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Toxicity of silver nanoparticles prepared by extract of *Eucalyptus* sp in some biological aspects of Citrus mealybug *Planococcus citri* (Risso), Hemiptera: Pseudococcidae

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Abstract:

This study was carried out to investigate the possibility of preparation of silver nanoparticles by aqueous extract of eucalyptus plant Eucalyptus sp and test their effect in some instars of mealybug P.citri. Results proved the possibility of preparation of silver nanoparticles by aqueous extract of Eucalyptus sp. The results showed that the aqueous extract rich in reducing, coated capping and stabilizer agents of nanoparticles. Images of scanning electron microscope for silver nanoparticles consisting of spherical and oval forms particles have ranged between (70.07 - 157.66) nm and an average of 113.87 nm. Toxicity tests for silver nanoparticles prepared showed that it was good efficacy in different instars for citrus mealy bug P.citri (eggs, crawlers and adult females) which saw the use of silver nanoparticles prepared in concentration 4250 ppm highest rates mortality were amounted to 76.67, 93.64 and 66.3% for the instar of mealybug respectively after 72 hours of treatment. LC50 values stood at an average of 1247.95, 1295.91 and 2235.99 ppm, respectively. relative effectiveness of silver nanoparticles prepared by eucalyptus *extract at semi-field conditions recorded the highest rate were 97.78, and 99.36% after 3 and 7 days of treatment in concentration 4250 ppm, while the lowest rate of the relative effectiveness of nanoparticles at the concentration 510 ppm, amounting to 64.89 and 56.35% after 3 and 7 days, respectively. The overall results show that the use of silver nanoparticles prepared in combating insect mealybug is a promising method will depend on nanotechnology in pest control and can be a component of integrated pest management.*

Key words: Silver Nanoparticles, *Eucalyptus* sp, *Planococcus citri*

INTRODUCTION

The citrus mealybug *Planococcus citri* (RISSO) is one of the most important economic pests of citrus trees and many other plant families causing significant economic losses in many countries of the world (Goldasteh et al, 2009; Polat et al, 2008). Importance of the insect in their high reproductive and diversity of plant families that affect them and their resistance to chemical pesticides (Seabra et al ,2013) as well as its contribution to the transfer of some viral plant diseases (Cid et al, 2010). The nanotechnology of the fastest technologies prevalent on a global scale has been dubbed the next technology revolution in various industrial, medical and agricultural fields and various other areas because of its great features (Lux, 2008). The concept is derived from the word Nanotechnology (Nano) which means dwarf in Greek language and in scientific terminology, the nano equals one (ppb) and nano particle are sized between (100-1) nm (Bhattacharyya et al, 2010). Agricultural applications occupies an advanced position in the hierarchy of priorities nanotechnology and expected that a huge revolution in the field of plant protection occur by relying on nanotechnology in the preparation of nanopesticides can provide solutions in the fight against agricultural pests and detection of pesticide residues and overcome their negative effects as well as improve the properties of Biological control agents and other applications that may contribute to reducing environmental pollution and reduce application cost (Ghormade et al ,2011; Ragaei&Sabry,2014). It is this spirit and given the importance of many of the research to the possibility of the use of silver nanoparticles in the fight against pests and thus provide new methods based on nanotechnology in the fight against pests this study was chosen.

MATERIALS AND METHODS

Preparation of laboratory culture of citrus mealy bug *P.citri*

Followed the method of rearing on potato sprouts for the purpose of rearing of mealy bug (Al-Ali, 1969). they work artificial infection for potato sprouts by different instar of *P.citri* and placed in the rearing room at a temperature of 26 ± 2 C° ,relative humidity 55-60% and Photo period (light: dark) 16: 8 hours.

