Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Abstract. Fractionation of the methanolic extract of *Pergularia tomentosa*, using column chromatography, resulted in the separation of 10 fractions and only the active one (that gave mortality > 70%) was kept and denoted the active fraction (AFPt). Adult female *Schistocerca gregaria* were treated with two concentrations of AFPt, 0.24 and 0.96 %, to investigate the effect on the female reproductive potential. Both concentrations of AFPt significantly increased the duration between adult emergence and first oviposition, as well as affected fecundity and fertility. Furthermore, the effects of AFPt on vitellogenesis and ecdysteroid titers were examined. Biochemical analyses revealed a significant decrease in protein, lipid and carbohydrate contents in ovaries. This study also showed that AFPt induced a significant decrease in the ovarian ecdysteroids levels, as well as the haemolymph ecdysteroid titers. The transcript levels of the ecdysteroid synthesis enzymes *Spook* and *Shadow* were significantly decreased when treated with the highest concentration of AFPt, while transcript levels of the ecdysteroid synthesis enzymes *Phantom* and *Disembodied* increased significantly in animals treated with AFPt. Treatment with AFPt also resulted in a significantly higher transcript level of both the ecdysone receptor (*EcR*) and its partner ultraspiracle/retinoid-X-receptor (*USP/RXR*).

Keywords, Ecdysone receptor, Retinoid-X-receptor, *Halloween* genes, Locust, *Pergularia tomentosa*, Reproduction.

Effects of *Pergularia tomentosa* active fraction on the oogenesis of the desert locust, *Schistocerca gregaria*: ovarian biochemical composition, and effects on the ecdysteroid pathway

MERIAM MILADI^{1*}. KHEMAIS ABDELLAOUI^{1*}. ELISABETH MARCHAL². CYNTHIA LENAERTS². AMEL BEN HAMOUDA¹. MERIEM M'SAAD GUERFALI³. MONIA BEN HALIMA KAMEL¹. JOZEF VANDEN BROECK²

¹Higher Agronomic Institute of Chott Mariem, Sousse University, Tunisia

²Molecular Developmental Physiology and Signal Transduction, KU Leuven, Naamsestraat 59, P.O. Box 02465, B-3000, Leuven, Belgium

³Laboratory of Biotechnology and Nuclear Technologies, LR16CNSTN01, National Centre of Nuclear Sciences and Technologies, Technopole Sidi Thabet, Tunisia.

Corresponding author

Dr. Khemais ABDELLAOUI : kemais_a@yahoo.fr

Telephone: 00216.73.327.544; Fax: 00216.73.327.591

* First and Second authors contributed equally to this work and have equal rank.

Short title: Natural compounds and locust physiology

Introduction

The desert locust, *Schistocerca gregaria*, is a serious menace to agricultural production in some countries, extending from tropical and subtropical areas of the west coast of Africa to the eastern border of India. Each swarm may contain billions of individuals eating everything in their path (FAO, 2009). Many approaches have been taken to explore chemicals that could disrupt physiological, biochemical or molecular processes, which are essential for the survival of this pest insect. Unfortunately, pest management practices are mainly based on chemical insecticides, and are likely to remain so, as long as these are the most efficient and economically favorable products available (Haynes, 1988; Rattan, 2010). However, long-term use of commercial synthetic pesticides has raised the incidence of pest resistance (William *et al.*, 1992) and the emergence and outbreak of new pests (Singh and Kaur, 2018). Consequently, there is a serious need to find reliable and efficient methods that substitute synthetic pesticides and safeguard the environment (Tapondjou *et al.*, 2005; Dris *et al.*, 2017). One of the most promising alternatives is the use of natural compounds that act as disruptors of endocrine regulatory processes (Acheuk *et al.*, 2012).

Among the plant families growing in Tunisia, *Pergularia tomentosa* (L) (Gentianales: Asclepiadaceae) is a perennial shrub, widespread in the Saharan and Sub-Saharan countries of North Africa (Gill, 1992; Gohar *et al.*, 2000). Previous phytochemical investigations resulted in the characterization of cytotoxic cardenolides and cardenolide glycosides, and steroidal toxins, which have become model systems in the development of theories for chemical ecology and coevolution (Agrawal *et al.*, 2012). Cardenolides have attracted attention as plant defense compounds against herbivorous insects (Agrawal and Fishbein, 2008; Rasmann *et al.*, 2009). The interference of these compounds with ecdysteroids patterns in invertebrates can be a very interesting and promising way for controlling pest species while reducing the effects on other animal groups and lowering the environmental burden (Carlson *et al.*, 2001). Moreover, plants producing cardenolides are actually synthesizing hormone analogs to ecdysones or juvenile hormone thus affecting pests in a highly specific manner (Dobler *et al.*, 2011).

Insect reproduction and its hormonal regulation represent potential targets for the development of biorational insecticides. In this perspective, the discovery of molecules that are able to interfere with the development and reproduction physiology of phytophagous insects has been the focus of several recent investigations. Butenandt and Karlson isolated the moulting hormone, ecdysone (E), for the first time in (1954) from the silkworm *Bombyx mori*, which was later reviewed by Karlson (1996). Ecdysteroid hormones were long thought to be only critical for moulting, metamorphosis and aspects of reproduction (Yamanaka *et al.*, 2013). However, it

is now well demonstrated that they interfere with many other aspects of insect life, such as diapause and innate immunity (Raikhel *et al.*, 2005; Verma and Tapadia, 2015).

