e-ISSN:2322-0139
p-ISSN:2322-0120

RESEARCH AND REVIEWS: JOURNAL OF PHARMACOLOGY AND TOXICOLOGICAL STUDIES

Larvicidal Potential of Fungi Based Silver Nanoparticles Against *Culex Quinquefasciatus* Larvae (II and III Instar).

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Research Article

Received: 05/08/2014
Revised: 21/08/2014
Accepted: 26/08/2014

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Keywords: Silver nitrate, *Penicillium notatum, Culix quinquefasciatus,* vector control, filariasis

ABSTRACT

Silver nanoparticles (AgNPs) were synthesized using *Penicillium notatum* and further characterized by UV-visible spectrophotometer, Scanning Electron Microscope (SEM), Energy Dispersive X-Ray Spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy to support the nanoparticles biosynthesis by fungi. The synthesized AgNPs were further investigated for its antibacterial and larvicidal activity in mosquitoes. AgNPs treatment caused considerable mortality rate against 2nd and 3rd instar larvae of *Culix quinquefasciatus* after 24 hours exposure. However, higher concentration of AgNPs was required to induce mortality against 3rd instar than 2nd instar. In general, lipid and protein contents were found to be reduced in larval tissues after AgNPs treatment; whereas level of carbohydrate was found to be increased. AgNPs exhibited antibacterial activity against *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Salmonella shigella.* Characterization studies reveal that *Penicillium notatum* biologically synthesized silver nanoparticles by reducing silver nitrate into nanosized silver ions. It can be concluded that rapid synthesis of fungi based silver nanoparticles will be helpful in developing a biological process for mosquito control using nanotechnology.

INTRODUCTION

Mosquitoes continue to be world's number one vectors to transmit human and animal diseases; and are prominent nuisance insect even after massive efforts of eradication or control [1]. *Culex* mosquitoes persistently bite and cause pain to both human and animals. *Culex quinquefasciatus* are especially responsible for the spread of filariasis. Lymphatic filariasis known as elephantiasis has largely affected nearly 1.4 billion people living in 73 countries Worldwide [2]. World health organization (WHO) has proclaimed that mosquitoes are human enemy number 1 and presently estimated that 50- 100 millions individuals are affected by the mosquito borne diseases [3]. Several methods including the use of chemical pesticides are adapted to control and eradicate mosquito population in the affected regions. However, synthetic chemical pesticides cause more harm to the environment, human life and other non target organisms as they are not easily degradable [4]. In addition, *Culex quinquefasciatus* mosquitoes unfortunately tend to develop more resistance against larvicides that are currently available. Fungi based biosynthesis of nanoparticle is one of the best biological methods to prepare silver nanoparticles [5]. Hence, silver nanoparticles, the benign materials exhibit numerous benefits in terms of eco-friendliness and better compatibility for the eradication of mosquitoes at their larval stage [6]. The mechanism of larvicidal activity of both natural and synthetic pesticides is still not clear to support the scientific evidences in vector control strategy. Therefore, investigation of basic biochemical parameters (protein, carbohydrate and lipid contents) in the pesticide treated insects is the need of the hour [7].

e-ISSN:2322-0139
p-ISSN:2322-0120

Pathogenic bacteria influence general quality of life and oral health leading to chronic conditions and systemic diseases [8]. Therefore, the present work was designated to investigate the antimicrobial effect of silver nanoparticles against microbes such as *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Salmonella shigella.*

MATERIALS AND METHOD

Synthesis and characterization of silver nanoparticles

Synthesis of silver nanoparticles: *Penicillium notatum* culture was grown in appropriate medium until it reached stationary phase. After 7 days, fungi culture was separated and grown in new medium for 3 days to prepare pure culture. After incubation, filtrate of pure fungi culture (4.5 ml) was added with 0.5ml of 1mM $AgNO_3$ and incubated in dark condition in shaker for biosynthesis of silver nanoparticles.

Characterization of nanoparticles:

UV-Vis spectral analysis was performed by using UV-Vis spectrophotometer, UV159 (Elico), equipped with matched quartz cuvettes. The reduction of pure silver ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 24 hours of incubation using small aliquot of the sample at 200-800nm. The synthesized nanoparticles were characterized by Scanning Electron Microscope (SEM), Energy Dispersive X-Ray Spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy.

