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Gossypol and related terpenoids: I -Toxicity and fertility effects

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ABSTRACT

Gossypol and related terpenoids are present throughout the cotton plant in glands of foliage, floral organs, and bolls as well as the roots. Gossypol; a polyphenolic binaphthalene-dialdehyde; and other terpenoids (generally referred as a terpenoid aldehydes) from cottonseed produce both general toxic and antifertility effects in mammals and insects. Acetylcholinesterase is the target site for organophosphates and carbamate insecticides and its role in cholinergic synapses is essential for life. *In vitro* inhibition, of partially purified AChE from *Torpedo* electric organ, kinetic parameters were 1.2 X 10^{-4} M for K_m, and 13.33 Δ ODλ_{412nm}. mg^{-1} protein. min^{-1} for the maximum activity (V_{max}) when gossypol was incubated with AChE for 5 min at 5X10^{-5} and 7.5X10^{-5} M. K_m value was found to be the same for the AChE activity in the absence of terpenoid, while the velocity of the enzyme reaction was depressed. The maximum velocity in the presence of terpenoid was found to be 11.11 and 6.89 Δ ODλ_{412nm}. mg^{-1} protein. min^{-1} at 5X10^{-5} and 7.5X10^{-5} M terpenoid respectively. These data indicate that gossypol is a non competitive inhibitor for *Torpedo* AChE with a K_i value of 50 µM. Purified terpenoid increased *Spodoptera* AChE activity, *in vitro*, up to 1X10^{-4}M. By increasing concentrations a slight inhibitory effect was show up at 5X10^{-3}M. The concept of such differences on the enzyme activities and inhibitions from two different sources (vertebrate vs. invertebrate) may play an important role in insecticide selectivity. However, the effect of purified terpenoid on percent eggs laid and % hatchability in treated pupae, adults and larvae of *S. littoralis* for three consecutive generations showed a significantly reduction of the number of egg laid and also the % hatchability.

Key words: Gossypol, Terpenoid aldehydes, *Spodoptera littoralis*, Hatchability, Infertility.

INTRODUCTION

Cotton has long been known as nature's food and fiber plant. It is produced worldwide in tropical and subtropical regions. Regardless of a continuous declining trend of the share of cotton fibers compared to that of chemical textile fibers since 1970's world demand and consumption of cotton fiber has been steady growing along with the worldwide economic growth. Meanwhile, the cottonseed production has increased in the same trend.

Cottonseed (a by-product) with an annual production of 7 million tons in USA and 36 million tons worldwide has long been processed into edible oil and protein feed meals. Due to the presence of gossypol, a known antinutritional factor to nonruminant animals, the protein meals are primarily used as feed to ruminants (Rojas *et.al.*, 2004).

Processing technologies have been developed to reduce the concentration of gossypol in the finished meal. A cottonseed meal produced by these processes can be used as a food ingredient as long as it contains no more than 450 ppm free gossypol (FG), a limited established by research effort. FAO/WHO has set limits of 600 ppm FG and 1200 ppm total gossypol (TG) for human consumption. Moreover, some additional caution was suggested concerning the presence of gossypol in both cottonseed meal and whole cottonseed when used in dairy rations (Arieli, 1998).

In addition to gossypol several other toxic terpenoids (generally referred to as terpenoid aldehydes) are found, including *p*-hemigossypolone *p*-hemigossypolone-6-methyl ether, heliocide H_1, heliocide H_2, heliocide H_3, heliocide H_4, heliocide B_1, heliocide B_2, heliocide B_3, and heliocide B_4.

Cotton leafworm, *S. littoralis* is one of the most destructive pests of numerous economic crops in Egypt and many other countries. Hatching larvae feed on tender leaves near the site of egg oviposition, then, as later instars, feed on and infest most parts of the host plants over the course of larval development. Different host plants or larval diets are known to affect susceptibility to insecticides (Sharma and Yadav, 2001; Zang *et al.*, 2005).