Preparation of aqueous extract of eucalyptus leaves

Selected eucalyptus leaves fresh and free of injuries and pathogenic infections, washed with distilled water to remove dust and dirt and then washing by deionized water for half an hour and placed under the fan air for the purpose of getting rid of the moisture washing for two hours. Cut off leaves a very small pieces by sterile scissors then weigh 100 g and put them in a glass flask 1000 ml capacity, and he finished size to 1000 ml deionized water. Preheat the solution at a temperature of 60 C° for ten minutes by a thermal heater (magnetic hot plate stirrer). The solution was cooled and filtered by Buchner suppress with filter paper (watman) No. 1. Collecting the filtrate in the beaker tightly and put in dark conditions in the incubator at a temperature of 5 ± 1 C° until use.

Preparation of silver nanoparticles

Aqueous solution of silver nitrate concentration of 50 mM as standard solution was attended by adding 8.4935 grams of silver nitrate powder AgNo3 (factory by Indian Alpha Company) into a glass beaker 1000 ml capacity and completed the volume to 1000 ml deionized water .preheated the solution at a temperature of 60 C° for 10 minutes while stirring then refund later to get ready for the preparation of silver nanoparticles. To prepare silver nanoparticles take 100 ml of an aqueous extract of eucalyptus leaves and add gradually to 900 ml of silver nitrate solution with heating and stirring heater (magnetic hot plate stirrer) at a temperature of 50 C° for a period of 20 minutes. Monitor occurrence of color changes of the solution, which indicates the biological reduction process and formation of silver nanoparticles. Cold solution and placed in a glass beaker tightly in the incubator at a temperature of 5 ± 2 C ° and under conditions of total darkness until use. For the purpose of purifying silver nanoparticles and get a silver nanoparticles as powder, centrifuge process for the solution of nanoparticles when the rotational speed of 4000 r / min for one hour and then took part sediment and add him deionized water and then underwent centrifugation process once again as the fore mentioned. Process restored many times visits to collect the Precipitate and put in a glass Petri and then placed in a convection oven at a temperature of 60 C ° for the purpose of drying for a period of 2-1 hours, then collects silver nanoparticles powder and save the electric incubator in glass bottles dark at 25 ° ± 2 C ° until use.

Characterization of silver nanoparticles

Studied the optical properties of silver nanoparticles, extract of eucalyptus and silver nitrate solution by device UV-VIS spectrophotometer type (Shimadzu). Identified the active groups (Functional groups) for the extracts of eucalyptus which could be involved and act also as reductive , capping and stabilizer agents for silver nanoparticles also identified, the active groups in the silver nanoparticles that were involved in the operations mentioned above solution by screening device FTIR (Fourier transform infrared) type (Shimadzu). as the structural characteristics of the nanoparticles formed in terms of shape and size were identified by scanning electron microscope (SEM).

Evaluating the efficiency of silver nanoparticles prepared in some Instars of citrus mealybug *P.citri*

Determined of mortality concentration of silver nanoparticles prepared by the extract of eucalyptus. Used deionized water for attended concentrations 4250, 3060, 2040, 1020 and 510 ppm. Treated eggs, crawlers and adult females of *P. citri* by concentration mentioned each separately by placing 20 eggs, 10 crawlers and 10 female adults on treated orange leaves by concentrations mentioned by dipping leaf method and placed in a plastic petri dish by three replicates while control treatment treated by deionized water. Put the dishes in the rearing room at a temperature of 27 ± 2 C°, relative humidity 60-50% and the photo periods light: the darkness (8:16) hours. Recorded mortality after 24, 48 and 72 hours, while the other hours were added to the treatment of eggs to overcome the incubation period for the eggs that have not hatched after 72 hours. Corrected mortality by Abbott formula (Abbott, 10). Determined of lethal concentration LC50 by Probit analysis program (Chi, 2015). In order to compare the effect of aqueous extracts of eucalyptus and solution of silver nitrate in instars of mealybug listed above, use the following concentrations of each extract 200000 ,400000, 600000 and 800000ppm.