In adults, ecdysteroidogenesis occurs mainly in the reproductive tissues (Raabe, 1973). Cholesterol and/or plant steroids act as a precursor to the biosynthesis of ecdysteroids, which in turn will be converted to E and (20E) through *Halloween* genes (as reviewed by Niwa and Niwa, 2014). These genes (*spook*, *spo*; *phantom*, *phm*; *disembodied*, *dib*; *shadow*, *sad*; *shade*, *shd*; ...) encode cytochrome P450 enzymes, which catalyze a series of hydroxylation steps resulting in the production of the active hormone, 20E (Lenaerts *et al.*, 2016). In *S. gregaria*, the *Halloween* genes *spook* (*Spo*), *phantom* (*Phm*) and *shade* (*Shd*) were previously characterized by Marchal and coworkers (2011). The response of ecdysteroids is essentially modified according to their binding to a heterodimeric complex of two nuclear receptors, the ecdysone receptor (*EcR*) and the retinoid X/ultraspiracle receptor (*RXR/USP*) (Lenaerts *et al.*, 2016). Protein subunits of the ecdysone receptor which belong to the nuclear receptor family whose members consist of up to six canonical domains, denoted A/B (transcriptional activation), C (DNA binding), D (linker) and E/F (ligand-binding) domains (Dinan, 2003). Interference with ecdysteroid-controlled processes, such as moulting and metamorphosis, is a promising strategy for the development of insecticides with low vertebrate toxicity (Palli *et al.*, 2005). In fact, it is shown that knock down of *EcR* and *RXR* in nymphs resulted in mortality at the time of moulting (Lenaerts *et al.*, 2016), and strongly affected the female reproductive physiology (Lenaerts *et al.*, 2019).

Our present study assesses the interference of the active fraction of *P. tomentosa* with the female reproductive success, fecundity and fertility, as well as its effects on oocyte maturation, biochemical composition of ovaries and the ecdysteroid levels in ovaries and haemolymph. Furthermore, we investigate the possible cross-talk of this fraction on ecdysteroid synthesis by investigating the relative mRNA levels of *Halloween* genes and the nuclear receptor components, *EcR* and *RXR*.

Materials and methods

Rearing of locusts

Desert locusts, *S. gregaria*, were taken directly from long-term reared gregarious cultures. Locusts were kept at (32 ± 1 °C), constant day/night cycle (13:11 h photoperiod) and ambient relative humidity between 40 % and 60 %. Each day they received fresh cabbage leaves, supplemented with dry oat flakes. Following mating, mature females were able to deposit their eggs in pots filled with slightly humidified sand/turf mixture. Every week these pots were collected and set apart in empty cages, where eggs were allowed to hatch into first instar

nymphs. The female locusts used in the described experiments, were collected immediately after emergence and kept in separate cages in order to determine their exact age.

Preparation of the active fraction of P. tomentosa

The Asclepediaceae *P. tomentosa* was collected during spring seasons (March-April 2016) from the region of Hergla (N: 36.1°, E: 10.3°), Tunisia. The active fraction preparation was carried out in the Laboratory of Insect Physiology and Molecular Ethology (KU Leuven, Belgium). The dried and powdered leaves and flowers of *P. tomentosa* (250 g) were extracted with 1250 mL MeOH. Extraction was performed at ambient temperature with the aid of repeated vortexing and sonication (4 x 15 min over a 24 h period) in a sonicator water bath (Branson, USA). The sample was then divided into four 500 mL polyvinyl chloride bottle and centrifuged at 530 g (2000 rpm) for 20 min each. The supernatants were then transferred to a fresh bottle and the solvent was evaporated in a Buchi Rotavapor (EL-131, Flawil, Switzerland). The obtained extract (18 g) was subjected to column chromatography on a silica gel column (63-200 µm, 70-230 meshes, Sigma Aldrich, Darmstadt, Germany). The column was initially eluted with hexane, followed by EtOAc and hexane mixtures (EtOAc:hexane, 25:75, 50:50, 75:25, 100:0). Then, the eluting solvent mixture was changed to MeOH:EtOAc (25:75, 50:50, 75:25, 100:0) and finally to 20 % aqueous acetic acid:MeOH (5:95, 10:90). 5 mL aliquots from each fraction were concentrated under reduced pressure in a Savant SpeedVac Concentrator (SVC 200H, Stratech Scientific, London, UK), the dried residues were dissolved in 5 mL EtOH and tested for their insecticidal activity against third instar nymphs. Only the MeOH:EtOAc (50:50) fraction where the mortality rate exceeds 70 % has been recovered and the solvent was evaporated. The dried active fraction of *P. tomentosa* (AFPt) (1.3 g) was kept at 4°C.

Treatments

The effects of the AFPt on the reproduction of *S. gregaria* were tested by topical application. The ethanolic solution (20 µL) was applied to a freshly emerged adult female (0 to 12 hours post emergence) on their dorsum with a microapplicator at concentrations of 0.24 and 0.96 % designed respectively AFPt C1 and AFPt C2. Concentrations were chosen in accordance with amounts used in previous work (Miladi *et al.*, 2019) when testing the effects of *P. tomentosa* crude extract against *Locusta migratoria* (Acrididae) nymphs. Four days later, the locusts were treated again with the same solution. Controls were given absolute ethanol.

Measurement of reproductive variables

Mating pairs were established for each newly emerged female (treated or control individual) using non-treated males of the same age. Control and experimental females (n = 10 for each concentration) were placed in separate boxes (20 cm³). The pairs were reared under the same

conditions described above for mass rearing. After mating, the females were supplied with pots filled with a humid sand/turf mixture, necessary for oviposition. These pots were checked daily for egg pods and the total number of eggs per egg pod was counted (to analyze fecundity). Eggs were allowed to hatch by placing them on the humid sand/turf mixture and covering them with approximately 1 cm of this mixture. The number of hatchlings was counted daily in order to determine hatchability percentage (to analyze fertility). To evaluate the effect of AFPt on oocyte development, the lengths of 5 basal oocytes in the treated and control groups were measured after 4, 8 and 12 days of adult emergence using an ocular micrometer fitted to a dissecting microscope (Leica LASEZ software V 3.1.0, Germany), adapted in a DM500 light microscope (Leica). The mean length was compared between treated and control.

