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Flaxseed Oil Micro Particles: It's Effect on Mulberry Silkworm, *Bombyx mori* L. Productivity and Antioxidant Activity

Rehab H. Taha[1] and O. H. Sadek[2]
[1] Plant Protection Research Institute, Agricultural Research Center, Egypt.
[2] Biochemistry Department, National Organization for Drug and Research, Egypt.

ABSTRACT

Increasing egg production is a necessary demand for silkworm rearing continuity in Egypt. Flaxseed plant has a compound that mimics estrogen hormone in vertebrates. Emulsions contain micro particles prepared from flaxseed oil were used in the present study as an exogenous source of phyto-estrogen. Micro particles size were measured by Zeta sizer and ranging from 1053 to 4087 nm with percentages 33.4 and 66.6 %, respectively. Fifth instar larvae were topically applied with flaxseed micro particles emulsion with three concentrations (0.25, 1 and 3 %) in addition to control every 48 hrs. The present study clarify that, 0.25% concentration have a tremendous effect on egg production and cocoon characters. As well as observations related to insect performance revealed maximum larval weight, effective rate of rearing (ERR), pupation percentage, pupal weight comparing with controls. On the other hand, deleterious effect was observed for concentrations (1 and 3%). Antioxidant activities were estimated by 2,2-diphenyl-1-picrylhryl free radical (DPPH*) scavenging activity reagent and the highest activity was recorded for 1 % treatment 36.947 %.

Keywords: *Bombyx mori* L, flaxseed oil, micro particles, phytoestrogen, insect performance, antioxidant activity, DPPH* scavenging activity

INTRODUCTION

Bombyx mori L. being monophagous insect and the quality of leaf consumed plays a significant role in cocoon yield and egg production. Recently in sericulture, the scientists are attempting to improve the silk production by using the nano/micro particles which may be exploited during the scarcity of leaf production (Pandiarajan *et al*., 2016). The scientific community was very much interested to study the physiological and biochemical impact of nano particles on silkworm *B. mori*. Nano materials posses particles with length scales ranged below 100 nm to 80,000 nm wide (Patil *et al*., 2016).

Phytoestrogens have potential protective effects against diseases and adverse conditions (Gilani and Anderson, 2002). Flaxseed contains high content of lignans, a type of phyto-estrogen or plant compound that can mimic estrogen hormone in vertebrates (Martinac, 2017). Flaxseed oil contain an essential fatty acid known as alpha-linolenic acid (ALA), which is one form of omega 3 fatty acids (Li, 2015). The other fatty acids, oleic acid and stearic acid are also constituted (Barre *et al*., 2009).

Analogs of vertebrate steroid hormones such as progesterone, testosterone and E2 have been recorded to exist in insect tissues (Bradbrook *et al*., 1990). The vertebrate estrogen hormone that develop female sexual characters have an analog in silkworm (Shen *et al*., 2015). The positive role of vertebrate hormones in silkworm biology, cocoon characters and larval growth were proved (Keshan and Ray, 2000 and Das 2016).

Free radicals/oxidants that known to cause various physiological disorders in living organisms (Singh and Singh, 2008), were combated by antioxidants that disturbing the free radical mediated oxidative processes (Cui *et al*., 2004). The balance between oxidants and antioxidants decides the organism vigour and health (Halliwell, 1996). 2,2-diphenyl-1-picrylhryl (DPPH) free radical scavenging reagent evaluate the antioxidant activity of a compound in a living organism (Kedare and Singh, 2011).

This study investigate the effect of emulsion contains flaxseed oil micro particles on larval weight (gm), effective rate of rearing, pupation percentage, single pupal weight (gm), cocoon weight (gm), cocoon shell weight (gm) and cocoon shell ratio. As well as fecundity and fertility per one female moth and the antioxidant activity of these micro particles in larval tissue for each treatment.