Acetylcholinesterase is of great interest because it is the target site for organophosphates (OPs) and carbamate insecticides in the central nervous system, and its role in cholinergic synapses is essential for life (Fournier and Mutero, 1994). Metabolic resistance to OPs has been associated with an increase in carboxylesterase activity; sequestration and slow turnover of phosphate by an overexpressed esterase are responsible for resistance. On the other hand, in some insects, resistance to OPs is associated with the decrease in carboxylesterase activity, such as in *Musca domestica*, where resistant strains have high ali-esterase, low OPs hydrolase, and intermediate malathion carbxylesterase (Campbell *et al.*, 1997).

The objective of this study was to determine the effect of terpenoid aldehydes alone or in combination with OPs and carbamate compounds on AChE from *Torpedo* electric organ and the cotton leafworm *S.littoralis* , in addition to its interaction with insect biometrics.

MATERIALS AND METHODS

Insect: Laboratory strain of Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) was reared on (i) a terpenoid aldehydes free artificial diet (Bakry *et al.*, 1973), (ii) the same artificial diet with terpenoid aldehydes (Meisner *et al.*,1978), and (iii) natural diet of castor oil leaves .

Terpenoid aldehydes: Terpenoid aldhydes were extracted and purified according to Boatner (1948) after soaking cottonseeds (Giza 89) in tap water for 12 hrs at room temperature, for softening of seed hull and to facilitate its removal, then hull-free kernels were left to dry at room temperature. Insoluble terpenoides were collected and dried at 80 ^{0}C for 8 hrs. The melting point was determined as a criterion of purity according to A.O.A.C. (1984), and it was found to be 185 ^{0}C.

Insecticides: Pure samples were obtained from Environmental Protection Agency (USEPA). Aminocarb; 4-dimethylamino-m-tolyl methylcarbamate, aldicarb; (EZ)-2-methyl-2-(methylthio)propionaldehyde O-methylcarbamoyloxime, dicrotophos; (E)-2-dimethylcarbamoyl-1-methylvinyl dimethyl phosphate and monocrotophos, dimethyl (E)-1-methyl-2-(methylcarbamoyl)vinyl phosphate were used in this study.

Acetylcholinesterase (AChE) Assay: AChE activity was measured in *Torpedo* electric organ and *Spodoptera* larvae preparations *using* a spectrophotometer (Sequoia-Turner (STC # 340-001)). Frozen Torpedo electric organ tissues were prepared according to Sherby *et al.* (1985). Twenty *Spodoptera* larvae (5^{th} instar) were starved for 24 hrs and then cut to heads and remaining bodies. Both were homogenized (5 parts per ml 0.1M phosphate PH 8.0) and centrifuged at 5000 rpm for 30 min and supernatant of heads or the remaining larval body were used as an enzyme source. The method of Ellman *et al.* (1961) as modified by Brownson and Watts (1973) was used for assaying AChE.

***In vitro* inhibition of acetylcholinesterase:** One ml of diluted *Torpedo* enzyme preparation was incubated with 10 µl of gossypol (at 10µM, 100µM, 2.5mM and 5.0 mM) and /or aminocarb (at 2.5, 7.5, 25, 50 and 75 µM) and aldicarb (at 1.0,2.5, 5.0, 7.5 and 50µM) for 5 min, while 10 min for monocrotophos and dicrotophos (10, 25, 50, 100,250 and 500 µM) was recommended. Absorbance value at λ_{412nm} was recorded, % inhibition was estimated and enzyme kinetic parameters (K_m, V_{max} and K_i) were calculated.

Effect of terpenoid on *Spodoptera* AChE: In a typical assay 10 µl of the *Spodoptera* larvae enzyme solution was added to 3ml of 1:1 mixture of 2mM substrate solution and 2mM dithiodinitrobenzoate (DTNB). The final concentration of substrate and DTNB in the assay mixture was 1 mM. Final enzyme protein concentration per assay was 100 µg of head capsules and 150 µg for the remaining body AChE. The change in absorption at 412 nm was monitored on Sequoia-Turner spectrophotometer in absence and presence of 10 µl of terpenoid (at 10, 100, 250 and 500 µM) after incubation for 5 min.

