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TOXICITY AND MODE OF ACTION OF STEROID AND TERPENOID SECONDARY PLANT METABOLITES AGAINST ECONOMICALLY IMPORTANT PEST INSECTS IN AGRICULTURE

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Thesis submitted in fulfilment of the requirements for the degree of

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WOORD VOORAF

Een thesis maak je niet in je eentje. Ik heb bij mijn werk veel hulp gehad van een groot aantal collega's en vrienden, en dat is het vermelden waard.

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TABLE OF CONTENTS

Chapter 1

Introduction to secondary metabolites, saponins and methods of pest control against insects

This chapter has been partially redrafted from:

De Geyter E, Lambert E, Geelen D and Smagghe G (**2007**) Novel advances with plant saponins as natural insecticides to control pest insects. *Pest Technology* 2, 96–105.

1.1. Plant secondary metabolites

Plants produce an enormous variety of natural products with highly diverse chemical structures. The evolution of chemical defences in plants is likely linked to the emergence of secondary metabolites, organic compounds that are not directly involved in the normal growth, development or reproduction of organisms, and are often produced as by-products during the synthesis of primary metabolic products. Although secondary metabolites were formerly regarded as "waste products" without physiological function for the plant, it has since become evident that they can fulfil important functions in the interaction between plants and their biotic and abiotic environment, providing protection against attack by herbivores and microbes and serving as attractants for pollinators and seed-dispersing agents. In addition to their physiological function in plants, natural products also have a strong impact on human culture, as humans exploit them as sources of pharmaceuticals, flavouring agents, pigments, fragrances, condiments, and for a wide range of other applications (Osbourn & Lanzotti, 2009).

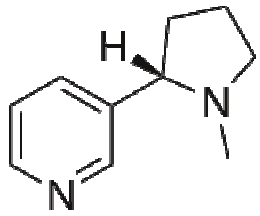
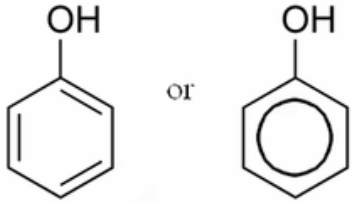
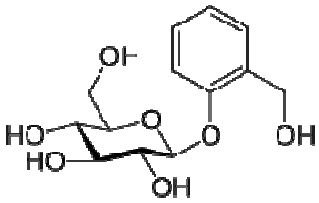
It has been known since the late 17th century that plants contain noxious chemicals that are avoided by insects. Man has used these chemicals as early insecticides; in 1690 nicotine was extracted from tobacco and used as a contact insecticide. In 1773, insect infested plants were treated with nicotine fumigation by heating tobacco and blowing the smoke over the plants (Ware & Whitacre, 2004). The formal study of plant resistance to herbivory was first covered extensively in 1951 by Reginald (R.H.) Painter, who is widely regarded as the founder of this area of research, in his book *Insect Resistance in Crop Plants* (Painter, 1951).

In later years, the applications of plant resistance became an important area of research in agriculture and plant breeding, particularly because they can serve as a safe and low-cost alternative to the use of pesticides (Smith, 2005). The use of botanical pesticides is widespread and notable examples include azadirachtin from neem *(Azadirachta indica)*, rotenone *(Derris)*, and pyrethrum (Russ, 2007).

Providing a full overview of the more than 200,000 structures of plant secondary metabolites known today is beyond the scope here, also because there is no rigid scheme for classifying natural products; their immense diversity in structure, function, and biosynthesis make it hard to fit them neatly into a few simple categories. In practice, however, they are often classified

into **six main classes** of natural products, as listed below (Fig. 1.1). Since secondary metabolites are often created by modified primary metabolite synthases, these categories should not be interpreted as saying that all molecules in the category are secondary metabolites, but rather that there are secondary metabolites in these categories (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Most important for this study are the terpenes and terpenoids, including steroids. This group will be discussed at the end, after a short overview of the other classes.

Alkaloids	**Phenols**	**Glycosides**
		
Ex. nicotine	Ex. phenol	Ex. salicin

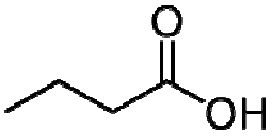
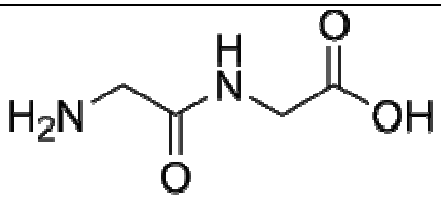
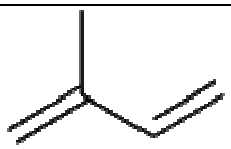
Fatty acids	**Peptides**	**Terpenes**
		
Ex. butyric acid	Ex. glycylglycine	Ex. isopreen

Figure 1.1. Overview of the six main classes of secondary metabolites with representative examples.

1.1.1. Alkaloids

Alkaloids are a group of naturally occurring chemical compounds that mostly contain basic nitrogen atoms. The term "alkaloid" is derived from the Arabic word "al-qali" that refers to potassium carbonate-containing ashes from plant material. Traditionally, alkaloids are defined as heterocyclic nitrogen compounds biosynthesized from amino acids; however, many other substances that do not exactly match this rule are classified as alkaloids, either for

historical reasons or due to their bioactivities. Besides carbon, hydrogen and nitrogen, molecules of alkaloids may contain sulphur and rarely chlorine, bromine or phosphorus (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

With currently more than 12,000 known structures, alkaloids present one of the biggest groups of natural products. Famous examples include nicotine, caffeine, morphine, and strychnine (Fig. 1.1). Alkaloids have pharmacological effects on humans and other animals and can be used as medications, as recreational drugs, or in entheogenic rituals. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke an aversively bitter taste (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Alkaloids have a wide **distribution in nature**: they are generated by various living organisms, especially by higher plants, about 10 to 25% of which contain alkaloids. The alkaloid content in plants is usually within a few percent and is inhomogeneous over the plant tissues. Depending on the type of plants, the maximum concentration is observed in the leaves (black henbane), fruits or seeds (*Strychnine* tree), root (*Rauwolfia serpentina*) or bark (cinchona). Furthermore, different tissues of the same plant may contain different alkaloids (Osbourn & Lanzotti, 2009).

Besides plants, they are found in certain types of fungi, such as psilocybin in the fungus of the genus *Psilocybe*, and in animals, such as bufotenin in the skin of some toads. Many marine organisms also contain alkaloids. Some amines, such as adrenaline and serotonin, which play an important role in higher animals, are similar to alkaloids in their structure and biosynthesis and are sometimes called alkaloids (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Ways of alkaloid **biosynthesis** are numerous and cannot be easily classified. However, there are a few typical reactions involved in the biosynthesis of various classes of alkaloids, including synthesis of Schiff bases and Mannich reaction. Biological precursors of most alkaloids are amino acids, such as ornithine, lysine, phenylalanine, tyrosine, tryptophan, histidine, aspartic acid, and anthranilic acid. Nicotinic acid can be synthesized from tryptophan or aspartic acid (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

1.1.2. Phenols & polyphenols

Phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) bound directly to an aromatic hydrocarbon group (6-carbon ring). The simplest example is phenol (C_6H_5OH) (Fig. 1.1).

Polyphenols are characterized by the presence of large multiples of phenol structural units. They are described as generally moderate water-soluble compounds, with molecular weight of 500–4000 Da, >12 phenolic hydroxyl groups, and 5–7 aromatic rings per 1000 Da, though the limits to these ranges are often somewhat flexible (Quideau *et al*., 2011).

Although similar to alcohols, phenols have unique properties and are not classified as alcohols (since the hydroxyl group is not bonded to a saturated carbon atom). They have higher acidities due to the aromatic ring's tight coupling with the oxygen and a relatively loose bond between the oxygen and hydrogen. Phenols range from simple tannins to the more complex flavonoids that give plants much of their red, blue, yellow, and white pigments (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Phenolic acids' **distribution in nature** includes mushroom basidiomycetes species (Barros *et al*., 2009). Volatile phenolic compounds are also found in plant resin, where they may attract benefactors such as parasitoids or predators of the herbivores that attack the plant. The most abundant polyphenols are the condensed tannins, found in virtually all families of plants, and comprising up to 50% of the dry weight of leaves (Osbourn & Lanzotti, 2009). They are polymers composed of 2 to 50 (or more) flavonoid molecules, inhibit herbivore digestion by binding to consumed plant proteins and making them more difficult for animals to digest, and by interfering with protein absorption and digestive enzymes (Van Soest, 1982; Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Other examples of phenols used for plant defence are lignin, silymarin and cannabinoids. Silica and lignins, which are completely indigestible to animals, grind down the mandibles of insects. Some phenols have antiseptic properties and are used in formulating disinfectants, while others possess estrogenic or endocrine disrupting activity (Osbourn & Lanzotti, 2009). Polyphenols can also be found in animals; in arthropods like insects and crustaceans, they play a role in epicuticle hardening (sclerotisation) (Dennell, 1947; Wigglesworth, 1988).

Notable sources for human consumption of natural phenols include berries, tea, beer, olive oil, chocolate or cocoa, coffee, pomegranates, popcorn, yerba maté, fruits and fruit based drinks (including cider and wine) and vegetables. Phenolic compounds in beverages have been shown to be helpful in the colour and sensory components, such as alleviating bitterness (Donovan *et al*., 1998). Herbs and spices, nuts (walnuts, peanuts) and algae are also potentially significant for supplying certain natural phenols. Such foods containing natural phenols are generally considered as health food. Plant polyphenols also have antioxidant action and may help to reduce tooth decay (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009; Ferrazzano *et al*., 2011).

In phenol **biosynthesis**, most of the natural phenols are derived from secondary plant metabolism of the shikimic acid pathway, the malic acid pathway or both. The aromatic amino acid phenylalanine, synthesized in the shikimic-acid pathway, is the common precursor of phenol containing amino acids and phenolic compounds. In plants, the phenolic units are esterified or methylated, and the polyphenols are submitted to conjugation. Many natural phenols are found in the glycoside form instead of the aglycone form; the glycosylated form increases the solubility (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

1.1.3. Glycosides & glucosinolates

A **glycoside** is a molecule in which a sugar is bound to a non-carbohydrate moiety, usually a small organic molecule. The sugar group is then known as the glycone and the non-sugar group as the aglycone or genin part of the glycoside. The glycone can consist of a single sugar group (monosaccharide) or several sugar groups (oligosaccharide) (Fig. 1.1).

Glycosides play important roles in living organisms: many plants store chemicals in the form of inactive glycosides. These can be activated by enzyme hydrolysis, which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medicines. In animals and humans, poisons are often bound to sugar molecules as part of their elimination from the body (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

The **glucosinolates** are a class of organic compounds that contain sulphur and nitrogen and are derived from glucose and an amino acid. They are water-soluble anions and belong to the glucosides (glycosides derived from glucose). Every glucosinolate contains a central carbon

atom, which is bound via a sulphur atom to the thioglucose group (making a sulphated ketoxime) and via a nitrogen atom to a sulphate group. In addition, the central carbon is bound to a side group; different glucosinolates have different side groups, and it is variation in the side groups that is responsible for the variation in the biological activities of these plant compounds (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

About 120 different glucosinolates are known to occur naturally in plants. They occur as secondary metabolites of almost all plants of the order Brassicales (including the families Brassicaceae, Capparidaceae and Caricaceae), but also in the genus *Drypetes* (family Euphorbiaceae) (Rodman *et al*., 1996; Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Because the use of glucosinolate-containing crops as primary food source for animals was shown to have negative effects, food crops that contain very low amounts of glucosinolates (e.g. canola) have been developed. The glucosinolate sinigrin, among others, was shown to be responsible for the bitterness of cooked cauliflower and Brussels sprouts (Van Doorn *et al*., 1998; Schonhof *et al*., 2004). On the other hand, plants producing large amounts of glucosinolates also are of interest because they can serve as sources for natural pesticides. Some of these compounds are also under investigation for mitigating cancer, with sulphoraphane from broccoli being the best known example (Nestle, 1998; Osbourn & Lanzotti, 2009).

1.1.4. Fatty acid synthase products

A **fatty acid** is a carboxylic acid with a long unbranched aliphatic tail (chain), which is either saturated or unsaturated (Fig. 1.1). Fatty acids are usually derived from triglycerides or phospholipids. When they are not attached to other molecules, they are known as "free" fatty acids. Fatty acids are important sources of energy because, metabolized, they yield large quantities of ATP (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Fatty acids without double bonds are called saturated, while fatty acids that have double bonds are known as unsaturated. The two carbon atoms at either side of the double bond can occur in a *cis* or *trans* configuration. A *cis* configuration means that adjacent hydrogen atoms are on the same side of the double bond, while a *trans* configuration means that the next two hydrogen atoms are bound to opposite sides. The differences in geometry play an important

role in biological processes, and in the construction of biological structures such as cell membranes (Fig. 1.2).

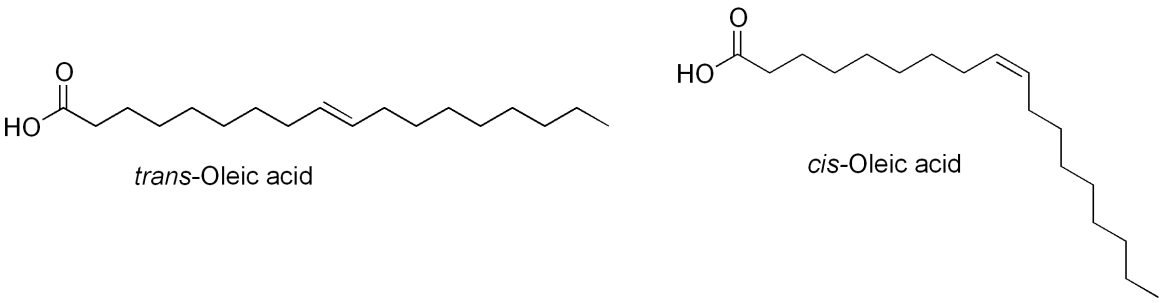


Figure 1.2. Comparison of the *trans* and the *cis*-isomer of oleic acid.

Fatty acids that are required by the human body, but cannot be made in sufficient quantity from other substrates (and therefore must be obtained from food) are called essential fatty acids. Humans lack the ability to introduce double bonds in fatty acids beyond carbons 9 and 10, as counted from the carboxylic acid side. Two essential fatty acids are linoleic acid (LA) and α-linolenic acid (ALA), which are widely distributed in plant oils (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

1.1.5. Nonribosomal peptides

Nonribosomal peptides (NRP) are a class of peptide secondary metabolites, usually produced by microorganisms like bacteria and fungi. They are a very diverse family of natural products with an extremely broad range of biological activities and pharmacological properties. Examples of nonribosomal peptides are antibiotics like actinomycin, antibiotic precursors, cytostatics and immunosuppressants (several of which are in commercial use), siderophores, pigments like indigoidine and toxins like microcystins and cyanotoxins from cyanobacteria (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Biosynthesis of nonribosomal peptides is done by specialized nonribosomal peptide-synthetase (NRPS) enzymes, which – unlike the ribosomes – are independent of mRNA. Each nonribosomal peptide synthetase can synthesize only one type of peptide. The enzymes are organized in modules that are responsible for the introduction of one additional amino acid.

The biosynthesis of nonribosomal peptides shares characteristics with the polyketide and fatty acid biosynthesis. Due to these structural and mechanistic similarities, some nonribosomal peptide synthetases contain polyketide synthase modules for the insertion of acetate or propionate-derived subunits into the peptide chain (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Epothilones, a new class of cancer drugs that prevent cancer cells from dividing by inhibition of microtubule function, are hybrids of the above two classes (Rosenberg *et al*., 2005).

1.1.6. Terpenes & terpenoids

Terpenes and their derivated **terpenoids** (also known as isoprenoids) are a class of hydrocarbons, produced by a wide variety of plants, particularly conifers. With over 55,000 entities being structurally identified, they represent the largest family of natural compounds. The name "terpene" is derived from the word "turpentine", since terpenes are the major components of resin and the turpentine produced from it (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. They contribute to the scent of eucalyptus and lavender, the flavours of cinnamon, cloves, and ginger, and the aroma and flavour of hops in beers. Aside from extensive use for their aromatic qualities, they also play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic, and other pharmaceutical functions. Well known examples include latex (natural rubber), menthol, vitamin A, cholesterol and the cannabinoids found in *Cannabis* (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

In terpene **biosynthesis,** all terpenes are derived from units of isoprene, which has the molecular formula C_5H_8 (Fig. 1.1). Terpene biosynthesis is mediated by two biosynthetic pathways: in eukaryotes, the cytosolic mevalonate (MVA) pathway is responsible for biosynthesis of the universal C5 building blocks of all isoprenoids. In prokaryotes, terpenes are derived from the plastid-located methyl-erythritol (MEP) pathway (also called desoxyxylulose phosphate or DXP pathway) (Eisenreich *et al*., 1998). In plants, both pathways operate. Both biosynthetic routes eventually lead to formation of the activated

isoprene units dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), the precursor from which all other terpenes are formed via head-to-tail or tail-to-tail linkage and subsequently can undergo cyclization and other modifications, e.g. oxidations or rearrangements (Dubey *et al*., 2003). Although MVA and DXP pathway are located in different compartments, there is an exchange between the two biosynthetic routes, especially from the plastidial to the cytosolic pathway. This has become particularly evident in the case of several sesquiterpenes, which are synthesized from DMAPP and IPP units provided by the DXP pathway, but not from MVA (Hemmerlin *et al*., 2003).

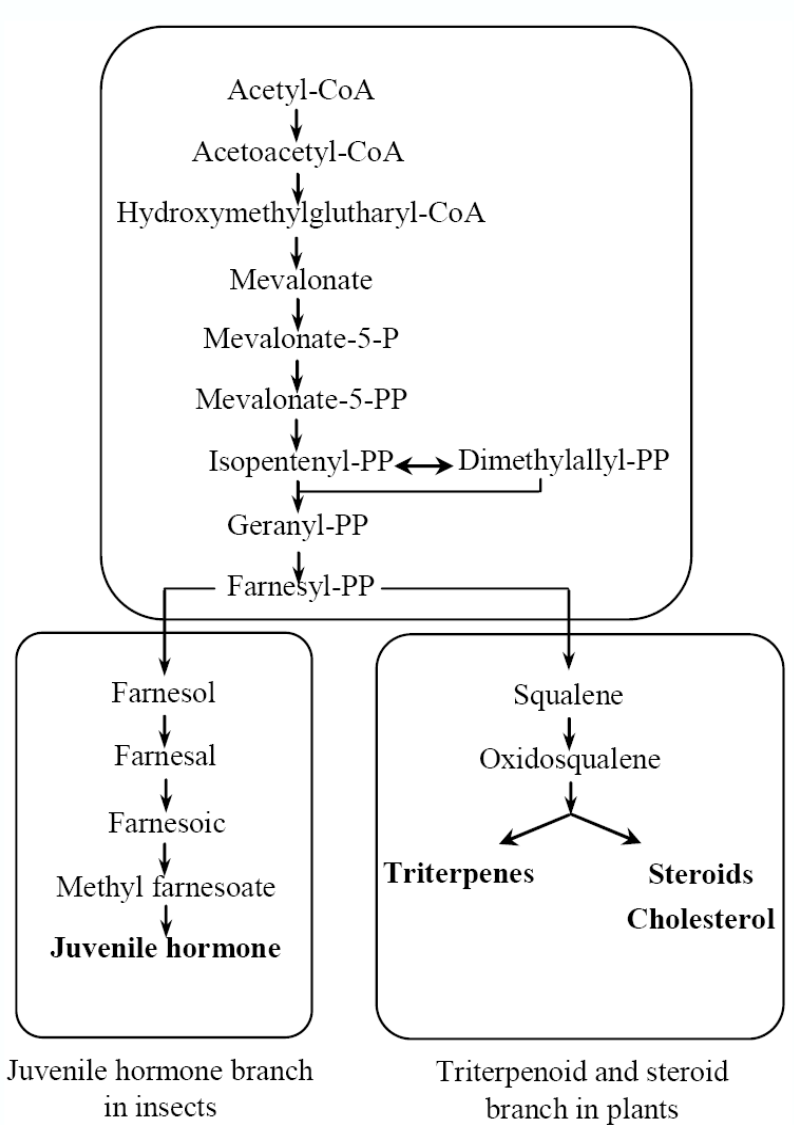


Figure 1.3. Flow diagram of the cytosolic mevalonate (MVA) pathway. The pathway is the same in plants and insects until formation of farnesyl diphosphate (FPP); from there on, the pathway leads to juvenile hormone synthesis in insects, and to steroid and triterpene synthesis in plants.

On the subject of plant defence against insects, it is interesting to note that the first part of the MVA pathway, until formation of farnesyl diphosphate (FPP), is exactly the same in insects and plants. However, in insects, FPP leads to formation of juvenile hormone, an insect hormone that plays a role in the regulation of moulting (and other functions) (Fig. 1.3). This branch of the terpene biosynthesis pathway is unique for insects (Belles *et al*., 2005). In plants, FPP leads to the formation of squalene, which is the common starting point for biosynthesis of triterpenes and steroids, e.g. cholesterol (Dubey *et al*., 2003). Insects lack this branch of the pathway, which means that they cannot produce endogenous cholesterol and have to extract the necessary precursors for cholesterol and other steroids from their food (Belles *et al*., 2005).

As chains of isoprene units are built up, the resulting terpenes are classified sequentially by size as hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, and tetraterpenes.

Steroids and sterols are also produced from terpenoid precursors, including cholesterol, vitamin D, and (steroidal) saponins.

1.1.6.1. Steroids

A **steroid** is a type of terpenoid compound that contains a specific arrangement of four rings that are joined to each other. Hundreds of distinct steroids are found in plants, animals, and fungi; examples include cholesterol, the sex hormones estradiol and testosterone, and the insect moulting hormone 20-hydroxyecdysone (Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

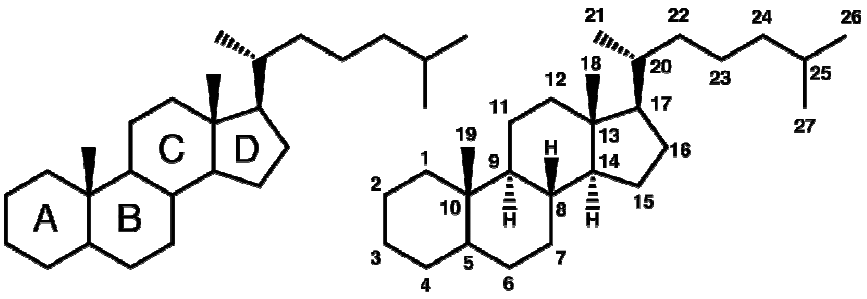


Figure 1.4. International Union of Pure and Applied Chemistry (IUPAC) recommended ring lettering (left) and atom numbering (right) of the steroid skeleton. The four rings (A-D) form a sterane core.

The sterane core of steroids is composed of seventeen carbon atoms bonded together to form four fused rings: three cyclohexane rings (designated as rings A, B, and C in the figure to the right) and one cyclopentane ring (the D ring). Steroids vary by the oxidation state of these rings, and by the number and nature of the functional groups attached to them (Fig. 1.4).

Steroid **biosynthesis** is an anabolic metabolic pathway that produces steroids from simple precursors. Sterols are special forms of steroids, with a hydroxyl group at position-3 and a skeleton derived from cholestane. Steroids are derived either from the sterol lanosterol (animals and fungi) or from cycloartenol (plants), with both lanosterol and cycloartenol being derived from the cyclization of the triterpene squalene. In contrast to animals, where cholesterol is the major sterol, many plant sterols are methylated or ethylated at C-24 of the side chain, e.g., campesterol and stigmasterol. These phytosterols are constituents of biomembranes in plants and influence their permeability. Phytosterols inhibit the absorption of cholesterol in animals; since they are more lipophilic than cholesterol, they are more readily incorporated into the micelles involved in fat digestion. Esters of phytosterols are therefore used as cholesterol-lowering food additives (Dewick, 2002; Belitz *et al*., 2004; Osbourn & Lanzotti, 2009).

In humans and other animals, the biosynthesis of steroids follows the mevalonate pathway that uses acetyl-CoA as building blocks to form dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) (Grochowski *et al*., 2006). As mentioned before, insects however are unable to biosynthese steroids by themselves, making steroid precursors an essential part of their diet (Belles *et al*., 2005).

1.1.6.1.1. Ecdysteroids and 20E

Ecdysteroids are a family of polyhydroxylated steroid hormones, which basic structure consists of cholesterol and a side chain. They are derived from enzymatic modification of cholesterol by p450 enzymes by a mechanism similar to steroid synthesis in vertebrates. They were first identified as insect moulting hormones by Butenandt and Karlson in 1954, and were later also found in crustaceans and arachnids (Lafont, 2000a). Their discovery in lower invertebrates made them known as general invertebrate steroid hormones, but they are also present in 5-6% of plant species in the form of phytosteroids, often in higher concentrations than those typically found in arthropods (Dinan, 1995).

One of the most well-known examples is the moulting hormone 20-hydroxyecdysone (20E), the archetypical ecdysteroid in insects and arthropods, which is the most commonly occurring and the most abundant (Lafont, 2000a). It regulates larval moults, onset of pupa formation, and metamorphosis in insects and other arthropods (Fig. 1.5).

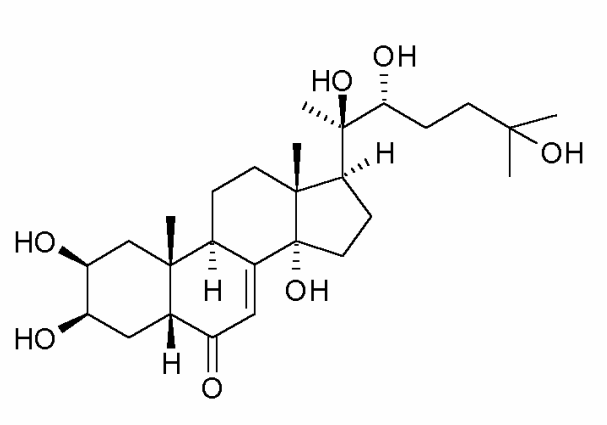


Figure 1.5. Chemical structure of 20-hydroxyecdysone.

Ecdysteroids fulfil diverse tasks as hormones, pheromones or insect deterrents (Nijhout, 1994). Their most frequent and prominent role is their function as moulting hormones, thereby controlling not only insect and arthropod moulting, but also reproduction and other physiological processes such as embryonic development (Riddiford *et al*., 2000; Spindler *et al*., 2001; Truman & Riddiford, 2002). In plants, they are generally considered secondary metabolites that protect against phytophagous insects either by feeding deterrency or toxicity (Lafont, 1997; Dinan, 2001). Several reviews about the various aspects of ecdysteroids exist in the literature (Lafont, 1997, 2000a, b; Dinan & Lafont, 2006).

The fact that the hormonal actions of ecdysteroids are specific to invertebrates, and that the ecdysteroids are chemically distinct from vertebrate steroid hormones, suggests that agents specifically disrupting ecdysteroid metabolism should not affect vertebrate steroid hormone systems. In this context, it has been suggested that phytoecdysteroids might be good candidates for development of new and safer strategies for crop protection against insects (Kubo *et al*., 1983; Soriano *et al*., 2004). However, they are less suitable as exogenous control agents (Dinan, 2001) due to their limited stability, high water solubility (washed away by the rain), limited activity by ingestion and high costs of production (compared to the synthetic compounds). Despite these limitations, ecdysteroids have high significance as model

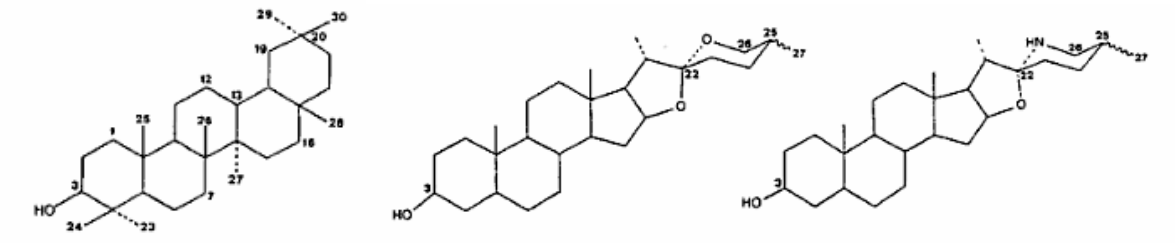
compounds for other interesting research areas such as medical gene switch applications or the characterisation of the ligand-binding site of ecdysteroid receptors (see 1.3.1.2) (Palli *et al.*, 2005).

1.2. Saponins

Saponins are a class of terpenoid secondary plant metabolites with divergent biological activities. They have anticarcinogenic, anti-inflammatory, antioxidant, hemolytic, immunostimulant and membrane-permeabilising properties, they affect food intake, growth and reproduction in animals, and they can be used as fungicides, molluscicides and pesticides, as well as against some bacteria and viruses (Francis *et al*., 2002; Sparg *et al*., 2004; Avato *et al*., 2006; Tava & Avato, 2006). It is generally assumed that they play an important role in plant defence against insects and diseases. They also have several pharmaceutical and medical applications (to lower cholesterol concentrations, as adjuvants in vaccines, to enhance penetration of macromolecules through cell membranes) and are used in the cosmetic (soap, emulsions) and feeding industry (food supplements) (Sparg *et al*., 2004).

1.2.1. Structural diversity and biosynthesis

The molecular structure of saponins consists of a hydrophilic sugar moiety linked to a hydrophobic aglycone. The combination of a hydrophilic (polar) and a hydrophobic (apolar) element into one molecule gives them soap-like properties: in aqueous solutions, they form foam (after shaking), diminish the surface tension and can aggregate into micelles. Their name comes from the Latin word for soap, "*sapo, -onis"*. The aglycone of a saponin (without the sugars) is also called a "sapogenin" (Hostettmann & Marston, 1995).



Triterpene Steroid Steroid alkaloid

Figure 1.6. The basic structure of triterpene, steroid and steroid alkaloid saponins, after Hostettmann & Marston (1995).

In general, they are divided into two main groups based on the structure of the aglycone: triterpene en steroidal saponins (Fig. 1.6). Triterpene aglycones consist of only 6-rings, with

30 C-atoms in total. Steroidal aglycones contain also 5-rings and have only 27 C-atoms. Some authors recognize a third group, that of the steroidal amines of steroidal alkaloids; they have the same basic structure as the steroidal saponins, but possess an NH-groep instead of an O-atom (Fig. 1.6).

Next to the aglycone, a saponin also contains one or more oligosaccharide chains (sugar moieties), which are glucosidally linked to the aglycone. 'Monodesmosidic' saponins only have one sugar group, usually at the C_3-position; 'bidesmosidic' saponins have a second one on the C_{26}- or C_{28}-position (Fig. 1.7). Tridesmosidic saponins and monodesmosidic saponins with the sugar chain on the C_{26}- or C_{28}-position are rare (Hostettmann & Marston, 1995; Sparg *et al*., 2004).

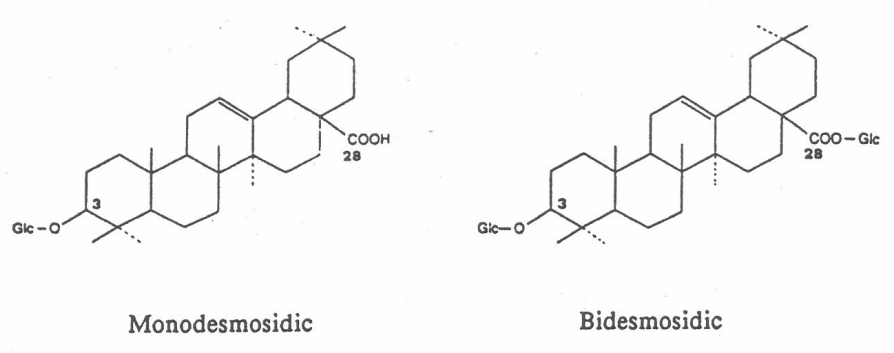


Figure 1.7. The basic structure of monodesmosidic and bidesmosidic saponins, after Hostettmann & Marston (1995).

The great complexity of saponin structure arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these moieties on the aglycone (Francis *et al*., 2002). Due to this complexity, saponins are difficult to classify. Because it is no longer customary to classify compounds based on their physicochemical or biological properties, a new classification based on the biosynthesis of the saponin carbon skeletons was proposed by Vincken *et al*. (2007).

Triterpene and steroid saponins are produced via terpene biosynthesis, of which the first steps were described above (see 1.1.6.1). Both triterpenoid and steroidal saponins originate from the same precursor-molecule, squalene (consisting of 6 isoprene units). Squalene is oxidized to oxidosqualene, and this is converted to cyclic derivatives. Cyclization of oxidosqualene to

saponins can proceed in two ways, either via the 'chair-chair-chair' or via the 'chair-boat-chair' conformation. An important difference between the two resulting skeletons lies in the stereochemistry. Triterpenes originate from the 'chair-chair-chair' conformation, while steroids arise from the 'chair-boat-chair' conformation. In the latter case, 3 C-atoms are split off in the MVA pathway (Vincken *et al*., 2007) (Fig. 1.3).

