

Efficiency of Green Synthesized Silver Nanoparticles with Sweet Orange, *Citrus sinensis* (L.) (Rutaceae, Sapindales) against *Tribolium confusum* Duval. (Coleoptera, Tenebrionidae)

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ABSTRACT

The post-harvest damage caused by several pests is a major problem in stored grain product industry. Recently, synthesizing NanoParticles (NPs) with plant extracts act as an alternative approach in reduction of this damage. An attempt was made to synthesize and test the mortality effect of green synthesized silver NanoParticles (AgNPs) with peels extract of sweet orange, *Citrus sinensis* (L.) (Rutaceae, Sapindales), against the adult tenebrionid beetle *Tribolium confusum* (Duval). The synthesized nanoparticles were characterized with UV-visible spectroscopy, X-ray Diffraction, and Transmission Electron Microscopy. Five bioassays were conducted by filter-paper residue and feeding methods with different concentrations of both green synthesizing and commercial AgNPs. Results showed that citrus extract by itself was not toxic against beetles, however, the green AgNPs showed 83-77% mortality in filter-paper residue and feeding methods. The impact of commercial AgNPs in both methods led to 75 and 40% mortality. The lowest *LC50* and *LT50* for green synthesized AgNPs were recorded (30.62 ppm and 9.92 day) for filter-paper residue tests. According to the results, the green silver nanoparticles, showed more efficiency than the commercial ones. This provides an evidence that green synthesized AgNPs have a great potential as an alternative option in stored products pest management.

Keywords: Filter-paper residue test, Stored grain pests, Tenebrionid beetle.

INTRODUCTION

Some insect pests cause heavy economic losses to stored grains throughout the world (Boxall *et al.*, 2002), with a damage rate of about 5-10% in temperate regions and 20-30% in tropical countries (Rajashekar *et al.*, 2010). Safety of food grains is one of the most important challenges confronting the

grain handling agencies and stored product entomologists worldwide (Lingampally *et al.*, 2013). Storage and upkeep of agricultural products are among critical important post-harvest activities (Abduz Zahir *et al.*, 2012). *Tribolium confusum* is one of the most serious cosmopolitan pests in stored grains and related products (Ranjashekar *et al.*, 2010). The confused flour beetle, *T. confusum* attacks stored grain

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products such as flour, cereals, meal, crackers, beans, spices, pasta, cake mix, chocolate, nuts, seeds and even dried museum specimens at home and grocery stores (Baldwin and Fasulo, 2017). This originally African beetle has a different distribution around the world, and mainly prefers cooler climates (Smith and Whitman, 1992). Several synthetic pesticides are applied in the form of contact insecticides and fumigants in management of these stored products (Devik and Devi, 2013). Obviously, application of pesticides, besides their positive role in controlling the pests, makes a lot of problems in terms of non-target organisms and environment pollution. To avoid the side effects of these pesticides, during the last decade, many efforts including using new technologies have been applied. Among the modern technologies, the nanotechnology has shown outstanding characteristics in terms of pesticide formulation and its application leading to use lesser amounts and more effective roles in pest management programs with marginal effects on the environment. The potential benefits of nanotechnology in agriculture have created a great interest in applying nanofertilizers, nanopesticides, nanosensors, and smart delivery systems for controlled release of agrochemicals (Mishra et al., 2017; Lade et al., 2017). Synthesizing NanoParticle pesticides (NPs) based on plants and microorganisms ingredients can be one of such techniques administered. Application of solid nanoparticles against rice weevil, *Sitophilus oryzae* led to killing nearly 70% of the insects (Pandey et al., 2012). An investigation on the insecticidal effect of silica and silver nanoparticles on the cowpea weevil *Callosobruchus maculatus* showed that both silica and silver NPs were highly effective on adults and larvae with 100 and 83% mortality, respectively (Rouhani et al., 2012a). A blend of these two NPs with two insecticides, cypermethrin and alpha Terhienyl and NPs zinc oxides and copper oxides effectively controlled the pest mite, *Epitrimerus pyri* (Wan and Zhang-Nian, 2005). Nowadays,

