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Effects of silver nanoparticles (AgNPs) and polymers on stored pests for improving the industry of packaging foodstuffs

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Abstract
The customers`s complaints of the contaminations of foodstuffs to stored pests such as insects, mold and toxic fungi polluted packagings after a short time of storing, were reasons to perform this research. Cereals are one of the most important stored products. In this study, we conducted batch experiments to assess the efficiency of silver nanoparticles incorporated into packaging polymer on the growth of fungi and insect pests. *Sitotroga cerealella* Ol. (Lep.: Gelechiidae) is tested because of economical importance of this pest in storing industry of packaged cereals. We applied AgNPs through the best packaging polymer (PP). The results showed that not only AgNPs could optimally omit toxic pathogens also but have a toxicant effect on insects. Thus, the property of antimicrobial and toxicity of AgNPs as a non-chemical pesticide investigated in the terms of storing cereals foods.

Keywords: Polymer, *Aspergilus flavus*, Penetration, AgNPs, Packaging, patho

Introduction
Storing of agricultural products and their infestation by stored-product pests poses an outstanding problem after harvesting. Storage in bulk or sacks is a usual method to control stored-product pests. These sacks are made of different materials such as sheeted polymers used for packaging agricultural products to prevent the entrance of pests and contamination [2]. Cereals are susceptible to mycotoxin [15]. There are few categories of mycotoxins regarding their chemical structure, sensitivity of certain organs and origin of fungi that produce them. Aflatoxin is a secondary metabolite produced by *Aspergillus flavus* [20]. Aflatoxin is potential to cause liver damage, cirrhosis, and liver cancer and aflatoxin B1 is the most dangerous toxin for animal and human health [33]. Cereals are the substrates mostly exposed to *Aspergillus flavus* attack and micromycete produced by them (Tabuc, *et al*., 2010). Moreover, *Sitotroga cerealella* Ol. (Lep.: Gelechidae) is one of the most important economical pest insects on agriculture products stored by feeding of cereals. This moth lay its eggs in anywhere, but at the time that it is on food containers, the larvae struggle to find any way to penetrate the foods after hatching the eggs. Some packaging polymers may offer virtually no resistance to insects that foodstuffs into the packages made of these polymers are infection source and a site for entering pathogen agents such as the microorganisms pointed in above [12]. *Aspergillus flavus* is an example of microorganisms that often contaminate stored foods [7]. The use of chemical control is very expensive and creates many dangers for living beings and environment. Hence, the probability of food poisoning and environmental pollutions rise. Therefore, access to the compounds which have the least risk, is very important in control of pests. The development of nanodevices and nanomaterials could open up novel applications in agriculture [28]. Nanophasic and nanostructured materials are attracting a great deal of attention because of their potential for achieving specific processes and selectivity, especially in biological and pharmaceutical applications (Pal, *et al*., 2007). Colloidal silver is of particular interest because of distinctive properties, such as good conductivity, chemical stability, catalytic and antibacterial activity [9]. In addition, Microbes are unlikely to develop resistance against silver, as they do against conventional and narrow-target antibiotics, because the metal attacks a broad range of targets in the organisms, which means that they would have to develop a host of mutations simultaneously to protect themselves (Pal, *et al*., 2007).
Surfactants and polymers modified AgNPs have advantages in antibacterial activities; however, their antibacterial actions are not fully understood [8]. Recent studies have demonstrated that specially formulated metal oxide nanoparticles have good antibacterial

activity [32] and antimicrobial formulations comprising nanoparticles could be effective bactericidal materials [10, 11]. The antibacterial and antiviral actions of silver, silver ion, and silver compounds have been thoroughly investigated [21, 22, 37].

In this research, silver nanaoparticles incorporated into polymer of packaging carried out for the first time to study the modified AgNPs incorporated to packaging polymer for investigating growth of *A. flavus* (producer aflatoxine B1) in packaged foodstuffs and showed that the aflatoxin will be optimally absorbed by silver nanoparticles. Information of permeability of polymers to pests and the effects of silver nanoparticles on pathogenic fungi and infestation of insects is necessary. Our study was performed to prove how polymer incorporated with silver nanoparticles could be effective in foodstuffs safety.