MATERIALS AND METHODS

Insect

Silkworm eggs of local hybrid were obtained from the Sericulture Research Department (SRD) of Plant Protection Research Institute (PPRI), Agricultural Research Center. Silkworms rearing was done at 26 ±2°C temperature , 80 ± 5 %relative humidity (Krishnaswamy , 1978). Local mulberry leaves (native variety) were fed to silkworms four times a day as recommended. After the fourth ecdysis, larvae were divided into five groups according to treatment doses (0.25 %, 1 % and 3 % of flaxseed micro particles) and two control groups {without treatment named negative control (-) and with liposome carrier named positive control positive (+)}. Four replicates with 50 larvae/ replicate were designed for each studied group. Emulsion contains flaxseed oil micro particles were topically applied upon dorsal side of larvae every 48 hours. The first treatment was after 24 hrs after the fourth moult, 2nd treatment was 72 hrs, 3rd treatment was 120 hrs and 4th treatment was 168 hrs. Insect performance in all treatments was assessed; single larval weight and effective rate of rearing (ERR). ERR was calculated according to (Chanu and Ibotombi, 2011). *ERR (%) = (no. of cocoon harvested / total no. of larvae reared) X 100*. As well as, single cocoon weight, shell weight and shell ratio according to (Ghosh, 1987). Total number of laid eggs per one female moth and number of fertile eggs were counted. The data were subjected to

statistical analysis system version 9.1 program proc. GLM (SAS Institute, 2003).

Preparation of flaxseed micro particles

Flaxseed oil from (Organo pharmaceutical company, Egypt), Chloroform (Scharlu Pharmaceutical Co. Spain), Carbopol 940 (Shaanxi Top Pharm Chemical Co., Ltd).

Method

On the basics of dissolution evaporation, in which oil could be decreased in size according to single or double emulsion process (McCall and Sirianni, 2013). But with some modification in which the oil is dissolved and the solvent evaporated to leave the oil particles with smaller size. Temperature increases the external pressure which decrease the oil particle. After that the oil phase is incorporated in aqueous phase to form emulsion, in addition a gelling agent was added to form emulsion gel (Shingel *et al*., 2008). This study was performed using liposomal composition composed of soya lecithin, cholesterol, flaxseed (10: 3:1), to form liposomal preparation. Liposomal preparation then incorporated with carbopol 940 to form liposomal gel applied to fifth larval instar.

30 mls of flaxseed oil were dissolved in 300 ml of chloroform, sonicated for 30 minutes, stirred for another 30 minutes (Khadka *et al*., 2014) then, the dissolved oil was put in rotary evaporator (*Xian Toption Instrument Co., China*), the chloroform part was completely evaporated. The oil stirring, sonication and evaporation ensure decrease in the oil particle size. The oil then incorporated in emulsion to form emulsion by stirring the oil in phosphate buffer (Na Cl, K Cl , Na_2HPO_4, KH_2PO_4) pH 6.5 for 30 minutes, triethanolamine was added as an emulsifying agent in a concentration of 2 % to add in formation of emulsion. After the emulsion was formed in a concentration of 0.25, 1, 3 % of flaxseed in buffer, carpobol 940 was added in a concentration of 2% to form emulsion gel or as a cream gel formula. The positive control was formed by the same previous method of preparation, instead the flaxseed oil was not incorporated in formula. The emulsion was compared with the oil microscopically to ensure the decrease in the particles size.

The particles were measured at the central lab, Regional Center for food and feed, Agricultural Research Center. Malvern, UK. Model: Zeta sizer nano series (Nano ZS).

Antioxidant activities of flaxseed oil micro particles using 2,2-Diphenyl-1-picrylhryl (DPPH*) free radical scavenging assay

The antioxidant activity for flaxseed oil micro particles were evaluated using the DPPH free radical (DPPH*) according to (MacDonald-Wicks *et al*., 2006; Moon and Shibamoto, 2009). 0.5 ml of homogenate of silkworm tissue was added to 1.5 ml of DPPH* reagent and incubated at 37̊ C in incubator for 30 minutes. The initial absorbance at 517 nm ($A_{initial}$) and the DPPH solution was measured with a spectrophotometer. The samples, then were read against air to find the decrease in the absorbances (A_{final}). Percent of DPPH* scavenging capacity was calculated; where $A_{initial}$ and A_{final} represent the absorbance of DPPH* spectrum without and with flaxseed oil micro particles at each concentration, respectively. The scavenging activity calculated as follow

$$\frac{\text{A Initial - A Final}}{\text{A Initial}} \times 100$$

RESULTS AND DISCUSSION

In the present study, Zeta sizer nano series (Nano ZS) was used to measure the particle size at 24.9 °C. Flaxseed particles size were ranged from 1053 and 4087 nm with density percentage 33.4 % and 66.6 %, respectively, as illustrated in Fig. (1).

