BIOACTIVITY OF LEMON EXTRACTS ON THE COTTON LEAFWORM, *Spodoptera littoralis* (Boisduval) (*Lepidoptera: Noctuidae*) I- ANTIFEEDANT EFFECT OF LEMON LEAF EXTRACTS AGAINST *S.littoralis* larvae.

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ABSTRACT

Five lemon leaf extracts were tested as antifeedants for the cotton leafworm, *Spodoptera littoralis* (Boisduval), where Chloroform, methanol, carbontetrachloride, ether and n-hexane were used. In choice tests; where larvae were offered treated and un-treated cotton leaf discs, extracts of the three latter solvents were very effective on 3rd instar larvae. The chloroform extract was effective, while the methanol extract had no such effect. In non-choice tests; where larvae were offered only treated cotton leaf discs, more than 97.0% feeding inhibition was observed with chloroform (weakly polar); 98.6, 83.0 and 95.7% for carbontetrachloride, ether, and n-hexane, (non-polar), respectively.

These results indicate that *Citrus limon* L. leaves contain chemicals that serve as antifeedants for *S.littoralis* larvae

Key words: *Spodoptera littoralis,* lemon extracts and Antifeedant effect.

INTRODUCTION

Lemon trees, *Citrus limon* L., are not known as host plants of the cotton leafworm, *Spodoptera littoralis* (Boisduval). They are well known as more resistant plants to citrus pests than any other citrus plants. On the other hand, several authors used fine-chopped lemon, or lemon juice in poison baits for the control of many insects such as locusts, grass-hoppers, the corn earworm, *Heliothis armigera* (Hubner), the fall armyworm, *S.frugiperda*, e.g., **Swenk (1918), Davis (1919), and Smith (1921). Roberto *et al.* (2002)** used limonoids obtained from *C. limon* seeds as antifeedants against larvae of *S .frugiperda.*

Moreover, various parts of citrus fruit trees, shrubs, are sources of citronella oil **(Bagdat, 2006 and Nhu-Trang *et al.*, 2006)**, which is well known to be a repellent to some insects such as mosquitoes. **Attaway *et al.* (1966)** identified 19 compounds in the leaf oil of *C. limon*, among them were some insect repellents.

Several authors used various extracts of different plants with low insect associations for antifeedant activity tests against many insect pests, e.g.; **Etman (1974)** for the native bud-worm of Australia, *Heliothis punctigera* Wallg., and the cluster caterpillar, *S.litura* (F.); **Widson *et al.* (1983)** for the corn earworm, *H.zea* (Boddie); **Abdel-Rahman and Al-Mozini (2007); Ballesta-Acosta *et al.* (2008)** for *S.littoralis* larvae.

Other authors, in intensive studies by using plant isolated chemical compounds for antifeeding activity tests, e.g.; **Kubo *et al.* (1976)** for the armyworms, *S.exempta*, and *S.littoralis*; **Bringmann *et al.* (1992), and Mannaa and Attia (1992)** for *S.littoralis*.

The objective of this investigation was to determine the feeding responses of the larvae of *S.littoralis*, to lemon leave extracts, and to asses the probability of the presence of insect antifeedants in lemon leaves.

MATERIALS AND METHODS

1- Insect culture:

Insects used in this study were obtained from laboratory colonies maintained in our Department, and reared on artificial medium by established procedures **(Etman *et al.*, 1983).** Rearing took place in a controlled environment room maintained at 28 ± 2 °C, with a natural light supplemented by artificial light for a 14 h photo phase, and natural dusk period.

2- Plant extraction:

Solvents used were methanol as a polar solvent; n-hexane, carbontetrachloride, and ether represent the non-polar solvents; and chloroform as a weakly polar solvent. Lemon leaves were collected fresh from 12-year-old trees, and dropped in the boiled solvent used to inhibit enzymatic activities. The lemon leaves were cut into small pieces and then minced with a food mencer, and thereafter the material (100 g) was homogenized in a Waring blender with 200 ml solvent. Solvents were removed by using a rotary evaporator at a temperature below 40 °C.

All plant crude extracts were kept below 0 °C. The crude extracts were bioassayed within a week. The final volume of the acetone extract solution was 25 ml.