Materials and Methods

Penetration test

In this study, we compared permeability of four kinds of transparent and flexible polymers against stored-insect pests that these are the same current polymers for foodstuffs packaging, including Polyethylene (PE), Polypropylene (PP), Polyvinylchloride (PVC) and Cellophane. These polymers were prepared in thickness of 29 µm [2]. These packages were flexible and completely without any pores. The tested insects (obtained from laboratory cultures) including 1st and 5th instar larvae of *S. crealella* [2]. These insects were collected from the field in one store of cereals in Sanandaj, Kurdestan, Iran on May 2011. We tested the insects in the conditions of without food on packaging polymers. The prepared packages were without any pores and each one of them with one thickness placed in a ca. 500 cc container vertically. Wheat used in the study originated from farmers in Sanandaj.

We applied 20 insects (larvae) in around of the packages for examination each polymer [2]. Each container was capped with a filter fine lace-mesh lid placed at the conditions of 25±1 °C, 75±5% RH and a 12L: 12D h cycle. More of the larvae penetrated in from < 24 h. Each hole on packages created by the insects around the packaging polymers and counted as penetration. When number insects reached maximum and no penetration accomplished later counting stopped. The most number of penetrated insects showed permeability percentage of used polymers. By increasing the insects into packages and stopping counting, we searched *A. flavus* produced aflatoxine into the cereal packages by chromatography (HPLC). Whatever a polymer had more resistance to pest penetration, the packaging had less contamination to pests and fungi. Hence, the percent of product`s pollution determined. Then in next step, the best polymer incorporated various concentrations of silver nanoparticles and examined to determine the toxicity percent on mycelium growth of tested fungus. Then, we studied the impressions of AgNPs on *S. cerealella* larvae polluted packaged cereals. In these tests, each polymer, each concentration of nanoparticles and each concentration of *A. flavus* was as one treatment. In addition, each treatment replicated three times. In these tests all, there was a control test and the nanoparticles size was 7nm. Statistical analysis of data carried out by MSTATC, SPPS16 and EXCEL software and the means compared by Duncan's mean test.

Preparation of different concentrations of *A. flavus* and AgNPs

There were many processes to synthesis silver nanoparticles e.g. electrochemical reduction, and (or) irradiation, etc [3, 4, 25, 27, 33, 38]. As we had the silver nanoparticle prepared of colloidal silver sulfide. For tests, we had different concentrations of nanosilver including: 550, 800, 1020 and 1300 ppm. Thus, the different concentrations of A. flavus were 0.1, 0.01, 0.001 and 0.0001 wt.

Using AgNPs incorporated into polymer

The effects of AgNPs on *A. flavus* in petri dish tests

We had packages made by the best polymer for packaging e. g. that had the least penetration to pests. Hence, Polypropylene mixed by the different concentrations of AgNPs in melting process as granule before extrusion. Also, mycelium pieces of *A. flavus* were separated in the diameter of 3 mm and placed in petri dishes which contained the different concentrations of AgNPs. We were cutting fine strings of packaging polymer (3mm) and longitudinally placed it in middle of agar into some petri dishes with different concentrations of fungus. This polymer was with silver nanoparticles. Also, we had a control test e.g. polymer without silver nanoparticles. Then, the petri dishes were incubated at 25±1 °C in darkness for times of 24, 48 and 72 hours. Therefore, the effect of AgNPs evaluated for preventing growth of *A. flavus*. Used method was by measurement of the growth of mycelium ray [26]:

[(A-B)/A]-100

A: diameter of growth area in control test

B: diameter of growth area in treatment

The effects of AgNPs on *A. flavus* in stored cereals packages

The AgNPs scattered the same on polymer surface to examine effects of the particles on polymer composite in anti-fungal on surface and around packaging polymer. The different concentrations of fungus tested for PP polymer with a constant of AgNPs that had at the most prevention of mycelium growth of *A. flavus*. Therefore, the polymer composited by AgNPs in determined concentrations of nanoparticles. The grains of wheat contaminated with the different concentrations of fungus prepartion placed into polymeric pouches those were made of polypropylene pieces at the size of 30×50-cm with the aid of a press plastic machine for packaging 500-gr wheat. These packages were completely devoid of any pores. The percent of mycelium growth was investigated in comparative to control test [26]. In beginning of tests until seventh day, when no *S. cerealella* penetrated again, we examined *A. flavus* growth into the packages in intervals of 12 h. Then, in the end of the seventh day, we compared the differences between the numbers of penetrated, living, and dead insects into packages with and without AgNPs to record the differences if there was. Also, we tested the perfect packages incorporated to AgNPs after 60 days for the period of seven days again. Therefore, we determined the effect of silver nano particles on the feeding of pest insects and inhibitory percent of *A. flavus* growth after two months. The reason is that after 60 days how the AgNPs could have affect on prevention and growth of pest insects and fungi. In the examinations, 5th instar larvae of *S. crealella* tested that had the most penetration and loss inside products. Then, the mortality percent of pest insects calculated by accounting dead insects into packages (with and without silver nano particles). The mycelium growth of *A. flavus* measured in the cereal packages at the first and last days of storage.

Results and Discussion

The results of this study should be viewed from two aspects: 1- suggestion of the best polymer among current polymers for packaging cereals, 2- suggestion of silver nanoparticles in

packaging as what prevent the fungi growth. So, this subject could help to sanitation of foodstuffs and should be effective for the consumer's healthy. Thus it would prevent spreading of contamination in stores. In these experiments, we determined the ability of one species of stored-product insects to penetrate and disperse contamination in stored cereals packages. Seven days after initiation of experiments, from among different polymers (PE, PP, PVC and Cellophane) with 29 µm thicknesses, *S. cerealella* 1st and 5th larva penetration rates in PE polymer was 27% and 87.5% of contamination respectively, while no penetration was observed in PP polymers with 29 µm (fig. 1).

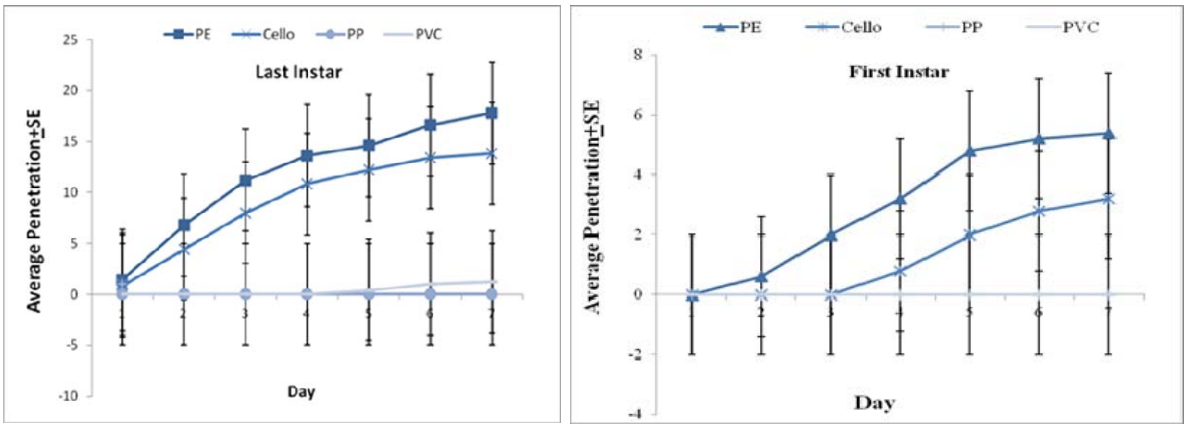

Fig 1: Number of first (1st) and last (5th) instar *S. cerealella* larvae in lack of food conditions that penetrated tested polymeric packages during the first 7-d period

Table 1 indicates average of penetration percentage of *S. cerealella* without food around the various polymers. In this table, the penetration average percentage in life stages is different as larvae in 5th instar penetration was more than 1st instar of larvae. Analysis of variance showed significant differences ($P \leq 0.05$) between the polymers permeability. Studied polymers in here rank generally from easiest to most difficult to penetrate: polyethylene, Cellophane, Polyvinylchloride and Polypropylene. The results of this study agree with findings of previous studies such as Cline (1978) that believes penetration of large larvae and adult insects of many species of stored pests to polyethylene and cellophane polymers with a thickness of less than 29 µm is possible. Proctor and Ashman (1972) suggest using of polyethylene layers with thickness of more than 65 µm in plastic bags and unsuitable using of bags with thickness of less than 40 µm [24]. Highland and Wilson (1981) believe that in this case polypropylene has a higher resistance than polyethylene (with equal thickness) [13].

