner *et al*., 2011).

A similar structure-activity relationship in GAs was obtained in the case of their membrane lytic activity (Milner *et al*., 2011; Keukens *et al*., 1996). The tested aglycones displayed negligible effect, the chacotriose-containing GAs (*α*-chaconine and solamargine) exhibited stronger membrane lytic

activity than solatriose-containing GAs (α-solanine and solasonine). α-Tomatine possessed the highest lytic activity.

Bergers and Alink (1980) examined effects of α-solanine and α-tomatine in beating rat heart cells. These ceased beating within few minutes after addition of α-solanine (80 μg/mL) or α-tomatine (20 μg/mL). At lower concentrations, both compounds induced increases in the contraction frequency of the heart cells.

GAs have also been tested for cardiotonic activities on isolated frog heart. This study showed that activity on heart was directly related to the kind of sugars contained in the GAs having a common aglycone. Moreover, compounds having different aglycones but the same sugars, such as α-tomatine and demissine, differed significantly with regard to cardiotonic potency. α-Tomatine had a greater activity than α-chaconine and α-solanine (Nishie *et al*., 1976).

Synergistic activity of major potato and black nightshade GAs

Our results indicated considerable synergistic interactions between main potato and black nightshade GAs in almost all tested combinations. The biological potency of the employed GAs mixture depended on their ratio. The most effective synergistic effects were noted for combinations 1 : 1 and 1.6 : 1 of both assayed mixtures. Interestingly, such ratios are similar to those effectively found in potato leaves (α-chaconine : α-solanine ≈ 1.6 : 1) and in black nightshade berries (solamargine : solasonine ≈ 1 : 1). This observation highlights the importance of the ratios existing between the two major GAs for plant defence mechanisms. Synergistic plant defence has been broadly defined as effect of multiple compounds that is greater than the effect expected and based on additive values of each individual compound (Nenaah, 2011a).

Studies of synergy were carried out by Roddick *et al*. (1988). They found that a mixture composed by 50% of α-chaconine and α-solanine led to a significant increase in membrane-disruptive activity. Moreover, ratio-dependent synergistic activity was observed in inhibition of snail feeding (Smith *et al*., 2001) and in toxicity on development of *Xenopus* frog embryos (Milner *et al*., 2011; Rayburn *et*

al., 1995). Synergistic interaction between the main potato GAs was also observed in a study of their toxicity against *Tribolium castaneum* and *Sitophilus oryzae* insects (Nenaah, 2011a).

Cardioinhibitory effects of extracts were always stronger than binary mixtures' effects. Probably, the synergistic effect is extended to other minor GAs contained in extracts. Stronger synergistic interactions among all GAs components could allow to the plant to maintain its resistance to pathogenic agents, even if it produces lower amounts of main GAs.

Effect of potato and tomato extracts on the pupae heartbeat

Potato and tomato extracts caused strong cardioinhibitory effects *in vitro* assays and displayed a positive chronotropic effect when they were injected in pupae. It is difficult to explain these different results. The discrepancies were most probably due to substantially different physiological conditions between the biological assays *in vitro* and the natural conditions within the body. The *in vivo* dorsal vessel occurs in the protective environment of various haemolymph proteins and amino acids and is kept in balance by homeostatic functions of the haemolymph. The most intensively investigated *in vivo* cardiostimulating peptides, proctolin and CCAP (crustacean cardioactive peptide) did not show any immediate direct cardiostimulating effect at concentrations up to 2×10^{-6} mol/L on heartbeat of *Manduca sexta* pupae (Sláma & Rosiński, 2005). Instead, injections of proctolin and CCAP in the range of concentration 10^{-9}–10^{-6} mol/L caused effects on heartbeat which were manifested only several hours after the injections (5 hours for proctolin). These delayed effects involved prolonged or even continuous periods of unidirectional, more efficient and faster anterograde pulsations (Sláma & Rosiński, 2005). The neuropeptide Zopat MS-2 inhibited contractions of the isolated heart but caused positive chronotropic effects in the anterograde phase of pupae heartbeat *in vivo* assays (Marciniak *et al*., 2010b). Probably, the pupae reacted to the stress conditions that were due to presence of the extracts increasing the heartbeat frequency and reducing the phases duration to reinstate normal conditions. Clearly further studies are needed to unravel the exact mechanism of action of tested extracts *in vivo*.