3- Specific extraction procedures:

a- Extraction with a mixture of methanol, chloroform and water:

This method was originally used by **Blight and Dyer (1959)** for the rapid extraction of lipids from fish. In 1970 it was used by Wayte and Southcott in isolating natural products from algae. The chloroform extract was evaporated and the residue dried with dry nitrogen. The residue was dissolved in acetone and transferred to a volumetric flask. The aqueous methanolic layer was filtered, and the extract evaporated at 50 °C to remove the methanol. Thenthe aqueous solution left was freeze dried. The residue dissolved in distilled water and the volume adjusted to 25 ml with acetone.

b- Carbontetrachloride extract:

The minced lemon leaves sample was extracted with a mixture of 250 ml carbon tetrachloride and 250 ml propanol 2, and 150 ml distilled water. The carbon tetrachloride phase was filtered, the solvent evaporated, and the residue dissolved in 25 ml acetone. The aqueous propanol solution was discarded.

c- Ether extract by Soxhlet extraction apparatus:

The plant sample was minced, 100 ml of water was added, and the plant material was freeze-dried. The dry residue was scrapped out and placed in the apparatus, which was left overnight until complete extraction was effected. The ether was evaporated, and the residue dissolved in acetone.

d- Extraction with n-hexane:

The plant material was blended with 500 ml n-hexane. The aqueous phase was discarded, the n-hexane phase filtered, the solvent evaporated, and the residue dissolved in acetone.

3- Bioassay for antifeeding activity:

The leaf-disc test used by **Munakata (1970)** was used in testing the lemon extracts for active principles with *S.littoralis*. Since 100 g of the lemon leaves were extracted and stored in 25 ml acetone, therefore the

concentration of the acetone solution of the extract was 4 g equivalent of fresh leaves per one ml acetone.

Round leaf-discs, 21 mm diameter, were punched out with a cork-borer, from the leaves of cotton plants. Treated (T) discs were immersed in the acetone solution of the lemon leaf extract, and the control (C) discs in pure acetone. After air drying, these discs were placed symmetrically in a polyethylene dish.

The larvae used for the experiments were removed from the rearing medium and placed onto cotton leaves for one hour to enable them to adapt more easily under the test conditions to the new type of food. The larvae then were starved for 2 h before the tests. Then 3rd-instar larvae were introduced in the center of the dish, covered, and left for 3 h. The experiments were conducted at 22 ± 2 °C. The "leaf-disk test" was repeated 12 times using 12 dishes for each leaf extract.

The data obtained were statistically analysed and any extract which showed significant results was repeated using two similar leaf-discs in the same dish, hence 3 different arrangements were used: Treated-control (T-C), Treated-Treated (T-T), and Control-Control (C-C). This enabled a study of the larval feeding responses where: (i) a choice between treated and untreated discs was offered, (ii) no choice between treated and untreated discs was offered, i.e., forced eaten feeding test. Each experiment was replicated 12 times. The graph paper method for measuring the leaf area consumed was used **(Dethier, 1947).**

RESULTS AND DISCUSSION

1- Choice experiments:

In these experiments, where larvae were given a choice between treated and control discs, Wilcoxon's signed-rank test were applied to treated and control data (actual area consumed) instead of a parametric analysis of variance. This was chose because the data did not appear to be normally distributed. Nevertheless, in the experiments where the observed difference was significant, the student's t-test was applied using the formula:

$$t = \frac{\bar{X} - \mu}{S / \sqrt{n}}$$

where μ is 20%
$\bar{X}$ is the mean of the feeding ratio (FR)
S is the S.D. of FR
N is the number of samples (12 in each experiment)

The feeding ratio was expressed as follows:

$$\text{Feeding ratio} = \frac{\text{Amount consumed of T disc}}{\text{Amount consumed of C disc}} \times 100$$

When the amounts consumed of the treated discs were less than 20% of those of the control discs, the plant extracts were considered to have strong feeding inhibitory activity. The 20% criterion was chosen because this amount of was thought to be of a possible economic value. The feeding inhibitory effect was expressed as follows:

$$1 - \frac{\text{Amount consumed of T discs}}{\text{Amount consumed of C discs}} \times 100$$

Since the tabulated t value for 11 degrees of freedom at 0.01 significance level is 3.11, and the calculated values for the carbon tetrachloride, ether, and n-hexane extracts are more than 3.11, it is evident that the feeding ratios in these experiments (means being 6.5%, 9.8%, and 10.5% for the three extracts, respectively) are significantly less than 20% at 1% level of probability. These highly significant differences and the results suggest that the above plant extracts have very strong feeding inhibitory activity against 3rd instar larvae of *S.littoralis* (table 1).