The relative effectiveness of silver nanoparticles against the mealybug *P.citri* which infects citrus seedlings

Studied the effect of silver nanoparticles prepared in mealy bugs which effects on orange seedlings. Brought orange seedlings two years age from of the General Company for Horticulture and Forestry / Ministry of Agriculture. In order to simulate the conditions of greenhouses were transferred to a rearing rooms at a temperature of 27 ± 2 C ^{o}, relative humidity of 55-60% and photo period light: dark (8:16) hour. Artificial infection was worked to orange seedlings by mealybugs instars include eggs and other instars. After getting a good infection of mealy bug. Before the spraying process recorded of population density of crawlers on seedlings then was sprayed seedlings by silver nanoparticles concentrations with a small hand sprayer with three replicates for each concentration while sprayed of control treatment by distilled water only. Recorded of population density after 3 and 7 days of treatment. Calculated of relatively effectiveness of silver nanoparticles by Abbott equation modified (modification of Abbott formula proposed by Henderson and Tilton (Henderson and Tilton, 1955).

$$Control\% = 100\left[1 - \frac{Ta \times Cb}{Tb \times Ca}\right]$$

Whereas*:*

Ta = number of insects in treatment after application

Cb = number of insects in control before application

Tb = number of insects in treatment before application.

Ca = number of insects in control after application

STATISTICAL ANALYSIS

Use of completely randomized design (CRD) for one factor experiment and Split Split plot design for multi factor experiment. Compared the differences between the means of treatments by less significance difference (LSD) at the level of probability of 0.05. analyzed the results according to the statistical program GenStat. 7.

RESULT AND DISCUSSION

Visual and spectral characteristics to the prepared silver nanoparticles

Resulting of add aqueous extract of eucalyptus to the silver nitrate solution to a reaction caused the appearance of discoloration settled down to a dark brown color Figure 1 c, which indicates the reduction of silver nitrate into silver nanoparticles. Characterized by the process of color change as fast as the big not exceed 20 minutes. Measured excitability surface plasmon silver nanoparticles in solution by a spectral analysis of UV. Its highest absorption at the wavelength (456.16) nm as other absorption values recorded at wavelengths (415.13, 459.10 and 464.99 nm) Figure 2. Record the highest absorption of particles silver nanoparticles prepared by the plant extract of Eucalyptus *Eucalyptus hybrida* at a wavelength of 412 nm, while the highest absorption of particles silver nanoparticles prepared by extract of eucalyptus-type *Eucalyptus macrocarpa* at a wavelength of 430 nm (Dubey et al ,2009**;** Rouhani et al, 2012) which refers to the variation of absorption peaks depending on the size and shape of nanoparticles formed as well as the difference in the effectiveness of the reducing agent in eucalyptus extracts.
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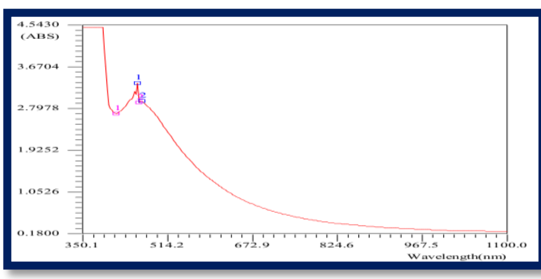
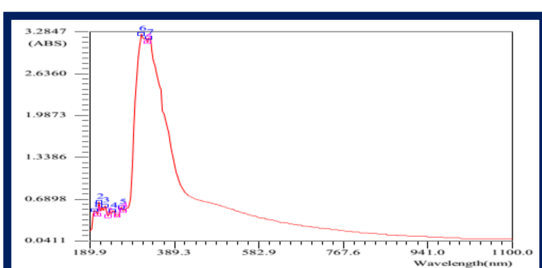
a **b** **c**

Figure 1: preparation of silver nanoparticles by aqueous extract of leaf Eucalyptus