Quantification of ovarian constituents

Ovaries were collected at three different time points, 4, 8 and 12 days after adult emergence, in order to obtain ovaries in the pre-vitellogenic and vitellogenic stages of oogenesis. Females ($n = 10$ for each concentration) and their ovaries were removed and weighed. Gonads were then homogenized in trichloroacetic acid (20%, w/v) and total carbohydrate, lipid and protein of ovaries were extracted according to Yuval *et al.*, (1998) with some modifications.

Ecdysteroid measurements using an enzyme immunoassay (EIA)

Ecdysteroid titers in *S. gregaria* haemolymph and ecdysteroid levels in the female gonads were measured 12 days after adult emergence, coinciding with the vitellogenic stage of oocyte development, using an enzyme immunoassay, modified from Porcheron *et al.*, (1989) and discussed by Pascual *et al.*, (1995) and Lafont *et al.*, (2005). Haemolymph (10 μ L) was collected from the base of the metathoracic leg of control and treated females using capillary tubes ($n = 15$). Each haemolymph sample was diluted in 100 μ L pure ice-cold ethanol and subsequently stored at -20°C until use. For the sampling of the gonads, one ovarium was dissected from each 12-day-old adult female locust and carefully rinsed in *S. gregaria* Ringer solution (1 L: 8.766 g NaCl; 0.188 g CaCl_2 ; 0.746 g KCl; 0.407 g MgCl_2 ; 0.336 g NaHCO_3 ; pH 7.2) and homogenized by means of a stick sonicator in 500 μ L pure ice-cold ethanol. All samples were then extracted three times with 100 % ethanol by cyclic centrifugation at 10000 g (10 min) each. All supernatants originating from the same sample were combined and dried in a Savant Speed Vac Concentrator (Thermo Scientific, until completely dry). The resulting pellets were dissolved in a sample buffer for Enzyme Immuno Assay (EIA) measurement (0.1 M phosphate buffer, pH 7.4) and stored at -20°C . In this EIA protocol a peroxidase conjugate of 20E was used as tracer together with rabbit L2 polyclonal antibodies. This L2 antiserum has a strong affinity for E, 3-deoxyecdysone and 2-deoxyecdysone and a 6- to 8-fold lower affinity for 20E.

Both serum and tracer were very kindly given by Prof. J. P. Delbecque (Université de Bordeaux, France). The standard curve used in all measurements was obtained with 20E, and therefore results are expressed as 20E equivalents.

RNA extraction and cDNA-synthesis

The other ovarium and fat body of each animal were dissected under a binocular microscope and rinsed in locust Ringer solution then immediately placed in MagNALyser Green Beads Tubes (Roche) as five independent pooled samples of three locusts per condition and snap-frozen in liquid nitrogen to prevent RNA degradation. The frozen samples were stored at -80°C until further processing. To purify total RNA from these samples, they were first homogenized by placing the tubes into the MagNALyser instrument (30 s, 6500 rpm; Roche, Mannheim, Germany). Subsequently, total RNA was extracted from these tissue homogenates by means of the RNeasy Lipid Tissue Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). In combination with this extraction procedure, a DNase treatment (RNase- Free DNase set, Qiagen) was performed to eliminate potential genomic DNA contamination. Purity and concentration of the resulting RNA samples were checked using a Nanodrop spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Inc.). From each total RNA sample, cDNA was made by reverse transcription of 500 ng of RNA with the PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, Invitrogen Life Technologies, Japan), using both random hexamer primers and oligo (dT) primers, according to the manufacturer's protocol. Before use, the resulting cDNA was diluted tenfold with Milli-QH water.

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) reactions were performed according to the Fast SYBR Green PCR Master Mix protocol (Applied Biosystems, Carlsbad, CA, USA). Primers sequences used in the qRT-PCR are given in Table 1. These primers were validated by designing relative standard curves for gene transcripts with serial dilutions (5x) of ovaries and fat body cDNA, as well as checking their dissociation curves. Each reaction contained 10 µL fast SYBR Green, 1 µL Forward and Reverse primer (10 nM), 3 µL Milli-Q water and 6 µL cDNA. The reactions were run in duplicate in 96-well plates on a Step One Plus™ Real-Time PCR System (Applied Biosystems) applying the following thermal cycling profile: holding stage at 95°C (10 min), followed by 40 cycles at 95°C (15 s) and 60°C (60 s). For each measured cDNA sample the normalization factor for the housekeeping genes relative to a calibrator sample was calculated and used to determine the normalized transcript levels of the target genes according to the comparative Ct method as previously described (Van Hiel *et al.*, 2009). Suitable reference genes were selected from a pool of candidate reference genes by means of the geNorm software (Van

Hiel *et al.*, 2009; Lenaerts *et al.*, 2016). The reference genes *GAPDH* and *RP49* were selected for the fat body and *GAPDH* and *CGI3220* for ovaries as having the most stable expression in the studied tissues according to (Lenaerts *et al.*, 2016). In order to test the transcript levels of the target genes, samples of five biologically independent pools were measured.

(Table 1)

Statistical analysis

The results are presented as the mean $\pm$ SD. GraphPad Prism 6 (San Diego, California, USA) was used to test the statistical significance of the observed differences. The normality of the distribution was tested using the Shapiro-Wilk test. For multiple comparisons, a parametric one-way and two-way analysis of variance (ANOVA) was performed on data along with Tukey's test. For the RNAi experiments, normalized relative quantities were log-transformed to allow the use of statistical tests. Differences were judged as significant at the 5% level.