GAs mechanism of action

GAs have shown to affect the physiological conditions of *Z. atratus* but other studies are necessary to know their mechanism of action. Interactions with receptors, neuromodulators or enzymes are possible, so as effects on cell membranes. One probable mechanism of action of GAs could be the direct or indirect alteration of active transport across membranes. The membrane potential of a cell is affected by the ionic concentrations inside and outside of the cell and by the permeability of carriers and ion pumps located in (or near) the cell membrane. If any of these are disrupted, the membrane potential across the cell can change (Blankemeyer *et al*., 1995; 1997).

It has been well established that contractions of muscles in insects as in other animals are induced by Ca^{2+} release from intracellular stores. In insect and other artropods the neuropeptide proctolin enhances contractures of muscles through the induction of Ca^{2+} release from stores and the increase of voltage-dependent Ca^{2+} channel activity by means of the activation of muscle membrane receptor (Philipp *et al*., 2006; Wilcox & Lange, 1995).

The action mechanism of GAs could be opposite to that of proctolin, that is, they could block the Ca^{2+} release. This hypothesis must be confirmed by other specific studies but it could explain the results showing the similar effects of structure-activity relationship and synergistic interactions of GAs on cardioactivity and membrane lytic activity.

Summarily, the physiological conditions of *Z. atratus* were seriously affected by Solanaceae plants' extracts and by the highest concentrations of pure GAs. The dose-dependent decrease in the frequency of the contractions of imago hearts was depended on extract composition and GA structure and it was stronger when the GAs were applied as binary mixtures. Interestingly, injections of concentrated tomato and potato extracts in the pupae caused opposite effects, namely reversible increases in the frequency of the contractions and reversible decreases in the duration of the both phases of pupa heartbeat. Further study to better clarify the action mechanisms of GAs in different pest species to diverse growth stages will give us useful information about the effective possibility of using in agriculture these novel molecules of natural origin having insecticidal action.

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Fig. 1 Structures of major potato, tomato and black nightshade glycoalkaloids.