Since the calculated value of " t " for the chloroform extracts is less than the tabulated value for 11 degrees of freedom at 5% level, there is no significant difference between the mean feeding ratio of this leaf extract (15.8%) and the 20% feeding ratio level. These results suggest that this extract has a strong feeding inhibitory activity against 3rd instar larvae of *S.littoralis.*

On the contrary, the methanol extract had no feeding inhibitory activity. This was demonstrated by the " not significant " results of Wilcoxon's signed-ranks test and the high feeding ratio mean (80.9%).

Table 1. Feeding responses of third-instar larvae of *S.littoralis* to cotton leaf-discs treated with lemon leaf extracts, and to alternative choices (controls) processed with acetone only Each extract was replicated 12 times, with 1 dish/ replicate and 10 larvae/ dish.

Solvent	Mean % area consumed ± S.E. T	C	Mean feeding ratio ± S.E.	Mean feeding inhibitory effect ± S.E.	Wilcoxon's test	Student's t-test Value (11 DF)
Chloroform	1.7 ± 0.6	10.9 ±1.3	15.8 ± 5.0	84.2 ± 5.0	S**	1.62
Methanol	7.5 ± 1.3	10.9 ± 1.4	80.9 ± 15.3	- -	N.S.	- -
Carbon-tet.	0.6 ± 0.2	10.5 ± 0,9	6.5 ± 1.9	93.5 ± 1.9	S**	6.15**
Ether	1.4 ± 0.5	17.0 ± 1.3	9.8 ± 3.3	90.2 ± 3.3	S**	4.32**
n-Hexane	0.7 ± 0.3	6.7 ± 1.0	10.5 ± 4.5	89.5 ± 4.5	S**	3.28**

S** = Significant at 1 % level of confidence.
N.S. = Not significant
C = Untreated control leaf disc
T = Treated leaf disc
Concentration used = 4 g equivalent of fresh leaf/1 ml

2-No choice experiments;

In order to study the larval feeding responses where no choice between treated and untreated discs were offered, the non-parametric Wilcoxon two-sample test was applied to the feeding ratios of the T-C divisions and the feeding ratios of the random pairs of sums of T-T and C-C divisions of the experiments.

Table 2. Feeding responses of third instar larvae of *S.littoralis* to cotton leaf-discs treated with lemon leaf extracts and to alternative choices (controls) processed with acetone only; and to treated or control leaf discs only, with no alternative choices. Each extract was replicated 12 times, with 3 dishes / replicate and 10 larvae / dish.

Solvent	Mean % area consumed ± S.E. *T	C	Mean feeding ratio ± S.E.	Mean % feeding inhibitory effect ± S.E.	Mean % area consumed ± S.E. T	T	C	C	Mean feeding ratio of random sums of (T+T/C+C x 100) ± S.E.	Mean % feeding inhibitory effect ± S.E.
Chloroform	0	10.9± 2.5	0	100	0.2± 0.09	0.13± 0.07	10.7± 1.0	9.6± 1.0	2.7±** 1.0	97.3± 1.0
Carbon-tet.	0	0.9± 1.3	0	100	0.2± 0.07	0.1± 0.03	9.8± 1.8	9.7± 2.0	1.4± 0.6	98.6± 0.6
Ether	0.5± 0.3	9.8± 0 8	5.2± 2.5	94.8± 2.5	1.7± 0.6	1.9± 0.9	9.4± 0.8	14.1± 2.0	17.0± 5.3	83.0± 5.3
n-Hexane	1.0± 0.3	4.8± 0.7	17.5± 6.1	82.5± 6.1	0.3± 0.1	0.4± 0.2	6.9± 0.8	5.8± 0.5±	4.3± 1.2	95.7± 1.2

* Every letter represents one leaf–disc. *Every pair of letters represents one dish.
**The result of Wilcoxon's two-sample test was significant.

