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Działanie ekstraktu z *Solanum nigrum* L. i jego głównych glikoalkaloidów, jako czynników zwiększających toksyczność insektycydu fenitrotionu w wybranych tkankach *Tenebrio molitor* L.

ang. Effects of *Solanum nigrum* L. extract and its main glycoalkaloids on selected tissues of *Tenebrio molitor* L. as factors increasing the toxicity of fenitrothion insecticide

Rozprawa doktorska wykonana pod kierunkiem

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Spis treści

1. Streszczenie

Rośliny należące do rodziny psiankowatych (Solanaceae), takie jak psianka czarna (*Solanum nigrum* L.), wytwarzają alkaloidy – wtórne metabolity, które znane są ze swoich toksycznych, ale także leczniczych właściwości. Wyniki licznych badań wskazują na letalne i subletalne efekty toksyczne względem wielu gatunków owadów, w tym szkodników magazynowych. Jednymi z najczęściej stosowanych na świecie grup związków w ochronie produktów spożywczych są pestycydy z grupy insektycydów fosforoorganicznych, do których należy fenitrotion, związek wciąż wykorzystywany w wielu rejonach świata. W niniejszej rozprawie doktorskiej zbadany został wpływ ekstraktu z niedojrzałych owoców psianki czarnej na wybrane aspekty fizjologii szkodnika magazynów zbożowych, mącznika młynarka (*Tenebrio molitor*: Tenebrionidae: Coleoptera), pod kątem możliwości wykorzystania go w zwalczaniu tego owada. Następnie określono, który z głównych składników ekstraktu: solasonina czy solamargina, jest odpowiedzialny za obserwowane efekty, przez podanie osobno dwóch glikoalkaloidów i porównanie z efektami powodowanymi przez ekstrakt. W kolejnym etapie badań określono toksyczne stężenia fenitrotionu dla larw mącznika młynarka, powodujące śmiertelność połowy badanej populacji (LC_{50}) oraz innych wybranych wartości współczynnika LC (LC_{40}). Ustalenie powyższych parametrów posłużyło do określenia wpływu ekstraktu z *S. nigrum* na toksyczność fenitrotionu, w dwóch różnych strategiach aplikacji. W pierwszej ekstrakt został podany w mieszaninie z fenitrotionem w proporcji 1:1, natomiast w drugiej, ekstrakt został podany na 24 h przed aplikacją fenitrotionu.

Otrzymane wyniki wskazały, że ekstrakt z *S. nigrum* powoduje u mącznika młynarka efekty subletalne, takie jak zmiany w kurczliwości narządów wewnętrznych, zmiany zawartości wybranych makromolekuł oraz zaburzenia w ultrastrukturze komórek jelita środkowego i ciała tłuszczowego. Glikoalkaloidy powodują subletalne efekty toksyczne u mącznika młynarka, a różnice w działaniu ekstraktu i pojedynczych glikoalkaloidów, sugerują synergistyczne działanie tych glikoalkaloidów. Ekstrakt z *S. nigrum* podawany równocześnie z fenitrotionem (LC_{50} = 400 µg/mL) w przypadku krótkotrwałej ekspozycji, nie zwiększał śmiertelności larw, w porównaniu do efektów powodowanych przez sam fenitrotion, jednak widoczne były efekty subletalne, na poziomie komórkowym. Natomiast druga strategia wykazała, że odpowiednio skorelowane podanie ekstraktu z psianki czarnej przed aplikacją fenitrotionu (LC_{40} = 300 µg/mL), może spowodować

istotny wzrost śmiertelności badanych szkodników oraz nasilić efekty subletalne, zarówno w porównaniu do stosowania równoczesnego obu związków, jak i samego fenitrotionu.

Przeprowadzone badania wskazują na możliwość wykorzystania ekstraktu z psianki czarnej jako naturalnego insektycydu, zwiększającego toksyczność fenitrotionu. Wzrost śmiertelności i intensywności efektów subletalnych sugeruje możliwość wykorzystania strategii skorelowanego podawania związków naturalnych oraz syntetycznych insektycydów w ochronie roślin i produktów spożywczych.

2. Summary

Plants that belong to the nightshades family (Solanaceae), such as black nightshade (*Solanum nigrum* L.) produce alkaloids, secondary metabolites, known for possessing their toxic but also therapeutic properties. Both alkaloids and their mixtures, as well as other compounds (i.e. extracts and other plant products), exhibit insecticidal properties against many insect species, including pests of stored food. One of the most popular groups of compounds used for food protection all over the world are organophosphate insecticides, of which fenitrothion is a member. Despite the fact of its withdrawal in many countries because of its high toxicity, it is still used in many regions of the world. The abovementioned matters are the basis for the presented doctoral dissertation. During the research, the influence of the black nightshade (*Solanum nigrum*: Solanaceae) unripe fruit extract on selected aspects of the physiology of a yellow mealworm (*Tenebrio molitor*: Tenebrionidae: Coleoptera) – one of the pests of stored food was tested, to estimate its potential usage as an insecticide against *T. molitor*. Next experiments were carried in order to determine, which of the main ingredients of the extract: solasonine or solamargine, is responsible for the observed effects, by a separate application of each glycoalkaloid, and comparison of the obtained results with the effects caused by the extract. As a next step, the description of toxic concentrations of fenitrothion for yellow mealworm larvae, causing the lethality of 50% studied population (LC_{50}) and approximate value (LC_{40}) were calculated. The determination of the mentioned parameters was used to carry out the last step of the research – the description of the influence of the *S. nigrum* extract on the toxicity of fenitrothion, in two different strategies of application. In the first one, the extract was mixed with fenitrothion in the ratio 1:1 and applied to the larvae, and in the second one, the extract was applied 24 h before the fenitrothion application.

The results showed, that the *S. nigrum* extract causes sublethal effects such as changes in the activity of visceral muscles, changes in the concentration of stored biomolecules, and disturbed the ultrastructure of the midgut and fat body cells in yellow mealworm. Glycoalkaloids present in the black nightshade extract caused sublethal toxic effects in yellow mealworm. The differences in the effects caused by the extract and single glycoalkaloids suggest the cooperative action of both glycoalkaloids. The *S. nigrum* extract applied at the same time with fenitrothion (LC_{50} = 400 µg/mL)

did not increase the lethality of *T. molitor* larvae, comparing to the effects caused by fenitrothion itself. However, it caused sublethal effects on subcellular and cellular levels. Next, application of the *S. nigrum* extract application before the application of fenitrothion (LC_{40} = 300 µg/mL) caused a significant increase of mortality of studied pests, and strengthened the sublethal effects, compared to both the fenitrothion, as well as to the mixtures of fenitrothion and extract, administered together.

The research that was carried, proved that the black nightshade extract can be used as a factor increasing fenitrothion toxicity. An increase in the mortality and intensity of sublethal effects suggested that the strategy of time-correlated application of natural substances and synthetic insecticides can successfully be used in plant and food protection.

3. Wprowadzenie

Rośliny należące do rodziny psiankowatych (Solanaceae), takie jak ziemniak (*Solanum tuberosum* L.), pomidor (*Solanum lycopersicum* L.), tytoń (*Nicotiana tabacum* L.) czy psianka czarna (*Solanum nigrum* L.), charakteryzują się wytwarzaniem alkaloidów – wtórnych metabolitów, które znane są ze swoich toksycznych, ale także leczniczych właściwości. Z racji swej toksyczności, alkaloidy mogą pełnić rolę obronną przed roślinożercami, na co wskazuje ich zwiększona produkcja podczas zgryzania oraz większa zawartość w młodych, niedojrzałych owocach i pędach roślin (Eltayeb i wsp. 1997).

Naturalne pochodzenie alkaloidów, powszechność ich występowania, a także różnorodność, skłaniają naukowców do badań nad praktycznym zastosowaniem tych związków w medycynie, farmakologii oraz ochronie upraw i produktów spożywczych. W ostatnich latach wzrosła ilość badań prowadzonych nad wpływem tych związków na owady będące szkodnikami upraw, wektorami chorób lub zapylaczami. Zarówno same alkaloidy, jak i mieszaniny substancji, w których są obecne (np. ekstrakty), wykazują działanie insektycydowe. Wyniki licznych badań wskazują na letalne i subletalne efekty toksyczne względem wielu gatunków owadów (Chowański i wsp. 2016). Co ciekawe, ten sam związek bądź mieszanina, może wykazywać wysoką aktywność względem danego gatunku owada, a tym samym brak toksyczności względem innego gatunku, co wskazuje na ich selektywność. Niewątpliwą zaletą potencjalnego zastosowania alkaloidów jako insektycydów jest ich szybka biodegradacja, powszechność występowania oraz niska toksyczność względem innych organizmów (Al Chami i wsp. 2003). Wiele danych literaturowych wskazuje na działanie przeciwnowotworowe, przeciwzapalne, antybakteryjne i przeciwwirusowe produktów pochodzenia roślinnego np. pozyskanych z *S. nigrum* (Atanu i wsp. 2011). Powyższe czynniki sugerują możliwość zastosowania produktów pochodzenia roślinnego, w tym alkaloidów, w zintegrowanej ochronie roślin, jako środków zwalczających szkodniki oraz ograniczających ich żerowanie, a zatem zwiększających plony oraz zysk producentów żywności. Należy zwrócić uwagę, że większość badań nad glikoalkaloidami ma charakter wysoce aplikacyjny. Koncentrują się one przede wszystkim na badaniu ostrych efektów letalnych, najważniejszych z punktu widzenia agrochemicznego. Natomiast ilość badań podstawowych, dotyczących subletalnych efektów działania

glikoalkaloidów na owady jest mniejsza, chociaż badania takie mogą być bardzo ważne dla określenia działania tych związków na organy, tkanki i komórki organizmu docelowego oraz zastosowania glikoalkaloidów (a także innych alkaloidów) w farmakologii, medycynie czy ochronie środowiska naturalnego oraz upraw. Dlatego zdecydowałam się na zbadanie wpływu subletalnych stężeń badanego ekstraktu i pojedynczych alkaloidów na wybrane aspekty funkcjonowania wybranego organizmu.

Ochrona plonów i produktów spożywczych należy do najbardziej wymagających gałęzi gospodarki pod względem restrykcji w stosunku do stosowania środków chemicznych. Przez wzgląd na ludzkie zdrowie oraz konieczność ochrony środowiska, zarówno naturalnego, jak i hodowanych roślin i zwierząt, dobierane środki chemiczne, a także ich metabolity powinny wykazywać się niską szkodliwością dla organizmów neutralnych, pożytecznych oraz konsumentów oraz krótkim okresem półtrwania, a zarazem wysoką skutecznością. Jednymi z najczęściej stosowanych na świecie grup związków w ochronie produktów spożywczych są pestycydy z grupy insektycydów fosforoorganicznych, do których należy fenitrotion. Mimo wycofania go z użytku w wielu krajach ze względu na toksyczność (ICSCS: fenitrothion), wciąż jest wykorzystywany w wielu rejonach świata. Fenitrotion stosowany jest od lat sześćdziesiątych, stąd dostępna jest obszerna wiedza na temat jego toksyczności i działania względem różnych grup organizmów (Wang i wsp. 2012), co pozwala na jej wykorzystanie w badaniach i analizie uzyskanych wyników.

Cele pracy

Wyżej omówione zagadnienia stanowią podstawę niniejszej rozprawy doktorskiej. Celem, który sobie postawiłam, było zbadanie **wpływu ekstraktu z niedojrzałych owoców psianki czarnej (*Solanum nigrum*: Solanaceae) na wybrane aspekty fizjologii szkodnika magazynów zbożowych, mącznika młynarka (*Tenebrio molitor*: Tenebrionidae: Coleoptera) (1)** pod kątem możliwości wykorzystania go w zwalczaniu tego owada. Kolejnym celem było **ustalenie, który z głównych składników ekstraktu: solanina czy solamargina, jest odpowiedzialny za obserwowane efekty, przez podanie osobno dwóch glikoalkaloidów i porównanie z efektami powodowanymi przez ekstrakt (2)**. Celem kolejnego etapu badań było **określenie toksycznych stężeń fenitrotionu dla larw mącznika młynarka, powodujących śmiertelność połowy badanej**

populacji (LC_{50}) oraz innych wybranych wartości współczynnika LC (LC_{40}) (3). Ustalenie powyższych parametrów posłużyło do przeprowadzenia ostatniego etapu badań, którego celem było **określenie wpływu ekstraktu z *S. nigrum* na toksyczność fenitrotionu w dwóch różnych strategiach aplikacji (4).**

1. Wpływ ekstraktu z owoców *S. nigrum* na fizjologię chrząszcza *T. molitor*

Skuteczność produktów pochodzenia roślinnego na szkodniki owadzie jest testowana przez wielu badaczy na całym świecie. W celu ograniczenia emisji szkodliwych pestycydów i ich negatywnych skutków na inne organizmy, testowane są pochodne roślin w postaci olejków eterycznych, ekstraktów, proszków czy oczyszczonych pojedynczych związków. Bardzo istotnym aspektem tych badań jest określenie efektów działania badanych substancji takich jak śmiertelność czy innych efektów fizjologicznych ograniczających liczebność szkodnika (efektów subletalnych). Powyższe czynniki mogą zależeć na przykład od sposobu aplikacji związków. Zagadnienia związane ze stosowaniem naturalnych pestycydów zostały zebrane i szczegółowo opisane w publikacji „Plant-Derived Substances Used Against Beetles – Pests of Stored Crops and Food – and Their Mode of Action: A Review." będącej częścią niniejszej rozprawy doktorskiej (Spochacz i wsp. 2018a).

Do tej pory opisano efekty toksyczne powodowane przez substancje zawarte w tkankach i organach *S. nigrum* jedynie względem kilku gatunków owadów (Obeng-Ofori i wsp. 2001, Ben-Abdallah i wsp. 2018, Chowański i wsp. 2018), co skłania do dalszych, bardziej szczegółowych analiz.

Prowadzone przeze mnie badania wykazały powstawanie efektów subletalnych zarówno u larw, jak i dorosłych chrząszczy *T. molitor*, pod wpływem działania ekstraktu z psianki czarnej (*S. nigrum)* oraz pojedynczych glikoalkaloidów, podawanych zarówno w diecie, jak i aplikowanych *in vitro* na izolowane tkanki. Wyniki powyższych eksperymentów zostały opublikowane w pracy „Sublethal Effects of *Solanum nigrum* Fruit Extract and Its Pure Glycoalkaloids on the Physiology of *Tenebrio molitor* (Mealworm)" będącej częścią niniejszej rozprawy doktorskiej (Spochacz i wsp. 2018b). Badania miały na celu określenie efektów zarówno na poziomie populacji (śmiertelność), organizmu (zmiany masy ciała, aktywność miotropowa, pomiar parametrów

biochemicznych), jak i komórkowym (obserwacja zmian ultrastruktury wybranych tkanek za pomocą transmisyjnej mikroskopii elektronowej) celem szerszego poznania mechanizmów działania badanych substancji na różnych poziomach organizacji życia. Wyniki przeprowadzonych analiz wykazały, iż ekstrakt z psianki czarnej, podawany topikalnie na półizolowany preparat sercowy, powoduje ujemny efekt chronotropowy i krótkotrwałe, odwracalne zatrzymanie aktywności miokardium. Zaobserwowano także wzrost aktywności skurczowej jajowodów, natomiast nie zanotowano wyraźnego wpływu na kurczliwość mięśni jelita tylnego. Powodowane przez ekstrakt zaburzenia w pracy narządów kluczowych dla funkcjonowania organizmu (serce) oraz biorących udział w procesach reprodukcji (jajowód) u *T. molitor*, wskazują na możliwość użycia ekstraktu z psianki czarnej w celu ograniczenia liczebności populacji mącznika młynarka.

Z uwagi na sposób podawania substancji owadom – drogą pokarmową – do przeprowadzenia analiz wybrane zostały: jelito środkowe odpowiedzialne za wchłanianie pokarmu oraz ciało tłuszczowe, które magazynuje substancje zapasowe, reguluje metabolizm i bierze udział w detoksykacji ksenobiotyków. Tkanki te są więc w istotny sposób narażone na działanie podawanych związków. Obserwacje prowadzone przy użyciu transmisyjnego mikroskopu elektronowego wykazały obecność zmian na poziomie komórkowym. Podanie ekstraktu, solasoniny lub solamarginy w pokarmie spowodowało zmiany w nabłonku jelita środkowego, takie jak rozwarstwianie błon jądrowych, zmiany w gęstości cytoplazmy i powstawanie na jej terenie obłonionych struktur, prawdopodobnie wakuol glikogenowych związanych ze zwiększonym metabolizmem glikogenu (Spochacz et al. 2018a). W komórkach ciała tłuszczowego po aplikacji ekstraktu stwierdzono obecność niehomogennych kropli lipidowych, które różniły się od kontrolnych, również nieregularnym kształtem (Spochacz et al. 2018a i 2018b). Powyższe obserwacje wskazują na powinowactwo glikoalkaloidów do zmagazynowanych lipidów oraz steroli tworzących błony biologiczne. Grupa steroidowa glikoalkaloidów pod względem budowy jest podobna do steroli błonowych, dlatego ich oddziaływanie może skutkować wbudowaniem się tych związków w błony biologiczne, a co za tym idzie, zaburzeniami ich ciągłości, a w efekcie funkcjonowania. Natomiast grupa cukrowa zawierająca ramnozę, może oddziaływać ze sterolami błonowymi, zmieniać ich przepuszczalność,

a także obniżać wzajemną adhezję komórek (Siddique i wsp. 2019, Roddick i wsp. 1990, Roddick i wsp. 2001, Keukens i wsp. 1996). Intensywność obserwowanych zaburzeń była zależna od stężenia i zwiększała się wraz ze wzrostem stężenia zastosowanego ekstraktu.

W wyniku pomiarów zmian masy ciała owadów zaobserwowano liniowe zwiększenie masy ciała larw, wraz ze spadkiem stężenia stosowanego ekstraktu, co wskazuje na słaby efekt stymulujący pobieranie pokarmu przez niskie stężenia ekstraktu i możliwe działanie inhibicyjne przez stężenia wyższe. Uważam jednak, że czas trwania eksperymentu był zbyt krótki, aby możliwe było zaobserwowanie wyraźnego wpływu ekstraktu na pobieranie pokarmu przez larwy (Spochacz i wsp. 2018b).

W kolejnych analizach mających na celu zmierzenie zawartości substancji zapasowych w ciele tłuszczowym, określenie zwartości glikogenu oraz lipidu całkowitego wykazały spadek ich ilości, po podaniu ekstraktu odpowiednio w stężeniach 0,1% i 1%. Może to wskazywać na inhibicję pobierania pokarmu przez składniki ekstraktu (Satake i wsp. 2000) lub wzrost zużycia energii podczas intoksykacji (Maliszewska i Tęgowska 2016).

2. Porównanie działania ekstraktu z psianki czarnej z czystymi glikoalkaloidami obecnymi w ekstrakcie

Solasonina i solamargina stanowiły odpowiednio 49,6% i 46,8% składu frakcji glikoalkaloidów badanego ekstraktu (Chowański i wsp. 2018), stąd hipoteza zakładająca ich największy udział w jego aktywności insektycydowej. Stężenia molowe obu glikoalkaloidów stosowanych w eksperymentach odpowiadały ich stężeniom w ekstrakcie. Dzięki temu, można było porównać efekty działania ekstraktu i pojedynczych glikoalkaloidów, określenie który z nich jest odpowiedzialny za obserwowany efekt, a także stwierdzenie występowania ewentualnego działania synergistycznego lub addytywnego. Dane literaturowe potwierdzają, że obecność dwóch różnych grup cukrowych może wpływać na występowanie synergizmu (Roddick i wsp. 2001).

W trakcie przeprowadzonych badań, zaobserwowałam, że podawanie pojedynczych glikoalkaloidów na izolowane tkanki (miokardium, jajowód i jelito tylne) nie powoduje żadnych istotnych zmian w pracy tych narządów. Wobec powyższego

stwierdziłam, iż zmiany widoczne przy aplikacji ekstraktu mogą być efektem działania synergistycznego badanych substancji jak i innych substancji biologicznie czynnych obecnych w ekstrakcie (Spochacz i wsp. 2018b).

Podobne wnioski przyniosły obserwacje wybranych tkanek z wykorzystaniem transmisyjnej mikroskopii elektronowej. Podanie solasoniny lub solamarginy w diecie larw *T. molitor* nie spowodowało żadnych wyraźnych zmian ultrastruktury nabłonka jelita środkowego (Spochacz i wsp. 2018b), natomiast efekty były widoczne po podaniu ekstraktu, wskazując na efekt synergistyczny lub będący skutkiem działania pozostałych glikoalkaloidów, stanowiących znacznie mniejszą część alkaloidów wchodzących w skład ekstraktu (3.6%). W komórkach ciała tłuszczowego, po podaniu solamarginy i solasoniny zaobserwowano niehomogenne krople lipidowe, zdegradowane zmagazynowane białka, elektronowo gęste jądra komórkowe oraz obniżenie gęstości cytoplazmy (Spochacz i wsp. 2018b). Wyniki te są zgodne z efektami obserwowanymi po podaniu ekstraktu, co wskazuje na udział tych dwóch glikoalkaloidów w zaburzaniu budowy struktur komórkowych, a także na możliwość kumulowania i ich efekty toksyczne w ciele tłuszczowym.

Odmienne skutki działania glikoalkaloidów i ekstraktu zaobserwowałam po zmierzeniu poziomu substancji zapasowych w ciele tłuszczowym larw. Ekstrakt obniżył zawartość glikogenu w komórkach ciała tłuszczowego, natomiast zarówno solasonina jak i solamargina, spowodowały wzrost jego zawartości w stosunku do kontroli. Zawartość lipidu całkowitego w ciele tłuszczowym spadła po podaniu larwom ekstraktu z psianki czarnej oraz solasoniny, natomiast wzrosła u larw karmionych pożywką z dodatkiem solamarginy (Spochacz i wsp. 2018b). Obserwowane różnice mogą wskazywać na odmienne efekty metaboliczne powodowane przez ekstrakt jak i pojedyncze glikoalkaloidy, a także na odmienne oddziaływanie na organizm owada.

3. Określenie toksycznych stężeń fenitrotionu dla larw mącznika młynarka

Aby określić wpływ ekstraktu na toksyczność fenitrotionu, w pierwszej kolejności wyznaczono stężenie tego syntetycznego insektycydu, powodujące śmiertelność połowy populacji larw mącznika młynarka (LC_{50}), a w dalszej kolejności jego efekty na funkcjonowanie owadów, przy krótkotrwałej ekspozycji (do 72 h). W wyniku

podjętych eksperymentów, wyznaczyłam stężenia 400 i 300 µg/mL fenitrotionu jako odpowiednio $LC_{50/24h}$ i $LC_{40/24h}$. Powyższe stężenia zostały użyte do dalszych doświadczeń, opublikowanych w pracy „*Solanum nigrum* Fruit Extract Increases Toxicity of Fenitrothion — A Synthetic Insecticide, in the Mealworm Beetle *Tenebrio molitor* Larvae", będącej częścią niniejszej rozprawy doktorskiej (Spochacz i wsp. 2020).

4. Wpływ ekstraktu z psianki czarnej na toksyczność fenitrotionu

Jak wspomniano wcześniej, sposób aplikacji związków może mieć wpływ na otrzymany efekt toksyczny (Spochacz i wsp. 2018a). Dlatego postawiłam kolejną hipotezę badawczą: efekt toksyczny działania naturalnego insektycydu oraz insektycydu syntetycznego może zależeć od odpowiedniego skojarzenia czasowego aplikacji obu związków. W celu weryfikacji hipotezy, przeprowadziłam szereg eksperymentów, w których równocześnie lub w przedziale czasowym podawano ekstrakt, powodujący efekty subletalne, z fenitrotionem aplikowanym w stężeniu odpowiadającemu wartości LC_{50}. W pierwszym wariancie, obie substancje podałam larwom jednocześnie, tworząc wcześniej ich mieszaniny w stosunku 1:1. Czas trwania eksperymentu był taki sam jak w poprzednich badaniach, czyli 5 dni, w tym 72 godziny aplikacji związków. Obecność ekstraktu w mieszaninie nie wpłynęła znacząco na wzrost śmiertelności larw w porównaniu ze śmiertelnością wywołaną przez podanie samego fenitrotionu (LC_{50}). Co więcej, z obliczeń przyrostu masy ciała larw wynika, że utrata masy była mniejsza w przypadku mieszanin niż samego fenitrotionu (Spochacz i wsp. 2020), co może sugerować niewielki potencjał ekstraktu wpływający na pobieranie pokarmu przez larwy, obserwowany we wcześniejszych analizach (Spochacz i wsp. 2018b). Warto zaznaczyć, że obserwowana utrata masy ciała nie jest w tym przypadku związana tylko z zatrzymaniem pobieraniem pokarmu, bowiem kontrolne, głodzone larwy charakteryzowały się istotnie mniejszym spadkiem masy ciała w porównaniu do larw zarówno w pierwszym jak i drugim wariancie eksperymentu. Dodatkowy czynnik podania substancji silnie toksycznej mógł spowodować zwiększenie nakładów energetycznych, koniecznych do detoksykacji organizmu lub zaburzoną gospodarkę wodną organizmu, co sugerują dane literaturowe (Maliszewska i Tęgowska 2016).

W kolejnych badaniach prowadzonych w tym wariancie eksperymentu, przeprowadziłam analizy biochemiczne ciała tłuszczowego, które wskazały jedynie

na wahania poziomu glikogenu, nieznaczny wzrost zawartości lipidów całkowitych, a także niewielki spadek ilości białka w ciele tłuszczowym pod wpływem mieszanin (Spochacz i wsp. 2020). Natomiast w obrazie mikroskopowym stwierdziłam różnice ultrastrukturze pomiędzy komórkami jelita środowego owadów traktowanych wyłącznie fenitrotionem, a traktowanych mieszaninami, zawierającymi dwa najwyższe stężenia ekstraktu. U tych ostatnich, zaobserwowałam ponadto rozdęcia siateczki śródplazmatycznej, co sugeruje zaburzenia w homeostazie jonowej oraz w funkcjonowaniu błon biologicznych na skutek działania glikoalkaloidów. Prawdopodobnie, jak sugeruje literatura, jest to związane z oddziaływaniem grup cukrowych glikoalkaloidów z błoną komórkową, co prowadzi do wzrostu jej przepuszczalności, a następnie do utraty jonów i zaburzeń w homeostazie jonowej (Dalhin i wsp. 2017). W komórkach ciała tłuszczowego obecne były nieregularne, niehomogenne, zmagazynowane białka oraz elektronowo gęsta chromatyna i cytoplazma. Podanie mieszanin spowodowało dodatkowe efekty w postaci zwiększenia przestrzeni międzybłonowej w otoczce jądrowej, zwiększenie przestrzeni międzykomórkowych, zmiany w homogenności kropli lipidowych oraz ich fuzję (Spochacz i wsp. 2020). Podobne efekty obserwowane były po podaniu samych glikoalkaloidów i ekstraktu (Spochacz i wsp. 2018b), co wskazuje, że jest to efekt związany z ich oddziaływaniem na struktury komórkowe.

Zastosowanie składników o potencjalnie różnym mechanizmie działania (ekstrakt i fenitrotion) i bogatszym składzie, mogłoby przyczynić się do zmniejszenia występowania odporności szkodników na insektycydy oraz zwiększenia skuteczności insektycydu, a poprzez zastosowanie mieszaniny tych związków, do ograniczenia liczby oprysków i emisji szkodliwych pestycydów do środowiska, a także pozytywnie wpłynąć na jakość produktów rolnych. Strategia wykorzystująca równoczesne podanie ekstraktu i fenitrotionu nie przyniosła jednak oczekiwanych rezultatów, stąd w drugim wariancie eksperymentu ekstrakt, w badanych wcześniej stężeniach procentowych, został podany na 24 godziny przed aplikacją fenitrotionu, w stężeniu odpowiadającemu wartości ustalonej jako LC_{50}, a następnie w niższym stężeniu, odpowiadającemu wartości ustalonej jako LC_{40}. Obniżenie stężenia miało na celu zmniejszenie toksyczności fenitrotionu. Wstępne badania wykazały bowiem, że przy stężeniu 400 µg/mL (LC_{50})

śmiertelność larw wzrasta bardzo gwałtownie, uniemożliwiając przeprowadzenie badań efektów subletalnych. Zgodnie z postawioną wcześniej hipotezą, w tym wariancie zaobserwowałam istotny wzrost śmiertelności larw. W ciągu 24 godzin od podania fenitrotionu śmiertelność wzrosła do 100%. Co ciekawe, podobnie jak w pierwszym wariancie eksperymentu, wykazano zmniejszoną utratę masy ciała larw po aplikacji ekstraktu jako czynnika poprzedzającego podanie fenitrotionu niż samego fenitrotionu. Badania biochemiczne wskazały na istotny spadek zawartości lipidu w ciele tłuszczowym w porównaniu do kontroli, jak i do samego fenitrotionu, co wskazuje na zaburzenia w gospodarce lipidowej, dodatkowo stymulowane czynnikiem w postaci glikoalkaloidów. Odnotowano także bardzo znaczący spadek zawartości glikogenu, wyraźnie pokazujący wysokie zapotrzebowanie energetyczne badanych owadów. Ponadto, obniżyła się zawartości białek w ciele tłuszczowym po zastosowaniu ekstraktu, a następnie fenitrotionu, podczas gdy ich zawartość wzrosła po podaniu samego fenitrotionu (LC_{40}).

W jelicie środkowym widoczne były rozbudowane systemy siateczki śródplazmatycznej, które ulegały rozdęciom, a także elektronowo gęsta cytoplazma. Aplikacja ekstraktu przed podaniem fenitrotionu spowodowała dodatkowe zmiany w postaci zwiększenia przestrzeni międzykomórkowych, co wskazuje na osłabienie ich wzajemnej adhezji. Zaobserwowałam także wzrost elektronowo gęstej chromatyny w jądrze komórkowym. Wyniki te wskazują na pojawienie się istotnego efektu toksycznego w komórkach badanych organizmów. Są one zgodne z danymi literaturowymi – uszkodzenia połączeń komórkowych są jednym ze skutków działania glikoalkaloidów (Roddick i wsp. 2001). W komórkach ciała tłuszczowego zarówno sam fenitrotion jak i skorelowana, następująca po sobie aplikacja ekstraktu i fenitrotionu, spowodowały zaburzenia adhezji komórek, wzrost zawartości elektronowo gęstej chromatyny, zmiany w homogenności kropli lipidowych oraz zmagazynowanych białek, a także spadek gęstości cytoplazmy otaczającej organelle. W przypadku aplikacji ekstraktu przed podaniem fenitrotionu, dodatkowo krople lipidowe w komórkach ulegały fuzji, co po raz kolejny wskazuje na powinowactwo glikoalkaloidów do błon biologicznych, prowadzących do zaburzeń w ich ciągłości.

Powyższe rezultaty uzyskane w drugim wariancie eksperymentu wskazują na zwiększoną toksyczność fenitrotionu w wyniku wcześniejszego, lecz nie równoczesnego, podania ekstraktu. Wzrost śmiertelności i nasilenie efektów subletalnych umożliwia potencjalne wykorzystanie tej metody jako elementu zintegrowanej ochrony roślin.

Podsumowanie

W opublikowanych przeze mnie pracach po raz pierwszy opisano wiele efektów działania badanych glikoalkaloidów oraz ekstraktu z *S. nigrum*, na poziomie organizmu i na wybranych suborganizmalnych poziomach organizacji życia. Uważam, że badania podstawowe, nie skupiające się jedynie na aspekcie aplikacyjnym, czyli efekcie letalnym, mogą wnieść wiele istotnych informacji wyjaśniających działanie glikoalkaloidów, ale także przyczynić się do skuteczniejszego ich stosowania w ochronie roślin i produktów spożywczych. Wzrost śmiertelności i intensywności efektów subletalnych sugeruje możliwość wykorzystania strategii skorelowanego podawania związków naturalnych oraz syntetycznych insektycydów w ochronie roślin i produktów spożywczych, ale równocześnie wskazuje na konieczność szczegółowych badań nad strategią stosowania zabiegów agrotechnicznych. Na uwagę, moim zdaniem, zasługuje możliwość zastosowania ekstraktu, a nie wyłącznie czystych substancji lub ich mieszanin. Uzyskanie takich ekstraktów jest tańsze, niż pozyskanie i stosowanie czystych związków. Uważam, że umożliwi to szersze stosowanie produktów naturalnych w rolnictwie, W konsekwencji, może się również przyczynić do obniżenia ceny produktów spożywczych.

Podsumowując, uzyskane wyniki pozwalają na sformułowanie następujących wniosków:

(1) Ekstrakt z *S. nigrum* powoduje u mącznika młynarka efekty subletalne, takie jak zmiany w kurczliwości narządów wewnętrznych, zmiany zawartości zmagazynowanych biocząsteczek i zaburzenia w ultrastrukturze komórek jelita środkowego i ciała tłuszczowego.

(2) Glikoalkaloidy obecne w ekstrakcie z psianki czarnej powodują subletalne efekty toksyczne u mącznika młynarka. Różnice w działaniu ekstraktu i pojedynczych glikoalkaloidów wskazują na synergistyczne działanie glikoalkaloidów.

(3) Stężenie fenitrotionu powodujące śmiertelność połowy badanej populacji ($LC_{50/24h}$) larw *T. molitor* wynosi 400 µg/mL, a $LC_{40/24h}$ jest równe 300 µg/mL.

(4) Ekstrakt z *S. nigrum* podawany równocześnie z fenitrotionem (LC_{50}) w przypadku krótkotrwałej ekspozycji, nie zwiększa śmiertelności larw, w porównaniu do efektów powodowanych przez sam fenitrotion, jednak widoczne są pewne efekty subletalne na poziomie komórkowym.

(5) Odpowiednio skorelowane podanie ekstraktu z psianki czarnej przed aplikacją fenitrotionu (LC_{40}) może spowodować istotny wzrost śmiertelności badanych szkodników oraz nasilić efekty subletalne, zarówno w porównaniu do stosowania równoczesnego obu związków, jak i samego fenitrotionu.

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4. Oświadczenia

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OŚWIADCZENIE DOKTORANTKI DOTYCZĄCE JEJ UDZIAŁU W POWSTANIU PRAC NAUKOWYCH STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

1. Plant-Derived Substances Used Against Beetles–Pests of Stored Crops and Food–and Their Mode of Action: A Review
(**Spochacz M.**, Chowański S**.**, Walkowiak-Nowicka K., Szymczak M., Adamski Z.)

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2. Sublethal Effects of *Solanum nigrum* Fruit Extract and Its Pure Glycoalkaloids on the Physiology of *Tenebrio molitor* (Mealworm)
(**Spochacz M.**, Chowański S., Szymczak M., Lelario F., Bufo S. A., Adamski Z.)

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   - Przygotowanie preparatów mikroskopowych i analiza ultrastruktury jelita i ciała tłuszczowego larw *Tenebrio molitor,* przygotowanie próbek i przeprowadzenie pomiarów parametrów biochemicznych ciała tłuszczowego, izolacja narządów kurczliwych i pomiar kurczliwości
   - Opracowanie wyników, analiza i interpretacja wyników wyżej wymienionych eksperymentów
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3. *Solanum nigrum* fruit extract increases toxicity of fenitrothion - a synthetic insecticide, in the mealworm beetle *Tenebrio molitor* larvae
(**Spochacz M.**, Szymczak M., Chowański S**.**, Bufo S.A., Adamski Z.)

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- Przygotowanie materiałów dodatkowych w postaci rycin i tabel

Marta Spochacz

Poznań, dnia 29.09.2020 .....

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Wydział Biologii
Zakład Fizjologii i Biologii Rozwoju Zwierząt
Laboratorium Mikroskopii Elektronowej i Konfokalnej

1. Plant-Derived Substances Used Against Beetles–Pests of Stored Crops and Food–and Their Mode of Action: A Review
(Spochacz M., Chowański S., Walkowiak-Nowicka K., Szymczak M., **Adamski Z.**)

2. Sublethal Effects of *Solanum nigrum* Fruit Extract and Its Pure Glycoalkaloids on the Physiology of *Tenebrio molitor* (Mealworm)
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Rola: Współautor

Jako promotor rozprawy doktorskiej mgr Marty Spochacz oświadczam, że mój wkład w powstanie wymienionych powyżej artykułów polegał na opisie działania prooksydantów i antyoksydantów pochodzenia roślinnego oraz opisie aplikacji tych substancji (artykuł 1), na udziale w tworzeniu koncepcji badań, częściowym udziale w opracowaniu danych, ich analizie oraz pisaniu i edytowaniu artykułów (artykuły: 2 i 3). Potwierdzam przeważający udział Doktorantki w przygotowaniu koncepcji badań, wykonaniu części eksperymentalnej, opracowaniu i analizie wyników, przygotowaniu artykułów oraz koordynowaniu procesu ich edytowania, aż do opublikowania.

Poznań, dnia 2020-09-24

Zbigniew Adamski

Prof. UAM dr hab. Zbigniew Adamski
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Oświadczam, że jako promotor pomocniczy rozprawy doktorskiej mgr Marty Spochacz, mój wkład w podane powyżej artykuły polegał przede wszystkim na planowaniu badań, analizie wyników i przygotowywaniu manuskryptów.
Zgodnie z powyższą kolejnością:

1. W przedstawionej pracy przeglądowej opisałam wpływ substancji pochodzenia roślinnego na zaburzenia w regulacji hormonalnej chrząszczy oraz możliwości zastosowania sproszkowanych roślin (w formie proszku) jako bioinsektycydy przeciwko chrząszczom.
2. W pracy oryginalnej „Sublethal Effects of *Solanum nigrum* Fruit Extract…" brałam udział w analizie danych oraz pisaniu i redagowaniu tekstu.
3. W pracy oryginalnej „*Solanum nigrum* fruit extract increases toxicity of fenitrothion…" brałam udział w walidacji i analizie wyników eksperymentów oraz w pisaniu i redagowaniu manuskryptu przed jego publikacją.