Table 1: Average permeability percentage of different polymers to *S. cerealella* without food

Polymer	Insects (20)[1]	Average penetrated insects ± SE	Duncan's test grouping
Polyethylene	1st	5.4±0.22	c[1]
	5th	18.2±0.2	ab[1]
Cellophane	1st	3.2±0.2	d[1]
	5th	18.2±0.2	b[1]
Polypropylene	1st	0.0±0.0	f[1]
	5th	0.0±0.0	e[1]
Polyvinylchloride	1st	0.0± 0.0	f[1]
	5th	1±0.31	f[1]

(1: Being Bilateral Effect and Duncan's Test Grouping, 2: Disbilateral Effect of Polymer and Thickness, F: First Instar Larvae, L: Last Instar Larvae)

According to the results of this research and some other studies a polymer cover of polypropylene is the most suitable one for stored cereals packaging to prevent insects' penetration. The present research shows polypropylene liners in 29 µm thickness are suitable for cereals and other grains packaging to prevent entering the pest insects to and protect the stored packaged products against contamination. On the other part, the results show that penetration percentage at first days is very quick but whatever number of the insects into packages would be more, insect penetration percentage decreased. This topic proves that insects always attempt to penetrate new food packages as their high activity is for availability to more food sources that it could cause to increase the probability of growth of pathogenic fungi all into the food packages. Hence, polymerization processes of polypropylene with silver nanoparticles avoided *A. flavus* contamination inside kernels. As in control tests (without AgNPs), packaged foodstuffs had a lot of contamination to the pest insects and 60 days after contamination, there was *A. flavus* grew in 99.8% of

the packaged samples. Furthermore, the results of tests on petri dishes by measurement of the diameter of mycelium growth showed in comparative to control, the AgNPs cause to decrease growth of the fungus, significantly ($P \leq 0.05$). In particular, silver nanoparticles have been shown to be a promising antimicrobial material [30]. Also, a research is performed for controlling pathogenic fungi on plants by Jo and their collaborations (2009) that antifungal activity of ionic or nanoparticle silver has a great potential for use in controlling spore-producing fungal plant pathogens [17]. The most effect of controlling the fungus by nanoparticles is in < 24h (Fig. 2). In addition, the different concentrations of silver nanoparticles controlled *A. flavus*, variously (Tables 2, 3, 4 and 5). Therefore, the concentration of nanoparticles and storage time is effective for controlling the fungi into foodstuffs packagings.

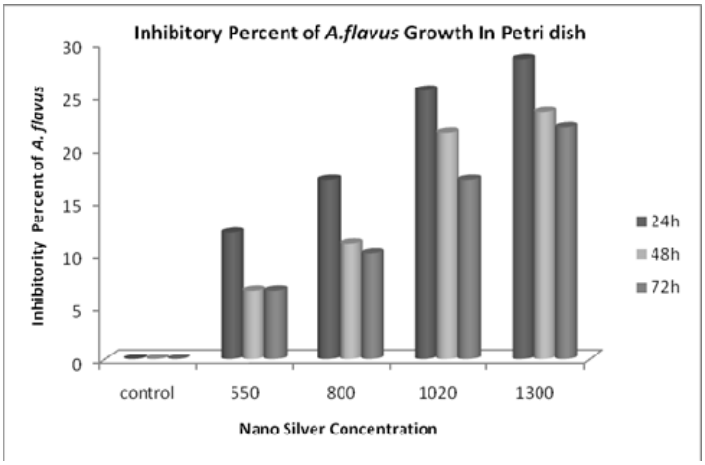

Fig 2: Inhibitory percent of *A. flavus* growth in petri dish tests

Table 2: Results of antifungal for 1% wt of silver salt

AgNPs concentration	Fungus concentration	The kind of packaging (29 µm)	Fungicide percent
Ag with 1% w for polymer	0.1	PP film	93.65%
		Nano PP film	96.43%
	0.01	PP film	91.5%
		Nano PP film	94.36%
	0.001	PP film	98.48%
		Nano PP film	99%
	0.0001	PP film	99.9%
		Nano PP film	99.9%

