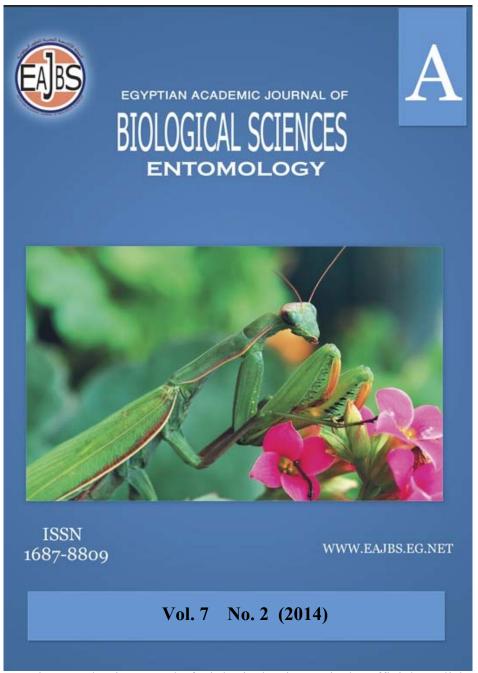
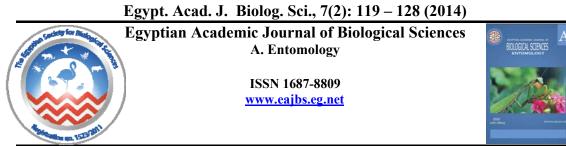
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Toxicological, biological and biochemical impact of some chitin synthesis inhibitors on the black cutwom, *Agrotis ipsilon* (Lepidoptera: noctuidae) (Hufn.)

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# ABSTRACT

The impact of two chitin synthesis inhibitors, chlorfluazuron and triflumuron against the black cutworm, *Agrotis ipsilon* (Hufn.) was studied. Feeding technique was adopted. Different effects of these two chitin synthesis inhibitors on the 4<sup>th</sup> instar larvae were investigated. Effect on biological aspects, larval duration, larval weight, percentage of pupation, pupal duration, pupal weight, percentage of adult emergence, adult longivity, total oviposition period, number of eggs per female, egg hatchability and sterility percentage were studied. Effect of both compounds on total proteins and glycogen levels was also investigated.

Keywords: Agrotis ipsilon- Lepidoptera- chitin synthesis inhibitors- biological aspects- total proteins - glycogen level.

# INTRODUCTION

The black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae) is a serious pest of corn and several agricultural crops in Egypt and many other countries of the world. This noctuid is almost polyphagous that attacks a large number of vegetable crops (Hill, 1983). The control of this pest has become a serious challenge facing applied entomologists nowadays regarding the widening circle of resistance and cross resistance to almost all available conventional insecticides, including organophosphorus and carbamate insecticides.

Insect growth regulators and chitin synthesis inhibitors (CSI) in particular have received wide attention in insect pest control strategies because of their unique action in interfering with chitin synthesis which confers a remarkable action specificity with very low mammalian toxicity, and also the lack of cross resistance between these compounds (Benzoylphenyl Urea compounds, BPU) and conventional insecticides. These compounds are particularly effective against lepidopterous immature insect stages, with relatively slow but strong action (Fahmy and Miyata, 1992 and Sammour *et al.*, 2008). The effect of these candidate compounds has not yet been fully investigated in many important insect pests.

The aim of this study is to investigate the effect of some CSI on different life aspects i.e toxicity, biology and biochemistry of the black cutworm, *Agrotis ipsilin* as a step towards a better understanding of the nature of their action aiming at extending

## their useful life in controlling many harmful pests.

**MATERIALS AND METHODS** 

## **Rearing technique and maintenance:**

The colony of the black cutworm, *Agrotis ipsilon* was maintained since 1982 in the laboratories of the Plant Protection Research Institute, Ministry of Agriculturem Dokki, Giza, without any insecticidal pressure. Rearing technique was the same adopted by (Abdin, 1979), and it was carried out under incubation at a constant temperature of 25° C and 70% relative humidity.