Poznań, dnia 02.10.2020

Monika Szymczak

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Rola: Współautor

Oświadczam, że mój wkład w wyżej wymienione artykuły polegał w przypadku pracy przeglądowej (1) na pomocy w zebraniu danych literaturowych dotyczących wpływu substancji o pochodzeniu roślinnym na układ nerwowy i wpływie olejków eterycznych na chrząszcze oraz ich opisie w odpowiednim rozdziale artykułu, a w przypadku prac oryginalnych (2 i 3) na pomocy przy prowadzeniu eksperymentów biochemicznych, analizie danych oraz edycji manuskryptu.

Szymon Chowański

Poznań, dnia 28.09.2020

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Rola: Współautor

Oświadczam, że mój udział w powyższej publikacji polegał na pomocy w zebraniu danych literaturowych dotyczących metod aplikacji substancji o pochodzeniu roślinnym oraz opisie tych metod w odpowiednim rozdziale artykułu.

Poznań, dnia 29.08.2020

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Università degli Studi della Basilicata
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The role: co-author

Hereby I declare that my participation in the above mentioned article was to contribute in (i) preparing and characterizing the extracts (ii) verifying the analytical methods; (iii) supervising the conclusions of researches.

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DOTTORATO DI RICERCA INTERNAZIONALE / *INTERNATIONAL PH.D PROGRAMME*
"APPLIED BIOLOGY AND ENVIRONMENTAL SAFEGUARD"
COORDINATOR PROF. SABINO AURELIO BUFO

DECLARATION

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The role: co-author

Hereby I declare that my participation in the above mentioned articles was to contribute in (i) conceiving of the presented ideas; (ii) verifying the analytical methods; (iii) supervising the conclusions of researches.

Potenza, 25/09/2020

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5. Publikacje wchodzące w skład rozprawy doktorskiej

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Comprehensive REVIEWS in Food Science and Food Safety

Plant-Derived Substances Used Against Beetles–Pests of Stored Crops and Food–and Their Mode of Action: A Review

Marta Spochacz, Szymon Chowański, Karolina Walkowiak-Nowicka, Monika Szymczak, and Zbigniew Adamski

Abstract: Plants are sources of numerous active substances that are used to protect crops. Currently, due to the limitations of using synthetic insecticides, plant products have attracted increasing attention as possible pesticides. In this review, we discuss some of the most interesting plant products (for example, Solanaceae, or Asteraceae extracts, *Artemisia absinthium* or *Citrus spp*. essential oils, and single compounds like α-chaconine, or α-solanine) that exhibit insecticidal activity against beetles that are pests of stored food products. Next, we describe and discuss the mode of action of these products, including lethal and sublethal effects, such as antifeedant or neurotoxic activity, ultrastructural malformation, and effects on prooxidant/antioxidant balance. Furthermore, the methods of application of plant-derived substances in food storage areas are presented.

Keywords: beetles, biopesticides, essential oil, plant extracts, stored food pests

Introduction

It is estimated that approximately 7% to 50% of all crops are destroyed by pests each year (Culliney, 2014; Oliveira, Auad, Mendes, & Frizzas, 2014; Pimentel, 2009; Sallam, 2013). To decrease such yield losses billions of kilograms of pesticides are used each year (Alavanja, 2009; Pimentel, 2005). Worldwide the usage of pesticides has increased, reaching 48000 tons of active ingredients in Germany, 24000 tons in Poland, more than 18000 tons in Great Britain, 62000 tons in Italy, and as much as 1.7 million tons in China (FAOSTAT, 2017). The economic significance of insecticides is very high, since insects are the most important crop pests. They can destroy crops and food products or contaminate them, making food unfit for human consumption. Insecticides are often not specific and also affect nontarget organisms.

Due to the phenomenon of development of resistance to insecticides, as well as the globalization of trade, which leads to the migration of insect pests, there is a constant need to introduce new substances used in plant protection. Because of the toxicity of insecticides to humans and the possible effects of their residues present in food, not every substance that possesses pesticidal activity can be used in crop protection. In particular, the usage of pesticides to protect stored products is prohibited by many governments. Several widely used pesticides, such as methyl bromide or ethylene dibromide, have been banned in many countries, due to their carcinogenicity and their role in the depletion of the ozone layer (Rajendran & Sriranjini, 2008).

More than 600 species are regarded as pests of stored products. Among the insects, cockroaches, moths, and beetles are the most important pests. Beetles are the largest taxon of *Insecta* and the most important order of pests of stored products (Culliney, 2014). Beetles may either eat the stored food or contaminate it while feeding on other organisms present in food storage facilities, such as fungi or invertebrates (Bousquet, 1990). Furthermore, this group of insects is often resistant to insecticides and can survive in dry conditions and absorb water from low-hydrated food. Therefore, their populations are difficult to control in food storage areas. Various groups of insecticides are used against coleopteran (beetles and weevils) crop pests, such as synthetic insecticides, fungal substances, and plant-derived substances (for example, essential oils, or alkaloids). Natural products make up a low percentage of current insecticides used (Yu, 2008). However, their usage will probably increase, due to the growing number of organic farms. Moreover, usage of plant-derived substances is among the strategies implemented in Integrated Pest Management.

The term "biopesticides" is still under debate. Many groups of factors are regarded as biopesticides, such as viruses, whole organisms (such as microbes, fungi, entomophagous invertebrates, parasitoids, and predators), and substances produced by living organisms (such as microbial or fungal toxins, insect pheromones, and plant secondary metabolites). Some researchers exclude plant extracts or even all chemical agents from bioinsecticides, whereas others classify these compounds as bioinsecticides, since organisms often act using toxic substances, and one cannot separate the

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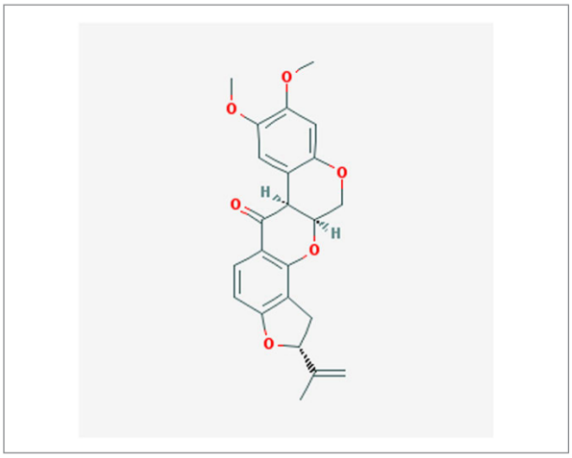

Figure 1–Rotenone, chemical structure (Pubchem, 2018d).

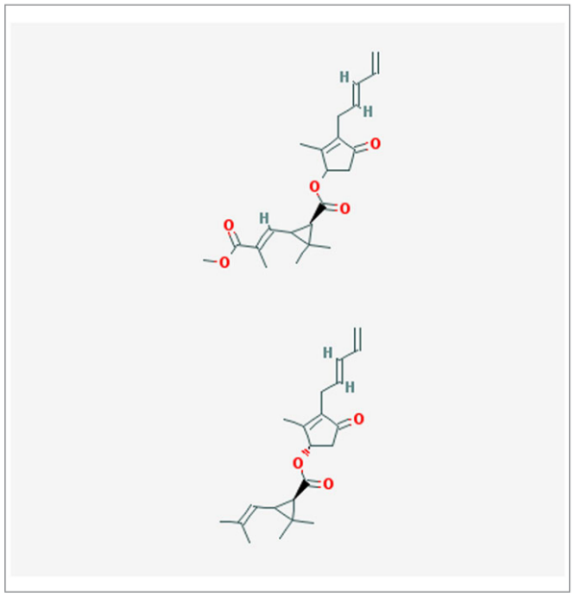

Figure 2–Pyrethrins, chemical structure (Pubchem, 2018c).

pesticidal activity of whole organisms from pest-produced substances (Glare, 2015). According to European Union regulations, bioinsecticides also cover plant extracts and foodstuffs (Marchand, 2017). Without a further exploration of the above-mentioned topic, we will focus on plant-derived substances, either pure or used as extracts that have been proven to have the potential to protect stored products against pests. In this sense, "biopesticide," "plant-derived insecticide," "natural insecticide," and other related terms will be used in our review in reference to pure substances produced by plants as well as plant extracts, which are often used by small farms.

Several classes of plant-derived chemicals can be distinguished: alkaloids, rotenoids, phenolic compounds, pyrethrins, oils, saponins, and some others. Some of them are already widely used, their activity is described in the subsequent chapters. For example, nicotine appears to be effective against various insects (aphids, leafhoppers, thrips, and whiteflies), but its usage is decreasing due to the high toxicity of nicotine to vertebrates. Rotenone (Figure 1) is used against mites and lice, although it has been found to be toxic to fish. Its toxicity is based on the malfunctioning of mitochondria both in invertebrates and vertebrates, thus posing a hazard to humans, also. Azadirachtin is also a well-known terpenoid substance that is used against stored-product pests (cockroaches). Sabadilla, which contains veratrine alkaloids, is used against pests of vegetables. Another compound–ryania–is used against beetles, moths, thrips, and aphids. Both sabadilla and ryania are neurotoxic. Pyrethrins (Figure 2) are used against flying insects, fortunately their toxicity to mammals at the concentrations used is infinitesimal (Isman, 2008; Yu, 2008).

The field of research describing the insecticidal activity of plant-derived substances and plant extracts is rapidly developing. *Brassicaceae*, *Asteraceae*, *Umbelliferae*, *Solanaceae*, and many other plant families contain substances showing toxic activity. Although the acute toxicity of pure plant-derived substances or plant extracts may be many times lower than the toxicity of synthetic insecticides (Derbalah, 2012), their subacute toxicity, including repellency or antifeedant activity has been described (Regnault-Roger, 2012; Said & Pashte, 2015). Moreover, these substances are usually low-toxic to humans, and, thus, some of them are even used in traditional cuisine, such as spices (Said & Pashte, 2015) and show low persistence, which limits residues in crops. Furthermore, plant-derived substances are relatively easy to obtain and to use as extracts. Due to the enormous variety of plant species, their metabolites, and the number of pests and possible responses to botanicals, new scientific reports are appearing constantly. Additionally, the methods of application of these substances for crop storage are still being developed.

In this review, we present a range of toxic effects caused by various plant-derived substances, mostly alkaloids and essential oils. We also show their mode of action and some important physiological reactions of insects exposed to plant-derived species. In few cases, we have not comprehensively discussed some active compounds due to inconsistency in the available data. However, a brief overview has been given, wherever necessary. We also attempt to discuss the possible methods of bioinsecticide usage for stored crop protection as well as perspectives for the future of plant-derived substances with respect to the protection of stored crops.

Mode of Action of Plant-Derived Substances

Due to the high number of used substances and target insects, there is a wide spectrum of observed effects. The insecticidal activity of nicotine suggests, that substances possessing insecticidal properties can be found in other *Solanaceae* plants. Indeed, many substances have been reported within this family (Chowański et al., 2016) and they can be both acutely and subacutely toxic against beetles. This acute lethal toxicity has drawn the attention of many researchers because the immediate insecticidal effect and possible application in agrochemistry. However, subacute effects are also important because they may limit the spread of the population (reduced fertility, fecundity, low vitality, or shorter lifespan) and reduce crop loss due to repellent, suppressant, or deterrent activity. These terms are often described with the more general term "antifeedant activity." On the other hand, there are few data that describe the mode of action of plant-derived substances at the

suborganismal level. Such basic research may be crucial for an explanation of the activity of bioinsecticides.

Various sublethal toxic effects of plant substances are presented in this review (Tables 1 to 6). For example, Ratnasekera and Rajapakse (2010) described a range of sublethal effects of vapors of certain plant oils (*Calophyllum inophyllum, Solanum indicum, Cinnamomum verum, Brassica juncea, Azadirachta indica, Madhuka longifolia, Ricinus communis, Cymbopogon nardus*, and *Sesamum indicum*) on the pulse beetle (*Callosobruchus maculatus, Bruchidae*). The oils decrease oviposition and adult emergence. Sublethal activity of various plant-derived substances were described. A wide range of other toxic sublethal effect exists: reduced weight, body size, decreased fertility, and altered behavior (Chowański et al., 2016). These effects are very important from the crop-owner's point of view. They limit crop infestation and slow down crop losses. Therefore, these sublethal toxicities should be considered equally important as lethal, acute toxicities.

The mode of action of toxic substances is much less studied than the effects at the population or single-organism level. Perhaps, as the applicatory effect is still the most important one, we still search for substances that can be used as new classes of insecticides; however an explanation of the activity of these compounds may help us to obtain a higher toxicity due to proper application (that is, time of exposure, concentration, and pest developmental stage), to explain the development of resistance within an exposed population, or to plan agrochemical strategies that include the synergistic action of 2 or more active compounds. In this paper, we try not only to show the current level of knowledge regarding plant-derived substances used in stored crop protection against beetle pests, but also to describe their mode of action and the possible methods of their usage and application.

Effects on the nervous system

Some of the tested substances are postulated to cause neurological effects. For example, there are alkaloids that reversibly inhibit the activity of acetylcholinesterase (AChE)—an important enzyme that regulates synaptic transmission, and, thus, they cause imbalanced functioning of the nervous system. The excitation of the nervous system is one of the most important modes of action of conventional insecticides as organophosphates and carbamates. Inhibition of AChE was reported for crude extracts from various plant families, including those that show insecticidal activity, for example, *Asteraceae* or *Solanaceae* (Niño, Hernández, Correa, & Mosquera, 2006). AChE inhibition was described as an effect of saponins in *Tribolium castaneum* (Tenebrionidae) larvae, where LC_{50} was 0.7 ppm (Sami, Bilal, Khalid, Nazir, & Shakoori, 2018). The authors of this work indicate, that sprays which they tested can be used in protection of stored food. Also various groups of alkaloids may bind to the specific amino acids in the active site of AChE (Deka, Kumar, Nayak, & Eloziia, 2017), which causes its inhibition. Two of the major glycoalkaloids of certain *Solanaceae* plants, namely, α-solanine (Figure 3) and α-chaconine, act synergistically with neuromuscular blocking drugs and cholinesterase inhibitors (McGehee et al., 2000). α-Chaconine was reported to be a strong AChE inhibitor in the Colorado potato beetle (*Leptinotarsa decemlineata*) (Wierenga & Hollingworth, 1992). On the other hand, nicotine and other structurally related alkaloids can mimic acetylcholine (Dayan, Cantrell, & Duke, 2009). Both types of activity lead to hyperactivity of the nervous system, which results, for example, in the alteration of muscle and gland activity. As a consequence of the neurotoxic activity of alkaloids, physiological processes of insects, such as larval growth and development (Weissenberg, Levy, Svoboda, & Ishaaya, 1998), heart activity (Ventrella et al., 2015), and food intake (Abbasipour, Mahmoudvand, Rastegar, & Hosseinpour, 2011; Singaravelan, Nee'man, Inbar, & Izhaki, 2005), can be disturbed, and crop losses may be limited. AChE inhibitors are also found among other plant families: *Fabaceae*, *Amaryllidaceae*, *Alliaceae*, *Papaveraceae* and *Ranunculaceae* (Howes & Houghton, 2009). These substances (for example, galantamine) may bind to choline-binding sites and acyl-binding sites, forming hydrogen bonds with the amino acids of the enzyme. Analysis of the bonds between AChE and bioinsecticidally active chemical compounds is a starting point for the formation of the modified alkaloids, which may act more effectively as insecticides (Greenblatt, Kryger, Lewis, Silman, & Sussman, 1999). In addition, some glycoalkaloids were reported to act with nicotinic but not muscarinic cholinergic receptors (Taveira et al., 2014). Alkaloids such as tomatine and tomatidine exhibit neurotoxic activity by disturbing the homeostasis of the endoplasmic reticulum and affecting the level of calcium in the cytoplasm, thus affecting synaptic transmission. Tomatine affects proteasomes within cells, leading to disturbed metabolism of nerve cells (da Silva, Andrade, Valentao, & Pereira, 2017). Tomato extract and tomato pure alkaloids were also reported to be factors that prevent mitochondrial membrane potential in nerve cells and, therefore, show neuroprotective activity (Taveira et al., 2014). Another mechanism of alkaloid action may involve an effect on sodium channels in the nervous system. For example, sabadilla exhibits neurotoxic activity by slowing the shutting down of Na^+-channels, disturbing membrane depolarization, and causing paralysis before death (Regnault-Roger, 2012). Similarly, evoked neurotoxicity was also noted in a case of aconitine isolated from plants from the genus *Aconitum* and veratridine extracted from *Liliaceae* plants (Dwivedy, 1988). Another alkaloid, ryania, interferes with nerve impulses at the Ca^{2+}-channel level and generates sustained contraction of the muscle and paralysis (Regnault-Roger, 2012).

Neurotoxic substances are also found among essential oils. Like alkaloids, some essential oils as well as their single compounds–monoterpenoids (Figure 4)–inhibit AChE activity (Chaubey, 2011; El-Sayed, El-Sheikh, Sherif, & Gouhar, 2015; Kim, Kang, & Park, 2013; Rajendran & Sriranjini, 2008) in correlation with repellent activity (Chaubey, 2011). This author suggests, that toxicity of tested EOs may appear due to AChE inhibition during fumigation. On the other hand, in some cases, the concentration must be very high—10^{-3} M—to obtain significant inhibition of AChE (Kostyukovsky, Rafaeli, Gileadi, Demchenko, & Shaaya, 2002). Perhaps, in this case mild neurotoxic effects would appear during exposure. The action of these substances may be linked with the malfunctioning of octopaminergic and GABA-ergic pathways; activation of octopaminergic metabotropic receptors may alter the activity of chloride channels (Isman, 2006), for example, through cAMP which is a secondary messenger in an octopaminergic pathways (Kostyukovsky et al., 2002). Octopamine is an interesting target for pesticidal substances, since it is a neurohormone and neuromodulator present in invertebrates but not vertebrates. A large number of physiological and behavioral processes are regulated by octopamine (Roeder, 1999). Among them, reproduction (Yamane, 2014) and feeding (Zhang, Branch, & Shen, 2013) may play important roles in the regulation of insect populations. Consequently, serious malfunctions of the nervous system may appear and disturb the functioning and development of invertebrate pests. In many cases, neurotoxic effects, as sublethal ones, were, by definition, possible to obtain, if the lethal insecticidal effects were obtained. For some pests, neurotoxic

Table 1—Effects of plant extracts on coleopterous insect pests of stored food products.

Plant species	Obtained	Pest	Stage	Endpoint	Toxicity	Reference
Acorus calamus var. *angustatus*	MetOH rhizome extract	*Lasioderma serricorne* (Anobiidae)	A	Mortality	90%: 72 hr; 3.5 mg/cm^2	(Kim, Park, Ohh, Cho, & Ahn, 2003)
Agastache rugosa	MetOH whole plant extract				100%: 48 hr; 3.5 mg/cm^2	
Allium sativum	MetOH bulb extract	*Attagenus unicolor japonicus*	L		93%: 7 days; 5.2 mg/cm^2	(Han, Kim, & Ahn, 2006)
Anacyclus pyrethrum	Aqueous root extract	*Callosobruchus maculatus*	A	Reduced longevity and fecundity, increased number of nonfertile eggs	doses: 0.5 to 2 g/50 seeds	(Khannouchi, Elhilali, & Zair, 2012)
Angelica dahurica	MetOH root extract	*A. unicolor japonicus*	L	Antifeedancy	100%: 30 days; 1.3 mg/cm^2	(Han et al., 2006)
Azadirachta indica	Water seeds extract	*Tribolium castaneum*	A	Mortality	$LD_{50;72hr}$ = 74.27 μg/insect (CT)	(Islam & Talukder, 2005)
	Neem oil emulsifiable concentrate	*Zabrotes subfasciatus (Bruchidae)*	A		93%: 30 days; 2000 ppm	(da Costa et al., 2014)
				Reduced oviposition	100%: 30 days; 1000 ppm	
Bauhinia purpurea	MetOH leaf extract	*Trogoderma granarium*	A	mortality	83%: 7 days; 100 mg/L	(Derbalah, 2012)
				Reduced progeny	98%: 7 days; 100 mg/L	
Caesalpinia gilliesii				mortality	93%: 7 days; 300 mg/L	
				Reduced progeny	60% 7 days; 300 mg/L	
Calotropis procera		*S.oryzae*	A	mortality	61%: 7 days; 5 mL/cm^2	(Nenaah, 2013)
				Reduced progeny	68%: 7 days; 5 mL/cm^2	
Carica papaya	Acetone leaf extract			mortality	76%: 24 hr; 0.5 mg/ cm^3 (FT)	(Rani & Devanand, 2011)
					37%: 24 hr; 0.5 mg/ cm^3 (CT)	
		T. castaneum	A		81%: 24 hr; 0.5 mg/ cm^3 (FT)	
					20%: 24 hr; 0.5 mg/ cm^3 (CT)	
Cassia fistula	MetOH leaf extract	*T. granarium*	A		57%: 7 days; 100 mg/L	(Derbalah, 2012)
				Reduced progeny	80%: 7 days; 100 mg/L	
Cassia senna				mortality	87%: 7 days; 100 mg/L	
				Reduced progeny	95%: 7 days; 100 mg/L	
Chrysanthemum frutescens	MetOH extract			mortality	50%: 7 days; 100 mg/L	
				Reduced progeny	62%: 7 days; 100 mg/L	
Cinnamomum cassia	MetOH bark extract	*L. serricorne*	A	mortality	100%: 24 hr; 3.5 mg/cm^2 (FT)	(Kim et al., 2003)
Cnidium officinale	MetOH rhizome extract	*A. unicolor japonicus*	L	Antifeedancy	100%: 30 days; 2.6 mg/cm^2	(Han et al., 2006)
Coccinia indica	Acetone leaves extract	*S. oryzae*	A	Mortality	71%: 72 hr; 0.5 mg/cm^3 (FT)	(Rani & Devanand, 2011)
					54%: 72 hr 0.5 mg/cm^3 (CT)	
		T. castaneum	A		62%: 72 hr 0.5 mg/cm^3 (FT)	
					57%: 72 hr 0.5 mg/cm^3 (CT)	
Cynodon dactylon	Water seeds extract				$LD_{50;72hr}$ = 152.3 μg/insect (CT)	(Islam & Talukder, 2005)
Decalepis hamiltonii	MetOH root extract	*Rhyzopertha dominica*	A	Acute toxicity, reduced progeny	$LD_{50;24hr}$ = 0.119 mg/cm^2	(Rajashekar, Gunasekaran, & Shivanandappa, 2010)
		Sitophilus oryzae	A		$LD_{50;24hr}$ = 0.119 mg/cm^2	
		Stegobium paniceum (Anobiidae)	A		$LD_{50;24hr}$ = 0.115 mg/cm^2	
		C. chinensis (Bruchidae)	A		$LD_{50;24hr}$ = 0.115 mg/cm^2	
Eucalyptus globules	Acetone leaf extract	*C. maculatus*	A	Hatching inhibition	39% less hatched eggs: 72 days; 2% v/v	(Rahman & Talukder, 2006)
Eugenia caryophyllata	MetOH bud extract	*A. unicolor japonicus*	L	Mortality	100%: 14 days; 2.6 mg/cm^2	(Han et al., 2006)
Euonymus japonicus	MetOH leaf extract	*T. granarium*	A	Mortality	80%: 7 days; 1300 mg/L	(Derbalah, 2012)
				Reduced progeny	90% 7 days; 1300 mg/L	

(Continued)

Table 1–Continued.

Plant species	Obtained	Pest	Stage	Endpoint	Toxicity	Reference
Foeniculum vulgare	MetOH fruit extract	*A. unicolor japonicus*	L	Mortality	100%: 28 days; 5.2 mg/cm^2	(Han et al., 2006)
	MetOH fruit extract	*L. serricorne*	A	Mortality	100%: 24 hr; 3.5 mg/cm^2	(Kim et al., 2003)
Humulus lupulus	Ethyl acetate cones extract	*S. oryzae*	A	Mortality	$LD_{50;48hr}$ = 29.2 μg (CT)	(Aydin et al., 2017)
		Sitophilus granarius	A		$LD_{50;48hr}$ = 15.4 μg (CT)	
		Acanthoscelides obtectus	A		$LD_{50;48hr}$ = 26.9 μg (CT)	
		T. castaneum	A		$LD_{50;48hr}$ = 21.1 μg (CT)	
		L. serricorne	A		$LD_{50;48hr}$ = 24.1 μg (CT)	
	Dichloromethane cones extract	*S. oryzae*	A		$LD_{50;48hr}$ = 33.1 μg (CT)	
		S. granarius	A		$LD_{50;48hr}$ = 21.9 μg (CT)	
		T. castaneum	A		$LD_{50;48hr}$ = 32.4 μg (CT)	
		L. serricorne	A		$LD_{50;48hr}$ = 32.2 μg (CT)	
	Acetone cones extract	*S. granarius*	A		$LD_{50;48hr}$ = 24.4 μg (CT)	
		T. castaneum	A		$LD_{50;48hr}$ = 29.6 μg (CT)	
		L. serricorne	A		$LD_{50;48hr}$ = 30.7 μg (CT)	
	Ethanol cones extract	*S. granarius*	A		$LD_{50;48hr}$ = 25.7 μg (CT)	
		L. serricorne	A		$LD_{50;48hr}$ = 3 4.0μg (CT)	
Illicium verum	MetOH fruit extract	*L. serricorne*	A	Mortality	100%: 24 hr; 3.5 mg/cm^2	(Kim et al., 2003)
Ipomoea sepiaria	Acetone leaf extract	*C. maculatus*	A	Hatching inhibition	39% less hatched eggs: 72; 2% v/v	(Rahman & Talukder, 2006)
Lysimachia davurica	MetOH whole plant extract	*A. unicolor japonicus*	L	Antifeedancy	100%: 30 days; 2.6 mg/cm^2	(Han et al., 2006)
Momordica charantia	Acetone leaf extract	*S. oryzae*	A	Mortality	100%: 24 hr; 0.5 mg/cm^3 (FT)	(Rani & Devanand, 2011)
					98%: 72 hr; 0.5 mg/cm^3 (CT)	
		T. castaneum	A		100%: 72 hr; 0.5 mg/cm^3 (FT)	
					98%: 72 hr 0.5 mg/cm^3 (CT)	
Murraya koenigii	Acetone leaf extract	*S. oryzae*	A		89%: 72 hr; 0.5 mg/cm^3 (FT)	(Mehrabadi, Bandani, Saadati, & Mahmudvand, 2011)
					75%: 72 hr 0.5 mg/cm^3 (CT)	
		T. castaneum	A		90%: 72 hr; 0.5 mg/cm^3 (FT)	
					68%: 72 hr; 0.5 mg/cm^3 (CT)	
Nardostachys chinensis	MetOH rhizome extract	*A. unicolor japonicus*	L	Antifeedancy	100%: 30 days; 1.3 mg/cm^2	(Han et al., 2006)
Punica granatum	MetOH seeds extract	*C. maculatus*	A	Inhibition of α-amylases	90% decreased activity	(Mehrabadi, Bandani, Saadati, & Mahmudvand, 2011)
Rhaponticum acaule	1% aerial parts MetOH/ petroleum/ ether/ chloroform/ ethyl acetate extracts	*Tribolium confusum*	L	Mortality	43% to 83%: 3 weeks (FT)	(Boussaada et al., 2008)
			A		≥33%: 16 days (FT)	
Rheum officinale	MetOH leaves extract	*S. granarius*	A	Inhibition of α-amylases	96% decreased activity: 10% v/v	(Mehrabadi et al., 2011)
		C. maculatus	A		95% decreased activity 10% v/v	
Rhus coriaria					95% decreased activity 10% v/v	
Tagetes erecta	Water seeds extract	*T. castaneum*	A	Mortality	$LD_{50;72hr}$ = 152.3 μg/insect (CT)	(Islam & Talukder, 2005)
Thespesia populnea	MetOH leaf extract	*T. granarium*	A		90%: 7 days; 300 mg/L	(Derbalah, 2012)
				Reduced progeny	63%: 7 days; 300 mg/L	
Vitex negundo	acetone leaf extract	*C. maculatus*	A	Hatching inhibition	64% less hatched eggs: 72 days; 3% extract	(Rahman & Talukder, 2006)
Zanthoxylum schinifolium	MetOH fruit extract	*A. unicolor japonicus*	L	antifeedancy	100%: 30 days; 2.6 mg/cm^2	(Han et al., 2006)

Abbreviation used in Table: CT, contact toxicity; FT, fumigant toxicity; A, adult; L, larva.

Table 2–Effects of alkaloid-containing plant extracts on coleopterous pests of stored food products.

Plant species	Obtained	Pest	Stage	Endpoint	Toxicity	Reference
Capsicum annuum	Acetone leaves extract	*S. oryzae*	A	Mortality, deterrence	$LC_{50;24hr}$ = 21.2 mg/ 20g diet (FT)	(Rani & Devanand, 2011)
				Mortality	$LC_{50;72hr}$ = 27.6 mg/20 g diet (CT)	
		T. castaneum	A		$LC_{50;24hr}$ = 24.8 mg/ 20g diet (FT)	
					$LC_{50;72hr}$ = 32.0 mg/ 20g diet (CT)	
Datura stramonium	MetOH seeds extract	*C. maculatus*	A	Inhibition of α-amylases	95.5% decreased activity: 10% v/v	(Mehrabadi et al., 2011)
		S. granarius	A		93.5% decreased activity: 10% v/v	
	Ethanol leaves extract	*T. castaneum*	A	Mortality	$LC_{50;48hr}$ = 3936 mg/L (CT)	(Abbasipour et al., 2011)
Jatropha curcas	Aqueous seeds extract	*Sitophilus zeamais*	A		≥70%: 48 hr; 10% v/v (CT)	(Silva et al., 2012)
		Ryzopherta dominic	A		100%: 48 hr; 5% v/v (CT)	
		T. castaneum	A		≥60%: 48 hr; 5 % v/v (CT)	
		Oryzaephilus surinamensis (Silvanidae)	A		≥90%: 48 hr; 5 % v/v (CT)	
Solanum melongena	Acetone leaves extract	*S. oryzae*	A		61%: 72 hr (CT)	(Rani & Devanand, 2011)
					100%: 48 hr (VT)	
		T. castaneum	A		61%: 72 hr (CT)	
					100%: 72 hr (VT)	
Solanum tuberosum	Total glycoalkaloid fraction	*T. granarium*	A		$LC_{50;48hr}$ = 16.7 μg/mg insect (CT)	(Nenaah, 2011a)
		S. oryzae	A		$LC_{50;24hr}$ = 38.6 $\mu g/cm^2$ (CT)	(Nenaah, 2011b)
		T. castaneum	A		$LC_{50;48hr}$ = 16.2 mg/kg grains (CT)	

Abbreviation used in Table: CT, contact toxicity; VT, vapor toxicity; DT, diet toxicity; A, adult.

Table 3–Effects of pure alkaloids on coleopterous pests of stored food products.

Compound	Pest	Stage	Endpoint	Toxicity	Reference
Capsaicin	*Tenebrio molitor*	L	Changes in behavioural thermoregulation	Tested concentrations: 10^{-7} to 10^{-4} M	(Olszewska & Tegowska, 2011)
α-Chaconine	*T. granarium*	A	Mortality	$LC_{50;96hr}$ = 18.1 μg/mg insect (CT)	(Nenaah, 2011a)
	S. oryzae	A		$LC_{50;24hr}$ = 48.2 $\mu g/cm^2$ (FT)	(Nenaah, 2011b)
Solamargine	*T. castaneum*	L	Larval growth inhibition	1 μmol/g of the diet (DT)	(Weissenberg et al., 1998)
α-solanine	*T. granarium*	A	Mortality	$LC_{50;96hr}$ = 22.5 μg/mg of insect (CT)	(Nenaah, 2011a)
	S. oryzae	A		$LC_{50;24hr}$ = 82.3 $\mu g/cm^2$ (FT)	(Nenaah, 2011b)
Solasodine	*T. confusum*	L	Developmental disruption and inhibition of metamorphosis	40% of larvae failed to pupae: 2.5% v/v (CT)	(Lingampally, Solanki, Anuradha, & Sabita Raja, 2014)
Solasonine	*T. castaneum*	L	Larval growth inhibition	1 μmol/g of the diet (DT)	(Weissenberg et al., 1998)
α-Tomatine	*S. oryzae*	A	Mortality	$LC_{50;24hr}$ = 52 $\mu g/cm^2$ (FT)	(Nenaah, 2011b)
	T. castaneum	L	Larval growth inhibition	1 μmol/g of the diet (DT)	(Weissenberg et al., 1998)

Abbreviation used in Table: CT, contact toxicity; FT, fumigant toxicity; DT, diet toxicity; A, adult; L, larva.

Table 4–Effects of plant powders on coleopterous pests of stored food products.

Plant species	Obtained	Pest	Stage	Endpoint	Toxicity	Reference
Azadirachta indica	Seed kernel	*Caryedon serratus* (Bruchidae)	A	Reduced egg laying	80% reduction: 2 weeks; 5% w/w	(El Atta & Ahmed, 2002)
				Reduced hatching	81% reduction: 2 weeks; 5% w/w	
	Leaves	*T. castaneum*	A	Residual toxicity	50.1 % inhibition ratio: 5% w/w	(Islam & Talukder, 2005)
	Seeds	*C. maculatus*	A	Mortality	1.3% to 31%: 7 days; 5 to 40 g of powder/kg of corn seeds	(Tofel et al., 2017)
				Reduced progeny	100% reduction: 7 days; 5 to 40 g of powder/ kg of corn seeds	
		S. zeamais	A	Mortality	22% to 93%: 7; 5 to 40 g of powder/ kg of corn seeds	
				Reduced progeny	100% reduction: 7 days; 5 to 40 g of powder/ kg of corn seeds	
Carum carvi	Seeds	*C. maculatus*	A	Mortality	10%: 7 days; 2% powder	(Ul Hasan Et Al., 2013)
Cynodon dactylon	5% leaves powder	*T. castaneum*	A	Residual toxicity	43% inhibition ratio	(Islam & Talukder, 2005)
Eucalyptus globules	Leaves	*C. maculatus*	A	Mortality	7.0 mortality rate: 7 days; 2% powder	(Ul Hasan Et Al., 2013)
Eugenia aromatica	Flower bud	*S. granarius*	A		$LD_{50;7days} = 0.369$ g of powder/20 g of wheat seeds	(Adarkwah et al., 2017)
		T. castaneum	A		$LD_{50;7days} = 1.416$ g of powder/20 g of wheat seeds	
		A. obtectus	A		$LD_{50;7days} = 0.088$ g of powder/20 g of wheat seeds	
Moringa oleifera	Leaves	*S. granarius*	A		$LD_{50;7days} = 0.686$ g of powder/20 g of wheat seeds	
		T. castaneum	A		$LD_{50;7days} = 1.38$ g of powder/20 g of wheat seeds	
		A. obtectus	A		$LD_{50;7days} = 0.179$ g of powder/20 g of wheat seeds	
Nigella sativa	Seeds	*C. maculatus*	A		8.0 mortality rate: 7 days; 2% powder	(Ul Hasan et al., 2013)
Plectranthus glandulosus	Leaves	*C. maculatus*	A	Reduced progeny	16% to 96% reduction: 5 to 40 g of powder/kg of corn seeds	(Tofel et al., 2017)
		S. zeamais	A		25% to 97% reduction: 5 to 40 g of powder/kg of corn seeds	
Tagetes erecta	Leaves	*T. castaneum*	A		57% inhibition ratio: 5% powder	(Islam & Talukder, 2005)

Abbreviation used in Table: A, adult.

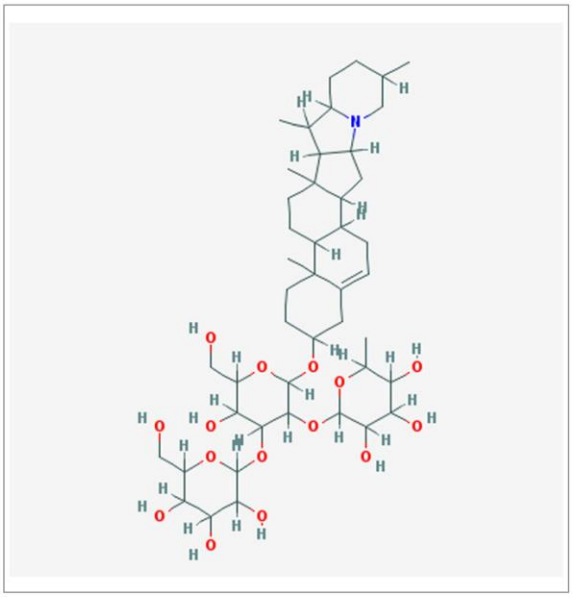

Figure 3–α-solanine, chemical structure(Pubchem, 2018g).

concentrations of, for example, glycoalkaloids did not exceed lethal ones, which could be applied as insecticides (Table 3).