Effect of terpenoid on *Spodoptera* biometrics: Pupae from *Spodoptera* lab strain reared on castor oil leaves, were taken, distributed at sex ratio of 1 male : 2 females , treated topically with acetone solution of different terpenoid concentrations and divided into three groups (treated males with untreated females, treated females with untreated males and treated males with treated females). After emergence, moths from treated pupae were retreated topically with the same concentration used for pupae and paired immediately. Each pair was placed in a separate cage with a 15% sugar solution in cotton squares .Egg-masses were collected daily and number of eggs and % hatchability were calculated. Neonates were transferred to the artificial diet contained 0.25, 0.5,

1.0 and 1.5 % terpenoid in a glass cup equipped with muslin covers. Neonates were grown through the entire larval stage on the artificial diet, pupae and moths were treated with the same terpenoid concentration for three consecutive generations and repeated twice (El-Sebae *et al.*, 1981).

In another experiment, neonates from treated moths were reared through the entire larval stage on castor oil leaves and moths produced treated with the same concentration of terpenoid. Eggs/ egg-masses and % hatchability were counted for three consecutive generations (Figure 1, 2, and 3).

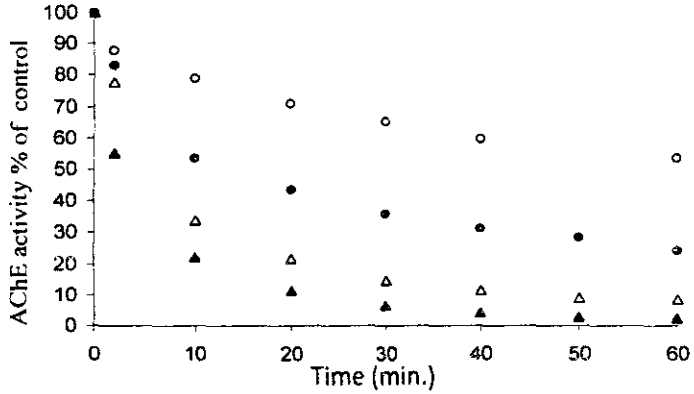

Figure 1: Time dependant inhibition of *Torpedo* AChE by 25 uM (o), 50 uM (•), 75 uM (Δ) and 100 uM(▲) of Gossypol. Values are means of triplicate measurements with SEM smaller than the size of the symbols.

Protein measurement: Protein measurement was determined according to the method of Lowery *et al.* (1951). Bovine serum albumin (BSA) was used as the standard protein.

RESULTS AND DISCUSSION

Effect of terpenoid on acetylcholinestrase (AChE) activity: Gossypol may affect enzymes in two ways; by reacting with the substrate thereby blocking the action of the enzyme and / or by combining with the enzyme itself. Gossypol combines with enzyme; it could cause loss of activity by complexing with the active site by changing the ionic character of the active site or by creating

steering hindrance (Abou- Donia, 1976). The present study; in part; was conducted to determine such interactions with AChE from different sources.

Progressive inhibition of AChE from *Torpedo* electric organ: Different terpenoid concentrations produced progressive inhibition of the membrane-bound AChE of *Torpedo* electric organ similar to the effects of the organophosphate DFP and the carbamate aldicarb (Bakry, *et.al.*,1986). The time- concentration dependence of terpenoid is shown in (figure1). In all cases, the degree of inhibition vs time was a curvilinear function, suggesting a first order kinetics. The % inhibition does not go to completion but achieves a steady state typical of that inhibitor concentration.