Eggs were kept in a clean glass jar till hatching. Newly hatched larvae were kept into new jars and fed on castor oil leaves, *Ricinus communis*. To avoid cannibalism, the larvae were reared individually in a separate unit of plastic cell tray (10.8 X 22.7 cm). Fresh castor oil leaves were offered daily till pupation. Pupae were then placed in glass jars until adult emergence. Food was provided daily by using a cotton pad soaked in 10% honey solution, and then eggs were daily collected.

## Chitin synthesis inhibitors (CSI):

i. Chlorfluazuron (IKI-7899): produced by Ishihara Sangyo Co., Ltd., Japan.

ii. Triflumuron (Alsystin): Produced by Bayer Co.

# **Bioassay technique**:

It is well known and documented by many investigators that chitin synthesis inhibitors are particularly effective against insect immature stages and especially during the larval intermoult periods. (Ishaaya and Ascher, 1977: Van Laecke *et al.*, 1989).

According to El Kady *et al.*, (1990), the newly moulted 4<sup>th</sup> instar larvae of *Agrotis ipsilon* were the most sensitive stage to CSIs. Therefore, newly moulted 4<sup>th</sup> instar larvae were used for bioassay tests in this study.

Leaf dipping technique was adopted to test the susceptibility of the black cutworm larvae to different CSIs (Abou- El-Ghar *et al.*, 1994). Castor oil leaves, *Ricinus communis* were dipped in insecticide solution, left to dry at room temperature and then offered to the newly moulted 4<sup>th</sup> instar larvae. Each concentration was replicated five times with 20 larvae each.

Mortality percentages were corrected using Abbott's formula (Abbott, 1925) as follows:

corrected mortality% =

Observed mortality % - Control mortality % ------ X 100 100 – Control mortality %

Data then were subjected to probit analysis (Finney, 1971) for determining  $LC_{50}$  values for each insecticide.

## **Biological Studies:**

Larvae treated with  $LC_{50}$  of the tested compound were examined daily to determine several biological aspects such as larval and pupal durations, weights, pupation percentage, adult emergence. Longivity and fecundity of adults were also determined and compared with those of untreated insects.

Fecundity was measured as the total number of eggs laid per female. The laid eggs were counted daily. The deterrent index was calculated according to Lundgren (1975). Fertility was estimated as the percentage of egg hatch. The corrected percentages of either fecundity or fertility were calculated using the formula described by Crystal (1968).

#### **Biochemical studies:**

Fourth instar larvae treated with  $LC_{50}s$  of chlorfluazuron or triflumuron were used to study the effect of these compounds on total protein and glycogen levels in the whole larval body extracts.

Total protein in the whole insect homogenate was determined using Biuret technique (Tufail *et al.*, 1994) and glycogen content was measured according to Roe and Dailey (1966).

## RESULTS

#### **Bioassay tests:**

The toxic effect of chitin synthesis inhibitors (CSIs) chlorfluazuron and triflumuron against the 4<sup>th</sup> instar larvae of the black cutworm, Agrotis ipsilon is shown in Table 1.

Susceptibility data showed that the  $LC_{50}$  values of both compounds were nearly the same. However, triflumuron was slightly more toxic than chlorfluazuron evidenced by the  $LC_{50}$  values which were 3.5 and 4.0 ppm for triflumuron and chlorfluazuron, respectively.

 Table 1: Susceptibility of the 4<sup>th</sup> instar larvae of the black cutworm, Agrotis ipsilon to chlorfluazuron and triflumuron.

Common name	Trade Name	LC <sub>25</sub> ppm	LC <sub>50</sub> ppm	LC <sub>90</sub> ppm
Chlorfluazuron	Atabron 10% EC	1.25	4,0	32.0
Triflumuron	Alsystin 5% EC	0.7	3.5	81.3

#### **Biological studies**:

Chlorfluazuron and triflumuron, benzoyl phenylurea compounds, are mainly effective against lepidopterous larvae, disrupting chitin formation, and they are very effective against immature insect stages with relatively slow but strong action.

In the present study, treatment of the lack cutworm, *Agrotis ipsilon* larvae with subleathal doses of chlorfluazuron and triflumuron not only exerted their action as larval death but they also caused an extended effect which was observed as pupal death, failure of emergence and even some adult death or malformations in cuticle, wings and appendages.

