IDENTIFICATION AND EFFECTS OF BIO-PRODUCT COMPOUNDS OF *Cyperus rotundus* ON THE BIOLOGICAL AND QUANTITY DNA IN *Tribolium castaneum* (Herbst.)(Tenebrionidae: Coleoptera)

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ABSTRACT

The plant pesticides have recently got great importance than the synthetic pesticides, against stored product pests because of their lesser hazards to the environment and other related biomass. The bio-product compounds were isolated after preparation of ethanolic extract from *Cyperus rotundus* rhizome by using soxhelt apparatus. The analysis of High-Performance Liquid Chromatography (HPLC) also showed identification of 2 compounds; Caffeine and 4-Hydroxy benzoic acid. The longest duration of both the larval stage and the pupal stage were 17 and 7 days, respectively at 2% F2. The mortality rate in larval stage reached 90% at 2% F3. The identification of *Tribolium castaneum* was carried out via designing specific primers with the use of PCR technique, the presence of more than 100 bp band specific for amplification of beta-tubulin intron gene of *T. castaneum* found in the negative control were absence in the positive controls helps to detect the effect of bio-product compounds at 2% of fractions on quantity of DNA in this insect.

Key words: *Tribolium castaneum*, *Cyperus rotundus*, PCR DNA.

INTRODUCTION

The damage caused by red flour beetle, *Tribolium castaneum* (Herbst.) (Coleoptera:Tenebrionidae) to various stored and food commodities like grain, flour and dried fruits is recorded to be 15-20% which is capable of measuring losses worth millions of Dolars every year in a developing country observed among damage proteins and fats of wheat, whereas, negative correlation was found in carbohydrate (Wakil *et al*., 2003). Control of these pests are prime important in order to meet the demands of increasing population. The outbreaks of these pests could be avoided either by protect in and/or by treatment of the stored commodities with chemicals. Protection includes all the prophylactic measures and disinfestation of stores, bins, bags and grains by using benzene hexachloride (BHC), baythion, diazinon, gardonaa and malathion etc. Perveen *et al*., (2013) reported these chemicals applying before the grains being stored in order to eliminate chances of future infestation of the pests. Treatment of grains, on the other hand, has to be carried out with fumigants when infestation of the

Recievied: 5/12/2016
Accepted: 16/7/2017

pests appears during the storage (Perveen and Shah, 2012). Fumigants such as phosphine and methyl bromide were used quick and effective tools for insect control in food commodities. Insect resistance, toxic residues and other factors on stored grain (Perveen and Shah, 2012; Riebeiro *et al*., 2003). Plant-derived substances, natural plant products and bio-insecticides have recently become of great interest owing to their versatile applications (Baris *et al*., 2006) for protection of agricultural commodities due to their low mammalian and vertebrate's toxicity and low persistence, no undesirable effects on animals and human beings (Raja and Albert, 2006). Therefore, development of bio-insecticides has been focused as a viable pest control strategy in recent years (Hashim and Devi 2003; Meena and Prates, 2006). The development of environmentally friendly insecticides, having specificity to insects has captured worldwide attention of scientists (Ishaaya and Degheele, 1998). Plants provide potential alternatives to currently used insect control agents. Photochemical studies have shown that the major chemical components of this herb are essential oils, flavonoids, terpenoids, mono-and sesquiterpenes. The plant contains cyprotena, cypera-2, 4-diene, & cyperene substances**.** The main objectives of this study were to:

1. Provide useful manage stored product insects via using diagnosed bio-product compounds in the plant extracts of *Cyperus rotundus*.
2. Identify and effects of these compounds on the test insect DNA quantity via molecular based methods.

MATERIALS AND METHODS

Extraction of plant

The study was carried out during 2015-2016 at Department of Plant Protection of Agriculture, College of Salahaddin University. The rhizome of medicinal herb *Cyperus rotundus* (200g) were rinsed and dried at 30∓1°C under shed in laboratory. Then were ground to fine powder using an electric grinder, extraction using a soxhelt apparatus for six hrs. and extracted with petroleum ether, ethanol and D.W. Fractionations were made according to (Perveen and Shah, 2012).