Effects on internal organs

Many stored plants and their parts contain oils or alkaloids that decrease insect feeding, which by itself reduces crop loss. A limited number of insects can cause significant loss of tobacco, due to the presence of alkaloids (nicotine) in stored plant organs (Edde, Eaton, Kells, Phillips, & Hagstrum, 2012). Insects develop a natural resistance to the alkaloids present in host plants. For example, the Colorado potato beetle is a pest resistant to potato glycoalkaloids (mainly α-solanine and α-chaconine). However, at the same time, *L. decemlineata* is not resistant to other *Solanaceae* plant extracts, as for example, *Solanum nigrum*, the extract of which causes acute toxicity to this insect (Gökçe et al., 2007). According to the research conducted by Armer (2004), *L. decemlineata* does not metabolize alkaloids, such as solanine and chaconine, from potato leaves because active alkaloids were found in the excreta of this beetle. However, the exact mode of action of alkaloids in insects is unknown. The given results can also be an effect of other components present in extract or their joint action.

Light microscopic studies revealed serious malformations of the midgut epithelium of the Khapra beetle (*Trogoderma granarium, Dermestidae*) exposed to caraway essential oil and its component, monoterpenoid carvone. The epithelial nuclei were deeply stained. Additionally, the gut musculature was thickened, and the regenerative cells were not pronounced (Osman, Swidan, Kheirallah, & Nour, 2016). These changes may be crucial for feeding and, consequently, limit crop losses.

Solanaeceae extracts may also cause ultrastructural changes, which may lead to decreased feeding and malfunctioning of insect organs. As our latest electron microscopy research has shown, larvae of the beetle *Tenebrio molitor* (Tenebrionidae) fed with nourishment containing an extract from *S. nigrum* unripe fruits,

Table 5–Effects of essential oils on coleopterous pests of stored food products.

Plant species	Plant part	Pest	Stage	Endpoint	Toxicity	Reference
Achillea fragrantissima	Aerial parts	*C. maculatus*	A	Mortality,	$LC_{50;48hr}$ = 19.2 μL/L air(FT) $LC_{50;48hr}$ = 56.3 μL/g of dust (CT)	(Nenaah, Ibrahim, & Al-Assiuty, 2015)
				inhibition of eggs development	$LC_{50;48hr}$ = 93.6 μL/L air(FT)	
	Aerial parts (nanoemulsion formulations)			Mortality	$LC_{50;48hr}$ = 12.7 μL/L air(FT)	
				Inhibition of eggs development	$LC_{50;48hr}$ = 31.2 μL/L air(FT)	
Ageratum conyzoides	Aerial parts	*C. maculatus*	A	Mortality	$LC_{50;48hr}$ = 19.2 μL/L air(FT) $LC_{50;48hr}$ = 38.4 μL/g of dust (CT)	
				Inhibition of eggs development	$LC_{50;48hr}$ = 71.6 μL/L air(FT)	
	Aerial parts (nanoemulsion formulations)			Mortality	$LC_{50;48hr}$ = 7.6 μL/L air(FT)	
				Inhibition of eggs development	$LC_{50;48hr}$ = 21.8 μL/L air(FT)	
Allium sativum	Aerial parts	*Alphitobius diaperinus*	L	Mortality, repellency, inhibition of AChE	$LC_{50;24hr}$ = 8.4 μL/L air(FT) $LC_{50;24hr}$ = 49% v/w (CT)	(Wang et al., 2014)
Artemisia herba-alba	Leaves	*Orysaephilus surinamensis*	A	Mortality	LC_{50} = 3.05 μL/L air (FT) LC_{50} = 2.242 μL (CT)	(Bachrouch, Ferjani, Haouel, & Jemãac, 2015)
		T. castaneum	A		LC_{50} = 27.76 μL/L air (FT) LC_{50} = 7.432 μL(CT)	
Artemisia absinthium	Leaves	*O. surinamensis*	A		LC_{50} = 5.22 μL/L air (FT) LC_{50} = 0.209 μL (CT)	
		T. castaneum	A		LC_{50} = 35.18μL/L air (FT) LC_{50} = 2.261 μL (CT)	
	Aerial parts	*S. granarius*	A		53%: 12 hr; 9 μL/L air (FT)	(Kordali, Aslan, Çalmaşur, & Cakir, 2006)
Artemisia dracunculus	Leaves	*A. diaperinus*	L	Reduction of body weight, inhibition of pupation and moulting	Tested concentrations: 0.25% to 1%	Szczepanik et al., 2018
Artemisia santonicum *Artemisia spicigera*	Aerial parts	*S. granarius*	A	Mortality	50%: 12 hr; 9 μL/L air (FT) 53%: 12 hr; 9 μL/L air (FT)	(Kordali et al., 2006)
Astrocaryum aculeatum	Seeds	*S. zeamais*	A		$LC_{50;24hr}$ = 53.4% v/v (FT)	(Santos, Fernandes, Lopes, & Sousa, 2015)
Azadirachta indica	Seeds	*C. maculatus*	A		99%: 6 mL of oil/kg of corn seed	(Tofel et al., 2017)
		S. zeamais	A		95% 6 mL of oil/kg of corn seed	
		C. maculatus	A		30% to 100%: 2 to 6 mL/kg food 100% of adult emergence: 2 to 6 mL/kg food	(Tofel et al., 2016)
		Sitophilus zeamais	A		15% to 100%: 2 to 6 mL/kg food 100% of adult emergence: 2 to 6 mL/kg food	
Brassica juncea	Aerial parts	*L. serricorne*	A		100%: 24 hr; 3.5 mg/cm^2 (FT)	(Kim et al., 2003)
Brassica rapa	Acetone solution	*C. maculatus*	A	Repellency	17.5: 4 hr; 1% v/v	(Fouad, 2013)
Carapa guianensis	Seeds	*S. zeamais*	A	Mortality	$LC_{50;24hr}$ = 60.3% (FT)	(Santos et al., 2015)
Carum copticum	Seeds	*S. oryzae*	A		$LC_{50;24hr}$ = 0.91 μL/L air (FT)	(Sahaf, Moharramipour, & Meshkatalsadat, 2007)
		T. castaneum	A		$LC_{50;24hr}$ = 0.91 (μL/L air (FT)	
Cinnamomum camphora	Fruit				$LC_{50;24hr}$ = 8.5 mg/L air (FT)	(Guo et al., 2016)
Cinnamomum cassia	Aerial parts	*L. serricorne*	A		100%: 24 hr; 3.5 mg/cm^2	(Kim et al., 2003)
Cinnamomum zeylanicum	Acetone solution	*C. maculatus*	A	Repellency	48%: 4 hr; 1% v/v	(Fouad, 2013)
	Commercial oils	*S. oryzae*	A	Mortality	$LD_{50;24hr}$ = 71.70 μL (FT)	(Jayakumar, Arivoli, Raveen, & Tennyson, 2017)
Citrus aurantium					$LD_{50;24hr}$ = 58.86 μL (FT)	
Citrus bergamia	Leaves	*S. zeamais*	A	Repellency	Significant repellency: 24 hr; 0.1% v/v	(Cosimi, Rossi, Cioni, & Canale, 2009)
		Cryptolestes ferrugineus	A		Significant repellency: 24 hr; 0.1% v/v	
		T. molitor	L		Significant repellency: 24 hr; 0.1% v/v	
Citrus limonum	Aerial parts	*A. diaperinus*	L	Mortality, repellency, inhibition of AChE	$LC_{50;24hr}$ = 33% v/w (CT) $LC_{50;24hr}$ = 11.8 μL/L air (FT)	(Wang et al., 2014)

(Continued)

Table 5–Continued.

Plant species	Plant part	Pest	Stage	Endpoint	Toxicity	Reference
Citrus reticulata	Fruit peels	*S. oryzae*	A	Mortality	$LC_{50;24hr}$ = 24.47 μL/100 μL acetone	(Mishra, Tripathi, & Tripathi, 2011)
		T. castaneum	L		$LC_{50;24hr}$ = 22.88 μL/100 μL acetone	
			A		$LC_{50;24hr}$ = 22.79 μL/100 μL acetone	
	Peel				$LC_{50;24hr}$ = 58.31 μL/100 μL acetone	(Saleem et al., 2013)
			L		$LC_{50;24hr}$ = 30.62 μL/100 μL acetone	
Cocholeria armoracia	Aerial parts	*L. serricorne*	A		100%: 24 hr; 3.5 mg/cm^2	(Kim et al., 2003)
Citrus sinensis	Peel	*T. castaneum*	L		$LC_{50;24hr}$ = 42.48 μL/100 μL acetone	(Saleem et al., 2013)
			A		$LC_{50;24hr}$ = 45.46 μL/100 μL acetone	
Coriandrum sativum	Leaves	*S. oryzae*	A		$LC_{50;24hr}$ = 18.11 μL/cm^2 (FT)	(Rani, 2012)
					$LC_{50;24hr}$ = 36.68 μL/cm^2 (CT)	
		Callosobruchus chinensis	A		$LC_{50;24hr}$ = 16.25 μL/cm^2 (FT)	
					$LC_{50;24hr}$ = 27.26 μL/cm^2 (CT)	
	Seeds	*S. granarius*	L		$LC_{50;48hr}$ = 100 μL/L air (FT)	(Zoubiri & Baaliouamer, 2010)
Crithmum maritimum	Leaves	*S. granarius*	L	Mortality, inhibition of AChE and BChE	100%: 24 hr; 10 μL of 10% v/v per 5 g of seeds (FT)	(Polatoğlu et al., 2016)
					10% 24 hr; 1 μL of 10% v/v per larva (CT)	
		S. oryzae	L		100%: 24 hr; 10 μL of 10% v/v per 5 g of seeds (FT)	
					47%: 24 hr; 1 μL of 10% v/v per larva (CT)	
		T. castaneum	L		19%: 24 hr; 1 μL of 10% v/v per larva (FT)	
		T. confusum	L		26%: 24 hr; 1 μL of 10% v/v per larva (CT)	
		R. dominica	L		5%: 24 hr; 1 μL of 10% v/v per larva (FT)	
					67%: 24 hr; 1 μL of 10% v/v per larva (CT)	
		O. surinamensis	L		91%: 24 hr; 1 μL of 10% v/v per larva (FT)	
					57%: 24 hr; 1 μL of 10% v/v per larva (CT)	
Croton anisatum	Commercial oil	*C. chinensis*		Oviposition inhibition	100% inhibition: 17.5 μL/30 seeds	(Chiluwal, Kim, Do Bae, & Park, 2017)
Cuminum cyminum	Dried fruits	*S. oryzae*	A	Mortality, repellency	$LC_{50;24hr}$ = 0.67 μL/cm^3 (FT)	(Chaubey, 2011)
				Inhibition of AChE	77% inhibition: 0.268 μL/cm^3	
	Seeds	*S. granarius*	A	Mortality	18%: 24 hr;16 μL/L air (pure oil) (FT)	(Ziaee et al., 2014)
					97%: 24 hr;16 μL/L air (as nanogel) (FT)	
		T. confusum	A		27%: 24 hr;16 μL/L air (as nanogel) (FT)	
					61%: 24 hr;16 μL/L air (as nanogel) (FT)	
Cupressus lusitanica	Leaves	*A. obtectus*	A		$LC_{50;24hr}$ = 4.08 μL/L air (FT)	(Bett et al., 2016)
					$LC_{50;24hr}$ = 0.11% v/w (CT)	
		S. zeamais	A		$LC_{50;24hr}$ = 29.11 μL/L air (FT)	
					$LC_{50;24hr}$ = 1.21% v/w (CT)	
		T. castaneum	A		$LC_{50;24hr}$ = 19.67 μL/L air (FT)	
					$LC_{50;24hr}$ = 0.18 % v/w (CT)	
Cymbopogon citratus	Leaves	*S. oryzae*	A	Mortality, repellency, nutritional, and feeding deterrence	$LC_{50;72hr}$ = 435.41 L/cm^2 (CT)	(Stefanazzi et al., 2011)
	Whole plant	*T. castaneum*	A	Mortality	$LC_{50;24hr}$: 4.2 mL/L air (FT)	(Bossou et al., 2015)
	Aerial parts	*O. surinamensis*	A		$LC_{50;12hr}$ = 33.1 mL/L air (FT)	(Hernandez-Lambraño, Pajaro-Castro, Caballero-Gallardo, Stashenko, & Olivero-Verbel, 2015)
		S. zeamais	A		$LC_{50;12hr}$ >604 mL/L air (FT)	
Cymbopogon giganteus	Whole plant	*T. castaneum*	A		$LC_{50;24hr}$ = 2.3 mL/L air (FT)	(Bossou et al., 2015)
Cymbopogon martinii	Aerial parts	*O. surinamensis*	A		$LC_{50;12hr}$ = 37.2 mL/L air (FT)	(Hernandez-Lambraño et al., 2015)
Cymbopogon martinii	Aerial parts	*S. zeamais*	A		$LC_{50;12hr}$ >604 mL/L air (FT)	
Cymbopogon nardus	Commercial oils	*S. oryzae*	A		$LD_{50;24hr}$ = 106.27 μL (FT)	(Jayakumar et al., 2017)
	Aerial parts	*S. zeamais*	A		$LC_{50;12hr}$ >604 mL/L air (FT)	(Hernandez-Lambraño et al., 2015)
		O. surinamensis	A		$LC_{50;12hr}$ = 46.9 mL/L air (FT)	

(Continued)

Table 5–Continued.

Plant species	Plant part	Pest	Stage	Endpoint	Toxicity	Reference
Cymbopogon schoenanthus	Whole plant	*T. castaneum*	A		$LC_{50;24hr}$: 2.1 mL/L air (FT)	(Bossou et al., 2015)
Drimys winteri	Leaves Stem bark	*T. castaneum*	A	Mortality, repellency	$LC_{50;24hr} = 8.96\ \mu g/L$ air (FT) $LD_{50;24hr} = 84.05$ g oil/mg insect (CT) $LC_{50;24hr} = 10.45\ \mu g/L$ air (FT) $LD_{50;24hr} = 75.14$ g oil/mg insect (CT)	(Zapata & Smagghe, 2010)
Elyonorus muticus	Leaves	*S. oryzae*	A	Mortality, repellency, nutritional, and feeding deterrence	$LC_{50;72hr} = 99.63\ \mu L/cm^2$ (CT)	(Stefanazzi et al., 2011)
Eucalyptus benthamii	Leaves	*S. zeamais*	A	Mortality	$LD_{50;24hr} = 0.16\ \mu L/cm^2$ (CT)	(Mossi et al., 2011)
Eucalyptus camaldulensis	Aerial parts	*C. maculatus*	A		$LC_{50;24hr} = 3.97\ \mu L/L$ air (FT)	(Negahban & Moharramipour, 2007)
		S. oryzae	A		$LC_{50;24hr} = 12.06\ \mu L/L$ air (FT)	
		T. castaneum	A		$LC_{50;24hr} = 33.50\ \mu L/L$ air (FT)	
Eucalyptus dunnii	Leaves	*S. zeamais*	A		$LD_{50;24hr} = 0.08\ \mu L/cm^2$ (CT)	(Mossi et al., 2011)
Eucalyptus globulus	Acetone solution	*C. maculatus*	A	Repellency	27%: 4 hr; 1% v/v	(Fouad, 2013)
	Commercial oils	*S. oryzae*	A	Mortality	$LD_{50;24hr} = 60.14\ \mu L$ (FT)	(Jayakumar et al., 2017)
	Leaves	*S. zeamais*	A		$LD_{50;24hr} = 0.25\ \mu L/cm^2$ (CT)	(Mossi et al., 2011)
Eucalyptus intertexta	Aerial parts	*C. maculatus*	A		$LC_{50;24hr} = 2.55\ \mu L/L$ air (FT)	(Negahban & Moharramipour, 2007)
		S. oryzae	A		$LC_{50;24hr} = 6.93\ \mu L/L$ air (FT)	
		T. castaneum	A		$LC_{50;24hr} = 11.59\ \mu L/L$ air (FT)	
Eucalyptus obliqua	Leaves	*S. oryzae*	A		$LC_{50;24hr} = 30.29\ \mu L/cm^2$ (FT) $LC_{50;24hr} = 52.77\ \mu L/cm^2$ (CT)	(Rani, 2012)
		C. chinensis	A		$LC_{50;24hr} = 21.70\ \mu L/cm^2$ (FT) $LC_{50;24hr} = 59.29\ \mu L/cm^2$ (CT)	
Eucalyptus saligna	Leaves	*A. obtectus*	A		$LC_{50;24hr} = 7.02\ \mu L/L$ air (FT) $LC_{50;24hr} = 0.02\%$ v/w (CT)	(Bett et al., 2016)
		S. zeamais	A		$LC_{50;24hr} = 26.85\ \mu L/L$ air (FT) $LC_{50;24hr} = 17.0\%$ v/w (CT) $LD_{50;24hr} = 0.10\ \mu L/cm^2$ (CT)	(Mossi et al., 2011)
		T. castaneum	A		$LC_{50;24hr} = 16.09\ \mu L/L$ air (FT) $LC_{50;24hr} = 0.19\%$ v/w (CT)	(Bett et al., 2016)
Eucalyptus sargentii	Aerial parts	*C. maculatus*	A		$LC_{50;24hr} = 3.87\ \mu L/L$ air (FT)	(Negahban & Moharramipour, 2007)
		S. oryzae	A		$LC_{50;24hr} = 12.91\ \mu L/L$ air (FT)	
		T. castaneum	A		$LC_{50;24hr} = 18.38\ \mu L/L$ air (FT)	
Eucalyptus viminalis	Leaves	*S. zeamais*	A		$LD_{50;24hr} = 0.79\ \mu L/cm^2$ (CT)	(Mossi et al., 2011)
Foeniculum vulgare	Leaves	*S. zeamais*	A	Repellency	Significant repellency: 24 hr; 0.1% v/v	(Cosimi et al., 2009)
		C. ferrugineus	A		Significant repellency: 24 hr; 0.1% v/v	
		T. molitor	L		Significant repellency: 24 hr; 0.1% v/v	
Gaultheria fragrantissima	Commercial oils	*S. oryzae*	A	Mortality	$LD_{50;24hr} = 116.15\ \mu L$ (FT)	(Jayakumar et al., 2017)
Illicium verum	Commercial oils	*C. chinensis*	A	Oviposition inhibition	100% inhibition: 17.5 μL/30 seeds	(Chiluwal et al., 2017)
Laurelia sempervirens	Leaves Stem bark	*T. castaneum*	A	Mortality, Repellency	$LC_{50;24hr} = 1.66\ \mu g/L$ air (FT) $LD_{50;24hr} = 44.05$ g oil/mg insect (CT) $LC_{50;24hr} = 1.63\ \mu g/L$ air (FT) $LD_{50;24hr} = 38.94$ g oil/mg insect (CT)	(Zapata & Smagghe, 2010)
Laurus nobilis	Leaves (Moroccan oil)	*R. dominica*	A	Mortality, Repellency	$LC_{50} = 68\ \mu L/L$ air (FT) $RD_{50} = 0.013\ \mu L/cm^2$	(Jemâa, Tersim, Toudert, & Khouja, 2012)
		T. castaneum	A		$LC_{50} = 172\ \mu L/L$ air (FT) $RD_{50} = 0.045\ \mu L/cm^2$	
	Leaves (Algerian oil)	*R. dominica*	A		$LC_{50} = 99\ \mu L/L$ air (FT)	

(Continued)

Table 5–Continued.

Plant species	Plant part	Pest	Stage	Endpoint	Toxicity	Reference
					$RD_{50} = 0.033\ \mu L/cm^2$	
		T. castaneum	A		$LC_{50} = 194\ \mu L/L$ air (FT)	
					$RD_{50} = 0.096\ \mu L/cm^2$	
	Leaves (Tunisian oil)	*R. dominica*	A		$LC_{50} = 113\ \mu L/L$ air (FT)	
					$RD_{50} = 0.036\ \mu L/cm^2$	
		T. castaneum	A		$LC_{50} = 217\ \mu L/L$ air (FT)	
					$RD_{50} = 0.139\ \mu L/cm^2$	
Lavandula hybrida	Leaves	*T. molitor*	L	Repellency	Significant repellency: 24 hr; 0.1% v/v	(Cosimi et al., 2009)
		S. zeamais	A		Significant repellency: 24 hr; 0.1% v/v	
		C. ferrugineus	A		Significant repellency: 24 hr; 0.1% v/v	
		T. molitor	L		Significant repellency: 24 hr; 0.1% v/v	
Lippia sidoides	Leaves	*S. zeamais*	A	Mortality	$LD_{50;72hr} = 7.10\ \mu g/mg$ (CT)	(Oliveira et al., 2017)
					$LD_{50;72hr} = 26.44\ \mu g/mg$ (CT) as nanoformulations	
Mauritia flexuosa	Seeds				$LC_{50;24hr} = 64.4\%$ v/v (FT)	(Santos et al., 2015)
Melaleuca alternifolia	Aerial parts			Mortality, inhibition of AChE, glutathione S-transferase and carboxylesterase	$LC_{50;72hr} = 8.42$ mg/L air (FT)	(Liao et al., 2016)
Mentha arvensis	Leaves	*S. oryzae*	A	Mortality	$LC_{50;24hr} = 45.5\ \mu L/L$ air (FT)	(Lee et al., 2001)
Murraya alata	Branches and leaves	*T. castaneum*	A	Repellency	76% to 92%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	(You et al., 2015)
Murraya euchrestifolia					20% to 98%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	
Murraya exotic					36% to 82%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	
Murraya koenigii					40% to 84%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	
Murraya kwangsiensis					42% to 86%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	
Murraya tetramera					62% to 98%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	
Myristica fragrans	Essential oil	*C. maculatus*	A	Mortality	$LC_{50;24hr} = 4.232\ \mu L/L$ air (FT)	(Alibabaie & Safaralizadeh, 2015)
Myrtus communis	Leaves	*A. obtectus*	A		$LC_{50;24hr} = 124.8\ \mu L/L$ air (FT)	(Ayvaz, Sagdic, Karaborklu, & Ozturk, 2010)
Oenocarpus bataua	Seeds	*S. zeamais*	A		$LC_{50;24hr} = 60.3\%$ v/v (FT)	(Santos et al., 2015)
Orbignya phalerata					$LC_{50;24hr} = 65.2\%$ v/v (FT)	
Origanum onites	Leaves	*A. obtectus*	A		$LC_{50;24hr} = 49.6\ \mu L/L$ air (FT)	(Ayvaz et al., 2010)
		A. diaperinus	L	Reduction of body weight, inhibition of pupation and moulting	range of tested concentrations: 0.25% to 1% v/v	(Szczepanik et al., 2018)
Pelargonium graveolens	Commercial oils	*S. oryzae*	A	Mortality	$LD_{50;24hr} = 92.68\ \mu L$ (FT)	(Jayakumar et al., 2017)
Pinus longifolia	Leaves				$LC_{50;24hr} = 47.88\ \mu L/cm^2$ (FT)	(Rani, 2012)
					$LC_{50;24} = 77.30\ \mu L/cm^2$ (CT)	
		C. chinensis	A		$LC_{50;24hr} = 33.11\ \mu L/cm^2$ (FT)	
					$LC_{50;24hr} = 74.95\ \mu L/cm^2$ (CT)	
Piper nigrum	Dried fruits	*S. oryzae*	A	Mortality, repellency	$LC_{50;24hr} = 0.58\ \mu L/cm^3$ (FT)	(Chaubey, 2011)
				Inhibition of AChE	75% inhibition: 0.232 $\mu L/cm^3$	
Ricinus communis	Seeds	*S. zeamais*	A	Mortality	$LD_{50} = 2{,}04$ mL/300 seeds	(Wale & Assegie, 2015)
	Acetone solution	*C. maculatus*	A	Repellency	1,5%: 4 hr; 1% v/v	(Fouad, 2013)
Rosmarinus officinalis	Commercial oils	*S. oryzae*	A	Mortality	$LD_{50;24hr} = 176.98\ \mu L$ (FT)	(Jayakumar et al., 2017)

(Continued)

Table 5–Continued.

Plant species	Plant part	Pest	Stage	Endpoint	Toxicity	Reference
Satureja bachtiarica	Aerial parts	*T. castaneum*	A	Mortality, repellency	$LC_{50,24hr}$ = 2.51 mg/L air 4.71 (FT) $LD_{50,24hr}$ = 40.60 μg/adult (CT) $LC_{50,24hr}$ = 2.51 mg/L air 2.51 (FT) $LD_{50,24hr}$ = 20.11 μg/adult (CT) $LC_{50,24hr}$ = 2.51 mg/L air 3.27 (FT) $LD_{50,24hr}$ = 34.2 μg/adult (CT)	(Taban et al., 2017)
Satureja khuzestanica						
Satureja rechinger						
Satureja thymbra	Leaves	*A. obtectus*	A	Mortality	$LC_{50,24hr}$ = 236.4 μL/L air (FT)	(Ayvaz et al., 2010)
Syzygium aromaticum	Acetone solution	*C. maculatus*	A	Repellency	32%: 4 hr; 1% v/v	(Fouad, 2013)
Tagetes filifolia	Aerial parts	*T. castaneum*	A	Mortality, increased peroxidation of lipids	$LC_{50,24hr}$ = 2.4 μL/mL (FT)	(Olmedo et al., 2015)
Tagetes minuta	Aerial parts	*C. maculatus*	A	Mortality	$LC_{50,48hr}$ = 161.9 μL/L air (FT) $LC_{50,48hr}$ = 88.4 μL/L air(CT)	(Nenaah et al., 2015)
	Aerial parts (nanoemulsion formulations)			Inhibition of eggs development Mortality Inhibition of eggs development	$LC_{50,48hr}$ = 77.8 μL/L air(FT) $LC_{50,48hr}$ = 40.7 μL/L air (FT) $LC_{50,48hr}$ = 65.9 μL/L air(FT)	
Tagites terniflora	Leaves	*T. castaneum*	A	Mortality, repellency, nutritional, and feeding deterrence	$LC_{50,72hr}$ = 362.82 μL/cm^2 (FT) $LC_{50,72hr}$ = 217.26 μL/cm^2 (CT)	(Stefanazzi et al., 2011)
		S. oryzae	A		$LC_{50,72hr}$ = 322.61 μL/cm^2 (FT) $LC_{50,72hr}$ = 146.58 μL/cm^2 (CT)	
Tanacetum vulgare	Leaves	*A. diaperinus*	L	Mortality, inhibition of larval growth, larval body weight, and pupation	48%: 10 days; 10 mg/mL in diet (DT)	(Szołyga et al., 2014)
Thuja occidentalis	Leaves				65%: 10 days; 10 mg/mL in diet (DT)	

Abbreviation used in Table: CT, contact toxicity; FT, fumigant toxicity; DT, diet toxicity; A, adult; L, larva.

Table 6–Effects of pure compounds derived from essential oils on coleopterous pests of stored food products.

Compound	Pest	Stage	Endpoint	Toxicity	Reference
Alloaromadendrene	*T. castaneum*	A	Repellency	30% to 80%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	(You et al., 2017)
(E)-anethole			Mortality, increased peroxidation of lipids, inhibition of AChE	$LC_{50;24hr} = 2.6$ μL/mL (FT)	(Olmedo et al., 2015)
	C. chinensis	A	Oviposition inhibition	100% inhibition: 17.5 μL/30 seeds	(Chiluwal et al., 2017)
Borneol	*S. granarius*	A	Mortality	70%: 6 hr; 1 μL/L air (FT)	(Kordali et al., 2006)
Bornyl acetate				70%: 6 hr; 1 μL/L air (FT)	
Camphene	*T. castaneum*	A	Repellency	16% to 60%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	(You et al., 2017)
D-camphor			Mortality	$LC_{50;24hr} < 2.3$ mg/L air (FT)	(Guo et al., 2016)
	L. serricorne	A		$LC_{50;24hr} = 2.4$ mg/L air (FT)	
Carvacrol	*C. maculatus*	A		$LD_{50;24hr} = 0.66$ μL/L air (FT)	(Ajayi et al., 2014)
Carvacrol	*S. oryzae*	A		$LC_{50;24hr} = 79.7$ μL/L air (FT)	(Lee et al., 2001)
			Inhibition of AChE	AChE inhibitory constant (K_i) = 0.050 mM	
α-caryophyllene	*T. castaneum*	A	Repellency	2% to 94%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	(You et al., 2017)
β-caryophyllene				22% to 88%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	
Caryophyllene oxide				44% to 98%: 2hr; concentration range 0.13 to 78.63 nL/cm^2	
1-8-cineole	*C. maculatus*	A	Mortality	$LD_{50;24hr} = 0.24$ μL/L air (FT)	(Ajayi et al., 2014)
	T. castaneum	A		$LD_{50;24hr} = 5.5$ mg/L air (FT)	(Guo et al., 2016)
	L. serricorne	A		$LD_{50;24hr} = 5.2$ mg/L air (FT)	
	S. granarius	A		70%: 6 hr; 1 μL/L air (FT)	(Kordali et al., 2006)
	S. zeamais	A		$LC_{50;72hr} = 6.61$ mg/L air (FT)	(Liao et al., 2016)
Cineole	*S. oryzae*	A	Mortality	$LC_{50;24hr} = 23.5$ μL/L air (FT)	(Lee et al., 2001)
			Inhibition of AChE	AChE inhibitory constant (K_i) = 0.084 mM	
Citronellal	*T. castaneum*	A	Mortality	$LC_{50;24hr}$: 1.2 mL/L air (FT)	(Bossou et al., 2015)
Estragole			Mortality, increased peroxidation of lipids, inhibition of AChE	$LC_{50;24hr} = 6.4$ μL/mL (FT)	(Olmedo et al., 2015)
β-eudesmol			Repellency	16% to 80%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	(You et al., 2017)
Isosafrole	*S. oryzae*	A	Mortality	$LC_{50;24hr} = 19.3$ μL/L air (FT)	(Lee et al., 2001)
			Inhibition of AChE	AChE inhibitory constant (K_i) = 0.71 mM	
(−)-linalool	*C. maculatus*	A	Mortality	$LD_{50;24hr} = 8.9$ μL/L air	(Ajayi et al., 2014)
Linalool	*C. chinensis*	A	Oviposition inhibition	100% inhibition: 15 μL/30 seeds	Chiluwal et al., 2017
	S. oryzae	A	Mortality	$LC_{50;24hr} = 39.2$ μL/L air (FT)	(Lee et al., 2001)
S-(−)-limonene	*C. maculatus*	A		$LD_{50;24hr} = 13.2$ μL/L air (FT)	(Ajayi et al., 2014)
Maslinic acid	*S. granarius*	A		$LC_{50;72hr} = 0.66$ mg/Petri (FT)	Kısa et al., 2018
	T. confusum	A		$LC_{50;72hr} = 0.61$ mg/ Petri (FT)	
	A. obtectus	A		$LC_{50;72hr} = 1.71$ mg/ Petri (FT)	
(−)-Menthone	*C. maculatus*	A		$LD_{50;24hr} = 1.2$ μL/L air (FT)	(Ajayi et al., 2014)
Menthone	*S. oryzae*	A		$LC_{50;24hr} = 12.7$ μL/L air (FT)	(Lee et al., 2001)
			Inhibition of AChE	AChE inhibitory constant (K_i) = 0.39 mM	
Methyleugenol			Mortality	$LC_{50;24hr} = 70.8$ μL/L air (FT)	
			Inhibition of AChE	AChE inhibitory constant (K_i) = 0.051 mM	
Methyl salicylate	*C. chinensis*	A	Oviposition inhibition	30% inhibition: 20 μL/30 seeds	(Chiluwal et al., 2017)
Oleanolic acid	*S. granarius*	A	Mortality	$LC_{50;72hr} = 8.391$ mg/ Petri (FT)	(Kısa et al., 2018)
	T. confusum	A		$LC_{50;72hr} = 2.57$ mg/ Petri (FT)	
	A. obtectus	A		$LC_{50;72hr} = 1.73$ mg/ Petri (FT)	
Oleuropein	*S. granarius*	A		$LC_{50;72hr} = 7.69$ mg/ Petri (FT)	
	T. confusum	A		$LC_{50;72hr} = 3.82$ mg/ Petri (FT)	
	A. obtectus	A		$LC_{50;72hr} = 0.34$ mg/ Petri (FT)	
Perillaldehyde	*S. oryzae*	A		$LC_{50;24hr} = 19.3$ μL/L air (FT)	(Lee et al., 2001)
			Inhibition of AChE	AChE inhibitory constant (K_i) = 0.71 mM	

(Continued)

Table 6–Continued.

Compound	Pest	Stage	Endpoint	Toxicity	Reference
α-pinene			Mortality	$LC_{50,24hr}$ = 54.9 μL/L air (FT)	
			inhibition of AChE	AChE inhibitory constant (K_i) = 0.44 mM	
(−)-α-pinene	*C. maculatus*	A	Mortality	$LD_{50,24hr}$ = 31.4 μL/L air (FT)	(Ajayi et al., 2014)
(−)-β-Pinene				$LD_{50,24hr}$ = 31 μL/L air (FT)	
Piperitone	*T. castaneum*	A		$LC_{50,24hr}$: 0.5 mL/L air (FT)	(Bossou et al., 2015)
Safrole				$LC_{50,24hr}$ = 4.7 μg/Adult (CT)	(Guo et al., 2016)
Spathulenol	*T. castaneum*	A	Repellency	38% to 100%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	(You et al., 2017)
α-Terpinene	*S. oryzae*	A	Mortality	$LC_{50,24hr}$ = 71.2 μL/L air (FT)	(Lee et al., 2001)
			Inhibition of AChE	AChE inhibitory constant (K_i) = 0.14 mM	
	T. castaneum	A	Repellency	4% to 82%: 2 hr; concentration range 0.13 to 78.63 nL/cm^2	(You et al., 2017)
α-Terpineol	*L. serricorne*	A	Mortality	$LC_{50,24hr}$ = 3.3 mg/L air (FT)	(Guo et al., 2016)
	S. granarius	A		70%: 6 hr; 1 μL/L air (FT)	(Kordali et al., 2006)
	S. zeamais	A		$LC_{50,72hr}$ = 4.21 mg/L air (FT)	Liao et al., 2016.
Terpinen-4-ol	*S. granarius*	A		70%: 6 hr; 1 μL/L air (FT)	(Kordali et al., 2006)
	S. zeamais	A		$LC_{50,72hr}$ = 3.22 mg/L air (FT)	(Liao et al., 2016)
α-Thujone	*A. diaperinus*	L	Mortality, inhibition of larval growth, larval body weight and pupation	60%: 10 days; 10 mg/mL in diet (DT)	(Szołyga et al., 2014)
				45%: 10 days; 10 mg/mL in diet (DT)	
β-thujone				$LC_{50,24hr}$ = 69.7 μL/L air (FT)	
Thymol	*S. oryzae*	A	Mortality	AChE inhibitory constant (K_i) = 0.57 mM	(Lee et al., 2001)
			Inhibition of AChE	$LD_{50,72hr}$ = 17.08 μg/mg (CT)	(Oliveira et al., 2017)
			Mortality	$LD_{50,72hr}$ = 20.75 μg/mg (CT) as nanoformulations	

Abbreviation used in Table: CT, contact toxicity; FT, fumigant toxicity; DT, diet toxicity; A, adult; L, larva.

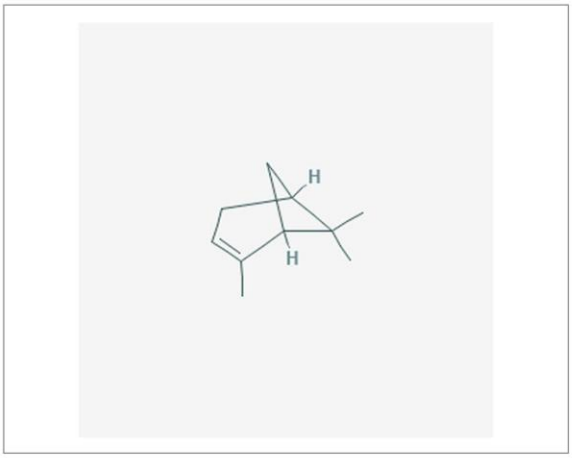

Figure 4–α-pinene, monoterpene, chemical structure (Pubchem, 2018f).

display ultrastructural malformations of the midgut after 3 days of treatment (Figure 5).

Tissues from the extract-exposed group showed disruption of biological membranes and changes in the density of the cytoplasm. This finding suggests a possible water imbalance as a consequence of poisoning with alkaloids. Similar results concerning alkaloids from the *Solanaceae* family were obtained by (Wink, 1998), who observed that solanine caused disruption in membrane permeability. This may be one of the ways the substance enters and harms the cells.

The data on the toxic effects of plant-derived substances on the ultrastructure of insects' cells are scanty. However, the research on other invertebrates, protozoans, and cell cultures proves that alkaloids and essential oils may cause cell vacuolization, permeabilization of cell membranes, and significant disturbances in mitochondrial ultrastructure, thus leading to abovementioned oxidative stress (Gao, Wang, & Ji, 2006; Holetz et al., 2003; Medina, Rodrigues, De Souza, Atella, & Barrabin, 2012; Pedroso et al., 2007). On the other hand, the activity of alkaloids may depend on their dose, and they may also act as a protectant of mitochondria (Taveira et al., 2014). Therefore, ultrastructural toxicity tests need further intensive studies, including a wide range of concentrations.