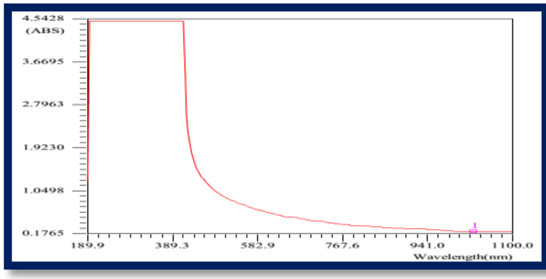
a. aqueous extract of eucalyptus; b. silver nitrate; c. suspension of silver nanoparticles



a



b



c

Figure 2. The absorbance spectra of silver nanoparticles synthesized with silver nitrate using of aqueous extract of eucalyptus plant
a. silver nanoparticles b. Silver nitrate c. eucalyptus extract

Determination of functional groups contribute to the formation of silver nanoparticles

Showed spectroscopy FTIR to extract eucalyptus Fig. 3 many units of energy peaks reflect the nature of the chemical complex of the plant and notes from the spectral analysis of the ethers (COC) most areas represent recording and therefore it is possible that the reductive factors or capping agents for nanoparticles followed halide group double C -Br second place, it can also lead carboxyl group (COOH) and amines NH and amides NH, which represents proteins and therefore can be proteins play an important role in the biological reduction of silver nitrate Ag^{+} to silver nanoparticles Ag° as well as its importance as capping, stabilizing agents and prevent conglomerate nanoparticles. It should be noted that the eucalyptus leaves content of *E.hybrida* kind of flavins and turbines have a significant role in stabilizing silver nanoparticles (Rouhani, 2012). in the context of the observed spectral analysis FTIR of silver nanoparticles prepared from eucalyptus leaf extract Figure 4 that a carboxylic acid groups which represent proteins believed to play a big role in the bio reduction of silver nitrate to silver nanoparticles as well as its role in capping and stabilizing of the silver nanoparticles and prevent them from gathering and this also applies to the halide group alkyl as well as the important role of amides and amines can also lead alcohols and phenols roles similar as will as total nitrogenous compounds (Nitro compounds) a source of protein.

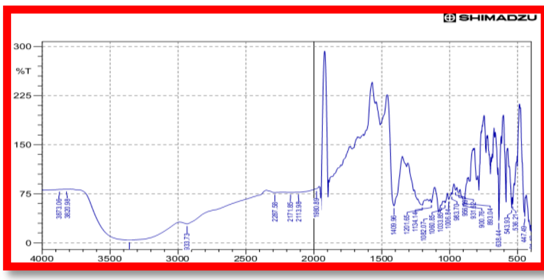


Figure 3: FTIR for aqueous extracts of eucalyptus leaves

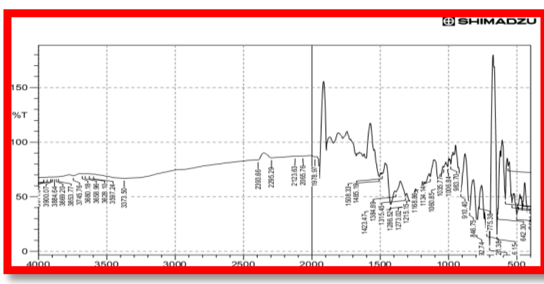


Figure 4: FTIR for silver Nano-particles prepared by extract of eucalyptus leaves

Determination of shape and size silver nanoparticles prepared

Showed of scanning electron microscope to be silver nanoparticles in different shapes and sizes. they showed size of silver nanoparticles prepared by extract of leaves eucalyptus ranges between (70.07 - 157.66) nm and an average of 113.87 nm and characterized these particles in spherical to oval shapes fig. (5). Noteworthy that the shape and size of nanoparticles an important role in determining of physical, chemical and biological properties.