Laboratory Insects rearing

The rearing method was adopted according to (Naqavi and Perveen, 1991) with some modifications. Adults of red flour beetle, *Tribolium castaneum* (Herbst.) were collected from the culture in Plant Protection Department in College of Agriculture and reared under controlled temperature at 30 ∓ 1˚C, RH 60 ∓ 2 . Ten pairs of adults of *T. castaneum* were taken in plastic bottles (height:

14 cm; dm: 8 cm) containing 300 g of wheat flour media (fine flour: wheat bran: Brewer's yeast; 7: 2: 1) and were tied with muslin cloth placed on a stand in laboratory to protect them from pests. They had incubation period 4-6 days, 6-7 larval instars and completed their life cycle in 22-25 days. When pure culture (after 4-5 generations) and sufficient population of uniform age and size were are experiments.

HPLC Analysis

The HPLC analysis was carried out by using ODSC18 column, 25cm x4.6x5µm. The mobile phase was (acetone 80+20 water), flow rate 0.5 ml/min and (Wave Length 280nm UV light) (Harborne, 1973).

Test insects

The (ten g) of flour treated with different concentrations (0.5, 1.5 and 2%) of the fractions, then dried overnight. The adults 1-2 days old were used in the experiment. Three replicates each consisted of ten insects were placed inside glass container that include 25g of flour. Each treated replicates were replaced in incubator and covered by muslin cloths. The studied features were as following:

1- Duration of larval stage and % larval mortality.

3- The number of emerged adults.

Preparation of controls

The positive control of the *T. castaneum* was prepared by extracting DNA from pure insect samples. The negative control was the DNA extracted from, treatment insects.

DNA extraction

DNA was extracted from the pure cultures of insects and the flour with a slight modification of a very simple extraction method called the high salt extraction method (Aljanabi and Martinez, 1997). Forty milligrams of the sample were crushed using 400 µL of sterile salt homogenizing buffer containing Tris, NaCl, and EDTA. Forty microliters of SDS (20%) and 8 µL of Proteinase K (20 mg/mL) were added and the samples were incubated at 65ºC overnight when 300 µL of 6M NaCl were added. DNA was recovered using isopropanol at half the volume of the supernatant and centrifuging at maximum speed for 10 s. The DNA was washed using 70% ethanol.

Primers

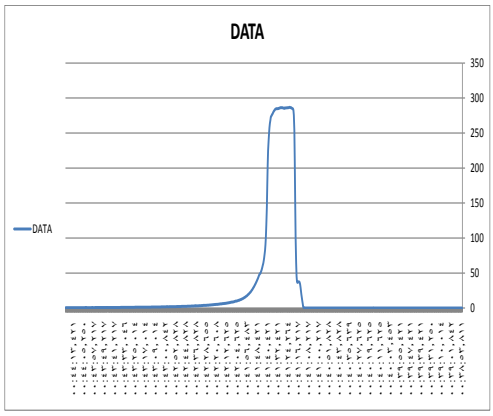
Insect nuclear primers were bought from the University of British Columbia, Vancouver, BC (http: //www.michaelsmith. ubc.ca/services/NAPS/Primer Sets/Primers_Oct2015.pdf) to identify insect specific primers. The primer combinations were tried as a screening process to randomly identify insect specific primers. There were 50 primers in the set. About 58 combinations were screened on all six insect species based on the details given about the primers and the genes they amplify. The initial conditions for the PCR for screening were maintained as: 94°C for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. Final extension was done at 72°C for 5 min after the 30 cycles. Once specific bands were obtained at low annealing temperature (56°C), the annealing temperature was then increased to 60°C to increase the stringency and to confirm the specificity of the bands. It was found that the primers designed to amplify the protein-coding gene, elongation factor 1-alpha, ATC TCC GGA TGG CAC GG (CT) GAC AA (EFS599); ACG TTC TTC ACG TTG AA (AG) CCA A (EFA923), were specific to *T. castaneum*. The amplified PCR product was purified with a QIAquick PCR purification kit (Qiagen ®). The assigned accession numbers was AY819656 (*T. castaneum*).