Prooxidant/antioxidant balance

The results describing the mode of action of plant-derived substances within insect cells are scarce. However, the cellular effects described for other animals suggest excessive formation of reactive oxygen species within the cytoplasm and in mitochondria *via* various metabolic pathways (Hasanain et al., 2015; Meng et al., 2016) as well as increased the peroxidation of tissues (Ayad, 2013). Plant-derived substances may disturb the prooxidant-antioxidant balance within cells (Neganova, Afanas'eva, Klochkov, & Shevtsova, 2012) and lead to serious alterations within cells. Usually, antioxidants enhance resistance to toxic substances. Prooxidants, in turn, may lead to decreased vitality and longevity of animals, due to various toxic effects, at the level of activity and structure of biomacromolecules. Due to the involvement of free radicals in the pathogenesis of various diseases, the antioxidant activity of plant-derived substances is more intensively studied than their prooxidant activity. Both crude extract and alkaloid fractions of *Mahonia aquifolium* showed radical-scavenging activity (Rackova, Oblozinsky, Kostalova, Kettmann, & Bezakova, 2007). Moreover, monoterpenoids, monoterpene hydrocarbons and phenols exhibit

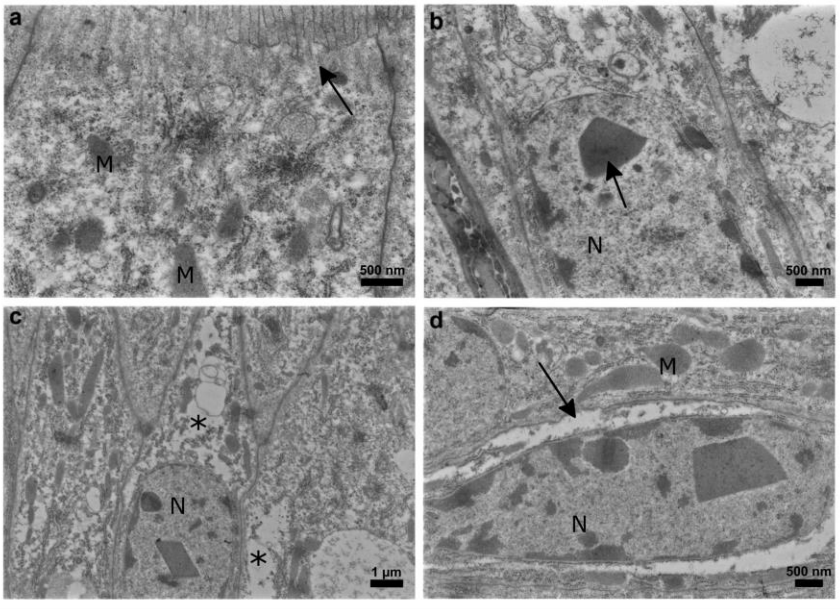

Figure 5–Ultrastructure of the *T. molitor* larvae midgut. Control cells (A and B) with the topical part of the cell and visible microvilli (A, arrow), dense cytoplasm and many mitochondria (M). Nuclei (N) may contain a protein crystal (B, arrow). Midgut cells of *T. molitor* larvae treated with 10% *S. nigrum* extract (C and D) show changes in cytoplasmic density (stars) and disruption of the nuclear membrane (D, arrow).

antioxidant activity (Kilic, Yesiloglu, & Bayrak, 2014; Lambert & Elias, 2010; Saleh, Clark, Woodard, & Deolu-Sobogun, 2010). Research on *Abrus precatorius* (Okoh, Asekun, Familoni, & Afolayan, 2014), *Fragaria x ananassa* (Wang, Wang, Yin, Parry, & Yu, 2007), and *Laurus nobilis* (Basak & Candan, 2013) essential oils proved that they possess antioxidant activity. Saleh et al. (2010) listed numerous compounds of essential oils that possess antioxidant activity, for example, camphor, cinnamic acid, carvacrol, or rosefuran. These substances affect various metabolic pathways connected with free-radical formation, such as release of nitric oxide and alteration of the activity of cyclooxygenase, lipoxygenase, and various kinases (Miguel, 2010). Thus, these substances may decrease the toxicity of xenobiotics, which act (among others) *via* the overproduction of free radicals, for example, some synthetic insecticides: fenitrothion, boric acid, or cypermethrin (Adamski, Ziemnicki, Fila, Zikic, & Stajn, 2003; Büyükgüzel et al., 2013b; Hyrsl, Buyukguzel, & Buyukguzel, 2007; Kolawole & Kolawole, 2014). Therefore, such alkaloids are not favored for use in crop protection. On the other hand, there are many essential oil ingredients, such as thujones, camphor, carvacrol or, santolina, that increase oxidative stress (Miguel, 2010). It is noteworthy, that some of these substances were reported as having both antioxidant and prooxidant activities. The same phenomenon was described for polyphenolic thujones (Lambert & Elias, 2010), flavonoids, ascorbic acid (Rahal et al., 2014), rosmarinic acid (Klimovich, Popov, Krivoshapko, Shtoda, & Tsybulsky, 2017), and some drugs such as aminoguanidine (Philis-Tsimikas, Parthasarathy, Picard, Palinski, & Witztum, 1995). Perhaps, their activity depends on the other substances present in plants, oxygen tension, or the concentration of transition metals, or the concentration of the substance may direct its bias toward pro- or antioxidant activity (Philis-Tsimikas et al., 1995; Rahal et al., 2014). Furthermore, alkaloids show antioxidant (Taveira et al., 2014) and prooxidant activities. (Büyükgüzel et al., 2013a) showed that when the diet is supplemented with a glycoalkaloid α-solanine an increase in tissue peroxidation of *Galleria mellonella* occurred.

The generation of free radicals and their effects on biomolecules may be important components of toxicity of this alkaloid against insects. Next, the metabolism of plant-derived substances, including detoxification, is often mediated by pathways that involve oxidation and reduction reactions and thus may generate free radicals (Hartmann et al., 2005; Macel, 2011; Sehlmeyer et al., 2010). These are common xenobiotic-detoxifying pathways that include, among others, enzymes that alter the oxidation state, such as cytochrome P-450, catalase, peroxidases, or glutathione *S*-transferase. Consequently, the production of free radicals may increase within the cells. Moreover, detoxification of various essential oils is mediated by the same pathways (Liao et al., 2016; Machial, 2010). Therefore, the prooxidant-antioxidant balance seems to be vulnerable to plant allelochemicals. Hence, glycoalkaloids and essential oils appear to be interesting agents that may be used in crop protection, either by acting directly as insecticides or as substances that synergistically increase the toxicity of other substances and thus decrease the usage of plant-protecting pesticides. However, due to their bimodal, pro-, and antioxidant actions, their activity should be carefully studied before their application as pesticides.

Disturbance of hormonal regulation

Many methods of plant protection focus on insect development. Juvenoids, chitinase inhibitors, PTTH-inhibitor insecticides, and other insecticides that affect the hormonal regulation of insect development are objects of research interest. Disturbance of development may not only kill insects but also significantly decrease the number of adult insects in the population. Consequently, the number of laid eggs may be limited. (Mitchell, Keogh, Crooks, & Smith, 1993) reported the disturbance of ecdysone 20-monooxygenase activity after exposure to several plant flavonoids. Interestingly, there were bimodal effects, that is, inhibition and stimulation of the monooxygenase by high and low concentrations of some flavonoids (kaempferol, morin, quercetin, and myricetin).

There is a close similarity in some metabolic pathways of plants and insects. For example, common polypody (*Polypodium vulgare*) produces ecdysteroids in a metabolic pathway that transforms ecdysone to 20-hydroxyecdysone. Thus, the plant may contain substances that affect metabolic pathways in insects that lead to the production of moulting-regulating hormones (Canals, Irurre-Santilari, & Casas, 2005). Next, terpenes may stimulate cytochrome P450 activity – which is crucial for the production of insect hormones, as well as pheromones. Consequently, the hormonal regulation of development and pheromone-regulated reproductive behavior may be disturbed (Brattsten, 1983). Notably, from the crop-protection point of view, toxicity may be species-specific, and the activity may differ even between closely related species. For example, terpenoid ethers and terpenoid ketones show juvenile hormone-like activity against *T. castaneum*, but not *Tribolium confusum* (Tenebrionidae), which appears to be resistant to these substances (Amos, Williams, Du Guesclin, & Schwarz, 1974).

Also, saponins affect development of insects, significantly altering their growth and molting (Ikbal, Ben, & Ben, 2006). To some extent, it may be due to their antifeedant activity (Nielsen, Nagao, Okabe, & Shinoda, 2010; Szczepanik, Biały, & Jurzysta, 2004; Szczepanik, Krystkowiak, Jurzysta, & Biały, 2001). However, Ikbal and coworkers drew attention to reduced amount of cholesterol absorbed by insects. That, in consequence, leads to decreased amounts of ecdysone, a hormone that plays crucial regulation in molting.

Application

The activity of plant-derived substances and plant extracts strongly depends on the solvent used by the researcher. The range and dynamic of the toxicity of extracts may double with the use of certain solvents (Boussaada et al., 2008). Next, various pests may be differentially susceptible to active substances delivered in various solvents (Ciepielewska, Kordan, & Nietupski, 2005). Furthermore, males and females may differ in their susceptibility to botanicals (Yeom, Kang, Kim, & Park, 2013). Therefore, the results may vary, which emphasizes the need for a wide range of tests of biological activity of the substances used in crop protection.

Feeding and contact toxicity

Tests of the activity of plant-derived substances are usually based on feeding or contact toxicity. In the 1st situation, the substance is added to the nutrient. In this case, insects may stop feeding for several days and avoid intoxication. In so-called choice tests, insects may choose nutrients with a tested compound and the control compound. These two are presented simultaneously to the pests in the same number of pieces, by turns, and the individuals are free to choose the piece of nutrient they feed on. Such a test enables to quickly check the effect of tested substance on feeding preferences. The results show if insects feed more on nontoxified food, which suggests deterrent activity of the tested substances (Boussaada et al., 2008). Szołyga, Gniłka, Szczepanik, and Szumny

(2014) conducted research with the usage of essential oils obtained from 2 plants, *Thuja occidentalis* and *Tanacetum vulgare*, against the lesser mealworm, *Alphitobius diaperinus* (Tenebrionidae). Isolated substances were provided to insects (after solvent evaporation) by incorporating them into the diet (oat flakes) as an acetone solution (10 mg/mL). It was found that *T. vulgare* and *Thuja* EOs possessed weak feeding deterrence activity. In the case of tansy oil, the authors reported deterrent activity in choice-test but the attractant activity when the no-choice test was used. *T. occinentalis* revealed deterrent activity, irregardless of the test type. Moreover, the effect was stage-dependent. For the 10-day-old insects, these substances were strong attractants and, in the same time, caused inhibition of growth, slowed development, and increased mortality (Szołyga et al., 2014). Research conducted on stored-grain pests *Sitophilus oryzae* (Curculionidae) (Stefanazzi, Stadler, & Ferrero, 2011) with the usage of hexane solutions of *Tagetes terniflora*, *Cymbopogon citratus*, and *Elionurus muticus* essential oils showed that all essential oils had strong feeding deterrent action. Oils not only reduced the food consumption rate but also significantly reduced the growth rate of insects.

Another way to administer essential oils is topical application. Within this procedure, the tested substance is applied directly on insect cuticle, on the defined structures (like, for example, sternites of thorax or prolegs, but not wings). Taban, Saharkhiz, and Hooshmandi (2017) used this method to test the activity of *Satureja rechingeri*, *Satureja bachtiarica*, and *Satureja khuzestanica* essential oils against the red flour beetle, *T. castaneum*. Dilutions were prepared as an acetone solution and applied topically and activity was tested after 24 hr. It was found that one of the oils, isolated from *S. khuzestanica,* exhibited higher activity (rapid insecticidal action) than did the two other investigated oils. Likewise, Bett et al. (2016) tested the activity of leaf essential oils from *Cupressus lusitanica* and *Eucalyptus saligna* against adult *T. castaneum*, *Acanthoscelides obtectus*, and *Sitophilus zeamais* (Curculionidae). A contact toxicity bioassay showed that the obtained leaf essential oils were moderate or strong toxicants to adult insects from all tested genera but depended on the duration of exposure and concentration and route of application. This is why research on the selection of appropriate botanical oil and the type of solvent, exposition time, and concentration is so important. The usage of botanical insecticides with foods in storage chambers may be ineffective if the substance is not spread evenly. Moreover, if substances show some toxicity against mammals, such as many synthetic insecticides, nicotine, and solanine do, their usage as a repellent will be restricted. Such effects were reported for cis-bifenthrin in concentration 20 μg/mL, which caused immunotoxic effects in murine macrophages (Wang et al., 2007), deltametrin, which was reported as a factor that disrupts endocrine and reproductive system of mice in dose 5 mg/kg body weight per day in 35 days of treatment (Ben Slima et al., 2017). Malfunctions of reproductive and endocrine system were observed in rats exposed to curcumin, quercetin combined with deltamethrin and cypermethrin (in doses 100 mg/kg body weight), as well as cypermethrin itself (1, 2, and 25 mg/kg body weight), impaired reproductive organs and affected fertility in rats (Sharma, Aslam Khan, & Singh, 2018; Singh, Bhagat, Raijiwala, Dighe, & Vanage, 2017). Thus essential oils with significant repellent activity against crop pests are interesting alternatives (Bett et al., 2016).

Plant-derived substances may also attract pests. That was proved, for example, for derivatives of methyl benzoate (Hammack & Petroski, 2004), 1,4-dimethoxybenzene (Marques et al., 2009), and (±)-linalool (Hammack, 2001). This kind of activity is used in environmental traps, mostly using pheromones. Additionally, such activity was reported for some essential oils and alkaloids. However, the repellent-attractant activity depends on the tested species, stereoisomeric structure, type of solvent used, and the concentration of the tested substance (Adamski et al., 2016; Boussaada et al., 2008; Hammack, 2001). Therefore, the usage of plant-derived attractants in crop protection should be carefully studied before their introduction to the market. Using attractants seems interesting, since it does not demand spread of the substance throughout the whole chamber. Next, botanicals can be used in traps not only as attractants but also as active ingredients in adhesive films. Marques et al. (2009) showed that traps with 1,4-dimethoxybenzene were significantly more successful in attracting *Diabrotica speciosa* beetles than the control traps were. However, this referred to the field crops. Such a technique can be relatively safe in closed rooms and does not demand spreading of the toxic substances on crops. On the other hand, the insecticidal effects of botanicals increases when no-choice tests are used (Boussaada et al., 2008). In such cases, the stored products should be covered/mixed with an active substance. Of course, the substance should not be harmful to humans or domestic animals and, moreover, should not have any effect on seed viability.

Application to commodities and surface treatments are relatively easy methods of protection. Because of the increasing resistance of pests to conventional insecticides, the efficacy of the insecticides decreases. Moreover, the application is limited due to the toxicity of many substances to humans or to reduced value of the stored crops (Arthur & Subramanyam, 2012), that is, solanine can be toxic to children in the dose of 20 mg per person (Izawa, Amino, Kohmura, Ueda, & Kuroda, 2010).

Fumigation

Fumigation is a method that uses gaseous substances to exterminate pests, as a strategy that can be used in the control of stored products. Indeed, several pros of this method are obvious–easiness of use, volatility of essential oils, ability to keep an effective concentration of insecticides within the closed space, and a high possibility to spread the substance evenly, considering even hard-to-reach places. Moreover, research of Rani and Devanand (2011) showed that the use of the same leaf extracts by two different methods, fumigation and contact toxicity, demonstrated a higher efficacy of the fumigation method. For example, the extract of *Carica papaya* yielded 46% mortality in *S. oryzae* after 72 hr of treatment when added to the diet, but the same extract as a vapor caused almost 100% mortality of these insects. As the data of studies conducted by Ajayi, Appel, and Fadamiro (2014) show, components of essential oils, such as 1-8-cineole, carvacrol, and eugenol, also possess a high fumigant toxic activity against the cowpea weevil *C. maculatus*, causing ≥90% mortality of adult beetles at low doses of 5 μL/L of air within 24 hr. Similar results were obtained by (Bett et al., 2016), who tested leaf essential oils from *C. lusitanica* and *E. saligna* against *A. obtectus*. In that study, *C. lu*sitanica oil caused 90.6% and 100% mortality of A. *obtectus*, respectively, 24 hr postfumigation, whereas the *E. saligna* essential oil, at 15 μL/L of air, caused 95% mortality of *A. obtectus* 24 hr postfumigation. Wang et al. (2014) presented a thesis that the essential oils of *Citrus limonum* and *Allium sativum* could serve as effective control agents of the darkling beetle, *A. diaperinus*. It was observed that the strongest fumigant activity appeared between 48 and 96 hr post-treatment, when 6th instars larvae were treated. The effects of fumigant toxicity of *C. lusitanica* and *E. saligna* essential oil components were also tested. They deterred oviposition, with results expressed as the number of

laid eggs and the number of adults. It was found that at the highest tested dose (60 μL/L) 1-8-cineole and carvacrol completely deterred oviposition. Among the oil treatments in lower doses that did not completely prevent oviposition, the tested essential oils completely inhibited adult emergence (Ajayi et al., 2014).

Nanoparticles and nanolayers

There are still some problems with the usage of some plant-derived substances, such as poor water solubility or aptitude for oxidation (Moretti, Sanna-Passino, Demontis, & Bazzoni, 2002). These authors proposed microencapsulation, with the use of gelatin, as a method to increase the usefulness of essential oils. Micro- or nanocapsules contain an oily core, whereas nanospheres are composites that form a matrix (São Pedro, Santo, Silva, Detoni, & Albuquerque, 2013; Zorzi, Carvalho, von Poser, & Teixeira, 2015). Nanoencapsulation of a carbamate insecticide methomyl with the aqueous core showed higher insecticidal activity than the traditional method, and the diffusion was controlled (Sun et al., 2014). Similar results were observed for sodium alginate nanoparticles loaded with the neonicotinoid insecticide imidacloprid (Kumar, Bhanjana, Sharma, Sidhu, & Dilbaghi, 2014) and for silica nanoparticles loaded with validamycin (Liu et al., 2006). Recently, modification of the fumigation method–nanoformulation–has also been suggested to significantly enhance the toxicity of natural substances against crop pests. Such a method, using poly(ethylene glycol) nanoparticles, significantly increased the toxicity of essential oils against *T. castaneum*, *Rhizopertha dominica (Bostrichidae)* (Gonzalez-Coloma et al., 2004), and the tomato borer (*Tuta absoluta*) (Campolo et al., 2017). Nanoformulations also showed increased activity of nanoparticles against other orders of insects (Gonzalez et al., 2016). Moreover, this technique increased the persistence of essential oils and slowed down their degradation (Sebaaly, Jraij, Fessi, Charcosset, & Greige-Gerges, 2015). Additionally, nanoformulations can solve problems related to essential oil volatility, their tendency toward oxidation, and insufficient water solubility (Campolo et al., 2017), and, importantly, nanoformulations have good storage stability under a broad range of temperatures (−10 °C to 55 °C) (Choupanian, Omar, Basri, & Asib, 2017). Nanoformulation was reported to be also a technique that increases the lethal toxicity of natural pyrethrins (Papanikolaou et al., 2017). The technique seems to be useful for gradually delivering natural active substances to the site of action, for example, in medicine (Chowański et al., 2017) or as antimicrobial agents (São Pedro et al., 2013), which simultaneously minimizes the toxic effects on nontarget organisms (Campolo et al., 2017). Several compounds have been used for the formation of microspheres or nanoparticles that are used to deliver plant-derived bioactive substances: liposomes (Moghimipour, Aghel, Zarei Mahmoudabadi, Ramezani, & Handali, 2012), oil-in-water nanoemulsions (Papanikolaou et al., 2017), Tween 20, chitosan combined with other substances (Ziaee, Moharramipour, & Mohsenifar, 2014), silica (Chaudhary et al., 2017), metals and metal oxides (Ali, Yousef, & Nafady, 2015), and proteins such as bovine or human serum albumins (Sokolik, Ben-Shabat-Binyamini, Gedanken, & Lellouche, 2018). Next, Athanassiou, Kavallieratos, Evergetis, Katsoula, and Haroutounian (2013) reported the usage of a silica gel with essential oils. These authors emphasized that silica had a low toxicity to mammals and could be easily removed from protected stored grains prior to processing. Adhesion of the carried substance to the carrier increases with its surface area. Therefore, the strategies that make a carrier (for example, silica) porous (Kim et al., 2017) or hydrophobic (Barik, Kamaraju, & Gowswami, 2012; Moro, Parneix, Cabane, Sanson, & d'Espinose de Lacaillerie, 2017) may increase the adhesion several times. To increase the adhesion of carried substances to the carrier, ultrasonication is used as a method to obtain expected formulas (Moustafa, Mohamad, & Torkey, 2015). Sonication decreases the size of particles in a time-dependent manner (Kumar et al., 2014). This finding suggests that nanoformulations can be successfully used to increase insecticidal activity and have a great potential in plant protection. Consequently, nanotechniques reduce the amount of used insecticides by isolating active ingredients from the environment, which limits the impact on the food/environment and reduces the cost of the application (Abreu, Oliveira, Paula, & de Paula, 2012). Polymer/layered silicate nanocomposites are also used as a matrix for fungal bioinsecticide formulations. Fungal conidia are very sensitive to external factors (abiotic). Thus, their encapsulation is necessary to ensure their marketing as bioinsecticides. Encapsulated conidia possess significantly higher efficacy in pest control (Batista et al., 2014).

Another method correlated with nanoparticles and nanolayers, used to control a number of pests, is the slow and controlled release of essential oils liberated from nanogels (Ziaee et al., 2014). (Werdin Gonzalez, Gutierrez, Ferrero, & Fernandez Band, 2014) suggest that this method is simple, cost-effective, and facile. More importantly, the method can be conducted at room temperature (Werdin Gonzalez et al., 2014). It was tested in accordance with nanogels of myristic acid-chitosan loaded with an essential oil extracted from *Cuminum cyminum* L. used against *Sitophilus granarius L.* (Curculionidae) and *T. confusum* (Ziaee et al., 2014). The insecticidal bioassay revealed that oil-loaded nanogels were more toxic than clear cumin oil. Moreover, cumin oil lost its insecticidal activity faster (after 12 days, cumin oils lost their insecticidal activity completely, whereas the nanogel lost maximally 60% of its activity). The activity of nanoparticles has not been tested against adult insects only. Additionally, their effect on eggs was evaluated. For example, in research conducted by Campolo et al. (2017), *T. absoluta* eggs were sprayed with adequate polyethylene glycol-lemon essential oils solutions and the mortality was estimated. It was found, that essential oils were lethally toxic to adult beetles but no such effect was observed with reference to embryos (Campolo et al., 2017).

There are many studies where the authors compare the activity of nonformulated and formulated botxoanical substances. A good example of such studies is a study conducted by Choupanian et al. (2017). They showed that an azadirachtin nanoemulsion demonstrated excellent lethal contact toxicity against *S. oryzae* (85% to 100%) and *T. castaneum* (74% to 100%), and compared to nonformulated neem oil the nanoemulsions were significantly more effective.

Additional methods that can support insecticidal properties of plant derivatives against stored pests are worth mentioning. An interesting approach was the usage of phytotoxins combined with exposure to UV light (Lee & Berenbaum, 1989). Consequently, the activity of antioxidant enzymes was altered, and the growth of larvae and food consumption were limited, which may lead to toxicity at the cellular and subcellular levels, as described above.

Next, an alternative technique for storing and protecting grains is hermetic storage combined with vacuum and gas hermetic fumigation (Freitas, Faroni, & Sousa, 2016). This method, in which polyethylene silo bags and polyethylene terephthalate (PET) bottles are used, was successfully utilized for *A. obtectus*. The success of this method is correlated with the creation of different environmental conditions such as decreased levels of oxygen and

increased levels of carbon dioxide, causing inhibition of feeding in insects, which then become inactive, and consequently, die from asphyxiation or desiccation (Freitas et al., 2016; Moreno-Martinez, Jiménez, & Vázquez, 2000; Murdock, Margam, Baoua, Balfe, & Shade, 2012). Mbata and Payton (2013) tested the combination of hermetic storage and usage of safe fumigants of botanical derivatives such as monoterpenoids on adults of *T. castaneum*, which caused 100% mortality. A similar effect of 100% mortality was also found in a case involving the hermetic method combined with the toxicity of garlic emulsion usage against *T. castaneum* and *R. dominica* (Jahromi, Asgar Pourmirza, & Hasan Safaralizadeh, 2012). An increased insecticidal effect of this combined method (vacuum storage in combination with a foliage powder of *Peumus boldus* and lime) against *S. zeamais* was also reported by (Ribeiro et al., 2014).

Plant-Derived Substances against Beetle Pests of Stored Food Commodities

Beetles are the most common pests of stored products. They are very resistant to the conditions in storage facilities and can survive in a very dry environment. Among insects that are storage pests, two groups are distinguished, considering the manner of infestation. Primary pests can infest intact grains, and their larvae can develop inside the grain, whereas secondary pests are not able to begin the infestation in optimal conditions. The insects of the 2nd group can attack already infested or damaged kernels, for example, those harmed by other insects or kept in nonoptimal conditions (Rajashekar, Rao, & Shivanandappa, 2012). Secondary pests are: *Attagenus unicolor japonicas (Dermestidae), A. diaperinus, T. molitor, T. castaneum* and *T. confusum* while the other stored beetles mentioned in this paper belong to the group of primary pests (Ahmed, 1983). All of the mentioned beetles not only are responsible for direct damage to grains but also contribute to fungal infections as the mechanical vectors (Birck, Lorini, & Scussel, 2006) and, hence, increase the risk of mycotoxin contamination. In this review, we present the research data for plant derivatives effects on stored-product beetle pests.

Plant extracts

As we have shown, plants are rich in active substances that can be used as potential bioinsecticides. Many plant families or particular species produce specific chemical compounds with unique characteristics. Considering only substances with insecticidal properties, pyrethrin can be mentioned, as a product of *Chrysanthemum cinerariifolium* or azadirachtin obtained from *A. indica*. Both of these compounds are already commercially available in many countries as natural insecticides (more details about the availability are presented in an article by El-Wakeil (2013). Azadirachtin A is the main component of A. indica oil. The EO was reported as lethal to *C. maculatus* and *S. zeamais* adults (Tofel, Nukenine, Stahler, & Adler, 2016). It is noteworthy to add that insects may develop tolerance to pure azadirachtin easier than to the extract (Isman, 2002). Extracts can be obtained from various plant parts, such as leaves, flowers, fruits, and underground bulbs and roots. The extraction process usually starts with freezing and drying of plant tissues, and then shredded tissues are dissolved in water or organic solvents with various polarities. This procedure may significantly influence the activity of extract ingredients (Rani & Devanand, 2011) and change the penetration of biological membranes, which next causes different physiological effects on tested animals. The choice of organic solvent type is very important during the isolation of pure compounds from plant extracts. As shown in the results of Aydin, Bayrak, Baran, and Cakir (2017), the concentration of pure xanthohumol isolated from *Humulus lupulus* cones increases with the polarity of the organic solvent. Sometimes, the usage of appropriate solvents and mechanical treatments such as stirring and shaking can be helpful for the release of active compounds from plant tissues (Cataldi, Lelario, & Bufo, 2005). Determination of the quantity and quality of extract ingredients can be conducted with high-performance liquid chromatography, gas chromatography, or mass spectrometry. This knowledge allows an evaluation of their potential function in further studies and enables choosing the most active substance that is responsible for physiological reactions. In Table 1, we present collected data about various plant extracts (mostly of unknown composition) that possess insecticidal properties against stored beetles.

Plant extracts containing alkaloids and pure alkaloids. Alkaloids are secondary metabolites produced by 20% of all flowering plant species (Wink, Schmeller, & Latz-Brüning, 1998). The representative of this group, α-solanine, is presented on Figure 3. Alkaloids are present in all plant organs, especially in unripe, young fruits and leaves, in various concentrations that may depend on many environmental factors. These allelochemicals, besides their natural role of interacting with other plants and animals (attractants for pollinators, feeding deterrents for herbivores), have been widely studied for their insecticidal, antibacterial, and antifungal properties. Alkaloids are considered for use as potential insecticides and are easily obtained from plant parts that are not used in agriculture. Their potential is very high due to increasing knowledge about the properties of alkaloids. One of the alkaloids, nicotine, is a 1st-generation botanical pesticide. It binds to acetylcholine receptors, both in invertebrates and vertebrates, causing direct toxicity. This effect speaks against the use of nicotine worldwide, hence its usage is limited in many countries (El-Wakeil, 2013). The mentioned knowledge regarding alkaloids was a starting point for the separation of this important group of compounds and extracts that contains them to another subchapter.

It has been proved that alkaloids, besides causing acute toxicity, lead to the disruption of biological membranes, malfunction of internal organs and metabolism, redox imbalance, and disturbances in the development and reproduction processes in insects or cause inhibition of food intake (Chowański et al. (2016), as also discussed in the chapters above). Both extracts and pure alkaloids are taken into consideration as biopesticides. However, according to research, the extract contains synergistically active compounds that increase insecticidal effectiveness, and the effect of using the pure compounds mixed together in different quantitative ratios may be modified (Ventrella et al., 2015). Moreover, the lack of response of *T. molitor* to a potato extract while the beetle from the same family *Zophobas atratus* showed a particular reaction (Marciniak et al., 2010) suggests the possibility of alkaloid selectivity. The two tables below present some data about plant derivatives containing alkaloids (Table 2) and pure alkaloids (Table 3) that affect stored beetles.

Powders from various plant parts

In some cases pesticidal agents can be applied as powders. They may be composed of various groups of potentially active substances. Obviously the science needs further, deeper research indicating the most active compounds. In addition to nonproteinaceous substances, plants also produce a variety of peptides and proteins that show insecticidal properties: lectins, ribosome-inactivating proteins, inhibitors of proteolytic enzymes, arcelins, chitinases, and ureases (Carlini & Grossi-de-Sa, 2002; Chowański, Kudlewska, Marciniak, & Rosiński, 2014; Dang & Van Damme,

2015; Walkowiak, Spochacz, & Rosiński, 2015). These proteins are active against coleopteran species, but the toxicity may differ among species (Hou & Fields, 2003). There are two main ways of their application: the proteins are added to a diet or they are produced within the tissues of genetically modified plants. The main problem of application in a diet is that the proteins may not be stable in the environment, especially at higher temperatures (Hou & Fields, 2003). GMO organisms, on the other hand, undergo strict regulations, which may differ between countries. Next, they protect only the modified crops, and not the stored non-GMO ones.

Powders obtained from native medicinal plants have been observed to be a promising alternative to protect stored crops. Ul Hasan, Sagheer, Bhatti, and Ali (2013) conducted research to check the effect of different doses of powders derived from eucalyptus (*Eucalyptus globules*), black onion seeds (*Nigella sativa*), and seeds of caraway (*Carum carvi*) on the mortality of adult *C. maculatus* after different time intervals (Table 4). The maximum mortality was observed after the application of caraway powder, then black onion seeds, and next eucalyptus powders. The powder concentration is another factor that increases mortality; the highest tested concentration (2%) caused the highest mortality. The percentage of dead insects was determined at the 3rd, 5th, and 7th days of exposure and the maximum mortality of every powder was observed at the 7th day of exposure (Ul Hasan et al., 2013).

Another research group confirmed the effectiveness of leaf powders toward a major stored-product pest, the red flour beetle (*T. castaneum*). The scientists used 5% leaf powders of *A. indica*, *Tagetes erecta*, and *Cynodon dactylon*, which showed residual toxicity and caused an approximately 50% inhibition rate, depending on the plant (Table 4). Plant powders might be useful as insect control agents, not only because of their nontoxicity to mammals, biodegradability, and lower risk of development of pest resistance, but also due to their ease of obtainment and their lower cost than those of commercial insecticides, which is especially important in developing countries (Islam & Talukder, 2005).

A. indica pulverized seeds and leaf powder of *Plectranthus glandulosus* were tested against the major insect pests *C. maculatus* and *S. zeamais*. Both plants caused mortality of beetles, but *P. glandulosus* was more potent, with 46% mortality at 40 g/kg of leaf powder. *A. indica* seed powder registered 34% mortality at the same dose. Moreover, within the two insect species, *S. zeamais* was more affected by the toxic effect of the *P. glandulosus* leaf powder. *P. glandulosus* leaf powder concentration at 10 g/kg caused 76% mortality of *S. zeamais*, while only 17% mortality was noticed in *C. maculatus*. Both botanicals also inhibited F_1 progeny production. *A. indica* powder completely reduced the emergence of progeny, even at the lowest dose of powder for both beetle species. In the case of *P. glandulosus*, the reduction of progeny production was dose-dependent, but in the highest amount of plant powder (40 g/kg), the inhibition of progeny production was 96% and 97% for *C. maculatus* and *S. zeamais*, respectively (Tofel, Kosma, Stähler, Adler, & Nukenine, 2017).

Adarkwah, Obeng-Ofori, Hörmann, Ulrichs, and Schöller (2017) investigated alternative control strategies of pests attacking stored grains. They used plant powder from *Eugenia aromatica* and *Moringa oleifera*, which caused significant mortality of the adult beetles (*S. granarius*, *T. castaneum* and *A. obtectus*) and reduction of F_1 adults compared with the control. They tested different doses of flower bud powders, from 0.25 to 2.5 g of powder per 20 g of wheat seeds. The dead were counted for 7 days. The most effective results were achieved after 7 days with the highest doses. More details (LD_{50} for flower bud or leaf powders and mortality of beetles) are presented in Table 4. *A. obtectus* was most susceptible toward the botanicals, followed by *S. granarius*. *T. castaneum* was the most tolerant for *E. aromatica* and *M. oleifera*.

Essential oils and their pure ingredients

Essential oils are complex mixtures of volatile compounds obtained by various types of distillation or by cold-pressing from fresh herbal organs. The composition of essential oils may differ, due to various procedures of their extraction, for example, various used solvents or boiling points (Baser & Buchbauer, 2015). Therefore, the number of essential oils is large and the data describing their activities have increased rapidly. Hence, Table 5 presents only a part of the research describing the insecticidal activity of essential oils. A large variety of toxic effects has been described, including lethality and various sublethal effects. These effects can be obtained not only through contact action but also through fumigation. This makes essential oils exceptionally suitable for crop protection as well as in food storage areas. We also present the effects of pure compounds obtained from essential oils (Table 6). An interesting study on the enhanced activity of a limonoid salannin was carried out by Yamasaki and Klocke (1989) who showed that antifeedant activity of this compound may be enhanced by several chemical modifications, like, for example, hydrogenation or substitution of the acetoxyl groups by methoxyl ones. Thus, native substances present in EOs are interesting not only as insecticides themselves, but also as "starting points" toward new, more active substances.

Other plant-derived products

The most extensively studied plant products have been described above. In addition to those previously mentioned, many other less-known compounds are used, also with proven insecticidal properties. Starting with the less processed products, crushed fresh bulbs of *A. sativum*, produce volatile substances that completely inhibit the emergence of adult *C. maculatus* from fumigated eggs and seed holing at a dose of 6.0 g per 20 g of cowpea seeds, while crushed bulbs from *Allium cepa* significantly reduced the emergence of adults (Ofuya, Olotuah, & Ogunsola, 2010). Mixed dried neem leaves (*A. indica*) have traditionally been used in rural India to prevent infestation in stored grains, and it is a common practice still (Saxena, 1995). Another interesting plant product is latex, which plays an important role in plants as defence mechanisms. It is produced by specialized cells, laticifers. The laticifer protein fraction (LP) isolated from *Calotropis procera* has been shown to have insecticidal properties. The effects of LP were tested on two coleopteran stored-product pests, *R. dominica* and *S. oryzae* (Nenaah, 2013). In a treated-filter bioassay, a concentration of 5 mL/cm^2 caused a mortality of 78% and 62% *S. oryzae* and *R. dominic*a adults, respectively, after 7 days of treatment. A similar substance from *C. procera* was examined in *C. maculatus*, causing mortality of larvae. The LD_{50} was calculated to be a dose equal to 0.14% (w/w) (Ramos et al., 2010). For this species, LP also caused weight loss of larvae and decreased adult emergence to 2% after 20 days of exposure after a dose of 0.5% LP (w/w). Another example of the experiment conducted on a protein fraction showed that a protein fraction from *Pisum sativum* seeds containing 60% protein and 30% starch decreased the number of *Cryptolestes ferrugineus (Laemophloeidae)* and *S. oryzae* adult beetles in wheat treated with concentrations of 0.01% to 10% (w/w) in a multiple-choice test (Fields, Xie, & Hou, 2001). *C. ferrugineus* was also sensitive to a seed fiber fraction containing 90% fiber and 3% protein in concentrations of 0.1% to 10% (w/w). In another two-choice bioassay, the protein fraction caused a significant decrease in the numbers of *C. ferrugineus,*

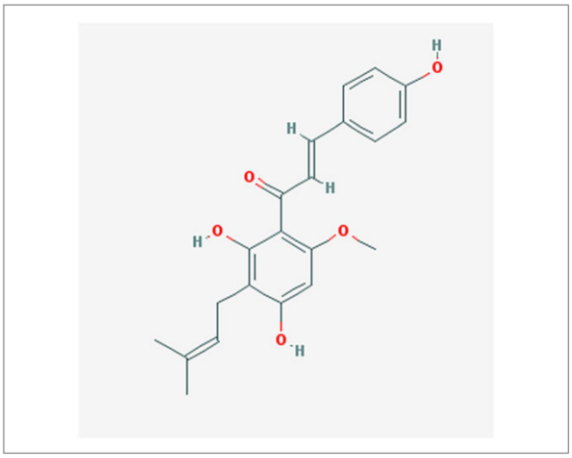

Figure 6–Xanthohumol, flavonoid, chemical structure (Pubchem, 2018e).