synthesizing NPs using plants is a novel and green approach to make ecofriendly pesticides. These products have also been studied for acute toxicity, antifeedant, or repellent, attractant, and fumigant effects, as well as inhibiting reproduction of many pest species (Mohammed, 2013). In addition, some plant wastes are a major source of bioactive compounds such as flavonoid compounds in citrus peels (Rahneem et al., 2018). NPs loaded with garlic essential oil worked effectively against *T. castaneum* (Yang et al., 2009). The synthesis of silver NPs using the peel extract and powder of naval *Cinnamon zeylanicum* has been reported (Sathishkumar et al., 2009). Soni and Prakash (2014) applied silver and gold NPs, synthesized based on a green method against malaria vector, *Anopheles stephensi* and filariasis vector, *Culex quinquefasciatus*. Their results have shown that *A. stephensi* was more susceptible than *C. quinquefasciatus*. Considering the importance of this pest, its short life span, and easy access, it is a good candidate for experiments on the effect of nanopesticides.

In this study, the mortality effects of the green nano silver synthesized from peel of *Citrus sinensis* on *T. confusum* have been investigated using two bioassay methods. Moreover, these biologically synthesized nanoparticles and aqueous extracts of *C. sinensis* have great potential to be used as an alternative pesticide.

MATERIALS AND METHODS

The effect of silver NPs against confused flour beetle, *T. confusum* Duval (Coleoptera, Tenebrionidae) was investigated under laboratory conditions.

Insect Rearing

Equal number of male and female adults of *T. Confusum*, obtained from the insectarium of the Entomology laboratory of the Islamic Azad University, were reared on wheat flour

in a container with a temperature of $27\pm 1^\circ\text{C}$ and $60\pm 5\%$ relative humidity, with wheat flour and yeast (10:1, w/w) as a regarding medium for a period of two months.

Synthesis of Green Silver Nanoparticle

Green synthesis of silver NanoParticles (AgNPs) was carried out using extract of *Citrus sinensis* (sweet orange) peels. 200 mL of deionized water was added to peel (40 mg) extracts to make a peel broth (Soni and Prakash, 2014). The mixture was boiled at 80°F , for 30 minutes. Then, the broth was filtered using whatman-1 filter paper. The aqueous extract of the peel was treated with 0.001M AgNO_3 with a ratio of 80:20 mL AgNO_3 to the aqueous peel. The solution was exposed to the sun for five minutes (Samadi, 2014; Lade and Patil, 2017). During bio-reduction of silver nitrate with peels' extract and stabilization of the synthesized AgNPs, a visible color change from faint yellow to brown was observed.

Nano Silver Preparation

A synthetic AgNPs solution in colloidal suspension form was also obtained from Nanosav (www.Nanosave.com). The size of the spherical AgNPs was between 15 to 20 nm.

Characterization of the Green Silver NPs

The size of the green synthesized AgNPs, was measured by TEM. The characterization

of the NPs was confirmed by scanning the reaction mixture using UV-Vis spectra at 350-550 nm. Additionally, 2 mg of chilled treated insects was mixed with 2 mL of hydrochloric acid (HCl) and 5 mL of HNO_3 (Nitric acid). Then, the mixture was put into a microwave for 15 minutes at 200°C and another 10 minutes at 200°C for digestion (Smith and Whitman, 1992; Park, 1934) to determine the absorbance value of the NPs using atomic absorption spectrophotometry. Also, an aqueous extraction containing AgNPs was applied for X-ray diffraction.

Bioassays

Under laboratory conditions ($30\pm 1^\circ\text{C}$, $75\pm 5\%$ RH), the toxicity of both the green synthesized and the commercial samples of AgNPs was tested through five bioassays (Table 1). As Table 1 shows, the tests were carried out using two methods including the filter paper residue test and oral feeding of adult confused flour beetles under a completely randomize design, with three replicates for each treatment. In the latter method, the filter papers were coated with five- milliliter solution of the AgNPs or citrus extraction. The solution was allowed to dry in room temperature for a period of 30 minutes. Then, the treated filter paper was put in inner side of petri dishes (9 cm diameter). Adult beetles were then released on the treated filter papers. In feeding method, the seed grains were soaked in AgNPs and allowed to evaporate at room temperature. The control was treated with distilled water.

Table 1. Five bioassay tests, treatment solutions, and bioassay methods.