Figure 5: Scanning electron microscope of silver nanoparticles prepared by eucalyptus extract

The effect of silver nanoparticles in instars of *P.citri*

Results indicate Entries in the table (1.2, 3) to contrast the effect of silver nano-particles prepared by aqueous extract of eucalyptus in instars of mealy bug (adults, crawlers and eggs) as it reached the highest rates of mortality when concentration 4250 ppm were 66.3, 93.64 and 76.67%, respectively, while the lowest rates of motality when concentration 510 ppm after 72 hours of treatment and the results of statistical analysis showed that there were significant differences between the concentrations in the events mortality at the possibility 0.05. Lethal concentration (LC50) were 2235.99, 1295.91 and 1247.95 ppm respectively Table 4. Characterized of silver nanoparticles unique attributes gained from nanoscale size, as the nanomaterials at this level behave quite different even from the parent material consisted of them that as these particles is characterized by the ability to overlap with many of the vital events of the insect because of its ability to penetrate the plasmic membrane and smashing many biomolecules such as enzymes as well as clotting proteins and the loss of plasmic membrane to his job and consequently cell death (Chakravarthy et al, 2012) as the insect exposed to particles of silver nanoparticles reduces the movement of larvae and makes the body wall of a strong and rigid as noted body bulge that becomes pasty and crisp and turn to dark brown (Abduz et al ,2012) which symptoms very similar to that observed for adults and nymphs mealy bugs treated with various silver nanoparticles in the current study. It is thus clear the importance of silver nanoparticles prepared by biological methods to influence a mealy bug and thus can be an important factor in the integrated pest management programs.

Table 1: The effect of silver nanoparticles in adult females of *P.citri*

Conc. (ppm)	**%correct mortality**			**Mean**
	24	**48**	**72**	
4250	**16.7**	**46.7**	**66.7**	**43.3**
3060	**6.7**	**36.7**	**63.3**	**35.6**
2040	**0.0**	**26.7**	**40.0**	**22.2**
1020	**0.0**	**23.3**	**36.7**	**20.0**
510	**0.0**	**3.3**	**10.0**	**4.4**
mean	**4.7**	**27.3**	**43.3**	**25.1±3.59**
LSD0.05	**Conc.= 7.03** **Time = 5.45** **conc. = ×Time = 12.18**			

Table 2 : Effect of silver nanoparticles in crawlers of *P.citri*

CONC. (ppm)	%correct mortality			Mean
	24	48	72	
4250	50.74	67.58	93.64	70.65
3060	48.60	28.95	50.62	42.72
2040	65.34	54.60	54.60	58.18
1020	32.36	21.55	38.0	30.64
510	16.12	9.66	33.77	19.85
mean	42.63	36.47	54.11	44.1
LSD0.05	5.18 Conc.= Time = 4.01 conc. = 8.97 × Time			

Table 3: The effect of silver nanoparticles in eggs of *P.citri*

Conc. (ppm)	% correct mortality after 120 h
4250	76.67
3060	75.0
2040	58.36
1020	46.65
510	28.39
LSD 0.05	0.98

Table 4: Toxicity of silver nanoparticles in instars of *P.citri*

Mealybug Instars	Lethal concentration (ppm)		95% confidence limits		Regression line equation	X2 Chi Seq.
	LC50	LC90	Lower	higher		
Eggs	1247.95	9636.19	997.8	1560.13	Y=0.53+1.44X	0.89
Crawlers	1295.92	6802.9	668.11	2502.98	Y=-0.54+1.78X	3.44
Female adult	2235.99	12567.18	1460.78	3426.44	Y=-0.73 +1.71X	2.33