1.2.2. Saponins in the plant kingdom

Saponins occur in a great number of non-related plant species (mainly Angiosperms and a few Pteridophyts), both wild plants and cultivated crops. Triterpenoid saponins are mostly found in dicotyledonous species, while many of the major steroidal saponins are synthesized by monocots, such as members of the Liliaceae, Dioscoraceae and Agavaceae families (Hostettmann & Marston, 1995; Osbourn & Lanzotti, 2009; Vincken *et al*., 2007). Despite the sometimes negative biological actions of saponins on animals and humans, they do occur in a wide variety of crops and edible plants: triterpenoid saponins have been detected in many *Leguminosae* (legumes), such as soybeans, beans, peas and alfalfa *(Medicago),* in *Chenopodiaceae* like sugar beet, spinach and quinoa, and in *Theaceae* (tea), while steroidal saponins can be found in grasses like oats, in *Solanaceae* like Capsicum peppers, aubergine, tomato and potato, in *Alliaceae* (alliums) such as leek, onion and garlic and in *Asparagaceae* (asparagus) (Francis *et al*., 2002). One plant often contains several kinds of saponins, depending on age, place of growth or variety, and of the location inside the plant (roots, leaves, …). For a secondary plant metabolite, they occur in remarkably high concentrations: 5-10% in the roots of *Primula*, 2-12% in glycyrrhizine, 10% in de bark of *Quillaja* and the seeds of *Camellia,* and up till 13% in the seeds of the horse chestnut (Hostettmann & Marston, 1995). There is no clear relationship between the plant origin and the type of saponin, nor is there evidence that specific saponins are associated with particular parts of plants (Vincken *et al*., 2007).

Although the vast majority of sources are from plants, saponins have also been isolated from marine organisms (Riguera, 1997), especially in the Cuvierian tubules of sea cucumbers. It was suggested that they store them as a form of chemical defence (Van Dyck *et al*., 2010).

Commercial formulations of plant-derived saponins – e.g., from the soapbark tree, *Quillaja saponaria*, and from other sources – are available via controlled manufacturing processes, which make them of use as chemical and biomedical reagents (Sigma-Aldrich, 2009). In the

Asian region (China), saponin powder and solutions from tea seeds are sold as natural insecticides; a good example is “Liquid Tea Saponin” from Hangzhou Choisun Tea Sci-Tech Co., Ltd.

1.2.3. Insecticidal activity of saponins

Although little is known about the exact functions of saponins, it is generally accepted that they play an important role in plant defence. They are said to have noticeable effects on vertebrate and invertebrate herbivores as well as on viral, bacterial or fungal infections (for a review, see Francis *et al*., 2002; Sparg *et al*., 2004; Tava & Avato, 2006). Apart from the abovementioned properties, saponins also possess clear insecticidal activities, exerting a strong and rapid-working action - different from neurotoxicity - against a broad range of pest insects (Table 1.1). The most observed effects are increased mortality, lowered food intake, weight reduction, retardation in development and decreased fecundity. As a consequence, these interesting plant components open new strategies to protect crops in modern agriculture and horticulture with Integrated Pest Management (IPM) programs against pest insects, either by spraying, or by selecting high-saponin varieties of commercial crops.

1.2.4. Mechanisms underlying the insecticidal activity

Saponins give rise to increased mortality levels, lowered food intake, weight reduction, retardation and/or disruptions in development and decreased reproduction in pest insects. The exact mechanism underlying these effects is still largely unknown, but it is likely that it is a combination of several activities. Potential modes of action found in literature include repellent or deterrent activity, reduced uptake of food through the gut, blocking sterol assimilation, antagonistic or competitive activity on the ecdysteroid reporter complex, membrane-permeabilising abilities and apoptosis-inducing activity.

1.2.4.1. Repellent or deterrent activity

Numerous of the above mentioned studies reported a lower food intake of insects fed on saponin-containing food (by measuring the area of leaves/amount of artificial diet consumed), although in nearly all cases the test insects made at least some attempts to feed on the plants/diets before rejecting them (Adel *et al*., 2000; Szczepanik *et al*., 2001; Shinoda *et al*., 2002; Agerbirk *et al*., 2003; Agrell *et al*., 2003; Szczepanik *et al*., 2004; Taylor *et al*., 2004; Golawska *et al*., 2006). In all these cases, the reduction of food intake was dose-dependent; however, two exceptions were reported: Hussein *et al*. (2005) found that the rate of diet

Table 1.1. Examples of studies supporting the insecticidal activity of various saponins against a broad range of insects.

Source of saponin	Insect order	Target insect	Reference
Alfalfa (*Medicago sativa*)	Coleoptera	Red flour beetle (*Tribolium castaneum*)	Shany *et al*. (1970)
Alfalfa (*M. sativa*)	Hemiptera	Pea aphid (*Acyrthosphon pisum*) & potato leafhopper (*Empoasca fabae*)	Horber *et al*. (1974)
Alfalfa (*M. sativa*)	Hemiptera	Pea aphid (*A. pisum*)	Pedersen *et al*. (1976)
Leek (*Allium porrum*)	Lepidoptera	Leek-moth (*Acrolepiopsis assectella*)	Harmatha *et al*. (1987)
aginosid from leek *(A. porrum)*	Lepidoptera	Variegated cutworm (*Peridroma saucia*) & bertha armyworm (*Mamestra configurata*)	Nawrot *et al*. (1991)
commercial saponins	Hemiptera	Migratory grasshopper (*Melanoplus sanguinipes*)	Westcott *et al*. (1992)
Alfalfa (*M. sativa*)	Lepidoptera	European corn borer (*Ostrinia nubilalis*)	Nozzolillo *et al*. (1997)
Fenugreek (*Trigonella foenum-graecum*)	Coleoptera	Red Flour Beetle (*Tribolium castaneum*) & bean weevil (*Acanthoscelides obtectus*)	Pemonge *et al*. (1997)
Alfalfa (*M. sativa*)	Lepidoptera	Cotton leafworm (*Spodoptera littoralis*)	Adel *et al*. (2000)
luciamin from potato vine *(Solanum laxum*)	Hemiptera	Greenbug/wheat aphid (*Schizaphis graminum*)	Soulé *et al*. (2000)
Alfalfa (*M. sativa*)	Coleoptera	Colorado potato beetle (*Leptinotarsa decemlineata*)	Szczepanik *et al*. (2001)
Quillaja bark saponins from soapbark tree (*Quillaja saponaria*)	Diptera	Yellow fever mosquito (*Aedes aegypti*) & northern house mosquito (*Culex pipiens*)	Pelah *et al*. (2002)
Yellow rocket (*Barbarea vulgaris*)	Lepidoptera	Diamondback moth (*Plutella xylostella*)	Shinoda *et al*. (2002)
Yellow rocket (*B. vulgaris*)	Lepidoptera	Diamondback moth (*P. xylostella*)	Agerbirk *et al*. (2003)
Medicago species	Coleoptera	Colorado potato beetle (*L. decemlineata*)	Szczepanik *et al*. (2004)
Field pies (*Pisum sativum*)	Coleoptera	Rice weevil (*Sitophilus oryzae*)	Taylor *et al*. (2004)
Alfalfa roots (*M. sativa*)	Coleoptera	Hairy rose beetle (*Tropinota squalida*)	Hussein *et al*. (2005)
Alfalfa (*M. sativa*)	Hemiptera	Pea aphid (*A. pisum*)	Golawska *et al*. (2006)
Balanites aegyptiaca	Diptera	Yellow fever mosquito (*A. aegypti*)	Wiesman & Chapagain (2006)
Quillaja bark saponins from soapbark tree (*Q. saponaria*)	Hemiptera	Pea aphids (*A. pisum*) & cotton leafworm (*S. littoralis*)	De Geyter *et al*. (2007a)
Red buckeye (*Aesculus pavia*)	Lepidoptera	Leafminer (*Cameraria ohridella)*	Ferracini *et al*. (2010)
various saponins	Lepidoptera	Corn earworm (*Helicoverpa zea*) & fall armyworn (*S. frugiperda*)	Dowd *et al*. (2011)

consumption was not affected (in spite of high mortality rates), and Soulé *et al*. (2000) saw an equal decrease in survival for all concentrations of saponins tested.

1.2.4.2. Slowing down the passage of food through the gut

According to Ishaaya (1986) saponins slow down the passage of food through the insect gut. Perhaps they reduce the digestibility of the food by inhibiting the secretion of digestive enzymes (proteases) (Ishaaya & Birk, 1965; Golawska *et al*., 2006) or by formation of sparingly digestible saponin-protein complexes (Potter *et al*., 1993). An obstruction of alimentary contents in the gut would limit or inhibit food uptake. Starvation, as well as disturbance of the digestion and assimilation processes, could reduce the insect growth rate. Adel *et al*. (2000) supported this hypothesis because in their experiment the treated larvae lost more weight than the controls just before pupation (gut purge).

1.2.4.3. Blocking sterol uptake

Insects are not capable of synthesizing sterol structures by themselves (Belles *et al*., 2005), but they do need these as precursors for the synthesis of steroids like cholesterol and the insect moulting hormone 20-hydroxyecdysone (20E). That means they have to gain them from their food: cholesterol or phytosterols from plants act as precursors (Belles *et al*., 2005). Shany *et al*. (1970) suggested that saponins in the diet block sterol uptake. Saponins can form insoluble complexes with sterols, thereby preventing their absorption. If all cholesterol in the food is bound to saponins, the insects cannot utilise it. Moreover, if larvae feed on a saponin-rich food, the ingested saponins may complex even cholesterol in their body, and thus suspend the biosynthesis of ecdysteroids. This could cause a disturbance of ecdysis (moulting failures) (Harmatha *et al*., 1987; Harmatha, 2000). Harmatha *et al*. (1987) reported an increase in ecdysial failures of leek-moth larvae (*Acrolepiopsis assectella*) on diets containing steroidal saponins. It was also observed that the effects of the saponins could be countered by adding a surplus of cholesterol or plant sterols to the diet (Shany *et al*., 1970; Harmatha *et al*., 1987).

1.2.4.4. Antagonistic or competitive activity on the ecdysteroid reporter complex

Because (steroidal) saponins have a steroid structure and show structural similarity to ecdysteroids - like the insect moulting hormone 20E - it has been suggested that they might exert an agonistic or antagonistic/competitive activity on the ecdysteroid reporter complex (EcR), the binding site for 20E. Some other secondary plant compounds with a similar structure (and even without) have been reported to have such effects (Dinan *et al*., 2001).

Such activity could disrupt the timing of moulting and metamorphosis (pupating), which is crucial for normal insect development. Aside from the studies already mentioned above (Shany *et al*., 1970; Harmatha *et al*., 1987), there are few, if any, indications supporting this hypothesis. Digitonin and aginosid, two steroidal saponins, were tested for their direct effect on the ecdysone reporter in a *Drosophila melanogaster* B-II assay by Harmatha & Dinan (1997), together with two additional leek flower saponins and some aglycones; but none of the compounds showed significant agonistic or antagonistic effects on EcR. In another BII cell bioassay for ecdysteroid agonist and antagonist activities (Dinan *et al*., 2001) there were no saponins showing agonist activity but few showed principal antagonistic activity on the EcR complex. Similar results for triterpenoid saponins could not be found in literature; it is likely that they have not often been tested because their structure only remotely resembles that of steroids.

1.2.4.5. Membrane-permeabilising ability

Another hypothesis often referred to in literature is that saponins disrupt the stability of the cell membrane on the cellular level. Not only has such effect been proven for hematocytes and a number of other cell types (Francis *et al*., 2002; Sparg *et al*., 2004), but saponins are also used in the medical industry as permeabilising agents (Mick *et al*., 1988; Humbel *et al*., 1998; Baumann *et al*. 2000). The primary action of membranolytic saponins upon the cell is to cause a general increase in permeability of plasma membranes. They can interact with and permeabilise the small intestine mucosal cells of animals, leading to a marked reduction in their ability to transport nutrients (Francis *et al*., 2002). This is suspected to result from their bipolar structure. The lipophilic component of the saponin could be easily integrated into the lipid fraction of the plasma membrane. The hydrophilic glycosidic portion that follows the lipid fraction presumably irreversible disorders the plasma membrane and disrupts its integrity. It is of interest to mention that this membrane-permeabilising ability of saponins can also have beneficial effects on animals (and humans): small quantities of dietary saponins may assist in the absorption of nutrients, drugs and toxins by increasing the permeability of the small intestine mucosa (Chuke, 1976; Oakenfull *et al*., 1979). However, so far there are no indications for beneficial effects on insects, as tests on insect cells in general are scarce.

1.4.2.6. Apoptosis-inducing activity

Several saponins have been reported to induce apoptosis in human cancer cells, explaining their lethal activity (Haridas *et al*., 2001; Chwalek *et al*., 2006; Niu *et al*., 2008). No studies

on insect cells are known to us, but it is possible that the same effect is responsible for reducing cell viability in insect cell culture lines. A serious disturbance of the cell growth/apoptosis balance could cause health problems and even death also in living insects.

Of course, one should always keep in mind that the situation under natural conditions might be a bit more complicated than in the laboratory. For example, an alternative hypothesis could be that saponins do not affect insects at all, but instead attack the microflora living in the insect gut. For most herbivore insects (invertebrates) the digestion of leaf material is mediated by symbiotic microorganisms that reside in the hindgut (Waterman, 1993), so any compound that kills off a critical amount of these supporting bacteria could seriously undermine the insect's digestive capabilities.

1.3. Targets for plant secondary metabolites in insects

1.3.1. EcR as target for insecticides

1.3.1.1. Ecdysone receptor (EcR)

The ecdysone receptor (EcR) is a nuclear receptor found in arthropods, where it controls development and contributes to other processes such as reproduction. It responds to pulses of 20-hydroxyecdysone (see 1.1.6.1.1) that occur during insect development. Since ecdysteroid hormones are hydrophobic, they can traverse lipid membranes and diffuse into cells. The active ecdysteroid binds on the EcR, a ligand-activated transcription factor found in the nuclei of insect cells, which forms a heterodimer with the ultraspiracle protein (USP) (Riddiford *et al*., 2000) (Fig. 1.8). This in turn leads to the activation of a cascade of many other genes, ultimately causing physiological changes that result in ecdysis (moulting) (Henrich, 2005).

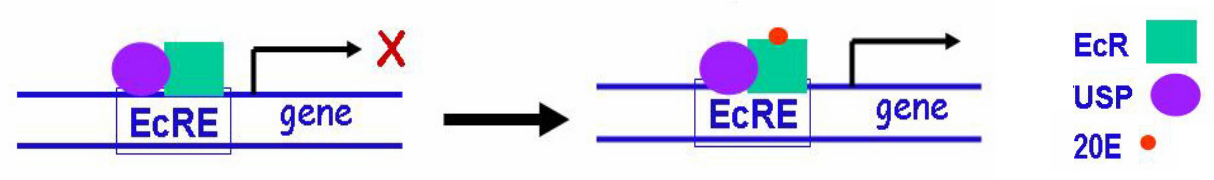


Figure 1.8. Schematic representation of the activation of the ecdysone reporter complex (EcRE) by 20E.

The EcR-USP/RXR heterodimer complex is a non-covalent heterodimer of two proteins, the EcR protein and ultraspiracle protein (USP) (Yao *et al*., 1993) or retinoid X receptor (RXR) (Hayward *et al*., 2003). These nuclear hormone receptor proteins belong to a superfamily or nuclear receptors (NRs) which mediate transcriptional responses to steroids and other lipophilic molecules; they are the insect orthologs of the mammalian farnesoid X receptor (FXR) and retinoid X receptor (RXR) proteins, respectively. EcR was first identified in the fruit fly, *Drosophila melanogaster* (Koelle *et al*., 1991) (Fig. 1.9), and has since then been cloned from several insects (Henrich, 2005), crustaceans (e.g. Asazuma *et al*., 2007; Chung *et al*., 1998) and a few chelicerates (Guo *et al*., 1997; Nakagawa *et al*., 2007). The structure differs slightly between different phylogenetic groups; based on sequence homology considerations (Hayward *et al*., 1999), most researchers reserve the term USP for the EcR partner from lepidopteran and dipteran insects (Mecopterida), and use RXR in all other instances (Bonneton *et al*., 2003; 2006).

Figure 1.9. Crystallographic structure of the ligand binding domain of the ecdysone receptor from *Drosophila melanogaster* complexed with ponasterone A (space-filling model, carbon = white, oxygen = red).

EcR and USP share the multi-domain architecture common to all nuclear hormone receptors, namely an N-terminal transcriptional activation domain (A/B domain), a DNA-binding domain (C domain, highly conserved between receptors), a linker region (D region), a ligand-binding domain (E domain, moderately conserved), and in some cases a distinct C-terminal extension (F-domain) (Koelle *et al.*, 1991). The DNA-binding domains of EcR and USP recognise specific short sequences in DNA, and mediate the binding of the heterodimer to these ecdysone response elements (EcREs) in the promoters of ecdysone-responsive genes. The ecdysteroid-binding pocket is located in the ligand binding domain of the EcR subunit, but EcR must be dimerised with USP (or with RXR) for high-affinity ligand binding to occur. In such circumstances, the binding of 20E (or an ecdysone agonist ligand) triggers a conformational change in the C-terminal part of the EcR ligand-binding domain that leads to transcriptional activation of genes under EcRE control (Bourguet *et al.*, 2000). There is also a ligand-binding pocket in the corresponding domain of USP, but its natural ligand remains uncertain, and USPs appear to be locked permanently in an inactive conformation (Clayton *et al.*, 2001).

1.3.1.2. Practical applications: insect cell screening systems & ecdysteroid-responsive cell lines

In scientific research, ecdysone receptors have two main fields of application:

- *Insecticides:* alternative methods of insect pest control aim for higher specificity, lower toxicity and increased potency. Ecdysteroid receptors are potential targets in the development of selective insect growth regulators because they work selectively against insects, and sometimes even a particular order or stage. Most commercial interest goes to the development of ecdysteroid agonists, that may or may not be steroids themselves; typically, such ecdysteroid agonist compounds mimic the activity of the natural insect moulting hormone (20E), inducing a lethal premature moulting in susceptible insects, especially Lepidoptera. Insect ecdysone receptors are currently better characterized than those from other arthropods, and mimics of ecdysteroids (like dibenzoylhydrazine compounds) are being used commercially as caterpillar-selective larvicides (Dhadialla *et al*., 1998, 2005).
- *Gene-switch:* 20E and other ecdysteroids are also used in biochemistry research as inducers in transgenic species. After putting a new gene into an animal/plant so that its expression is under the control of an introduced ecdysone receptor, adding or removing 20-hydroxyecdysone or ecdysone agonists from the nutient source gives a convenient way to turn the inserted gene on or off. As such it allows for controlled gene expression in scientific research, agriculture, and medicine, including potential use in gene therapy (Graham, 2002; Palli *et al*., 2005).

In the first field, **insect cell lines cultures** are useful tools in the study of the effects and mode of action of a wide range of substances in a rapid and controlled way. Especially on the issue of hormone signalling and metabolic pathways, use of cell system bioassays has several advantages. Insect cell line cultures are easy to cultivate, so growing them is less work-intensive, faster and less expensive than the rearing of living insects, especially in large quantities. Bioassays on cells are also less time consuming than those carried out with whole insects. Second, because they form an isolated system, there is less interference from other cells and tissues and no influence of endogenous hormones and/or other potential active substances, which allows for a more controlled environment and less unwanted variation in results. In addition, they tolerate high concentrations of metabolic by-products and high expression levels can be reached with relative ease. Also, cloning of cDNAs for various genes from a cell line is comparatively easier than using whole animals due to less

complexity of RNA (Palli *et al*., 1995; Smagghe, 2007).

Presently, insect cell lines have been of great assistance to researchers working on many multidisciplinary fields of science such as physiology, morphogenesis, virology, pathology, biochemistry, genetics and other fields of biology and medicine. In the area of toxicology and insecticide research, they are good tools for screening purposes and identifying the mode of action of various test compounds, while providing the researchers a large amount of homogeneous material and a simpler model system (Smagghe, 2007). Additionally, the easy handling and the fast response of the cell culture systems facilitate their use as a fast predictive method in the field of the development of insecticide resistance, offering new opportunities to scientists.

Aside from the normal insect cell culture lines, a large number of **ecdysteroid-responsive cell lines** are available. They are designed to contain markers to visualize the activity of the ecdysteroid receptor (EcR) and form irreplaceable assets to study the mode of action of compounds that mimic the action of ecdysteroids (Dinan *et al*., 2001; Smagghe, 2007). High-throughput screening systems are very important in the search and identification of compounds that disrupt the insect endocrine system, because they allow scientists to test a large number of potentially active compounds in a very limited amount of time. A good example is the transformed silkmoth (*Bombyx mori*)-derived Bm5 cell line that responds to the addition of ecdysone-like substances through the expression of the green fluorescent protein (GFP) /luciferase reporter, which leads to the appearance of green fluorescence /luminescense (Fig. 1.10). Because the amount of green fluorescence can be easily quantified in individual wells of a 96 well plate by a fluorescence plate reader, the engineered cell lines can be used for the screening of compounds with ecdysteroid mimetic activity in high-throughput format.

Figure 1.10. Schematic representation of the gene sequence inserted in an ecdysteroid-responsive cell line, leading to the expression of the green fluorescent protein (GFP) gene /luciferase reporter gene and the appearance of green fluorescence /luminescense after activation by 20E.

While most of the current high-throughput screening system targets lepidopteran insects (moths and butterflies) (Dhadialla *et al*., 1998), future work should aim at the expansion of the technology to cell lines that originate from other insect orders, such as Diptera (flies and mosquitoes) and Coleoptera (beetles). The availability of such insect order-specific screening systems is predicted to assist significantly in the search of new compounds that target the insect endocrine system with increased specificity and potency.

1.3.1.3. Successes in pest control: synthesizing ecdysone agonists

Pest management strategies have evolved over the years from broad-spectrum to target specific narrow spectrum pesticides. Neurotoxic insecticides such al DDT, organophosphates, and carbamates have been replaced with chemicals that are more insect specific. A serendipitous discovery at Rohm and Haas Company in Spring House, Pennsylvania, led to the development of differently substituted dibenzoylhydrazine (DBH) compounds as unique chemicals that act as ecdysone agonists with enormous potential for development as insect-specific control agents with little or no effect on non-target species (reviewed in Dhadialla *et al*., 1998, 2005). Surprisingly, these compounds bear no structural resemblance to the steroid moulting hormone, 20E, but yet they act by binding on the EcR.

Four DBH ecdysone agonists are currently available on the market as insecticides: tebufenozide (RH-5992), methoxyfenozide (RH-2485) and chromafenozide (ANS-118) are highly lepidopteran specific (Dhadialla *et al*., 1998, 2005; Yanagi *et al*., 2000). Halofenozide (RH-0345) is used to control coleopteran (scarabid larvae) and lepidopteran insects in turf and ornamentals (Dhadialla *et al*., 1998, 2005). Like 20E, they transactivate a succession of moult initiating transcription factors that, in turn, induce the expression of a group of moult-related genes. As a result of the expression of these up-regulated genes, the larva undergoes premature apolysis and head capsule slippage and takes on the appearance of the pharate larva. However, unlike 20E, which is cleared at this juncture, allowing the down-regulated genes to be expressed, these synthetic agonists are not cleared easily. Therefore, all the down-regulated events that occur as the titre of 20E decreases are repressed by the presence of the ecdysone agonist. The result is that the insect remains trapped in the moulting process and dies slowly from starvation and desiccation (Dhadialla *et al*., 1998, 2005).

The success of these compounds in insect control programs validates EcR as a valuable target for the development of environmentally friendly insecticides (Nakagawa, 2005). However, as

mentioned above, current available ecdysone agonists mainly target lepidopteran insects together with a limited number of coleopteran insects (Dhadialla *et al*., 1998). For the discovery of ecdysone agonists that target other insect groups, efficient screening systems based on activation of the EcR are needed.

1.3.2 The cell membrane as target for insecticides

1.3.2.1 The (insect) cell membrane

The cell membrane or plasma membrane is a biological membrane that surrounds the cytoplasm of a cell and, in animal cells, physically separates the intracellular components from the extracellular environment. It consists of the lipid bilayer of phospholipids with embedded proteins. Cell membranes maintain the cell potential and are involved in a variety of cellular processes such as cell adhesion, ion conductivity and cell signalling. They also play a role in anchoring the cytoskeleton to provide shape to the cell, and in attaching to the extracellular matrix and other cells to help group cells together to form tissues (Alberts *et al*., 2002) (Fig. 1.11).

The cell membrane is selectively permeable to ions and organic molecules and able to regulate what enters and exits the cell, thus facilitating the transport of materials needed for survival (fungi, bacteria and plants also have the cell wall which provides a mechanical support for the cell and precludes the passage of larger molecules). The cell membrane thus works as a selective filter that allows only certain things to come inside or go outside the cell, either actively (by transport proteins or endocytosis/exocytosis) or passively (by diffusion/osmosis).

1.3.2.1.1. Structure and composition

According to the **fluid mosaic model** of Singer and Nicolson (1972), biological membranes can be considered as a two-dimensional liquid in which all lipid and protein molecules diffuse more or less easily. Although the lipid bilayers that form the basis of the membranes do indeed form two-dimensional liquids by themselves, the plasma membrane also contains a large quantity of proteins, which provide more structural complexity. Examples of such structures are protein-protein complexes, pickets and fences formed by the actin-based cytoskeleton, and potentially lipid rafts.

Lipid bilayers form through the process of self-assembly. The cell membrane consists primarily of a thin layer of amphipathic phospholipids which spontaneously arrange so that the hydrophobic ‘tail’ regions are isolated from the surrounding polar fluid, causing the more hydrophilic ‘head’ regions to associate with the intracellular (cytosolic) and extracellular faces of the resulting bilayer and forming a continuous, spherical lipid bilayer (Fig. 1.11C). Despite

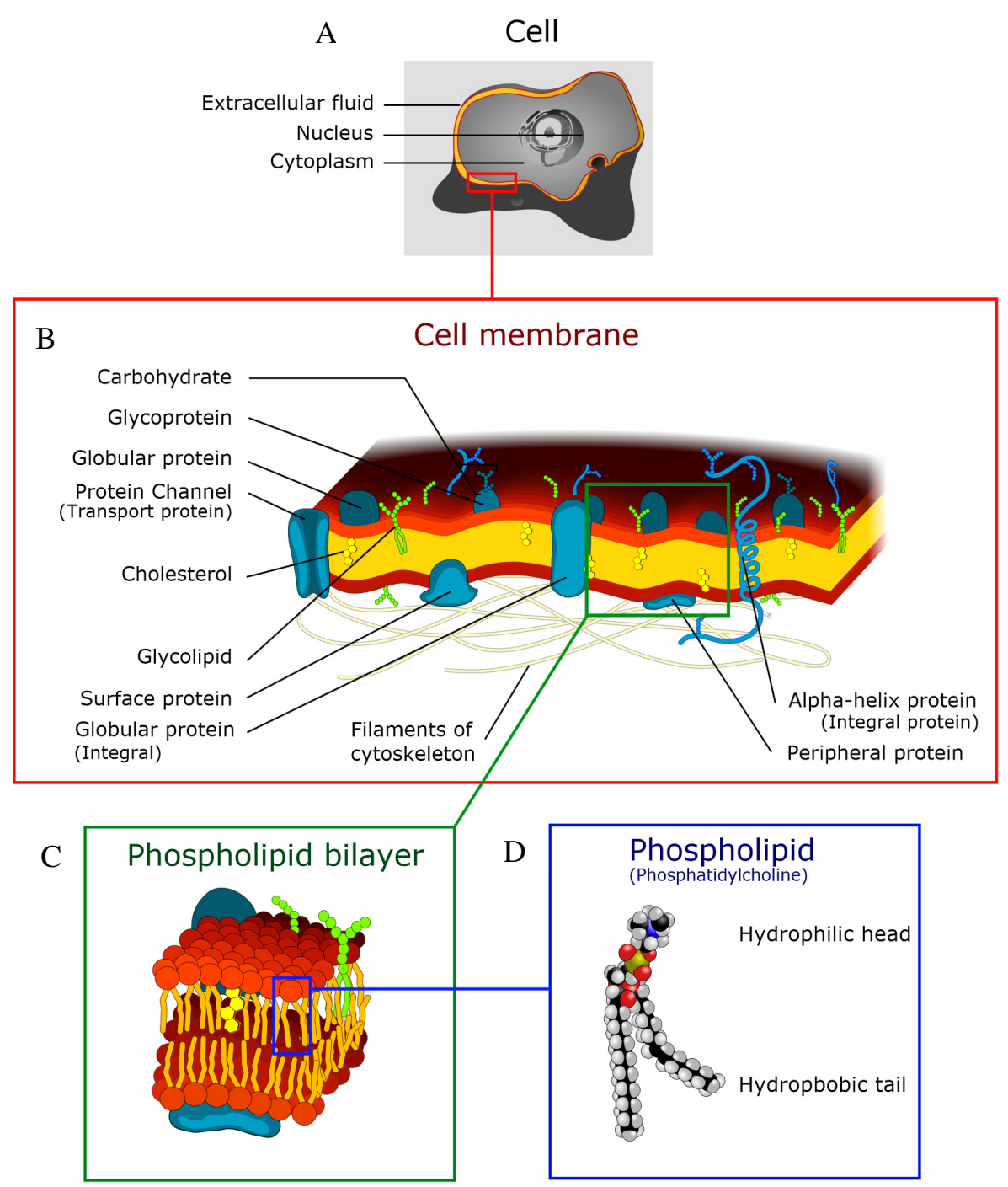


Figure 1.11. Illustration of a eukaryotic cell membrane. (A) General outline of the cell, (B) cell membrane with several kinds of proteins, (C) detail of the lipid bilayer, with the arrangement of the amphipathic lipid molecules and (D) molecular structure of a single phospholipid with its hydrophilic head and hydrophobic tail.

its function as the barrier, the structure is quite fluid and not fixed rigidly in place, as the entire membrane is held together via non-covalent interaction of hydrophobic tails. However, the exchange of phospholipid molecules between intracellular and extracellular leaflets of the

bilayer is a very slow process. Lipid rafts and caveolae are examples of cholesterol-enriched microdomains in the cell membrane.

Lipid bilayers are generally impermeable to ions and polar molecules. The arrangement of hydrophilic heads and hydrophobic tails of the lipid bilayer prevents polar solutes (e.g. amino acids, nucleic acids, carbohydrates, proteins, and ions) from diffusing across the membrane, but generally allows for the passive diffusion of hydrophobic molecules. This gives cells the ability to control the movement of these substances via transmembrane protein complexes such as pores, channels and gates.

The cell membrane is composed out of three main elements: lipids, carbohydrates, and proteins (Alberts *et al*., 2002):

- Lipids: The cell membrane consists of three classes of amphipathic lipids: phospholipids, glycolipids, and cholesterols. The amount of each depends upon the type of cell, but in the majority of cases phospholipids are the most abundant (Lodish *et al*., 2004). In red blood cell studies, 30% of the plasma membrane is lipid. In animal cells, cholesterol is normally found dispersed in varying degrees throughout cell membranes, in the irregular spaces between the hydrophobic tails of the membrane lipids, where it confers a stiffening and strengthening effect on the membrane (Alberts *et al*., 2002).
- Carbohydrates: Plasma membranes also contain carbohydrates, predominantly glycoproteins, but with some glycolipids (cerebrosides and gangliosides). They are responsible for formation of the glycocalyx, an extracellular polymeric matrix external to the plasma membrane, consisting of the carbohydrate moieties of membrane glycolipids and glycoproteins. It is an important feature in all cells, especially epithelia with microvilli. Recent data suggest the glycocalyx participates in cell adhesion, lymphocyte homing, and many others.
- Proteins: Proteins within the membrane are key to the functioning of the overall membrane. These proteins mainly transport chemicals and information across the membrane. Every membrane has a varying degree of protein content of different types (Table 1.2). The amount of protein differs between species and according to function, but the typical amount in a cell membrane is 50%. These proteins are undoubtedly important to a cell: approximately a third of the genes in yeast code specifically for them, and this number is even higher in multicellular organisms (Lodish *et al*., 2004).

Table 1.2. The different types of membrane proteins (Alberts *et al*., 2002).

Type	Description	Examples
Integral proteins or *transmembrane proteins*	Span the membrane and have (1) a hydrophilic cytosolic domain, (2) a hydrophobic membrane-spanning domain that anchors it within the cell membrane and (3) a hydrophilic extracellular domain. They interact widely with hydrocarbon chains of membrane lipids and can be released by agents that compete for the same nonpolar interactions.	Ion channels, proton pumps, G protein-coupled receptor
Lipid anchored proteins	Covalently bound to single or multiple lipid molecules; they hydrophobically insert into the cell membrane and anchor the protein. The protein itself is not in contact with the membrane.	G proteins
Peripheral proteins	Attached to integral membrane proteins or associated with the peripheral regions of the lipid bilayer by electrostatic interactions and hydrogen bonding with the hydrophilic phospholipid heads. These proteins tend to have only temporary interactions with biological membranes, and will easily dissociate to carry on their work in the cytoplasm.	Some enzymes, some hormones

The cell membrane, being exposed to the outside environment, is an important side of cell-cell communication. As such, a large variety of protein receptors and identification proteins, such as antigens, are present on the surface of the membrane. Functions of membrane proteins can also include cell-cell contact, surface recognition, cytoskeleton contact, signalling, enzymatic activity, or transporting substances across the membrane.