The results of the analysis showed that there was a significant difference between the feeding responses of 3rd instar larvae of *S.littoralis* to chloroform lemon leaves extract where a choice was offered (0.0%), and their feeding responses where no choice was offered (2.7%). In this case larvae ate more of the treated discs where no choice was offered. Similarly, larvae ate more of the treated discs of the carbontet. extract, bor the difference was not significant, (the mean feeding ratios were 0.0 and 1.4% for choice and no choice tests, respectively (table 2).

In the other two extracts i.e. ether and n-hexane, there were no significant differences between the feeding responses of 3rd *S.littoralis* larvae where a choice or no choice was offered. The mean feeding ratios were 5.2% and 17.0% for ether estract; 17.5% and 4.3% for n-hexane, for choice and no choice tests, respectively (table 2). In other words, in the no-choice tests more than 97% protection was offered by the two weakly polar extract used i.e. chloroform whereas 98.6, 83.0 and 95.7% protection by the non-polar extracts i.e. carbon tet., ether and n-hexane, respectively.

These results suggest that the above lemon leaf extracts have very strong feeding inhibitory activity against 3rd instar larvae of *S.littoralis*. The above results have been confirmed by applying Kruskal-Wallis test, which gave the same results as did Wilcoxon's signed-ranks test, approximately.

Etman (1974) reported the presence of two aldehydes, an alkaloid, a glycoside and an organic acid in lemon leaf extracts. **Attaway *et al.* (1966) and Nhu-Trng *et al.* (2006)** analysed the lemon leaf-oil and found that they contain citronellal, which is a mixture of stereo-isomeric aldehydes **(Merek, 1960). Kamiyama (19670** stated that trans-2-hexenal (a leaf-aldehyde) is a compound of the leaf-oil of rough lemon. In the same analogy, Ruberto et al. (2002) confirmed that natural limonoids obtained from seeds of Citrus limon, i.e. limonin and obacunone showed highly significant antifeedant activity against larvae of *S.frugiperda*; while Chang **MingChow *et al.* (2004)** reported the presence of ascorbic acid in the lemon leaf methanol extract.

The inhibitory activity observed with *S.littoralis* 3rd instar larvae here may stem from the aldehydes, alkaloids, glycosides, and the organic acid compounds together, or it may be due to the action of some of these compounds individually.

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استخدام مستخلصات أوراق نبات الليمون كمواد مانعة لتغذية يرقات دودة ورق القطن

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تم اختبار خمسة مستخلصات لأوراق الليمون ضد العمر الثالث ليرقات دودة ورق القطن وهي مستخلصات الكلوروفورم، الكحول الميثيلي، رابع كلوريد الكربون، الإثير، وان- هكسان، لدراسة مدى تأثيرها كمستخلصات تحتوي على مركبات لمواد مانعة للتغذية، وقد أوضحت تجارب التغذية الاختيارية أن المستخلصات الخام لرابع كلوريد الكربون والاثير وان-هكسان لها خصائص مانعة للتغذية قوية جدا ضد يرقات العمر الثالث التي غذيت على أوراق قطن معاملة بتلك المستخلصات. وكذلك أظهرت التجارب أن مستخلص الكلوروفورم له خاصية مانعة قوية. في حين أن مستخلص الكحول الميثيلي لم يكن له أية خواص مانعة للتغذية.

وعندما أعيدت التجارب باستخدام طريقة التغذية الإجبارية التى لم تعطى فيها اليرقات خيارات تفضيلية بين أوراق نبات القطن المعاملة والغير معاملة فأنه تم الحصول على٩٧,٣% وقاية لأوراق القطن المعاملة بمستخلص الكلوروفورم في حين أن مستخلصات رابع كلوريد الكربون والأثير وان- هكسان أعطت ٩٨,٦% و٨٣% و٩٥,٧% وقاية على الترتيب.

وقد أدت هذه النتائج إلى الاستنتاج بأن مستخلصات أوراق الليمون بالمذيبات غير القطبية تحتوي على مواد تمنع تغذية العمر الثالث ليرقات دودة القطن وبدرجة قوية جدا.