The effect of two sublethal doses,  $LC_{50}$  and  $LC_{25}$  of both chitin synthesis inhibitors on the following aspects was investigated:

#### Larval duration:

Table 2 Shows that the treatment of the 4<sup>th</sup> instar larvae of the black cutworm with both tested benzoylphenyl urea compounds resulted in a significant prolongation in the larval duration. this prolongation was more obvious with triflumuron treatments than chlorfluazuron and at  $LC_{50}$  values than with  $LC_{25}$  values.

## Larval weights:

Seventy two hours post treatment of the 4<sup>th</sup> instar larvae with the sublethal doses,  $LC_{50}$  and  $LC_{25}$  of both compounds, a segnificant reduction of larval body weight compared to the control insects was observed (Table 2). This reduction in body weight was more apparent with chlorfluazuron treatments than triflumuron treatments. This reduction reached more than 25% with chlorfluazuron  $LC_{50}$  treatments as compared to control, this effect was 45% higher with  $LC_{50}$  treatments than with  $LC_{25}$  treatments.

		Mean larval duration	Mean larval weight (mg.)	Pupation %
Insecticide	Dose	(days)	(95% c.i.)	-
		(95% c.i.)		
Chlorfluazuron	LC <sub>50</sub>	28.3	0.125	28.9
		(23.1-35.2)	(0.09-0.17)	
	LC <sub>25</sub>	27.0	0.126	31.2
		(21.9-29.7)	(0.08-0.16)	
Triflumuron	LC <sub>50</sub>	30.7	0.171	35.3
		(24.9-34.1)	(0.12-2.1)	
	LC <sub>25</sub>	29.0	0.179	39.0
		(25.1-33.0)	(0.080.191)	
Control	00.00	26.5	0.232	98.3
		(23.1-28.2)	(0.16-0.28)	

Table 2: Effect of chlorfluazuron and triflumuron on the larval duration, larval weight and pupation percentage of *Agrotis ipsilon*.

## **Pupation percentage:**

the percentage of pupation resulted from treatment of  $4^{\text{th}}$  instar larvae with LC<sub>50</sub> of both chitin synthesis inhibitors was highly reduced compared to the control insects (Table 3). This reduction has reached about 70% and 60% with chlorfluazuron and triflumuron, respectively as compared to control insects.

Table 3: Effect of chlorfluazuron and triflumuron on pupal duration, pupal weight and percentage of adult emergence of *Agrotis ipsilon*.

Insecticide	Dose	Mean pupal duration (days)	Mean pupal weight	Adult emergene
		(95% c.i.)	(mg) (95% c.i.)	%
Chlorfluazuron	LC <sub>50</sub>	12.0	0.191	18.3
		(10.5-14.1)	(0.012-0.25)	
	LC <sub>25</sub>	10.0	0.198	26.7
		(9.1-12.0)	(0.13-0.24)	
Triflumuron	LC <sub>50</sub>	13.6	0.199	29.7
		(11.6-15.1)	(0.15-0.26)	
	LC <sub>25</sub>	11.5	0.203	35.5
		(9.7-13.2)	(0.18-0.25)	
Control	0.00	9.8	0.368	86.6
		(8.1-12.1)	(0.30-0.43)	

#### **Pupal duration:**

Results shown in Table 3 indicate that the two compounds have increased the mean pupal duration of the black cutworm pretreated as 4<sup>th</sup> instar larvae. this reduction has reached more than 35% and 20% with triflumuron and chlorfluazuron, respectively as compared to control.

## Pupal weight:

Table 3 shows that the tested chitin synthesis inhibitors caused a reduction of the mean pupal weight of pupae resulted from treated larvae. again this reduction was more obvious with  $LC_{50}$  than  $LC_{20}$  treatments. This reduction has reached 45% less than control insects with both compounds.

#### Percentage of adult emergence:

The percentage of adult emergence resulted from pretreated  $4^{\text{th}}$  instar larvae has been highly reduced. Emerged adults derived from treated larvae were 75% and 60% less than control insects with chlorfluazuron and triflumuron, respectively (Table 3).

## Adult longivity:

Results obtained showd a significant reduction in adult longivity of the black cutworm derived from pretreated 4<sup>th</sup> instar larvae with both benzoylphenyl urea compounds used in this study. This reduction was more apparent with LC<sub>50</sub>s than LC<sub>25</sub>s treatments (Table 4). It has reached about 45% and 25% with chlorfluazuron and triflumuron LC<sub>50</sub>s, respectively as compared to control insects.