Screening of larvicidal activity of silver nanoparticle

2^{nd} and 3^{rd} in star larvae of *Culex quinquefasciatus* were procured from National Centre for Disease Control, Mettupalam, Coimbatore, Tamilnadu. The larvae were maintained in trays containing distilled water and supplied with yeast. The larvicidal activity of fungi synthesized AgNPs was evaluated as described by World Health Organization method with slight modifications [9]. Different test concentrations of AgNP for 2^{nd} instar larvae (0.5ppm 0.7ppm, 0.9ppm, 1.1ppm and 1.3ppm) and 3^{rd} instar larvae (1ppm, 2ppm, 3ppm, 4ppm and 5ppm) in 200ml of distilled water were prepared. Five replicates each containing 20 larvae was subjected to larvicidal bioassay for all the test concentration and control group (distilled water). Mortality rate was recorded after 24 hour exposure period.

Estimation of biochemical parameters

After the treatment of 24 hours, larvae were removed from the test solution and washed with chilled normal saline. Larval tissue homogenate (10%) was prepared in 0.25M chilled sucrose solution by homogenizer. The homogenate was centrifuged at 700x g for 10 minutes to remove all the cell debris. Supernatant was adopted for estimation of total carbohydrates, lipids, proteins, alkaline phosphatase and acid phosphatase. All the parameters were carried out in triplicate.

Estimation of total proteins

Lowry's method was adopted to estimate protein content in the larvae. Reaction of protein with Folin-Coicalteu become purple blue proportional to the amount of proteins and read at 620 nm. Further protein concentration was calculated with optical density [10].

Estimation of total carbohydrates

Carbohydrate was estimated as described in the method of Nelson [11]. Proteins were removed from the tissue homogenate and the filtrate containing glucose only as reducing substrate was heated with alkaline copper reagent and subsequently treated with Arsenomolybdate reagent. The blue color thus developed was read at 540 nm and protein content was calculated.

Estimation of lipids

Total lipids present in the larval tissue were estimated following the method of Bragdon [12]. Lipid content was separated from the non-lipid components by chloroform- methanol solution and lipid in the aqueous phase was reduced by sulphuric acid- dichromate mixture. The resultant green colour was measured at 600 nm and the concentration of lipid was calculated.

e-ISSN:2322-0139
p-ISSN:2322-0120

Antimicrobial activity of AgNP

In-vitro antimicrobial screening of silver nanoparticles was performed by disc diffusion method using *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Salmonella shigella* [13]. Muller Hinton Agar (MHA) obtained from Hi-media (Mumbai) was used for the preparation of medium. Kanamycin (30µg per disc) was used as a positive control.

RESULT AND DISCUSSION

UV- Visible Spectroscopy

Reduction of Silver nitrate into Silver nanoparticles during exposure of fungi was indicated by a gradual increase in color development from colorless to almost reddish brown (Figure 1). Silver nanoparticles (AgNPs) synthesized by *P.notatum* were primarily characterized by UV- Visible Spectroscopy. AgNPs present typical spectrum having maximum absorption in the range of 250 to 400 nm. The spectra attribute to the surface Plasmon response (SPR) properties of metal nanoparticles when electrons are conducted on AgNPs surface. These unique and tunable optical properties due to the SPR depend on size, shape and distribution of nano sized particles of silver ions [14]. Reduction of silver ions is particularly measured and monitored using UV-Visible spectrum analysis with diluted silver nanoparticle sample (Figure 2).

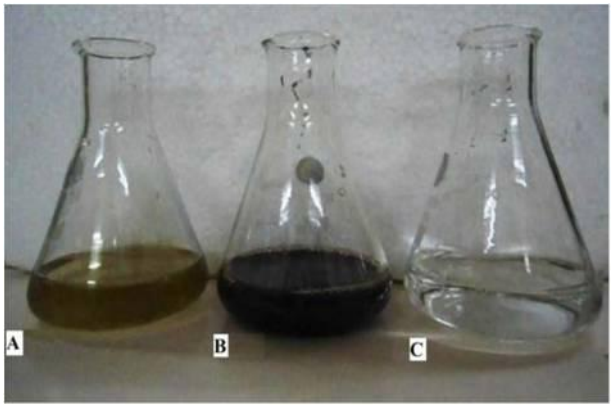

Figure 1: Reduction of Silver Nitrate to Silver Nanoparticles by *P.notatum* [A- fungal filtrate B- AgNO nanoparticles, C- 2.0 mM $AgNO_3$].