1.3.2.1.2. Variation

The cell membrane has different lipid and protein compositions in distinct types of cells. While human cells have been studied extensively, not so much is known about the specificities of insect cells, or the differences between species. In general, insect cell membranes seem to function largely the same as in mammalian cells; notable differences are that (1) insects were reported to have a unique enzyme secretion system that consists of splitting of membrane vesicles from the microvilli (Terra *et al*., 2006), and (2) the presence or absence of cholesterol can influence insect cell grow and susceptibility to certain viruses (Umashankar *et al*., 2008), as well as a number of other functions, since insect cell lines contain a rather low quantity of cholesterol in their plasma membrane (Opekarová & Tanner,

2003) and cannot produce cholesterol without precursors (Belles *et al*., 2005), which could potentially be a bottleneck for the overexpression of sterol-requiring membrane proteins (Opekarová & Tanner, 2003).

1.3.2.2 Insect alimentary canal

One of the ways insecticides can effect insect cell membranes is through ingestion, by effects on the insect alimentary canal. The gastrointestinal tract in insects consists of three parts, the fore-, mid- and hindgut (Fig. 1.12). In general, the foregut is involved in facilitating the uptake, storage, and physical processing of food. It is lined with a chitin-containing cuticle that is part of the insect exoskeleton. A valve separates the foregut and midgut. The midgut is the major site of food digestion and lacks a cuticle, but is lined with the peritrophic membrane, an anatomical structure that envelops the food bolus in the majority of insects (Lehane & Billingsley, 1996; Terra & Ferreira, 2005). Hemipterans are characterized by the absence of the peritrophic membrane; however, the microvillar membranes of many hemipteran midgut cells are not in direct contact with the food bolus due to the existence of the so-called perimicrovillar membrane, which covers the microvilli extending into the gut lumen (Silva *et al*., 2004).

The peritrophic membrane is composed of chitin, mucopolysaccharides, and proteins and it separates ingested vegetation from the midgut epithelium. It is probably meant to protect the gut surface from damage caused by abrasive food material and to limits the access of microorganisms. It also allows the transfer of liquid and digested substances to the midgut epithelial cells, but prevents the passage of larger food particles. It is worn out by the passage of food, but constantly regenerates from the epithelial cells. The anterior region of *A. pisum* has cells with an apical complex network of lamellae (apical lamellae) instead of the usual regularly-arranged microvilli. These apical lamellae are linked to one another by trabulae. Modified perimicrovillar membranes are associated with the lamellae and project into the lumen, but they start to disappear towards the end of midgut (Cristofoletti *et al*., 2003). A valve separates the midgut and the hindgut, which is lined with a cuticle similar to the foregut and is also involved in uptake of digested material, although to a lesser extent. Undigested material is then excreted through the rectum and the anus.

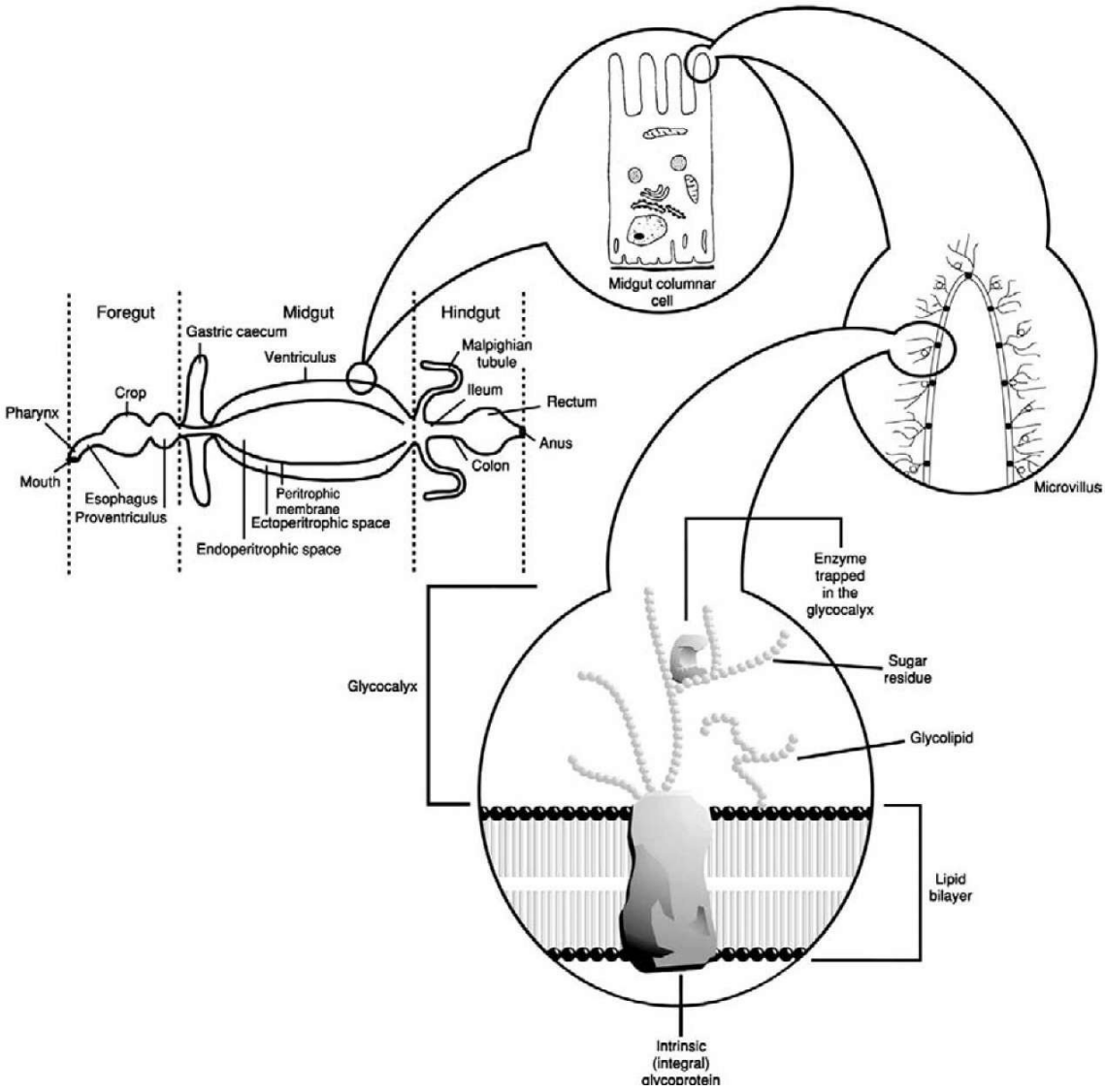


Figure 1.12. Schematic representation of the insect gut compartment at the level of tissue, cell and molecules. The apical surfaces of epithelial cells are dense with actin-based finger-like projections known as microvilli, which increase cell surface area and thereby increase the absorption rate of nutrients (Terra & Ferreira, 2005).

Digestion of food molecules, which are mostly polymers, happens in three steps: the first one reduces the big molecules to smaller oligomers, the second breaks them down even further to dimers, and the third turns them to monomers. Digestion usually occurs by the action of digestive enzymes from the midgut, with little or no participation of salivary enzymes. Frequently, initial digestion occurs inside the peritrophic membrane, intermediate digestion in the ectoperitrophic space, and final digestion at the surface of midgut cells by integral microvillar enzymes or by enzymes trapped into the glycocalyx. In addition, digestion may be facilitated by micro-organisms in the gut (Chapman, 1998).

The midgut is involved in enzyme secretion and absorption of digested food and has a gradient of pH values. The pH of the contents of the midgut is one of the most important internal environmental properties that affect digestive enzymes. It is usually in the 6-7.5 range, the main exceptions being the very alkaline midgut contents of Lepidoptera (pH 9-12) (Dow *et al*., 1992) and the acid posterior region of the midgut of Hemiptera (Clark, 1999), which may be related to their lysosome-like digestive enzymes (cysteine and aspartic proteinase).

1.3.2.3 Successes in pest control: *Bacillus thuringiensis*

Bacillus thuringiensis Berliner (or Bt) is a Gram-positive, soil-dwelling bacterium, commonly used as a biological pesticide (Madigan & Martinko, 2005). During sporulation, many Bt strains produce crystal proteins (proteinaceous inclusions), called δ-endotoxins that have insecticidal activity (Fig. 1.13). This has led to their use as insecticides, and more recently to genetically modified crops using Bt genes (Bravo *et al*., 2011).

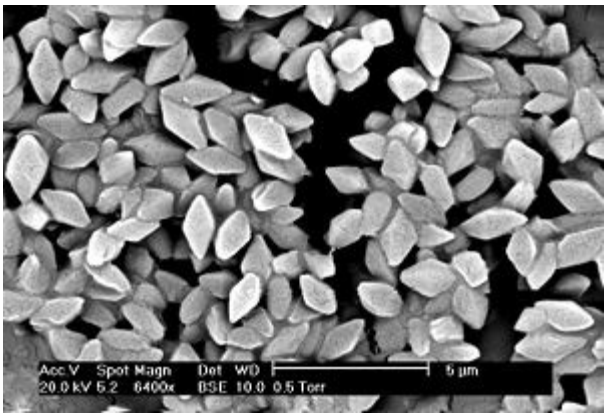


Figure 1.13. Spores and bipyramidal crystals of *Bacillus thuringiensis* morrisoni strain T08025.

B. thuringiensis was first discovered in 1901 by the Japanese biologist Shigetane Ishiwatari. In 1911, it was rediscovered in Germany by Ernst Berliner, who isolated it as the cause of a disease called *Schlaffsucht* in flour moth caterpillars. In 1976, Robert A. Zakharyan reported the presence of a plasmid in a strain of *B. thuringiensis* and suggested the plasmid's involvement in endospore and crystal formation (Zakharyan *et. al*., 1979; Cheng, 1984). Upon sporulation, *B. thuringiensis* forms crystals of proteinaceous insecticidal δ-endotoxins (called crystal proteins or Cry proteins), which are encoded by *cry* genes. In most strains of *B.*

thuringiensis, the *cry* genes are located on the plasmid (Stahly, 1984; Beegle & Yamamoto, 1992; Xu *et al*., 2006).

Spores and crystalline insecticidal proteins produced by *B. thuringiensis* have been used to control insect pests since the 1920s (Lemaux, 2008, Bravo *et al*., 2011). They are being used as specific insecticides under trade names such as Dipel and Thuricide. Because of their specificity, these pesticides are regarded as environmentally friendly, with little or no unwanted effects on humans, wildlife, pollinators, and most other beneficial insects.

Cry toxins have specific activities against insect species of the orders Lepidoptera (moths and butterflies), Diptera (flies and mosquitoes), Coleoptera (beetles), Hymenoptera (wasps, bees, ants and sawflies) and nematodes. *B. thuringiensis*-based insecticides are often applied as liquid sprays on crop plants, where the insecticide must be ingested to be effective. The solubilized toxins are thought to form pores in the midgut epithelium of susceptible larvae. Thus, *B. thuringiensis* serves as an important reservoir of Cry toxins for production of biological insecticides and insect-resistant genetically modified crops. When insects ingest toxin crystals, the alkaline pH of their digestive tract activates the toxin. Cry toxin gets inserted into the insect gut cell membrane, forming a pore. The pore results in cell lysis and eventual death of the insect (Dean, 1984).

1.3.2.3.1. Genetically engineered Bt crops

The Belgian company Plant Genetic Systems was the first company (as a spin-off of Ghent University) to develop genetically engineered plants (tobacco) with insect tolerance by expressing *cry* genes from *B. thuringiensis* (Höfte *et al*., 1986; Vaeck *et al*., 1987). In 1995, potato plants producing Bt toxin were approved safe by the US Environmental Protection Agency (http://www.epa.gov), making it the first pesticide-producing crop to be approved in the USA, where it is currently grown on a large scale. Bt crops (mainly corn and cotton) were planted on 281,500 km² in 2006 (165,600 km² of Bt corn and 115,900 km² of Bt cotton). This was equivalent to 11.1% and 33.6%, respectively, of global plantings of corn and cotton in 2006 (Brookes & Barfoot, 2006).

Environmental impacts appear to be positive during the first ten years of Bt crop use (1996–2005): one study concluded insecticide use on cotton and corn during this period fell by 35.6 million kg of insecticide active ingredient, which is roughly equal to the amount of pesticide

applied to arable crops in the EU in one year. Using the environmental impact quotient (EIQ) measure of the impact of pesticide use on the environment, the adoption of Bt technology over this ten-year period resulted in a respective reduction of 24.3% and 4.6% in the environmental impact associated with insecticide use on the cotton and corn area using the technology (Tabashnik *et al*., 2003; Brookes & Barfoot, 2006).

Bt toxins are a potential alternative to broad-spectrum neurotoxic insecticides. The toxicity of each Bt type is limited to one or two insect orders; it is nontoxic to vertebrates and many beneficial arthropods, because Bt insecticidal cytotoxins work by binding to the appropriate receptor on the surface of midgut epithelial cells. Any organism that lacks the appropriate receptors in its gut cannot be affected by Bt (Gill *et al*., 1992; Knowles, 1994).

1.3.2.3.2. Limitations of Bt

Constant exposure to a toxin creates evolutionary pressure for pests resistant to that toxin. In November 2009, the Monsanto Company (http://www.monsanto.com) found the pink bollworm had become resistant to (first generation) Bt cotton in parts of Gujarat, India. This was the first instance of Bt resistance confirmed by Monsanto scientists anywhere in the world. Nowadays, many more cases are known, as reviewed in Bravo *et al*. (2011).

One method of reducing resistance is the creation of non-Bt crop refuges to allow some non-resistant insects to survive and maintain a susceptible population. The aim is to encourage a large population of pests so any genes for resistance are greatly diluted. This technique is based on the assumption that resistance genes will be recessive.

Another problem is the expansion of secondary pests to replace the controlled ones. Several studies have documented surges in 'sucking pests' (which are not affected by Bt toxins) within a few years of adoption of Bt cotton. In China, the main problem has been with mirids (Lu *et al*., 2010), which have in some cases "completely eroded all benefits from Bt cotton cultivation" (Wang *et al*, 2008). Similar problems have been reported in India, with both mealy bugs and aphids (Stone, 2010).

1.4. Insects of this study

In agriculture and horticulture, insect pests are a very important factor of loss. As an average, they account for the destruction of almost 20% of the world's annual crop production (Oerke & Dehne, 2004). Additionally, many insects are carriers of plant viruses and pathogens, which further increase damage. At present the control of insects requires the use of a wide range of systemic and contact insecticides, most of which are based on a neurotoxic mechanism. According to the international "Insecticide Resistance Action Committee" (IRAC), there are currently >550 species of pest insects, resistant against most current insecticide groups, implying a high demand for novel insecticide targets. For the development of novel tactics for pest control, most attention is currently going to Integrated Pest Management (IPM), which consists of a combination of chemical (insecticides) and biological means (beneficial organisms) - this accordingly to the current European guidelines for agri- and horticulture.

1.4.1. Aphids

Aphids are among the world's most important groups of pest insects. They are small (1-10 mm), hemimetabolous insects which belong to the order of the Hemiptera. They are herbivores with a piercing-sucking feeding technique: with their proboscis they penetrate through the leaf tissue towards the phloem and feed on the plant sap. There are about 4000 known species, each with their own specific host plants, at least 250 of which are a treat to crops and cultivated plants (Van Emden & Harrington, 2007).

The life cycle of aphids is rather complicated and can vary between species. In general sexual reproduction takes place in autumn, with winged female laying eggs on the winter host plant (usually woody plants). In spring the first adults migrate to the summer host plant(s); during summer they multiply mostly by asexual reproduction (parthenogenesis) by unwinged females that are viviparous (Fig. 1.14). Some species do not change host plants.

Aphids often cause serious damage to crops, sometimes with 30-50% loss, causing both direct damage to plants via feeding and indirect damage by transmitting viruses that can devastate agricultural crops (Alford, 2000). Many populations have already acquired resistance towards a number of traditional and modern insecticides, making a search for alternative strategies imperative (Elbert *et al*., 2008). In addition, aphids are not sensitive to the entomotoxins of Bt (Sharma *et al*., 2004). Together with increasing public concern and awareness about pesticide

safety and possible damage to the environment, this had spurred much attention for the search for new alternative aphicides (Edwards *et al*., 2008).

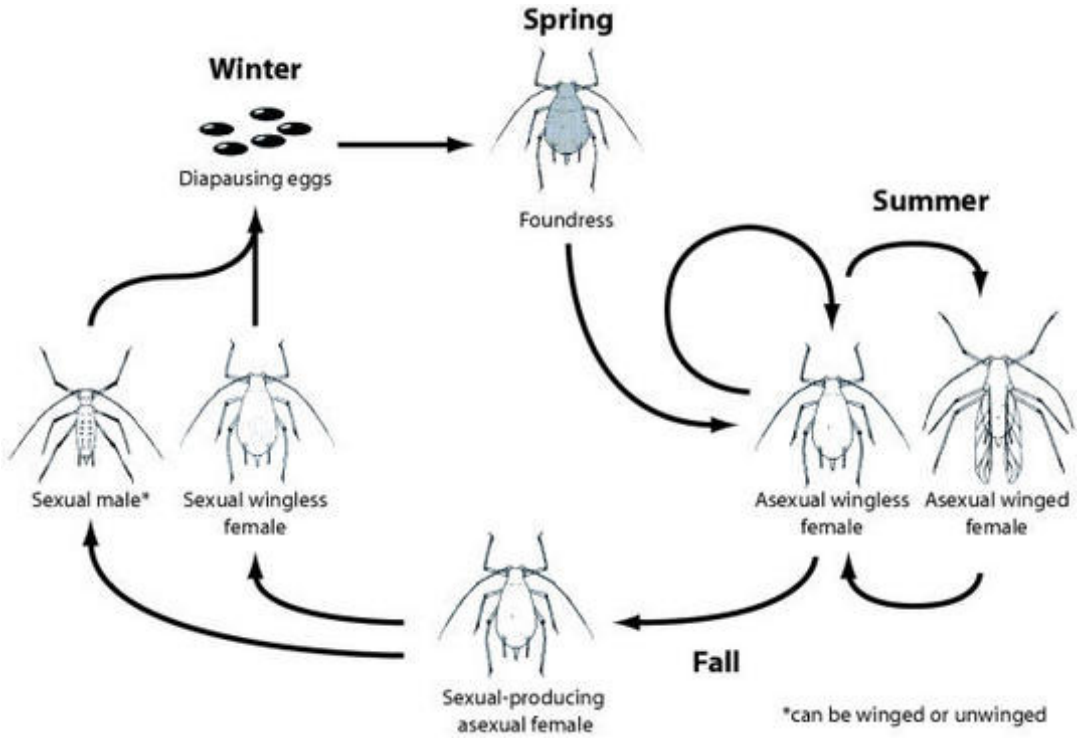


Figure 1.14. Schematic overview of the reproductive cycle of aphids (*Acyrthosiphon pisum*) from Shingleton *et al*. (2003).

Of particular significance is the pea aphid ***Acyrthosiphon pisum*** (Harris) (Hemiptera, Aphidiae), a major pest which causes hundreds of millions of euros of crop damage every year (Van Emden & Harrington, 2007) (Fig. 1.15).

The species feeds on a wide range of legume plants, including important forage and vegetable crops, like peas, beans and alfalfa. It is also an important vector of over 30 plant viruses (Alford, 2000; Blackman & Eastop, 2000). The species has been studied extensively as a model organism in scientific research, and the entire genome has recently been sequenced by the International Aphid Genomic Consortium, providing a resource for the discovery of new targets for control (http://www.aphidbase.com; Richards *et al*., 2010). Adding to that, its short life span and fast reproduction make it an excellent choice as a test organism in this study, being itself an important pest insect and as a model for the piercing-sucking insects.

Phylum: Arthropoda
Subphylum: Uniramia
Superclassis: Hexapoda
Classis: Insecta
Subclassis: Pterygota
Ordo: Hemiptera
Subordo: Homoptera
Familia: Aphidina
Genus: Acyrthosiphon
Species: Acyrthosiphon pisum

Figure 1.15. Taxonomic position and photo of the pea aphid.

1.4.1.1. Life cycle and maintenance of the colony in the lab

In nature, aphids follow a complicated life cycle with sexual and asexual stages (see above). In the laboratory, however, they are kept under constant summer conditions, which means only asexual reproduction takes place. Adult (wingless) females will produce offspring by viviparous parthenogenesis, at a rate of 4 to 12 nymphs a day, about a hundred in her lifetime. These nymphs are exact genetic replicates of their mother, and are all females. The adult life span is around 30 days. The nymphs will undergo four moults - taking about 7 to 10 days - before reaching sexual maturity, at which point they will immediately start producing offspring of their own. In the laboratory, colonies are kept on living *Vicia faba* (lab bean) plants, under standard conditions of 23 ± 5°C, humidity 65 ± 5% and a photoperiod of 16h light and 8h darkness.

1.4.2. Lepidoptera

Another very important group of pest insects is the Lepidoptera, one of the most diverse groups of leaf-chewing insects. The order has more than 180,000 species in 128 families and 47 superfamilies (http://www.ucl.ac.uk/taxome/). Their life cycle has four stages: egg, larvae (caterpillar), pupa and adult (butterfly/moth). This is typical for holometabolous insects with a whole metamorphosis process. Many species are polyphagous and can do massive damage to a wide variety of crops, causing severe losses to the world's food production.

For this project we have been working with the Egyptian cotton leafworm, ***Spodoptera littoralis*** (Fig. 1.16), because it is a very important pest species by itself and additionally serves as a model organism representative for insects with biting-chewing mouthparts.

Phylum: Arthropoda
Subphylum: Uniramia
Superclassis: Hexapoda
Classis: Insecta
Ordo: Lepidoptera
Subordo: Heteroneura (Frenatae)
Sectio: Heteroceura
Familia: Noctuidae
Subfamilia: Amphipyrinae
Genus: Spodoptera
Species: Spodoptera littoralis

Figure 1.16. Taxonomic position and photo of the cotton leafworm.

The species is considered a major agricultural pest in many parts of the world (mainly Africa and Asia, but also Europe), with a host range of over 40 plant families including several major crops like cotton, corn, tobacco, beets and other vegetables (Alford, 2000). Due to excessive selection pressure by the intensive use of insecticides on these crops, many populations have developed high degrees of resistance against most groups of commercially available insecticides (Ishaaya *et al*., 1995; Smagghe & Degheele, 1997; Pluschkell *et al*., 1998) and

even Bt (Strizhov *et al*., 1996). Its broad range of host plants and easy rearing make it a good candidate for lab studies on efficacy tests of novel insecticide compounds.

1.4.2.1. Life cycle and maintenance of the colony in the lab

The life cycle of the cotton leafworm comprises 9 stages: egg, 6 larval stages, pupa and adult. Under laboratory conditions, the whole cycle takes approximately 40 days. The eggs take about 4 days to hatch; the resulting larvae are less than 1 mm thick and only 3-4 mm long. The different larval stages are distinguished by head capsule sizes. Each takes about 3 days, except for the 6^{th} and last stage: in this one, the larvae will continue to feed during the first 3-4 days, then enter a wandering phase in which they stop feeding, clean their gut, and prepare for transition to pupa. The pupal stage takes about 10 days, during which the pupae are disinfected by short exposure to 40% formaldehyde vapours to avoid the transfer of infections. The adult stage also takes about 10 days, during which females will produce 1500-2000 eggs in 1-2 days. During all this time, the cultures are kept under standard conditions of 23 ± 5°C, humidity 65 ± 5% and a photoperiod of 16h light and 8h darkness. Larvae are grown on a wheat-germ based artificial diet (modified from Poitout *et al*., 1972), while the adults are fed honey water (10%).

1.5. Goals and outlines

The main aim of this work was to investigate the toxicity and mode of action of a number of steroidal and non-steroidal plant metabolites against two economically important pest insect groups, in regard to better understand the mechanism behind the insecticidal activity and to explore their potential as alternative natural insecticides. The project focused on phytoecdysteroids and saponins on the one hand, and on two major pest insect orders, aphids (Hemiptera) and caterpillars (Lepidoptera), on the other. In the different experiments, we used both *in vivo* tests with living insects and *in vitro* bioassays with insect cell cultures. In addition, a cell-based reporter screening system was established to investigate agonist and antagonist interactions with the receptor (EcR) of the steroidal insect moulting hormone, 20-hydroxyecdysone (20E).

Below, the different goals of this multidisciplinary project are listed in detail.

- Testing of a cell-based reporter bioassay that allows for fast and easy screening of a large number of compounds for ecdysteroid agonistic and antagonistic effects on the EcR. Then, with this *in vitro* bioassay, a number of ecdysteroid agonists (e.g. dibenzoylhydrazines) and antagonists was tested in order to better understand their mode of action and efficacy against insects (chapter 2).
- In-depth study of the effects and mode of action of two steroidal and two non-steroidal saponins on three insect cell lines from different orders and tissues, namely embryonic S2 cells from *Drosophila melanogaster* (Diptera), ovarian Bm5 cells from *Bombyx mori* (Lepidoptera) and midgut CF-203 cells from *Choristoneura fumiferana* (Lepidoptera), by researching the following hypotheses (chapter 3):
    - screening for ecdysteroid agonistic and antagonistic effects by using the 20E response bioassay as described above. Here the sensibility of cells of different species and tissue origin was also investigated;
    - assessing the cell viability and membrane integrity of the cells after treatment with saponins by using MTT and trypan blue viability assays, respectively;
    - checking for induction of apoptosis in the cells by caspase-3 like activity and DNA-fragmentation assays;
    - studying the possibility that addition of cholesterol to the cell medium can counter the negative effects of saponins by testing a parallel series of 20E response and

viability bioassays after adding cholesterol to the cell medium alongside with the saponin, and the interaction and role of cholesterol in abovementioned mechanisms.

- Determining the insecticidal activity of *Quillaja saponaria* saponin *in vivo* in insects as *Acyrthosiphon pisum* pea aphids and *Spodoptera littoralis* cotton leafworm caterpillars fed on saponin-enriched diet and leaves (chapter 4).
- Investigating potential repellent and deterrent activities of *Q. saponaria* saponin by observing insect behaviour on treated glassplates and quantifying the preference of insects for saponin-treated or untreated food in choice-experiments, respectively (chapter 4).
- Examining the midgut-specific effects of *Quillaja saponaria* saponins with use of primary midgut cell cultures from *S. littoralis* and making microscopic slides of the *A. pisum* aphid gut (chapter 4).

Finally, chapter 5 summarizes the main findings of this study and provides some future perspectives with a scientific research model associated with the development of a plant-based natural insecticide within the context and criteria used by the European and Mediterranean Plant Protection Organization (EPPO) describing how field trials should be conducted in order to test the efficacy of plant protection products that are candidate chemicals for being proposed for registration (http://www.eppo.org/).

Chapter 2

A cell-based reporter assay for screening for EcR agonist/antagonist activity of natural ecdysteroids in Lepidoptera (Bm5) and Diptera (S2) cell cultures, followed by modelling of EcR interactions

This chapter has been redrafted from:

De Geyter E, Swevers L, Rougé P, Coll J, Geelen D and Smagghe G. A cell-based reporter assay for screening for EcR agonist/antagonist activity of natural ecdysteroids in Lepidoptera (Bm5) and Diptera (S2) cell cultures, followed by modelling of EcR interactions. *Insect Science*, in preparation.

2.1. ABSTRACT

Ecdysteroid signal transduction is a key process in insect development and hence an important target for insecticide development. We employed an *in vitro* cell-based reporter bioassay for screening potential ecdysone receptor (EcR) agonistic and antagonistic activity. Natural ecdysteroids were assayed for their ability using ecdysteroid-responsive cell line cultures that were transiently transfected with the reporter plasmid ERE-b.act.luc. We used the dipteran Schneider S2 cells of *Drosophila melanogaster* and the lepidopteran Bm5 cells of *Bombyx mori* insect cell lines as these represent robust cell systems for ecdysteroid-inducible luciferase reporter gene analysis.

Measurements showed an EcR agonistic activity only for cyasterone, both in S2 (IC_{50} = 3.3µM) and Bm5 cells (IC_{50} = 5.3µM). However, the activity was very low compared to that of the commercial insecticide tebufenozide. An antagonistic activity was found for castasterone in S2 cells, with an IC_{50} of 0.039µM; in Bm5 cells this effect only became visible at much higher concentrations (IC_{50} = 18µM) and might be due to general cell toxicity rather than true antagonistic activity. To gain more insight in the interaction with the EcR receptor, three-dimensional modelling of dipteran and lepidopteran EcR-LBD was performed. Modelling of the interaction of the ecdysteroids with the EcR receptor supported binding of cyasterone, but not of castasterone.

In conclusion, we show that the cell-based reporter bioassay tested here is a useful and practical for screening candidate EcR agonists and antagonists, but it does not provide sufficient evidence for direct interaction with the receptor.

2.2. INTRODUCTION

Ecdysteroids are a group of steroid hormones that control moulting and reproduction in arthropods. Whether they fulfil hormonal functions in other invertebrate groups is still a matter of debate (Trenin & Volodin, 1999). The most studied representatives are ecdysone and 20-hydroxyecdysone (20E), the insect moulting hormone. They have been detected in ca. 6% of plant species analysed so far, and more than 300 different ecdysteroids have been isolated from animal and plant sources (Dinan *et al*., 2001). Starting from the 60's, the possibility to extract similar molecules from plants (phytoecdysteroids) has made them easily available in large amounts, and this was the beginning of a new field of studies specifically targeting the ecdysone reporter (EcR), the receptor site for 20E. This is an interesting target for insect pest control, because compounds acting on this receptor will only affect insects and arthropods, which makes them interesting targets for the search for safer and more specific insecticides. This has led to the development of a number of successful EcR agonist insecticides, namely tebufenozide, methoxyfenozide, chromafenozide and halofenozide (see 1.3.1.3).

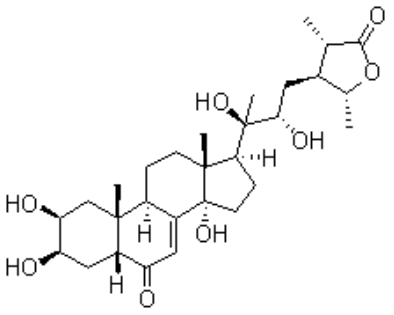
However, in order to successfully search for new compounds that disrupt the insect endocrine system, there is need for high-throughput screening systems. To address this need, we tested a method using transfected *Drosophila*-derived S2 and *Bombyx mori*-derived Bm5 cell lines that respond to the addition of ecdysone-like substances through the expression of the luciferase reporter and the appearance of luminescence. Because the amount of luminescence can be easily quantified in individual wells of a 96 well plate by a luminescence plate reader, the transfected cells can be used for the screening of compounds with ecdysteroid mimetic activity in high-throughput format.

For this project, we tested three substances at various concentrations: cyasterone, inokosterone en castasterone (Fig. 2.1A). All three are relatively small molecules with a steroid core and a tail, which makes them structurally similar to 20E. This is very different from the abovementioned commercial EcR agonists like tebufenozide, which do not resemble steroids at all (Fig. 2.1B).

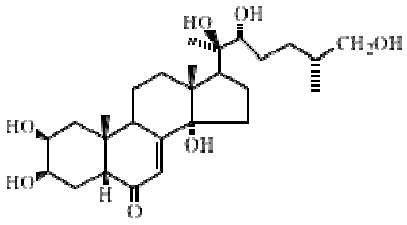
We used two insect cell line cultures, Schneider S2 cells from *Drosophila melanogaster* (Diptera) and Bm5 cells from *Bombyx mori* (Lepidoptera). Both insect orders contain a

number of very important pest species, against which many of the current insecticides are no longer effective. Since both groups have different classes of insecticides working against them, comparing the two could give us an indication about the specificity of the observed effects. We also did a three-dimensional modelling of dipteran and lepidopteran EcR-LBD to better understand the mechanism of action of the ecdysteroids.

A



cyasterone



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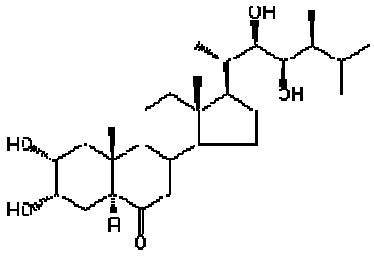
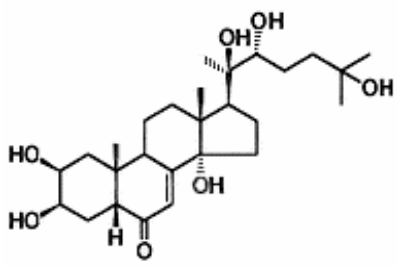
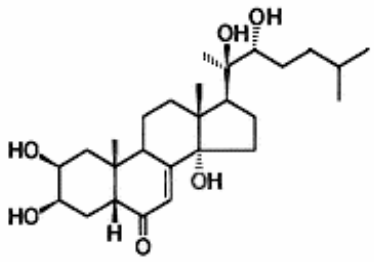


castasterone

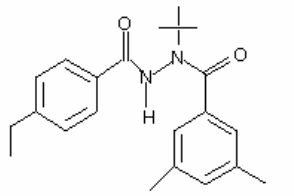
B



20-hydroxyecdysone



ponasterone A



tebufenozide

Figure 2.1A, B. Three natural ecdysteroids: cyasterone, inokosterone and castasterone (A) and three known EcR agonists: 20E and ponasterone A, natural ligands for the edysone receptor, and tebufenozide, a commercial insecticide (B).

2.3. MATERIALS AND METHODS

2.3.1. Chemicals

Cyasterone, inokosterone and castasterone (>97%, HPLC) were gifts from Prof. Dr. Josep Coll, Dept. of Biological Organic Chemistry, CID-CSIC, E-08034 Barcelona, Spain. All other products are analytical grade unless otherwise mentioned.