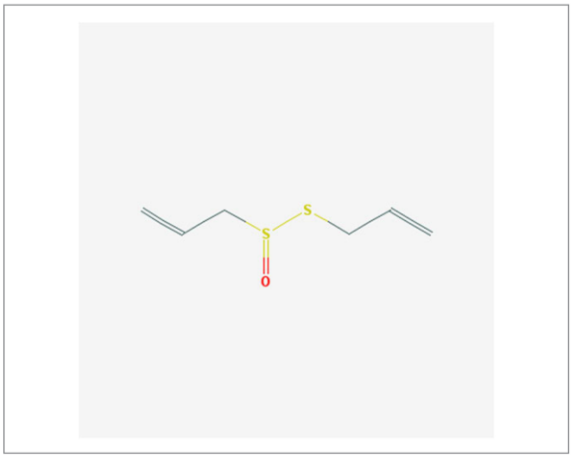

Figure 7–Allicin, chemical structure (Pubchem, 2018a).

S. oryzae, S. zeamais, T. castaneum, and *T. confusum*. Proteins found in plants are a very interesting option as inhibitors of insect digestive enzymes. An α-amylase inhibitor isolated from *Withania somnifera* seeds affected the activity of α-amylases from *T. castaneum* adults. It also caused a decrease in the consumption and growth of this insect at a dose of 1.6 mg/g (Kasar et al., 2017).

Other groups of pure compounds isolated from plants were also studied in stored beetles. Flavonoids are plant secondary metabolites that play an important role in insect deterrence. According to (Nenaah, 2013), a crude flavonoid fraction isolated from *C. procera* at a concentration of 5.0 mL/cm^2 caused 86% mortality of *S. oryzae* and 64% mortality of *R. dominica* after 7 days of treatment. Particular flavonoid glycosides from the fraction, such as quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside, and 5-hydroxy-3,7-dimethoxyflavone-4'-*O*-beta-glucopyranoside, were also tested against these two beetles, but the results showed a lower mortality percentage than that of the crude flavonoid fraction. In the research of Kotkar et al. (2002), flavonoids were isolated from an aqueous extract of *Annona squamosa*. At a concentration of 0.07 mg/mL, these compounds showed insecticidal activity against *C. chinensis* (Kotkar et al., 2002). Xanthohumol (XN) is a prenylated flavonoid isolated from hop cones (*H. lupulus*). The chemical structure of this compound is presented on Figure 6. As shown in the results of Aydin et al. (2017), a XN methanol solution and extracts from hop cones exhibited insecticidal activity against stored product pest beetles. The LD_{50} of XN was <5 μg in the case of *S. granarius* and *L. serricorne*, <10 μg for *S. oryzae*, and much higher, specifically 26.4 μg and 22.3 μg, for *A. obtectus* and *T. castaneum*, respectively. In all cases, extracts from *H. lupulus* were more active (with activity described above in Table 2). The other study with 1.0% XN ethanol solution caused a medium deterrent activity for *S. granarius* adults and *T. confusum* larvae and weak feeding deterrent activity against *T. confusum* adults and larvae of *T. granarium* (Jackowski et al., 2015). In this study, it was also noticed that the activity of the hop extract was stronger than that of pure XN. Changes in the chemical structure of XN by cyclization, isomerization, oxidation, and reduction increased feeding deterrence (Jackowski et al., 2017). Isoxanthohumol (IXN) showed good deterrent activity (*T*-value = 103.4) for *S. granarius* adults, which was 13.4 points higher than that of XN; the *T*-value of xanthohumol flavone (FXN) was 22.9 points higher and that of α,β-dihydroxanthohumol was 22 points higher than the *T*-value of XN. Moreover, FXN also increased the deterrence activity for other species such as *T. confusum* larvae (*T*-value 58.1 points higher than that of XN) and *T. granarium* larvae (*T*-value 57 points higher than that of XN).

Withanolides are another group of secondary metabolites found in the *Solanaceae* family. Their antifeedant activity against stored beetles was investigated already in 1987 by (Ascher et al., 1987). Nicalbin A and a 1:1 mixture of withanicandrin (isolated from *Nicandra physaloides*) and daturalactone A (isolated from *Datura quercifolia*) were active against *T. castaneum* larvae. Nicalbin A caused 99% mortality, whereas a mixture of withanicandrin and daturalactone A caused 68% mortality, both at a concentration of 500 ppm. Both examined substances have also shown antifeedant properties. Three withanolides isolated from *Salpichroa origanifolia* salpichrolides A, C, and G were tested on *T. castaneum* (Mareggiani et al., 2002). The highest used concentration (2000 ppm) incorporated into the diet of larvae caused a significant delay in development, while salpichrolides A and G showed lethal effects. To continue the description of lactone group compounds, coumarins isolated from *M. alata* (You et al., 2017) are worth to mention. Meranzin, phebalosin and muralatin Krevealed significant repellent activity against *T. castaneum*. The value of the percent repellency (PR%) by meranzin was 100% at a concentration of 78.63 μg/cm^2 after 2 hr of treatment, while the PR% of phebalosin and muralatin K was 76 and 82%, respectively.

Sulphur-containing amides were isolated from *Glycosmis lucida* by Yang et al. (2015). *N*-[2-(4-hydroxyphenyl)-ethyl]-3-methanesulfonyl-*N*-methyl-propionamide (A), methylgerambullin (B) and *N*-{2-[4-(6,7-hydroxy-3,7-dimethyl-oct-2-enyloxy)-phenyl]-ethyl

}-3-methanesulfonyl-*N*-methyl-acrylamide (C) demonstrated significant feeding deterrent activity against *T. castaneum*. EC_{50} values for compounds A, B, and C were 311.6, 244.9, and 108.3 ppm, respectively.

Allicin (ally-2-propenthio sulfinate) is an organic, sulfur-containing compound. The chemical structure of this compound is presented of Figure 7. It is one of the characteristic compounds isolated from garlic. Lu, Zhong, Wang, Liu, and Wan (2013) isolated allicin, impregnated it with hexane and tested it against 3

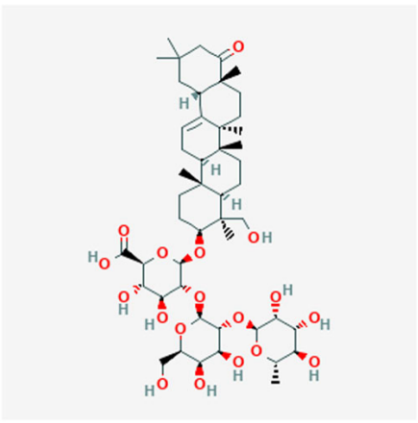

Figure 8–Dehydrosoyasaponin I, chemical structure (Pubchem, 2018b).

stored beetles, both larvae and adults. A fumigation bioassay resulted in mortality of *T. castaneum* (LD_{50} = 0.38 μL/L for adults after 6 days of exposure, 0.11 μL/L for larvae after 3 days of exposure), *O. surinamensis* (Silvanidae) (LD_{50} = 0.51 μL/L for adults after 6 days of exposure, 0.12 μL/L for larvae after 3 days of exposure) and *C. ferrugineus* (LD_{50} = 0.51 μL/L for adults after 6 days of exposure, 0.36 μL/L for larvae after 3 days of exposure).

Saponins are glycosides with a broad spectrum of action, including insecticidal activity against various insect families (De Geyter, Lambert, Geelen, & Smagghe, 2007; Singha & Kaur, 2018). These substance may inhibit growth and act as antifeedant but also they may cause lethal effects (Nielsen et al., 2010; Szczepanik et al., 2004, 2001). Taylor, Fields, and Sutherland (2004) isolated saponins from protein-rich fraction derived from pea flour. One of the most active saponin with insecticidal properties was dehydrosoysaponin I (Figure 8), that in dose 1.4 mg decreased the food consumption of *S. oryzae* to 17%. Additionally toxic activity of saponins differ between coleopteran species. Da Silva and co-workers found different toxicity of saponins present in *Medicago truncatula* seeds to *T. castaneum* and *S. oryzae* (Silva, Faroni, Sousa, & Freitas, 2012). 3-GlcA-28-AraRhaxyl-medicagenate caused high toxicity for *S. oryzae* where LT_{50} of 100 μg was 16.57 days. While for *T. castaneum* this compound showed no toxicity. Therefore, saponins are promising tools in plant protection as agents that not only limit feeding but also decrease pest population by causing acute and chronic effects. In 2012, (Rajashekar, Rao & Shivanandappa) described a new class of insecticide, namely, decalesides. These trisaccharides isolated from edible roots of *Dendrocalamus hamiltonii* turned out to have insecticidal activity targeting insects gustatory sites probably by sodium pump inhibition (Rajashekar & Shivanandappa, 2017). At the same time, as natural trisaccharides, they undergo rapid hydrolization in mammalian organisms, which makes these compounds safe for humans. Decaleside II methanol extract was tested on *R. dominica, S. oryzae, T. castaneum*, and *C. chinensis* (Rajashekar & Shivanandappa, 2014). The dose of 100 mg/kg of grain caused 100% mortality of all tested insects after 7 days of treatment, whereas the toxicity of decaleside II was dose-dependent. Furthermore, the same dose almost completely prevented progeny from emerging for a period of 3 months.

Conclusions–Perspectives Regarding Botanical Insecticides for Stored Crop Protection

In this review, we tried to show and describe some of the important issues concerning plant-derived substances and their usage in the protection of stored crops. As we have shown, there is an enormous variety of substances that exhibit insecticidal activity which can be used in crop protection. They differ in chemical structure, toxicity, mode of action, and possible application. It is also notable that tests on extracts, compared to single compounds, indicate that there are still many substances awaiting a description of their activity. Furthermore, research findings on extracts and blends indicate that they can also be used as relatively cheap plant protection. Our review also showed that the natural substances often possess a lower toxicity than the synthetic commercial substances. However, the sublethal effects are also very important and may result in decreased crop loss, due to lower vitality, fecundity, or antifeedant activity. Together with the lower toxicity of plant-derived substances to humans, the final gain of crop protection by natural substances may be higher than in the case of synthetic insecticides. In addition, the modern methods for spreading substances may increase their toxicity. This concept seems to be very interesting and has attracted the attention of many researchers. Additionally, chemical modification of natural substances may lead to the invention of new classes of insecticides.

On the other hand, there is a very wide range of pests that differ in their susceptibility to various compounds. As we mentioned, the usage of synthetic insecticides for stored crops protection is limited. Therefore, the number of tests on natural substances that can be applied as insecticides is constantly growing. The number of "bioinsecticide—pest" interactions is enormous. Some of them may be unique, due to the continuing evolution of plants and their pests.

In summary, natural plant-derived substances are still an open field for a range of studies by many researchers: chemists, biochemists, cell biologists, psychologists, entomologists, and ecotoxicologists. We are sure that the number of investigations in this field will increase over the next several years and that the application of bioinsecticides will also become an increasingly important branch of the protection of stored products against beetle pests.

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Authors' contributions

Marta Spochacz was responsible for the detailed description of the ultrastructure and physiological effects on the internal organs, the influence of plant extracts, plant extracts containing alkaloids and pure alkaloids and other plant products on beetles, and general coordination of manuscript composition. Szymon Chowański participated in the detailed description of the effects of plant derivatives on the nervous system and influence of essential oils on beetles and editing the manuscript. Karolina Walkowiak-Nowicka participated in the description of application methods. Monika Szymczak participated in the description of the effects on hormonal regulation and influence of the powders obtained from plants on beetles. Zbigniew Adamski participated in the description of prooxidant and antioxidant activity of plant derivatives, and application of these substances in crop protection.

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Sublethal Effects of *Solanum nigrum* Fruit Extract and Its Pure Glycoalkaloids on the Physiology of *Tenebrio molitor* (Mealworm)

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Sublethal Effects of *Solanum nigrum* Fruit Extract and Its Pure Glycoalkaloids on the Physiology of *Tenebrio molitor* (Mealworm)

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Abstract: Background: *Solanaceae* plants produce glycoalkaloids (GAs) that affect various physiological processes of herbivorous insects and they are being tested as potential alternatives for synthetic pesticides. They cause lethal and sublethal effects. Nevertheless, their mode of action remains unclear. Therefore, we examined the effects of *Solanum nigrum* fruit extracts and pure glycoalkaloids on a model beetle, *Tenebrio molitor*. Methods: Plant extracts or pure alkaloids were added to the food of the larvae for three days. The lipid, glycogen, and protein content in the fat body and the midgut were determined, and the contractility of the heart, hindgut, and oviduct muscles was tested using the video-microscopy technique. Finally, the ultrastructure of the fat body and the midgut was observed using electron microscopy. Results: No lethal effects were noted. Sublethal changes were observed in the content of biomolecules, malformations of organelles, chromatin condensation, and heart and oviduct contractility. The observed effects differed between the tested glycoalkaloids and the extract. Conclusions: Both the extract and pure GAs have a wide range of effects that may result in impaired development, food intake, and reproduction. Some early effects may be used as bioindicators of stress. The effects of the extract and pure alkaloids suggest that the substances produced by the plant may act additively or synergistically.

Keywords: *Solanum nigrum* extract; *Tenebrio molitor*; ultrastructure; midgut; fat body; biochemistry; contractility; heart; oviduct; glycogen; lipids

Key Contribution: *Solanum nigrum* extract and its pure glycoalkaloids given with food caused sublethal changes in the larvae of *Tenebrio molitor*. The effects that were caused by the extract differed from those that were caused by pure glycoalkaloids.

1. Introduction

In recent years, the knowledge about the potential alternatives for synthetic pesticides, such as plant derivatives, has significantly increased [1–3]. Natural products are already in use on the markets worldwide, for example, in organic agriculture [4]. Some of the active compounds from extracts have been changed structurally to obtain more persistent substances, such as the neem tree (*Azadirachta*

indica) extract, which has progressively become increasingly popular [5], or pyrethrins obtained from *Chrysanthemum cinerariifolium*, which in the 1970s became a source for the third class of synthetic pyrethroids [6]. One of the most difficult aspects of crop protection is the application of substances to stored commodities. This step requires easily degradable compounds that are relatively nontoxic to mammals. From an economic point of view, substances that are used in crop protection should be inexpensive and their emission should not lead to their environmental accumulation or to toxic effects on nontarget organisms. Natural substances that are produced by plants to deter herbivores meet the criteria outlined above. They not only are effective in the control of pest insect populations, causing up to 100% mortality at concentrations as low as 0.5 mg/cm^3 after 24 h of fumigation [7], but also exhibit selective action against various species [8,9]. The effectiveness of plant derivatives may strongly depend on the dosage, method of extraction and solvent [10–12], as well as the application method [13]. Given that there are an enormous number of possible plant extracts containing active ingredients and that there are many solvents that can be used, there is a great need to study the possible combinations to obtain the most effective substances for specific species. The majority of research has focused on direct toxic effects, which can be used to limit pest populations [1]. However, understanding the mode of action of plant derived substances can be useful in planning strategies for pest control. The nature of the effects on both the target and nontarget tissues can be tested by exposure of the target species to low doses of the plant-derived substances. Although the effects may be subtle and even statistically non-significant, some discrete malformations and malfunctions may be observed before the massive toxic effects appear when low concentrations of these substances are used [14–16]. Furthermore, the sublethal doses and concentrations can reveal the first effects and the mode of action at the level of organs, tissues, or even cells.

In this study, we tested the extract that was obtained from the unripe fruits of *Solanum nigrum*, a plant commonly distributed in Europe, which is known to produce glycoalkaloids (GAs). Previous studies have demonstrated that the extract caused larvicidal effects in mosquitoes (Diptera), such as *Culex vishnui* [17], *Culex quinquefasciatus* [18], *Culex pipiens*, *Aedes caspius* [19], *Anopheles culicifacies*, *Aedes aegypti* [20], and *Anopheles stephensi* [21]. Toxic effects have also been found in the fruitfly (*Drosophila melanogaster*: Diptera) [22] and the Colorado potato beetle (*Leptinotarsa decemlineata*: Coleoptera) [23]. *S. nigrum* extract, in addition to its toxic effects, was reported to have promising anticancer [24] and antimicrobial properties [25]. The tested extract contains 10 GAs, but two, solasonine and solamargine, are present in greatest amounts [22]. Studies have shown not only the toxic influence of glycoalkaloids on animal health [26], but also the beneficial effects, such as anticancer properties [27,28]. Since alkaloids have been reported as promising tools for pest management (for review see: [1,2]), we decided to examine the extract from *S. nigrum* fruits as well as pure solasonine and solamargine and to compare their effects on a model organism in ecotoxicological studies and a pest of stored products—the yellow mealworm beetle *T. molitor*.

We addressed the following research questions:

- Do the extract and the pure GAs cause lethal toxic effects and disturb the development of *T. molitor* larvae?
- Do the tested substances cause malformations of the cells in the exposed tissues?
- Do the tested substances affect the biochemical parameters of the exposed tissues?
- Do the tested substances affect the physiological parameters of *T. molitor* larvae?
- Do the effects of the extract differ from the effects of pure GAs, and (if yes) what aspects of the toxicity may be caused by solasonine, solamargine or other compounds of the extract?

To answer these questions, we conducted some observational studies and tests of various levels of biological organization. This study included an analysis of the general toxic activity of the *S. nigrum* extract given in the food on the growth of *T. molitor* larvae. Since we had already observed some ultrastructural changes in response to exposure to *Solanaceae* plant extracts [2,29], we decided to test the ultrastructure of the midgut and fat body, which are important tissues for the ingestion and

distribution of toxic agents within insect bodies. The midgut was directly exposed to the agents present in the ingested feed. To complement the changes that were observed with electron microscopy, biochemical assays of parameters, such as the content of lipids, glycogen, and proteins in the fat body were conducted. Next, further studies included the analysis of the influence of the extract and pure glycoalkaloids on the visceral muscles and myocardium contractile activity under in vitro conditions, to check their utility as possible factors affecting muscle activity. The modulation of muscle contractility of organs, such as the heart, hindgut, or oviduct may result in impaired development, food intake, and reproduction. Hence, the above mentioned parameters may be crucial for better understanding the toxic mode of action of the tested alkaloids, and they may also contribute to the more efficient application of plant derived substances in plant protection. Consequently, this may lead to the decreased use of both synthetic and natural substances in plant protection, with the benefits of limiting treatment of crops and food products and reducing environmental pollution.

2. Results

2.1. Changes in Body Mass

The average percentage gain in body mass by the control larvae during the experiment was 15.7 ± 0.8% with n = 139 (Table 1). None of the larvae died during the experiment. The lowest mean percentage weight gain (13.4 ± 1.61%) was obtained after solasonine application to the diet at a concentration of 7.52 × 10^{-6} M (Table 2), and the highest (19.1 ± 1.28%) after the application of solamargine in the concentration 7.23 × 10^{-6} M.

Table 1. The percentage gain in body mass by *T. molitor* larvae after application of the extract, solamargine, solasonine and saline B (control) into to the diet. The data are shown as the mean ± SEM. ANOVA, Tukey's test.

Concentration / Gain inBody Mass (%)	0.01%	0.1%	1%	10%	Control	
Extract	18.6 ± 1.28	18.7 ± 1.42	17.5 ± 1.33	15.2 ± 1.5		
Solamargine	18.2 ± 1.31	19.1 ± 1.28	17.0 ± 1.05	16.8 ± 1.34	15.7	± 0.75
Solasonine	13.9 ± 1.38	13.4 ± 1.61	16.5 ± 1.63	16.7 ± 1.42		

2.2. Effects on Visceral Muscle Contractility In Vitro

2.2.1. Heart Activity

The extract that was applied to the heart caused a negative chronotropic effect, the strength of which increased with increasing concentration (Figure 1). The strongest effect was observed after application of the 0.1% and 1% extracts. In these cases, an average percentage regarding the lowering of the heart rate of −8.3 ± 1.61% and −40.4 ± 4.58%, respectively, was observed. 1% solution also caused the reversible inhibition of heart activity (Figure 2). None of the tested GAs caused a significant effect on heart activity.

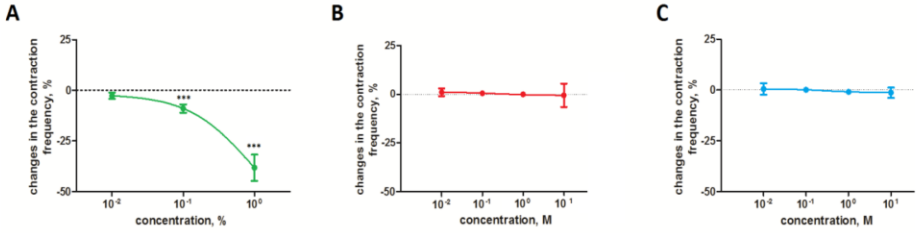

Figure 1. The contraction frequency of *T. molitor* heart after the application of the *S. nigrum* extract (**A**) and pure glycoalkaloids (solamargine (**B**) and solasonine (**C**)). *** Statistical significance at $p \leq 0.001$, Kruskal-Wallis test with Dunn's test.

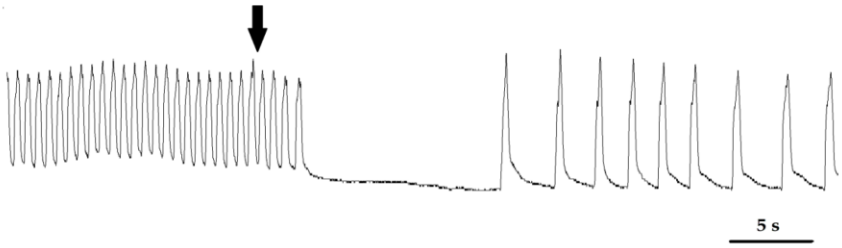

Figure 2. Sample myocardiogram of an adult *T. molitor* beetle. The arrow shows the moment the 1% *S. nigrum* extract was applied.

2.2.2. Oviduct Contractility

In contrast to the heart, the 1% extract applied to the oviduct increased the contraction frequency of this organ by an average of 152.7 ± 47.79%. The observed effect was dose dependent, and the intensity of the response increased with an increasing extract concentration (Figure 3A). In the case of solamargine, we also observed a slight increase in the oviduct contraction frequency after application of the glycoalkaloid (Figure 3B). However, the relationship between the strength of the observed effect and concentration was opposite to that caused by the extract.

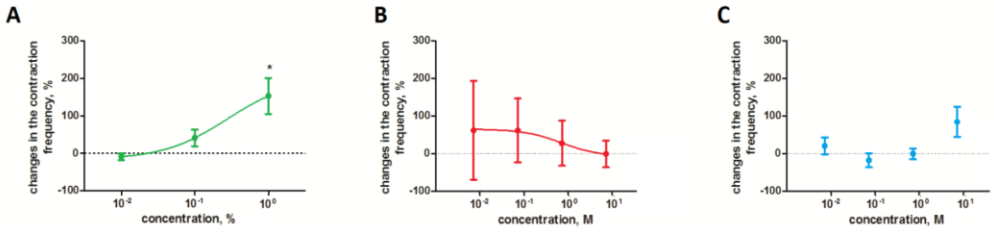

Figure 3. Effect of the *S. nigrum* extract (**A**) and pure glycoalkaloids (solamargine (**B**) and solasonine (**C**)) on contractile activity of *T. molitor* oviduct. * Statistical significance at $p \leq 0.05$, Kruskal-Wallis test with Dunn's test.

2.2.3. Hindgut Contractility

Similar to the oviduct, the *S. nigrum* extract increased the frequency of the hindgut contraction; nevertheless, the observed effect was definitely slighter (Figure 4A). None of the pure alkaloids that were applied on the isolated hindgut caused a significant effect (Figure 4B,C).

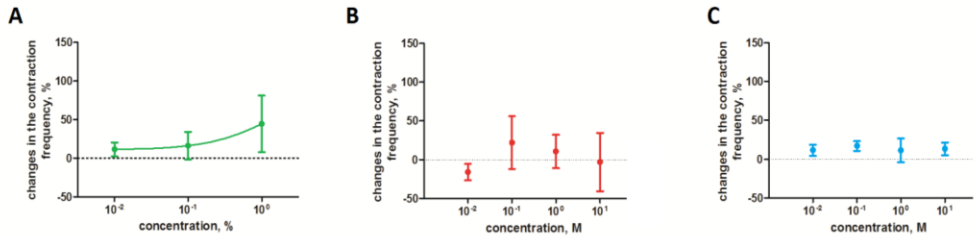

Figure 4. Effect of the *S. nigrum* extract (**A**) and pure glycoalkaloids (solamargine (**B**) and solasonine (**C**)) on contractile activity of *T. molitor* hindgut. ANOVA with Tukey's test or Kruskal-Wallis test with Dunn's test.

2.3. The Influence on the Fat Body and the Midgut Ultrastructure

2.3.1. Midgut

The columnar midgut cells of *T. molitor* (Figure 5) are characterized by nuclei surrounded by cytoplasm containing rough endoplasmic reticulum (RER), Golgi bodies, and elongated shaped

mitochondria. Each nucleus usually contains a single or double protein crystal (Figure 5, No. 1) [30]. The apical part of the cell includes long microvilli that take part in the absorption of digested food. When considering the high metabolic rate and function of midgut cells, the apical zone contains many mitochondria, smooth endoplasmic reticulum (SER), pinocytotic vesicles, and lysosomes. Special attention was paid to the abovementioned zone of the cells because it is the first zone to have contact with digested compounds (in this study, potentially the administered glycoalkaloids).

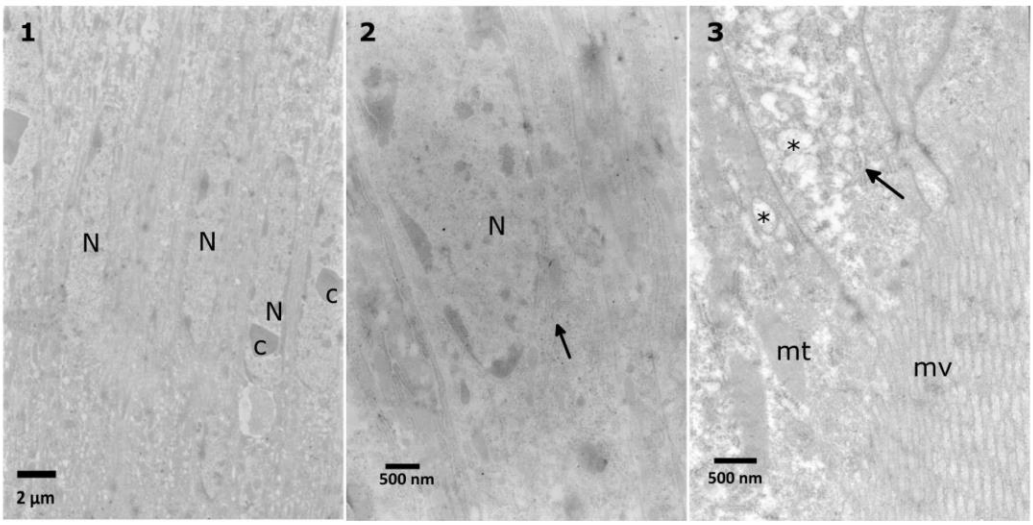

Figure 5. Control cells of the *T. molitor* larvae midgut. The nuclei (N) with protein crystals (c) (**1**) are surrounded by cytoplasm rich in endoplasmic reticulum (arrows) (**2**). The apical part of the cells (**3**) contains many mitochondria (mt), pinocytic vesicles (asterisks) and microvilli (mv).

The lowest extract concentration did not cause any evident changes in the ultrastructure in comparison to the control observations. First, mild effects, such as the disruption of the nuclear membranes and swollen perinuclear space, were observed after application of the 0.1% extract (Figure 6 No. 5). After the application of 1% extract, the same effect was observed, with additional changes in the density of the cytoplasm (Figure 7, No. 7). The strongest effects were observed after the application of the 10% extract (Figure 7, No. 8, 9). The nuclear membranes were separated in the basal part of the cells (Figure 7, No. 7, 9). Additionally, a decrease in the cytoplasm density was observed, especially around the nuclei, with the presence of single-membranous structures, most likely glycogen vacuoles (Figure 7, No. 8, Glv), which may be associated with glycogen redeployment [31]. When solasonine or solamargine were added to the diet, no significant changes were observed in the ultrastructure of midgut cells at any tested concentration.

The tested substances did not significantly alter the amount of electron dense chromatin within the nuclei (Figure 8). The correlations between the substance concentration and heterochromatin ratio for the extract, solamargine, and solasonine were 0.06, 0.60, and 0.43, respectively. Only the relationship between the solamargine concentration and heterochromatin ratio could be regarded as a moderate positive correlation.

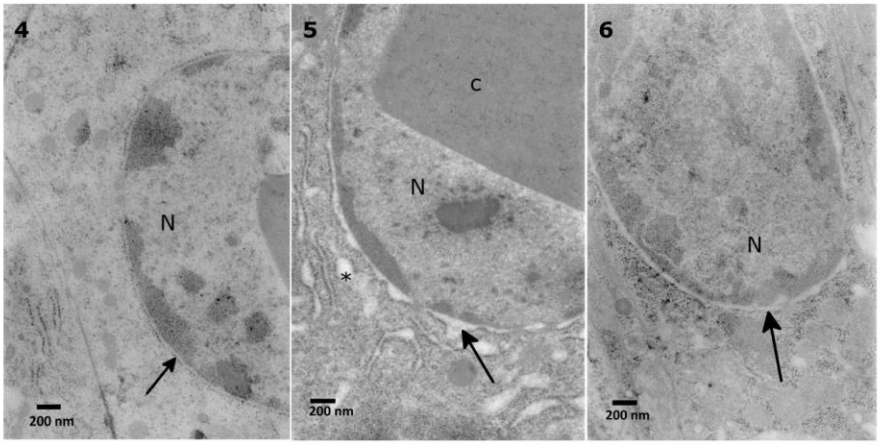

Figure 6. Nuclear (N) membranes in the *T. molitor* midgut cells. The membranes in the control cell (**4**) adhere to each other (**4**, arrow). The membranes are disturbed after application of 0.1% (**5**, arrow) and 1% *S. nigrum* extract (**6**, arrow). The disturbance is also observed in the endoplasmic reticulum (**5**, asterisks).

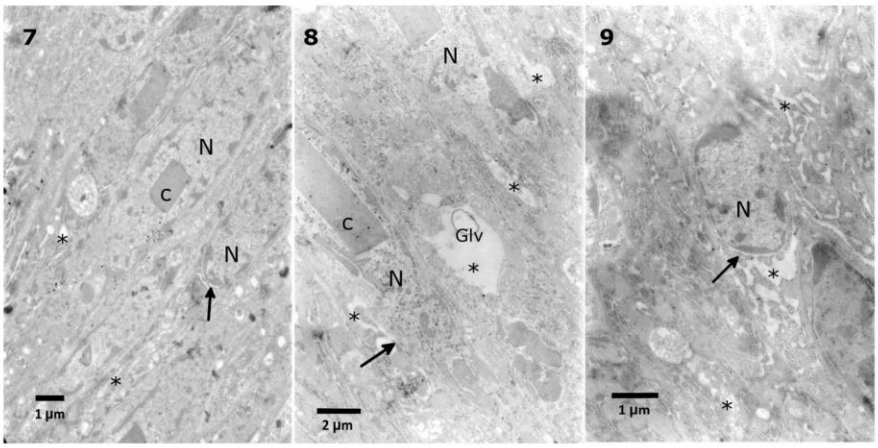

Figure 7. Malformations of nuclear membranes (**7**, arrow) and vacuolization in the cytoplasm (**7**, asterisks) after application of a 1% *S. nigrum* extract in the *T. molitor* midgut cells. The 10% extract caused vacuolization in the cytoplasm (**8**, **9** asterisks) with the appearance of glycogen vacuoles (**8**, Glv) and disturbance of nuclear membranes (**8**, **9**, arrows).

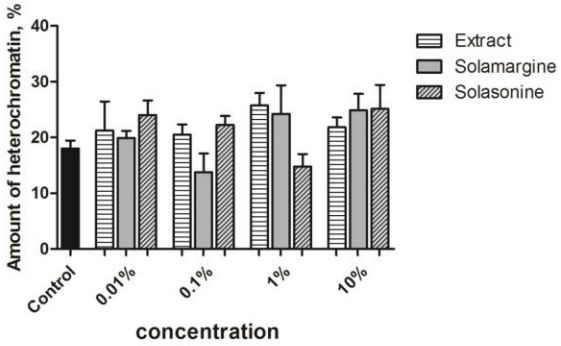

Figure 8. Heterochromatin ratio in the columnar midgut nuclei exposed to various concentrations of tested substances. Solasonine and solamargine were used in concentrations equivalent to their concentrations in the extract (Table 2). Kruskal-Wallis test with the Dunn's test.

2.3.2. Fat Body

The observed trophocytes (Figure 9, No. 1) possessed regularly shaped lipid droplets of various sizes, stored proteins, and cytoplasm filled with glycogen granules. The control cells had regular nuclei, with heterochromatin patches being located in the center and in the vicinity of the nuclear envelope (Figure 9, No. 2). The glycogen granules in the cytoplasm of the control cells appeared to be uniformly distributed. After the application of the extract at a concentration of 0.1%, some of the lipid droplets lost their homogeneity and regularity in shape (Figure 10, No. 4). Increasing the extract concentration to 1% caused a disruption of stored proteins and a decrease in the cytoplasm density (Figure 10, No. 5, 6). As in the case of the midgut cells, the most visible effects were observed after the application of the 10% extract, where the appearance of disrupted proteins and lipids were the most prominent changes. In many cases, nuclei with very dense nucleoplasm were observed (Figure 11, No. 7).

The application of solamargine at a concentration of 7.23×10^{-7} M caused an increase in the cytoplasm density, but with areas of vacuolization and a change in the homogeneity of the stored proteins (Figure 12, No. 10). An increase in the applied concentration to 7.23×10^{-5} M caused slight changes in the lipid droplets homogeneity and an increase in the cytoplasm density. Some observed nuclei showed the increase of the nucleoplasm density (Figure 12, No. 11). The strongest concentration of solamargine, 7.23×10^{-4} M, also caused changes in the lipid droplet homogeneity (Figure 12, No. 12).

Solasonine, at a concentration of 7.52×10^{-7} M, caused changes in the lipid droplet homogeneity (Figure 13, No. 13). Similar observations were noted after application of the 7.52×10^{-4} M concentration (Figure 13, No. 15). This concentration also caused the disintegration of the stored proteins. The decrease in the cytoplasm density with areas of vacuolization was observed after the application of solasonine at a concentration of 7.52×10^{-5} M (Figure 13, No. 14).

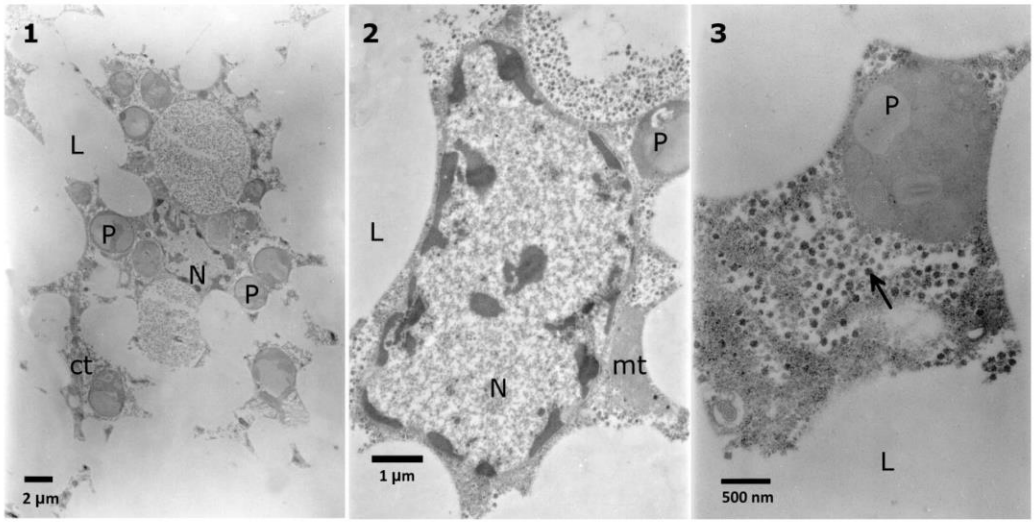

Figure 9. Control cells of the *T. molitor* larval fat body. The cells contain a nucleus (N) (**1**,**2**), lipid droplets (L), stored proteins (P), mitochondria (mt) (2), and cytoplasm (ct) with glycogen granules (**3**, arrow).

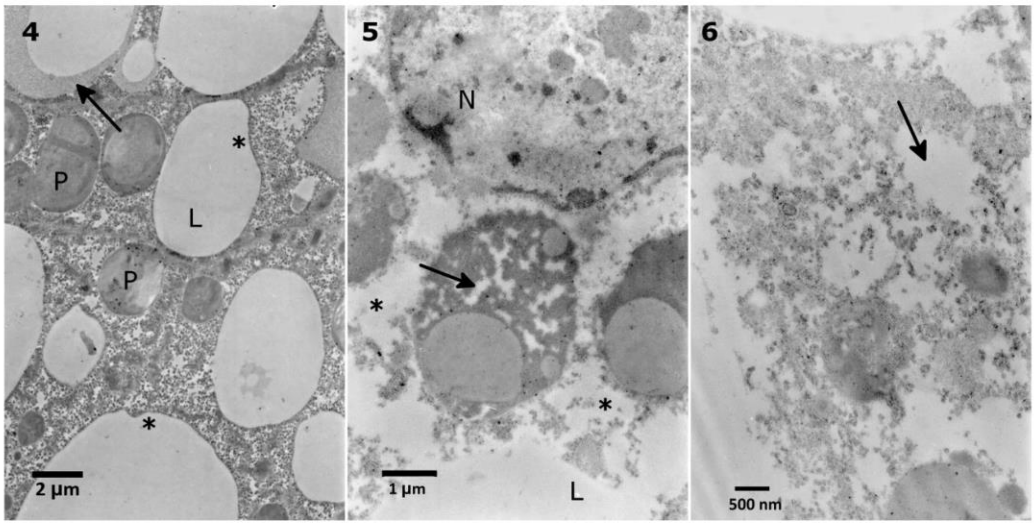

Figure 10. Fat body cells of the *T. molitor* larvae treated with 0.1% *S. nigrum* extract showed changes in the lipid droplet homogeneity (**4**, arrow) with changes in shape regularity (**4**, asterisks). The 1% extract caused disintegration of the stored proteins (**5**, arrow), and vacuolization of the cytoplasm (**5**, **6**, arrows).