Results

Measurement of reproductive variables

The results reported in Table 2 show the effect of AFPt on the fecundity and fertility of *S. gregaria*. It appears that the treatments of newly emerged females with the two tested concentrations resulted in a significant reduction of the fecundity ($F_{(2, 20)} = 10.73$; $P = 0.0007$). The most marked result was noted with AFPt C1 where fecundity decreased from 158.2 ± 53.4 eggs per female in the control groups to 71.29 ± 36.57 . The treatment also significantly reduced fertility ($F_{(2, 27)} = 77.79$, $P < 0.0001$), particularly with the highest concentration, AFPt C2, for which hatching did not exceed 44.21 ± 5.59 % even after extending the incubation period. In control individuals, hatching success rate was 89.22 ± 7.64 % (Table 2).

This adverse influence of AFPt was also demonstrated by a significant reduction in the number of eggs by egg-pod ($F_{(2, 27)} = 10.78$, $P = 0.0004$) and the number of hatchling by egg-pod ($F_{(2, 27)} = 47.06$, $P < 0.0001$). We reported in control individuals 56.8 ± 9.39 eggs by egg-pod that decreased to 42.7 ± 8.38 in treated individuals with AFPt C1 (Table 2).

(Table 2)

We further investigated if days between adult emergence and first laying (Fig. 1A) and longevity (Fig. 1B) were affected by the AFPt treatment. The treatment significantly increased the duration between adult emergence and first oviposition ($F_{(2, 24)} = 10.96$, $P = 0.0004$) up to 24.75 ± 4.02 and 21.44 ± 2.4 days in females treated with AFPtC1 and AFPtC2 respectively compared to 18.9 ± 0.8 days in control animals. We observed no significant AFPt effects on female longevity ($F_{(2, 27)} = 1.457$, $P = 0.2506$) (Fig. 1B).

(Fig 1)

Oocyte size

The mean oocyte length for treated groups, four days post adult emergence was 0.71 ± 0.09 mm and 0.63 ± 0.05 mm for respectively AFPtC1 and AFPt C2 treatment. This was significantly smaller than that for control group (1.69 ± 0.05 mm) (Fig. 2). This smaller oocytes length was maintained 8 and 12 days after post adult emergence. In fact, ovaries of treated females with AFPt C1 contained oocytes ranging in size from 2.5 ± 0.62 mm 8 days post treatment to 3.77 ± 0.54 mm 12 days post treatment. Similarly, for the group treated with AFPt C2, the mean length of oocytes passed from 2.69 ± 0.14 mm at day 8 to 3.01 ± 0.38 mm at day 12. The analysis of variance, with the AFP concentrations as classification criterion, showed a significant difference among treatments for all the dates tested ($F_{(2, 15)} = 432.9$, $P < 0.0001$; $F_{(2, 15)} = 64.57$, $P < 0.0001$; $F_{(2, 15)} = 99.61$, $P < 0.0001$, for respectively 4, 8 and 12 days post adult emergence).

(Fig 2)

Quantification of ovarian contents

Change in protein content

Biochemical data related to protein content are summarized in Figure 3A. Results revealed that ovarian protein levels gradually increased in control and AFPt C1 treated females, but decreased with the highest concentration tested (AFPt C2) in 8-days-old females. The analysis of variance of ovarian protein levels showed a significant difference among treatments at different time points ($F_{(2, 9)} = 9.89$, $P = 0.0053$; $F_{(2, 7)} = 10.25$, $P = 0.0083$; $F_{(2, 10)} = 12.69$, $P = 0.0018$, respectively 4, 8, and 12 days after adult emergence). For instance, the ovarian protein levels recorded in 4-day-old females treated with the AFPt C1 and AFPt C2 were of 0.39 ± 0.09 $\mu\text{g}/\text{mg}$ and 1.36 ± 0.34 $\mu\text{g}/\text{mg}$ respectively, against 1.19 ± 0.44 $\mu\text{g}/\text{mg}$ for the control group. The protein content continued to increase with age for AFPt C1 and control group. In fact, we recorded at day 8-post adult emergence 1.65 ± 0.47 $\mu\text{g}/\text{mg}$ for control locusts and 0.6 ± 0.22 $\mu\text{g}/\text{mg}$ for AFPt C1 treated locust. However, it decreased for the group of locust treated with AFPt C2 and we recorded 0.89 ± 0.17 $\mu\text{g}/\text{mg}$.

After 12-days post adult emergence, the protein content significantly increased for all concentrations tested. We measured 1.72 ± 0.69 and 1.45 ± 0.39 $\mu\text{g}/\text{mg}$ respectively for AFPt C1 and AFPt C2 against 3.39 ± 0.62 $\mu\text{g}/\text{mg}$ for control females of the same age. In fact, the ANOVA showed that the effect of the AFPt on protein contents varied by age ($F_{(2, 10)} = 11.05$, $P = 0.0029$; $F_{(2, 8)} = 2.65$, $P = 0.13$, respectively for the AFPt C1 and AFPt C2). In control individuals, the mean protein concentration increased significantly during the experimental period ($F_{(2, 8)} = 19.08$, $P = 0.0009$).