The effect of silver nitrate and aqueous extracts in some mealy bugs instars

The results showed reduced the effect of silver nitrate and aqueous extracts of eucalyptus in the instars mealybug (eggs, crawlers and adult females) table 5. Where the highest rates of mortality when using of silver nitrate in concentrate 800,000 ppm amounting to 13.56, 21.45 and 20.0% respectively, while lower mortality recorded when using concentration 200,000 ppm. the highest rates of mortality for instars of mealy bugs mentioned when using aqueous eucalyptus at concentration 800 000 ppm as ratios were of 7.36 and 16.0% for eggs and crawlers respectively while 0.0% in adult females .the lowest rates of mortality recorded at concentration of 200,000 ppm. the results showed superiority of silver nitrate to against mealybug instars compared to the effect of aqueous extract of eucalyptus with significance difference when the level of probability of 0.05. The results showed increased rates of mortality with increased concentration. And despite the decline in mortality the results indicated that crawlers were more affected than the rest of the other instars then the eggs and adult with significance difference when level of probability of 0.05. In similar studies outweigh the impact of silver nanoparticles on the effect of silver nitrate and aqueous extract of the plant *Euphorbia prostrata* against insect weevil rice *S.oryzae* at concentration of 250 mg / kg grain were mortality 70.0% after 72 hours of treatment while caused aqueous extract of the plant and silver nitrate at a concentration of 1000 mg / l stood mortality 40 and 37% respectively (Abduz et al ,2012). Also concentration 10 mg / L from silver nanoparticles the latest mortality 100% of the larvae of mosquitoes while require concentration 50 mg / ml of an aqueous extract of the plant *Eclipa prostrate* to recorded mortality 83%, indicating a significant superiority for silver nanoparticles (Rajakumar& Rahuman , 2011)

Table 5: the effect of silver nitrate and an aqueous extract of eucalyptus in some instars of mealybug

Treatment	Conc. ppm	%correct mortality Eggs	crawlers	Adult female	mean
Silver Nitrate	800000	13.56	21.45	20.0	18.34
	600000	16.88	17.77	20.0	18.22
	400000	11.8	14.33	3.36	9.83
	200000	1.70	7.17	0.0	2.95
	Mean	10.99	15.18	10.84	12.34
Eucalyptus Aqueous Extract	800000	7.360	16.000	0.000	7.79
	600000	4.060	11.000	0.000	5.02
	400000	5.230	8.000	0.000	4.41
	200000	3.667	4.000	0.000	2.56
	Mean	5.08	9.75	0.0	4.95
LSD0.05	Treat.= 0.50 inst.= 0.37 trat ×conc.= 0.69 treat.×inst.=0.69 Conc.= 0.31 conc. ×inst.=0.68 trat.×conc.×inst. =1.38				

Relative effectiveness of silver Nano particles against the mealy bug *P.citri*

The results showed in the table (6) explained contrast the relative effectiveness of silver nanoparticles prepared by aqueous extract of eucalyptus *Eucalyptus* sp in the population density of crawlers mealybug. the highest rate amounted to Relative effectiveness of silver nanoparticles at the concentration of 4250 ppm, as was the relative effectiveness average of 97.78% after three days of treatment while the lowest rate of the relative effectiveness reached when the concentration 510 ppm which stood at an average of 64.89% after three days of treatment also the results showed that the relative effectiveness of silver nanoparticles has maintained its percentage at high concentrations after 7 days reaching the highest rate of the relative effectiveness of 99.36% at a concentration of 4250 ppm while the lowest rate of relative effectiveness amounted to 56.35% at 510 ppm concentration. The findings revealed influenced by the relative effectiveness to silver nanoparticles over time after 3 and 7 days respectively with significant differences at the level of probability of 0.05 and has attributed the low impact after 7 days in the rest of concentration to probability of loss of silver nanoparticles to the ideal characteristics which is impossibility stay act as nanoparticles after arriving the ocean for a long time because of a assembly and lose their biological properties (George, 2008). the results showed to the importance of silver nanoparticles because good efficacy in killing of mealy bug instars as well as the lack of stay for a long time in the environment so they are environmentally friendly which can be adopted in integrated pest management programs.

Table (6): The relative effectiveness of silver nanoparticles against *P.citri*

Treatment	Conc.(ppm)	%relative	Efficiency	Mean
		3 day	7 day	
Silver nanoparticles	4250	97.78	99.36	98.57
	3060	92.49	89.77	91.13
	2040	93.69	90.01	91.85
	1020	93.48	70.55	82.02
	510	64.89	56.35	60.62
	Mean	88.47	81.21	84.84
LSD0.05	Conc.=0.98 Time= 0.39 Conc. ×Time =1.14			

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