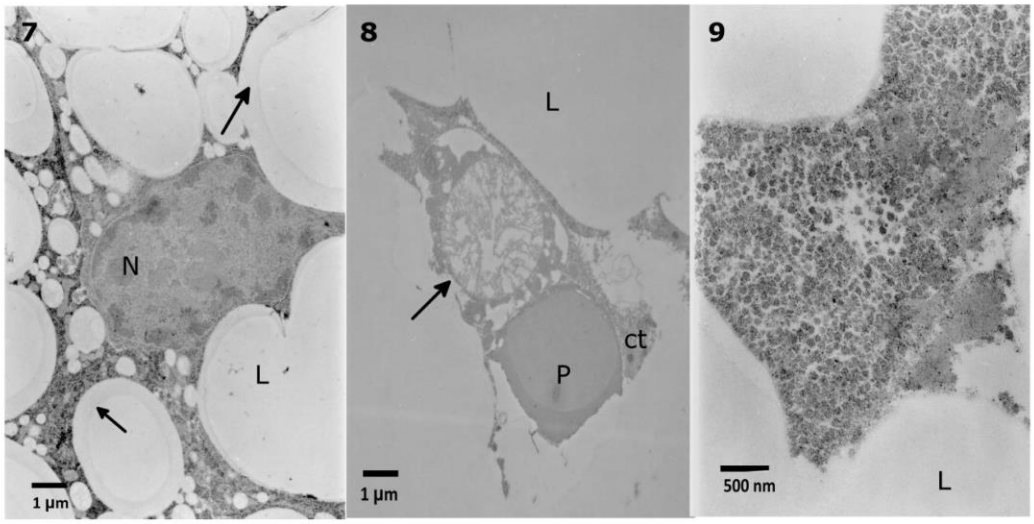

Figure 11. The nuclei (N) of the fat body cells after application of the 10% *S. nigrum* extract to the *T. molitor* larvae showed increased density. In the lipid droplets (L), the homogeneity decreased (**7**, arrow), with the appearance of other nonhomogeneous structures (**8**, arrow). The cytoplasm density increased (**9**).

In the case of the extract and solamargine, we noted a statistically non-significant, but evident increase in the heterochromatin ratio within the nuclei that is positively correlated with the increasing concentration of the tested substance (Figure 14). In the case of solasonine, the tendency was clear for the three lower concentrations but the low ratio that was calculated for the highest one weakened the overall trend. Additionally, the values of the correlation coefficients indicated a strong positive correlation between the extract and solamargine concentrations, and the heterochromatin ratio (0.75 and 0.78, respectively). The correlation coefficient for solasonine showed a very strong correlation ranging from 0.01% to 1%, but the highest concentration drastically decreased the coefficient to a negative value (−0.77).

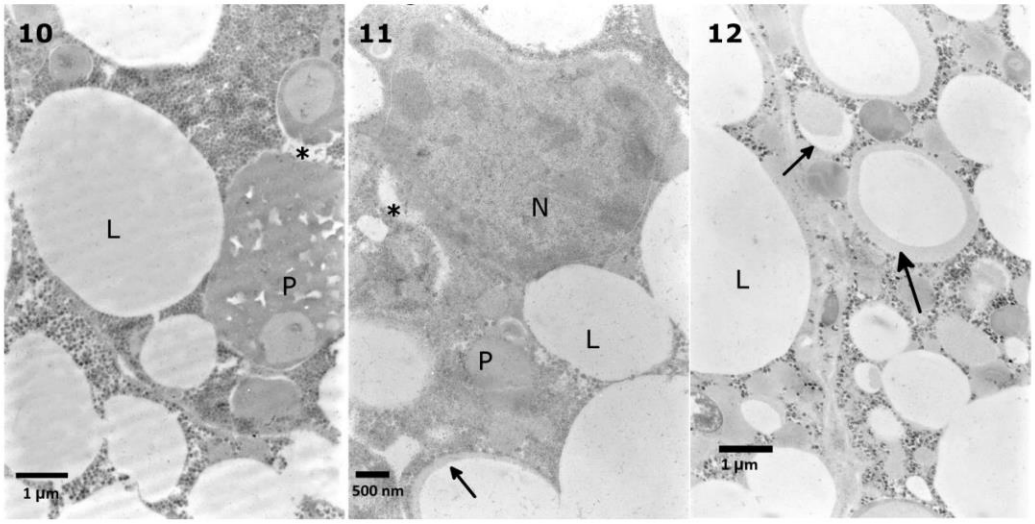

Figure 12. The fat body cells of the *T. molitor* larvae showed changes in the stored protein homogeneity (**10**, P) after application of solamargine at a concentration of 7.23×10^{-7} M and in the lipid droplet homogeneity (**11**, arrow) after the application of the 7.23×10^{-5} M concentration. Similar changes in the lipid droplets homogeneity were observed after the application of a concentration of 7.23×10^{-4} M. Slight changes in the cytoplasm density were observed after the application of solamargine at concentrations of 7.23×10^{-7} M and 7.23×10^{-5} M (**11**, **12**, asterisks).

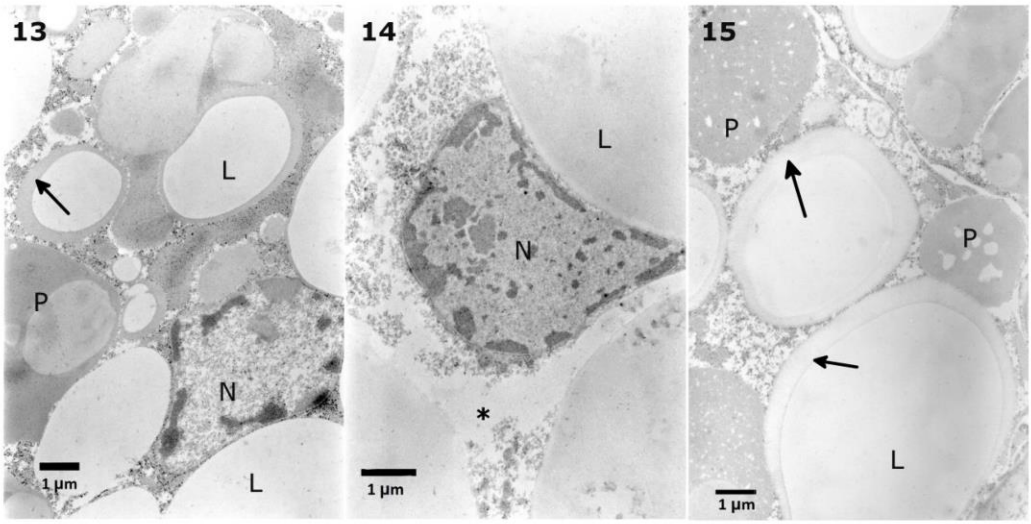

Figure 13. Solasonine given in the diet of the *T. molitor* larvae caused changes in the lipid droplet homogeneity after the application of concentrations of 7.52×10^{-7} M (**13**, arrow) and 7.52×10^{-4} M (**15**, arrows). The concentration 7.52×10^{-5} M caused a decrease in the cytoplasmic density (**14**, asterisk). After the application of a concentration of 7.52×10^{-4} M, initial disintegration of the stored proteins was observed (**15**, P).

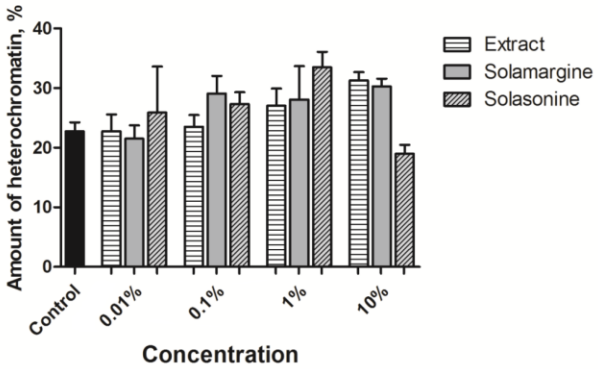

Figure 14. Heterochromatin ratio in the nuclei of the fat body cells exposed to various concentrations of tested substances. Solasonine and solamargine were used in concentrations equivalent to their concentrations in the extract (Table 2). Kruskal-Wallis test with Dunn's test.

2.4. Biochemical Assays of the Fat Body Cells

2.4.1. Glycogen

Both the extract and solamargine caused changes in the glycogen level of the fat body as compared to the control (Figure 15). No significant changes were observed after solasonine application to the diet of the larvae. The average amount of glycogen in the fat body that was isolated from the control insects was 57.1 ± 6.73 µg/mg of dry mass of the tissue. The extract caused a significant decrease in the glycogen content in the fat body to 22.3 ± 6.27 µg/mg after the application of the 0.1% concentration. Solamargine significantly increased the amount of the glycogen in the fat body at concentrations ranging from 7.23×10^{-7} M to 117.7 ± 16.47 µg/mg, and 7.23×10^{-5} M to 119.2 ± 19.41 µg/mg as compared to the control. The application of solasonine increased the glycogen content in the fat body. The strongest effect was observed at the lowest tested concentration, which caused an almost two-fold increase in the glycogen content nevertheless, the change was statistically non-significant. Between the effects that are caused by the extract and the testes pure GAs, in general, the glycogen level was higher after the application of either of the GAs than that of the extract application. A significant difference was achieved at a concentration of 0.1%, where the extract decreased and solamargine and solasonine increased the level of glycogen in the fat body (Figure 15).

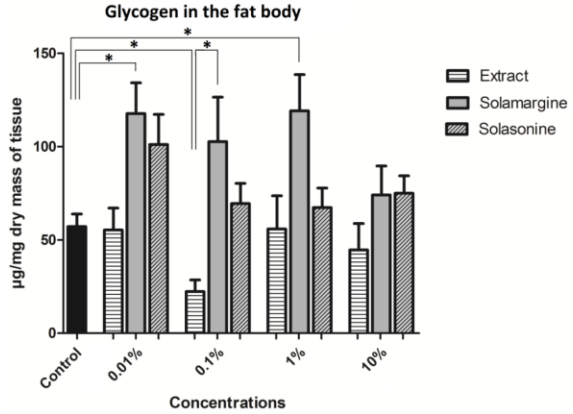

Figure 15. The level of glycogen in the fat body of the *T. molitor* after the application of extract from *S. nigrum* and solasonine and solamargine in their molar concentrations equal to their concentrations in the applied extract concentrations. * Statistical significance at $p \leq 0.05$; Kruskal-Wallis test with Dunn's test, $n \geq 12$.

2.4.2. Lipids

Lipids are the main ingredient of the fat body, representing more than 50% of the dry weight of the tissue [32]. The measured mass of the lipids in this study included not only the components of the lipid storing vacuoles, but also, for example, membrane lipids. However, the inclusion of the other lipids in the obtained results was negligible. In the control, the average lipid content in the dry mass of the fat body was 0.69 ± 0.02 mg/mg of dry tissue (Figure 16). At a concentration of 1%, the extract lowered the lipid content in the fat body after application to 0.57 ± 0.02 mg/mg. Solasonine also significantly decreased the lipid content in the fat body to 0.6 ± 0.02 mg/mg and 0.56 ± 0.02 mg/mg in comparison to that of the control at concentrations ranging from 7.52×10^{-6} M to 7.52×10^{-4} M, respectively. Differences between the extract and solamargine were observed for concentrations 0.1% ($p \leq 0.05$) and 1% ($p \leq 0.01$), and the lipid amount in the fat body was lower after extract application than after solamargine application. At all of the tested concentrations, significant differences were observed between solasonine and solamargine, where a higher content of lipids was present after solamargine application and lower after solasonine application.

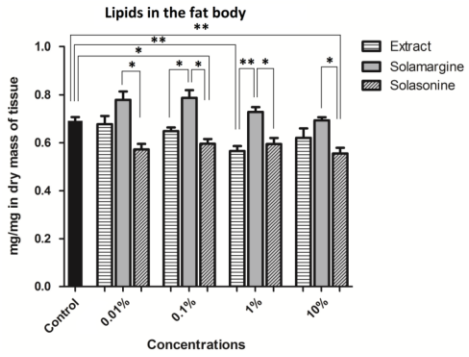

Figure 16. The content of lipids in the fat body of the *T. molitor* larvae after application of the extract from *S. nigrum* and solasonine and solamargine in their molar concentrations equal to their concentrations in the applied extract concentrations. ** Statistical significance at $p \leq 0.01$, * $p \leq 0.05$, one-way ANOVA, Tukey's test, $n \geq 9$.

2.4.3. Proteins

The content of the soluble proteins in the fat body did not show significant changes after application of the extract or GAs. In the control, the average protein content was 0.06 ± 0.008 mg per mg of dry mass of the tissue (Figure 17).

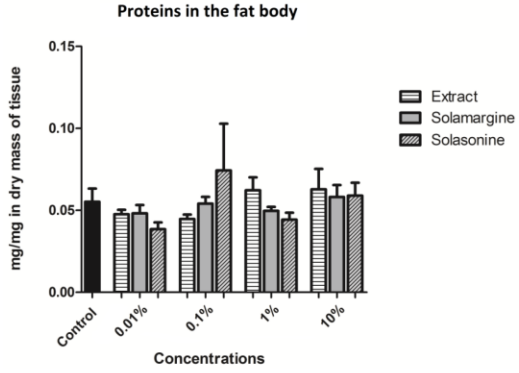

Figure 17. The content of the soluble proteins in the fat body of the *T. molitor* larvae after application of the extract from *S. nigrum* and solasonine and solamargine in their molar concentrations equal to their concentrations in the applied extract concentrations. Kruskal-Wallis test with Dunn's test, $n \geq 13$.

3. Discussion

In the present study, an expanded description of the effects that are caused by the extract of *S. nigrum* and pure GAs on *T. molitor* physiology was conducted. The tested substances do not appear to have acute toxic effects on the *T. molitor* larvae. In addition, the results differed between the extracts and pure GAs. The results showed that the exposure of the larvae to the tested substances caused slight changes in the body mass, ultrastructure of the midgut and fat body, and biochemical parameters of the fat body. However, it must be noted that these results were obtained over the relatively short observation periods that were used for the experiments. Hence, the observed effects often showed a trend, but the changes were not always statistically significant.

At the subcellular level, one can observe the very early effects, which appeared only in some cells. Therefore, the observed malfunctions and malformations can be used as bioindicators of stress caused by toxic substances. Perhaps, extended exposure or increased dosage would have given more significant effects. However, the aim of the study was to examine the direct, immediate effects and the mechanisms of the toxicity of *S. nigrum* extract and pure GAs. Furthermore, sublethal effects are often very important, they can limit crop or stored food loss [2] or affect the cost-to-benefit ratio similar to that obtained with synthetic insecticides [33].

The weight of the larvae did not change significantly but some tendencies were observed. It appears that the plant-derived substances may cause bimodal effects. For example, exposure to the low concentrations of solasonine slightly decreased the larval gain in body mass, while higher concentrations did not cause such an effect. On the other hand, only the highest concentration of the extract decreased the body mass, and the lower concentrations increased the body mass. Alkaloids in high concentrations may deter herbivores from feeding, as usually they are present in unripe fruit. Glycoalkaloids, such as solamargine and solasonine, have been tested by Weissenberg et al. [8] on *Tribolium castaneum*, belonging to the same family (Tenebrionidae) as *T. molitor*. The results showed that these compounds acted as growth inhibitors in larvae, but the experiment lasted 15 days. This finding also proves that GAs may be useful in plant protection in sublethal doses, thus limiting pest feeding. The results indicate that the plant must produce other substances that are responsible for the limitation of feeding and the growth of herbivores.

The effects on muscles differed between the tested substances. The application of the extract altered the contraction of heart, oviduct, and hindgut musculature, whereas the effects of solasonine and solamargine were much weaker, with solasonine decreasing the oviduct activity. These data suggest that neither solasonine or solamargine was responsible for the observed effects after application of the extract and indicate that plant extracts must contain other active substances in addition to the main GAs, perhaps alkaloids present in a lower concentrations, which affect muscle physiology. Both the glycoalkaloids may influence each other or interact with other ingredients of the extract. A stronger reaction of the insects to extracts than to single compounds has been described previously [12,34]. Moreover, the opposite effect was observed after the application of the extract to the heart, where the contraction frequency decreased, as compared to that on the oviduct, where the frequency increased. This finding suggests that the extract has a different mode of action on the two muscle tissues, and its components may interact with different receptors. The obtained results are similar to those that were obtained by Ventrella et al. [34], where black nightshade extract was used on the heart of *Zophobas atratus* (Tenebrionidae), and at a concentration of 0.5 mM, it caused a reversible negative chronotropic effect.

The microscopic observations revealed the differences between the effects caused in both of the tissues. Perhaps the differences were caused by a longer time of exposure of the fat body than of the midgut. Columnar cells transport ingested substances to other tissues, and trophocytes store substances and play crucial roles in detoxification. Therefore, these cells can reveal more drastic effects. A similar phenomenon was observed in the case of exposure to other toxic substances, such as boric acid [35] and tomato or potato leaf extract [36]. Although the intensity of the ultrastructural malformations differed between the tested organs, species, and substances, some of those effects were

similar: swollen nuclear envelopes and endoplasmic reticulum, cytoplasmic vacuolization, or swollen mitochondria have been reported for many toxic substances, including synthetic pesticides [16,37] or plant-derived substances [15,29,38,39]. These ultrastructural changes may be due to the increased production of reactive oxygen species, which has been reported for various toxic substances [35,40–42]. It is noteworthy that the biochemical parameters of the fat body depend on the biochemical parameters of the hemolymph, which transfers compounds to and from the fat body. Therefore, in the near future, we plan to further observe of the hemolymph biochemical parameters, such as sugar and lipid levels, and to correlate the obtained results with those of the fat body biochemistry and ultrastructure. To compare the effects that are caused by the *S. nigrum* extract and its pure GAs, both of the substances were given to the larvae and changes in the ultrastructure of the midgut and fat body cells were observed. While the extract caused a disruption of the nuclear membranes and cytoplasm vacuolization in the midgut, solasonine, and solamargine did not show any visible effects on these cells. This suggests that other glycoalkaloids present in the extract may be responsible for the observed effect or that solasonine and solamargine act synergistically in the extract or that the other compounds found in the extract play crucial roles in the process of membrane lysis by GAs.

Ultrastructural studies showed changes in the chromatin condensation that could lead to the altered expression of genes. The results suggest that solamargine is mostly responsible for chromatin condensation. For both tested concentrations of solamargine, the correlations with the effects on chromatin condensation were the strongest observed in this study. On the other hand, bimodal effects were observed in the case of solasonine in the fat body cells. Therefore, the correlation coefficient cannot be treated here for the whole range, as it measures linear relationships. The condensation of chromatin was also reported for cancer cells that were exposed to solamargine [43]. Solasonine and solamargine are both glycosides of solasodine (aglycone, the true alkaloid). They are characterized by sugar moieties and aglycone moieties. The electrophilic behavior of these compounds is regulated by the polarity of the sugar moieties and by the presence of oxygen and nitrogen ion pairs in the aglycone moiety, which does not contain aromatic rings. These features are responsible for GA-induced membrane disruption and interactions with nucleic acids, which result in DNA malformations [44,45]. Again, the weak effects that were observed in this study probably reflect the relatively low doses of applied substances that affected the nuclei in both the tested tissues. However, the teratogenic activity of GAs has been proven [46–48] and it may be due to the disruptive effect of GAs on membranes and nucleic acids.

The results of the biochemical studies showed that the level of glycogen decreased significantly after the application of the extract at only one concentration 0.1% (Figure 15). Glycogen is the first source of glucose during periods of starvation or detoxification; hence, one can suppose that the extract might have had a significant effect on the larvae. However, the weight gain of the larvae treated with this extract concentration was the highest among the used concentrations, which did not confirm the theory of starvation. Rather, glycogen, as an energy source, might have been used for detoxification and the vitality of the insects was not decreased. A slight decrease in the glycogen content of the fat body was also observed after application of the extract at a concentration of 10%, and the percentage of the larval weight gained seemed to decline. Perhaps a longer exposure would demonstrate whether this effect could be intensified. Such an effect suggests that *S. nigrum* produces substances that can be used as deterrents. Satake et al. [49] observed a decrease in the fat body glycogen content after starvation of the larvae of *Bombyx mori*, and that can be an early bioindicator of the inhibition of food intake. Another place, where carbohydrates are stored by insects and are transported to or from the fat body, is the hemolymph, which was not studied in our research. Previous studies claimed that the hemolymph glucose level can be an indicator of fat body carbohydrate metabolism [50]. The correlation between these factors results from the inhibition of glycolysis in the fat body by the key enzyme regulating this process present in the hemolymph—fructose-2,6-biphosphate. Solamargine caused an increase in the glycogen content, as compared to that of the control. The difference was significant after application of the concentrations of 7.23×10^{-7} M and 7.23×10^{-5} M. The increase

in the glycogen amount may be connected with the increase in the food intake by the larvae, which corresponds with the percentage body mass gain after solamargine application. The results showed the highest body mass gain in the case of solamargine. However, in general, the content of glycogen in the fat body was higher after the application of both GAs than after the extract application. A significant difference was obtained between the extract 0.1% concentration and the equivalent concentration of solamargine, which suggests different modes of action in the extract.

S. nigrum extract significantly decreased the lipid content in the fat body (Figure 16) after application of the 1% concentration. This result does not correspond with the gain in body mass, which increased when compared to that of the control. One supposes that the reason for this discrepancy may be, again, not the observed level of lipids in the hemolymph. Lipids from the fat body could have been moved to the hemolymph, which may have caused the decrease in the lipid levels of the fat body, without changing the body mass of the insects. Lipid mobilization is activated during starvation stress [50], which supports the theory that food intake is inhibited by alkaloids. Significant differences were present not only between the level of lipids in the control and extract treatment groups, but also between the extract and solamargine treatment groups. These data show, similar to the case of glycogen, that in the extract, other substances can be present that modulate its mode of action and play a crucial role in the toxic action of the major GAs. Significant differences in the lipid content were obtained between both the GAs at all the tested concentrations. These results show different influences of both the GAs on the lipid metabolism in the fat body.

No significant changes in the protein content were observed after the application of the tested substances. Only slight changes in the amount of protein were present when compared to that of the control. However, the quantity of protein was measured, but their profiles were not assessed. We do not know if some proteins are specifically produced, which would not necessarily influence the general protein content of the fat body, but might greatly alter its metabolism. This is a very interesting question that we will address in future studies. Furthermore, the observed changes in the chromatin density of the fat body, as well as its other ultrastructural alterations, suggest that the metabolism of the fat body may change dramatically during exposure to the GAs and their detoxification.

The difference between the effects that were observed with the transmission electron microscope and the biochemical analyses is also of interest. The altered homogeneity of the lipid droplets, cytoplasm density, and therefore glycogen content, as well as the stored protein disintegration observed by microscopy, did not strongly correspond with the results of the biochemical analyses. The main reason for this difference is that the biochemical analyses were conducted on dry tissue. In addition, it is very likely that the ultrastructural changes occurred before they could be analyzed biochemically and before significant lethality appeared. These results suggest that the use of transmission electron microscopy as a tool for early changes in detection, especially when employing short tests periods is highly justifiable. Furthermore, this implies that ultrastructural malformations may be used as environmental and functional bioindicators of exposure to the low concentrations of stressors, or they may represent the early stage of exposure.

4. Conclusions

The results indicate that both the extract and pure GAs have a wide range of sublethal effects. Although the effects do not cause mortality in the larvae, they may disturb the insects' development and metabolism at various levels. The observed modulation of muscle contractility of such organs as the heart, hindgut, or oviduct may result in impaired development, food intake, and reproduction. Hence, the above mentioned parameters may be crucial for better understanding the mode of toxicity of the tested alkaloids. Furthermore, these studies may also contribute to the more efficient application of plant-derived substances for plant protection. Consequently, this may lead to a decreased usage of both synthetic and natural substances in plant protection, which may limit the pollution of the environment, crops, and food products. It is noteworthy that some effects were observed very early, after exposure. Therefore, they may be useful as bioindicators of stress. Moreover, they may be used

to limit the pest population by decreasing the vitality of insects. Consequently, they may decrease herbivory. Interestingly, the effects of the extract and pure alkaloids differ from each other. These data suggest that substances produced by the plant may act additively or synergistically. Hence, the effect of the extract may be more intense than that of pure glycoalkaloids. Furthermore, this suggests that plant extracts not only are an interesting source of new insecticides, but also may be used as relatively inexpensive tools in plant protection, especially when integrated pest management strategies are applied.

5. Materials and Methods

5.1. Insects

Tenebrio molitor larvae and adult beetles were obtained from the breeding culture at the Department of Animal Physiology and Development (AMU) under laboratory conditions; the experimental specimen were maintained at 26 °C and a 60% relative humidity in a 12 h light to 12 h dark photoperiod. For the experiments, four-week-old adults and larvae after molting with weights of 120–140 mg were used. Determining insect weight allowed us to choose the larvae with the same metabolic rate and to control for weight gain.

5.2. Extraction and Analyses

Extracts were obtained from *S. nigrum* unripe berries. The voucher specimens were deposited at the Herbarium Lucanum (HLUC, Potenza, Italy), with the ID Code: 2320. The extraction method was previously described by Cataldi et al. [51] and Adamski et al. [41]. The berry samples were lyophilized and ground to a fine powder using a laboratory mill. The samples (1.5 g) were placed in 20 mL of 1% acetic acid aqueous solution. The suspension was stirred for 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. Two supernatants were subsequently mixed together. The extract was filtered through a single-use 0.22 µm nylon filter (Whatman, Maidstone, UK) and then injected into the LC/MS system. The chemical analysis was conducted at the Department of Sciences, University of Basilicata by Prof. Sabino Bufo's team.

The extracts at concentrations of 0.01, 0.1, 1, and 10% were diluted in physiological saline A (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$, 5 mM glucose, and 5 mM HEPES, pH 7.0) for in vitro experiments or in saline B (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$) for in vivo experiments. Pure solasonine and solamargine were purchased from Glycomix (Glycomix Ltd, Compton, Berkshire, UK). The standard glycoalkaloids were diluted in the physiological saline A or B for in vitro and in vivo experiments, respectively. The concentrations of both glycoalkaloids were calculated as an equivalent of their quantity in the tested extract (Table 2).

Table 2. Calculation of the glycoalkaloid concentrations in the extract.

S. nigrum Extract Concentration (%)	Solamargine (M)	Solasonine (M)
0.01%	7.23×10^{-7}	7.52×10^{-7}
0.1%	7.23×10^{-6}	7.52×10^{-6}
1%	7.23×10^{-5}	7.52×10^{-5}
10%	7.23×10^{-4}	7.52×10^{-4}

5.3. Exposure of the Larvae to the Tested Substances

5.3.1. In Vitro Heart Bioassay

The in vitro effects of the tested extract and GAs on *T. molitor* heart were measured with a microdensitometric technique [52]. Anesthetized four-week-old adult insects were decapitated and their legs and wings were cut off. The ventral cuticle was removed with narrow stripes left the

on sides. The visceral organs were carefully removed to expose the myocardium. In the next step, the semi-isolated hearts were placed in a superfusion chamber with the open-perfusion system being mounted in the microdensitometer MD-100 (Carl Zeiss, Jena, Germany) and were perfused with saline A. The flow rate of saline A was 300 µL/min, wherein the solution was continuously removed from the superfusion chamber by chromatographic paper (Whatman No. 3, Sigma-Aldrich, St. Louis, MO, USA). Ten microliters of the tested substances were applied with a Hamilton syringe through the application port placed 70 mm above the superfusion chamber. After recording the control heart activity for 0.5 min, the tested compounds were applied, and an additional 2 min of heart activity was recorded. The recording of new-isolated heart activity was preceded with 10 min of preincubation with saline A to stabilize the heart rate. The calculation of changes in the heart rate was conducted according to a previously described method [53], as the percentage change between the heart activity recorded before and after application. The obtained data were analyzed with LARWA and ANALIZA software (Both programs have been written in 2004 for our Department, Poznań, Poland). For each concentration, 5–12 larvae were used.

5.3.2. In Vitro Oviduct and Hindgut Bioassay

To analyze the changes in the contractile activity of the oviduct and hindgut treated with the tested compounds, a video microscopy technique was used [54,55]. Four-week-old insects of *T. molitor* beetle were anesthetized for 8 min. Next, the insects were decapitated and the legs and wings were removed. The isolation of the oviduct with ovaries was conducted after removing the dorsal cuticle. Next, the preparation was carefully cleaned of undesirable tissues, such as the fat body and Malpighian tubules with microsurgical forceps. The oviducts with ovaries were placed on the Sylgard elastomer and attached with Minuten pins. A similar procedure was used for the isolation of the hindgut. The organs were placed in the incubation chamber with a constant flow rate of 300 µL/min of saline A. The chamber was placed on an Olympus SZX12 stereomicroscope that was equipped with a Pixeling 662 camera. Similar to the heart bioassay, the recording was preceded by 10 min of preincubation. Each recording lasted 2 min; after 30 s of control recording, 10 µL of the tested substances was applied through the port with the Hamilton syringe. The obtained data were analyzed with the AnTracker (PreOptic, Warsaw, Poland) software. The changes in the oviduct and hindgut contraction frequency were calculated as the percentage change between the frequencies recorded before and after application of the tested compounds. For each used concentration, 6–13 larvae were used.

5.3.3. Determination of Changes in Larval Weight

The larvae were kept separately in the flasks. The day after collection, insects were fed for three days with a recipe prepared according to David et al. [56] containing 10 µL of the tested substances. The control larvae were fed with the same mixture containing 10 µL of saline B. On the fourth day of the experiment, the larvae were weighed and the samples were collected according to the further description.

The larvae fed for three days with the extract, solasonine, or solamargine, were weighed before and after the experiment. The number of larvae used varied from 32 to 139 individuals per concentration used. The difference between the weights before and after the experiment of larvae that werefed with the tested substances was compared to that of the control larvae.

The change in body mass was calculated according to the following Equation:

$$\Delta = \left(\frac{b \times 100}{a}\right) - 100 \quad (1)$$

where '*a*' is the mass of larva before and '*b*' is the mass after the experiment.

5.3.4. Transmission Electron Microscopy

The larvae that were fed with the extract or pure alkaloids were chosen randomly (three per concentration) and anesthetized with carbon dioxide. Then, they were dissected and the samples of the fat body and midgut were isolated, washed in physiological saline B, and cleaned of other structures, such as the Malpighian tubules and tracheoles. The preparation and fixation of the samples were carried out according to the methods that were described by Adamski et al. [14], as follows: the samples were placed in 2% glutaraldehyde in 0.175 M cacodylate buffer for 2 h, postfixed with 1% osmium tetroxide for 2 h, and finally dehydrated and embedded in Spurr resin (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections were cut with a Leica ultramicrotome and stained with uranyl acetate and lead citrate. Samples were observed under JEOL 1200EX II JEM (JEOL, Tokyo, Japan) transmission electron microscope. We focused on midgut columnar epithelial cells and fat body trophocytes, as the most frequent cells, which are also crucial for the ingestion, storage, detoxification, and regulation of physiological processes.

The heterochromatin ratio (i.e., electron dense surface to whole nucleus surface area) were calculated using the classic stereological method with the computer programme STEPanizer [57]. Digital grids (1024 squares per picture) were plotted on the images and the number of squares over the electron dense and electron lucent chromatin were counted. Then the ratio was calculated. The mean from a minimum of 8 nuclei per concentration for each test variant was calculated and compared. Next, the correlation between the concentration of the tested substances and the heterochromatin ratio was calculated. Values between -0.3 and 0.3 were regarded as having no linear relationship, values between $0.3 < x \leq 0.5$ and $-0.3 > x \geq -0.5$ indicated a weak (positive/negative) relationship, values between $0.5 < x \leq 0.7$ and $-0.5 > x \geq -0.7$ were regarded as having a moderate (positive/negative) relationship, values between $0.7 < x \leq 0.9$ and $-0.7 > x \geq -0.9$ indicated a strong (positive/negative) relationship, and values between $0.9 < x \leq 1$ and $-0.9 > x \geq -1$ indicated a full (positive/negative) relationship.

5.3.5. Biochemical Analysis of the Fat Body

The samples of the fat body (1–3 mg) after isolation were placed in Eppendorf tubes, then dried under vacuum conditions (-0.9 atm) at 60 °C, and weighed. Next, the glycogen, lipid, and protein content in the samples were determined. The amount of analyzed substances was expressed as milligrams of substances per milligram of dry mass of the tissue. The number of individuals used for each concentration for each test was at least nine.

- **Determination of theglycogen content**

Isolation and determination of glycogen, as described previously by Chowański et al. [58], was carried out according to the procedure of van Handel [59] and Dubois et al. [60], respectively, as follows. Next, 500 µL of 30% KOH was added to the samples and incubated for 15 min at 90 °C to lyse the tissues. After lysis, 50 µL of saturated solution of Na_2SO_4 and 800 µL of 96% ethanol were added to precipitate the glycogen. Next, the samples were centrifuged at 10,000 rpm for 10 min and the obtained pellet was washed three times with 70% ethanol. After evaporation of residual ethanol at 74 °C, 500 µL of purified water was added. The pellet was shaken for 5 min at 80 °C and then centrifuged for 5 min at 10,000 rpm. The obtained solution was used to determine the glycogen amount. As a standard oyster, glycogen (Sigma-Aldrich, St. Louis, MO, USA) was used.

- **Determination of the lipid content**

The isolation of the fat body lipids was conducted according to the Folch et al. [61] method described previously by Chowański et al. [62]. The tissues were homogenized in 1000 µL of chloroform-methanol mixture (2:1, v/v) and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to new Eppendorf tubes and washed three times with 220 µL of 0.29% NaCl. The remaining solution was evaporated at 30 °C under vacuum (-0.9 atm). The pellet was dissolved

in 1000 µL of chloroform-methanol mixture and 500 µL of the solution was transferred to the new Eppendorf tubes. After drying under vacuum (30 °C, −0.9 atm), the mass of the residual lipids was measured gravimetrically.

- **Determination of the soluble protein content**

After drying, the samples were homogenized in saline B on ice. Next, they were centrifuged at 10,000 rpm for 5 min. Two microliters of the intranatant was placed on the PTFT membrane, dried, and measured with a Direct Detect® Infrared Spectrometer (Merck Millipore, Burlington, MA, USA).

5.4. Statistical analysis

All the data are presented as the mean values ± SEM of n number of replicates. The statistical significance of differences between the control and treatment values was determined using appropriate statistical test: one-way ANOVA Tukey's test, Student's t-test, or, if there was not a normal distribution, the nonparametric Kruskal-Wallis test and Dunn's Multiple Comparison test. The statistical analyses were conducted using GraphPad Prism 5 software (GraphPad Software Inc, Version 5.01, MacKiev, La Jolla, CA, USA, 1992–2007). Differences were considered to be statistically significant if $p \leq 0.05$ (*), $p \leq 0.01$ (**), or $p \leq 0.001$ (***)

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Solanum nigrum Fruit Extract Increases Toxicity of Fenitrothion—A Synthetic Insecticide, in the Mealworm Beetle *Tenebrio molitor* Larvae

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Abstract: Synthetic insecticides are widely used for crop protection both in the fields and in the food stored facilities. Due to their toxicity, and assumptions of Integrated Pest Management, we conducted two independent experiments, where we studied the influence of *Solanum nigrum* unripe fruit extract on the toxicity of an organophosphorus insecticide fenitrothion. In the first variant of the experiment, *Tenebrio molitor* larvae were fed with blended fenitrothion (LC_{50}) and the extract in four concentrations (0.01, 0.1, 1 and 10%) in ratio 1:1 for 3 days. In the second variant, a two-day application of fenitrothion (LC_{40}) was preceded by a one-day extract treatment. The first variant did not show any increase in lethality compared to fenitrothion; however, ultrastructure observations exhibited swollen endoplasmic reticulum (ER) membranes in the midgut and nuclear and cellular membranes in the fat body, after application of blended fenitrothion and extract. An increased amount of heterochromatin in the fat body was observed, too. In the second variant, pre-treatment of the extract increased the lethality of larvae, decreased the level of glycogen and lipids in the fat body and disrupted integrity of midgut cellular membranes. *S. nigrum* extract, applied prior to fenitrothion treatment can be a factor increasing fenitrothion toxicity in *T. molitor* larvae. Thus, this strategy may lead to decreased emission of synthetic insecticides to the environment.

Keywords: *Solanum nigrum* extract; fenitrothion; *Tenebrio molitor*; beetles; transmission electron microscopy; glycogen; protein; lipid; fat body; midgut

Key Contribution: *Solanum nigrum* fruit extract containing glycoalkaloids increases toxicity of fenitrothion, a synthetic insecticide, in the mealworm *Tenebrio molitor* in two different strategies of application.

1. Introduction

The protection of food products imposes the rigorous usage of chemicals like insecticides, which usage worldwide increases annually. From 2011, the amount of emitted pesticides reached over 4 million tons and still increases [1]. In Poland, besides increasing usage of synthetic pesticides,

the usage of insecticides from the organophosphorus group increased in the past years to 1467.4 t, where at the same time, the amount of used botanical products and biologicals decreased to only 1.1 t (data for 2017, [1]). However, considering the harmful effects of synthetic pesticides, their usage should be limited, and substitute methods such as biopesticides should be implemented. Such methods of stored products' protection are the focus of intensive studies (for review see: [2]).