## **Total oviposition period:**

Total oviposition period of adults derived from  $4^{\text{th}}$  instar larvae treated with both compounds was significantly reduced. This reduction was more than 75% and 65% for chlorfluazuron and triflumuron LC<sub>50</sub> treatments, respectively compared to control tests (Table 4).

Table 4: Effect of chlorfluazuron and triflumuron on the adult longivity and total oviposition period of	
Agrotis ipsilon.	

Insecticide	Dose	Mean adult longivity (days) (95% c.i.)	Total oviposition period (days) (95% c.i.)
Chlorfluazuron	LC <sub>50</sub>	7.2	2.3
	20	(6.1-8.2)	(1.7-3.1)
	LC <sub>25</sub>	11.5	7.1
		(9.97-13.1)	(5.8-7.9)
Triflumuron	LC <sub>50</sub>	9.8	3.4
		(7.9-11.2)	(2.9-3.9)
	LC <sub>25</sub>	10.7	9.8
		(8.8-12.1)	(9.4-10.1)
Control	0.00	13.25	11.1
		(11.1-14.8)	(10.8-11.5)

## Number of eggs per female:

A significant reduction in the number of eggs laid per female derived from treated 4<sup>th</sup> instar larvae has resulted (Table 5). This reduction reached about 90% and 88% with chlorfluazuron and triflumuron treatments, respectively as compared to control insects.

#### Egg hatchability percentage:

Table 5 shows that hatchability of eggs laid by females derived from treated larvae was, almost, completely inhibited. Hatchability% ranged from zero-8% with both compounds compared to 93% for control tests.

 Table 5: Effect of chlorfluazuron and triflumuron on the number of eggs per laid per female, percent hatchability and sterility of Agrotis ipsilon.

 Inserticid
 Description

Insecticide	Dose	Number of eggs / female (95% c.i.)	Hatchability %	Sterility %
Chlorfluazuron	LC <sub>50</sub>	133.1	0.0	100
		(125-139)		
	LC <sub>25</sub>	181.6		
		(175-190)	4.3	99.9
Triflumuron	LC <sub>50</sub>	201.0	0.0	100
		(195-210)		
	LC <sub>25</sub>	216.9		
		(209-221)	7.59	99.9
Control	0.00	1854	93.68	0.00
		(1845-1872)		

#### Sterility percentage:

The hatchability data abtained and shown in Table 5 indicated complete sterility of females derived from 4<sup>th</sup> instar larvae of the black cutworm treated with either chitin synthesis inhibitors. No strility has resulted in the control insects.

## **Biochemical studies:**

Changes in total protein mg glycogen contents throughout the course of insecticide poisoning was studied.

## **Total protein:**

Changes in total proteins of the whole body extract of the  $4^{\text{th}}$  instar larvae of *Agrotis ipsilon* at 24, 48 and 72 hours post treatment with LC<sub>50</sub>s chlorfluazuron and triflumuron are shown in Table 6.

Untreated larvae showed a remarkable and gradual increase in total protein levels with time. Total protein level was significantly higher in treated larvae 24 and 48 hours after exposure with both chitin synthesis inhibitors, compared to the untreated. However, 72 hours post treatment, total protein level showed a remarkable decrease that even reached a value less than that of the untreated larvae at the same time.

Table 6: Effects of treatments with sublethal dose ( $LC_{50}$ ) of chlorfluazuron and triflumuron on the total protein content of whole body extract of the 4<sup>th</sup> instar larvae of *Agrotis ipsilon*.

protein content of whole b	ody extract of the 4 linstal	latvae of Agrons ipsuon.
Insecticide	Time post treatment	Total protein (µg/mg) (95% c.i.)
Chlorfluazuron	24 hours	66.93
		(61.1 – 72.2)
	48 hours	122.04
		(115.9 – 129.0)
	72 hours	124.86
		(121.5 – 127.6)
Triflumuron	24 hours	98.74
		(94.1 – 103.2)
	48 hours	128.17
		(123.2 – 133.4)
	72 hours	124.86
		(119.3 – 130.1)
Control	24 hours	51.66
		(46.9 – 55.7)
	48 hours	97.40
		(92.3-102.4)
	72 hours	140.15
		(134.9 – 146.1)

## **Glycogen content:**

Table 7 shows the changes in glycogen content of the  $4^{th}$  instar larvae of the black cutworm at 24, 48 and 72 hours after treatments with LC<sub>50</sub>s of chlorfluazuron and triflumuron.