Change in carbohydrate content

Figure 3B shows changes in ovarian carbohydrate concentrations in *S. gregaria* adult females upon treatment with AFPt. On the fourth day after treatment, the carbohydrate content was not significantly different for both control and treated females ($F_{(2,7)} = 3.32$, $P = 0.096$). We recorded 6.83 ± 1.5 and 9.45 ± 3.42 $\mu\text{g}/\text{mg}$ respectively for AFPt C1 and AFPt C2 against 11.87 ± 2.84 $\mu\text{g}/\text{mg}$ for control females of the same age. However, the variance analysis of concentrations showed a significant difference between the treatments and controls 8 and 12 day post adult emergence respectively ($F_{(2,8)} = 31.13$, $P = 0.0002$; $F_{(2,9)} = 26.22$, $P = 0.0002$). In fact, ovarian carbohydrate eight days after the emergence in treated females measured 9.97 ± 3.67 $\mu\text{g}/\text{mg}$ and 11.37 ± 1.97 $\mu\text{g}/\text{mg}$, for respectively AFPt C1 and AFPt C2, versus 26.26 ± 2.85 $\mu\text{g}/\text{mg}$ in control ovaries. The total carbohydrate continued to increase with age and for all concentrations tested. For control females 12-days post adult emergence 3.39 ± 0.62 $\mu\text{g}/\text{mg}$ was measured, against 1.72 ± 0.69 and 1.45 ± 0.39 $\mu\text{g}/\text{mg}$ respectively for AFPt C1 and AFPt C2 of the same age.

The ANOVA analysis also showed that the effect of the AFPt on carbohydrate varied among age for the control group and females treated with AFPt C1 respectively ($F_{(2,7)} = 56.08$, $P < 0.0001$; $F_{(2,9)} = 7.60$, $P = 0.01$). This is not the case for females treated with AFPt C2 where no significant difference was recorded when considering the age as classification criterion ($F_{(2,8)} = 3.13$, $P = 0.09$).

Changes in lipid content

As illustrated in Figure 3C, ovarian lipid levels continued to increase during sexual maturation until day twelve after adult emergence for all the concentrations tested. Our analysis showed that total lipid contents of ovaries sampled from AFPt-treated females on day 12 decreased significantly ($F = 13.04$, $\text{df} = 2$, $P = 0.0022$) compared to the untreated control insects. In fact, the concentration of lipids in treated 12-days-old females was respectively 7.84 ± 2.38 and 7.25 ± 1.55 for AFPt C1 and AFPt C2, compared to the control group measuring at 13.54 ± 1.7 $\mu\text{g}/\text{mg}$. The ANOVA showed that the effect of the AFPt on lipid concentration varied with age ($F = 80.11$, $\text{df} = 2$, $P < 0.0001$; $F = 21.45$, $\text{df} = 2$, $P = 0.0002$; $F = 23.75$, $\text{df} = 2$, $P = 0.0004$), respectively for control, AFPt C1 and AFPt C2.

(Fig 3)

Ecdysteroid measurements using an enzyme immunoassay (EIA)

To investigate the effect of AFPt on ecdysteroid synthesis, ecdysteroid titers in the haemolymph of 12-day-old female locusts were measured using EIA (Fig. 4A). At the same day, ovaries

were dissected, ecdysteroids were extracted and measured with EIA. The resulting ecdysteroid levels per mg tissue are presented in Fig. 4B.

Results revealed that ecdysteroid titers measured in treated locusts (Fig. 4A) were significantly lower ($F_{(2, 13)} = 93.39$, $P < 0.0001$) compared to those measured in control locusts seeing that on day 12 of the first gonadotrophic cycle, the females are in full vitellogenesis. In ovaries, total ecdysteroid levels were significantly lower in treated females ($F_{(2, 16)} = 30.66$, $P < 0.0001$) compared to the control of the same age (Fig. 4B). It should be noted that for this experiment the treated locusts were still in the previtellogenic stage of oocyte development, while the controls were already in the vitellogenic stage.

(Fig 4)

Transcript level studies

To further investigate the role of AFPt in the regulation of the ecdysteroid biosynthesis, tissue transcript levels of *S. gregaria* orthologues of the ecdysteroid signaling pathway were determined using qRT-PCR.

Transcript levels of five known *Halloween* genes, *Spook* (*Spo*), *Phantom* (*Phm*), *Disembodied* (*Dib*), *Shadow* (*Sad*) and *Shade* (*Shd*) (Marchal *et al.*, 2011, Marchalet *et al.*, 2012) which are involved in the last steps of ecdysteroid biosynthesis by the female gonads were measured (Fig. 5B). A significant decrease in transcript levels of *Spo* was observed in animals treated with the highest concentration AFPt C2 ($F_{(2,8)} = 5.81$, $P = 0.027$) compared to controls. In fact, the transcript level of *Spo* dropped from 1.57 ± 0.52 in control locust to 0.23 ± 0.4 after treatment with AFPt C2. Similarly for *Sad* ($F_{(2,8)} = 9.73$, $P = 0.0007$), a relative transcript level of 0.13 ± 0.08 was recorded in the AFPt C2 group versus 0.84 ± 0.17 in the control group. The transcript levels of *Phm* ($F_{(2,8)} = 22.51$, $P = 0.0005$) and *Dib* ($F_{(2,8)} = 39.3$, $df = 2$, $P < 0.0001$) increased significantly in AFPt treated animals compared to control locusts. For *Phm*, we measured relative transcript levels of 2.27 ± 0.69 in the control group against 8.16 ± 0.74 and 4.85 ± 1.63 for respectively AFPt C1 and AFPt C2. For *Dib*, we recorded a significant increase only with AFPt C2 which reached 3.7 ± 0.81 against 0.77 ± 0.14 in control group. No significant change was observed for *Shd*.