2.3.2 Insect cell lines

Cell line cultures were maintained in the Laboratory of Agrozoology (Ghent University, Belgium) at a temperature of 27°C. *D. melanogaster* Schneider S2 cell line cultures were maintained in HyQ SFX-Insect™ Medium (Perbio Science, Erembodegem, Belgium) (Mosallanejad & Smagghe, 2009). *B. mori* Bm5 cells were cultured in IPL-41 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Bornem, Belgium) and additional minerals (Soin *et al*., 2008). Only during the intermediate stages of the transfection, serum free medium was used (Swevers *et al*., 2004).

2.2.3. Transfection of insect cells

The compounds were assayed for their ability to activate transcription of an ecdysteroid-inducible luciferase reporter gene using ecdysteroid-responsive S2 or Bm5 cells. Prior to exposure, the cells were transiently transfected with the reporter plasmid by lipofection according to the manufacturer's instructions (Invitrogen) as described in Soin *et al*. (2008). Briefly, the desired number of wells of a 6-well plate was filled with 3×10^6 S2 or 2×10^6 Bm5 cells. The cells were given time to attach to the bottom and washed once or twice with serum free medium. Lipofectin was first pre-incubated alone for 45 min in culture medium and then 15 min together with 1.5 µg of the reporter plasmid ERE-b.act.luc (Fig. 2.2) before adding to the cells. The ERE-b.act.luc reporter construct is composed of seven copies of the ecdysone response element (ERE) derived from the *Drosophila* hsp27 promoter (Riddihough & Pelham, 1987), a *B. mori*-derived basal actin promoter (b.act), followed by the reporter gene for firefly luciferase (luc) and a termination signal. The cells were incubated at 27°C for 5 h, after which the transfection medium was removed and replaced by normal culture medium (with serum).

2.2.4. Bioassays for EcR agonist and antagonist responsiveness

For the bioassays, transfected cells were treated with ecdysteroids at final concentrations of 5-

500 µM (prepared in DMSO). A cell solution with a density of $2x10^6$ cells/ml was prepared for S2 cells, $2\text{-}3x10^5$ cells/ml for the lepidopteran cells. Next, 1 µl of the compound was added at the bottom of the required wells of a white 96-well microtitre plate (Greiner labortechnik, Frickenhausen, Germany) with a micropipette, after which 100 µl of the cell solution was added into the wells.

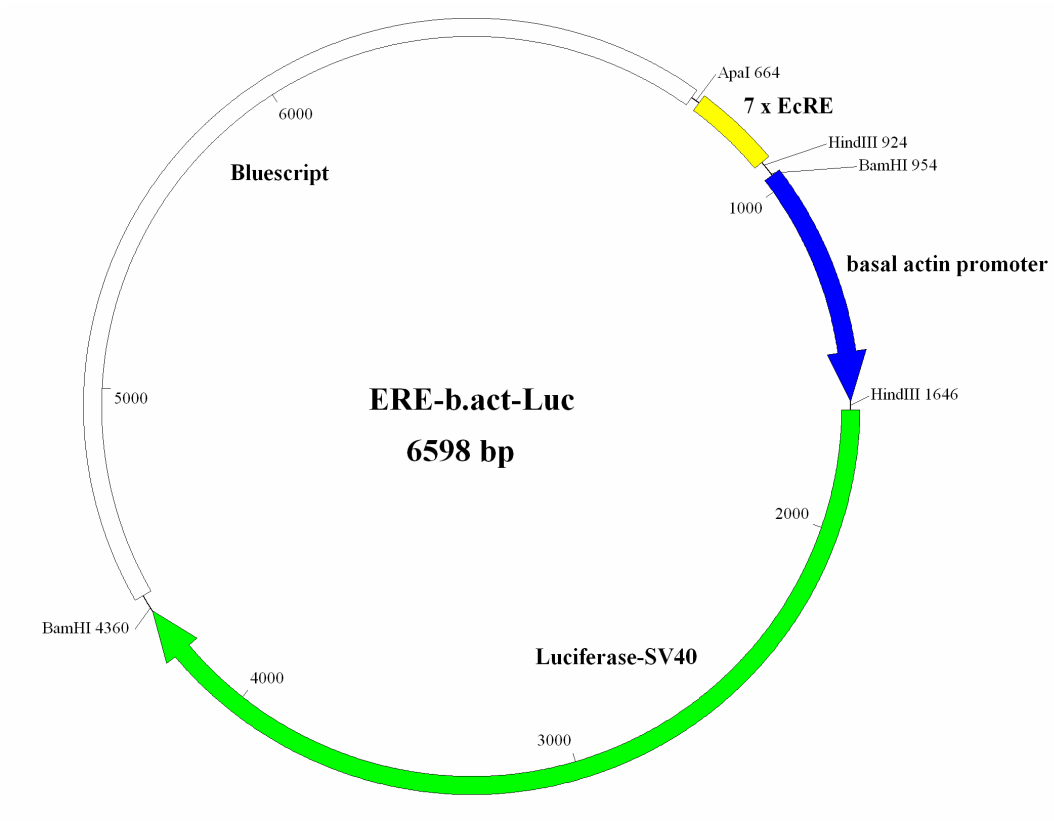


Figure 2.2. Plasmid with the ERE-b.act.luc reporter construct, composed of seven copies of the ecdysone response element (ERE) derived from the *Drosophila* hsp27 promoter, a *Bombyx mori*-derived basal actin promoter (b.act), followed by the reporter gene for firefly luciferase (luc) and a termination signal.

For testing EcR agonist activity, 1 µl of a 50 µM-solution of 20E (final concentration = 500 nM) was added only to the positive control. The plates were sealed with parafilm and incubated at 27°C for 24 h before reading. For EcR antagonist activity, the plates (without 20E) were first incubated at 27°C for 24 h, then 1 µl of a 50 µM solution of 20E was added to each well except for the negative control, and then the plates were incubated for another 24 h. For measuring the luciferase expression, the Steady-Glo luciferase assay system kit (Promega, Leiden, the Netherlands) was used: 100 µl luciferase substrate was added to each well and the

luminescence was measured with a Tecan M200 luminometer (Tecan, Mechelen, Belgium). For each concentration 3 replicates were done, and each experiment was repeated 2 or 3 times.

Data were presented as a percent response compared to 500 nM of 20E (i.e. positive control). Respective EC_{50}'s (median effective concentration values on reporter gene induction) and IC_{50}'s (medium inhibitory concentration values) with corresponding 95% confidence limits were calculated with Prism v4 (GraphPad Software Inc., La Jolla, CA); the accuracy of data fitting to the sigmoid curve model was evaluated through examination of R^2 values.

2.2.5. Three-dimensional modelling of dipteran and lepidopteran EcR-LBD

Homology modelling of the ligand-binding domain (LBD) of the EcR from *Bombyx mori* (BmEcR-LBD) and *Drosophila melanogaster* (DmEcR-LBD), was performed with the YASARA Structure program (Krieger *et al*., 2002) running on a 2.53 GHz Intel core duo Macintosh computer. Different models were built from the X-ray coordinates of the EcR of the Lepidoptera *Heliothis virescens* in complex with synthetic ligands (RCSB Protein Data Bank code 3IXP), the RXR-USP receptor of the Coleoptera *Tribolium castaneum* bound to ponasterone A (PonA) (PDB Code 2NXX) (Iwema *et al*., 2007), the EcR-LBD of the Hemiptera *Bemisia tabaci* complexed to PonA (PDB code 1Z5X) (Carmichael *et al*., 2005), the EcR-USP of *Heliothis virescens* in complex with 20E (PDB code 2R40) (Browning *et al*., 2007) and the human KXR ligand binding domain (PDB code 4DK8) (Kopecky *et al*., 2012), used as templates, respectively. Finally, a hybrid model was built up from the four previous models. PROCHECK (Laskowski *et al*., 1993) was used to assess the geometric quality of the three-dimensional model. In this respect, all of the residues of BmEcR-LBD and DmEcR-LBD were correctly assigned on the allowed regions of the Ramachandran plot (result not shown). ANOLEA (Melo & Feytmans, 1998) was also used to evaluate the quality of the models. Molecular cartoons were drawn with YASARA and PyMol (W.L. DeLano, http://pymol.sourceforge.net). Docking of the steroids 20E, PonA, cyasterone and inokosterone, the antagonist castasterone and the non-steroidal agonist tebufenozide to both EcR-LBD was performed with the YASARA structure program. Clipping planes of BmEcR-LBD and DmEcR-LBD complexed to 20E, PonA, cyasterone, inokosterone, castasterone, and tebufenozide were also rendered with PyMol.

2.3. RESULTS AND DISCUSSION

2.3.1. EcR agonistic activity

Luciferase reporter assays in S2 cells showed an EcR agonistic activity for cyasterone only (IC_{50} = 3.3µM) and for the reference EcR agonist tebufenozide (IC_{50} = 0.71 µM) (Table 2.1). Tebufenozide is a non-steroid EcR agonist insecticide with specific activity against Lepidoptera that has been on the market for over ten years (Dhadialla *et al*., 1998). The activity in cyasterone was about 5 times lower than for tebufenozide and 100 times lower than for 20E (Table 2.1). Considering that tebufenozide is reported to be largely inactive against Diptera, this efficiency is low compared to existing commercial products.

In Bm5 cells, the activity of cyasterone was similar to that in S2 cells (IC_{50} = 5.3µM). However, the effect of the Lepidoptera-specific tebufenozide is 10000 times higher than cyasterone (Fig. 2.3). While such activity is interesting from a scientific point of view, it is clear that the necessary concentrations would be too high to make it commercially applicable, especially since the effect is probably non-specific as it works on both dipteran and lepidopteran cells alike.

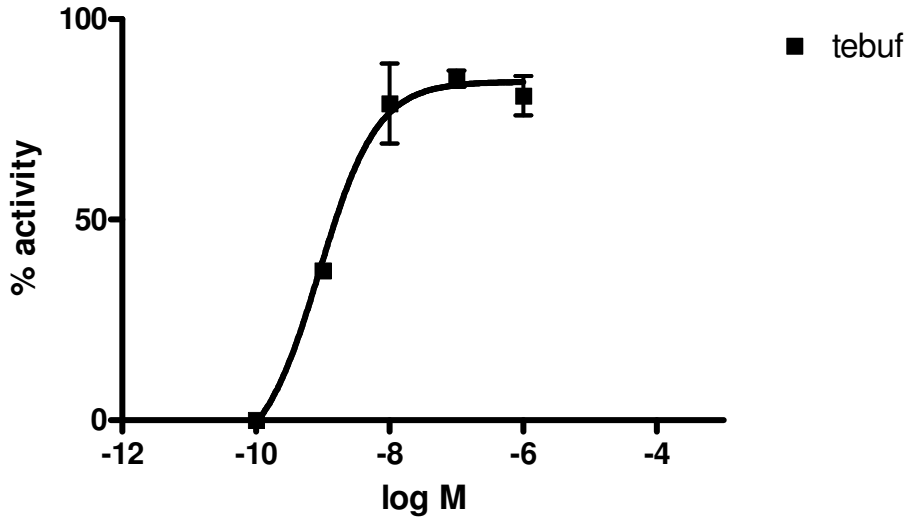
Table 2.1. EcR agonistic activity for three known EcR agonists and three newly tested ecdysteroid compounds for *Drosophila melanogaster* S2 and *Bombyx mori* Bm5 cells.

	S2	**Bm5**
	IC_{50} (95% CL; R^2) (µM)	**IC_{50} (95% CL; R^2) (µM)**
20E	0.040 (0.027-0.060; 0.91)	0.017 (0.0097-0.030; 0.84)
ponasterone A	0.054*	0.011*
tebufenozide	0.71 (0.35-1.41; 0.83)	0.00089 (0.00023-0.0035; 0.90)
cyasterone	3.3 (-- ; 0.92)	5.3 (2.1-13.5; 0.90)
inokosterone	inactive	inactive
castasterone	inactive	inactive

*Data from Soin, 2009.

Data are given as median response values together with the 95% confidence interval (both in µM) and the R^2 as accuracy of data fitting to the sigmoid curve model after Prism v4.

A



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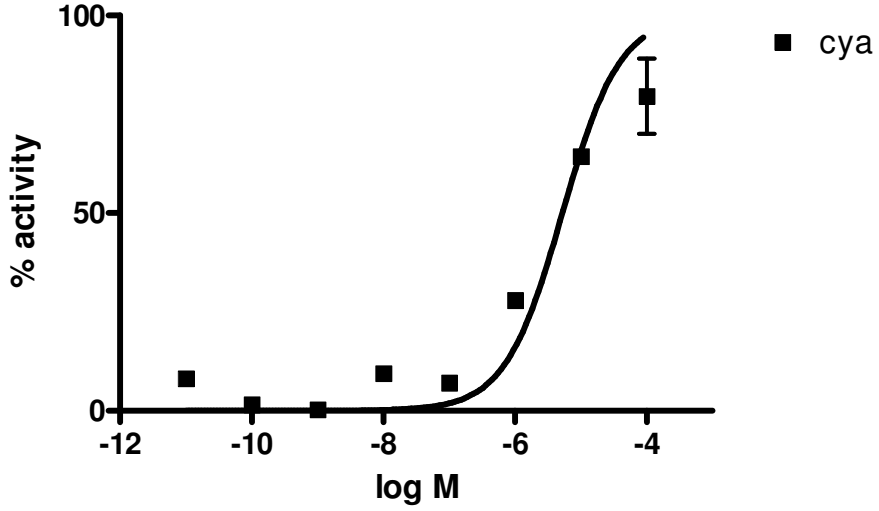


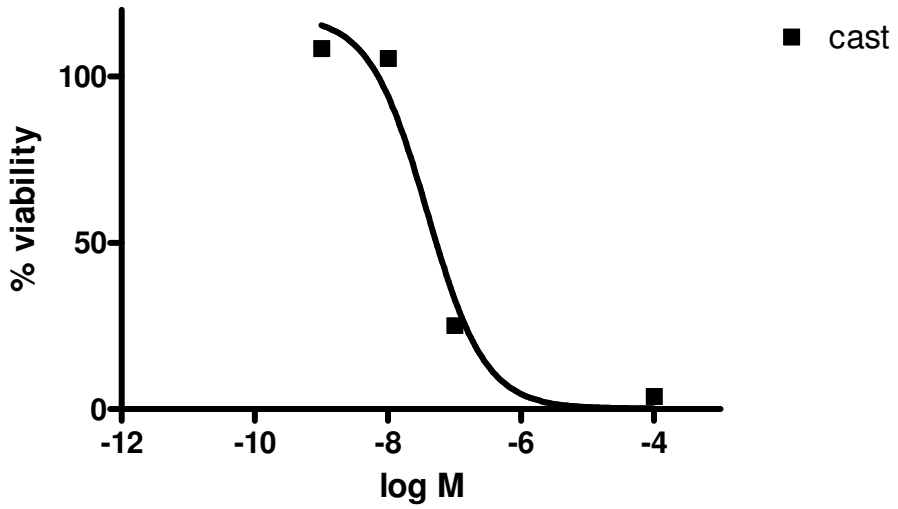
Figure 2.3A, B. EcR agonistic activity of tebufenozide (A) and cyasterone (B) for lepidopteran *Bombyx mori* Bm5 cells.

2.3.1. EcR antagonistic activity

An antagonistic activity was found for castasterone in S2 cells, with an IC_{50} of 0.039µM (Table 2.2). In contrast, in Bm5 cells this effect only became visible at much higher concentrations (IC_{50} = 18µM) (Fig. 2.4). Given the sensitivity of Bm5 cells to a number of other steroid compounds (data not shown), it is possible that the lack of EcR activity at this

concentration was due to general cell toxicity rather than a direct antagonistic activity on the receptor.

A



B

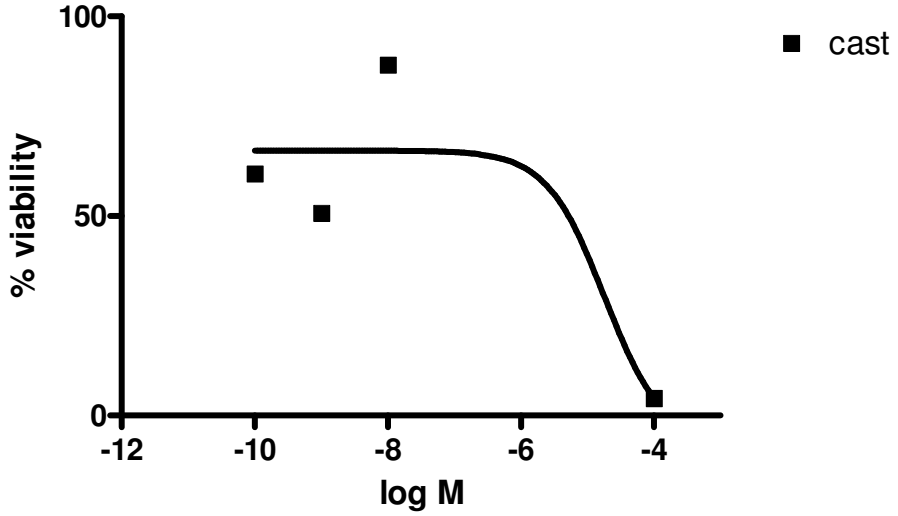


Figure 2.4A, B. EcR antagonistic activity of castasterone for (A) dipteran *Drosophila melanogaster* S2 and (B) lepidopteran *Bombyx mori* Bm5 cells.

Table 2.2. EcR antagonistic activity for 20E, tebufenozide and three newly tested ecdysteroid compounds for *Drosophila melanogaster* S2 and *Bombyx mori* Bm5 cells.

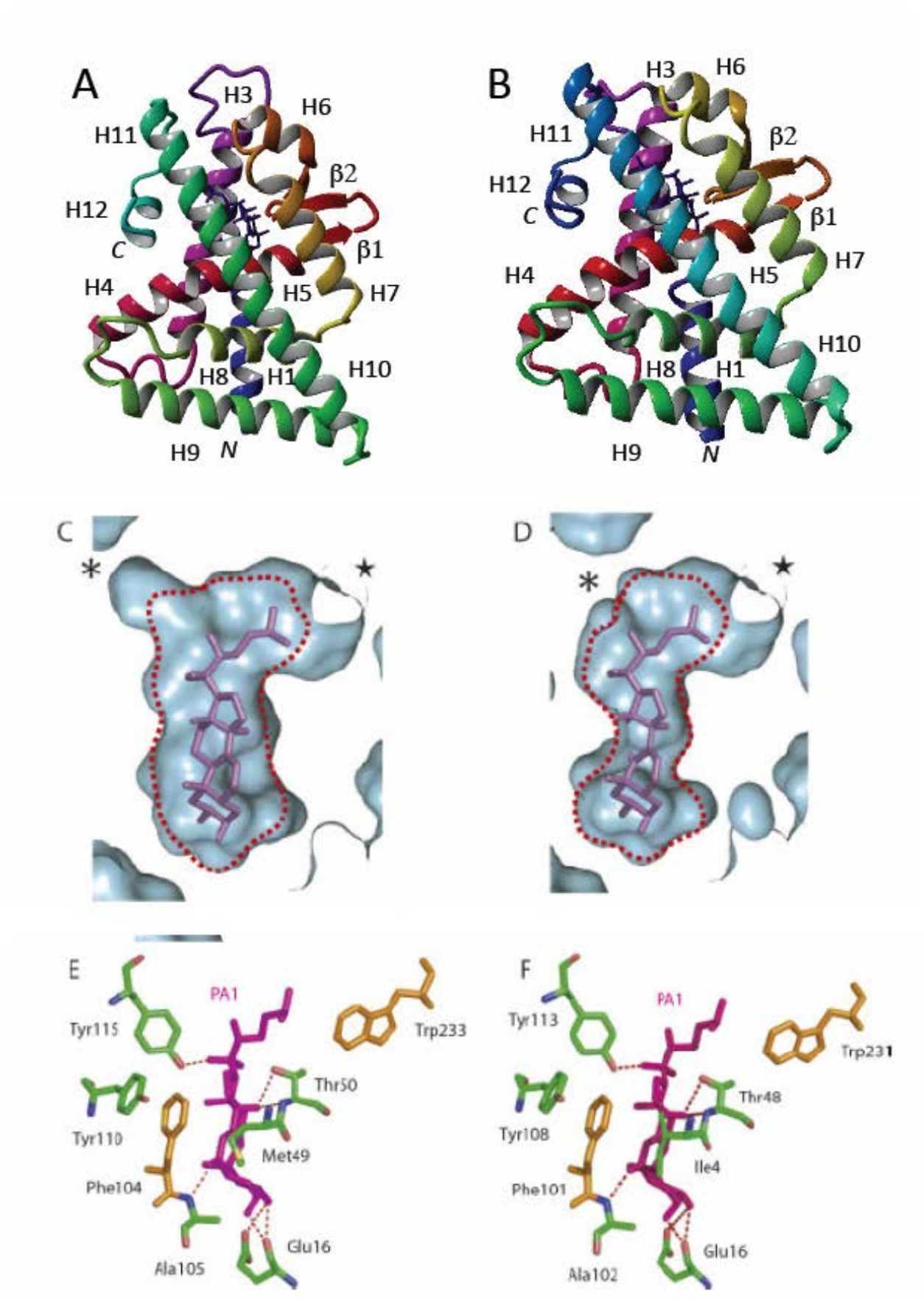
	S2	**Bm5**
	IC_{50} (95% CL; R^2) (µM)	**IC_{50} (95% CL; R^2) (µM)**
20E	inactive	inactive
tebufenozide	inactive	inactive
cyasterone	inactive	inactive
inokosterone	inactive	inactive
castasterone	0.039 (0.0000019-818; 0.97)	18 (-- ; 0.97)

Data are given as median response values together with the 95% confidence interval (both in µM) and the R^2 as accuracy of data fitting to the sigmoid curve model after Prism v4.

2.3.2. Three-dimensional modelling of dipteran and lepidopteran EcR-LBD

2.3.2.1. Binding of PonA and 20E

Ponasterone A and 20E are insect steroid hormones involved in regulating metamorphosis, and the natural ligands for inducing the ecdysone reporter system. The modelled BmEcR-LBD and DmEcR-LBD both consist of the canonical three-dimensional structure of the ecdysteroid receptors, built up from 12 α-helices tightly packed around a ligand-binding pocket that specifically anchors PonA and other ecdysteroids (Fig. 2.5A, B). Upon binding of PonA to the receptors, the aliphatic chain of PonA becomes anchored to the large lobe located at the bottom of the pocket (Fig. 2.5C, D) via hydrophobic interactions involving residues Met120, Met214, Leu229 and Trp233 of BmEcR-LBD and Met118, Met212, Leu227 and Trp231 of DmEcR-LBD. A series of hydrogen bonds connects ponA with hydrophilic residues (Glu16, Thr50, Ala105 and Tyr115 of BmEcR-LBD, and Glu16, Thr48, Ala102 and Tyr113 of DmEcR-LBD). Together with stacking interactions with aromatic residues (Phe104 and Tyr110 of BmEcR-LBD, Phe101 and Tyr 108 of DmEcR-LBD) the hydrogen bonds promote the binding of PonA (Fig. 2.5E, F). This binding scheme is similar in the different insect EcR's resolved.


A
H3
H6
H11
β2
H12
C
β1
H4
H5
H7
H8
H1
H10
H9
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B
H3
H6
H11
β2
H12
C
β1
H4
H5
H7
H10
H8
H1
H9
N
C
D
E
PA1
Tyr115
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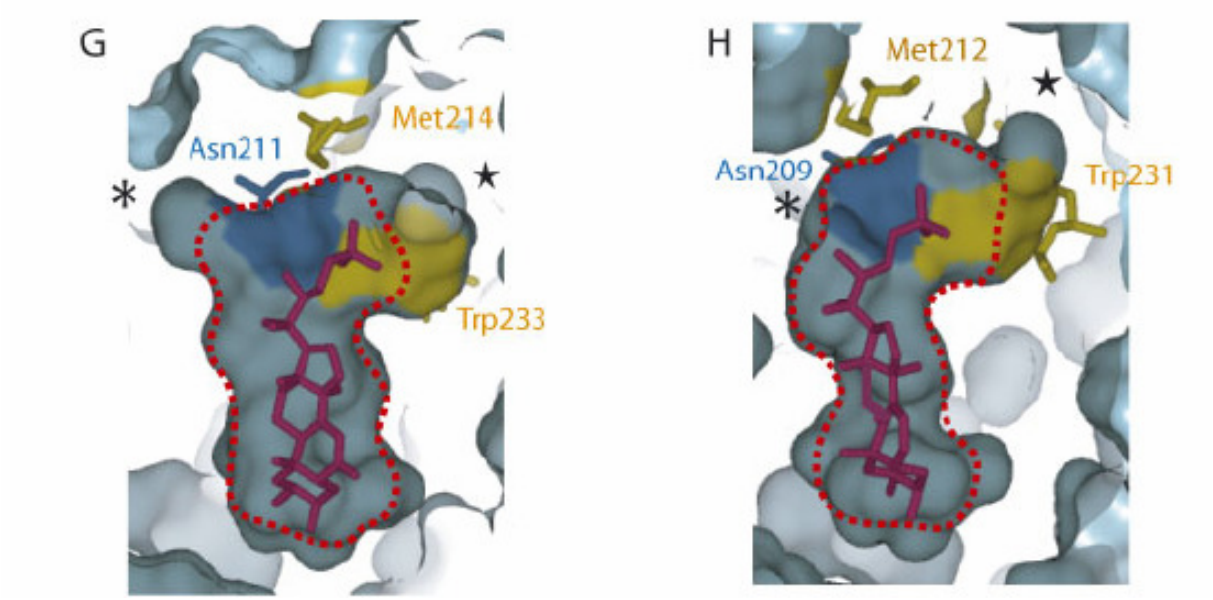


Figure 2.5A, B. Ribbon diagrams of the modelled BmEcR-LBD (A) and DmEcR-LBD (B). The 12 α-helices building the three-dimensional fold of the receptors are differently coloured and numbered H1 to H12; the two short strands of b-sheet are coloured purple and numbered β1 and β2. *N* and *C* correspond to the *N*- and *C*-terminus of the polypeptide chains respectively. Ponasterone A (PonA) complexed to the EcR-LBD is represented as blue stick.

C, D. Clips showing the binding of PonA to the ligand-binding pocket of BmEcR-LBD (C) and DmEcR-LBD (D). The two lobes located at the bottom of the pocket of BmEcR-LBD are indicated by a star (★) and an asterisk (*).

E, F. Residues of the ligand-binding pocket of BmEcR-LBD (E) and DmEcR-LBD (F) interacting with PonA (pink stick) by direct hydrogen bonds (coloured red) and stacking interactions (residues in orange sticks) are labelled.

G, H. Clips showing the binding of 20E to the ligand-binding pocket of BmEcR-LBD (C) and DmEcR-LBD (D). The two lobes located at the bottom of the pocket are indicated by a star (★) and an asterisk (*). Hydrophobic residue interacting with the methyl groups at C26 and C27 of 20E are in yellow sticks. The Asn209 and Asn211 residues H-bound to the hydroxyl group at C25 of 20-hydroxyecdysone (20E) are in blue sticks.

Docking with 20E yielded an anchoring pattern similar to that of PonA. However, in addition to the hydrophobic interactions occurring between methyl groups at C26 and C27 of 20E and hydrophobic residues of BmEcR-LBD (Met120, Met214, Leu229 and Trp233) and DmEcR-LBD (Met118, Met212, Leu227 and Trp231), the hydroxyl group at C25 of 20E creates an additional hydrogen bond with residue Asn211 of BmEcR-LBD and Asn209 of DmEcR-LBD respectively (Fig. 2.5G, H). This additional H-bond strengthens the interaction of the ecdysone receptors with 20E.

2.3.2.1. Binding of tebufenozide and ecdysteroids

In contrast to the natural ligands, tebufenozide binds to the ecdysone-binding pocket of BmEcR-LBD in a quite different orientation (Fig. 2.6A). The ethyl-phenyl ring of tebufenozide accommodates a second less extended lobe occurring at the bottom of the ecdysone-binding pocket of BmEcR-LBD at the opposite side of the lobe harbouring the aliphatic chain of PonA or 20E. This binding pattern essentially consists of hydrophobic interactions with hydrophobic residues (Ile, Leu, Met) and stacking interactions with aromatic residues (Phe, Tyr). In this respect, DmEcR-LBD, which lacks this second lobe at the bottom of the ecdysone-binding pocket, readily differs from BmEcR-LBD (Fig. 2.6A,B) and does not provide an anchor for the ethyl-phenyl ring of tebufenozide (Fig. 2.6B). Hence tebufenozide is not suitable for binding the ecdysone-binding pocket of DmEcRLBD. Since the presence of this second lobe is typical for Lepidoptera, this explains why the insecticide is highly specific against Lepidoptera, but much less so against Diptera and other insect orders.

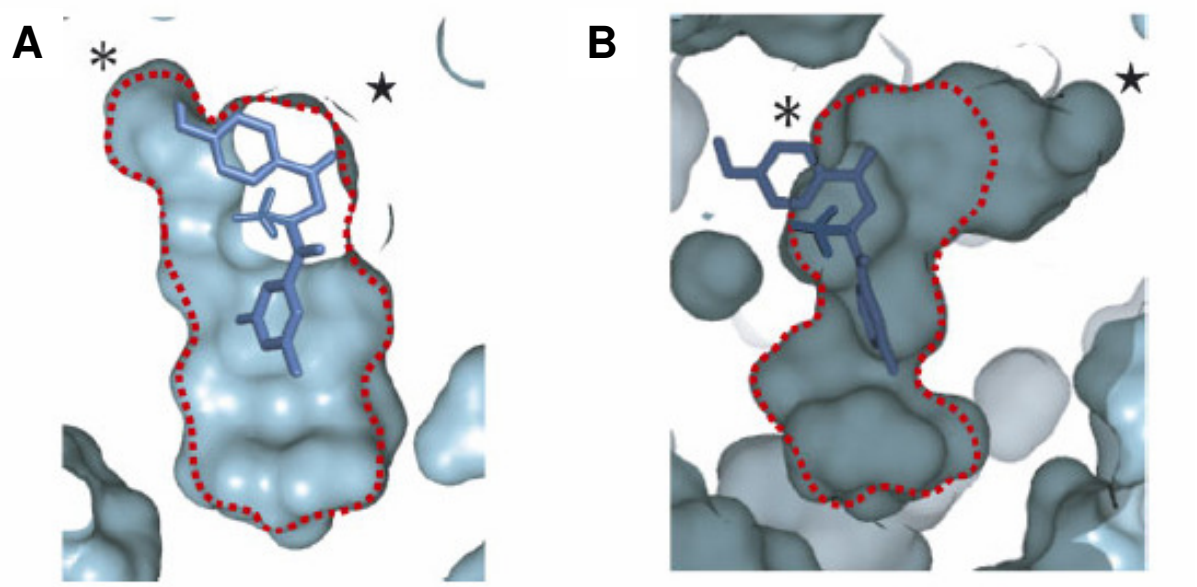


Figure 2.6A, B. Clips showing the binding of tebufenozide to the ligand-binding pocket of BmEcR-LBD (A) and DmEcR-LBD (B). The two lobes located at the bottom of the pocket of BmEcR-LBD are indicated by a star (★) and an asterisk (*). The lack of the second lobe (asterisk, *) at the bottom of the ecdysone-binding pocket of DmEcR-LBD prevents the ethyl-phenyl ring of tebufenozide from being accommodated by the receptor.

The ecdysteroids bind to the receptor in yet another way. Upon docking to the ligand-binding pocket of BmEcR-LBD (Fig. 2.7A) and DmEcR-LBD (Fig. 2.7B), cyasterone adopts a

position within the binding-pocket that is slightly different from that of 20E or PonA, due to its distinct, but similar conformation compared to both hormones. Although the position is not entirely the same, it is close enough to act as an EcR agonist, though with much lower activity than 20E and PonA.

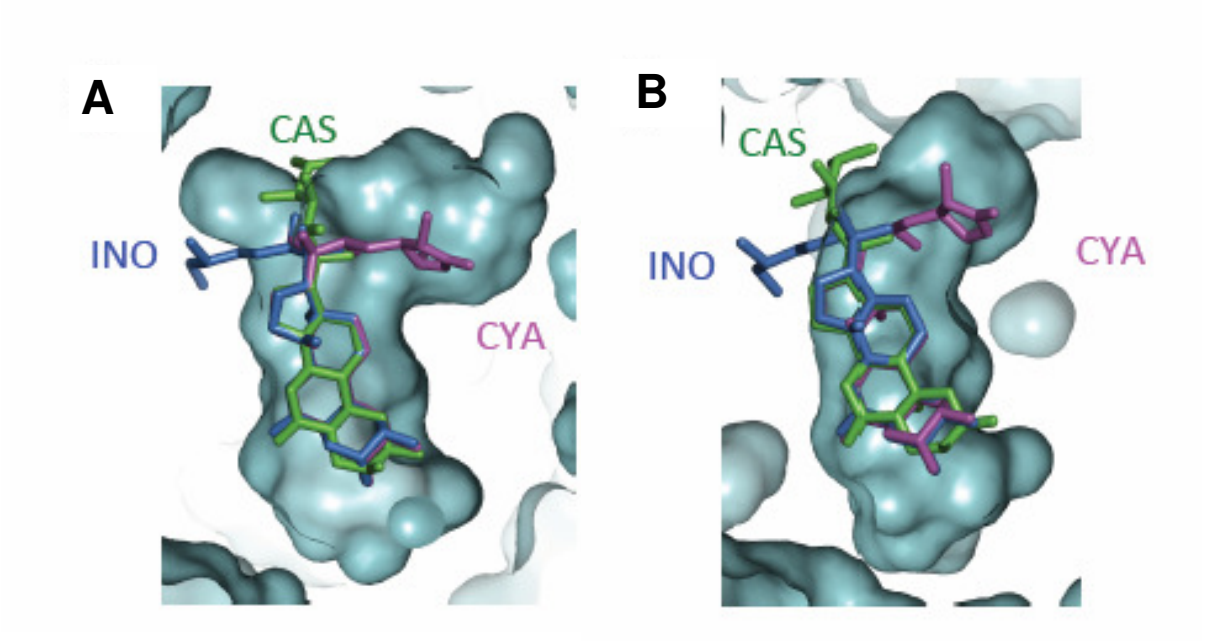


Figure 2.7A, B. Docking of cyasterone (CYA) (pink stick), inokosterone (INO) (blue stick) and castasterone (CAS) (green stick) to the ligand-binding pocket of BmEcR-LBD (A) and DmEcR-LBD (B). Note the inability of inokosterone and castasterone to correctly accommodate the ligand-binding pocket of both EcR-LBD.