Thermal cycler

PCR was performed on a total volume of 25 µL using a Techne thermocycler (Techne, Burkhardtsdorf, Germany). One half microliters of DNA (20 ng/µL) were used with 19.5 µL of PCR mix containing 2.5 µL of 10X PCR buffer, 2 µL of DNTP's (each at 10 mM), 0.2 µL of Taq polymerase 5 units/µL and 0.75 µL of magnesium chloride 50 mM. Two microliters each of forward and reverse primer (20 pmoles /25 µL) were added to make the final volume to 25 µL. The thermo cycler program used for the specific primers was 94°C for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. Final extension was done at 72°C for 5 min after the 30 cycles. The above program was used the insect specific primers was identified.

RESULTS

The analysis of High-Performance Liquid Chromatography (HPLC) showed identification of two active compounds caffeine, P-Hydroxy benzoic acid 60.45-79.65%, respectively. Only the P-Hydroxy benzoic acid in F2 was recorded 70.34%, Table 1.



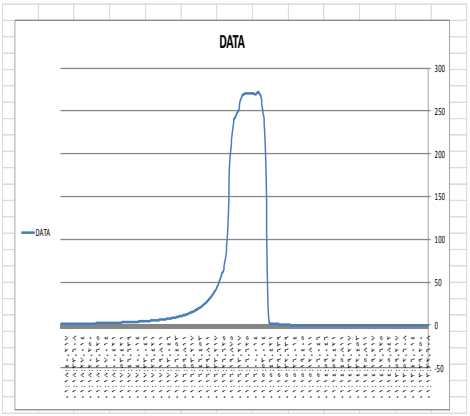


Fig. 1. Peak of Caffeine **Fig. 2. Peak of P-Hydroxy benzoi acid**

Table 1. The compounds which diagnostic via HPLC.

Fractions	Caffeine		P-Hydroxy benzoic acid	
	Rt	%	Rt	%
F2			2.33	70.34
F3	2.74	60.45	2.90	79.65
Standard compounds	2.74		2.23	

The Table 2 shows the significantly effects of the fraction F2 which isolated by (Column Chromatography) of the ethanolic extract of *Cyperus rotundus* rhizome on duration of larval stage which reached 16 day in treatment with concentration of 2%. The mortality rates were 25, 30 and 50% for 0.5, 1.5 and 2 percent concentrations. The duration of pupal stage with concentration as it was higher in the highest concentration 6, 7 and 7 days at 0.5, 1.5 and 2% concentration. The maximum concentration (2%) caused reduction in number of the adults 9, 8 and 5 at 0.5, 1.5 and 2%, respectively.

Table 2. The Effect of F2 on the biological features of *T. castaneum*

Concentration, %	The period Larval /day	Mortality % in larval stage	The period pupal /day	Number of the adult
0.5	12 c	25 c	6 c	9 b
1.5	13 b	30 b	7 b	8 b
2	16 a	50 a	7 b	5 c
Control	13 b	10 d	13 a	50 a

Within a column means followed by a same letter are not significantly different at 5% level.

The Table 3 shows the significantly effects of the fraction F3 which isolated by (CC) of the ethanolic extract of *Cyperus rotundus* rhizome on duration larval stage which reached 14 days in treatment with concentration 2%. The mortality rates were 65, 70 and 90% at 0.5, 1.5 and 2% concentrations. The duration pupal stage varied with concentration as it was higher in the highest concentration 8, 9

and 7 days at 0.5, 1.5 and 2% concentration. The maximum concentration (2%) caused reduction in the adult's population 2.

Table 3. Effect of F3 on the biological features of *T. castaneum*

Concentration, %	The period Larval stage/day	Mortality % in larval stage	The period pupal stage /day	Number of the adult
0.5	15 a	65 c	8 b	3 c
1.5	14a	70 b	9 b	4 b
2	14a	90 a	7 c	2 d
Control	13 b	10 d	13 a	50 a

Within a column means followed by a same letter are not significantly different at 5% level.