Treatment solution	Bioassay method
Peal <i>Citrus</i> extraction	Filter paper residue test
Green AgNPs synthesized by peal <i>Citrus</i> extraction	Filter paper residue test
Green AgNPs synthesized by peal <i>Citrus</i> extraction	Feeding method
Commercial AgNPs	Filter paper residue test
Commercial AgNPs	Feeding method



Citrus Peels Extraction Bioassay

Fifteen adults of *T. confusum* reared under laboratory conditions were exposed to filter papers impregnated with five different concentrations of sweet citrus peel aqueous extracts (4,000, 2,000, 1,000, 500, 250, 125 ppm). The adult mortality was checked in 2-day intervals for a period of 20 days according to the method of Debnath *et al.* (2010).

Green AgNPs Bioassays Filter Paper Residue Test and Feeding Method

Five concentrations (169, 100, 50, 25, and 12.5 ppm) of the green synthesized AgNPs were tested for their mortality on 15 adults of the red flour beetle through filter paper residue test and feeding method. For the oral feeding bioassay, 5 mL of different concentrations of green synthesized AgNPs were mixed with 0.5 g of wheat grains. The dead adults were counted in 2-day intervals for a period of 14 days.

Commercial AgNPs Bioassays through Filter Paper Residue Test and Feeding Method

In these two bioassay tests, the adults were

treated with five synthetic AgNPs concentrations (500, 250, 169, 100, 50, and 12.5 ppm) using the filter paper residue test and the oral feeding method. For the two bioassay methods, 5 mL of different concentrations were mixed with 0.5 g wheat grains. The mortality of dead adults was checked in 2-day intervals for a period of 20 days.

Statistical Analysis

The two Lethal Concentrations (LCs 50 and 90) were estimated using Polo Plus (version 2.0). The mortality correction was carried out using Abbott's formula. The percentage mortality was calculated by dividing the number of dead adults to the number of adults introduced (Elemike *et al.*, 2017).

RESULTS

Characterization on the Synthesized Green AgNPs

The monitoring of AgNPs formation with absorbance in the range of 350-500 nm was carried out using UV-VIS spectrophotometer (Figure 1). The sweet

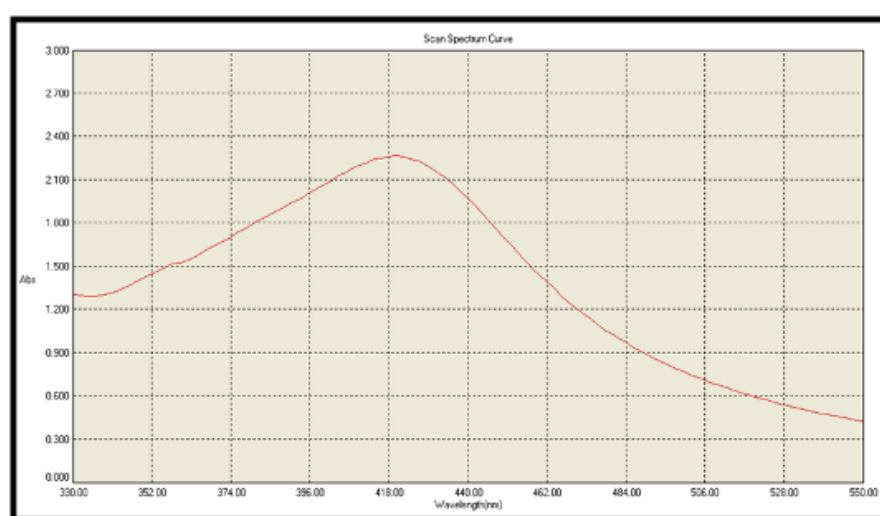


Figure 1. UV-VIS absorption spectra of AgNPs synthesized by *C. sinensis* peel extract.

citrus peel extract with AgNPs showed change in color from light yellow to brown implying the formation of the NPs.

Transmission Electron Microscopy (TEM)

The determination of the size, shape and morphology of the green AgNPs was carried out using a transmission electron microscopy (Figure 2). The TEM micrograph shows the size of spherical nanoparticles as 50 nm. The nanoparticles dispersion pattern indicates stabilization by capping agents.