A yellow mealworm beetle (*Tenebrio molitor* L.) is considered a cosmopolitan pest, destroying grains and flour food products. Additionally, the insects pollute the food with their frass and moulted exoskeletons, making them unfit for human consumption. The insects can cause losses up to 15% of grains and flour production worldwide [3–5] and may act as vectors of other pests, e.g., fungi [6]. For these reasons, the yellow mealworm beetle is a model organism in many studies concerning its susceptibility to plant derivatives [7–11]. In previous reports, we checked the sensitivity of yellow mealworm beetle to *Solanum nigrum* fruit extract (EXT) and its main glycoalkaloids, given in the diet and in in vitro tests. We described the altered amount of biomolecules, ultrastructural malformations, increased chromatin condensation, and altered heart and oviduct contractility effects [12,13], which showed that the extract and also its main glycoalkaloids solasonine and solamargine might have affected the insect metabolism, development, and reproduction. Hence, even if natural substances may not have a lethal effect, they may significantly decrease the vitality of the insects. For the usage of *S. nigrum* extract and both glycoalkaloids speaks their impact on human health. The extract possesses medicinal properties [14] including anticancerous ones [15]. Solasonine and solamargine also have anticancer properties proved in many studies [16–18], having low toxicity [19,20]. Additionally, the persistence in the environment of glycoalkaloids is short [21]. Therefore, these data became a starting point to test the *S. nigrum* extract as a potential factor increasing the toxicity of synthetic insecticides. As a consequence, the amount of synthetic insecticides in use would be decreased.

For the studies, we chose fenitrothion (FN), a synthetic insecticide from the group of organophosphates, that despite its toxicity and latest withdrawal from sale by the law in European countries, is commonly used in food storage facilities in many countries worldwide. Moreover, FN is very well-studied for its chemical and physiological properties, as evidenced by numerous studies, including reports about its influence on insects [22–25], rodents [26–28], and humans [29–31]. Therefore, this pesticide is a good model for the tests of organophosphates' toxicity. Also, the use of organophosphates, as a group of synthetic insecticides, are among those pesticides, should be limited due to their low selectivity and high toxicity to non-target animals.

The midgut is the first barrier between the consumed food and the hemocoel. It is built of a single layer of longitudinal cells covered with microvilli. Very often, the insecticidal substances enter the organism though the digestive tract with the diet—hence our attention was focused on this tissue. On the other hand, the fat body is the main tissue responsible for fat storage, important for proper endocrine physiology, development, and detoxification. Therefore, we decided to focus on these two tissues due to their importance for entrance, metabolism and storage of toxic substances in the organism. The effects of the *S. nigrum* extract in used concentrations on the midgut and fat body cells were previously described [12].

The aim of our study was to determine the influence of the EXT on FN toxicity and to find the most suitable strategy of the extract application with FN to reach the most effective results, i.e., to obtain high toxicity for the tested pest species and the low usage dose. The hypothesis to verify was: The usage of extract may increase the susceptibility of insects to FN and, possibly, decrease the amount of used FN, which causes the same effect as when only FN is used.

2. Results

2.1. Lethality of Larvae after Fenitrothion Treatment

After the first 24 h of treatment, the lethality of larvae was estimated. The values for $LC_{50/24h}$ (lethal concentration) and $LC_{40/24h}$ where 400 µg/mL and 300 µg/mL, respectively (Figure 1).

These concentrations caused 100% mortality of larvae after 72 h of treatment and were chosen for further experiments. The lowest used concentrations (40 and 50 µg/mL) caused reversible paralysis of insects. After the application of the concentrations of 200 µg/mL and 100 µg/mL, the mortality after 72 h of treatment did not reach 100%. As a control, 70% ethanol was used, and did not show any lethal effects on larvae.

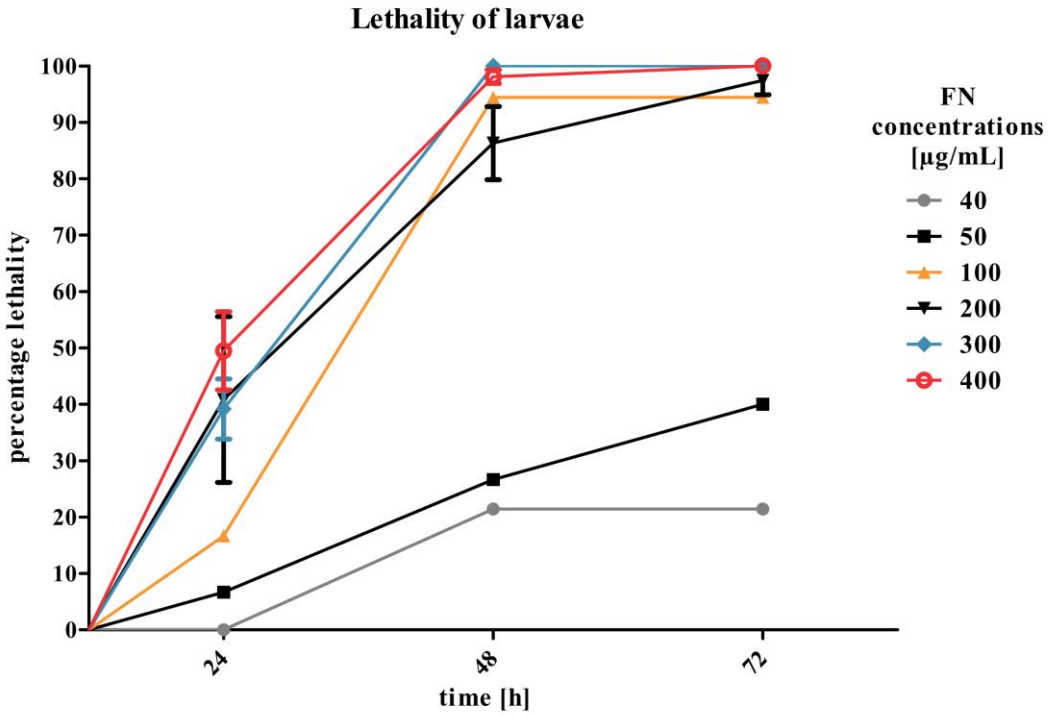

Figure 1. Lethality of *T. molitor* larvae (±standard error of measurement (SEM)) after application of fenitrothion (FN) in the diet in 6 increasing concentrations 40, 50, 100, 200, 300 and 400 µg/mL, $n \geq 15$ per treatment.

2.2. Lethality and Changes in the Body Mass in Larvae Treated with the Mixtures of Fenitrothion and Solanum nigrum Extract

The larvae were weighed before and after the experiment and the change of body mass (delta—Δ) was calculated. The values obtained for FN and the FN–EXT blends were compared to the control, and to starving larvae. Also, the FN–EXT blends and FN-only in the concentration of 400 µg/mL, were compared. The body mass of starving larvae dropped after 3 days of starvation, but the results were not statistically significant compared to the control larvae. The difference in the body mass of larvae treated with FN compared with FN–EXT blends was not statistically significant. Highly significant statistical differences were observed between groups treated with FN and FN–EXT in relation to control and starvation treatments (Table 1.). In the previous study, the delta (Δ) was calculated for larvae treated with EXT only. To compare, the values were (±SEM): 0.01%: +18.62 ± 1.28; 0.1%: +18.43 ± 1.42; 1%: +17.03 ± 1.4; 10%: +15.16 ± 1.5; $n \geq 53$ (Spochacz et al., 2018b).

Table 1. Changes in the body mass of *T. molitor* larvae after application of blended FN (400 μg/mL) with the *S. nigrum* extract (EXT).

Δ Delta, Mean Value ± SEM		**Control**	**Starvation**	**FN (400 μg/mL)**	**FN (400 μg/mL) + EXT 0.01%**	**FN (400 μg/mL) + EXT 0.1%**	**FN (400 μg/mL) + EXT 1%**	**FN (400 μg/mL) + EXT 10%**
		+18.97 ± 7.53	**−5.59 ± 1.04**	**−21.28 ± 6.11**	**−18.09 ± 5.46**	**−16.9 ± 12.35**	**−18.71 ± 8.33**	**−18.14 ± 4.23**
n		30	20	45	23	37	36	38
Difference in rank sum, significance Compared to control Compared to starvation	a)	-	31.7 ns	142.8 ***	115.2 ***	115.1 ***	119.5 ***	112.8 ***
	b)	31.7 ns	-	111.1 ***	83.45 ***	83.39 ***	87.8 ***	81.1 ***

Dunn's Multiple Comparison test, *** $p < 0.001$. The increased body mass marked as "+", decreased body mass marked as "-".

Comparing the lethality of larvae after application of FN–EXT blends to FN only, a slight, insignificant increase of lethality of larvae can be observed when the two compounds were applied. While the lethality reached 50 ± 6.9% (±SEM) after application of FN in the concentration of 400 µg/mL, the FN–EXT blends increased the mortality to 68.7 ± 6.9%, 66.6 ± 5.28% and 61.6 ± 10.2% when FN was in the mixtures with 0.1, 1 and 0.01% of the EXT, respectively. The lowest lethality of larvae after 24 h of treatment appeared after the application of the mixture of FN 400 µg/mL with the EXT at a concentration of 10% (43 ± 15.6%) (Figure 2). There was no lethality when the 70% ethanol and saline B were applied.

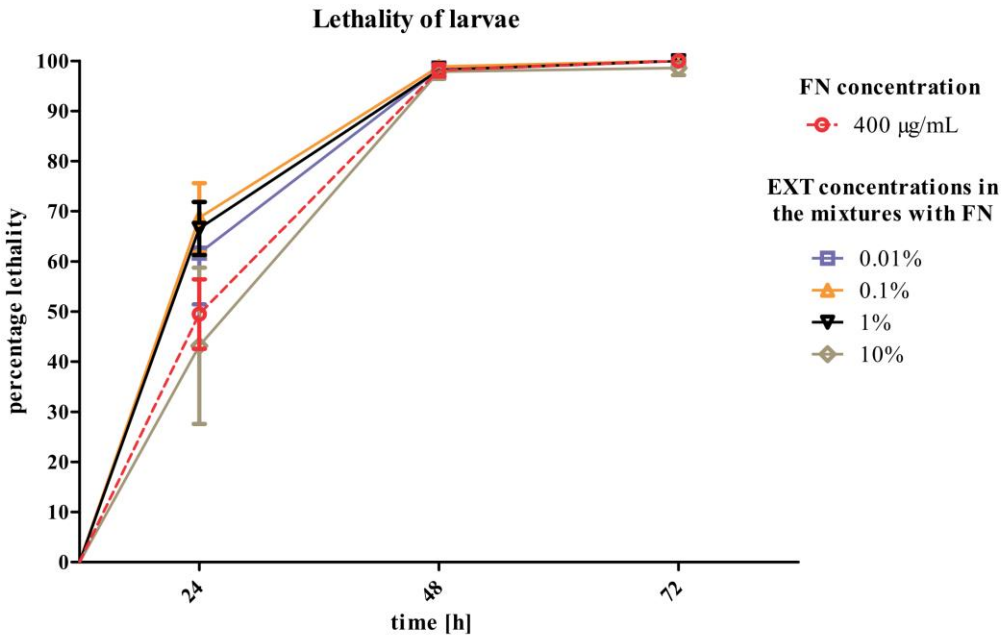

Figure 2. Lethality of *T. molitor* larvae after application of fenitrothion (FN) in the concentration of 400 µg/mL and its blends with *S. nigrum* extract (EXT) in four concentrations. Mantel–Cox test, $n \geq 46$ per treatment. Changes were not statistically significant compared to fenitrothion (400 µg/mL).

2.3. Biochemical Analysis of the Fat Body of Tenebrio molitor Larvae Treated with the Mixtures of Fenitrothion and Solanum nigrum Extract

Obtained results are presented as a percentage change in lipids, glycogen and soluble proteins content in the fat body in comparison to the control larvae (100%). The mean content of lipids in the fat body is equal to 0.74 ± 0.018 mg/mg (±SEM) of the dry mass. Despite the fact that the larvae treated with FN, as well as the insecticide and its mixtures with EXT, significantly lost weight (Table 1), the lipid content in the fat body has only slightly changed compared to the control (Figure 3A). The highest changes were observed after the application of the mixture of FN (400 µg/mL) and EXT at concentrations of 0.1 and 1%. In those variants, fat body contained about 8 ± 4.4% and 7.3 ± 3.16% more lipids than the control, respectively.

Glycogen is stored in the fat body as a source of energy. We checked its level in the fat body, and the mean content of glycogen in that tissue in control was 16.4 ± 5.95 µg/mg of dry tissue. The glycogen level decreased after the application of the FN mixtures with the lowest (0.01%) and the highest (10%) EXT concentrations (Figure 3B). The average content of glycogen after application of mentioned FN–EXT blend was lower than in the control, about 43 ± 34.3% and 38 ± 37.3%, respectively. Whereas a slight increase in glycogen content in the fat body was noted in insects treated with a mixture of FN (400 µg/mL) and EXT at a concentration of 0.1 and 1%.

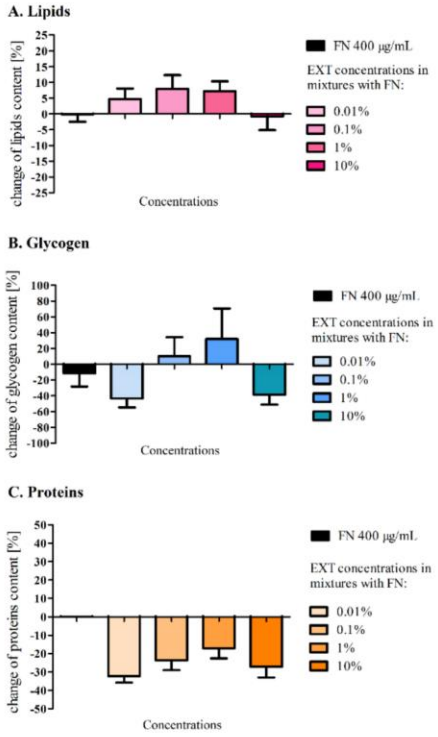

Figure 3. The content of lipids (**A**), glycogen (**B**) and soluble proteins (**C**) in the fat body of *T. molitor* larvae after the treatment with fenitrothion (FN) in the concentration of 400 µg/mL and its mixtures in ratio 1:1 with *S. nigrum* extract (EXT) as percentage change compared to the control larvae. Dunn's Multiple Comparison test, $n \geq 9$ per treatment. Changes were not statistically significant.

After the application of FN in the concentration of 400 µg/mL and its mixtures with the EXT, the decrease in the protein level in the fat body of larvae was observed (Figure 3C). Tissue from control insects contained mean 0.08 ± 0.008 mg of proteins in 1 mg of the dry mass of tissue. The more drastic drop was observed within tissues exposed to blends, than the FN alone. The average protein content was lower at about 32 ± 10.5% and 27 ± 5.9% than the control, when the FN mixtures with the lowest (0.01%) and the highest (10%) EXT were applied, respectively.

2.4. The Influence on the Midgut and the Fat Body Ultrastructure

2.4.1. Midgut

The final effects, observed under TEM, are presented in Figure 4 and summarized in Table A1. FN (400 µg/mL) caused an increase of electron-dense chromatin and cytoplasm, cells were rich in raw endoplasmic reticulum (RER) (Figure 4, No. 2) compared to control cells (Figure 4, No. 1). Similar effects were observed after the application of blended FN and EXT (Figure 4, No. 3–5). However, some additional changes such as swollen ER were present (Figure 4, No. 4 and 5) when FN–EXT (1 and 10%) were applied into the larvae diet.

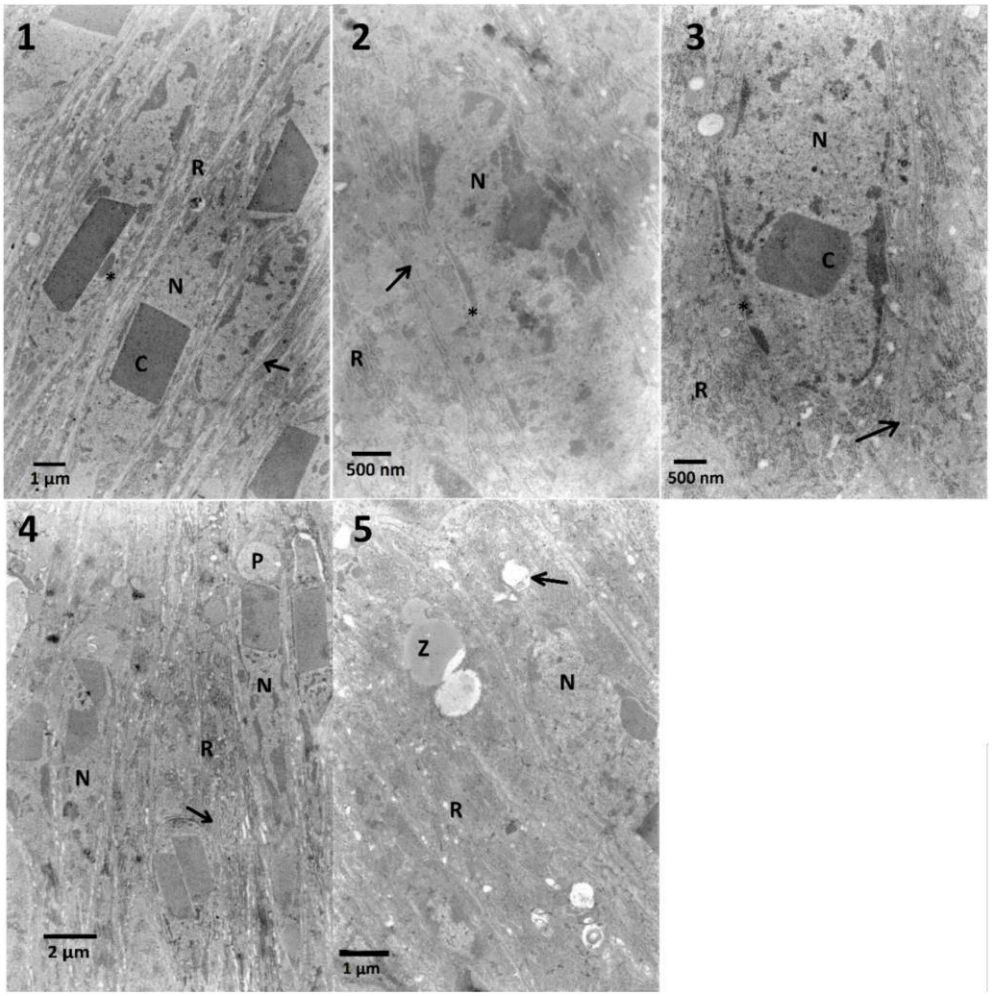

Figure 4. The ultrastructure of the midgut cells of *T. molitor* larvae after the treatment of the first variant, mixtures of FN (400 µg/mL) and EXT. **No. 1**—Control cells: nuclei (N) with protein crystal (C) are present. The presence of the protein crystals in the midgut nuclei is a specific feature of this species. Endoplasmic reticulum (R) surrounded by cytoplasm. Regular cell membrane and nuclear membrane are marked with an arrow and an asterisk, respectively. **No. 2**—Larvae fed with FN (400 µg/mL): an increase of cytoplasm density can be observed with an increase of ER (R), no irregularity in cellular and nuclear membranes can be observed (arrow and asterisk, respectively). **No. 3**—Larvae fed with blended FN and EXT in the concentration of 0.1%: dense cytoplasm with high amount of ER (R). An arrow points the cellular membrane. **No. 4**—1%: nuclei (N) with increased amount of dense chromatin, swollen ER (R, arrow) and undefined structures (P) present next to the nuclei, possibly vacuoles indicating beginning of cell degeneration, necrosis. **No. 5**—10%: beside swollen ER (R, arrow) in the cytoplasm other electron-dense and electron-lucent structures can be observed (Z).

2.4.2. Determination of Chromatin Density in the Midgut Cells

The density of chromatin was calculated and presented in Figure A1. In all tested concentrations, the mean value of percentage amount increased compared to the control treated with a mixture of ethanol and saline B (mean ± SEM: 27.8 ± 1.85%). The highest increase was observed after the application of FN (400 µg/mL), which was equal to 37.3 ± 3.0%. Although clear, the changes were not statistically significant. The correlation coefficient for the first variant of treatment in the midgut cells showed moderate negative correlation: −0.65.

2.4.3. Fat Body

Trophocytes present in the fat body are responsible for storing lipids in droplets, glycogen in granules deposited in the cytoplasm, and proteins in varied sizes of oval structures. Between lipid droplets filling the majority of the cell volume, a nucleus is present with the electron-dense nucleoplasm, placed in the center and close to the envelope (Figure 5, No. 1). Ultrastructural changes observed in the fat body cells treated with FN and its blends with EXT were put in order in Table A2.

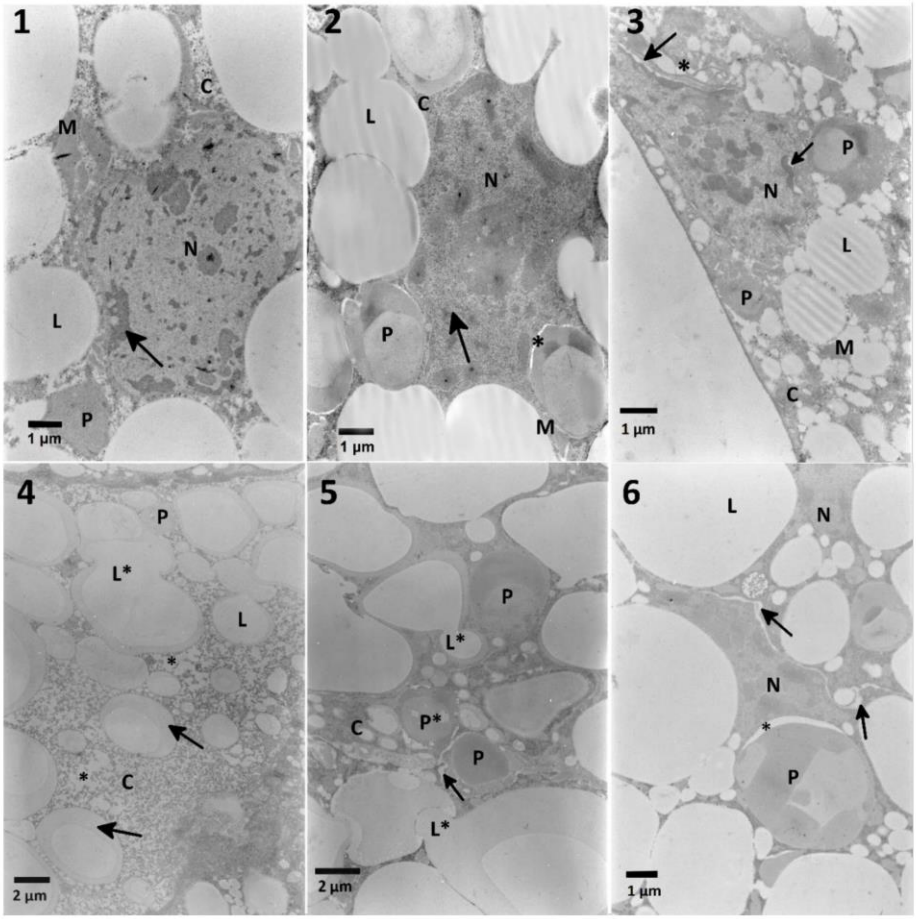

Figure 5. The ultrastructure of the fat body cells of *T. molitor* larvae. **No. 1**—Control cells: the regular nucleus (N) with electron-dense nucleoplasm located peripherally (arrow), dense cytoplasm with granules of glycogen (C), lipid droplets (L), stored proteins (P) and regular mitochondria (M). **No. 2**—The fat body cells of larvae treated with FN (400 µg/mL). Note an increased condensation of chromatin with large nucleolus in the center of the nucleus (arrow) and appearance of electron-lucent space between the cytoplasm and stored proteins (asterisk), and cytoplasm and mitochondria (M). The density of the cytoplasm is increased (C). **No. 3**—The fat body cells of larvae treated with the mixture of EXT (0.01%) and FN (400 µg/mL). Note that the swelling of the intermembraneous space of nuclear envelope (arrows) can be observed. **No. 4**—0.1%: changes in the lipid droplets homogeneity (arrows), the fusion of the droplets (L*) and the decrease of the cytoplasm density (C and asterisk); **No. 5**—1%: stored proteins are degraded (P*). Between cells, the increased intercellular space can be observed (arrow). The highest concentration of the EXT mixed with FN caused even more prominent increase of the intracellular space, disruption of the cellular membranes (arrows); **No. 6**—10%: note increased electron-dense chromatin within nuclei (N), electron-lucent space between stored protein (asterisk) and between cells (arrows).

Fenitrothion in the concentration of 400 µg/mL given to larvae for 3 days caused an increase of the nucleoplasm density (Figure 5, No. 2). The cytoplasm around stored proteins created electron-lucent space, which may suggest vacuolization and imbalanced osmotic conditions.

Blended FN and the EXT (0.01%) (Figure 5, No. 3) caused swelling of the intermembrane space of the nuclear envelope. What is more, in the places where the envelope was disturbed, dense nucleoplasm appeared.

A tenfold increase of the EXT concentration in the mixture with FN caused the decrease of the cytoplasm density, which could be observed together with the vacuolated areas, and fusion of the lipid droplets with their homogeneity diminished (Figure 5, No. 4). Similar effects were observed after the application of the blended FN and 1% EXT (Figure 5, No. 5). Besides the lipid droplets merging and changes in their homogeneity, part of stored proteins also showed a decrease in their homogeneity.

The highest used concentration of the FN–EXT blends caused significant disturbance of cellular membranes with no visible effects on the membranes inside the fat body cells (Figure 5, No. 6). As previously described, cytoplasm was vacuolized around stored proteins.

2.4.4. Determination of Chromatin Density in the Fat Body Cells

Electron-dense nuclei observed under transmission electron microscopy suggested potential changes of the heterochromatin-electron lucent chromatin ratio. Therefore, the nuclei on the electronograms were analyzed for the amount of heterochromatin. In each used concentration, the increase of the amount of heterochromatin was observed (Figure A2) compared with control (25.0 ± 2.35%). Statistical significance was noticed in FN (400 µg/mL) and its blends with 1 and 10% EXT concentrations reaching value 35.4 ± 1.9%, 38.0 ± 1.96% and 38.9 ± 3.7%, respectively. The correlation coefficient for the first variant of treatment in the fat body cells showed a strong positive correlation: 0.72. Mortality and changes in the body mass of larvae exposed to fenitrothion pre-treated with *S. nigrum* extract.

The differences in the mass gain were calculated (Table 2). FN (300 µg/mL) caused the highest loss of the larval body mass. Both FN (300 µg/mL) and the EXT-pre-treated groups showed significant differences in the body mass compared to the control, and to starving larvae. What is more, when EXT (0.01 and 10%) was applied before FN, the body masses were significantly different from the FN-treated group. The body mass of larvae after treatment with EXT alone are presented in Section 2.2.

Table 2. The changes in the body mass of *T. molitor* larvae after pre-treatment with the S. *nigrum* EXT, prior to the fenitrothion-treatment in the concentration of 300 μg/mL, compared to the control, starving larvae and pure FN (300 μg/mL).

Δ Delta, Mean Value ±SD		**Control**	**Starvation**	**FN (300 μg/mL)**	**EXT 0.01% + FN (300 μg/mL)**	**EXT 0.1% + FN (300 μg/mL)**	**EXT 1% + FN (300 μg/mL)**	**EXT 10% + FN (300 μg/mL)**
		+14.13 ± 9.39	**−5.59 ± 1.04**	**−19.83 ± 3.53**	**−15.21 ± 5.94**	**−16.28 ± 5.50**	**−15.34 ± 6.83**	**−14.95 ± 5.13**
n		21	20	32	26	17	22	21
Studentized rage distribution (q), significance,	a)	-	15.5 ***	29.7 ***	24.56 ***	22.89 ***	23.72 ***	23.15 ***
a) Compared to control b) Compared to starvation	b)	15.5 ***	-	12.27 ***	7.94 ***	7.96 ***	7.75 ***	7.35 ***
c) Compared to FN 300 μg/mL	c)	29.7 ***	12.27 ***	-	4.3 *	ns	ns	4.27 *

Tukey's Multiple Comparison Test, * $p < 0.05$, *** $p < 0.001$.

What seems to be the most important result in the process involving the EXT applied before FN is the significantly raised mortality of larvae (Figure 6). The *S. nigrum* extract in concentrations 0.01, 0.1, 1 and 10% increased mortality from 40 to 100, 90, 79 and 96%, respectively.

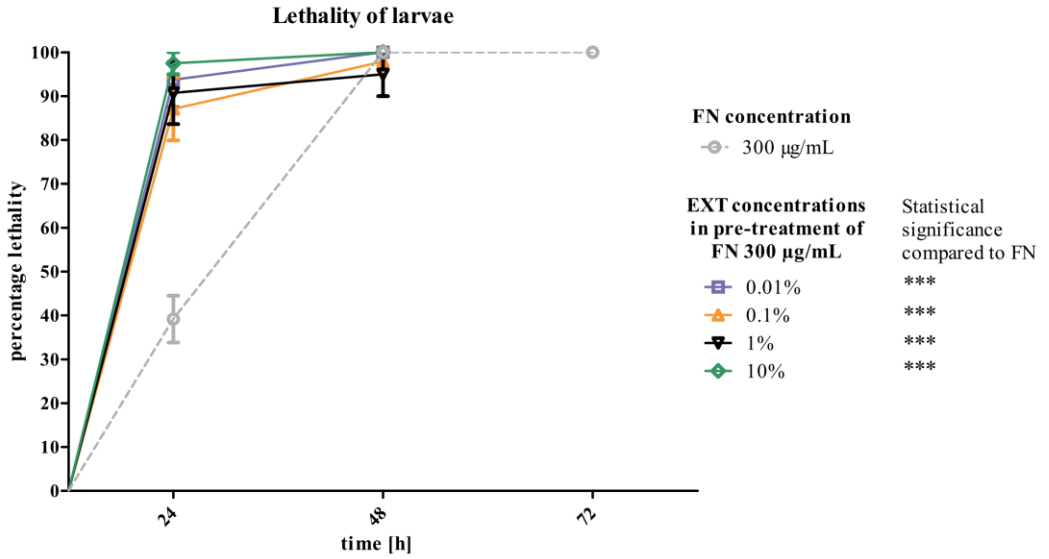

Figure 6. Lethality of *T. molitor* larvae after pre-treatment of *S. nigrum* EXT in four concentrations in the first day of treatment and FN in the concentration of 300 µg/mL in the second and third day of treatment compared to FN in the concentration of 300 µg/mL. Mantel–Cox test, *** $p \leq 0.01$, $n \geq 30$ per treatment.

2.5. Biochemical Analysis of the Fat Body of Tenebrio molitor Larvae after Application of the Solanum nigrum Extract as a Preceding Factor before Fenitrothion Application

The biochemical analyses were conducted similarly as in the first variant of the experiment. Three main types of stored substances such as lipids, glycogen and soluble proteins were isolated and their concentration in the dry mass of tissue was measured. The content of lipids in the fat body after 3 days of treatment with the EXT and FN decreased significantly (Figure 7A) compared to the control (0.63 ± 0.08 mg/mg, ±SEM) but in the case of FN in the concentration of 300 µg/mL, the level of lipids scarcely, insignificantly increased (0.68 ± 0.05 mg/mg). The highest decrease was observed when the EXT in the concentration of 1% was applied before FN (0.42 ± 0.02 mg/mg). In each variant with using the EXT as a preceding factor, the average amount of lipid in the fat body was significantly lower (*** $p < 0.001$) from FN in the concentration of 300 µg/mL.

The decrease in the level of glycogen in the fat body was significant after application of all tested compounds (Figure 7B). In the control larvae, after 3 days of the experiment, the average level of glycogen in the fat body was 29.9 ± 6.15 µg/mg (±SEM). The highest decrease in the glycogen level was observed in the larvae treated with FN (300 µg/mL) and larvae treated with 0.1% EXT and FN, where glycogen level dropped to 1.5 ± 0.19 µg/mg and 1.6 ± 0.39 µg/mg, respectively.

The fraction of soluble proteins in the control larvae was 0.09 ± 0.007 mg/mg (±SEM) of dry tissue mass. The significant increase of protein (0.12 ± 0.01 mg/mg) in the fat body was observed in larvae treated with FN (300 µg/mL) (Figure 7C). It also increased after the treatment with the extract in the 10% concentration, which was 0.11 ± 0.01 mg/mg.

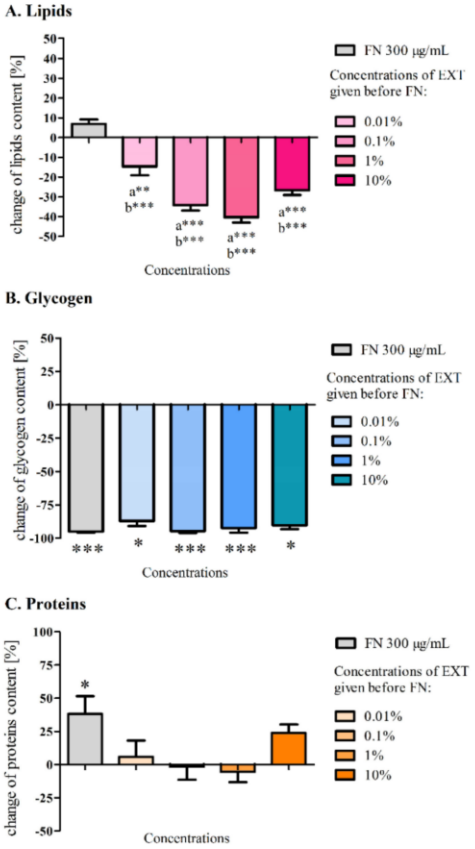

Figure 7. Content of lipids (**A**), glycogen (**B**) and soluble proteins (**C**) in the fat body of *T. molitor* larvae with FN (300 µg/mL) that were pre-treated with *S. nigrum* EXT in the concentrations of 0.01%. 0.1, 1 and 10% one day before FN treating. The values were compared to control and to FN with Dunn's Multiple Comparison Test (**A**,**C**) or Dunnet's Multiple Comparison Test (B), * $p \leq 0.05$, ** $p \leq 0.1$, *** $p \leq 0.01$ compared to control (**B**,**C**) or in case of lipids (**A**) significant changes were obtained not only compared to control (a) but also to FN (b),$n \geq 10$ per treatment.

2.6. The Influence on the Midgut and the Fat Body Ultrastructure

2.6.1. Midgut

The ultrastructure of midgut cells showed electron-dense cytoplasm, an increase of the amount of RER in the cytoplasm (Figure 8, No. 6), and its swelling after application of all used concentrations compared to control (Figure 8, No. 1 and 2). The results are summarized in Table A3. Cellular membranes were significantly separated in the apical part of the cell (Figure 8, No. 4 and 5) and an increase of electron-dense chromatin was observed in this variant with pre-treating with EXT (Figure 8, No. 3).

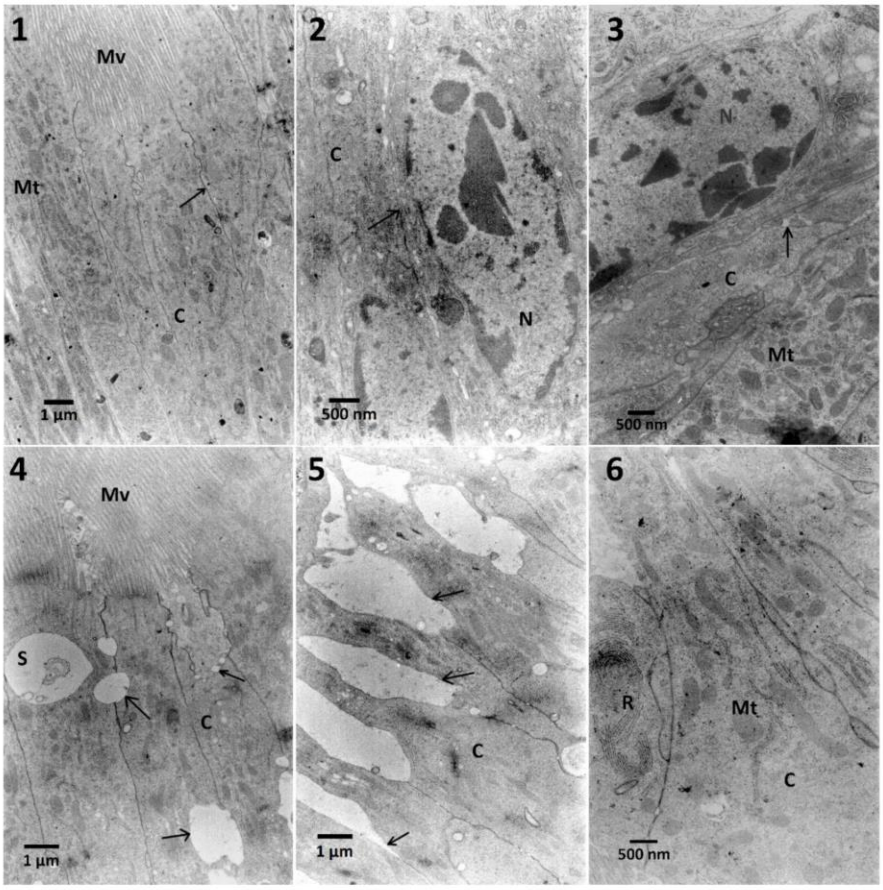

Figure 8. The ultrastructure of midgut cells of *T. molitor* larvae. **No. 1**—Control cells with marked microvilli (Mv), cytoplasm (C), and adherent cell membrane (arrow). **No. 2**—Midgut cell of larvae fed with FN (300 µg/mL). Nucleus (N) and cell membrane (arrow) are marked. Cytoplasm (C) increased its density. **No. 3**—Midgut cells of larvae treated with FN (300 µg/mL) with pre-treatment of 0.01% extract. Cytoplasm (C) and chromatin in the nucleus (N) are electron-dense, free space between cell membranes (arrow), and numerous mitochondria (Mt) can be observed. **No. 4**—Midgut cells of larvae treated with FN (300 µg/mL) with pre-treatment of 1% extract. Beside the disruption of cellular membranes (arrows) close to microvilli (Mv), the swelling of intermembrane space can be observed (S). The density of cytoplasm (C) increased. **No. 5**—Midgut cells of larvae treated with FN (300 µg/mL) with pre-treatment of 1% extract. In the apical part of the cells the disruption of cellular membrane is significant (arrows). **No. 6**—Midgut cells of larvae treated with FN (300 µg/mL) with pre-treatment of 10% extract. In higher magnification, extensive ER system (R) is present with mitochondria (Mt) in electro-dense cytoplasm (C).