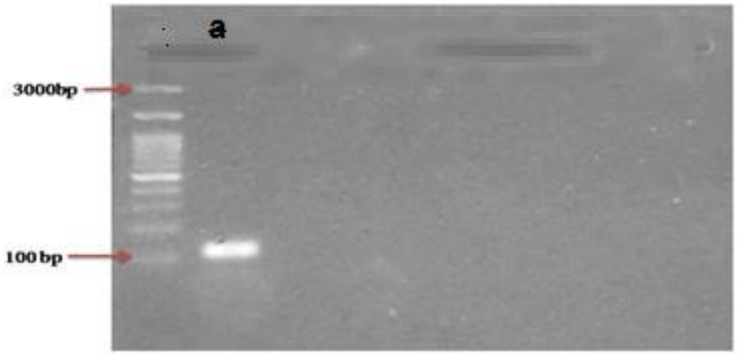


Fig.1. Gel plate PCR product of pure red flour beetle DNA using elongation factor 1-Alpha primers (EFS599; EFA923)

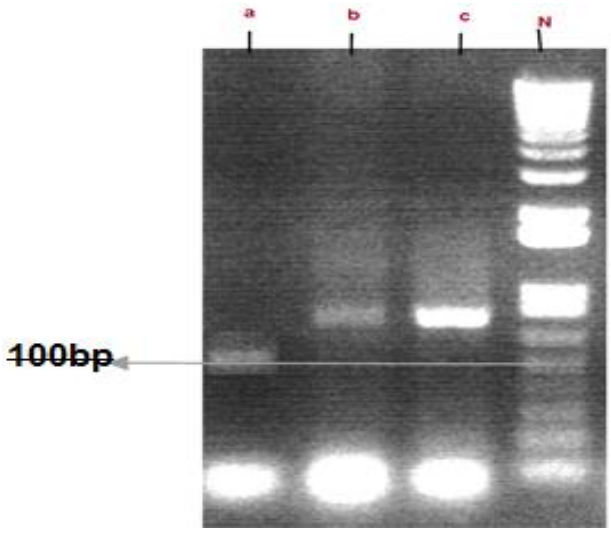


Fig. 2. Gel plate PCR product of (a) positive control, Negative controls of Treatment insect withF2 at 2% (b) Treatment insect withF3 at 2% (c) of *T. castaneum*.

DISCUSSION

The bio-products are more significant for controlling the stored grains and agricultural pests due to their lesser harmful effects compared to chemical pesticides. The high salt extraction method (Aljanabi and Martinez, 1997) was adopted to extract DNA from the pure insect DNA extraction using the promega

wizard genomic DNA extraction kit which extracted DNA from a single beetle that was successfully amplified by the insect-specific primer. The size of the band was Approximately 100 bp and the repeatability was 100% (Fig.2). The DNA extracted from the insects treated with fractions was amplified by PCR. From Fig (2) the negative controls, insects treated with F2 (b) and F3(c) had bands of sizes varying from 110-3000 bp but the positive control of the pure insect (a) DNA alone had a PCR product at 100 bp. Thus the presences of these bands in the negative control were absence in the positive control helps to detect the effect of bio-product compounds at 2% of fractions.

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تشخيص المركبات الفعالة لنبات السِعِد *Cyperus rotundus* في حياتية وكمية DNA لخنفساء الطحين الحمراء *Tribolium castaneum*(Herbst.) (Tenebrionidae: Coleoptera)

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المستخلص

لقيت مبيدات الآفات ذات الأصل النباتي اهتماما كبيرا مقارنة بالمبيدات الصناعية ضد آفات الحبوب المخزونة نظرا لأنها لا تلحق أضرارا كبيرة بالبيئة. المركبات الفعالة التي شخصت بتقانة HPLC في المستخلص الكحولي لرايزومات نبات السعد *Cyperus rotundus* هي caffeine 4.Hydroxy benzoic acid. تم اختبار تأثير الأجزاء المفصولة في حياتية خنفساء الطحين الحمراء *Tribolium castaneum* حيث أظهر الجزء F2 تأثير في كلا فترتي الدورين اليرقي والعذري حيث بلغ 17 و 7 على التوالي عند المعاملة بالتركيز 2%. أظهر الجزء F3 بتركيز 2% تأثيرا في الدور العذري حيث بلغت نسبة الموت 95% . تم تشخيص الحشرة باستخدام تقنية PCR وظهور حزمة أكبرمن 100 bp في المجموعة الضابطة السلبية، كمؤشر لتأثير المنتجات الطبيعية على كمية DNA فيها.

الكلمات المفتاحية: خنفساء الطحين الحمراء، نبات السعد *Cyperus rotundus*.