Table 7: Effects of treatments with sublethal dose  $(LC_{50})$  of chlorfluazuron andmtriflumuron on the total glycogen content of whole body extract of the 4<sup>th</sup> instar larvae of *Agrotis ipsilon*.

Insecticide	Time post treatment	Total glycogen
	-	(mg gl./100 gm tissue) (95% c.i.)
Chlorfluazuron	24 hours	16.91
		(14.6 - 18.8)
	48 hours	20.98
		(16.3 - 25.2)
	72 hours	94.59
		(89.4 – 99.1)
Triflumuron	24 hours	32.01
		(29.9 – 35.1)
	48 hours	24.41
		(19.1 – 29.3)
	72 hours	88.18
		(80.9 – 95.5)
Control	24 hours	17.89
		(14.9 - 20.7)
	48 hours	13.07
		(11.9 – 15.3)
	72 hours	59.19
		(52.4 - 65.6)

Generally speaking, glycogen levels were almost higher in the treated larvae than the untreated ones with both insecticides and at all time intervals.

Glycogen level was higher with triflumuron treatment at 24 hours and 48 hours post treatment than with chlorfluazuron at the same time intervals. However, the situation was reversed at 72 hours where glycogen level was higher with chlorfluazuron treatment that with triflumuron.

## DISCUSSION

Benzoylphenyl urea compounds are not only action specific as chitin synthesis inhibitors (CSIs) but also stage specific (Gagou *et al.*, 2002) as they only effect insect immature stages and particularly the moulting stages at the critical time of chitin formation (Belinato *et al.*, 2013).

Newly moulted  $4^{th}$  instar larvae of *Agrotis ipsilon*, used in this study have been reported as the most sensitive larval instar to CSIs (Abdel-Azeem, 1989; El-Kady *et al.*, 1990 and Abou El-Ghar *et al.*, 1994).

Both chitin synthesis inhibitors, chlorfluazuron and triflumuron, exhibited remarkable toxicities against 4<sup>th</sup> instar larvae of Agrotis ipsilon and triflumuron showed slightly higher toxicity than chlorfluazuron. Similar observations were also reported by Mostafa (1998) on the same insect.

Chlorfluazuron is characterized by a slight latent effect than other chitin synthesis inhibitors (Haga *et al.*, 1984). In other words, toxic effects of chlorfluazuron start later and last longer than triflumuron. This is due to the longer half-life time and/or slower detoxification rate inseide insect body (Neumann and Guyer, 1987).

The significant decrease in treated larval body weights compared to the untreated larvae was dose dependent and can be explained by the decreased or improper feeding rate which was reported to be due to the weak or malformed mandibles resulted from the poor scirotization with chitin. This may lead to retardation of larval development or even starvation to death (Neumann & Guyer, 1987 and Guyer & Neumann, 1988).

Only one third of treated larvae were able to pupate successfully. These pupae, derived from small sized and weaker larvae will normally be less in weight compared to untreated insects. Also due to the lack of proper sclerotization of the newly formed puparium, evaporation of body fluids may also take place which will also lead to decreased pupal weight, this explains the results obtained in this study.

Adult emergence percentage also showed a significant, dose dependant, reduction and did not exceed 20-35% compared to 87% in control insects with appearance of pupal adult intermediates. Failure of adult emergence was more drastic with chlorfluazuron treatments due to its longer latent effect. Similar results were also obtained by other investigators. (Degheele, 1990, Khattar, 2014, and Tabozada, 2014).

The suppression of egg production of females developed from treated larvae obtained in this study may in part be due to interference of the tested chitin synthesis inhibitors with oogenesis and vitellogenesis processes as reported by Perveen and Miyata (2000). The significant reduction in egg production can also be due to the sharp decline in oviposition period of females coming from treated larvae, 2-3 days compared to 11 days in untreated insects.