Table 3: Results of antifungal for 1.5% wt of silver salt

AgNPs concentration	Fungus concentration	The kind of packaging (29 µm)	Fungicide percent
Ag with 1.5% w for polymer	0.1	PP film	96.32%
		Nano PP film	97.15%
	0.01	PP film	96.47%
		Nano PP film	96.02%
	0.001	PP film	99.15%
		Nano PP film	99.9%
	0.0001	PP film	99.9%
		Nano PP film	99.9%

Table 4: Results of antifungal for 2% wt of silver salt

AgNPs concentration	Fungus concentration	The kind of packaging (29 µm)	Fungicide percent
Ag with 2% w for polymer	0.1	PP film	99.28%
		Nano PP film	99.86%
	0.01	PP film	99.9%
		Nano PP film	99.9%
	0.001	PP film	99.81%
		Nano PP film	99.9%
	0.0001	PP film	99.9%
		Nano PP film	99.9%

Table 5: Results of antifungal for 3% wt of silver salt

AgNPs concentration	Fungus concentration	The kind of packaging (29 µm)	Fungicide percent
Ag with 3% w for polymer	0.1	PP film	99.9%
		Nano PP film	99.9%
	0.01	PP film	99.9%
		Nano PP film	99.9%
	0.001	PP film	99.9%
		Nano PP film	99.9%
	0.0001	PP film	99.9%
		Nano PP film	99.9%

The other results indicated many packaging with AgNPs were free from *A. flavus* and therefore, probably there was no aflatoxinB1 in storing process in comparative to control test ($P \leq 0.05$) so that AgNPs eliminated the various concentrations of the fungus (Tables 2, 3, 4 and 5). Cereals packages were made of polymers with AgNPs in the concentration of 1300 ppm, so that there was a significant difference among the treatments and control tests ($P < 0.05$) in preventing of growth of *A. flavus* (fig. 2). These results are according to the results of research of Jo and collaborations (2009) that believe there is a decrease in diameter of clones of *B. sorokiniana* and *Mognaporthe grisea* in the concentration of 100 ppm of AgNPs [17]. These nanoparticles disturb the transportation system such as ionic flows. The difference of effective concentrations in related to the kind of fungus and the structure of nanoparticles [36].

Hwang and collaborations (2008) believe that the antibacterial mode of action is due to the silver nanoparticles, the silver ions or a combination of both. These compounds can hurt to Lipids, Proteins and Nucleic acids [14]. Also, the antifungal effect of silver nanoparicles on *A. flavus* was very fast in first but by the passing of time in next days the nanoparticles effect on inhibitory of fungus growth went down and so approximately fixed (Fig. 2) but it was not due to resistant them to AgNPs because microbes are unlikely to develop resistance against

silver, as they do against conventional and narrow target antibiotics (Pal, *et al*., 2007). During a period of 7 days, the effect of nanoparticles on *A. flavus* had been caused to significantly decrease the growth of fungus in comparison to control test ($P<0.05$) (Fig. 3).

Fig 3: the effect of nanoparticles for preventing the penetration of insects to foodstuffs packagings in the first and the last seven days of tests

Multiple modes of action targeting a broad range of biological pathways of microbes provide an important benefit for avoiding the development of resistance, which has been increasingly important in terms of current issues for the chemical management of many plant fungal diseases. Since the efficacy of silver is greatly influenced by application timing, preventative applications of silver ions and nanoparticles work better before spores penetrate and colonize within the plant tissue [17]. On the other hand, in comparative to control tests, some insects penetrated to cereals packages with AgNPs were not able to continue any growth and then died after several hours (Table 6).

Table 6: The probit analysis of *Sitotroga cerealella* mortalities in 5th instar larvae through packaging by PP incorporated to nanoparticles against determined concentrations of phosphine gas

Developmental Stage	LC50 (mg/L)	LC95 (mg/L)	χ2	P	Slope (b)	Intercept (a)
Larvae	1439.94 (1264.53-1803.65)	4271.5 (2939.15-8770.21)	0.18	0.91	3.48	-6

So, an interesting point is that AgNPs have toxicity`s influence on insects penetrated through packages (fig. 4). AgNPs incorporated to PP polymer probably can be toxic for insect pests in two ways: 1- contact, 2- stomach. As silver nanoparticles can bind to different tissues and can cause potential toxic effects like cell activation, producing reactive oxygen species, which are more toxic to tissue, inflammation and finally all these processes gradually lead to cell death [16]. Prolonged exposure to silver shows toxic effects on CNS such as cerebral ataxia [1].