Kinetic study of *Torpedo* AChE activity: in a duple reciprocal plot for substance concentrations; acetylthiocholine iodide (ASChI); and *Torpedo* AChE activity, the velocity (v) is expressed as λ_{max} (nm.mg $^{-1}$ protein. min $^{-1}$), while the mM acetylthiocholine iodide concentrations were varied over the identical range. According to Lineweaver and Burk (1943), K_m was calculated to be 1.2 X 10 $^{-4}$ M, and the maximum activity was 13.33 nm. mg $^{-1}$ protein. min $^{-1}$ in absence of gossypol. On the otherhand, when gossypol was incubated with AChE for 5 min at 5X10 $^{-5}$ and 7.5X10 $^{-5}$ M , K_m value was found to be the same for the AChE activity, while the velocity of the enzyme reaction was depressed 11.11 and 6.89 nm mg $^{-1}$ protein. min $^{-1}$, respectively. These data indicate that gossypol is a non competitive inhibitor for *Torpedo* AChE (Warton and Carty 1972).

***In vitro* Inhibition of AChE Activity from *Torpedo* Electric Organ**: Partially purified AChE of *Torpedo* electric organ was inhibited to a different extent with terpenoid, two organophosphates (monocrotophos and dicrotophos) and two carbamate insecticides (aminocarb and aldicarb). Terpenoid was tested alone and at its I_{50} value with different concentrations of the previously mentioned insecticides. Incubation period of terpenoid – enzyme complex and terpenoid – enzyme -insecticides complex was within the linear response of the enzyme toward these compound. Table 1 represents the *in vitro* inhibition of *Torpedo* AChE by terpenoid, two organophosphate, and two carbamate insecticides. It is interesting to mention that; based on the I_{50} values terpenoid has an inhibitory power to AChE equal to that of monocrotophos, and ½ the

potency of dicrotophos while aminocarb and aldicarb were more potent as anticholinesterases. By treating *Torpedo* AChE with terpenoid - insecticides mixtures, the inhibitory power increased twice.

Table 1: *In vitro* inhibition of *Torpedo* electric organ acetylcholinestrase (AChE) by some compounds.

Compound	I_{50} (M)
Gossypol (G)	$5.0X10^{-5}M$
Aminocarb	$7.0X10^{-6}M$
Aldicarb	$5.0X10^{-6}M$
Monocrotophos	$5.8X10^{-5}M$
Dicrotophos	$3.0X10^{-5}M$
Aminocarb + G at $5X10^{-5}M$	$3.0X10^{-6}M$
Aldicarb + G at $5X10^{-5}M$	$2.0X10^{-6}M$
Dicrotophos + G at $5X10^{-5}M$	$2.4X10^{-5}M$
Monocrotophos + G at $5X10^{-5}M$	$1.8X10^{-5}M$

Interaction of terpenoid with AChE activity from *Spodoptera* larvae: The effect of terpenoid on activity of AChE from *Spodoptera* larvae head capsules and the remaining body homogenate was investigated. The data (Table 2) showed that the purified terpenoid increased AChE activity up to $1X10^{-4}M$. By increasing concentrations a slight inhibitory effect was show up at $2.5X10^{-3}M$. It is clear that I_{50} value is less than 5mM. Interaction of spinosad (Abu-Taleb, 2005) with *Spodoptera* head capsules AChE and imidacloprid at 0.75 X LD_{50} (Abou-Donia, 2008) with rat brain AChE showed same effect that we recorded here. Increased AChE activity results in decreased acetylcholine (ACh) and less than optimal function of ACh receptors. Increased AChE expression was reported to induce neurodegeneration *in vivo* and *in vitro* (Day and Greenfield, 2003). This possible mechanism involves influx of calcium ions following activation of α7 nAChR and induction of neuronal apoptosis (Zbarsky, *et.al.*, 2004). In addition, it was suggested that an increase in AChE protein may reflect enhanced axonal repair and synaptic modeling (Bigbee *et. al.*, 2000). The concept of such differences on the enzyme activities and inhibitions from two different sources (vertebrate vs. invertebrate) may play an important role in insecticide selectivity (Casida, and Quistad, 2004).