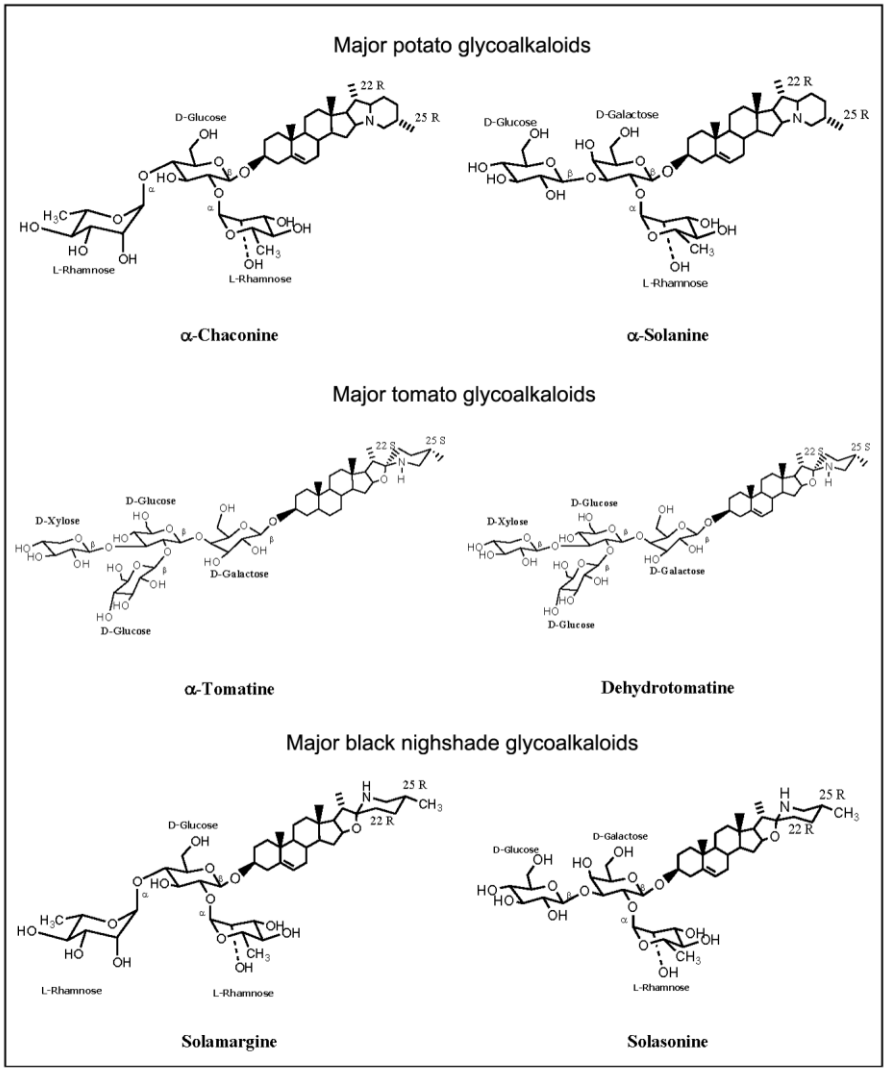

Fig. 2 Changes in the *in vitro* heartbeat frequency (ΔFreq) of adult *Z. atratus* after application of black nightshade extract at concentration of 0.5 mmol/L (#) and of each other tested sample at concentration of 1 mmol/L. Results are expressed as mean of six replicates ± SD. Significant differences ($P < 0.05$) from control (saline A, $\Delta Freq_{control} = 0.11 \pm 1.67$) are indicated by asterisks (Student *t*-test). All extracts concentrations are referred to their major GA.

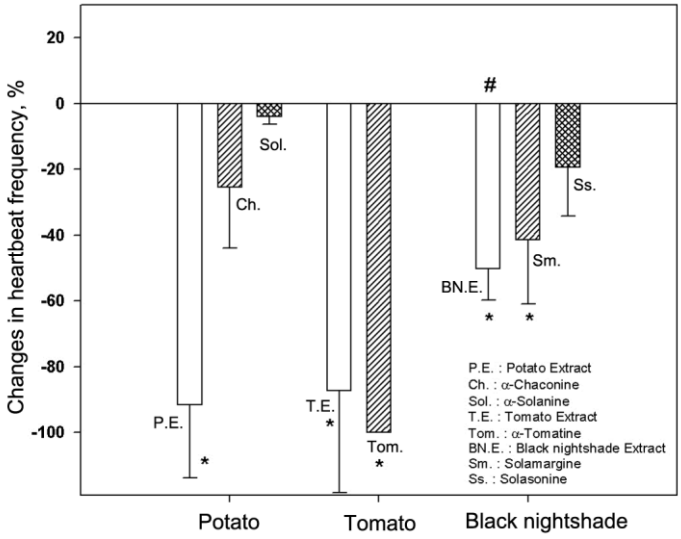

Fig. 3 Myograms displaying three different responses of the *Z. atratus* heart activity to the tested samples *in vitro*: (A) no effect produced by α-solanine 1 mmol/L; (B) reversible cardiac arrest caused by α-chaconine 1 mmol/L; (C) irreversible cardiac arrest caused by α-tomatine 1 mmol/L. Samples applications are indicated by arrows.

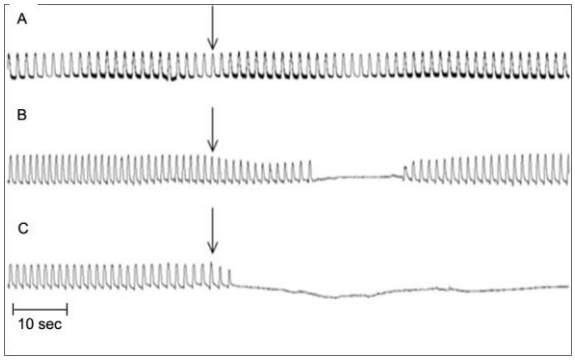

Fig. 4 Dose-response curves for the effect of GA extracts on the contractions frequency of *Z. atratus* hearts *in vitro*. Results are expressed as mean of six replicates ± SD. Significant differences ($P < 0.05$) from control (saline A, $\Delta Freq_{control}$ = 0.11 ± 1.67) are indicated by asterisks (Student *t*-test). All extracts concentrations are referred to their major GA.