The resulted reduction in egg hatchability due to larval treatments with chitin synthesis inhibitors may be attributed to the improper chitin formation during the emeryonic development which was observed and reported by many investigators (Adel, 2012, and Farinos et al., 1989).

Generally speaking, very high degrees of sterility are very well documented in many insect species due to treatment with several benzoylphenyl urea compounds (Guadalupe Rojas and Morales-Ramos 2004, Sáenz-de-cabezón *et al.*, 2006, and Adel, 2012).

Proteins and glycogen are generally the major biochemical components necessary for an organism to develop, grow and perform its vital activities. Moreover, they are the main components for chitin formation. Thus it may be of interest to study the changes in total protein ang glycogen contents throughout the course of insecticide poisoning.

Insect carbohydrate reserves are present as glycogen and trehalose which can be readily converted to glucose for chitin synthesis (Chippendale, 1978 and Mohamed, 1998). Glycogen content levels showed the same pattern of a slight increase 48 hours post treatment. However, 72 hours post treatment, glycogen levels showed a sharp increase which was not observed in untreated insects. In untreated insects glycogen was converted to glucose which was incorporated in the synthesis of new cuticle. However, in treated insects this did not take place due to inhibition of chitin synthesis and consequently glycogen levels remained high.

Protein levels of treated insects showed a significant increase during the first time interval *i.e.* 24 hours post treatment with both compounds. This increase can be explained by the natural increase of protective hydrolytic and detoxifying enzymes that usually take place after exposure to xenobiotics (Ishaaya and Yablonski, 1987).

On the other hand, the sharp decline in total protein levels 72 hours post exposure can be explained by the inhibition of protein synthesis as a first sign of cell death as reported by Deloach *et al.*, (1981). Similar observations were also reported by Farag (2001).

We need to extend the useful life of such candidate insecticides through the wise use of minimum concentrations to suppress pest populations and not iradicating them in order to avoid resistance development.

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#### **ARABIC SUMMERY**

التأثيرات السمية و البيولوجية و الكيموحياتية لبعض مثبطات تخليق الكيتين على الدودة القارضة السوداء *أجروتيس أبسيلون* (حرشفيات الأجنحة: الليليات)

> **عادل رمــزى فهمـــى** قسم علم الحشــرات – كلية العلوم – جامعة عين شمس- القاهرة

تعتبر مثبطات تكوين الكيتين من اكثر المجموعات أمنا على الأنسان و الحيوانات و الكائنات النافعة المحيطة و فى الوقت ذاتة تخصصية التأثير. و عدم وجود مقاومة خلطية بينها و بين المبيدات التقليدية جعلها من أهم و أقوى البدائل التى تحظى بالأهتمام و الدراسة على مستوى العالم. و لفهم مزيد من الجوانب حول هذة المجموعة تم اختيار مركبين من أكثر أعضائها فاعلية و هما الكلور فلواز ورون و الترايفلومورون و دراسة تأثير هما على واحدة من أهم الأفات الزراعية خطورة فى مصدر و مناطق كثيرة من العالم و هى الدودة القارضة السوداء. و قد تمت دراسة النقاط الأتية: 1. السمية و تأثير ها على كثير من المناحى البيلوجية للحشرة.

٢. التأثير على محتوى البروتينات و الجليكوجين داخل جسم الحشرة (عنصرى تكوين الكيتين) و بمعاملة العمر اليرقى الرابع حصلنا على النتائج الأتية: أ. كان الترايفلومورون هو الأسرع تأثيرا و لكن الكلورفلوازورون كان الأبقى نظرا لطول نصف العمر داخل

ا. كان الترايفلومورون هو الأسرع تاتيرا و لكن الكلورفلوازورون كان الابقى نظراً لطول نصف العمر داخل جسم الحشرة

ب. أظهرت النتائج انخفاض واضح في أوزان البرقات و العذاري و النسب المئوية لكل من تكوين العذاري و خروج الحشرة الكاملة و النقص الواضح لمعدل وضع البيض و كذلك النسبة المئويي للفقس. في نفس الوقت زادت المركبات المستخدمة من متوسط العمرين البرقي و العذري.

ج. تأثر محتوى البروتينات و الجليكوجين نتيجة المعاملة بالمركبين.