Inokosterone and castasterone, however, readily differ from cyasterone by a different orientation of the aliphatic chain linked to the D steroid ring. As a result, they are unable to properly accommodate the ligand-binding pockets in both EcR-LBD (Fig. 2.7A,B) in this model. The lobe of the BmEcR-LBD ligand-binding cavity that is opposite to the one harbouring the aliphatic chain linked to the D ring of cyasterone is not large enough for the differently oriented aliphatic chain linked to the D ring in inokosterone. As seen in Fig. 2.5B, the lack of a second lobe in the ligand-binding pocket of DmEcR-LBD also prevents the binding of inokosterone. Due to steric hindrance, inokosterone is thus unable to bind to the receptor and is agonistic nor antagonistic.

The aliphatic chain linked to the D ring in the ecdysone antagonist castasterone is also oriented in such a way that no binding can occur to any of the lobes in both ligand-binding

pockets. Based on this, we can expect that the antagonistic activity observed in the measurements is not immediately linked to interaction with the EcR receptor, but rather an indirect effect of other processes in the cell that could lead to a loss in EcR response, like lower metabolism or general cell toxicity.

2.4. CONCLUSION

Although we were able to confirm that the cell-based reporter bioassay tested here is useful for screening for EcR agonistic and antagonistic activities, the test compounds included in this study were not very promising for the development of new, specific insecticides. Cyasterone, the only compound with EcR agonistic activity, binds at concentrations 10000 times lower than the commercial insecticide tebufenozide in Bm5 cells. Castasterone showed an antagonistic activity in S2 cells, but modelling with the ecdysone receptor indicated that the effect is not due to a specific interaction on the level of the receptor. Therefore, rather than to pursue this line of work, we decided to look for alternatives in other groups of secondary plant metabolites.

Chapter 3

Saponins do not affect the ecdysteroid receptor complex but cause membrane permeation in insect culture cell lines

This chapter has been redrafted from:

De Geyter E, Swevers L, Soin T, Geelen D and Smagghe G (**2012**). Saponins do not affect the ecdysteroid receptor complex but cause membrane permeation in insect culture cell lines. *Journal of Insect Physiology* 58,18-23.

De Geyter E, Swevers L, Caccia S, Geelen D and Smagghe G (**2012**). Saponins show high entomotoxicity by cell membrane permeation in Lepidoptera. *Pest Management Science*, doi:10.1002/ps.3284.

3.1. ABSTRACT

This project studied the effects of four saponins with a triterpenoid (*Quillaja saponaria* saponin and aescin) or steroid structure (digitonin and diosgenin, which is the deglycosylated form of dioscin) in one dipteran and two lepidopteran insect cell lines, namely Schneider S2 cells of *Drosophila melanogaster*, ovarian Bm5 cells of *Bombyx mori* and midgut CF-203 cells of *Choristoneura fumiferana*, respectively. A series of different experiments were performed to investigate potential mechanisms of action by saponins with regard to ecdysteroid receptor (EcR) responsiveness, cell viability, cell membrane permeation, and induction of apoptosis with DNA fragmentation and caspase-3 like activity.

Major results were that (1) exposure of S2 or Bm5 cells containing an EcR-based reporter construct to a concentration series of saponin scored no EcR activation, while (2) a loss of ecdysteroid signalling was observed with median inhibitory concentrations (IC_{50}'s) of 10-50 µM for S2 and 3-10 µM for Bm5 cells. In parallel, (3) a concentration-dependent loss of cell numbers for S2 cells in MTT cell viability assays with median effective concentrations (EC_{50}'s) of 50-700 µM was observed. Both lepidopteran cell lines also show a high sensitivity to all four saponins, with EC_{50}'s of 7-200 µM. (4) A trypan blue assay with *Q. saponaria* saponin confirmed the cell membrane permeation effect leading to cell toxicity with a median lethal concentration (LC_{50}) value of 44 µM for S2 cells, and interestingly this effect was very rapid. (5) Exposure to 20E at 500 nM as used in the EcR-based report assay induced caspase-3 like activities which may help to explain the discrepancies between loss of EcR-responsiveness and cell viability. (6) Low concentrations of saponins induced DNA fragmentation and caspase-3 like activities, confirming their potential to induce apoptosis, and (7) the saponin effects were counteracted by addition of cholesterol to the culture medium.

In general the data obtained provide evidence that saponins exert a strong activity on both dipteran and lepidopteran cells; however, the anti-ecdysteroid action by saponins is not based on a true antagonistic interaction with EcR signalling, but can be explained by a cytotoxic action due to permeation of the insect cell membrane.

3.2. INTRODUCTION

Saponins are a class of steroidal or triterpenoid secondary plant metabolites with diverse biological properties, such as hemolytic, anticarcinogenic, anti-inflammatory, molluscicidal and antifungal activities (Francis *et al*., 2002; Sparg *et al*., 2004). In insects, they also pose strong detrimental effects on survival, growth and reproduction of a broad range of pest insects (Ishaaya, 1986; De Geyter *et al*., 2007b), including Lepidoptera (Harmatha *et al*., 1987; Adel *et al*., 2000), Hemiptera (Soulé *et al*., 2000; Golawska *et al*., 2006) and Coleoptera (Shany *et al*., 1970; Szczepanik *et al*., 2001). Although strong interesting potencies were reported, most studies did not investigate the mechanism(s) behind the insecticidal action.

As steroidal saponins show structural similarities to the insect moulting hormone 20-hydroxyecdysone (20E), it has been suggested that they could exert an agonistic or antagonistic/competitive activity on the ecdysteroid reporter complex (EcR) (Dinan *et al*., 2001). Such activity would disrupt the timing of moulting and metamorphosis, which is vital for normal insect growth and development. In support of this hypothesis, Harmatha *et al*. (1987) reported ecdysial failures in leek-moth larvae (*Acrolepiopsis assectella*) fed on diet containing steroidal saponins.

Another mode of action often found in literature is that saponins disrupt the stability of the cell membrane. Indeed, such effect has been demonstrated for hematocytes and a number of other cell types, including yeasts and bacteria (Francis *et al*., 2002; Sparg *et al*., 2004). Hence, saponins are used in the medical industry as permeabilising agents to facilitate uptake of other substances into the cell (Mick *et al*., 1988; Humbel *et al*., 1998; Baumann *et al*., 2000). Additionally, a number of triterpenoid saponins have also been associated with the induction of apoptosis in human cancer cells, which may explain the lethal activity (Haridas *et al*., 2001; Chwalek *et al*., 2006; Niu *et al*., 2008). However, to our knowledge, experiments on insect cells are scarce.

Here we report on the effects of four saponins, two with a triterpenoid structure (*Quillaja saponaria* saponin and aescin) and two with a steroid structure (digitonin and diosgenin, which is the deglycosylated form of dioscin) (Fig. 3.1). We tested them on one dipteran and two lepidopteran cell lines of different tissue and origin: embryonic Schneider S2 cells of *Drosophila melanogaster* (Diptera), ovarian Bm5 cells of the silkworm *Bombyx mori*, and

midgut CF-203 cells of the spruce budworm *Choristoneura fumiferana*. To understand the saponin mechanisms of action, tests included an ecdysteroid receptor (EcR) assay, a MTT cell viability assay and a trypan blue cell permeation assay to distinguish between EcR activation and cytotoxicity effects for S2 and Bm5 cells. CF-203 cells were tested for cell viability and DNA fragmentation. We also investigated whether the four saponins can cause cell death via apoptosis in insect cells by performing caspase-3 like activity and DNA fragmentation assays. In addition, as it was reported that addition of cholesterol to the insect diet can counter the negative effects of saponins (Harmatha *et al*., 1987), we tried adding cholesterol to the cell medium alongside with the saponins and evaluated its effects on EcR-interaction, cell viability and membrane permeation.

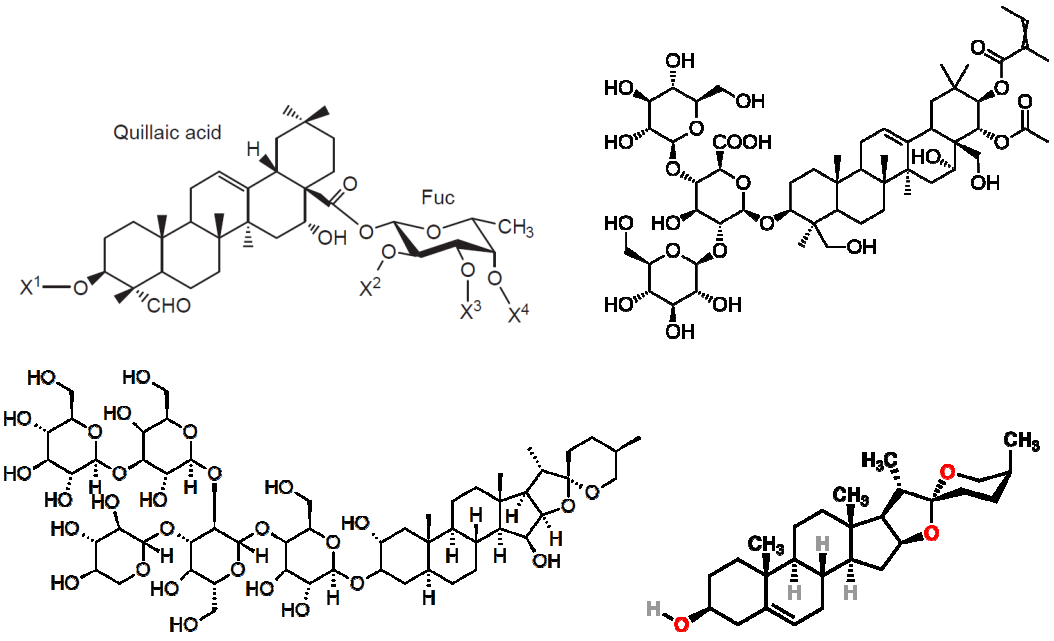


Fig. 3.1. Basic structure of *Quillaja saponaria* saponins (upper left), aescin (upper right), digitonin (lower left) and diosgenin (lower right).

3.3. MATERIALS AND METHODS

3.3.1. Chemicals

Purified powder of *Quillaja saponaria* Molina bark saponins and aescin (both ≥95%), tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], trypan blue, Actinomycin D and proteinase K were purchased from Sigma-Aldrich (Bornem, Belgium), digitonin and diosgenin (the deglycosylated form of dioscin) (≥99%) from

Chromadex Inc. (Irvine, CA), and cholesterol (>99%) from MP Biomedicals (Solon, OH). The commercial *Q. saponaria* bark saponin consists of a heterogeneous mixture of molecules varying both in their aglycone and sugar moieties, with the main aglycone (sapogenin) moiety being quillaic acid, a triterpene of predominantly 30-carbon atoms (hydrophobic) of the D12-oleanane type. The aglycone is bound to various sugars (hydrophilic) including glucose, glucuronic acid, galactose, xylose, apiose, rhamnose, fucose and arabinose (http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/1/s4521pis.Par.0001.File.tmp/s4521pis.pdf). We used an average molar mass of 1900 g/mol. All other products are analytical grade unless otherwise mentioned.

3.3.2 Insect cell lines

Cell line cultures were maintained in the Laboratory of Agrozoology (Ghent University, Belgium) at a temperature of 27°C. *Drosophila melanogaster* Schneider S2 cell line cultures were maintained in HyQ SFX-Insect™ Medium (Perbio Science, Erembodegem, Belgium) (Mosallanejad & Smagghe, 2009). *Bombyx mori* Bm5 cells were cultured in IPL-41 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Bornem, Belgium) and additional minerals (Soin *et al*., 2008). Only during the intermediate stages of the transfection, serum free medium was used (Swevers *et al*., 2004). *Choristoneura fumiferana* CF-203 cells were cultured in Insect-Xpress medium (Bio-Whittaker-Cambrex Bioscience, Walkersville, MD) supplemented with 2.5% FBS (Sigma-Aldrich, Bornem, Belgium) (Vandenborre *et al*., 2008).

3.3.3. Treatment of insect cells with saponins

For determining the effects of saponins on S2, Bm5 and CF-203 cells, the cells were treated with saponins at final concentrations of 5-500 µM (prepared in 50% ethanol). A cell solution with a density of $2x10^6$ cells/ml was prepared for S2 cells, $2\text{-}3x10^5$ cells/ml for the lepidopteran cells. Next, 1 µl of the compound was added at the bottom of the required wells of a 96-well microtitre plate (Greiner labortechnik, Frickenhausen, Germany) with a micropipette and left to dry for a few minutes, after which 100 µl of the cell solution was added into the wells. The plates were sealed with parafilm and incubated for 1 or 2 days at 27°C, and used for one of the following experiments. For each concentration 3 replicates were done, and each experiment was repeated 2 or 3 times.

3.3.4. Assay with transfected cells for EcR agonist and antagonist responsiveness

The compounds were assayed for their ability to activate transcription of an ecdysteroid-inducible luciferase reporter gene using ecdysteroid-responsive S2 or Bm5 cells. Prior to exposure, the cells were transiently transfected with the reporter plasmid by lipofection according to the manufacturer's instructions (Invitrogen) as described in Soin *et al*. (2008). Briefly, the desired number of wells of a 6-well plate was filled with 3×10^6 S2 or 2×10^6 Bm5 cells. The cells were given time to attach to the bottom and washed once or twice with serum free medium. Lipofectin was first pre-incubated alone for 45 min in culture medium and then 15 min together with 1.5 µg of the reporter plasmid ERE-b.act.luc (Fig. 2.2) before adding to the cells. The ERE-b.act.luc reporter construct is composed of seven copies of the ecdysone response element (ERE) derived from the *Drosophila* hsp27 promoter (Riddihough & Pelham, 1987), a *B. mori*-derived basal actin promoter (b.act), followed by the reporter gene for firefly luciferase (luc) and a termination signal. The cells were incubated at 27°C for 5 h, after which the transfection medium was removed and replaced by normal culture medium (with serum).

For testing EcR agonist activity, transfected cells were plated as described in 2.3 in white 96-well plates and 1 µl of a 50 µM-solution of 20E (final concentration = 500 nM) was added only to the positive control. The plates were incubated at 27°C for 24 h before reading. For EcR antagonist activity, the plates (without 20E) were first incubated at 27°C for 24 h, then 1 µl of a 50 µM solution of 20E was added to each well except for the negative control, and then the plates were incubated for another 24 h. For measuring the luciferase expression, the Steady-Glo luciferase assay system kit (Promega, Leiden, the Netherlands) was used: 100 µl luciferase substrate was added to each well and the luminescence was measured with a Tecan M200 luminometer (Tecan, Mechelen, Belgium). Data were presented as a percent response compared to 500 nM of 20E (i.e. positive control). Respective EC_{50}'s (median effective concentration values on reporter gene induction) and IC_{50}'s (medium inhibitory concentration values) with corresponding 95% confidence limits were calculated with Prism v4 (GraphPad Software Inc., La Jolla, CA); the accuracy of data fitting to the sigmoid curve model was evaluated through examination of R^2 values.

3.3.5. Assay for cell viability with MTT

The viability of the treated cells was determined in accord to Decombel *et al*. (2004). Briefly, after treatment and incubation as described in 3.3.3, 100 µl of cell solution was transferred to an Eppendorf microtube and 100 µl of a 1 mg/ml-MTT solution was added. After 3 h

incubation at 27°C, the formazan crystals were collected by centrifugation for 7 min at 20,000 g at 4°C; then, the supernatant was removed and the formazan crystals were dissolved in 220 µl isopropanol. For the next 30 min, the microtubes were rotated using a test tube rotator (Labinco, Breda, the Netherlands). After centrifugation of the resulting solution for 7 min at 20,000 g, 200 µl supernatant out of each Eppendorf tube was transferred into a transparent 96-well plate (one sample per well) and the absorbance was measured at 560 nm in a microtitre plate reader (PowerWare X340, Bio-Tek Instruments Inc., Winooski, VT). The results were presented as the percentage of active cells in comparison to the control batch and the percentage effect was calculated. EC_{50}'s (median effective concentration values on cell proliferation) with corresponding 95% confidence limits and R^2 values were calculated with Prism v4 as described above.

3.3.6. Assay for cell membrane permeation with trypan blue

The trypan blue method was performed according to Soin *et al*. (2008). Briefly, after treatment and 48h incubation as described in 2.3, 50 µl cell suspension of S2 or Bm5 cells was mixed with 50 µl trypan blue solution (0.4%) and incubated for 5 min. The numbers of blue dead (permeated) and white living (intact) cells were counted in a Bürker cell counter, and the percentage of permeated dead cells was calculated. LC_{50}'s (median lethal concentration values on cell mortality) and corresponding 95% confidence limits and R^2 values were calculated with Prism v4 as described above.

In addition, to investigate the rapidness of the cell toxicity effects, cells were incubated for short time intervals of 15 s to 3 h with various concentrations of *Q. saponaria* saponins. In parallel, to evaluate whether the cells were able to recover from the exposure to saponins, cells were treated with 500 µM *Q. saponaria* saponins for 15 s, and then the medium was removed and replaced by fresh untreated culture medium. Samples of cells of which the medium had not been replaced (incubated for the same amount of time) were counted simultaneously as a reference. In the two experiments, the numbers of dead and living cells were counted after different time intervals with use of trypan blue as above.

3.3.7. Assay to measure induction of caspase-3 like activity

Caspase-3 like enzyme activities, as a measure of cell death by apoptosis, were determined according to the protocol of Promega (http://www.promega.com.cn/techserv/tbs/TB241-550/tb323.pdf). Briefly, S2 cells were treated with saponins as described in 2.3 (2 ml per

treatment). A concentration of 10 nM actinomycin D was used as a positive control; in the negative control cells were incubated in untreated culture medium. Per treatment, three replicates were performed. After 24 h, the cells were collected into Eppendorf tubes (one for each treatment). The tubes were centrifuged at 1,000 g for 10 min (3x) and washed with phosphate buffer saline (PBS) twice. Cells were redissolved in 60 µl lysis buffer (50mM HEPES, pH 7.4, 0.1mM EDTA, 0.1% CHAPS, 5mM DTT) and centrifuged for 10 min at 10,000 g, after which the supernatant was collected. Total protein concentration was determined using the Bradford method, by pipetting 2.5 µl of the lysate and 7.5 µl lysis buffer into a 96-well plate (3 replicates per treatment) and adding 250 µl Coomassie blue to each well (becomes blue in reaction to protein). The absorbance was measured at 595 nm in a microtitre plate reader (PowerWare X340, Bio-Tek Instruments Inc.). From the results, the concentration of protein and the according volume of lysate needed for the caspase-3 activity reading were calculated and a black 96-well plate was filled with the appropriate volumes, diluted to 90 µl with assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol). Just before reading, 10 µl of Ac-DEVD-AFC substrate (Ac-Asp-Glu-Val-Asp, labeled with 7-amino-4-trifluoromethyl coumarin) was added to each well and the intensity of fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm using a spectrofluorometer (TECAN, Infinite M200, Switzerland) for 60 min (one measurement a minute). In addition, we equally repeated the reading in the presence of 0.1 mM caspase-3 inhibitor, Ac-DEVD-CHO (Sigma-Aldrich) to confirm caspase-3 specificity.

3.3.8. Assay to assess DNA fragmentation

As described above in 2.3, S2, Bm5 and CF-203 cells (2-3×10^5 cells/ml, 2 ml per treatment) were treated with saponins and collected and washed after 24 and 48 h. A concentration of 10 nM actinomycin D was used as a positive control; in the negative control cells were incubated in untreated culture medium. DNA was extracted as described in Shahidi-Noghabi *et al.* (2010a): cells were homogenized and mixed in DNA extraction buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA-NaOH, 0.1% SDS, pH 8.0) on ice. Homogenized cells were treated with 20 mg/ml RNase for 30 min at 37°C. Subsequently, 100 mg/ml of proteinase K was added and cells were incubated at 50°C for 60 min. DNA was extracted using a standard phenol-chloroform extraction method. DNA samples (5 µg) were run on a 1.5% agarose gel at 100 V and visualized by staining with ethidium bromide.

3.3.9. Assay to assess counteraction of saponin effects by cholesterol in insect cells

To determine if addition of cholesterol to the cell medium could influence the effects of saponins on insect cells, the ecdysteroid responsiveness (3.3.4), MTT (3.3.5) and trypan blue (3.3.6) bioassays were repeated with S2 and Bm5 cells in culture medium containing equal weight amounts of saponin and cholesterol. The experiments were performed and analyzed as described above.

3.4. RESULTS

3.4.1. Saponins pose no EcR agonistic activity but inhibit EcR responsiveness

A series of experiments showed that none of the saponins showed any agonistic activity at the concentrations tested (data not shown). The 20E response of the treated cells was never significantly higher than that of the non-treated cells.

The saponins did, however, exert a strong concentration-dependent EcR antagonist activity, with a total loss of response for all four saponins at concentrations of 100-200 µM for S2 and 10-50 µM for Bm5 cells. Results showed a rapid decrease in EcR response with increasing saponin concentrations, with an IC_{50} of 17 µM for *Q. saponaria* saponins, 30 µM for aescin, 49 µM for digitonin and 39 µM for diosgenin for S2 cells (Table 3.1; Fig. 3.2A). For Bm5 cells, sigmoid curve fitting in Prism calculated an IC_{50} of 6.8 µM for *Q. saponaria* saponins, 8.3 µM for aescin, 3.7 µM for digitonin and 7.2 µM for diosgenin (Table 3.1; Fig. 3.2B).

Table 3.1. Biological activity of the four saponins (*Quillaja saponaria*, aescin, digitonin and diosgenin) in *Drosophila melanogaster* S2 and *Bombyx mori* Bm5 cells for EcR antagonism (IC_{50}).

	S2	**Bm5**
Saponin	**IC_{50} (95% CL; R^2) (µM)**	**IC_{50} (95% CL; R^2) (µM)**
Q. saponaria	17 (14-20; 0.92)	6.8 (6.1-7.7; 0.98)
aescin	30 (23-40; 0.88)	8.3 (1.9-3.7; 0.80)
digitonin	49 (40-61; 0.95)	3.7 (2.1-6.5; 0.93)
diosgenin	39 (26-60; 0.80)	7.2 (1.1-46; 0.98)

Data are given as median response values together with the 95% confidence interval (both in µM) and the R^2 as accuracy of data fitting to the sigmoid curve model after Prism v4.

3.4.2 Saponins reduce cell viability in insect cells

Incubation with any of the four tested saponins caused clear harmful concentration-dependent effects on S2 cells as well as in both lepidopteran cell lines. There was a decrease in viability with increasing saponin concentrations: for S2 cells, the dose-response curves resulted in an EC_{50} of 51 µM for *Q. saponaria* saponin, 350 µM for aescin, and 104 µM for digitonin (Table 3.2; Fig. 3.2A). For diosgenin, a decrease of 24% was obtained with the highest concentration of 480 µM, and an LC_{50} of 699 µM (237-2067) was extrapolated. For Bm5 cells, the dose-response curves resulted in an EC_{50} of 17 µM for *Q. saponaria* saponin, 34 µM for aescin, 7.5 µM for digitonin, and 48 µM for diosgenin (Table 3.2; Fig. 3.2B). CF-203 cells followed a similar pattern with respective EC_{50} values of 13, 25, 15 and 202 µM for *Q. saponaria,* aescin, digitonin and diosgenin (Table 3.2).

Table 3.2. Biological activity of the four saponins (*Quillaja saponaria*, aescin, digitonin and diosgenin) in one dipteran and two lepidopteran cell lines (embryonal *Drosophila melanogaster* S2, ovary *Bombyx mori* Bm5 and midgut *Choristoneura fumiferana* CF-203 cells) for cell viability in a MTT assay (EC_{50}).

	S2	**Bm5**	**CF-203**
Saponin	**EC_{50} (95% CL; R^2) (µM)**	**EC_{50} (95% CL; R^2) (µM)**	**EC_{50} (95% CL; R^2) (µM)**
Q. saponaria	51 (46-55; 0.89)	17 (14-20; 0.88)	13 (9.0-19; 0.83)
aescin	350 (260-480; 0.64)	34 (25-46; 0.96)	25 (14-43; 0.68)
digitonin	104 (89-120; 0.94)	7.5 (4.1-14; 0.92)	15 (9-22; 0.97)
diosgenin	480 µM (24%)*	48 (32-73; 0.81)	202 (122-332; 0.71)

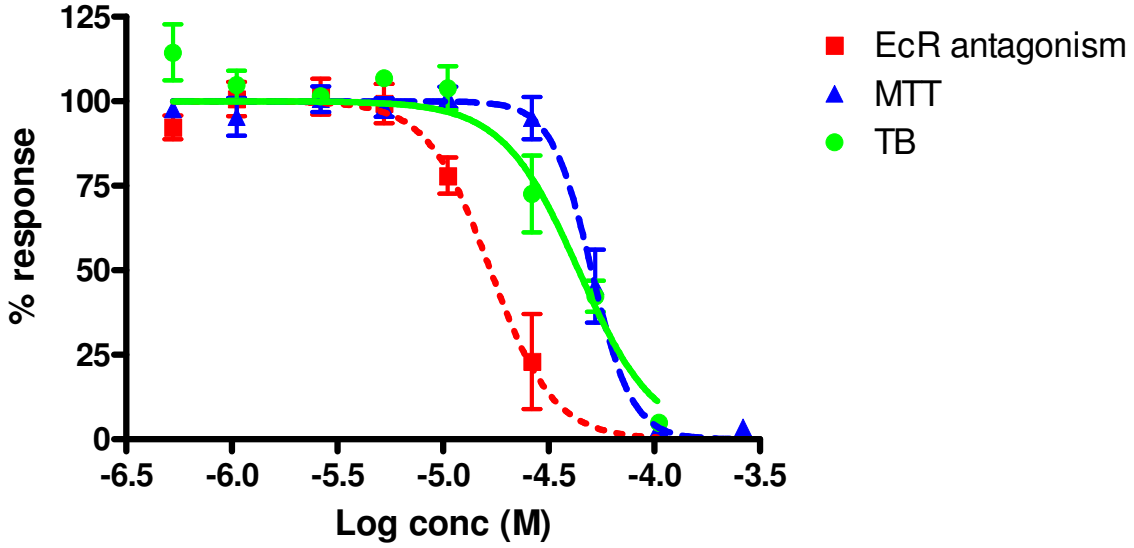
* The highest concentration tested (480 µM) resulted in 24% loss of cell viability.

Data are given as median response values together with the 95% confidence interval (both in µM) and the R^2 as accuracy of data fitting to the sigmoid curve model after Prism v4.

3.4.3 Saponins cause cell membrane permeation

S2, Bm5 and CF-203 cells exposed during 48 h to *Q. saponaria* saponin showed clear signs of membrane permeation leading to cell death. The effect was dose-dependent with an LC_{50} of 44 µM (95% CL: 36-53 µM; R^2 = 0.88) for S2 cells (Fig. 3.2A), 11 µM (95% CL: 8.4-14 µM; R^2=0.90) for Bm5 (Fig. 3.2B) and 8.6 µM (95% CL: 7.8-9.5 µM; R^2=0.98) for CF-203 cells.

A



B

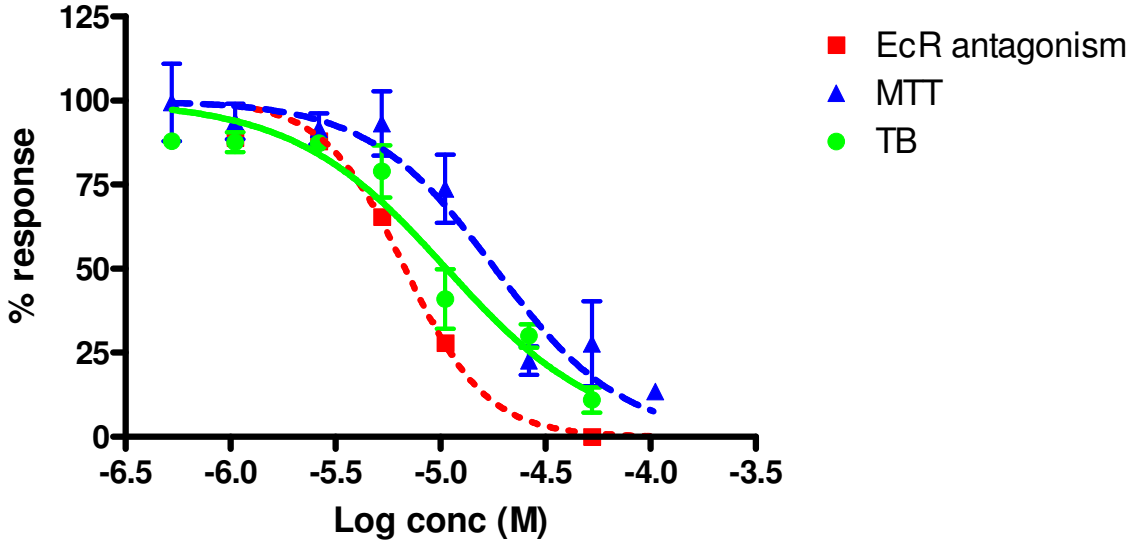


Figure 3.2A, B. Dose-response curves on the biological activities of *Quillaja saponaria* saponin in *Drosophila melanogaster* S2 (A) and *Bombyx mori* Bm5 cells (B) for EcR antagonism (IC_{50}), cell viability in a MTT assay (EC_{50}), and cell permeation in a trypan blue (TB) assay (LC_{50}). Data are given as median response values together with the 95% confidence interval (both in µM) after sigmoid curve fitting in Prism v4.

For experiments with shorter periods of time, exposure of S2 cells to *Q. saponaria* saponins at 500 µM for 15 s already resulted in 62.2±6.5% cell toxicity compared to the untreated control

cells. After 1 min this had further increased to 99.7±0.1%, confirming the cell permeation effect was very rapid. After 15 min, cell toxicity was complete (100±0.0%). Lepidopteran Bm5 and CF-203 cells were also incubated during short intervals of 1 min, and these experiments gave an immediate total mortality at 50 µM for both cell lines, confirming the rapid cell permeation effects of *Q. saponaria* saponins.

When the medium containing 500 µM saponin was removed after 15 s and replaced with saponin-free medium, an S2 cell toxicity rate of 62.2±6.5% was recorded which did not significantly ($p>0.05$) change for at least 3 h after treatment. Even at 24 h after the medium change, the percentage of dead cells had not significantly changed (53.0±16.2%; $p=0.6$), indicating that the permeation effects by saponin were permanent and could not be reversed by removing the saponins from the medium.

3.4.4. 20-Hydroxyecdysone at 500 nM and saponins at low concentrations induce caspase-3 like activities

Incubation of S2 cells with 20E at 500 nM resulted in a significant increase of the caspase-3 like enzyme activity to 456% of control cells (Table 3.3). In parallel, saponins at low concentrations of 10-20 µM caused a low but significant ($p<0.05$) increase in caspase-3 like activity: for *Q. saponaria* saponins (at 10 µM) the activity was 36% higher than in the untreated control cells, for aescin (18 µM) 85%, for digitonin (16 µM) 23% and finally for diosgenin (48 µM) 62% (Table 3.3).

Table 3.3. Increase in caspase-3 like activities as percentage of the untreated control (=100%) upon exposure during 24 h of *Drosophila melanogaster* S2 cells to 20-hydroxyecdysone at 500 nM and the four saponins *Quillaja saponaria* (10 µM), aescin (18 µM), digitonin (16 µM) and diosgenin (48 µM).

Treatment	mean activity (%)	st. dev.	p-value
20E	456	208	0.03
Q. saponaria	136	8	0.002
aescin	185	18	0.001
digitonin	123	8	0.01
diosgenin	162	16	0.003

Data are expressed as mean ± SD based on three replicates together with the p-value after a Student's t-test.

3.4.5. Saponins cause DNA fragmentation upon exposure at low concentrations

As shown in Fig. 3.3, DNA isolation of S2 cells exposed to 20E (500 nM) and low concentrations of saponins, as described above for caspase-3 like activity induction, confirmed that these concentrations caused DNA fragmentation, leading to apoptosis. The effect was strongest for *Q. saponaria* saponins and aescin, but was observed for all four saponins.