Considering the smaller oocyte size of treated females, we examined the expression of two vitellogenin genes (*Vg1* and *Vg2*) in the fat body of 12-days old females of *S. gregaria* (Fig. 5D). The ANOVA analysis showed no significant difference in the transcript levels of *Vg1* ($F_{(2, 11)} = 1.97$, $P = 0.185$) and *Vg2* ($F_{(2, 10)} = 0.209$, $P = 0.814$).

To gain a better understanding of the relationship between ecdysteroid levels and hormone receptor expression, we treated female adults at the first day after molt and then four days later

with the AFPt. The transcriptional responses assayed by RT-qPCR 12-days post adult emergence revealed a significant increase in the relative mRNA levels of both *EcR* ($F_{(2, 12)} = 87.85$, $P < 0.0001$) as well as *RXR* ($F_{(2, 12)} = 5.465$, $P = 0.024$) (Fig 5C).

(Fig 5)

Discussion

Effect of AFPt on the reproductive physiology

Secondary metabolites present in plants apparently function as defense (toxic), which inhibits reproduction and other processes in phytophagous insects (Rattan, 2010). Adult fecundity and fertility (egg hatchability or viability) are very important reproductive parameters for investigating the effects of some plant compounds (Shaalán *et al.*, 2005; Acheuk *et al.*, 2012). Likewise, their interaction with reproduction and its hormonal regulation represent potential targets for the development of botanical insecticides (Abdellaoui *et al.*, 2018). The analysis of the result for several variables measured here, revealed that the treatment of *S. gregaria* females during the previtellogenic period with AFPt, significantly reduced both fecundity and fertility and increased the duration between emergence and first oviposition. However, no significant effect was observed on longevity. It is also found that the ingestion of AFPt has an effect on ovarian development and oogenesis by decreasing the size of basal oocytes. Similar reduction in the reproductive potential following the application of other plant extracts, such as a methanolic extract of *Haplophyllum tuberculatum*, has been reported in the migratory locust, *Locusta migratoria* (Acheuk *et al.*, 2012). Similarly, Abdellaoui *et al.* (2018) reported that treatment of adult females during the pre-ovipositional phase with olive leaf extract elicited a significant adverse effect on the reproductive potential of *L. migratoria* by significantly reducing both fecundity and fertility.

The different reproductive potential disturbances observed in the treated females could be associated with molecules present in AFPt extract. Indeed, the Asclepiadaceae family, notably in the genera *Asclepias*, *Pergularia*, *Gomphocarpus* and *Calotropis* are known cardenolides-containing plants (Gohar *et al.*, 2000). It is conceivable that cardenolides compounds contained in the AFPt play a fundamental role in this activity. They constitute one of the most common and widespread group of defensive compounds playing a major role in host plant resistance against herbivorous insects and which have a significant adverse influence on their development and reproduction (Agrawal *et al.*, 2012). Therefore, these chemicals appear to be involved in the effects described here, but additional studies will be necessary to identify the bioactive molecule(s) originating from this plant and to allow further clarity on their sites and modes of action.

The impact of AFPt on the reproductive potential of *S. gregaria* females may be also attributed to antifeeding effect associated with molecules present in this plant especially the cardenolides. In fact, Green *et al.* (2011) demonstrated that cardenolides deter feeding, typically in nonadapted insects, using laboratory choice tests. The same author reported a feeding deterrent activity of six cardenolides from *P. tomentosa* against *Spodoptera littoralis*. Similarly, Akhtar and Isman (2003) noted an antifeedant effect of two cardenolides (digitoxin and cymarín) against the cabbage loopers *Trichoplusia ni* larvae.

Effect of AFPt on the biochemical composition of ovaries

It also found that upon treatment with the AFPt, a significant decrease in the amounts of ovarian constituents (proteins, carbohydrates and lipids) compared to control females, particularly at day 12. This period coincides with the vitellogenetic stage of oocyte growth and development, where the synthesis and incorporation of different metabolites in oocytes are at their maximum. It must be emphasized that the decline in the amount of ovarian constituents correlates well with the significant decrease in the size of basal oocytes from the pre-vitellogenic AFPt-treated females. The antigonadotrophic activity of AFPt appears to be a result of its interference with the vitellogenesis, especially during the incorporation of different metabolites in oocytes. Indeed, protein uptake by the ovaries from the haemolymph is regulated by the Juvenile hormone (JH). The JH acts on the follicle cells surrounding the vitellogenic oocytes, bringing about a reduction in their volume and resulting in the appearance of extensive intercellular spaces between the cells, through which the vitellogenin in the haemolymph gains access to the oocyte surface. The development of these spaces, or patency, is an important means by which the rate of vitellogenesis is controlled. The synthesis of JH of the female is regulated by signals from the brain, where relevant information from other tissues is integrated. The signals to initiate JH synthesis are generally related to nutrients. In the locust, mating and some food odours act via neurosecretory cells in the brain to initiate the synthesis and release of JH. We suggest that the antifeeding effect exerted by AFPt appears to inhibit the synthesis of JH by the *Corpora allata*, which limits the activity of the fat body, leading to a lower synthesis of vitellogenin and a disturbance of their incorporation in the oocytes.