2.6.2. Determination of Chromatin Density in the Midgut Cells

In the nuclei of midgut cells treated with FN (300 µg/mL) the amount of heterochromatin decreased (23.7 ± 1.69%) compared to the control (27.8 ± 1.85%) (Figure A3). The highest mean values were observed under the influence of FN with pre-treatment of 0.1% EXT (30.7 ± 2.0%). The correlation coefficient for the second variant of treatment in the midgut cells exhibited no signs of correlation: −0.18.

2.6.3. Fat body

Cells of the fat body treated with FN (300 µg/mL) showed abnormalities of the cellular membranes adhesion, increased of the cytoplasm and chromatin electron-density as well as changes in homogeneity of stored proteins and lipid droplets (Figure 9, No. 3) compared to control cells (Figure 9, No. 1 and

2). When EXT was applied as a preceding factor before FN (Figure 9 No. 4–6), similar effects were observed, but with different intensity (Table A4).

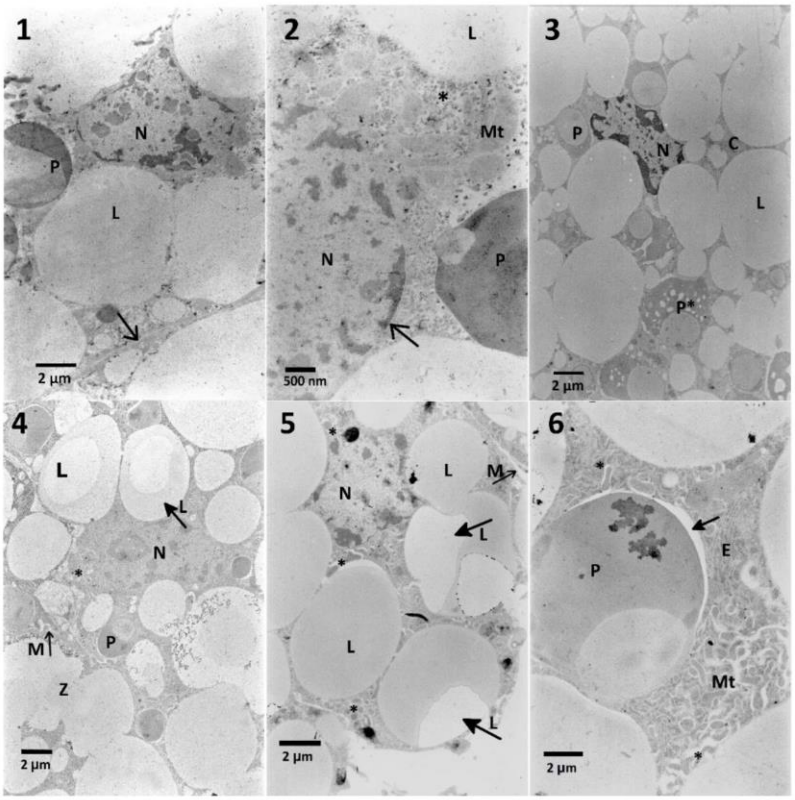

Figure 9. Ultrastructure of the fat body cells of *T. molitor* larvae after treatment with FN (300 µg/mL) and pre-treatment with the *S. nigrum* EXT. **No. 1,2**—Control cells. Nuclei (N) with nuclear membrane (2, arrow), lipid droplets (L), stored proteins (P) in the cytoplasm with glycogen granules (2, asterisk) with mitochondria (Mt) are visible. Cell membrane is marked with arrow (1). **No. 3**—Exemplary fat body cell treated with FN (300 µg/mL). In the center, nucleus (N) with electron-dense chromatin is present, electron-dense cytoplasm (C) and stored proteins that starts degraded (P*). **No. 4**—Pre-treatment of the lowest concentration of the EXT (0.01%) caused changes in the homogeneity of lipid droplets (arrow L) and their fusion (Z), vacuolization and decreased cytoplasmic electron density around stored proteins (P), and mitochondria (asterisk), increased density of chromatin in the nucleus (N) and disturbed cellular membranes (arrow M). **No. 5**—Inhomogeneous and irregular lipid droplets (arrows L) and mitochondria (asterisk) and disturbed cell membrane (arrow M) visible after application of 1% EXT before FN. **No. 6**—Pre-treatment of the highest concentration of the EXT (10%) caused vacuolization of the cytoplasm surrounding stored protein granules (arrow), mitochondria (M), and what is more, the distention of the endoplasmic reticulum (E).

2.6.4. Determination of Chromatin Density in the Fat Body Cells

The amount of heterochromatin (±SEM) showed an increasing tendency after application of each concentration compared to the control (25.08 ± 7.42%) (Figure A4). The highest difference from control was observed after application of EXT in the concentration of 10% in the pre-treatment (35.2 ± 2.13%), nevertheless, noticed differences were not statistically significant. In the fat body cells treated with FN with EXT pre-treatment, the correlation coefficient showed strong positive correlation: 0.7.

2.7. Discussion

The first strategy of application assumed that the EXT added to the FN may increase the lethality of *T. molitor* larvae by additive action on the tissues crucial for the absorption and detoxification processes, such as fat body and midgut. What is more, we planned to mimic agrochemical application that would not last for more than two-to-three days. Therefore, we focused on the first results and acute toxicity effects. However, the larvae did not show any significant increase in mortality after 24 h of treatment. In addition, in the highest concentration of EXT (10%) mixed with FN, a slight decrease in mortality was observed (Figure 2). Due to the observed effects, the strategy of application has been changed and the EXT in four concentrations was given to the nourishment for 24 h before application of FN in the concentration of 300 µg/mL, which was a concentration causing 40% of lethality of *T. molitor* larvae after 24 h of treatment. The second variant of the experiment showed that the mortality of larvae increased significantly after application of FN, when the EXT was applied 24 h before (Figure 6). That may prove the increase of the sensitivity of larvae to synthetic insecticide under the influence of the EXT. In case of this strategy, the potentiation of the lethal toxicity of FN by EXT was observed.

The loss of the body mass of larvae treated with this mixture was significantly lower from the loss of the body mass of starving larvae (Table 1) which suggests high energy expenditure for the detoxification process [32,33]. Also, this proves that the lethal effect was not caused by deprived feeding. The loss of the weight by the larvae could be the result of water loss through open spiracles as a consequence of the paralysis and an increase in the metabolic rate after the insecticide exposure [34]. Similar results appeared in the second variant of the experiment.

Sublethal effects on the fat body of *T. molitor* larvae caused by *S. nigrum* extract were previously described [12]. The EXT caused a significant decrease in the glycogen level at a concentration of 0.1% and decrease of lipids content at a concentration 1% in the fat body cells. Further biochemical analyses of the levels of glycogen, lipids and proteins (Figure 3) in the first variant of the experiment did not show any significant changes compared to the control, which may be a confirmation of a short and strong neurotoxic, lethal effect caused by FN in the chosen concentration and its mixtures with the EXT, rather than other metabolic effects. It can also indicate slight changes of quantity or quality of particular compounds among tested substances that might have not been visible, due to the short period of exposure. Perhaps, the changes would be significant after longer exposure. However, such a strategy of application would not mimic the mode of agrochemical treatments we wanted to follow. This is confirmed by the work of Wojciechowska et al. [35], where the composition of the fat body changed in terms of the quality of compounds such as amino acids, fatty acids, cholesterol and carboxylic acids, in larvae treated with various types of insecticides. However, in our studies, particular fractions of lipids were not analysed. In the second variant of the experiment, the comparison of the amount of lipids after application of FN (300 µg/mL) and FN (300 µg/mL) with pre-treatment of EXT gave significant differences. The additive effect can be confirmed by the previous observations of lipid content in the fat body after the EXT application [12]. In this case, the acute toxic effect was postponed, and some detoxification mechanisms could appear. Additionally, lipids are used as the first energetic substrates [32] in cases such as intoxication.

Glycogen levels varied widely in the first variant of the experiment, which can be explained not only by the different state of food intake by each larvae [36], but also by the acute toxicity, which was tested in our experiment, did not allow for the development of the extensive detoxifying strategy. Perhaps, that is the reason why the observed alterations were not statistically significant. Possibly, larvae might have used energy for detoxification from another source, such as trehalose from hemolymph [37,38], which level was not examined in our studies. In the case of the second variant of our experiment, each used concentration decreased glycogen level significantly. FN in the sublethal and lethal doses given in the diet of the silkworm *Bombyx mori* was proven to decrease the glycogen level in the fat body [37] which confirms that this compound can change the energetic metabolism in insects.

Protein level in the fat body increased notably after feeding the larvae with FN (300 µg/mL). Other research reports decrease of protein level after application of FN to *B. mori* [39] and increase when insecticides from other groups and Deodar oil were applied to *T. molitor* [10]. These insects may use different compensatory mechanisms in response to stress, also, different used substances and strategies of their application may develop different reaction.

In our previous study, the EXT in the concentration of 0.01% given for 3 days to larvae, did not show any visible changes in the midgut cells ultrastructure. The concentration of 1% was responsible for the disruption of nuclear membranes and 10% EXT additionally for the appearance of glycogen vacuoles, a sign of changes in its metabolism in the midgut cells [12]. The above-mentioned observations were neither visible after the application of FN and FN–EXT blends nor in the midgut cells from larvae pre-treated with the EXT for 24 h and then FN for 48 h. Possibly, the exposure on the EXT was to short and as a result of an acute toxic action of FN, EXT did not penetrate the cells. However, this proposal is difficult to agree with, because the effects of FN–EXT blends were present in the fat body, which points that both glycoalkaloids and FN or their metabolites had to cross the midgut epithelium and reach the fat body causing malformations. Similar results were observed by Büyükgüzel et al. [40], where boric acid caused much more significant alterations of trophocytes of the fat body, than of the epithelial cells of the midgut.

In the case of midgut cells in the first variant of the experiment, neither synergy, nor additive effect between FN and EXT were observed (Table A1). The calculation of the correlation coefficient in the midgut cells for determination of chromatin density (Figure A1) showed moderate negative correlation which suggests the possible inhibition of FN activity by EXT in these cells. Midgut cells also exhibited electron-dense cytoplasm and abundance of RER, where in the two highest concentrations its swelling was observed (Table A1, Figure 4). However, in the second variant, different effects appeared, such as cellular membrane disruption. Cells were also rich in RER in all tested concentrations, and the swelling of ER was present (Table A3). This can be explained by the fact, that synthetic insecticides can cause disturbance of ER membranes and cell membranes in the midgut cells as well as mentioned chromatin condensation [41]. Both synthetic and bio-insecticides are responsible for oxidative and nitrosative stress [42], which can result in observed effects.

Effects observed in the fat body cells obtained from insects treated only with the *S. nigrum* extract were limited to the decreased cytoplasmic density and the homogeneity of lipid droplets and an increased electron-density of nuclei. Additionally, solasonine and solamargine were responsible for disturbance of lipid droplets and stored proteins [12]. The effects of cell disruption were observed in the ultrastructure of the fat body (Figure 5, Table A2) in both variants of experiment. The mixtures of *S. nigrum* extracts caused additional effects such as fusion and loss of homogeneity of lipid droplets (Figure 5, No. 4,5) suggesting some additive effect. What is interesting, these changes were not noted in the lowest and the highest used concentrations of the extract in mixtures. The fat body cells of larvae treated with EXT and then FN (Table A4; Figure 9) showed different signs of intoxication, such as vacuolization of cytoplasm around mitochondria, stored proteins, and endoplasmic reticulum, which can be symptoms of disruption of cellular membranes caused by the EXT.

Most found nuclei in the fat body cells, and also midgut cells from larvae treated with FN mixed with the EXT featured with electron-dense chromatin. Observed effects can be a sign of the response of insects to contact with xenobiotics, and may indicate the early beginning of pyknotic processes, which are characterized by shrunken and electron-dense nuclei [43,44]. The ultrastructural malformations of fat droplets are most probably due to high lipophilic properties of FN. Besides, lipids are the first reserves, used before glycogen or other energy-rich substrates [32]. Strong positive correlation obtained in the chromatin density of fat body cells treated with FN–EXT blends, suggest the increase of FN toxicity caused by increasing concentration of the EXT, especially well visible in the highest used concentration of EXT in blends (Figure 5, No. 6).

The malformation of biological membranes caused by glycoalkaloids (solasonine and solamargine) has been previously described [45]. They cause easier penetration of toxic substances to the cell [46],

which could be an additive factor to increase FN toxicity. The changes in the chromatin condensation in the fat body after application of FN and with pre-treatment of EXT also suggests a potentiation effect (Figure A3), confirmed by calculated correlation coefficient, which showed strong positive correlation. Also, some pure glycoalkaloids induce liposome disruption and hemolysis [47]. Therefore, the observed malformations of cellular membranes and lipid droplets could be observed in our research. Next, FN may increase polarity and lead to enhanced water penetration [48]. In consequence, osmolarity of cytoplasm, shrinkage or swelling of intramembranous space of membrane-bound organelles may be observed.

Abovementioned observations suggest (1) relatively fast and unharmful transfer of toxic substances such as FN that is a small, non-polar molecule and is able to migrate through the cell membrane or by the paracellular pathway [49], and (2) their longer persistence in the fat body, which is the main detoxifying organ in larvae. Besides, due to high lipid content, it enables a high accumulation of toxins within fat body cells. This manifests in the disturbance of cellular and nuclear membranes, and an increase of the chromatin and cytoplasm electro-density, which next can lead to further changes in metabolism [50].

In several studies, plant-derived substances were used in binary mixtures with synthetic insecticides on insects due to increase of toxic properties, mainly by extension of mode of action on different targets obtaining synergistic effects. In many studies, the application of both plant-derived and synthetic insecticide with obtained synergism or additivity, were performed without creating their mixtures before the application [51,52]. First, we focused on a more practical aspect, where the mixtures were created to avoid multiplication of the field application. A similar strategy was used in studies of Maurya et al. [53], where imidacloprid mixed with crude petroleum ether *Ocimum basilicum* leave extract in ratio 1:1 showed synergistic properties against *Anopheles stephensi* than imidacloprid applied alone. Fenitrothion blended in ratio 1:1 with *Callitris glaucophylla, Daucus carota* or *Khaya senegalensis* extract was effective against *Culex annulirostris* [54]. Note that in both cases, FN and LC_{25} extracts were calculated and the final summary of effectiveness of both used substances could be predicted and compared with results. In our studies, we used EXT, which did not cause lethal effects [12], which could result in no increase in lethality in the first variant of the experiment. However, other studies of Maliszewska and Tęgowska [55] shows that capsaicin which does not have insecticidal properties, added to an organophosphorus insecticide, methidathion, increased its toxicity. Therefore, the effects may be alkaloid-specific. We decided to perform the second variant of the experiment and avoid blending used chemicals, which resulted in a massive increase of larvae lethality, proving the potentiation—the effect when non-lethal substance increases lethality of a toxic compound. A similar strategy was used on a mosquito (*Aedes aegypti*) with 4 h exposition on essential oils vapors before deltamethrin topical application, which also resulted in toxicity growth [56]. The effects prove that the strategy of application may significantly affect results of the agrochemical strategies.

To sum up our experiment results, we can agree that increased lethality, significant changes in the levels of glycogen and lipids and ultrastructure malformations prove that the second variant of the experiment is more effective. We assume that the mode of action of the extract depends on its sublethal effects [12], such as the membranes malformations that increase the FN permeation. Second, the detoxication of previously applied EXT may decrease the energy resources necessary for FN detoxication. And last, observed changes in the lipids stored in the fat body may decrease the ability to store the lipophilic xenobiotics (such as FN) in the fat body and their spreading in other tissues, i.e., nerve tissue.

3. Conclusions

Literature data and our observations suggest that the *S. nigrum* extract's mode of action contributed to changes in the prooxidant/antioxidant balance, which disturb lipid peroxidation in stored lipid droplets, in the fat body, cellular membranes stability, and facilitated fenitrothion intoxication. According to the obtained results, *S. nigrum* extract is a potentiating factor for fenitrothion,

with a subsequent 1-day application period on *T. molitor* larvae. Even though it does not cause acute lethality, it may significantly increase toxicity of fenitrothion. Pre-treatment with plant-derived products—in this case, a common plant from the Solanaceae family—can be beneficial for human health and environmental protection. The same effect can be achieved with the use of lower dose of synthetic insecticide, which had a positive effect on the decrease of its emission. Also, the observed effect can be obtained with use of relatively low concentrations of EXT. That may limit costs and perhaps the extracts can be made of plant parts which are not used by farmers as sold products. A mildly persistent natural synergist with rich composition may also prevent from increasing resistance development among insect pests. The obtained results suggest that proper strategies are crucial for increasing the insecticidal effects.

4. Materials and Methods

4.1. Materials

4.1.1. Insects

T. molitor larvae were obtained from the breeding culture at the Department of Animal Physiology and Development. The larvae were bred under laboratory conditions at 26 °C and 60% of humidity in a 12:12 h dark to light photoperiod. For the experiments, larvae after molting with weights of 120–140 mg were used. The insect's weight allowed to choose the larvae with a similar metabolic rate.

4.1.2. Substances

Fenitrothion (Purity: 98.3 ± 0.2% (*m/m*)) was purchased from the Institute of Organic Industrial Chemistry, Annopol, Warsaw, Poland. Because of their low solubility in water, fenitrothion solutions were prepared in 70% ethanol for the lethality calculation.

The *S. nigrum* unripe berries extract was obtained from the research group of prof. Bufo, S.A. from the University of Basilicata, Potenza, Italy. The voucher specimens were deposited at the Herbarium Lucanum (HLUC, Potenza, Italy), with the ID Code: 2320. The extraction method was conducted according to the method previously described by Cataldi et al. [57]. The chemical analysis was conducted at the Department of Sciences, University of Basilicata by Prof. Sabino Bufo's team. The extracts at concentrations of 0.01, 0.1, 1, and 10% were diluted in physiological saline B (274 mm NaCl, 19 mm KCl, 9 mm $CaCl_2$).

The concentration of 70% ethanol solution of fenitrothion and *S. nigrum* extract were mixed together in the ratio 1:1 creating the final concentration of 400 µg/mL of fenitrothion and 0.01, 0.1, 1 and 10% extract solution in 10 µL of applied substance, what imitates a simultaneous application of both substances.

4.2. Methods

The lethal concentrations (LC_x) of fenitrothion were calculated for *T. molitor* larvae. Solutions were prepared and applied into the nourishment for each larva. The lethality of larvae was checked after 24, 48 and 72 h of treatment. For each used concentration (40, 50, 100, 200, 300 and 400 µg/mL) a minimum of 15 larvae were used in each of the three repetitions of the experiment.

4.2.1. Methods of Application

Due to the results obtained in the first variant of the experiment, the strategy of application of fenitrothion and the extract has been changed (Table 3). The *S. nigrum* extract was considered as a preceding factor that may suddenly weaken the larvae, disturb the physiology of larvae and ease the fenitrothion penetration. The extract in one of four concentrations of 0.01%, 0.1%, 1%, and 10% was given to the larvae in the first day of treatment and then fenitrothion in the concentration of 300 µg/mL was applied. The lethality was counted from the day when fenitrothion was applied and

compared to lethality caused by fenitrothion in the concentration 300 μg/mL. The higher concentrations of fenitrothion (400 μg/mL) were not used due to the their strong acute effects.

Table 3. The schematic presentation of the experiments conducted in the study.

Days of Experiment/Hours of Treatment	1/-	2/0	3/24	4/48	5/72
Variant 1	Choosing larvae 120–140 mg after moulting	mixtures 1:1 Fenitrothion 400 μg/mL and *S. nigrum* extract in four concentrations: 0.01, 0.1, 1, 10%	mixtures 1:1 Fenitrothion 400 μg/mL and *S. nigrum* extract in four concentrations: 0.01, 0.1, 1, 10%	mixtures 1:1 Fenitrothion 400 μg/mL and *S. nigrum* extract in four concentrations: 0.01, 0.1, 1, 10%	Weighing the larvae after the experiment, sampling
Variant 2		*S. nigrum* extract in four concentrations: 0.01, 0.1, 1, 10%	Fenitrothion 300 μg/mL	Fenitrothion 300 μg/mL	

"-" defines the lack of treatment in the first day of experiment.

a) Application of extract mixed with fenitrothion

The first method assumed the additive action of the *S. nigrum* extract and fenitrothion. The substances were mixed together in a ratio of 1:1, creating a final concentration of 400 μg/mL of fenitrothion, and the extract in one of four concentrations: 0.01, 0.1, 1, and 10%. The larvae were fed with these blends for 3 days. The mortality was noted every 24, 48 and 72 h. The results were compared with the control (saline) and the fenitrothion in the concentration of 400 μg/mL.

b) Fenitrothion application with pre-treatment of extract

Due to the results obtained in the first variant of the experiment, the strategy of application of fenitrothion and the extract has been changed (Table 3). The *S. nigrum* extract was considered as a preceding factor that may sublethally weaken the larvae, disturb the physiology of larvae and ease the fenitrothion penetration. The extract in one of four concentrations of 0.01, 0.1, 1 and 10% was given to the larvae in the first day of treatment and then fenitrothion in the concentration of 300 μg/mL was applied. The lethality was counted from the day when fenitrothion was applied and compared to lethality caused by fenitrothion in the concentration 300 μg/mL. The higher concentrations of fenitrothion (400 μg/mL) was not used due to the very strong acute effects.

c) Starvation

Additionally, a group of larvae was selected and not fed for the time of experiment due to the examination of the body mass loss during starvation. Larvae were weighed before and after the experiment, and the delta was calculated.

4.2.2. Lethality and Mass Gain

The *T. molitor* larvae weighing 120–140 mg were kept separately in the glass flasks. The day after collection, insects were fed for three days with the artificial diet prepared according to David et al. [58]. Each portion of the nourishment contained 10 mL of tested substances or the solvents of the used substances such as saline (274 mm NaCl, 19 mm KCl, 9 mm $CaCl_2$) or/and 70% ethanol as a control. On the fourth day, the mortality of larvae was counted, and the alive larvae were weighed and samples of the fat body and midgut were collected. The difference between the weight before and after the experiment was used to calculate the change in the body mass according to the following equation:

$$\Delta = (b \times 100)/a - 100 \quad (1)$$

where 'a' is the mass of larva before and 'b' is the mass after the experiment.

The lethality was noted after 24, 48 and 72 h. Larvae were considered dead when the paralysis was irreversible and blocked the vital functions. For the experiments the $n \geq 30$ individuals per concentration.

4.2.3. Biochemical Analysis of the Fat Body

Small pieces (1–3 mg) of the fat body were washed with saline and placed in weighed Eppendorf tubes. All samples were dried under vacuum conditions (−0.9 atm.) at 60 °C and weighed. The content of glycogen, lipids and proteins were determined and the amount of substances was expressed as milligrams or micrograms of substance per milligram of dry mass of the tissue. Isolation and determination of glycogen, lipids and proteins content was described previously [12] for the fat body of *T. molitor* larvae treated with *S. nigrum* extract. For each analysis at least nine individuals were collected from three repetitions.

4.2.4. Determination of Glycogen Level

Isolation and determination of glycogen level were carried out according to procedures of van Handel [59] and Dubois et al. [60] respectively as follows. The samples were incubated for 15 min at 90 °C with 500 µL of KOH to lyse the tissues. Then 50 µL of a saturated solution of Na_2SO_4 and 800 µL of 96% ethanol were added to precipitate the glycogen. The obtained suspension was centrifuged at 10,000 rpm for 10 min and the supernatant was rejected. The pellet was washed with 70% ethanol three times. After the evaporation of residual ethanol at 74 °C, 500 µL of purified water was added. The pellet was shaken for 5 min at 80 °C and then centrifuged for 5 min at 10,000 rpm. The obtained solution was used to determine the glycogen amount spectrophotometrically (Eppendorf BioSpectrometer, Hamburg, Germany). As a standard oyster glycogen (Sigma-Aldrich, St. Louis, MO, USA) was used.

4.2.5. Determination of the Lipid Content

The isolation of the fat body lipids was conducted according to the Folch et al. [61] method. Dry tissues were homogenized in 1000 µL of chloroform-methanol mixture (2:1 *v/v*) and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new Eppendorf tube and washed three times with 220 µL of 0.29% NaCl. The remaining solution was evaporated at 30 °C under vacuum (−0.9 atm). The pellet was dissolved in 1000 µL of chloroform-methanol mixture (2:1 *v/v*) and 500 µL of the solution was transferred to the new weighed Eppendorf tubes. After drying under vacuum (30 °C, −0.9 atm), the mass of the residual lipids was measured gravimetrically and counted as milligrams of lipids in a milligram of dry mass.

4.2.6. Determination of the Soluble Protein Content

Dry samples were mixed with 200 µL of saline and homogenized on ice. Samples were centrifuged at 10,000 rpm for 5 min at 4 °C. Next, 2 µL of the infranatant was placed on the PTFT membranes and measured with a Direct Detect® Infrared Spectrometer (Merck Millipore, Burlington, MA, USA).

4.2.7. Transmission Electron Microscopy

The larvae chosen randomly were anesthetized with carbon dioxide. Pieces of the fat body and midgut were washed with saline and fixed in 2% glutaraldehyde. The procedure was carried out according to Adamski et al. [62]. Pieces of the fat body and cleaned midgut were placed in 2% glutaraldehyde in 0.175 M cacodylate buffer for 2 h and postfixed with 2% osmium tetroxide for 2 h. Samples were subsequently dehydrated with increasing concentrations of ethanol and then embedded in Spurr Low—Viscosity Embedding Media (Polysciences, Inc., Warrington, PA, USA). Ultrathin sections of the resin were cut with a Leica ultramicrotome and stained with uranyl acetate and lead citrate. Samples were observed under JEOL 1200EX II JEM (JEOL, Tokyo, Japan) transmission electron microscope. For each analysis of the fat body and the midgut, three insects were used. The results presentation contains the most representative, exemplary electronograms with visible

changes. The summary of observed changes were graded (+ slight changes, ++ intense changes, - absent) and placed in tables.

4.2.8. Determination of Chromatin Density

The chromatin ratio was calculated using the stereological method counting the electron dense surface to whole nucleus surface with the computer program STEPanizer [63]. The digital grids (1024 squares pre-picture) were plotted on the images of nuclei (at least 10 per each concentration). The number of squares over the electron dense and electron lucent chromatin were counted and the ratio was calculated.

Next, the Pearson correlation coefficient concerning the concentration of the tested substances and the heterochromatin ratio was calculated. Values between −0.3 and 0.3 were regarded as having no linear relationship, values between $0.3 < \times \leq 0.5$ and $-0.3 > \times \geq -0.5$ indicated a weak (positive/negative) relationship, values between $0.5 < \times \leq 0.7$ and $-0.5 > \times \geq -0.7$ were regarded as having a moderate (positive/negative) relationship, values between $0.7 < \times \leq 0.9$ and $-0.7 > \times \geq -0.9$ indicated a strong (positive/negative) relationship, and values between $0.9 < \times \leq 1$ and $-0.9 > \times \geq -1$ indicated a full (positive/negative) relationship.

4.2.9. Statistical Analysis

All the data are presented as the mean values ±SEM of n number of replicates. The statistical significance of differences between the control and treatment values was determined using statistical tests: one-way ANOVA Tukey's test and Dunnet's Test, or if there was not a normal distribution, the nonparametric Kruskal–Wallis test and Dunn's Multiple Comparison Test. The Mantel–Cox test was used to compare lethality. The statistical analyses were conducted using GraphPad Prism 5 software (GraphPad Software Inc, Version 5.01, MacKiev, La Jolla, CA, USA, 1992–2007).

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Abbreviations

FN—fenitrothion; EXT—*Solanum nigrum* extract; FN–EXT blends—mixtures of fenitrothion and the *Solanum nigrum* extract; LC—lethal concentration; TEM—transmission electron microscopy; ER—endoplasmic reticulum; RER—raw endoplasmic reticulum.

Appendix A

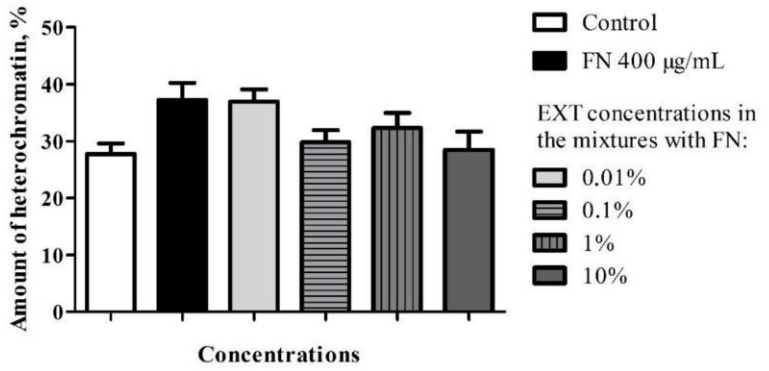

Figure A1. Amount of heterochromatin in the midgut cells from larvae treated with FN–EXT blends. Tukey's Multiple Comparison Test, $n \geq 10$ per treatment. Changes were not statistically significant.

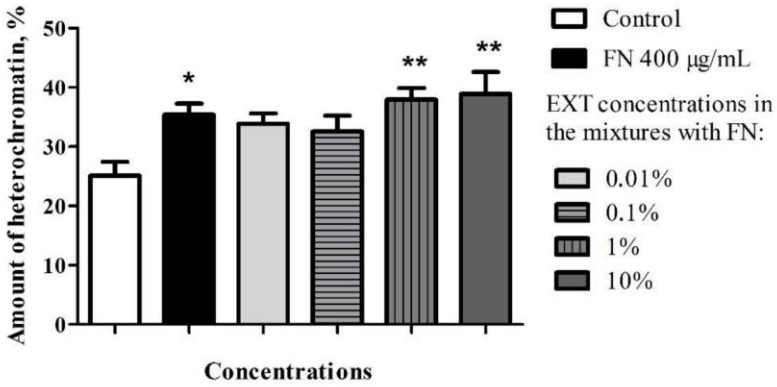

Figure A2. Amount of heterochromatin in the fat body cells from larvae treated with FN–EXT blends. Tukey's Multiple Comparison test, changes significant compared to control, $n \geq 9$ per treatment. ** $p < 0.01$, * $p < 0.05$.

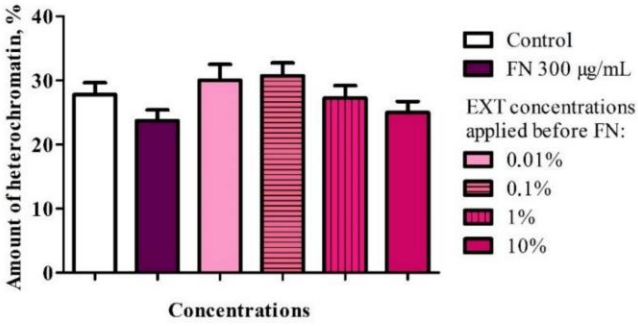

Figure A3. Changes in the amount of heterochromatin in midgut cells of larvae treated with FN (300 µg/mL) pre-treated with EXT at concentration 0.01, 0.1, 1 and 10%. Dunn's Multiple Comparison test, $n \geq 10$ per treatment. Changes were not statistically significant.

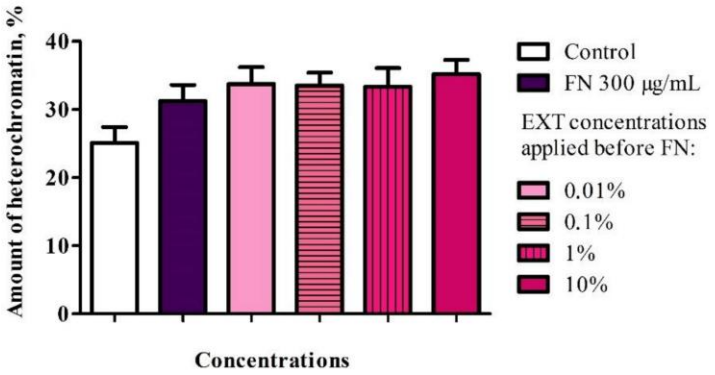

Figure A4. Amount of the heterochromatin in the fat body cells. Tukey's Multiple Comparison test, $n \geq 10$ per treatment. Changes were not statistically significant.

Table A1. Effects of the application of FN in the concentration of 400 µg/mL and its mixtures with *S. nigrum* extract (EXT) in four concentrations on the midgut ultrastructure, compared to control.

	Exemplary Electronogram Number	Concentrations				
		FN (400 µg/mL)	FN (400 µg/mL) EXT 0.01%	FN (400 µg/mL) EXT 0.1%	FN (400 µg/mL) EXT 1%	FN (400 µg/mL) EXT 10%
Disruption of the cellular membranes	2,3	-	-	-	-	-
Increase of electron dense chromatin	4	++	+	+	++	++
Swollen intracellular space of nuclear membrane	-	-	-	-	-	-
Electron-dense cytoplasm	2–5	++	++	+	++	++
Vacuolization of cytoplasm	-	-	-	-	-	-
Rich in RER	2–5	++	++	+	++	++
Swollen ER	4,5	-	-	-	+	++

Types of observed effects: + slight changes, ++ intense changes, - absent.

Table A2. Effects of the application of fenitrothion in the concentration of 400 µg/mL (FN 400) and its mixtures with *S. nigrum* extract (EXT) in four concentrations on the fat body ultrastructure.

Types of Changes.	Exemplary Electronogram Number	Concentration				
		FN (400 µg/mL)	FN (400 µg/mL) EXT 0.01%	FN (400 µg/mL) EXT 0.1%	FN (400 µg/mL) EXT 1%	FN (400 µg/mL) EXT 10%
Disruption of the cellular membranes	5,6	–	–	+	+	+
Increase of electron dense chromatin	2,6	++	+	++	++	++
Swollen nuclear membrane	3,6	–	++	–	+	–
Changes of the lipid droplets homogeneity	4,5	–	++	++	++	–
Fusion of the droplets	4,5	–	–	+	+	–
Increase of the cytoplasm density	2,3,4,6	++	–	++	++	++
Irregular homogeneity/degradation of protein granules	5	+	–	–	+	–
Vacuolization of cytoplasm	4	–	–	+	–	–

Types of observed effects: + slight changes, ++ intense changes, – absent.

Table A3. Effects of the application of FN in the concentration of 300 µg/mL and with pre-treatment of *S. nigrum* EXT in four concentrations on the midgut ultrastructure.

Types of Changes	Exemplary Electronogram Number	Concentration				
		FN (300 µg/mL)	EXT 0.01% + FN (300 µg/mL)	EXT 0.1% + FN (300 µg/mL)	EXT 1% + FN (300 µg/mL)	EXT 10% + FN (300 µg/mL)
Disruption of the cellular membranes	4,5	-	++	++	++	+
Increase of electron dense chromatin	3	-	++	++	+	+
Swollen intracellular space of nuclear membrane	-	-	-	-	-	-
Electron-dense cytoplasm	2–6	+	++	++	++	++
Vacuolization of cytoplasm	-	-	-	-	-	-
Rich in RER	6	++	++	++	++	++
Swollen ER	4	+	+	++	+	++

Types of observed effects: + slight changes, ++ intense changes, - absent.

Table A4. Effects of the application of FN in the concentration of 300 µg/mL and with pre-treatment of *S. nigrum* EXT in four concentrations on the fat body ultrastructure.

Types of Changes	Exemplary Electronogram Number	Concentration				
		FN (300 µg/mL)	EXT 0.01% + FN (300 µg/mL)	EXT 0.1% + FN (300 µg/mL)	EXT 1% + FN (300 µg/mL)	EXT 10% + FN (300 µg/mL)
Disruption of the cellular membranes	4,5	+	+	+	++	+
Increase of electron dense chromatin	3,4	+	++	+	++	++
Swollen nuclear membrane	-	-	-	-	-	-
Changes of the lipid droplets homogeneity	4,5	+	++	+	+	+
Fusion of the droplets	4	-	+	-	+	-
Increase of the cytoplasm density	3–6	+	++	++	++	++
Irregular homogeneity/degradation of protein granules	3	++	++	++	+	-
Vacuolization of cytoplasm	4–6	+	++	+	++	++

Types of observed effects: + slight changes, ++ intense changes, – absent.

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