DNA isolation of Bm5 and CF-203 cells exposed to concentrations of saponins that induced low percentages of cytotoxicity in the MTT bioassays (*Q. saponaria* 10 µM, aescin 18 µM, digitonin 16 µM and diosgenin 48 µM) also confirmed that these concentrations caused DNA fragmentation, leading to apoptosis. Here, the effect was strongest for *Q. saponaria* saponin and digitonin (for both cell lines), but was observed for all four saponins (data not shown).

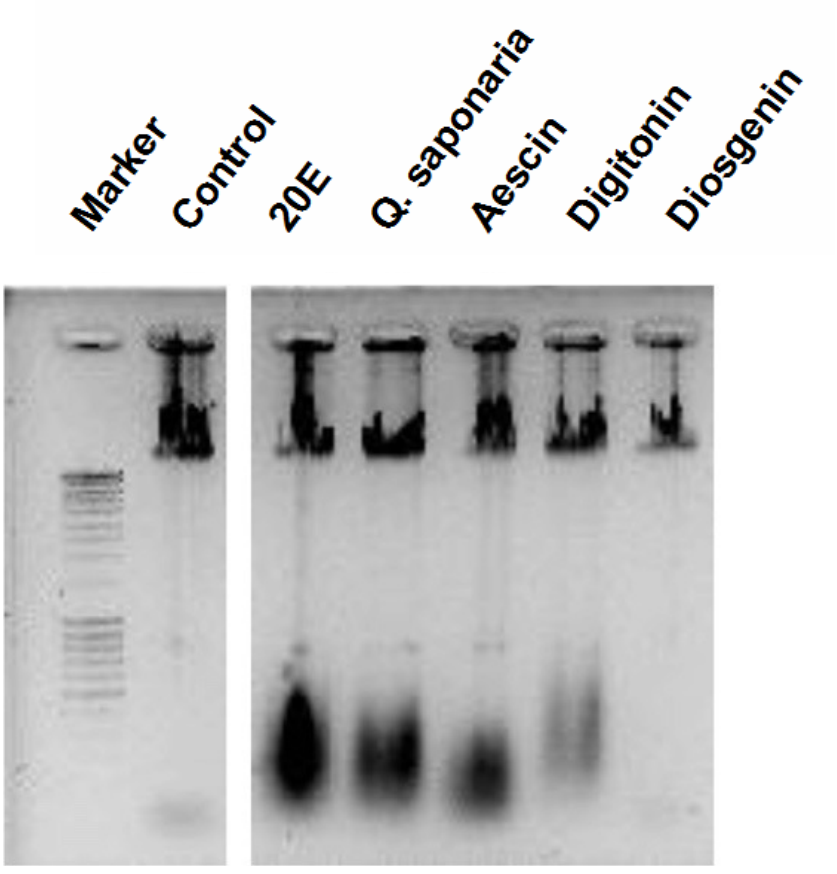


Figure 3.3. Agarose gel electrophoresis pattern of *Drosophila melanogaster* S2 cells showing DNA fragmentation upon exposure for 24 h to the insect moulting hormone 20E at 500 nM and the four saponins *Quillaja saponaria* (10 µM), aescin (18 µM), digitonin (16 µM) and diosgenin (48 µM).

3.4.6. Addition of cholesterol counteracts the effects of saponins

In both the EcR response and the MTT and trypan blue viability tests, addition of cholesterol in a 1:1 saponin:cholesterol ratio was seen to partially reduce the effects of saponins on S2 and Bm5 cells (Table 3.4). Based on medium response values (IC_{50}'s and EC_{50}'s), in the presence of cholesterol the detrimental effects of saponins became visible at about 4 times higher concentrations than with saponins alone. Addition of cholesterol by itself had no effect.

Table 3.4. Biological activity of the four saponins (*Quillaja saponaria*, aescin, digitonin and diosgenin) when combined at 1:1 with cholesterol in *Drosophila melanogaster* S2 and *Bombyx mori* Bm5 cells for EcR antagonism (IC_{50}) and cell viability in an MTT assay (EC_{50}).

	S2	**Bm5**
Saponin	**IC_{50} (95% CL; R^2) (µM)**	**IC_{50} (95% CL; R^2) (µM)**
Q. saponaria	74 (41-136; 0.44)	15 (12-20; 0.93)
aescin	113 (73-174600; 0.68)	
digitonin	160 µM (15%)*	
diosgenin	114 (68-190; 0.76)	
Saponin	**EC_{50} (95% CL; R^2) (µM)**	**EC_{50} (95% CL; R^2) (µM)**
Q. saponaria	100 µM (44%)*	47 (24-96; 0.52)
aescin	--	
digitonin	959 (335-2745; 0.71)	
diosgenin	480 µM (12%)*	

* The highest concentration tested (in µM) resulted in % loss of cell viability as given between brackets.

Data are given as median response values together with the 95% confidence interval (both in µM) and the R^2 as accuracy of data fitting to the sigmoid curve model after Prism v4.

3.5. DISCUSSION

Saponins are known to have a broad range of divergent biological activities. They have anticarcinogenic, anti-inflammatory, antioxidant, hemolytic, immunostimulant and membrane-permeabilising properties, can affect feed intake, growth and reproduction in animals, and are used as fungicides and molluscicides, as well as against some bacteria and viruses (Francis *et al*., 2002; Sparg *et al*., 2004). More importantly to this study, they have strong detrimental effects on insects, causing mortality, growth retardation and decreased fecundity (De Geyter *et al*., 2007b). It is generally expected that they play an important role in plant defence against predators and diseases. However, the exact mechanisms behind this insecticidal action are not yet completely elucidated.

Steroidal saponins show structural similarities to the ecdysteroid insect moulting hormone 20E. As various other plant derived secondary compounds have been shown to display agonistic, or antagonistic/competitive activity on the EcR receptor site for 20E, it has been proposed that saponins might trigger or inhibit EcR signalling and thereby interfere with insect ecdysis (Harmatha *et al*., 1987; Dinan *et al*., 2001). So far there is little proof supporting this hypothesis: two steroidal saponins, digitonin and aginosid, together with two additional leek flower saponins and some aglycones were tested for their direct effect on the EcR in a *D. melanogaster* B_{II} assay by Harmatha & Dinan (1997), but none of the compounds showed significant agonistic or antagonistic activity. In another B_{II} cell bioassay for ecdysteroid agonist and antagonist activities (Dinan *et al*., 2001), there were no saponins showing agonist activity, but few showed principal antagonistic activity. For both B_{II} bioassays, it should be remarked that cell viability was scored as a measure of effect. In accordance to the latter data, our current experiments using ERE-dependent reporter constructs in transfected S2 and Bm5 cells clearly demonstrated that neither the two triterpenoid saponins (*Q. saponaria* saponin and aescin) nor the two steroidal saponins (digitonin and diosgenin) have any agonistic activity on the EcR complex. By contrast, the four saponins did show a strong antagonistic activity, significantly lowering the EcR response starting from concentrations of about 5-10 µM onwards; however, the experiment did not allow to conclude whether this is due to a true antagonistic activity on the receptor (EcR), or to other effects like loss of cell viability. In a similar case, data reported by Soin *et al*. (2008) did not support JH analogs (JHAs) acting through a direct modulation of the activity of the EcR transcription complex, because the 'antagonism' of EcR activity by JHAs correlated with

cytotoxicity.

In order to confirm whether the antagonistic activities observed with the four saponins could be the result of a more general cytotoxicity effect, cell viability assays were conducted using MTT as a substrate (Decombel *et al*., 2004). The MTT method is a colourimetric method that measures the reduction of a tetrazolium component (MTT) into a formazan product by viable cells. Metabolism in viable cells produces 'reducing equivalents' like NADH and NADPH. At death, cells rapidly lose the ability to reduce tetrazolium products; the production of the coloured formazan product is therefore proportional to the number of cells in culture (unless metabolic processes in the cells have changed). In our observations, the decreases in EcR response and in cell viability with increasing saponin concentrations were very similar for both S2 and Bm5 cells (Fig. 3.2), which led to the conclusion that the lowered EcR response in the insect cells is due to general cell toxicity rather than to a true antagonistic activity. As a consequence, we propose that older reports on EcR-antagonism by diverse saponins should be reinvestigated taking into account cytotoxicity effects.

To explain more in detail the mechanism of loss of cell viability by saponins, we tested the effects of *Q. saponaria* saponins in a trypan blue permeability assay. Trypan blue dye can enter cells only through damaged plasma membranes, staining the entire cell blue. Many saponins have been shown to permeabilise the plasma membrane in yeast, bacterial and mammal cells by interacting with membrane sterols (Polacheck *et al*., 1991; Leung *et al*., 1997; Armah *et al*., 1999; Levavi-Sivan *et al*., 2005). Digitonin and its analogs were shown to be able to cause membrane damage by binding to the cholesterol in liposomes (Nishikawa *et al*., 1984). But although saponins are broadly used as permeabilizing agents in the medical sector (Mick *et al*., 1988; Humbel *et al*., 1998; Baumann *et al*., 2000), the exact mechanism by which they allow the entrance of molecules into the cell and the extent of reversibility of this process are still being discussed (Melzig *et al*., 2001; Levavi-Sivan *et al*., 2005). The formation of non-specific 'pores' seems to be the most generally recognized mode of action (Armah *et al*., 1999), but Levavi-Sivan *et al*. (2005) suggested rearrangement of membranes rather than pore formation as the mechanism of action of *Q. saponaria* saponins. Also, in many cases, different saponins show different or specific effects (Sung *et al*., 1995; Leung *et al*., 1997; Menzies *et al*. 1999; Levavi-Sivan *et al*., 2005). In our results, the MTT viability tests clearly indicated that both steroidal and triterpenoid saponins have strong cytotoxic effects on the embryonic S2 cells at concentrations of 50-500 µM. Lepidopteran cell lines

show an even higher sensitivity to all four saponins under examination, with a concentration-dependent loss of cell viability with EC_{50} values of 7-200 µM for both Bm5 and CF-203 cells. In accordance with these observations, the trypan blue experiments confirmed that the saponins cause a permeabilisation of the cell membrane because the dye could enter the cells. These effects work very fast and can be perceived in S2 cells after less than 15 seconds. Here, it was also of interest that replacement of the *Q. saponaria* saponin-treated medium with fresh untreated medium after 15 s of incubation did not allow the insect cells to recover, suggesting a pore formation rather than a rearrangement of the cell membrane by the saponin treatment. In conclusion, the current data are strong indicatives that permeabilisation is the primary cause of the cytotoxicity -- or a direct result of it -- as the numbers of perforated cells in the trypan blue assay correspond closely with the loss of metabolic activity in the MTT assays (Fig. 3.2).

A second interesting observation associated with the current experiments was that there was a small discrepancy between the dose-response curves for EcR responsiveness and the cell viability assays. According to the apoptosis experiments (see below), this can be explained by the caspase-3 like activity caused by 20E. Indeed at 500 nM, which is the concentration used for activating the ecdysteroid response in the EcR-antagonist bioassay, 20E caused an increase of caspace-3 like activity to 456% of untreated control cells. Thus, because of the induction of caspase-3 like activity, 20E could 'sensitize' the cells to the effects of the saponins.

To test the ability of saponins for inducing caspase-3-dependent apoptosis, as reported before in mammalian cells (Haridas *et al*., 2001; Chwalek *et al*., 2006; Niu *et al*., 2008), we exposed the cells to concentrations of saponins that induced low percentages of cytotoxicity in the MTT bioassays and tested for caspase-3 like activities as well as for induction of DNA fragmentation. We found increased caspase-3 like activity for all four saponins in S2 cells, indicating an additional apoptosis-inducing effect of saponins. This is backed up by the results of the DNA fragmentation assay, where all saponins show signs of fragmentation in all three cell lines (Fig. 3.2).

Apart from the two abovementioned, several other potential modes of action to explain the insecticidal activity of saponins have been brought forward in literature. One of them is that saponins can bind (phyto)sterols in the food, thus hindering the uptake of necessary sterol precursors by the insect (Shany *et al*., 1970) and interrupting the biosynthesis of ecdysteroids

(Harmatha, 2000). According to Harmatha *et al*. (1987), adding extra cholesterol (or other sterols) to the food can counter the effects of saponins. In our experiments, addition of cholesterol in a 1:1 saponin:cholesterol ratio was proven to successfully counteract the effects of saponins on the cell cultures, but only within a limited range: if the concentration of saponins and cholesterol was increased by four times or more, the protective effect of the cholesterol was lost and we saw a dose-dependent decrease in cell viability like before. Once again, the mode of action for this 'postponing' effect is unclear. Since saponins are known to form insoluble complexes with cholesterol in water (Mitra & Dungan, 2000), we presume that the additional cholesterol in the medium will bind with the saponins and thus prevent them from affecting the cells. But since the ratio saponin:cholesterol stayed the same in all treatments, this does not explain why the effect was lost at higher concentrations. Another hypothesis is that the availability of cholesterol in the medium helps to maintain the integrity of the cell membranes: since cholesterol is an essential part of the membrane, perhaps early damage caused by binding of saponins can be restored more easily in the presence of high cholesterol levels. This is in agreement with the observation that the mechanism of action of certain saponins on fungi has been found to be due to the detergent function via interaction with sterols in the cell membrane (Armah *et al*., 1999), and that fungi without sterols in the membrane are highly resistant to saponins (Arneson & Durbin, 1967). It would explain the limited range in which cholesterol works, since there is a limit to the cells' recuperation capacities; however, to successfully determine the exact nature of the interactions between saponins and cholesterol, further research will be needed.

The concurrence between the results on *Drosophila* S2 cells and on lepidopteran Bm5 and CF-203 cells supports the hypothesis that the basic mechanism of action of saponins on insect cells is universal and does not depend on insect order or tissue origin of the cells, although the sensitivity of the cells to the saponins differs significantly, with both lepidopteran cell lines being about 2-4 times more sensitive than the dipteran S2 cells. It is possible that this is due to larger cells having a larger membrane surface and thus being more susceptible to the permeating effects of saponins; both Bm5 and CF-203 cells are relatively large with a diameter of 20-25 µm, whereas the smaller S2 cells measure only 5 µm. Another possibility is that the cell lines differ in membrane composition, for example a higher concentration of membrane sterols; but unfortunately, we do not possess the chemical data for an in-depth analysis.

As a general conclusion, our results do not support a role for steroidal and triterpenoid saponins acting directly as agonists, nor as true antagonists on the EcR complex. Instead, our data confirmed that these saponins cause cytotoxicity by a rapid and stringent permeation of the insect cell membrane. The discrepancy between the dose-response curves for EcR antagonism and cell viability can be due to the induction of caspase-3 like activity, leading to cell death, by the 500 nM of 20E used in the EcR responsiveness assays. We also found that low concentrations of saponins can induce caspase-3 like activity and DNA fragmentation (to be shown) in exposed insect cells, and finally, addition of cholesterol to the cell medium can counteract the effects by saponins.

Chapter 4

Triterpene saponins of *Quillaja saponaria* show strong aphicidal and deterrent activity against the pea aphid, *Acyrthosiphon pisum*, and the cotton leafworm, *Spodoptera littoralis*

This chapter has been redrafted from:

De Geyter E, Smagghe G, Rahbé Y and Geelen D (**2012**). Triterpene saponins of *Quillaja saponaria* show strong aphicidal and deterrent activity against the pea aphid (*Acyrthosiphon pisum*). *Pest Management Science* 68, 164-169.

De Geyter E, Swevers L, Caccia S, Geelen D and Smagghe G (**2012**). Saponins show high entomotoxicity by cell membrane permeation in Lepidoptera. *Pest Management Science*, doi:10.1002/ps.3284.

4.1. ABSTRACT

Saponins are a class of secondary plant metabolites consisting of a sugar moiety glycosidically linked to a hydrophobic aglycone (sapogenin) that often possess insecticidal activities. Four saponins were selected: two triterpene saponins, *Quillaja saponaria* saponin and aescin, and two steroidal saponins, digitonin and diosgenin (the deglycosylated form of dioscin). The triterpene *Q. saponaria* bark saponin received special attention because of its high activity and availability. Their effects were investigated on two important pest species: a model piercing-sucking insect, the pea aphid *Acyrthosiphon pisum,* and a biting-chewing insect, the cotton leafworm *Spodoptera littoralis*. Aphids were challenged by oral and contact exposure to demonstrate aphicidal activities, and in choice experiments to support use as a natural deterrent. In addition, we tested the entomotoxic action of *Q. saponaria* saponin with primary midgut cell cultures and larval stages of *S. littoralis*.

When aphids were exposed to supplemented artificial diet for 3 days, a strong aphicidal activity was recorded for three of the four saponins, with an LC_{50} of 0.55 mg/ml for *Q. saponaria* saponin, 0.62 mg/ml for aescin and 0.45 mg/ml for digitonin. The LT_{50} values ranged between 1 and 4 days, depending on the dose. For diosgenin, only low toxicity (14%) was scored for concentrations up to 5 mg/ml. In choice experiments with treated diet, a deterrence index of 0.97 was scored for *Q. saponaria* saponin at 1mg/ml. In contrast, direct contact showed no repellent effect. Spraying of *Vicia faba* bean plants with *Q. saponaria* saponin resulted in an LC_{50} of 8.2 mg/ml. Finally, histological analysis in aphids fed with *Q. saponaria* saponin demonstrated strong aberrations of the aphid gut epithelium. In *S. littoralis*, *Q. saponaria* saponin caused cytotoxicity in primary midgut cell cultures of *S. littoralis* (EC_{50} 4.7 µM or 0.009 mg/ml) and killed 70-84% of larvae at pupation at a dose of 30-70 mg/g, while lower concentrations retarded larval weight gain and development.

The present insect experiments provide strong evidence that saponins, as tested here with triterpene *Q. saponaria* saponin, can be useful as natural insecticides and deterrents. Furthermore, primary midgut cell cultures of *S. littoralis* and histological analysis of the aphid midgut suggested the insect midgut epithelium to be a primary target of saponin activity.

4.2. INTRODUCTION

The pea aphid *Acyrthosiphon pisum* (Harris) (Hemiptera, Aphidiae) is an important cosmopolitan pest that feeds on a wide range of legume plants, including many agricultural crops in large parts of the world. In addition, it can act as a vector for more than 30 plant virus diseases (Blackman & Eastop, 2000). The species is frequently used as a model organism and representative for the aphids, and recently its entire genome sequence has been published (The International Aphid Genomics Consortium, 2010). Pest control of *A. pisum* is challenging because the long-term use of synthetic broad-spectrum insecticides has led to the widespread development of resistance; in addition, aphids are considered to be insensitive to treatments with the insecticidal toxins of *Bacillus thuringiensis* Berliner (Sharma *et al*., 2004). Therefore, together with an increasing public awareness and concerns regarding pesticide safety and possible damage to the environment, scientists and companies are on a constant search for new, natural insecticides (Edwards *et al*., 2008).

The insect order of the Lepidoptera also contains many very important pest species, against which the current insecticides are poorly effective due to development of high levels of resistance; therefore, demands for new insecticides are high. Here in this project, *S. littoralis* was used as representative of the Lepidoptera as a major group of pest insects in agriculture, but by itself it is also an important cosmopolitan pest, causing high losses in agriculture due to its high polyphagous character with >40 host plants and the fact that many populations show high levels of resistance to nearly all insecticide groups (Alford, 2000).

A potentially interesting class of natural molecules consists of the saponins, a group of secondary plant metabolites comprising a sugar moiety glycosidically linked to a hydrophobic aglycone (sapogenin), which may be triterpene or steroidal. They occur in a great number of plant species (mainly angiosperms), both wild plants and cultivated crops, and possess divergent biological activities, including a clear non-neurotoxic insecticidal activity against a broad range of pest insects (Sparg *et al*., 2004; De Geyter *et al*., 2007b; Vincken *et al*., 2007). The most commonly observed effects on insects are increased mortality, lowered food intake, weight reduction, retardation in development and decreased fecundity. Although the exact mode of action is not known, it is likely that they work through a combination of several activities.

In order to study the effects of saponins on the pea aphid, two triterpene saponins, *Quillaja saponaria* saponins and aescin, and two steroidal saponins, digitonin and diosgenin (the deglycosylated form of dioscin), were selected for this project (Fig. 3.1). After a first screening for toxicity, additional toxicity tests and choice bioassays were performed with the triterpene *Q. saponaria* saponins, which originate from the bark of the soapbark tree *Q. saponaria*, to examine in more detail the potential against aphids. The effects on the insect midgut *in vivo* were also evaluated by making microtome slides of intoxicated aphid guts.

Finally, we determined the entomotoxic action of *Q. saponaria* saponins with primary midgut cell cultures and larval stages of the cotton leafworm *Spodoptera littoralis,* to confirm the insect midgut as a primary target tissue for entomotoxicity by saponins and the potential use of saponins as natural insecticides in the control of pest Lepidoptera.

4.3. MATERIALS AND METHODS

4.3.1. Chemicals

Commercially available powder of *Quillaja saponaria* Molina bark saponins (see 3.3.1) and aescin (≥95%) were purchased from Sigma-Aldrich (Bornem, Belgium), and digitonin and diosgenin (the deglycosylated form of dioscin) (≥99%) from Chromadex Inc. (Irvine, CA). Other chemicals were of analytical grade unless otherwise mentioned.

4.3.2. Insects

Continuous colonies of *Acyrthosiphon pisum* and *Spodotera littoralis* are maintained under standardized conditions (23 ± 5°C, 65 ± 5% relative humidity, 16 h photoperiod) in the Laboratory of Agrozoology, Ghent University, Belgium. The aphids are reared on *Vicia faba* (L.) bean plants, and all bioassays were carried out using newborn nymphs of 0–12 h age (Christiaens *et al*., 2010). *S. littoralis* is kept on an agar-based artificial diet as described before (Iga & Smagghe, 2011); for experiments, newly moulted third instar catterpillars were used.

4.3.3. Insect bioassays

4.3.3.1. Aphid bioassays with oral exposure via artificial diet without and with choice

For the no-choice aphid survival assays, artificial diet test cages were prepared as described

before (Sadeghi *et al*., 2007) by stretching a layer of parafilm over a hollow Plexiglas tube (diameter = 3 cm; height = 3 cm). On this first layer of parafilm, 100 µl of artificial diet was placed, and then a second layer of parafilm was stretched over the first to keep it sealed. The saponins were tested at different concentrations in the artificial diet, ranging between 0.01 and 70 mg/ml for *Q. saponaria* saponins, 0.01 and 10 mg/ml for aescin, 0.1–10 mg/ml for digitonin and 0.1 and 5 mg/ml for diosgenin. Ten neonate (<12 h old) aphids were placed on this sachet, and a hollow plastic ring with a ventilation lid was placed on top of the tube to prevent insect escape (Fig. 4.1). The tubes were placed upside down in a six-well plate. For each treatment, three replicates were performed. Survival (expressed as the percentage of living aphids out of the total number of aphids treated) was scored at 24 h intervals for 3 days. The medium lethal concentrations (LC_{50}) and medium lethal times (LT_{50}) and the corresponding 95% confidence intervals (95% CI) were calculated in GraphPad Prism v.4 (GraphPad Software, La Jolla, CA), as described previously (Smagghe *et al*., 2010); the accuracy of data fitting to the curve model was evaluated through examination of R^2 values.

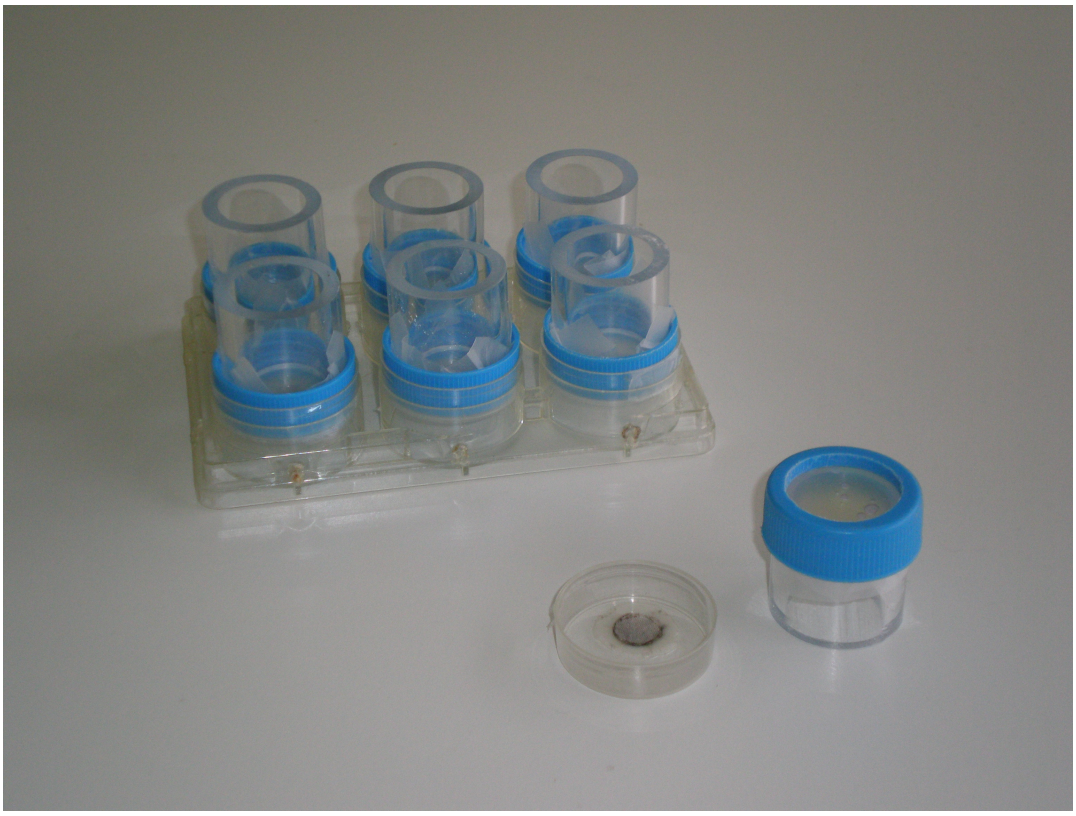


Figure 4.1. No-choice toxicity bioassay test cages for *Acyrthosiphon pisum*. Aphids are placed inside the blue ring, between the double parafilm containing artificial diet and the lid. The cages are placed upside down in the incubator.

In addition to the above-mentioned no-choice assays, artificial diet test cages were prepared for the choice experiments as described above, except that two cages were connected to each other: one cage contained normal untreated artificial diet and the other contained artificial diet containing 0.1, 1 and 10 mg/ml of saponins. The two cages were placed with the openings opposite to each other, forming a 6 cm long tube with the sachet of artificial diet on both sides. Ten neonate (<12 h old) aphids were placed inside each tube, and the tubes were placed into the incubator in a horizontal position so that the aphids could easily walk from one side to the other. For each concentration, three replicates of ten aphids starting on the diet with saponin and three replicates of ten aphids starting on the control diet were performed. After 24 h, the number of living aphids and the number of aphids on each sachet (saponin-containing versus control diet) were counted. A deterrence index

$$DI = (C - T) / (C + T)$$

where C is the number of aphids on the control diet and T is the number of aphids on the saponin-containing diet, was calculated as described previously (Zapata *et al*., 2010).

4.3.3.2. Aphid bioassays for repellent/deterrent activity on contact exposure

To investigate the repellent/deterrent activity of *Q. saponaria* saponins via contact, neonate (<12 h old) aphids were exposed to the saponin by direct contact on inert material. Glass plates (10 × 10 cm) were painted with a paintbrush on half their surface with an aqueous 10 or 100 mg/ml saponin solution, and on the other half with water, and then left to dry at room temperature in a fume hood. Subsequently, ten neonate aphids were placed in the arena, and a petri dish (diameter = 9 cm) was placed upside down on the glass plate to prevent insect escape. For each concentration, three replicates of ten aphids were performed with insects put on the saponin-treated surface, and a further three replicates of ten aphids with insects put on the water-treated control surface. The numbers of aphids on the saponin-treated and water-treated control sides were counted every 15 min in the first hour, and then at 60 min intervals up to 7 h. The DI was then calculated as described above in Section 2.4.1.

4.3.3.3. Aphid bioassays with exposure after spraying of bean plants without and with choice

To evaluate the use of *Q. saponaria* saponins when applied to living plant material, freshly cut *V. faba* bean leaves were sprayed by hand with an aqueous saponin solution (1, 10 and 100 mg/ml) and then left to dry in a fume hood at room temperature. Subsequently, the leaves were placed individually in a petri dish (diameter = 9 cm) with wet cotton wool, as described

before (Shahidi-Noghabi *et al*., 2008). Ten neonate (<12 h old) aphids were placed on each leaf, and three replicates were undertaken for each concentration in this no-choice experiment.

In parallel, a choice experiment was set up with two freshly cut *V. faba* bean leaves, where one was sprayed with *Q. saponaria* saponin solution and the other with water. The leaves were placed as above in a petri dish (diameter = 9 cm) with wet cotton and with a 2 cm distance between each other. Ten neonate (<12 h old) aphids were placed on each leaf. For each concentration, three replicates (n = 10 aphids) starting on saponin-sprayed leaves and a further three replicates of ten aphids starting on water-sprayed leaves were performed. After 24 h, the number of living aphids and the number of aphids on each leaf were counted, and the DI was calculated as above.

4.3.3.4. Caterpillar bioassays with oral exposure via artificial diet without choice

Newly moulted (0-6 h) third instars of *S. littoralis* were fed on Stonefly Heliothis artificial diet containing different concentrations of *Q. saponaria* saponin (at 10, 20, 30, 50 and 70 mg/g) until pupation; control series were fed with untreated diet (Iga & Smagghe, 2011). At the start of the experiment, the mean fresh weight of the third instars over the different series was 2.5±0.5 mg ($P>0.05$). Insects were followed until pupation and adult formation, and data were analysed as before with a Student's t-test (Smagghe & Degheele, 1994). Per concentration of saponin, three replicates of 10 larvae were scored. In addition, the median toxicity concentration (LC_{50}), which is the concentration needed to kill 50% of the insects treated, with corresponding 95% CL and R^2 values were calculated with Prism v4 as described above.

4.3.4. Histological analysis of midgut aberrations in intoxicated aphids

Neonate aphids were fed on artificial diet containing 10, 1 or 0.1 mg/ml of *Q. saponaria* saponins for 24 h. Subsequently, the insects were fixed in 4% formaldehyde solution for 48 h, dehydrated in an ethanol series (70, 95, 99 and 100%) and butanol and finally embedded in paraffin. Serial sections of 10 µm thickness were cut using a microtome (Jung AG, Heidelberg, Germany), essentially as described before (Smagghe & Degheele, 1994). After dewaxing and mounting, slides were analysed under an Olympus BX51 fluorescence microscope (Olympus, Aartselaar, Belgium). Digital images were acquired using an Olympus Color View II camera (Olympus, Belgium) and further processed with Olympus analySIS cell-F software (Olympus Soft Imaging Solutions, Münster, Germany).

4.3.5. Primary culture of midgut cells of *S. littoralis*

Primary midgut cell cultures were prepared from actively feeding fourth instars of *S. littoralis*. Briefly, dissected midguts were obtained as described in Hakim *et al*. (2009) and cells dissociated for 1.5 h with 2 mg/ml of collagenase (Type I-AS, Sigma) in insect physiological solution (Cermenati *et al*., 2007). Cells were recovered and resuspended in unsupplemented Grace's insect cell culture medium (Gibco) and incubated for 15 h at 23°C with different concentrations of *Q. saponaria* saponins; control cells were incubated with equal amounts of PBS. After incubation, two aliquots of each sample were tested for viability with PrestoBlue Cell Viability Reagent (Invitrogen) according to manufacturer's instructions (Invitrogen). LC_{50}'s (median lethal concentration values on cell viability) and corresponding 95% CL and R^2 values were calculated with Prism v4 as described above.

For confocal analysis, intoxicated and control cells, after 5 h of incubation, were rinsed in PBS, fixed 15 min with 4% para-formaldehyde in PBS, rinsed 3 times with PBS, permeabilised 5 min with 0.1% (v/v) Triton X-100 and rinsed again 3 times with PBS. Actin filaments were labelled by incubating cells 30 min with 4 µg/ml TRITC-phalloidin (Sigma). After 3 rinses with PBS, samples were mounted in Vectashield Mounting Medium (Vector Laboratories) and examined under a confocal laser scanning microscope (Nikon A1r; Nikon Instruments Inc., Paris, France) as described before (Staljanssens *et al*., 2011).