X-Ray Diffraction (XRD)

The XRD pattern of AgNPs produced by sweet orange peel extracts is presented in Figure 3. The range being 5-90. It exhibits a broad peak at 38. The pattern also shows peaks at 2θ of 38.26° , 44.47° , 64.71° , 77.7° , and 81.90° which are attributed to (111),

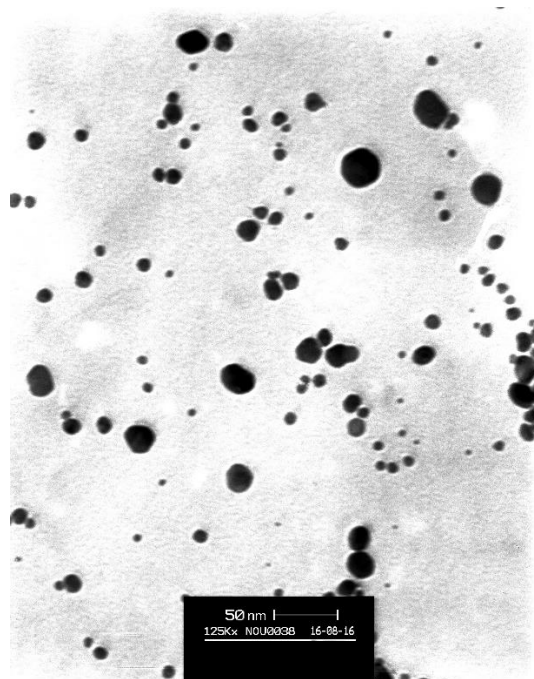


Figure 2. Transmission electron microscopy image of the green AgNPs synthesized with *C. sinensis* peel extract.

(200), (220), (311), and (222) planes of Ag₂O nanocrystals (JCPDS No.006-5378). A more intense and predominant orientation was that of (111). The broadening of the peaks indicates which particle is in nano regime. Furthermore, the peaks are in standard model showing the presence of silver in aqueous extracts. The atomic absorption revealed sufficient accumulation of 3.75 mg L^{-1} consumed by green silver nanoparticles in adults.

Bioassays Results

Adult mortality test with sweet orange peel extraction using filter paper residue bioassay showed no toxicity effects on adult beetles after two weeks exposure, and all beetles remained alive.

The results of the bioassay of adult beetles with green synthesized AgNPs are presented in Table 1. Complete mortality of adults was not observed in none of the 2-day intervals. The *LC*₅₀ values of synthesized AgNPs were 64.16 and 30.62 ppm for the oral administration and the filter paper residue tests, respectively. The *LT*₅₀ values for the tests were 10.65 and 9.92 days, respectively. The mortality rate of adult red flour beetles was 83% for the filter paper residue test, while for the feeding test the value was 77% at their highest dosage of green silver nanoparticles.

The results showed the highest *LC*₅₀ (1,087.9 ppm) of adults at 18.58 day in the feeding method, while the value in the filter paper residue test was 72.13 ppm at 16.45 day (Table 2). The mortality percentage at the highest dose for the feeding and filter paper residue tests were 40 and 75%, respectively. The variance analysis of different bioassays are shown in Table 3.

DISCUSSION

This study focused on the impact of AgNPs on some structural and biological aspects of Flour Confused Beetles (CFB), *T. confusum*,

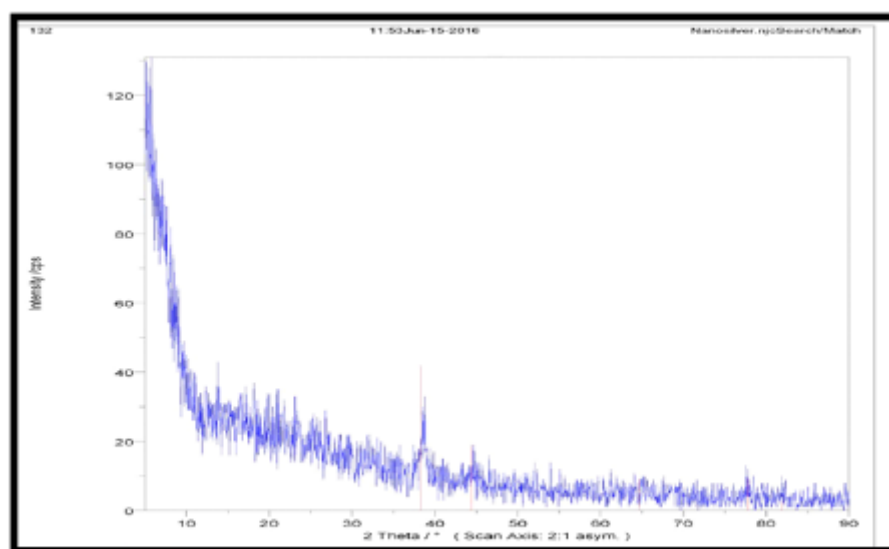
**Table 2.** LC50, LC90, LT50 and other statistical analysis of aqueous peel extract of *C. Sinensis*, and synthesized Ag NPs against the adults of *T. Confusum*.^a