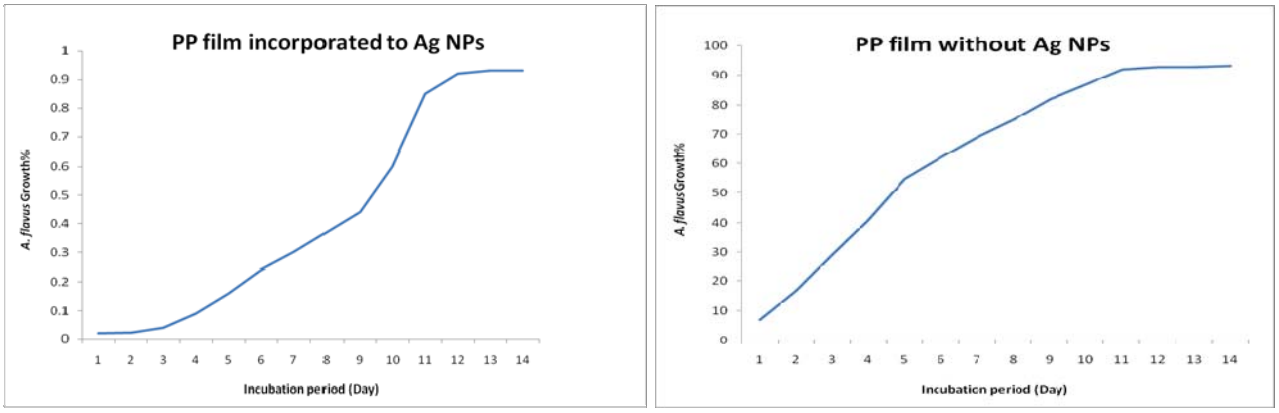

Fig 4: Growth pattern of *A. flavus* in different times for the cereals packagings without and with the concentration of 1300 ppm s of silver nanoparticles

The other reason for death of these insects is the alveolar region that in there, the AgNPs can be submersed into the surfactant lining of alveoli. By this submersion, nanoparticles can produce surface radicals and reactive oxygen species, which are more toxic to alveolar surfaces [4]. Some recent studies indicated that exposure to nanoparticles can induce oxidative stress in lung epithelial cells [18, 19, 29] and also in alveolar macrophage cells [30, 31]. These toxic effects are due to their intensive catalytic activity [19]. Hence, when we observed the safe products after two months into many packages, those had not any loss of fungi and insects; we did the next step of tests for a period of 7 days, once more. However, in later times

after two months, the effect of nanoparticles had more effect to prevent the penetration of insects. Therefore, in fact the AgNPs have the repellent virtues that the property may be due to the magnetic function of AgNPs that effect on surrounding medium especially at long times.

The minimum and maximum contaminations limits of *A. flavus* by silver nanoparticles in the treatments are 102 and 1010 spore/mm^2. In the concentrations, it could be assumed that the difference between contaminated and uncontaminated polymers are significant. Therefore, in the number of 102 spore/mm^2, it could be assumed that the samples (packages with AgNPs) are free from *A. flavus*. Thus, Aflatoxin B1 produced by *A. flavus* was mostly found (more than 20 ppm) in foodstuffs packagings without AgNPs. However, the kernels into these packages with AgNPs could be maintained well and nearly be without pollution as the treatments still resulted in abatement of *A. flavus* level after two months of storage although the level was still safe (<10 spore/mm2). Basically, mold needs a relatively high water activity (aW) (>0.70) for its growth [34] and it could be created by insects as the excess frass and spittle that sometimes webbing by larvae cause to provide this moisture. On such conditions, mycotoxins easily attack the foodstuffs in dry conditions to produce fungi and in particular *A. flavus* which can produce aflatoxins. The results showed that the growth of *A. flavus* on cereals contaminated from penetration of *S. cerealella* through cereal packagings without silver nanoparticles. When the number of *A. flavus* comes to 104 spore/mm^2 up, larvae penetration was stopped. So, in the control packages the most loss was because of presence of *A. flavus* and aflatoxine B1 measured of 500 gr wheat sometimes run to 600 ppm. Analysis of data showed there is a significant difference among the products into the polymer packages incorporated to AgNPs and control samples (polymer packages without AgNPs). So, decreasing the foodstuffs contamination in order to process of pest insect`s penetration could be minimized by coating these products through pp polymer incorporated to silver nanoparticles (Fig.5).