Effect of AFPt on ecdysteroid biosynthesis and the ecdysone receptor complex

The classic insect hormone, 20E, controls not only moulting and metamorphosis but also other key aspects in the life cycle of insects including oogenesis (Raikhel *et al.*, 2005). In adult females, ecdysteroids are produced by the ovaries, where they may act to regulate ovarian development and oocyte production or can be incorporated as conjugates in eggs and act as a source of ecdysteroids during embryogenesis (Lagueux *et al.*, 1977; Lagueux *et al.*, 1981). It is

therefore of great interest to further exploring the role of ecdysteroids in the more ancestral panoistic type of ovaries. Treatment of *S. gregaria* females with the AFPt, had a significant impact on the female reproductive physiology in locusts. The decreased size of basal oocytes correlates well with the significantly reduced ecdysteroid levels in ovaries and haemolymph and the progress of vitellogenesis in AFPt-treated locusts. Our observations suggest that the AFPt-treated locusts were not able to produce ecdysteroids and enter the vitellogenic stage of oocyte maturation. Similarly, Abdellaoui *et al.* (2015) correlated the very small amounts of ecdysteroids of *L. migratoria* with young ovaries. The ecdysteroid level increases clearly by the end of terminal oocyte maturation in adult females. This increase is obvious at the time when the terminal oocytes reach a length ranging from 6.0 to 6.5 mm (Lagueux *et al.*, 1977). The other possible explanation for this observation may be that cardenolides, contained in the AFPt can bind to the ecdysteroids receptor and induce a negative feedback control. Indeed, injection of the 20E agonist tebufenozide into isolated abdomens of *B. mori* stimulated vitellogenesis, but resulted in the arrest of oocyte growth prior to choriogenesis, indicating that an absence of the 20E signal is required during late oogenesis in this species (Swevers and Iatrou, 1999 and Hult *et al.*, 2015). Similar results conducted in crustaceans have shown that exogenous 20-hydroxyecdysone (20E) as well as RH-5849, a nonsteroidal ecdysteroid agonist, reduced ecdysteroid synthesis significantly when injected into inter moult animals (Dell *et al.*, 1999).

The effects of botanical insecticides on ecdysteroid levels and biosynthesis have received little attention. For instance, azadirachtin was demonstrated to result in an inhibition of reproduction by change in the ecdysteroid titers, inhibiting both oogenesis and ovarian ecdysteroid synthesis in *L. migratoria* females, as reported by Rembold and Sieber (1981). Azadirachtin was also able to inhibit ecdysone secretion and induce its accumulation in brain-ring gland complexes of *Calliphora vicina* (Koolman *et al.*, 1988).

Most recent research on the role of ecdysteroids in the female reproductive physiology focused on the ecdysone receptor and its downstream response genes in the meroistic ovaries of Holometabola (Lenaerts *et al.*, 2019). The transcript levels of the *Halloween* genes and the receptor *EcR/RXR* were measured to better understand the involvement of AFPt in the regulation of ecdysteroid biosynthesis. The obtained results revealed a significant decrease in transcript levels of *Spo*, (encoding an enzyme involved in the rate-limiting black box of ecdysteroidogenesis) in animals treated with the highest concentration. However, transcript levels of *Phm* and *Dib* increased significantly in experimental animals. *EcR* and *RXR/USP* isoforms are differentially expressed in the tissues of many insects and are known to mediate

different responses to ecdysteroids during development and reproduction, though much of the work has focused on *Drosophila melanogaster* (Diptera, Drosophilidae) and *Aedes aegypti* (Diptera, Culicidae) (Hult *et al.*, 2015). The transcript levels of both *EcR* and *RXR/USP* were significantly higher compared to controls. There is no positive, but rather an apparently negative correlation between hormone levels and hormone receptor transcript levels. Nonetheless, the underlying mechanisms of such interactions have never been revealed, we suggest that the slight increase in ecdysteroids induces a positive response to the *EcR* gene, whereas high hormone levels are repressive (Mello *et al.*, 2014). In hemimetabolous insects like, cockroach *Diploptera punctata* results suggest that *DpRXR* and *DpEcR* are not required early in the reproductive cycle when events are JH-dependent, but do mediate critical ecdysteroid feedback to the *Corpora allata* late in the gonadotropic cycle. The receptor complex is activated upon 20-hydroxyecdysone (20E) binding and initiates a cascade of gene transcription (Yao *et al.*, 1993).

For *Manduca sexta*, *EcR* expression was induced by low concentrations of 20E while high concentrations repressed the expression of this gene (Jindra *et al.*, 1996). Like our current results, this suggests the presence of another mechanism through which the formation of ecdysteroid receptor activity can be regulated is a competitive binding of other factors to either *EcR* or *USP* (Raikhel *et al.*, 2005).

A number of quite disparate chemicals have been found to bind to ecdysone receptors, including ecdysteroids, BAHs, tetrahydroquinolines, acylaminoketones and most recently methylene lactams (Dinan, 2003). Thus, the receptor appears to be capable of adopting a number of robust conformational states, each able to bind a particular chemical group, rather than exhibiting a continuum of varying conformations in response to each new chemical group (Dobler *et al.*, 2011). Indeed, several studies have demonstrated the extraordinary ability of ecdysteroid receptor complexes to recognise a range of disparate chemistries. Since plants and vertebrates lack these receptors, these molecules could provide safe and attractive insecticides that disrupt endocrine control of pest development (Hill *et al.*, 2012). It may be also of interest to check whether expression of the *Halloween* genes correlates with the timing of the peak in gonadal ecdysteroid secretion in insects and further characterize the feedback loops in the ecdysteroid biosynthetic pathway.

Conclusion

Given the continued need to explore novel alternatives of pest control, phytochemical biomolecules would be a new pathway. In fact, due to their multiple mechanisms of action and their different receptor sites, these molecules could be used to optimize the efficiency and

specificity of future insecticide design with specific or multiple target sites, while ensuring economic and ecological sustainability.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Oligonucleotide sequences for primers used in qRT-PCR.