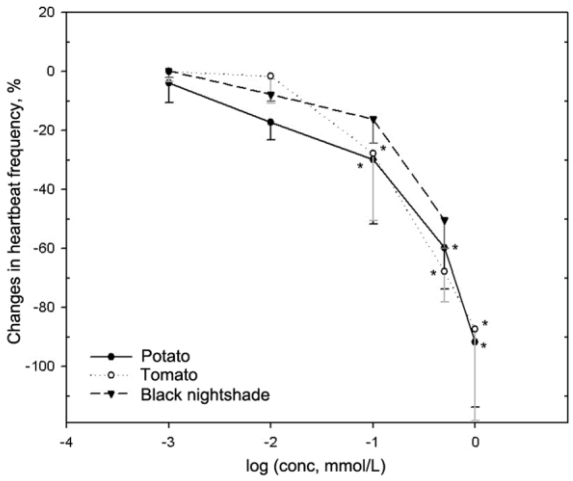

Fig. 5 Synergistic effect on heartbeat frequency of binary mixtures at different combinations tested *in vitro*: (A) α-chaconine + α-solanine; (B) solamargine + solasonine. The *x*-axis of each graph reports the concentration of GAs, either alone or contained in assayed binary mixtures. Results are expressed as mean of six replicates ± SD. Significant differences ($P < 0.05$) from control (saline A) are indicated by asterisks (Student *t*-test).

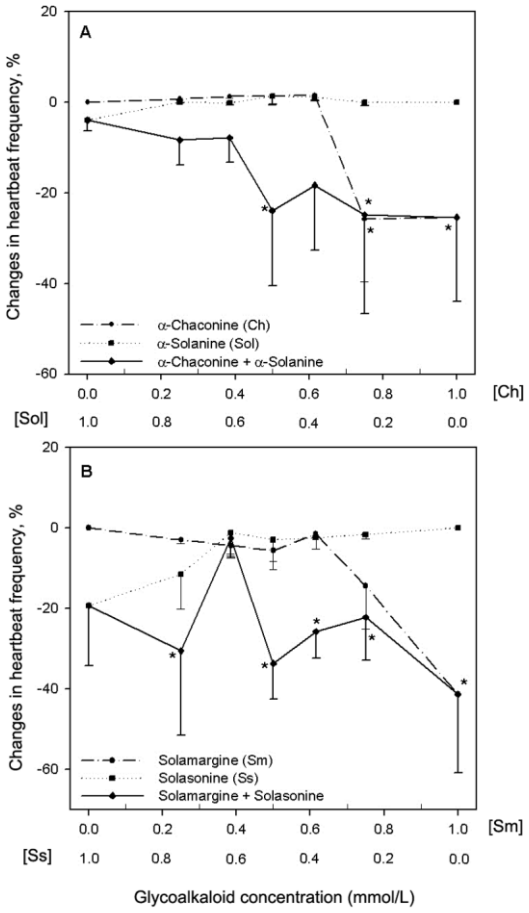

Fig. 6 Optoelectronical recordings of *in vivo* 1-day old *Z. atratus* pupae heartbeat after injection of (A) physiological saline B (control), (B) tomato extract (containing 1 mmol/L of α-tomatine) and (C) potato extract (containing 1 mmol/L of α-chaconine).