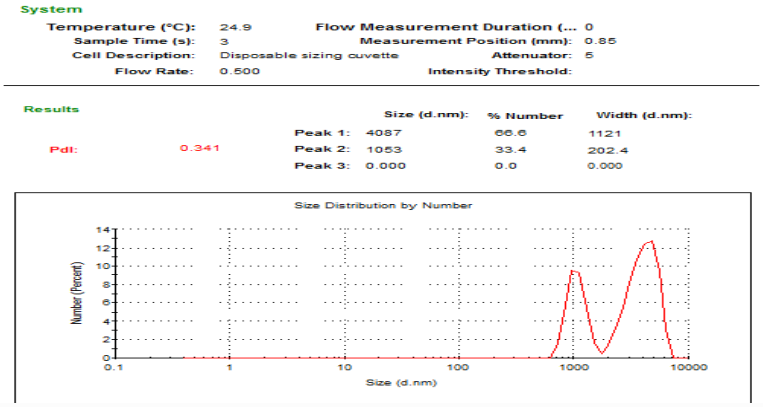

Fig. 1. Particle size distribution curve for flaxseed oil micro particles at 24.9°C using a Malvern Zeta Sizer Nano ZS

The size of synthesised particles played an important role in the mortality levels of treated *B. mori* larvae with biologically synthesised silver nano particles (Rajasekharreddy and Usha Rani, 2012). Otherwise it is not desired to reduce the particle size sharply as concluded by (Linkov *et al*., 2008). It was observed that, the size reduction of the nano particles (> 20 nm) may lead to an increase in particle surface area, which

in turn may permit more chemical metabolites to attach the surface and then the reactivity is enhanced leading to an increase in the toxic effects (Suh *et al*., 2009). Kato *et al*. (2012) proposed that nano particles size is important for particle target and effect.

Silkworm larvae are very sensitive to exogenous hormones, the effect is dependent on the larval age and hormone dose (Dai *et al*., 1985). Estrogen hormone can penetrate the larval cuticle effectively as suggested by Keshan and Ray (2001). The liposomal carrier was used in the present study because of its characteristic magnificent way to deliver the drug and to improve the bioavailability of drug (Mutalik *et al*., 2014).

Data in the present study showed that, single larval weight increased significantly in 0.25% emulsion treated group upon (2.289 gm) comparing with both negative and positive controls (1.940 and 2.198 gm, respectively). The same trend was achevied for effective rate of rearing, pupation ratio and single pupal weight (94 %, 96 % and 0.653 gm, respectively). While larval group treated with 3 % emulsion recorded lower values for the same parameters. Single larval weight was (1.63 gm), effective rate of rearing was 38 %, pupation ratio was 52 %, and single pupal weight was 0.475 gm. This is may be due to treatments with higher doses of micro particles, the particles were absorbed into most larval tissues and disturbed the metabolism and resulted in the death of the larvae as suggested by Rajasekharreddy and Usha Rani (2012). The toxicity of higher doses of ethanolic plant extracts of white Kwao-Krua (*Peuraria mirifica*) a phytoestrogen - rich plant on Thai multivoltine silkworm, *B. mori* was recorded in Chawna *et al*. (2007) studies. There were a reduction in larval, pupal weights, silk filament and egg lied per female moth at 5% and 10% w/v treatments. While the improved effect was recorded for cocoon characters at 1% w/v treatment. As well as, silkworms treated with high doses of *P. mirifica* showed slow move, no-appetite and diarrhea. In the present study, cocoon characters for 0.25 % treated group were also improved significantly, single cocoon weight, single cocoon shell weight and single cocoon shell ratio recorded (0.802 gm, 0.156 gm and 19.472 %, respectively) comparing with either negative control (0.684 gm, 0.112 gm and16.033 %, respectively) or positive control (0.745 gm, 0.126 gm and 16.995 %, respectively). Ohnishi *et al*. (1985) proposed that phytoestrogens modulate growth and reproduction by interacting with steroid hormone system and high doses may disturb this system leading to malformations and death. Keshan and Ray (1998) demonstrated the effect of topical application of E2 [0.05 to 4.0 µg/g body weight] on the first and second day of the fifth larval instar. Silkgland weight, fibroin content, DNA and RNA contents and glutamate–pyruvate transaminase activity in the posterior silkgland were affected depending on dose. Keshan and Ray (2000) supposed that, cocoon shell weight increment after estradiol treatment resulted from the activity of silkgland cells. Patil *et al*. (2016) stated that, 300 ppm dose is the most suitable small size of nano gold particles that directly stimulates receptor part of the posterior silkgland, resulting in more fibroin content in treated silkworms. Das and Ray (1996 and 1997) demonstrated the efficiency of estradiol-17β on the activity of steroidogenic and lipogenic enzymes in silkworms ovary and fat bodies. As well as increased amount of vitellogenin-like protein in the haemolymph and ovary was recorded. Estradiol-17β may act in a nuclear-mediated way (Keshan, 1999). Das and Ray (2014) suggested the involvement of exogenous estradiol injection in the synthesis vitellogenin in *B. mori* (BmVg). BmVg is the precursor of the vitellin (BmVn), the major egg-yolk protein, responsible for female characters (Xiang *et al*., 2005).