Target genes	Forward primer	Reverse primer
SgEcR	5'-AAGGTTGATAATGCGGAATATGC-3'	5'-GTGATGGGCGCTCTGAAAAT-3'
SgRXR	5'-AATGCCTCGCTATGGGAATG-3'	5'-TCCTTTGTTCGCTGCCTTTC-3'
SgSpo	5'-CAACATCTTCACCAGCTACATGTG-3'	5'-GGGTCGTCGTAGTCGAAGGA-3'
SgPhm	5'-CGCAGAGCCCGGACAAC-3'	5'-CGAACATGTCGGCCATGA-3'
SgDib	5'-TAGCTGGAATGGACACAACATC-3'	5'-CTGGGTCTGGAAAATACTCTGG-3'
SgSad	5'-ATCGTGGCCGAGATTACGAA -3'	5'-AGCACCATCTCCGGATCCT -3'
SgShd	5'-CCGCCGTCATTGACTTCATA-3'	5'-GTGAGCTCCCAAGCGTGG-3'
SgVg1	5'-CCGCTGAACATCACTGCAAT-3'	5'-ACTTGGGCCAAATGGATGAG-3'
SgVg2	5'-GCTACCCGCAATCTGTAAAATACA-3'	5'-CGACTGTGAAAGGGCATTGA-3'

Abbreviations: EcR = ecdysone receptor, RXR = retinoid-X-receptor, Spo = spook, Phm = phantom, Dib = disembodied, Sad = shadow, Shd = shade, Vg = Vitellogenin.

Table 2. Effect of AFPt on fecundity and fertility of *Schistocerca gregaria*.

	Fecundity	Ne/Ep	Ha/ Ep	Fertility (%)
Control	158.2 ± 53.4	56.8 ± 9.39	50.7 ± 9.36	89.22 ± 7.64
AFPt C1	71.29 ± 36.57 **	42.7 ± 8.38 ***	25 ± 6.972 ****	58.25 ± 10.71 ****
AFPt C2	76.57 ± 30.35 **	54.8 ± 1.87	24.2 ± 2.93 ****	44.21 ± 5.59 ****
F	F _(2, 20) = 10.73	F _(2, 27) = 10.78	F _(2, 27) = 47.06	F _(2, 27) = 77.79
P	0.0007	0.0004	P < 0.0001	P < 0.0001

The data are represented as means ± SD. Females (n = 10 for each condition) received each 20 µL of the AFPt at two different times, the first was just the day of ecdysis and the second four days later. Control animals were treated similarly, but with ethanol only. Statistical analysis consisted of one way ANOVA. Significant differences (P < 0.01; P < 0.001 and P < 0.0001) are indicated by asterisks (**; *** and **** respectively) (Tukey's test). Ne/Ep, number of eggs by egg-pod; Ha/Ep, number of hatchling by egg-pod.

Figure legends

Fig. 1. Effect of the AFPt on the days between adult emergence and first egg laying (A) and longevity (B) in *S. gregaria* females. Locusts were treated with two concentrations of AFPt denoted (AFPt C1= 0.24%) and (AFPt C2 = 0.96%) or absolute EtOH (Control), at one and four days after molting to the adult stage. The data are represented as means $\pm$ SD. Number of females (n = 10 for each condition). Statistical analysis consisted of one way ANOVA. Significant difference ($P < 0.001$) is indicated by asterisks (***) (Tukey's test).

Fig. 2. Changes in the oocyte size of 4, 8, and 12-days-old of control and treated females. The data represent mean $\pm$ SD of 10 animals per condition. Statistical analysis consisted of one way ANOVA. Significant differences ($P < 0.0001$) are indicated by asterisks (****) (Tukey's test). Asterisks represent comparison between concentrations for the same date.

Fig. 3. Changes in ovarian protein (A), carbohydrate (B) and lipid (C) levels ($\mu\text{g}/\text{mg}$ of ovaries) in 4, 8, and 12-days-old control and treated females. The data represent mean $\pm$ SD of five independent animals per treatment (AFPt C1 and AFPt C2). Statistical analysis consisted of two-way ANOVA. Significant differences ($P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$) are indicated by asterisks (* /+; **/++, ***/+++ and ****/++++ respectively) (Tukey's test). Asterisks represent comparison between control and concentrations for the same date and crosses represent comparison between the date 4 and other dates for the same treatment.

Fig. 4. Effect of the AFPt on ecdysteroid titres in the haemolymph (A) and total ecdysteroid levels in the ovaries (B) of 12-day-old adult females. 20 μL of the AFPt was topically applied on the female adult at the first day after ecdysis. Four days later, the locust was treated again with the same solution. Control animals were treated similarly, but with ethanol only. Haemolymph samples were taken twelve days post treatment and the ecdysteroid contents were measured by EIA. The data represent mean $\pm$ SD of five independent pools of three animals. Statistical analysis consisted of one way ANOVA. Significant differences ($P < 0.0001$) are indicated by asterisks (****) (Tukey test).

Fig. 5. Effect of the AFPt treatments on the ecdysteroid biosynthesis in 12-day-old female *S. gregaria*. (A) Overview of the ecdysteroid biosynthesis pathway in larval *D. melanogaster* (Lenaerts *et al.*, 2016). Transcript levels of the *Halloween* genes, *Spo*, *Phm*, *Dib*, *Sad* and *Shd*, (B) and the nuclear receptors, *EcR* and *RXR* (C) were measured in ovaries of *S. gregaria* female using qRT-PCR. (D) Relative transcript levels of two vitellogenins in fat body of 12-day-old control and treated adult females. Results were obtained by analyzing five independent pooled

755 samples of three locusts per condition and were represented as means $\pm$ SD. Statistical analysis
756 consisted of one way ANOVA. Significant differences ($P < 0.05$, $P < 0.01$, $P < 0.001$ and $P <$
757 0.0001) were indicated by asterisks (*, **, *** and **** respectively) (Tukey's test).

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