4.4. RESULTS

4.4.1. Insect bioassays

4.4.1.1. Aphid bioassays with oral exposure via artificial diet without and with choice

Exposure of *A. pisum* to saponins incorporated into artificial diet without choice affected aphid survival in a concentration-dependent manner. After 3 days, 46 and 100% mortality were scored with *Q. saponaria* saponins at 1 and 3 mg/ml respectively; sigmoid curve fitting estimated an LC_{50} of 0.55 mg/ml (Table 4.1; Fig. 4.2). Concentrations of 1 mg/ml also caused sublethal effects, with the remaining aphids being smaller (ca 50%) than the controls. For aescin and digitonin, the respective LC_{50} values were 0.62 and 0.45 mg/ml after feeding for 3 days on artificial diet (Table 4.1). With aescin, concentrations of 1 and 10 mg/ml caused 78 and 100% mortality, whereas 76 and 100% mortality were realised with digitonin at 1 and 5 mg/ml respectively. In contrast, only 14% mortality was scored for the highest concentration

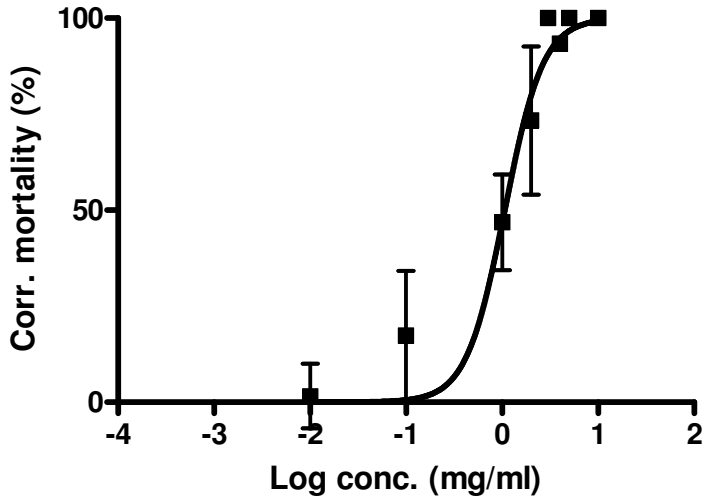


Figure 4.2. Induction of aphid mortality by *Quillaja saponaria* saponins in the pea aphid *Acyrthosiphon pisum*. Concentration-response curve after three days of feeding on treated artificial diet. Mortality percentages are based on 2-3 repeated experiments, each consisting of three groups of 10 neonates each; a total of 60-90 aphids was tested per concentration. Data are corrected for mortality in the controls (0-20%) using Abbott's formula. Statistical analysis and graph were generated with the GraphPad Prism 4.0 software.

Table 4.1. Aphid mortality against the pea aphid *Acyrthosiphon pisum* for two triterpene saponins (*Quillaja saponaria* saponins and aescin) and two steroidal saponins (digitonin and diosgenin).

Saponin	**LC_{50} (mg/ml)**	**95% CI (mg/ml)**	**R^2**
Q. saponaria	0.55	0.32 - 0.96	0.94
aescin	0.62	0.43 - 0.91	0.91
digitonin	0.45	0.18 - 1.12	0.84
diosgenin	(18% with 5 mg/ml) *	-	-

* % toxicity with highest concentration tested (given between brackets).

Data are expressed as as median response (LC_{50}) values (as mg/ml in the artificial diet) together with the corresponding 95% confidence interval and the R^2 as accuracy of data fitting with Prism4.

of diosgenin tested (5 mg/ml). Under these no-choice conditions in treated artificial diet, the *Q. saponaria* saponins also proved to work rapidly, with an LT_{50} of 1.3 days calculated for 3 mg/ml (0.9–1.7; R^2 = 0.93). For lower concentrations, 50% mortality of exposed aphids was reached after 1.8 days (1.4–2.3; R^2 = 0.89) with 2 mg/ml and after 3.7 days (3.9–4.3; R^2 =

0.77) with 1 mg/ml.

In the choice experiments with treated and control artificial diet, a high DI value of 0.96–0.97 was measured after 24 h for *Q. saponaria* saponins at 1 and 10 mg/ml. No or very few aphid neonates (0–1 aphids) were present on the diet containing saponins. Interestingly, the DI effects were equal when aphids were placed on the saponin-containing diet or on the water-treated control diet at the start of the assay. Lower concentrations of 0.1 mg/ml of *Q. saponaria* saponins induced a lower, but still significant, deterrent activity of 62% (DI = 0.62), while control experiments showed a balanced distribution (DI = 0.10 ± 0.09).

4.4.1.2. Aphid bioassays for repellent/deterrent activity on contact exposure

In the experiments where glass plates were painted with *Q. saponaria* saponins on one side and water on the other side, the aphid neonates showed no signs of repellent/deterrent activity in any of the cases (DI= −0.1) (data not shown).

4.4.1.3. Aphid bioassays with exposure after spraying of bean plants without and with choice

When fresh *V. faba* bean leaves were sprayed with an aqueous solution of 100 mg/ml of *Q. saponaria* saponins in a no-choice set-up, 78% aphid mortality was already achieved within the first day, and 100% after 2 days. After 72 h, lower concentrations of 3 and 10 mg/ml caused 37 and 51% mortality, respectively. An LC_{50} of 8.2 mg/ml (3.8–17.8; R^2 = 0.84) was calculated for the exposure of aphids to *V. faba* bean leaves sprayed with *Q. saponaria* saponins after 3 days.

In the choice experiments, none of the leaves sprayed with 100 mg/ml gave any aphid neonate, and this effect was visible immediately from the first day up to the end of the experiment after 3 days. For 10 mg/ml of saponin, the DI after 24 h was 0.53, whereas for concentrations of 3 mg/ml, or lower, no significant deterrent activity was observed (DI = 0.09) (data not shown). As above, in the choice experiments with treated artificial diet, the DI effects were equal when aphids were placed on the saponin-sprayed leaf and when they were placed on the water-sprayed control leaf at the start.

4.4.1.4. Caterpillar bioassays with oral exposure via artificial diet without choice

When third instars of *S. littoralis* were fed with *Q. saponaria* saponins at a dose of 30-70 mg/g in the diet, there was a significant reduction ($P<0.0001$) of about 45-58% of the larval weight gain already after 1 day of treatment, and this negative effect continued during subsequent feeding. For instance, after 5 days the individual fresh weight of larvae treated with 30-70 mg/g saponin yielded only 36±4 mg (representing a significant average reduction in weight gain of 50%; $P<0.0001$) as compared to 69±7 mg in the controls. It was striking that there was also a slight reduction in weight gain (18-20%; $P<0.01$) after 5 days of feeding on diet supplemented with 10-20 mg/g saponin, even though those concentrations caused less than 10% mortality. The effect was most pronounced after 10-11 days, just before the control larvae started losing weight (Fig. 4.3 and 4.4).

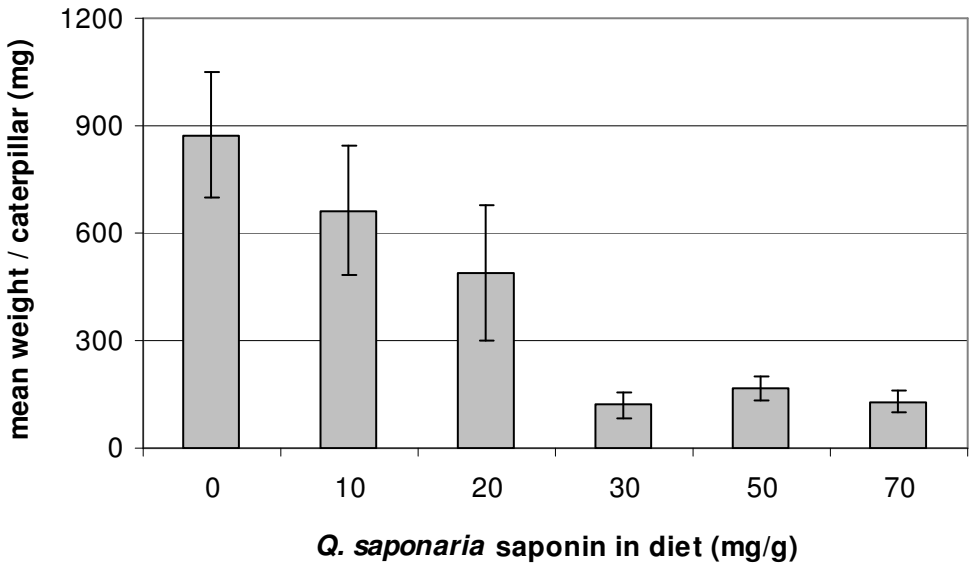


Figure 4.3. Effect of different concentrations of *Quillaja saponaria* saponins on *Spodoptera littoralis* larvae after 10 days. Data are given as mean + SD for one experiment with three replicates.

Together with a reduction in growth, the development of the larvae was also delayed. In the control series, all larvae had moulted into the fifth instar at day 6, and this was also the case in treatments with 10-20 mg/g saponin (92-100%), while conspicuously lower percentages of <30% of larvae treated with 30-70 mg/g saponin had moulted into the fifth instar. Two days later, at day 8, all larvae in the control had moulted in the last sixth instar, while this was the case with none exposed to a dose of 30-70 mg/g saponin. Later, at the moment of successful

pupation in the controls, i.e. after 2 weeks, there was 70-84% mortality at pupation for concentrations of 30-70 mg/g saponin. With the lower concentrations of 10-20 mg/g, there was no loss of survival at pupation above the control; however, we observed a retardation in development in these larvae as it took 4-5 days longer from the third instar to the pupal stage. Sigmoid curve fitting using the percentages of loss of survival at pupation estimated an LC_{50} of 44 mg/g (95% CL: 26-75 mg/g; R^2=0.91) for *Q. saponaria* saponins in the diet.

Later, after 1 month, the pupae developed into adults with successful hatching percentages of 75-88% for the pupae of the control series and those fed with 10-20 mg/g saponin. In contrast, the pupae from the treatments with 30-70 mg/g were clearly affected as only 27-45% of these developed into the adult stage.

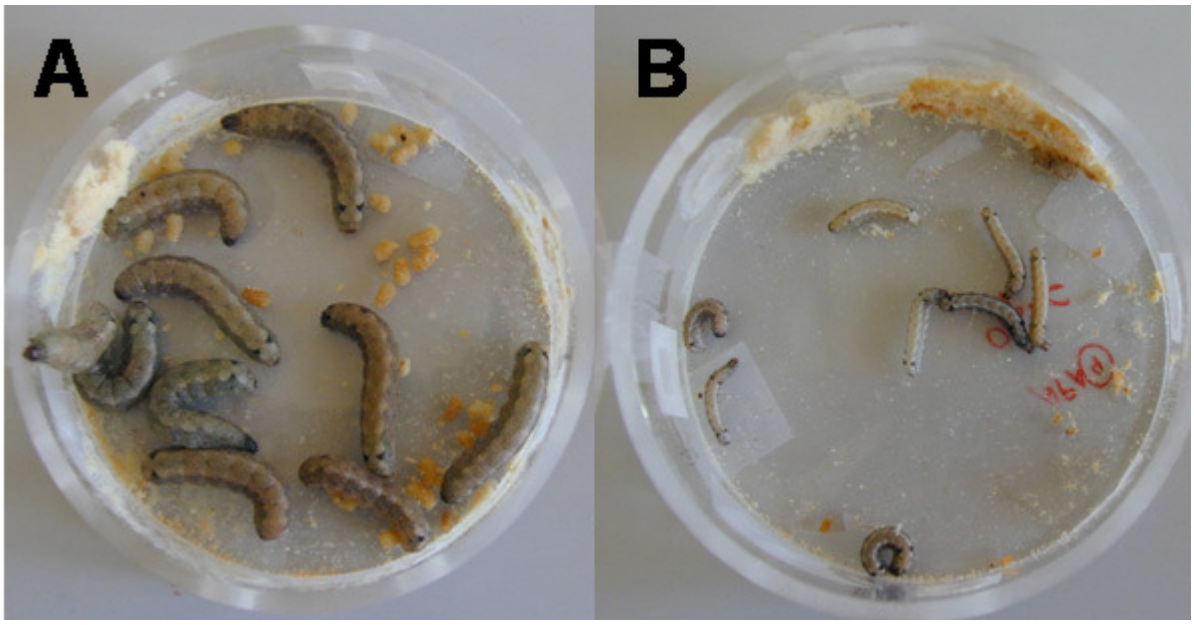


Figure 4.4. Feeding of *Quillaja saponaria* saponins at 30 mg/g in the diet caused a strong inhibition of larval growth in *Spodoptera littoralis* (B) compared to controls (A). The photos were taken at 11 days of treatment.

4.4.2. Histological analysis of midgut aberrations in intoxicated aphids

As shown in Fig. 2, the integrity of the insect midgut epithelium of aphids fed on 10 mg/ml of *Q. saponaria* saponins was lost. The epithelium cell had collapsed and did not show defined cellular structures such as the nucleus or a plasma membrane. In contrast, the epithelium of the control sample was intact (Fig. 4.5). In aphids fed on the lowest saponin concentration of

0.1 mg/ml, the epithelium did not show any damage and was similar to that of the control aphids.

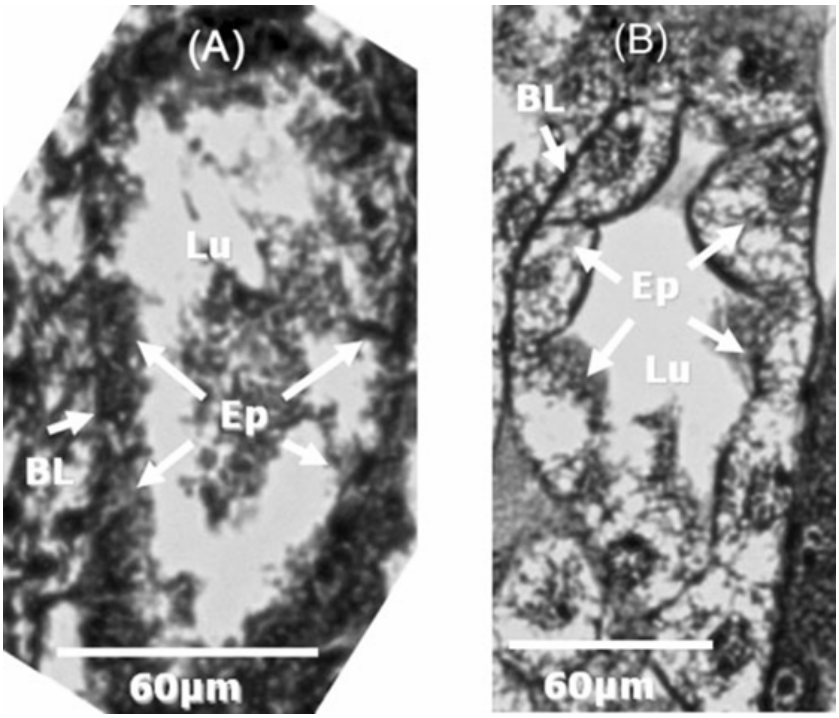


Figure 4.5. Cross section through the thorax of first instars of the pea aphid (*Acyrthosiphon pisum*) after feeding for 24 h on artificial diet containing 10 mg/ml *Quillaja saponaria* saponins (A) as compared to untreated control diet (B). The arrows indicate that the midgut epithelium (Ep) was seriously damaged in the intoxicated aphids as the cell membranes were broken and the cell content spilled into the gut lumen (Lum), while the midgut epithelium in the control samples was intact. BL=basal lamina; bar = 60 µm.

4.4.3. *Q. saponaria* saponin caused cytotoxicity in primary midgut cell cultures from *S. littoralis* larvae

With primary midgut cell cultures of *S. littoralis*, *Q. saponaria* saponins caused clear symptoms of cytotoxicity. The effect was concentration-dependent, and sigmoid curve fitting allowed to calculate an EC_{50} of 4.7 µM (95% CL: 3.1-7.1 µM; R^2=0.93) or 0.009 mg/ml.

Under the confocal fluorescence microscope, the cells exposed to 100 µM (0.2 mg/ml) of *Q. saponaria* saponins for 5 h showed a strong blebbing and damaged basolateral membrane, as shown by the absence of an intact actin network next to the basolateral cell edge. In contrast the control cells showed a large nucleus in the cell centre and intact membrane all around the

cell as confirmed by the actin staining. The intoxicated cells were not swollen and the actin staining confirmed the presence of the microvilli, as was observed in the controls (Fig. 4.6).

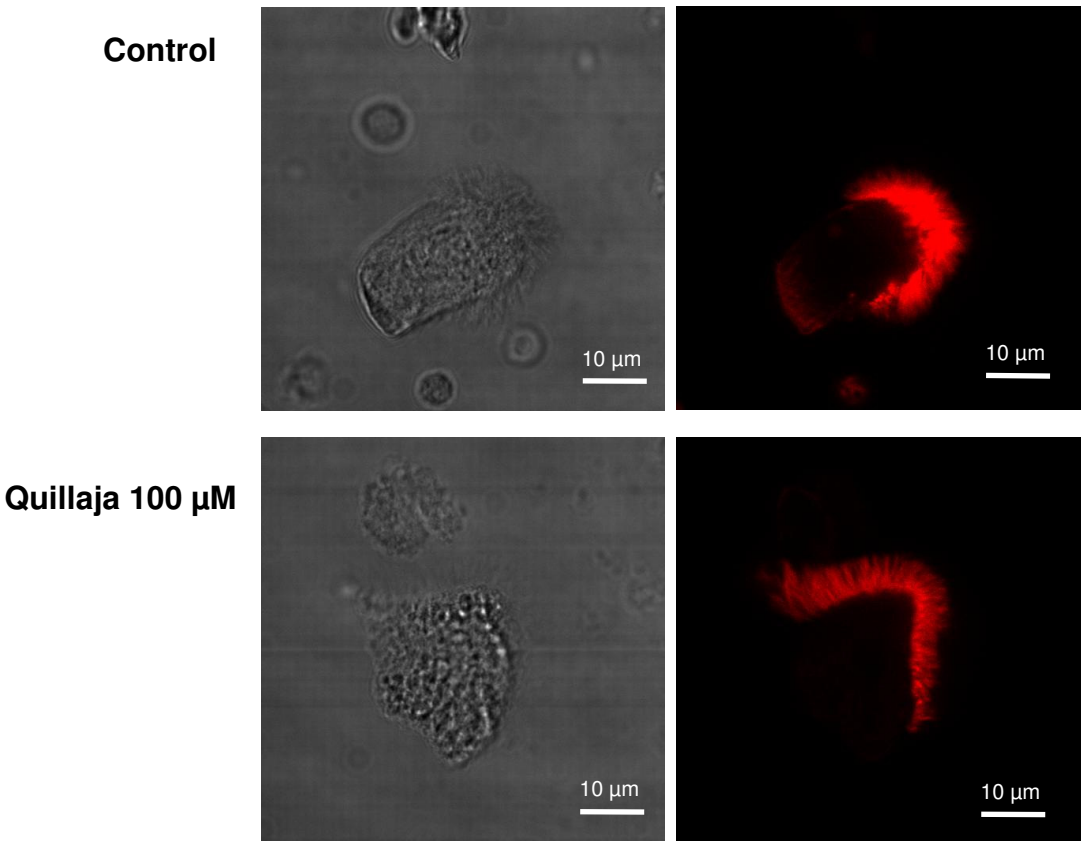


Figure 4.6. Primary midgut cell cultures of last instars of *Spodoptera littoralis* under the confocal fluorescence microscope, after exposure with 100 µM (0.2 mg/ml) of *Quillaja saponaria* saponins. Treated cells show clear symptoms of cytotoxicity, most notably a distorted membrane, blebbing and apoptotic bodies. In contrast the control cells showed a large nucleus in the cell centre and intact cell membrane. The intoxicated cells were not swollen and the actin staining confirmed the presence of the microvilli, as was observed in the controls.

4.5. DISCUSSION

In the light of the ever-growing problem of insect resistance against the most commonly used groups of insecticides, there is much interest in the development of new pesticides to slow down the trend towards resistance build-up. Botanical insecticides are often seen as good alternatives for synthetic chemical products because they often have lower mammalian toxicity and environmental persistence, and therefore pose fewer risks to non-target organisms and human health (Isman, 2006).

Saponins cause a rapid and significant decline in aphid survival, with LC_{50} values ranging between 0.45 and 0.65 mg/ml for three of the four saponins tested. In comparison, the two most notable selective aphicides on the market today are pymetrozine and flonicamid (applied as the formulated products Chess and Teppeki); both compounds act specifically against aphids as feeding inhibitors and have respective LC_{50} values of 0.24 and 0.01 µg/ml in artificial diet (Sadeghi *et al*., 2009b). Imidacloprid (LC_{50} = 0.03 µg/ml), a broad-spectrum and strongly systemic neonicotinoid insecticide targeting the nicotinic acetylcholine receptor (nAChR), is also often used against a large variety of pest insects, including sucking pest insects such as aphids and whiteflies; however, its intensive use has been leading to high levels of resistance in many cases (Elbert *et al*., 2008). In addition, all these commercial aphicides have strong systemic properties which can lead to detrimental side effects towards beneficial insects and pollinating insects (Tomlin, 2003; Mommaerts *et al*., 2010). In recent studies, Sadeghi *et al*. (2009a) reported, in similar assays, an LC_{50} of 0.35 and 0.70 mg/ml for two insecticidal mannose-binding lectins (GNA from snowdrop *Galantus nivalis* L. and ASA from *Allium sativum* L.) after feeding for 3 days on treated diet. Similarly, Shahidi-Noghabi *et al*. (2010) recorded an LC_{50} of 0.37 mg/ml for the entomotoxic type-2 ribosome-inactivating protein *Sambucus nigra* L. lectin SNA-I after 3 days.

Similarly, saponins also cause high and rapid *in vivo* entomotoxic effects on larvae of *S. littoralis* (with 70-84% mortality at pupation for concentrations of 30-70 mg/g saponin), and in concentrations comparable to those which can be found in nature. In edible crops, saponins were reported in concentrations as high as 100 mg/g dry weight in tea seeds, 56 mg/g in chick peas, 40-60 mg/g in soybeans, 47 mg/g in spinach and 20-35 mg/g in pigeon peas (De Geyter *et al*., 2007b; Belitz *et al*., 2004; Kohata *et al*., 2004). In sublethal concentrations, they caused reduced weight gain and retardation of development.

These findings underlines the first objective of the present study, namely to confirm the intrinsic insecticidal activity of saponins, and are in accordance with many other studies on a large number of insect species that date back to the 1970s (for a review, see De Geyter *et al.* 2007b). Although the required concentrations of saponins are higher than those of the above-mentioned synthetic products, the fact that the saponins show rapid and high activities against *A. pisum* aphids and larvae of *S. littoralis* in the same order of magnitude as some entomotoxic proteins such as lectins under comparable conditions in the laboratory suggests that they represent potentially valuable leads for alternative agents in the control of aphids and other pest insects. Additionally, it can be expected that there are no or reduced risks for side effects in pollinating insects such as bees and bumblebees, as the non-systemic characteristics of saponins will prevent them from accumulating in the pollen and nectar in the way that compounds such as imidacloprid accumulate; however, such risk assessments still need to be completed. Thirdly, saponins have a mode of action that is different from all other insecticide groups, which is important if cross-resistance development is to be avoided in the struggle against insecticide resistance, of particular importance in aphids. Combined with their low mammalian toxicity, this makes them promising as potential natural alternatives for exploitation in pest control.

In addition to insect toxicity, a strong deterrent activity from *Q. saponaria* saponins was also noticed. Deterrents (or antifeedants) are popular in the search for ecologically friendly insecticide alternatives, as they work by changing the behaviour of the insect pest and pose no threat to beneficial insects or natural enemies. Such behaviour-changing insecticides have been receiving more attention in recent years (Isman, 2006; Cook *et al*., 2007; Koul, 2008; Nerio *et al*., 2010). Products that combine a deterrent activity with toxicity effects are even more interesting, because they can both prevent new insects from settling and reduce populations that were already on the plant. When faced with a choice between artificial diet with or without harmful amounts of *Q. saponaria* saponins, *A. pisum* showed an immediate and very pronounced preference for diet without saponins, as demonstrated in the choice experiments. The effect is even visible (although less pronounced) at concentrations that have no known effects on survival or development, suggesting that the aphids are more sensitive to the taste effect of the saponins than to their toxicity. Nawrot *et al*. (1991) had already reported strong deterrent effects of aginosid, a steroidal saponin, against two species of caterpillars. The present results confirm that the effect is also apparent for triterpene saponins, even at sublethal concentrations. Correspondingly, Ferracini *et al*. (2010) recently proposed that

extracts containing saponin from the leaves of *Aesculuspavia* L. can prevent the infestation of *Aesculus hippocastanum* L. trees by leafminers (*Cameraria ohridella* Deschka & Dimic).

Spraying *Q. saponaria* saponins of *V. faba* bean leaves confirmed the present results, demonstrating both a toxic and a deterrent action of *Q. saponaria* saponins against aphids. Szczepanik *et al*. (2001) reported earlier that dipping potato leaves for 5 s in aqueous solutions at various concentrations of extracted alfalfa saponins caused reduced food intake, growth rate and survival in larvae of the Colorado potato beetle (*Leptinotarsa decemlineata* Say). Similarly, Pemonge *et al*. (1997) could reduce destruction of stored products by two important Coleopteran pest species (*Tribolium castaneum* Herbst and *Acanthoscelides obtectus* Say) with the use of extracts from *Trigonella foenum-graecum* L., and found that the same effects could be achieved by topical application or addition to the diet of powdered leaves/seeds. These data prove that practical application of saponins is possible in the control of different pest insects; however, more experiments with different crops, products and methods of application and under practical conditions are needed before making firm claims.

In spite of a long history and numerous studies on the subject, the exact mode of action of saponins has not been exactly determined, but it is likely that they work on more than one level. The present work pointed to the insect midgut as the primary target for saponin toxicity: the cells of the midgut epithelium were damaged or completely destroyed in aphids fed on *Q. saponaria* saponin (Fig. 4.5), and primary midgut cell cultures from *S. littoralis* larvae also showed high sensivity towards this saponin (Fig. 4.6). The insect midgut is an interesting target tissue because any detrimental effect on the midgut epithelium will lead to starvation, implying lower insect damage, and finally death of the intoxicated insect (Hakim *et al*., 2010). As saponins have been reported to cause lysis of erythrocytes and bacteria cells (Sparg *et al*., 2004), and the present authors' own experiments confirmed negative effects of *Q. saponaria* saponins on aphid midguts and primary midgut cell cultures from *S. littoralis* larvae, it is postulated that this midgut epithelium damage is due to destruction of the cell membranes, and that the subsequent insect mortality is the result of gut failure and starvation. Since this mechanism is different from the mode of action of *Bacillus thuringiensis* (*Bt*) in midgut cells, it can also be of help in the management of resistance against Bt (Tabashnik, 2008). In addition, because aphids are not sensitive to the toxins of *Bt* (Sharma *et al*., 2004), the current data suggest that saponins can represent important leads in the development of new alternate, environmentally friendly aphid control agents.

In conclusion, *Q. saponaria* saponins have a strong and fast-acting effect on the pea aphid *A. pisum* and the cotton leafworm *S. littoralis*, which is most probably due to a deterrent activity and the destruction of the cells of the insect midgut epithelium, causing the insect to starve and finally die. The results presented here provide further support for saponins playing a role in plant resistance to insect pest species and having potential as new, natural insecticides. However, substantial field testing will be required to verify the applicability of saponins in the control of pest insects, as well as an evaluation of the possible risks to beneficials and natural enemies within IPM.

Chapter 5

Conclusions & Future Perspectives

The main aim of this work was to investigate the toxicity and mode of action behind the insecticidal activity of a number of steroidal and non-steroidal plant metabolites, and to explore their potential as alternative natural insecticides against two economically important pest insect orders: aphids (Hemiptera) and caterpillars (Lepidoptera). A number of ecdysteroids were screened using an *in vitro* cell-based reporter system to search for active compounds that showed agonist and antagonist interactions with the ecdysteroid receptor (EcR, the receptor of the steroidal insect moulting hormone 20E), followed by an in-depth study of the effects and mode of action of two steroidal and two non-steroidal saponins on three insect cell lines, and *in vivo* tests on living insects.

5.1. Cell-based reporter bioassay for screening EcR activity

In chapter 2, we employed an *in vitro* cell-based reporter bioassay for screening potential EcR agonistic and antagonistic compounds. We used insect cell lines of Schneider S2 cells of *Drosophila melanogaster* (Diptera) and Bm5 cells of *Bombyx mori* (Lepidoptera) that were transiently transfected with the reporter plasmid ERE-b.act.luc. We were able to confirm the validity of the bioassay by identifying one EcR agonistic (cyasterone) and one antagonistic compound (castasterone), and modelling their interaction with the ecdysone receptor confirmed these findings. However, the EcR activity of before mentioned compounds was low compared to commercial insecticides.

5.2. Effects of saponins on insect cell cultures

In chapter 3, we tried to identify the mode(s) of action of saponins on insect (cell)s, and to assess their potential as alternative insecticides. We investigated the effects of four commercially available saponins on one dipteran and two lepidopteran insect cell lines of different tissue origin, namely embryonic Schneider S2 cells of *Drosophila melanogaster*, ovarian Bm5 cells of *Bombyx mori* and midgut CF-203 cells of *Choristoneura fumiferana*, respectively.

5.2.1 Saponins have no direct effect on the EcR receptor, but cause cell toxicity through cell membrare permeation

Screening the saponins for ecdysteroid agonistic and antagonistic effects by using the cell-based EcR reporter bioassay mentioned above showed that they have no agonistic activity. In contrast, all four saponins did cause a strong concentration-dependent loss of EcR response, starting from concentrations of about 5-10 µM onwards and leading to a total loss of activity

at concentrations of 100-200 µM for S2 and 10-50 µM for Bm5 cell lines, with median inhibitory concentrations (IC_{50}'s) of 10-50 µM (S2) and 3-10 µM (Bm5).

However, after comparing with an MTT cell viability assay (Decombel *et al*., 2004), we found that all four saponins also caused concentration-dependent cytotoxicity in all three cell lines. For the dipteran S2 cells, a strong cytotoxic effect was observed with EC_{50} values of 50-700 µM; the two lepidopteran cell lines showed an even higher sensitivity to the saponins, with 50% loss of cell viability at 7-200 µM. Since with increasing saponin concentrations, decreases in EcR response and in cell viability were similar for both S2 and Bm5 cells, we concluded that the lowered EcR response in insect cells is probably due to general cell toxicity rather than a direct antagonistic effect on the receptor.

In addition, trypan blue bioassays revealed that incubating S2, Bm5 and CF-203 cells with *Q. saponaria* saponin for 48 h caused membrane permeation leading to cell death, and the effect was dose-dependent with an LC_{50} of 44 µM for S2 cells, 11 µM for Bm5 and 8.6 µM for CF-203 cells. Furthermore, S2 cells subjected to 500 µM *Q. saponaria* saponin for shorter periods of time suffered 62.2±6.5% cell toxicity after exposure of 15 s; after 1 min this had further increased to 99.7±0.1%. For the lepidopteran cell lines, total mortality was achieved after 1 min at 50 µM for both cell lines. These results confirm a strong and rapid cytotoxic effect due to permeation of the cell membrane.

5.2.2 Saponins can cause caspase-3 like activity and DNA fragmentation at lower concentrations

To identify additional causes of cytotoxicity, we exposed the cells to low concentrations of saponins and tested for induction of apoptosis by caspase-3 like activity and DNA-fragmentation. We found a low but significant ($p<0.05$) increase in caspase-3 like activity in S2 cells for all four saponins at concentrations of 10-20 µM, as well as for 20E at 500 nM.

In parallel, cells collected for DNA extraction confirmed that low concentrations of saponin cause DNA fragmentation leading to apoptosis. The effect was observed for all four saponins in all three cell lines, but it was strongest for *Q. saponaria* saponins and aescin in S2 cells and for *Q. saponaria* saponin and digitonin in Bm5 and CF-203 cells.

5.2.3 Addition of cholesterol can counteract the effects of saponins on insect cells

To determine if the presence of cholesterol can influence the effects of saponins on insect cells, the EcR responsiveness, MTT and trypan blue bioassays were repeated with S2 and Bm5 cells in culture medium containing equal weight amounts of saponin and cholesterol. We found that addition of cholesterol in a 1:1 saponin:cholesterol ratio could successfully counteract the effects of saponins in all three tests, but only within a limited range: if the concentration of saponins and cholesterol was increased by four times or more, the protective effect of the cholesterol was lost and we saw a dose-dependent decrease in cell viability like before.

5.2.4 Conclusions on cell cultures

As a general conclusion on insect cell lines, our results do not support a role for steroidal and triterpenoid saponins as agonists, nor as direct antagonists acting on the EcR receptor. Instead, our data confirmed that these saponins cause cytotoxicity by a rapid and stringent permeation of the insect cell membrane. The small discrepancy between the dose-response curves for EcR antagonism and cell viability could be explained by induction of caspase-3 like activity by the 500 nM of 20E used in the EcR responsiveness assays. We also found that low concentrations of saponins can induce caspase-3 like activity and DNA fragmentation in exposed insect cells. Finally, addition of cholesterol to the cell medium can partially counteract the effects of the saponins.

The concurrence between the results on *Drosophila* S2 and lepidopteran Bm5 and CF-203 cell lines supports the hypothesis that the basic mechanism of action of saponins on insect cells is universal and does not depend on insect order or tissue origin of the cells, although the sensitivity of the cells to the saponins differs significantly, with both lepidopteran cell lines being about 2-4 times more sensitive than the dipteran S2 cells. It is possible that this is due to both Bm5 and CF-203 cells being 4-5 times larger in diameter than the S2 cells, since having a larger membrane surface could make cells more susceptible to the permeating effects of saponins.

5.3. Effects of saponins on living insects

In chapter 4, we studied the effect of saponins *in vivo* in two important pest insects, the pea aphid *Acyrthosiphon pisum* and the cotton leafworm *Spodoptera littoralis*. We used the same four saponins as before, with a focus on *Q. saponaria* bark saponin for its high activity and

availability. Aphids and larval stages of *S. littoralis* were challenged by oral and contact exposure with artificial food and sprayed plant leaves to determine the insecticidal activity.