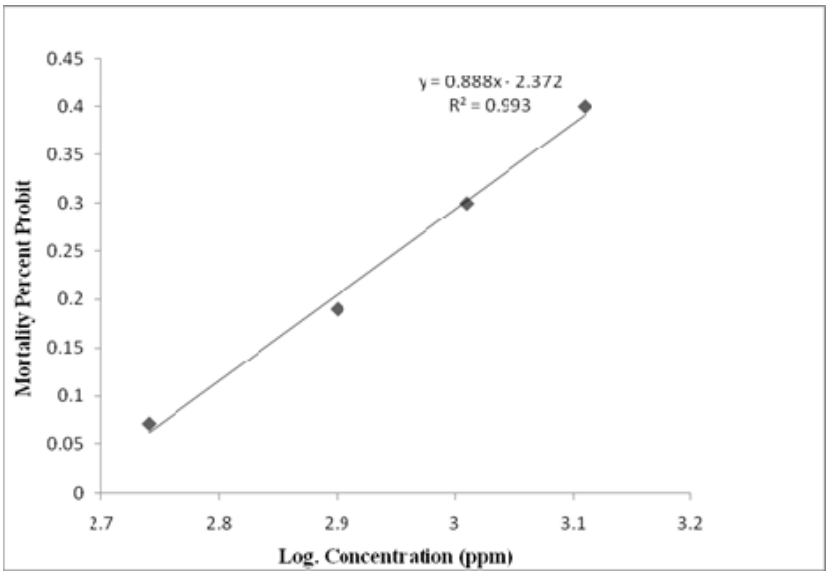

Fig 5: The relationship between logarithm of concentration of silver nanoparticles and probit of mortality percent of *S. cerealella* larvae

Thus, Silver nanoparticles may be less toxic to humans and animals than synthetic fungicides. Furthermore, it could be some toxic for pest insects and has insecticide properties. It has reported that silver nanoparticles can bind to proteins and enzymes in cells. These silver nanoparticles can make adhesive interactions with cellular membranes and produce highly reactive and toxic radicals like reactive oxygen species, which will cause inflammation and destroy cells like mitochondria. Subsequently they produce apoptogenic factors that cause cell death and necrosis in the cellular environment. Silver nanoparticles can show intensive toxic effects on the mitochondrial function and cell viability [35]. So, according to the results of this research and other studies, silver nanoparticles could be introduced as a pesticide that control fungi such as *A. flavus* and larvae populations of *S. cerealella*, in warehouses, greenhouses and farms that this topic is very important in crop protection and health.

Conclusion

This study demonstrated the possibility of use biological synthesized silver nanoparticles and their incorporation in materials, providing them sterile properties. The pp polymer incorporated with these silver nanoparticles exhibited antibacterial activity against *A. flavus* and *S. crealella*.

The use of unfit materials for packaging foodstuffs, placing packaged foodstuffs in polluted stores and also foods with contamination inside packages in during storage could be a where pest insects grow there and cause to produce so much aflatoxins. Therefore, the results of study can reduce big economic losses in two parts; attack of pest insects and growth of toxic fungi as yearly lead to become lost huge quantities of foodstuffs in storage industries. Suggested polymer in this study polymerizated with suitable concentration of silver nanoparticles reduced matchlessly the customers' complaints arising from contamination of cereals to pest insects and toxic fungi. Such a change would undoubtedly reduce the using chemical pesticides in stores of food products because this kind of packaging also was toxic for the insects. Therefore, this study can be one of the most effective solutions in helping to sanitation and increase of foodstuffs for companies whose want to have foodstuffs with much high quality. So, we coated the polypropylene with silver nanoparticles as polymeric cover on products, it could severely help to decrease the toxicity effect of silver nanoparticles on packaged foodstuffs. So, polymerization of polypropylene for packaging agriculture

products is the best method to apply silver nanoparticles to protect foodstuffs.

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