Nanoparticles bioassay	LC50 (PPM)	Limits 90 (PPM)	LC90 (PPM)	Limits 90 (PPM)	Slope (±SE)	χ^2	LT50
Filter paper residue (Green AgNPs)	64.16	(44.6 - 93.5)	627.56	(303.12 - 3235.9)	1.3±0.31	2.3	10.65
Feeding method (Green AgNPs)	30.62	(15.51 - 46.94)	276.03	(158.74 - 821.8)	1.2±0.31	1.1	9.92
Filter paper residue (AgNPs)	1087.9	(462.8 - 13211.7)	73.03	(7872.3 - 9986)	0.4±0.16	0.3	18.58
Feeding method (AgNPs)	72.13	(21.95-168.738)	26760	(3436 – 27120)	0.7±0.26	4.5	16.45

^a LC50: Lethal Concentration that kills 50% of the exposed adults, LC90: Lethal Concentration that kills 90% of the exposed adults, χ^2 Chi-square.

Table 3. Variance analysis of mortality percentage of different bioassay of *T. confusum*.

Mean square		12.5 ppm	25 ppm	50 ppm	100 ppm	169 ppm
Source	DF					
Nanosilver	3	190.44	144.75	144.63	189.41	344.00
Error	8	1033	19.25	10.75	15.08	53.83
F		142.8	7.52	13.92	12.56	6.39
Pr> F		<0.001	0.01	0.001	0.001	0.01
% CV		7.69	25.43	15.67	20.00	17.08

**Figure 3.** XRD Pattern of green Ag nanoparticles synthesized by *C. sinensis* peel extract.

including its mortality. The filter paper residue and the orally feeding applications of AgNPs on *T. confusum* showed mortality rates up to 83% according to the bioassay tests. In the first bioassay using the extract of *Citrus sinensis* peels on the adults of CFB, no mortality was observed in all three replicates during the 2-day intervals. Zewde and Bekelle (2012) obtained the same results while investigating the impact of AgNPs against the Mexican bean weevil, *Zabrotes*

subfasciatus. Adding the essential oil of this citrus species to peel extracts showed degrees of mortality after 24 hours, which could be related to the presence of D-limonene in essential oil of citrus peels (Sharaby, 1988). In a bioassay tests on the mortality of the carpenter ants (*Camponotus nearcticus*) with the volatile oils of sweet orange (*Citrus sinensis*), lime (*Citrus aurantifolia*) and lemon (*Citrus Limon*) carried out by Adusei-Mensah et al. (2014),

the result showed that the former was less effective in terms of repellency and lethal activity with an LC_{50} of 17.02%. In the second bioassay, the adults of *T. confusum* were exposed to the filter papers impregnated with different concentrations of AgNPs, synthesized by citrus peel extracts. The adults were also fed orally with the AgNPs in the third bioassay. The results confirmed 83 and 77% mortality in the filter-paper residue test and the feeding method, respectively. Abdu Zahir *et al.* (2012) studied the efficiency of aqueous leaves extracts of *Euphorbia prostrata*, as a green material against adults of *Sitophilus oryzae* L. Their results suggested the synthesized AgNPs obtained from the leaves extracts as ideal eco-friendly controlling method of *S. oryzae*. The effectiveness of silver nanoparticles synthesized with capping the leaf extracts of different plants including citrus was studied on *Drosophila melanogaster* (Araj *et al.*, 2015). Results showed that the silver NanoParticles (AgNPs) had a high mortality impact on larvae, pupae, and adults besides their role as egg deterrence. Moreover, a combination of green AgNPs and the extracts of *Cinnamomum zeylanicum* (Chowdappa and Shivakumar, 2013) and *Ficus racemosa* (Velayutham *et al.*, 2013) against mosquito larva, *Anopheles stephensi* and *Culex quinquefasciatus* made 100% mortality on these two medically important insect pests. This obviously implies that the combination of AgNPs with plants' extracts are more effective than their mere application. This is in congruent with the work of Elemike *et al.* (2017) in which the silver nanoparticles combined with extracts of *C. sinensis* peels were found very toxic against third and fourth instars of *Culex quinquefasciatus* larvae, with LC_{50} values of 148.5 and 95 ppm, respectively. The impact of different concentrations of synthesized AgNPs without the peel extract of citrus species led to 75 and 40% mortalities, respectively in both filter paper residues and feeding applications, during the 4th and 5th bioassays. The effectiveness of AgNPs on larval stages