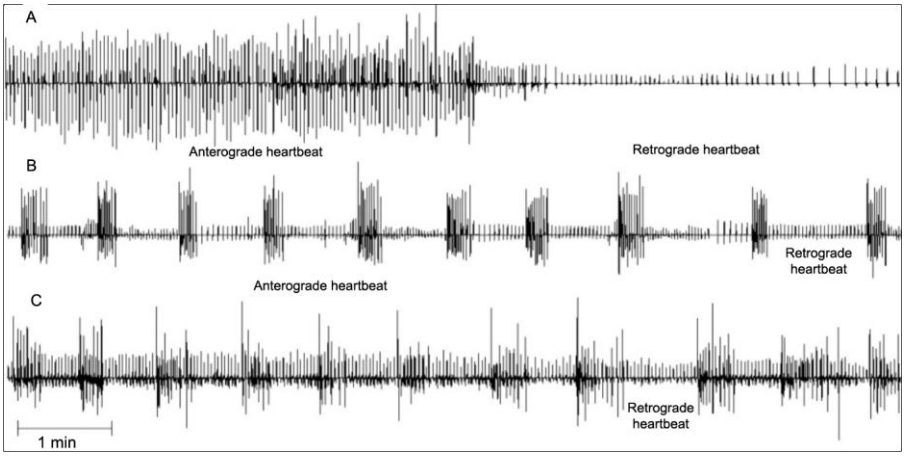

Fig. 7 The *in vivo* heartbeat contraction frequency in anterograde and retrograde phases of 1-day old pupae of *Z. atratus* after injection of (A) saline B, (B) tomato extract and (C) potato extract *in vivo* bioassays. Results are expressed as mean of five replicates ± SD. Significant differences ($P < 0.05$) from control (saline B) are indicated by asterisks (Student *t*-test).

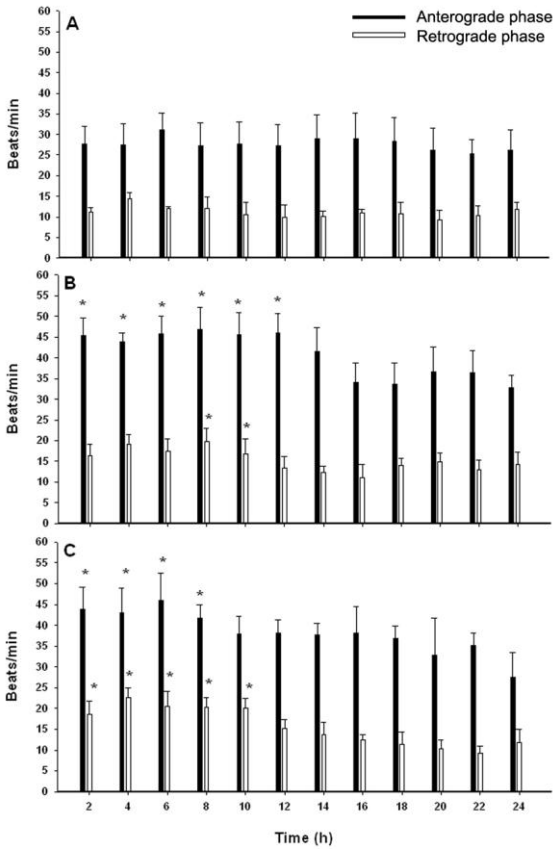

Fig. 8 *In vivo* duration of heartbeat anterograde and retrograde phases and diastasis periods of 1-day old pupae of *Z. atratus* after injection of (A) saline B, (B) tomato extract and (C) potato extract *in vivo* bioassays. Results are expressed as mean of five replicates ± SD. Significant differences ($P < 0.05$) from control (saline B) are indicated by asterisks (Student *t*-test).