The obtained results revealed that treatment with 0.25% showed highest number of laid eggs per female moth (505 eggs) followed by control positive group (470 eggs). Fertilized eggs per female moth was significantly improved in 0.25 % treated group significantly, comparing with the other treated groups (479 eggs). Shen *et al*. (2015) found that exogenous E2 increased BmVn in the female moth eggs because it regulates BmVg expression in fat bodies.

Table 1. Biological and economical characters of *B. mori* upon the effect of flaxseed micro particles emulsions 0.25, 1% and 3 % (mean±SD).

parameters	Control (-)	Control (+)	0.25 %	1 %	3 %	$LSD^{1\%}$
Larval wt (gm)	1.940±0.06 ^{ab}	2.198±0.40 ^{a}	2.289±0.15 ^{a}	1.857±0.16 ^{b}	1.630±0.07 ^{b}	0.566
ERR	80±12 ^{c}	88±10 ^{b}	94±12 ^{a}	76±11 ^{d}	38± 19 ^{e}	2.612
Pupation %	84±14 ^{c}	92±11 ^{b}	96±13 ^{a}	78±16 ^{d}	52±17 ^{e}	2.687
Cocoon wt (gm)	0.684±0.02 ^{b}	0.745±0.02 ^{ab}	0.802±0.01 ^{a}	0.735±0.01 ^{ab}	0.570±0.06 ^{c}	0.090
Shell wt (gm)	0.112±0.003 ^{b}	0.126±0.005 ^{b}	0.156±0.016 ^{a}	0.121±0.007 ^{b}	0.071±0.007 ^{c}	0.024
Shell %	16.033±0.59 ^{b}	16.995±0.39 ^{ab}	19.472±1.93 ^{a}	16.552±0.96 ^{ab}	12.633±1.03 ^{c}	3.001
Pupal wt (gm)	0.560±0.02 ^{b}	0.604±0.03 ^{ab}	0.653±0.02 ^{a}	0.633±0.02 ^{a}	0.475±0.04 ^{c}	0.073
Fecundity/female	417±29 ^{b}	470±13 ^{ab}	505±17 ^{a}	422±44 ^{b}	318±20 ^{c}	83
Fertility/female	298±35 ^{b}	388±37 ^{ab}	479±35 ^{a}	382±40 ^{ab}	304±20 ^{b}	148