Table 2: Effect of gossypol on AChE from *Spodoptera littoralis* larvae

Gossypol concentration in(mM)	AChE from *Spodoptera* head capsules		AChE from *Spodoptera* remaining body	
	% Activity of control	% Inhibition	% Activity of control	% Inhibition
0.00	100.00	0.00	100.00	0.00
0.01	112.00	0.00	110.55	0.00
0.10	112.00	0.00	113.12	0.00
2.50	81.54	18.55	92.19	7.81
5.00	38.72	61.28	54.93	45.07

Effect of terpenoid on oviposition and hatchability of the Egyptian cotton leafworm: Adults of *S. littoralis* were treated topically at different concentrations, the experiment was designed to study the contact effect of this compound on the mating behavior, oviposition and hatchability of the egg laid. It is clearly noticed that increasing concentration significantly reduces the number of eggs laid and the percent of egg hatching. The effect is accumulated as an adult treated in consecutive generations (Figure 2).

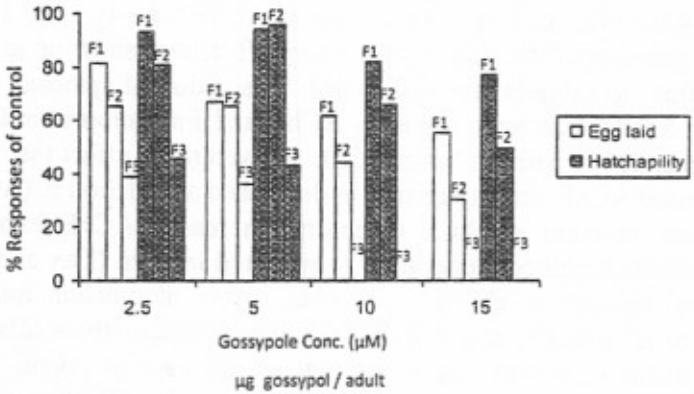

Figure 2: Interaction of gossypole with *Spodoptera* adult biometrics; (□) egg laid and (▤) Hatchapilty for three (F_1, F_2, and F_3) consective generations.

The effect of purified terpenoid on percent eggs laid and % hatchability in treated pupae, adults and larvae of *S. littoralis* for three consecutive generations showed a significantly reduction of the number of egg laid and also the %

hatchability (figure 3). The data showed that the accumulative effect is quite clear and are in a good agreement with the finding of Sherby (1979) when he worked with gossypol buffered with sodium salt. The interrelationship between the effect of gossypol on the number of eggs laid and their hatching is biologically clear. In each gossypol concentration used the number of eggs and the percent hatchability decreased significantly and reached to zero % of control after the 3rd generation.

Figure 3: Interaction of gossypole with *Spodoptera* pupae, adults and larvae biometrics; (□) egg laid and (▩) Hatchapilty for three (F_1, F_2,and F_3) consective generations.

Pigment glands contain a variety of terpenoid chemicals of which the yellow pigment gossypol is the best known and most studied (Adams *et al.*, 1960). Terpenoids from pigment glands in cottonseed, discolor cottonseed oil and meal, denature protein in the meal (Berardi and Goldblatt, 1969) and act as toxins to monogastric animals. Moreover, there were several investigations concerning the possible interaction of gossypol with the biology of the major cotton pest. The effect of gossypol on the toxicity of several insecticides against major cotton insects has been studied (Shaver and Wolfenbarger, 1976 and Meisner *et al.*, 1978).