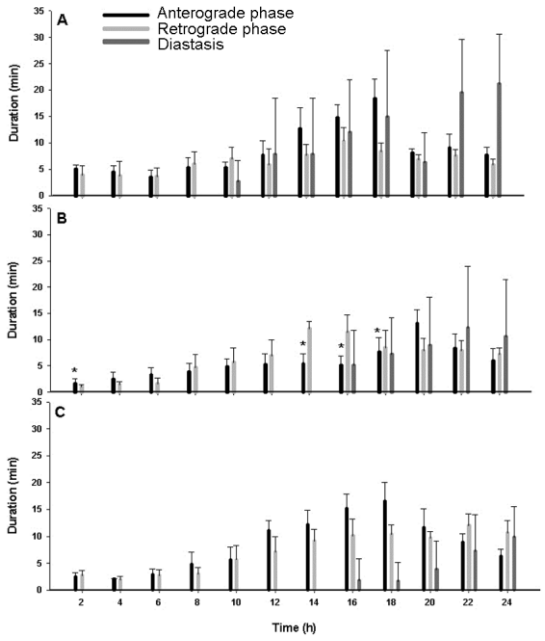

Fig. 9 *In vivo* changes in amplitude of the heartbeat contractions (referred to the maximum amplitude for each heart) in anterograde and retrograde phases of 1-day old pupae of *Z. atratus* after injection of (A) saline B, (B) tomato extract and (C) potato extract *in vivo* bioassays. Results are expressed as mean of five replicates ± SD. Significant differences ($P < 0.05$) from control (saline B) are indicated by asterisks (Student *t*-test).

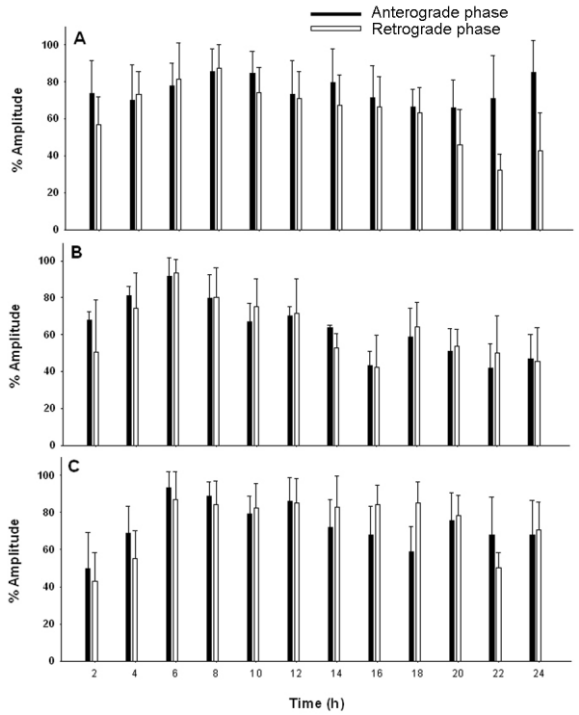

Table 1 Synergistic activity of major potato and black nightshade GAs *in vitro*. Changes in the heartbeat frequency (ΔFreq) of adult *Z. atratus* after application of single GAs and binary mixtures at different combinations (having a more intense effect).

Binary mixture [X]+[Y]=1mmol/L	Combination	ΔFreq $_X$, %	ΔFreq $_Y$, %	ΔFreq $_{(X+Y)}$, %	Coactivity coefficient CA
X + Y: *α*-Chaconine + *α*-Solanine	1 : 1	1.42 ± 2.04^{a}	1.35 ± 1.76^{a}	$-23.90 \pm 16.57^{bc}*$	−8.63
	1.6 : 1	1.54 ± 0.97^{a}	1.16 ± 0.84^{a}	-18.30 ± 14.44^{abc}	−6.78
	3 : 1	$-25.81 \pm 13.75^{bc}*$	-0.05 ± 0.17^{a}	$-24.89 \pm 21.84^{bc}*$	0.96
X + Y: Solamargine + Solasonine	1 : 3	-0.68 ± 0.46^{ab}	-11.46 ± 8.71^{abc}	$-30.57 \pm 20.97^{bc}*$	2.52
	1 : 1	-5.62 ± 4.71^{ab}	-2.96 ± 5.39^{ab}	$-33.64 \pm 8.83^{c}*$	3.92
	1.6 : 1	-1.56 ± 2.87^{ab}	-1.27 ± 0.79^{ab}	$-25.76 \pm 6.64^{bc}*$	9.10
	3 : 1	-14.44 ± 10.66^{abc}	-0.84 ± 0.94^{ab}	$-22.17 \pm 10.64^{bc}*$	1.45

Results are expressed as mean of six replicates ± SD. Significant differences ($P < 0.05$) from control (saline A) are indicated by asterisks (Student *t*-test). Means followed by the same letters are not significantly different ($P < 0.05$, Tukey test).