5.3.1 Saponins cause high mortality in A. pisum *aphids and* S. littoralis *caterpillars*

Survival bioassays with artificial diet showed that saponins cause a rapid and significant concentration-dependent decline in aphid survival, with LC_{50} values ranging between 0.45 and 0.65 mg/ml for three of the four saponins tested. For caterpillars, third instars of *S. littoralis* fed with *Q. saponaria* saponins at a dose of 30-70 mg/g in the diet showed a significant reduction in larval weight gain already after 1 day of treatment, and this negative effect continued during subsequent feeding. This eventually resulted in 70-84% mortality at pupation (sigmoid curve fitting estimated an LC_{50} of 44 mg/g). With lower concentrations of 10-20 mg/g, there was no loss of survival at pupation above the control; however, we did observe a retardation in development in these larvae, as it took 4-5 days longer to go from the third instar to the pupal stage.

These findings confirm the intrinsic insecticidal activity of saponins, and are in accordance with many other studies on a large number of insect species that date back to the 1970s (see Table 1.1, De Geyter *et al.*, 2007b). Although the required concentrations of saponins are higher than for many synthetic products, the fact that saponins show rapid and high activities against *A. pisum* aphids and larvae of *S. littoralis* makes them potentially valuable leads for alternative agents in the control of aphids and other pest insects.

5.3.2 Q. saponaria *saponins have a deterrent effect on* A. pisum *aphids in choice-experiments using artificial diet, but no repellent activity by contact*

To investigate the potential repellent and deterrent activities of *Q. saponaria* saponins, we performed a number of choice experiments, observing insect behaviour on treated glass plates and their preference for saponin-treated vs untreated food. Results clearly showed that, when faced with a choice between artificial diet with or without harmful amounts of *Q. saponaria* saponins, *A. pisum* showed an immediate and very pronounced preference for diet without saponins for *Q. saponaria* at 1 and 10 mg/ml after 24 h. Further testing showed that lower concentrations of 0.1 mg/ml *Q. saponaria* saponins induced a lower, but still significant, deterrent activity of 62% (DI = 0.62). The fact that the effect is still visible at concentrations that have no known effects on survival or development suggests that the aphids are more sensitive to the taste than to the toxicity of the saponins, which might make them even more

promising candidates for crop protection.

Next to testing deterrent activity through food, we also tried testing repellent activity on contact by exposing aphids to glass plates that were painted half with an aqueous *Q. saponaria* saponin solution, half with water. In contrast to the previous setup, we found that the test aphids in this case showed no preference for either side (DI= −0.1), not even at the highest concentration of 100 mg/ml saponin.

5.3.3 Q. saponaria *saponins also cause aphid mortality and deterrent activity when sprayed on bean plants leaves*

To evaluate the effects of *Q. saponaria* saponins when applied to living plant material, freshly cut *Vicia faba* bean leaves were sprayed with aqueous saponin solution. We found a very high activity for concentrations of 100 mg/ml saponin, where 78% aphid mortality was achieved within the first day and 100% on the second. Lower concentrations caused less pronounced effects; an LC_{50} of 8.2 mg/ml was calculated for exposure to *V. faba* bean leaves sprayed with *Q. saponaria* saponins after 3 days. This is higher than for artificial food, but the effect is still significant.

In parallel, a choice experiment was set up with two freshly cut *V. faba* bean leaves, one sprayed with *Q. saponaria* saponin solution and the other with water. We found no aphid neonates on leaves sprayed with 100 mg/ml, and this effect was visible immediately from the first day up to the end of the experiment after 3 days. For 10 mg/ml saponin, we found a DI of 0.53 after 24 h, which is again lower than for artificial food, but still proves that saponins are effective when sprayed on leaves.

5.4. Histological analysis of midgut aberrations in intoxicated *A. pisum* and primary midgut cell cultures of *S. littoralis* confirms the midgut as a primary target for saponin activity

We examined the midgut-specific effects of *Q. saponaria* saponins using primary midgut cell cultures from *S. littoralis* and microscopic slides of the *A. pisum* aphid gut. Results showed that *Q. saponaria* saponin caused clear symptoms of cytotoxicity in primary midgut cell cultures of *S. littoralis*, and this effect was concentration-dependent with an EC_{50} of 4.7 µM (0.009 mg/ml). Under the confocal fluorescence microscope, cells exposed to 100 µM (0.2 mg/ml) of *Q. saponaria* saponin for 5 h showed strong blebbing and damaged basolateral

membranes, as shown by the absence of an intact actin network next to the basolateral cell edge. In contrast, the control cells showed a large nucleus in the cell centre and intact membrane all around the cell as confirmed by the actin staining.

In order to observe the effect of saponins on the midgut of *A. pisum* treated with *Q. saponaria* saponin, aphids were fed on artificial diet containing saponins for 24 h. Microscopic observation of the microtome slides of aphids fed on 10 mg/ml of *Q. saponaria* saponin showed that the integrity of the insect midgut epithelium had been disrupted: the epithelium cells had collapsed and did not show defined cellular structures such as the nucleus or plasma membrane. In contrast, the epithelium of the control sample was intact. In aphids fed on low saponin concentrations of 0.1 mg/ml, the epithelium did not show any damage and was similar to that of the control aphids.

These results clearly point to the insect midgut as the primary target for saponin toxicity: the cells of the midgut epithelium were damaged or destroyed in aphids fed on *Q. saponaria* saponin. The insect midgut is an interesting target tissue because detrimental effects on the midgut epithelium will lead to starvation, implying lower insect damage, and finally death of the intoxicated insect (Hakim *et al*., 2010).

5.5. Future perspectives

In this study, it has been shown that the tested saponins have detrimental effects on at least two different species and orders of insects. Many more examples with various insects and saponins have been reported in literature (for a review, see Table 1.1; De Geyter *et al*., 2007b). However, the vast majority of these studies were done in the lab; field tests are rare, and tests that include spraying even rarer (although suggestions for practical applications have been made, like in Ferracini *et al*., 2010).

In order to really establish the potential of saponins as new botanical insecticides, not only do the most promising compounds need to be identified (as it is worth remembering that not all saponins share the same efficiency, or even the same mode of action (Levavi-Sivan *et al*., 2005)), but there is also need for additional tests with more different types of insects (including beneficials) and plants under natural conditions. In order to determine the usefulness of saponins in the light of Integrated Pest Management (IPM), one needs to know their effect on natural enemies like predators, parasites and parasitoids; not only the direct

impact of spraying is important, but also the effects that may occur futher down the food chain.

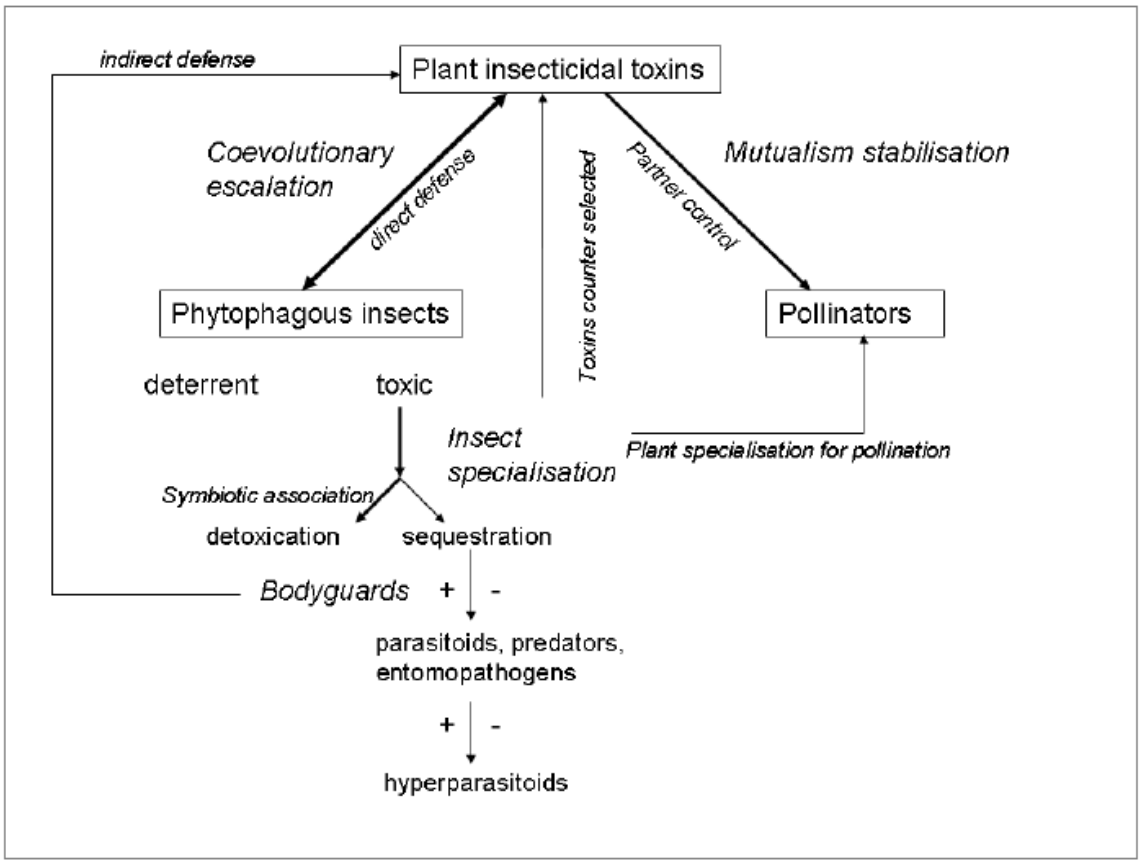


Figure 5.1. Schematic representation of the possible roles of plant insecticidal toxins in ecological networks. Positive and negative effects of plant toxins on higher tropic levels are indicated by + or – signs.

As illustrated in Fig. 5.1, the ecology of ecosystems is not limited to plant-insect interactions. The total effect of insecticidal compounds (both natural defences and insecticides) on plants and insects depends on their community-level consequences. Insecticides can have a negative impact on parasite/parasitoid/entomopathogen fitness; these effects may occur directly, when they affect either the adults in the field or the eggs/developing larva or viral/bacterial multiplication on or inside the herbivore body, or indirectly, when the parasitoid/pathogen suffers from low host size or quality, or from enhanced insect immunity (Ojala *et al.*, 2005). Predators can also be affected by accumulation of toxins present in their prey.

A good example of such effects can be seen in the generalist pollinator *Bombus impatiens,* which feeds on a large range of nectariferous plants, including some plants producing toxic nectar. It was shown that nectar containing a high concentration of the alkaloid gelsemine could affect bumble bee fecundity (Manson & Thomson, 2009). The negative impact of lingering toxins was even observed in a four-trophic-level interaction involving plants–herbivores–parasitoids and hyperparasitoids: the hyperparasitoid *Lysibia nana* parasitising the endoparasitoid *Cotesia glomerata,* which itself infects the caterpillar *Pieris brassicae,* was found to be negatively affected by high concentrations of glucosinolates in the diet of *P. brassicae* (Harvey *et al*., 2003). With this in mind, a key challenge is to integrate the different ecological consequences of insecticide application on plants and in strategies for dealing with the effects on all the trophic levels. Optimal IPM strategies should take into account not only the target insect pest throughout its development, but also the whole ecological context, including the insect's competitors, predators/parasitoids, pathogens and symbionts.

Next to the actions on insects, plants and the ecological network, the effects of saponins on the broader environment will need to be addressed. Regulations of the European Council (Regulation No 1107/2009 79/117/EEG and 91/414/EEG of 21 October 2009, among others) dictate that – for commercial insecticides – "upon application, the compound or its residues should have no harmful effects on the health of humans or animals, nor on the groundwater or the environment." Tests should account for:

- possible leftover residues in human or animal food;
- the effects on non-target species, both on their survival and behaviour;
- possible effects on (diversity of) the ecosystem;
- the behaviour and dissipation of the compound in the environment, with special attention for the contamination of water (including estuarine and groundwater), air and soil. These effects should be studied over large distances to account for potential spreading patterns;
- known cumulative/synergist effects.
- In addition, specific safety instruction should be developed as to not impede the safety or health of the users.

Saponins are usually considered a low-hazard substance to humans (Oakenfull, 1981), so the chances of them forming a direct risk to the population are slim. However, they are known to be potentially harmful to fish (Sparg *et al*., 2004), so accumulation in rivers could be a risk. Saponins are generally biodegradable, but cases of mass fish mortality as a result of saponin

pollution have occurred (Grib *et al*., 2006). Furthermore, given their use as adjuvants in medicine (Sparg *et al*., 2004), is it highly possible that they will interact with other insecticides. This might open new possibilities, as a synergetic effects could facilitate the uptake of insecticidal compounds.

In short, further studies on saponins should focus on tests under field conditions, with special attention to the effects on natural enemies/beneficials and the ecosystem. The idea of testing different saponins and/or combinations with other inseciticides might also be worth exploring.

5.6. Final conclusions

We confirmed that the *in vitro* cell-based reporter bioassay using ecdysteroid-responsive S2 or Bm5 cells that were transiently transfected with the ERE-b.act.luc reporter plasmid (Soin *et al*., 2008) is useful for screening potential EcR agonistic and antagonistic compounds. However, the compounds we tested were not optimal for development into new insecticides.

In our investigation of saponins, we concluded that they (especially *Q. saponaria* saponins) have a strong and fast-acting effect on the pea aphid *A. pisum* and the cotton leafworm *S. littoralis*, most likely due to a combination of deterrent activity and cell membrane permeation leading to destruction of the cells of the insect midgut epithelium, causing the insect to starve and die. Additionally, an apoptosis-inducing activity of the saponins could be identified, though the effect was rather minor in comparison. A similar deterrence and mortality effect could be observed when saponins were sprayed on plant leaves, but at higher concentrations. We believe that these data provides further support for saponins playing a role in plant resistance to insect pest species and having potential as new, natural insecticides. However, to verify the applicability of saponins in the control of pest insects within IPM, trials under field-related conditions are needed as well as an evaluation of the possible risks to natural enemies and beneficials, the environment and the human health.

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Summary / Samenvatting

SUMMARY

In the light of the ever-growing problem of insect resistance against the most commonly used groups of insecticides, there is much interest in the development of new alternatives to slow down the trend towards resistance build-up. In order to search for new insect-specific pesticides, we employed an *in vitro* cell-based reporter bioassay for screening potential EcR agonistic and antagonistic activity in natural ecdysteroids. Test compounds were assayed for their ability to activate transcription of an ecdysteroid-inducible luciferase reporter gene using ecdysteroid-responsive cell line cultures that were transiently transfected with the reporter plasmid ERE-b.act.luc. We used one dipteran and one lepidopteran insect cell line, Schneider S2 cells of *Drosophila melanogaster* and Bm5 cells of *Bombyx mori*, respectively.

Measurements showed an EcR agonistic activity only for cyasterone, both in S2 (IC_{50} = 3.3µM) and Bm5 cells (IC_{50} = 5.3µM). However, the activity was very low compared to that of commercial insecticides. An antagonistic activity was found for castasterone in S2 cells, with an IC_{50} of 0.039µM; in Bm5 cells this effect only became visible at much higher concentrations (IC_{50} = 18µM) and might be due to general cell toxicity rather than a direct antagonistic activity on the receptor. Three-dimensional modelling of the interaction with the EcR receptor also indicates that there is no direct binding with the receptor.

Although the test compounds included in this study were not very promising for the developing into new, specific insecticides, we were able to confirm that the cell-based reporter bioassay tested here is useful for screening for EcR agonists and antagonists. With this method, it is possible to screen large numbers of potentially active ecdysteroids and other compounds in a short amount of time.

Another potentially interesting class of natural molecules are the saponins, a group of secondary plant metabolites consisting of a sugar moiety glycosidically linked to a hydrophobic aglycone (sapogenin) that often possess insecticidal activities. Four saponins were selected: two triterpene saponins, *Quillaja saponaria* saponin and aescin, and two steroidal saponins, digitonin and diosgenin. The triterpene *Q. saponaria* bark saponin received special attention because of its high activity and availability. We studied their effects on one dipteran (embryonal Schneider S2 cells) and two lepidopteran insect cell lines (ovarian Bm5 cells and midgut CF-203 cells of *Choristoneura fumiferana*). A series of different experiments

were performed to investigate potential mechanisms of action by saponins with regard to ecdysteroid receptor (EcR) responsiveness, cell viability, cell membrane permeation, and induction of apoptosis with DNA fragmentation and caspase-3 like activity.

Major results were that exposure of S2 and Bm5 cells containing an EcR-based reporter construct to a concentration series of saponin caused no EcR activation, but did result in a dose-dependent loss of ecdysteroid signalling with median inhibitory concentrations (IC_{50}'s) of 10-50 µM for S2 and 3-10 µM for Bm5 cells. In parallel, we saw a similar loss of cell activity in MTT cell viability assays with median effective concentrations (EC_{50}'s) of 50-700 µM for S2 cells; both lepidopteran cell lines showed an even higher sensitivity to all four saponins, with EC_{50}'s of 7-200 µM. A trypan blue assay with *Q. saponaria* saponin confirmed that the effect was due to cell membrane permeation leading to cell toxicity, with a median lethal concentration (LC_{50}) value of 44 µM for S2 cells, and this effect became apparent within minutes. Exposure to 20E at 500 nM as used in the EcR-based report assay also induced caspase-3 like activities, which may help to explain the discrepancies between the EcR-responsiveness and cell viability assays. Likewise, low concentrations of saponins induced DNA fragmentation and caspase-3 like activities, confirming their potential to induce apoptosis. Eventually, we found that the saponin effects were counteracted by the addition of cholesterol to the culture medium.

The data provide evidence that saponins exert a strong activity on both dipteran and lepidopteran cells; however, the anti-ecdysteroid action by saponins is not based on a direct antagonistic interaction with EcR signalling, but rather on a cytotoxic effect due to permeation of the insect cell membrane. An additional apoptosis-inducing activity of the saponins was identified.

In another series of experiments, we investigated the effects of saponins *in vivo* on two important insect pest species: a model piercing-sucking insect, the pea aphid *Acyrthosiphon pisum* (Hemiptera), and a biting-chewing insect, the cotton leafworm *Spodoptera littoralis* (Lepidoptera). Aphids were challenged by oral and contact exposure to demonstrate aphicidal activities, and in choice experiments to support use as a natural deterrent. In addition, we tested the entomotoxic action of *Q. saponaria* saponin with primary midgut cell cultures and larval stages of *S. littoralis*.

When aphids were exposed to supplemented artificial diet for 3 days, a strong aphicidal activity was recorded for three of the four saponins, with an LC_{50} of 0.55 mg/ml for *Q. saponaria* saponin, 0.62 mg/ml for aescin and 0.45 mg/ml for digitonin. The LT_{50} values ranged between 1 and 4 days, depending on the dose. For diosgenin, only low toxicity (14%) was scored for concentrations up to 5 mg/ml. For caterpillars, third instars of *S. littoralis* fed with *Q. saponaria* saponin at a dose of 30-70 mg/g in the diet showed a significant reduction in larval weight gain of about 45-58% already after 1 day of treatment, and this negative effect continued during subsequent feeding. After 2 weeks, at the moment of successful pupation in the control, there was 70-84% mortality at pupation for concentrations of 30-70 mg/g saponin; sigmoid curve fitting estimated an LC_{50} of 44 mg/g. With lower concentrations of 10-20 mg/g, there was no loss of survival at pupation above the control; however, we observed a delay in development in these larvae as it took 4-5 days longer from the third instar to the pupal stage.

In choice experiments with saponin-treated versus untreated artificial diet, a deterrence index of 0.97 was scored for *Q. saponaria* saponin at 1 mg/ml, meaning that none or very few aphids were found on the treated diet. In contrast, direct contact with saponins applied on glass plates showed no repellent effect. Spraying of *Vicia faba* bean plants with *Q. saponaria* saponin resulted in an LC_{50} of 8.2 mg/ml, and confirmed the deterrent effect, though the concentrations needed to achieve the same activity were higher.

Looking for the cause behind the insect toxicity, we examined the midgut-specific effects of *Q. saponaria* saponin using both primary midgut cell cultures from *S. littoralis* and microscopic slides of the *A. pisum* aphid gut. Results showed that *Q. saponaria* saponin caused clear symptoms of cytotoxicity in primary midgut cell cultures of *S. littoralis*, and this effect was concentration-dependent with an EC_{50} of 4.7 µM (0.009 mg/ml). Under the confocal fluorescence microscope, cells exposed to 100 µM (0.2 mg/ml) of *Q. saponaria* saponin for 5 h showed strong blebbing and damaged basolateral membranes, as shown by the absence of an intact actin network next to the basolateral cell edge. In contrast, the control cells showed a large nucleus in the cell centre and intact membrane all around the cell as confirmed by the actin staining.

Histological analysis of aphids fed on artificial diet containing 10 mg/ml of *Q. saponaria* saponin for 24 h demonstrated strong aberrations of the aphid gut epithelium: the epithelium cells had collapsed and did not show defined cellular structures such as the nucleus or a

plasma membrane. In contrast, the epithelium of the control sample was intact. In aphids fed on low saponin concentrations of 0.1 mg/ml, the epithelium did not show any damage and was similar to that of the control aphids. The effect is similar to that observed in primary midgut cells from *S. littoralis,* suggesting the insect midgut epithelium to be a primary target of saponin activity.

We concluded that saponins (especially *Q. saponaria*) have a strong and fast-acting effect on the pea aphid *A. pisum* and the cotton leafworm *S. littoralis*, most likely due to a combination of deterrent activity and cell membrane permeation leading to destruction of the cells of the insect midgut epithelium, causing the insect to starve and die. A similar deterrence and mortality could be observed when saponins were sprayed on plant leaves, though higher concentrations were required here. These observations provide strong evidence that saponins are natural insecticides and deterrents. For them to be successful in the control of pest insects within Integrated Pest Management, trials under field conditions are needed as well as an evaluation of the possible risks to natural enemies and beneficials, the environment and the human health.

SAMENVATTING

Met het oog op het steeds groter wordende probleem van resistentie tegen de meest gebruikte soorten insecticiden, bestaat er een grote interesse in de ontwikkeling van nieuwe alternatieven om deze toenemende resistentie het hoofd te bieden. Op zoek naar nieuwe, insectspecifieke pesticiden maakten we gebruik van een *in vitro* celgebaseerde biotoets om producten te screenen op EcR-agonistische en -antagonistische activiteit, met name in natuurlijke ecdysteroïden. Producten werden getest op hun vermogen om de transcriptie van een ecdysteroïd-geïnduceerd luciferase reporter gen te activeren in cellijnen getransfecteerd met het reporter plasmide ERE-b.act.luc. We gebruikten één diptere en één lepidoptere cellijn, Schneider S2 cellen van *Drosophila melanogaster* en Bm5 cellen van *Bombyx mori*, respectievelijk.

Volgens de metingen was er enkel een EcR-agonistische activiteit in cyasterone, zowel in S2 (IC_{50} = 3,3µM) als in Bm5 cellen (IC_{50} = 5,3µM). Deze activiteit was echter laag in vergelijking met commerciële insecticiden. Een antagonistische activiteit werd gevonden in castasterone in S2 cellen, met een IC_{50} van 0,039µM; in Bm5 cellen werd dit effect pas zichtbaar bij veel hogere concentraties (IC_{50} = 18µM) en dit zou te wijten kunnen zijn aan een algemeen cytotoxisch effect en niet aan een rechtstreeks antagonistisch effect op de receptor. Driedimensionale modellen van de interactie met de EcR-receptor gaven aan dat er geen rechtstreekse binding op de receptor plaatsvindt.

Hoewel de geteste producten in deze studie niet erg geschikt leken voor de ontwikkeling van nieuwe, selectieve insecticiden waren we wel in staat om te bevestigen dat de hier geteste celgebaseerde biotoets nuttig is voor de screening van EcR-agonisten en -antagonisten. De methode maakt het mogelijk om grote aantallen potentieel actieve ecdysteroïden en andere producten te testen in korte tijd.

Een andere interessante soort natuurlijke moleculen zijn de saponines, een klasse secundaire plantmetabolieten bestaande uit een suikergroep in glycosidische binding met een hydrofobisch aglycon (sapogenin) die vaak insecticidale eigenschappen bezitten. Voor deze studie werden vier saponines geselecteerd: twee triterpene, *Quillaja saponaria* saponine en aescine, en twee steroïdale, digitonine en diosgenine. Het triterpene *Q. saponaria* schors saponine werd extra onder de loep genomen omwille van haar hoge activiteit en

beschikbaarheid. We bestudeerden de effecten van de saponines op één diptere (embryonale Schneider S2 cellen) en twee lepidoptere cellijnen (eierstok Bm5 cellen en middendarm CF-203 cellen van *Choristoneura fumiferana*). Een reeks experimenten werden uitgevoerd om het werkingsmechanisme van saponines te onderzoeken met betrekking op EcR-activiteit, celviabiliteit, celmembraanpermeatie en inductie van apoptose via DNA fragmentatie en caspase-3-gelijkende activiteit.

De voornaamste resultaten waren dat het blootstellen van S2 en Bm5 cellen met een ingebouwd EcR-gebaseerd reporter construct aan een concentratieserie van saponine geen EcR-activatie veroorzaakte, maar wel een concentratieafhankelijk verlies aan ecdysteroïdactiviteit, met 'median inhibitory concentrations' (IC_{50}'s) van 10-50 µM voor S2 en 3-10 µM voor Bm5 cellen. We zagen een gelijkaardig verlies aan celactiviteit in MTT celviabiliteitstoetsen, met 'median effective concentrations' (EC_{50}'s) van 50-700 µM voor S2 cellen; de twee lepidoptere cellijnen vertoonden een nog hogere gevoeligheid t.o.v. alle vier de saponines, met EC_{50}'s van 7-200 µM. Een trypaanblauwbiotoets met *Q. saponaria* saponine bevestigde dat het effect te wijten was aan celmembraanpermeatie leidend tot celtoxiciteit, met een 'median lethal concentration' (LC_{50}) waarde van 44 µM voor S2 cellen, en dit effect was na een paar minuten al zichtbaar. Blootstelling aan 20E aan concentraties van 500 nM, zoals gebruikt in de EcR-biotoets, leidde ook tot inductie van een caspase-3-gelijkend effect, hetgeen de discrepantie tussen de EcR activiteit en celviabiliteitscurves zou kunnen verklaren. Op dezelfde manier vonden we ook dat lage saponineconcentraties DNA fragmentatie en caspase-3-gelijkende effecten induceerden. Tot slot zagen we ook dat de effecten van saponines konden worden tegengewerkt door toevoegen van cholesterol aan het celmedium.

De data zijn het bewijs dat saponines een sterk effect uitoefenen op zowel diptere als lepidoptere cellen; maar de anti-ecdysteroïd activiteit waargenomen in de testen is niet gebaseerd op een rechtstreeks effect op de EcR-receptor, maar wel op een cytotoxisch effect door permeatie van de celmembraan. Een bijkomende apoptose-inducerende activiteit van de saponines werd eveneens geïdentificeerd.

In een andere reeks experimenten onderzochten we het effect van saponines *in vivo* op twee belangrijke pestinsecten: een model voor de stekend-zuigende insecten, de erwtenbladluis *Acyrthosiphon pisum* (Hemiptera), en een model voor de bijtend-kauwende insecten, de

katoenuil *Spodoptera littoralis* (Lepidoptera). Bladluizen werden getest via orale toediening en direct contact, en in keuze-experimenten om een eventuele afschrikkende/afwerende activiteit van saponines te bepalen. Daarnaast testten we het entomotoxisch effect van *Q. saponaria* saponine met primaire middendarmcellen en larvale stadia van *S. littoralis*.

Wanneer bladluizen werden blootgesteld aan een artificieel dieet aangevuld met saponines voor 3 dagen zagen we een sterke aphicidale activiteit voor drie van de vier saponines in het experiment, met een LC_{50} van 0,55 mg/ml voor *Q. saponaria* saponine, 0,62 mg/ml voor aescine en 0,45 mg/ml voor digitonine. De LT_{50} waarden varieerden tussen 1 en 4 dagen, afhankelijk van de dosis. Voor diosgenine werd slechts een lage toxiciteit gevonden (14%) voor concentraties tot 5 mg/ml. Rupsen in het derde ontwikkelingsstadium van *S. littoralis* gevoerd met 30-70 mg/g *Q. saponaria* saponine in het dieet vertoonden een significante reductie in gewichtstoename van 45-58% vanaf de eerste dag van de behandeling, en deze negatieve trend zette zich voort doorheen het experiment. Na twee weken, op het moment van verpopping in de controle, was er een mortaliteit van 70-84% voor concentraties van 30-70 mg/g saponine; 'sigmoid curve fitting' schatte de LC_{50} op 44 mg/g. Met lagere concentraties van 10-20 mg/g was er geen additionele mortaliteit bij verpopping, maar wel een vertraging in de ontwikkeling: de rupsen hadden 4-5 dagen langer nodig om het popstadium te bereiken.

In keuze-experimenten met saponine-behandeld versus onbehandeld artificieel dieet vonden we een 'deterrence index' van 0,97 voor 1 mg/ml *Q. saponaria* saponine, met geen of heel weinig bladluizen op de behandelde voeding. Direct contact met saponines aangebracht op een glasplaat daarentegen had geen afschrikkend effect. *Vicia faba* boonplanten besproeien met *Q. saponaria* saponine resulteerde in een LC_{50} van 8,2 mg/ml, en bevestigde de afwerende activiteit, hoewel er hogere concentraties nodig waren om hetzelfde effect te bereiken.

In een volgende stap onderzochten we de middendarmspecifieke effecten van *Q. saponaria* saponine met behulp van primaire middendarmcellen van *S. littoralis* en histologische darmcoupes van *A. pisum*, op zoek naar de oorzaak van de insecticidale activiteit. De resultaten toonden aan dat *Q. saponaria* saponine symptomen van cytotoxiciteit veroorzaakte in primaire middendarmcelculturen van *S. littoralis*, en dat dit effect concentratieafhankelijk was met een EC_{50} van 4,7 µM (0.009 mg/ml). Onder de confocale fluorescentiemicroscoop zagen we duidelijke tekenen van 'blebbing' en beschadigde basolaterale membranen bij cellen

blootgesteld aan concentraties van 100 µM (0.2 mg/ml) *Q. saponaria* saponine voor 5 uur, als aangetoond door de afwezigheid van een intact actinenetwerk naast de basolaterale rand van de cel. In de controlecellen daarentegen vonden we een grote celkern in het midden van de cel en intacte membranen rondom, als bevestigd door de actinekleuring.

Histologische analyse van bladluizen gevoerd met artificieel dieet met 10 mg/ml *Q. saponaria* saponine voor 24 h toonde sterke afwijkingen in het darmepithelium: de epitheliumcellen waren zwaar beschadigd en er waren geen afgelijnde structuren zichtbaar zoals de kern of het plasmamembraan. Het epithelium van het controlestaal was wel in goede staat. Bij bladluizen gevoerd met lagere saponineconcentraties van 0,1 mg/ml was het epithelium niet beschadigd en leek op dat van de controle. Het effect is gelijkaardig aan dat geobserveerd in de primaire middendarmcellen van *S. littoralis,* en suggereert een belangrijke rol voor het middendarmepithelium als (hoofd)doelwit voor saponineactiviteit.

We concluderen dat saponines (vooral *Q. saponaria*) een sterk en snel effect uitoefenen op de erwtenbladluis *A. pisum* en de katoenuil *S. littoralis*, hoogstwaarschijnlijk te wijten aan een combinatie van een afwerende activiteit en celmembraanpermeatie leidend tot de vernietiging van de cellen van het middendarmepithelium, wat ervoor zorgt dat het insect verhongert en sterft. Een gelijkaardige afwerende en lethale activiteit werd waargenomen bij bladeren besproeid met saponines, al waren de benodigde concentraties hier wel hoger. Deze observaties verschaffen bewijs dat saponines actief zijn als natuurlijke insecticiden en afweerstoffen. Om te zien of ze toepasbaar zijn in het kader van Integrated Pest Management zijn wel nog extra testen nodig, met name proeven onder veldcondities en een evaluatie van de mogelijke risico's t.o.v. natuurlijke vijanden, gewenste insecten, het milieu en de volksgezondheid.

Curriculum Vitae

PERSONAL DETAILS

Name: Ellen De Geyter
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EDUCATION

1996 – 2002 Latin-Mathematics, Koninklijk Lyceum II, Ghent

2002 – 2006 Bachelor & Master in Biology, with great distinction at Ghent University; Master thesis: Bottom-up en top-down controles in estuariene nematoden-gemeenschappen (promotor: Prof. dr. Tom Moens), Department of Biology, Marine Biology Research group

2007 – 2012 PhD student at Ghent University, Departments of Crop Protection and Plant Production

Additional training

14-day training on the procedure of making histologal coupes under tutoring of Professor Yvan Rahbé at the Institut National de la Recherche Agronomique, Lyon (INSA-Lyon, UMR203 INRA BF2I, Biologie Fonctionnelle Insectes et Interactions, F69621 Villeurbanne Cedex, France).

SCIENTIFIC CONTRIBUTIONS

A1 publications

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De Geyter E, Geelen D and Smagghe G (2007). First results on the insecticidal action of saponins. *Communication in Agricultural and Applied Biological Sciences*, Ghent University, **72**/3, 645-648.

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59th International Symposium on Crop Protection, 22 May 2007, Ghent, Belgium: De Geyter E, Rahbé Y, Geelen D and Smagghe G, Insecticidal activity of saponins.

60th International Symposium on Crop Protection, 20 May 2008, Ghent, Belgium.

61th International Symposium on Crop Protection, 19 May 2009, Ghent, Belgium.

62nd International Symposium on Crop Protection, 18 May 2010, Ghent, Belgium.

64th International Symposium on Crop Protection, 22 May 2012, Ghent, Belgium.