The chemistry, physiology, and metabolism of gossypol in different animal species answered several questions in each case, but the possible internal reaction of this compound remain unclear in most cases. There is indirect evidence that aromatic hydrocarbons (such as benzene, polyphones and naphthalene) can inhibit AChE by the charge transfer complex (ctc) formation and that most aromatic carbamates owe much of their affinity for the enzyme (not, of course, their carbomylating activity) to the ctc formed by their aromatic moieties. The evidence in both cases is based on parallelisms between variations in the ability of series of inhibitors to from ctc with model acceptor (Hetnarski and O' Brien 1975) and variations in their ability to combine with the enzyme. As well known that gossypol possess a particular chemical structure enhance such kind of interrelationships with the AChE macromolecule these based on its reaction with several amines to form stable derivatives or function of phenolic and carbonyl group in the chemical structure. In this case it should be stressed that the evidence for ctc formation with AChE is still indirect; spectral evidence is necessary to prove it, and because only of the thousands of amino acids in the enzyme is affected, such evidence may be hard to get.

The incorporation of gossypol whether applied topically or into the food media, suppressed the larval weight (Meisner *et al* ., 1978 and sherby *et al*, 1987), prolonged the larval stage, reduced the number of eggs laid with a drastic morphological changes and decreased the percent hatchability significantly. The biochemical data referred to the possible direct and indirect interaction of gossypol with several targets within the larvae, but we should concentrate on the possible genetic variability, which might be carried directly by gossypol. The combination of the biochemical interactions and the biological effect of gossypol will open a new era of understanding such effects on genetic bases.

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الجوسيبول والتربينويدات الشبيهه : 1- السمية والتأثير على الخصوبة

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جامعة الإسكندرية- كلية الزراعة- قسم كيمياء وتقنية مبيدات الأفات

يتواجد الجوسيبول والتربينويدات الشبيهه فى نباتات القطن فى غدد تنتشر بجميع أجزاء النبات ويؤدى تواجدها إلى تأثيرات سامة كما تؤثر على خصوبة كلا من الحشرات والثدييات ولقد تم إستخلاص هذ المكونات من بذور نباتات القطن لدراسة تأثيرها على إنزيم الأسيتيل كولين إستيراز المستخلص من العضو الكهربائى لسمك التوربيدو ومخ يرقات دودة ورق القطن حيث يعمل الإنزيم كهدف للمبيدات الفسفورية العضوية والكارباماتية الحشرية ويلعب دورا حيويا فى حياة الكائنات الحية وخلصت الدراسة إلى أن التثبيط الخارجى للإنزيم المستخلص من السمك يمتلك ثابت ميكائيل قيمته $10X1.2^{-4}$ مولار وقيست السرعة القصوى للتفاعل الإنزيمى وذلك فى غياب وفى وجود الجوسيبول بعد تحضينه مع الإنزيم لمدة خمس دقائق عند تركيزات $10x5^{-5}$ مولار و $10x7.5^{-5}$ مولار ولم تتغير قيمة ثابت ميكائيل بينما إنخفضت السرعة القصوى للتفاعل بزيادة التركيز بما يبين أن التأثير لا تنافسى وأن قيمة ثابت التثبيط حوالى 50 ميكرو مولار.

أما فيما يخص الإنزيم المعزول من رؤوس يرقات دودة ورق القطن فقد أدت المعاملة إلى زيادة نشاط الإنزيم المختبر خارجيا حتى تركيز بلغ $10X1^{-4}$ مولار وهذا التباين فى الإستجابة للإنزيمين المعزولين من السمك ومن يرقات دودة ورق القطن قد يوحى بإستغلال هذا التنوع فى زيادة إختيارية الفعل الإبادى الحشرى كما وأن إستخدام هذه المركبات المستخلصة من بذور نبات القطن فى دراسات على خصوبة دودة ورق القطن من خلال التأثير على نسب البيض الذى تضعه إناث الحشرة وعلى نسب الفقس نتيجة معاملة الإطوار الحشرية المختلفة بطرق معاملات متنوعة سواء للعذارى أو اليرقات أو الحشرات الكاملة على مدار أجيال متعاقبة قد أوضحت وبشدة تأثيرا خافضا لكلا من نسب البيض ونسب الفقس والتحول إلى الأطوار الحشرية المختلفة.