and adults of *Callosobruchus maculatus* feeding on cowpea seed in the contact bioassay method showed an LC_{50} value of 2.06 g kg⁻¹ cowpea on adults and 1.00 g kg⁻¹ cowpeas on larvae, respectively (Rouhani *et al.*, 2012a). In addition, the insecticide NPs was calculated as 424 mg mL⁻¹ (Rouhani *et al.*, 2012b). The same result was obtained while using this nanoparticle on the two-spotted spider mite *Tetranychus urticae* through a leaf dipping method (Jalalizand *et al.*, 2013). Commercial and photosynthesized AgNPs were applied on red flour beetle adult stages by contact and feeding methods. It is well clear from the results of the present study that the commercial and photosynthesized AgNPs have entomotoxic potential against red flour beetle adult stages, but they were more effective in contact method than feeding method. This may be due to the ability of nano-particles to penetrate into insect cuticle in the contact method faster than entering the digestive system in feeding method. Previous investigation revealed the intake of nanoparticles by attachment to the insect cuticle due to physical phenomena and the resulting dehydration in insects (Stadler *et al.*, 2017). Silver nanoparticles could penetrate larval membrane and cause death (Sap-Iam *et al.*, 2010).

In conclusion, the *T. confusum* is a key pest in stored grain products. The impact of Ag NPs on the mortality rate of these pest, especially in combination with peel citrus extracts, led to its significant control. This study could lead to open an alternative and ecofriendly approach to control stored grain pests based on nanotechnology.

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اثر نانو ذرات نقره گیاهی ساخته شده با گیاه پرتقال (*Citrus sinensis* (L.)
Tribolium confusum Duval. علیه سوسک (Rutaceae, Sapindales)
(Coleoptera, Tenebrionidae)

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چکیده

خسارت وارده به مرحله پس از برداشت توسط بسیاری از آفات یکی از اساسی ترین مشکلات در صنعت انبارداری محصولات دانه ای انباری است. اخیرا ساخت و تولید نانو ذرات توسط عصاره گیاهی بعنوان یک روش جایگزین در کاهش این گروه از خسارت ها وارد شده مورد توجه قرار گرفته است. در این مطالعه تلاش بر این بود که علاوه بر تولید نانو ذرات، میزان مرگ و میر حاصل از نانو ذرات نقره گیاهی ساخته شده از عصاره پرتقال، بر علیه سوسکهای بالغ *Tribolium confusum* تعیین گردد. نانو ذرات تولید شده با اسپکتروفوتومتری، پراش اشعه X و میکروسکوپ الکترونی گزاره مورد ارزیابی قرار گرفت. پنج زیست سنجی با دو روش آغشته سازی با کاغذ صافی و خوراکی با غلظت های مختلف دو محلول نانو نقره تجارتي و گیاهی صورت گرفت. نتایج نشان داد که عصاره استخراج شده از گیاه پرتقال به تنهایی برای حشرات بالغ سمی نبوده است. در حالیکه نانو ذرات نقره گیاهی (AgNPs) ۷۷-۸۳ درصد مرگ و میر در دو روش آغشته سازی و خوراکی از خود نشان داد. اثر نانو ذرات نقره تجاری با دو روش به ترتیب ۷۵ درصد در آغشته سازی و ۴۰ درصد به روش خوراکی مرگ و میر از خود نشان داد. کمترین LC50 و LT50 برای زیست سنجی با نانو ذرات نقره گیاهی (۳۰/۶۲ppm و ۹/۹۲ روز) با روش آغشته سازی کاغذ صافی گزارش شد. بر اساس نتایج حاصله، نانو ذرات نقره گیاهی بیشترین تاثیر را در حشره کشی بر *T. confusum* در مقایسه با نوع تجاری از خود نشان داد. نانو ذرات نقره گیاهی دارای اثرات بالقوه ای بعنوان یک عامل جایگزین در مدیریت آفات انباری دارد.