The same small letters in a raw means there is no significance

In the present study, DPPH* is a free radical reagent and UV spectrum is used to estimate the antioxidative efficiency of micro particles prepared with different concentrations. Antioxidant compound commonly stabilizes the free radicals DPPH* through proton– electron transfer pathway, thus, the DPPH* can be stabilized and then forms non-radical species of DPPH, which is undetectable in UV spectrum (Moon and Shibamoto, 2009). Fig. (2) illustrate the change of the oxidant DPPH* reagent in different flaxseed oil

micro particles concentrations. This implied that DPPH* radicals were scavenged and decreased with increasing flaxseed emulsion concentrations. Control (+) and 0.25 % samples increase the scavenging activity to the free radicals significantly comparing to control (-) and recorded (27.108, 27.308 and 14.457 %, respectively). While; 1 % sample recorded the highest activity among all tested samples (36.947%). The increase in scavenger activity in 1% treatment may disturb the physiological state and metabolism inside insect body that resulted in larval death. For 3 % sample the activity suddenly decreased significantly to (6.626 %). The results are supported by Wongkrongsak *et al*. (2016) findings as they concluded that, by increasing water soluble silk fibroin (WSSF) nano particles concentration, the UV absorbance of DPPH* reduced.

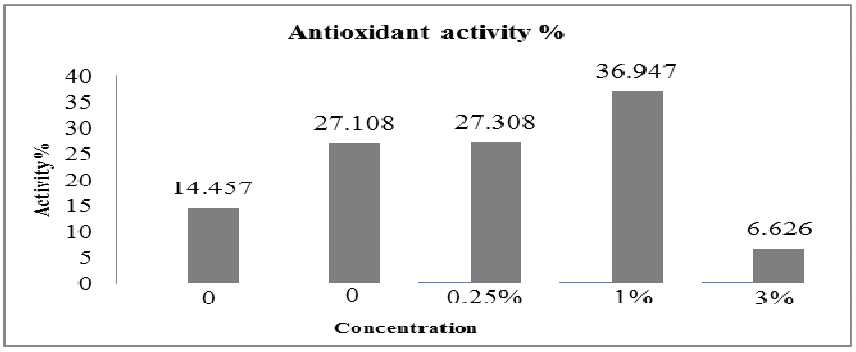

Fig. 2. Antioxidant activity % for *B. mori* larval tissues upon treatment with flaxseed micro particles emulsions (0.25, 1 and 3 % concentrations).

It may be concluded from this study that, applying flaxseed oil micro particles emulsions with low doses to *B. mori* fifth larval instar improve insect performance, cocoon characters and egg production.

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تأثير جسيمات الميكرو المصنعه من زيت بذر الكتان على إنتاجية يرقات الحرير التوتيه (*Bombyx mori* L.) ونشاطها المضاد للأكسده

رحاب حسني طه [١] و أسامه حسن صادق [٢]

[١] معهد بحوث وقاية النباتات – مركز البحوث الزراعيه – مصر .

[٢] قسم الكيمياء الحيوية – الهيئة القومية للرقابة و البحوث الدوائية – مصر .

زيادة إنتاج البيض هو أحد المتطلبات الضرورية لإستمرار تربية يرقات الحرير التوتية في مصر . يحتوي نبات الكتان على مركب يحاكي هرمون الإستروجين في الفقاريات . في هذه الدراسه تم تحضير مستحلب يحتوي على جزئيات ميكرو مصنعة من زيت بذر الكتان بثلاث تركيزات (0.25, 1 , 3 %) كمصدر نباتي لهرمون الإستروجين . تم قياس حجم الجزيئات وتراوحت من ١٠٥٣ إلى ٤٠٨٧ نانومتر بنسب ٣٣,٤ و ٦٦,٦ ٪ على التوالي . و تم تطبيق هذا المستحلب علي يرقات الطور الخامس للحشره كل ٤٨ ساعه . وتوضح الدراسة الحالية أن تركيز ٠,٢٥ ٪ له تأثير إيجابي على زيادة إنتاجية البيض ومواصفات الشرنقة. بالإضافة إلى ذلك سجلت هذه المعامله أعلى وزن للبرقات و أعلى معدل لكفاءة التربيه و أعلى نسبة تعذير وكذلك وزن العذراء مقارنة بالكنترول . و من إتجاه أخر لوحظ التأثير الضار للتركيزات (١ و ٣ %) على اليرقات و كل القياسات تحت الدراسه. كذلك تم دراسة نشاط هذه التركيزات كمضادات للاكسده بإستخدام المركب 2,2-diphenyl-1-picrylhryl free radical (DPPH*) و أعلى نشاط تم تسجيله وجد في عينات يرقات المجموعة المعامله ب تركيز ١ % و كانت ٣٦,٩٤٧ ٪ .