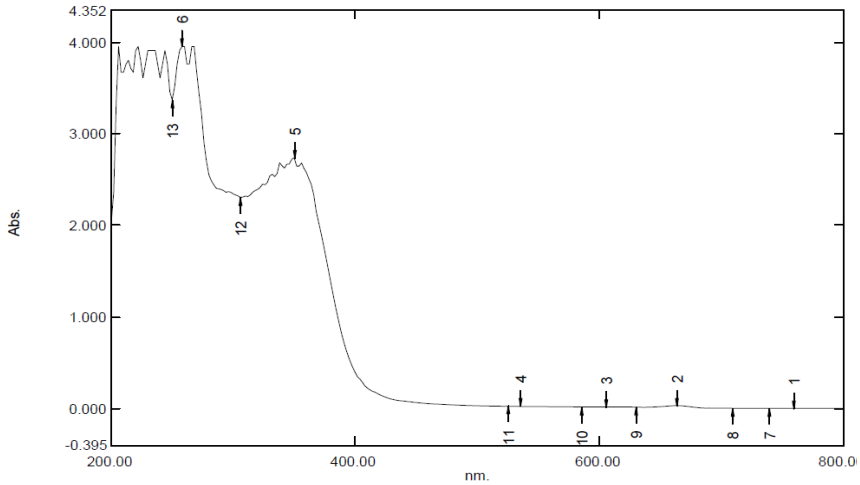

Figure 2: UV-Visible Spectrum indicating the presence of nanosized silver ions.

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FT-IR

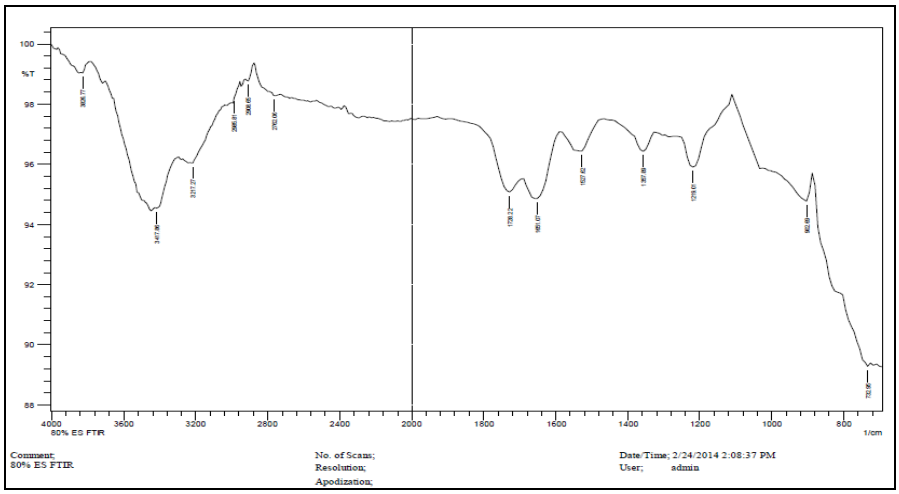

Figure 3: Fourier Transform Infrared Spectroscopy spectra

The spectrum exhibits the band at 1635.64 cm^{-1} corresponding to primary amide groups (strong peak); similarly the presence of bands at 1381.64 cm^{-1} represents the nitro compounds including primary (CN) and secondary amines (NH) stretch vibration of proteins. Strong bands of phenyl ring compounds indicate the occurrence of proteins with silver nanoparticles. The role of proteins is necessary for the reduction and capping around the nanoparticles synthesized by *P.notatum*. FTIR analysis clearly suggests that protein and other bioorganic compound from *P.notatum* might be involved in the formation and stabilization of AgNPs. *P.notatum* releases the extra cellular proteins and enzyme molecule to stabilize nano silver ions in aqueous medium [15].

Scanning Electron Microscopy Analysis

Characterization of silver nanoparticles using SEM analysis revealed that the average size of Silver ions ranged from 70 nm to 90 nm. SEM results also demonstrate the existence of rods and hexagonal shapes of AgNPs synthesized by *P.notatum*.

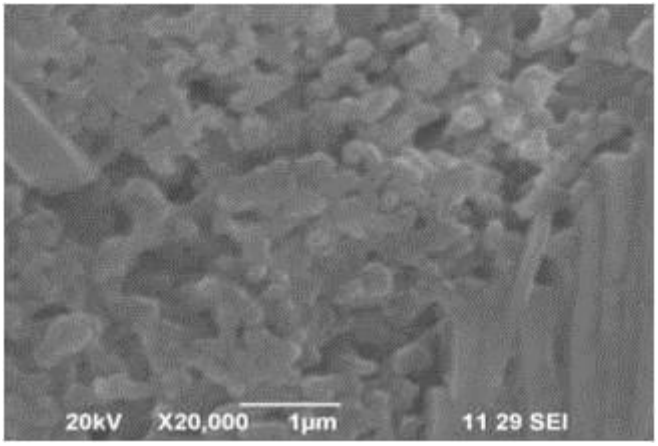

Figure 4: Scanning Electron Microscope picture of silver nanoparticles

Energy Dispersive X-Ray Spectroscopy

The EDAX spectrum exhibits the peaks indicating the silver, chloride, calcium, sodium and oxygen species. This proves the generation of silver nanoparticles along with other ions by the action of fungi in the medium.

e-ISSN:2322-0139
p-ISSN:2322-0120

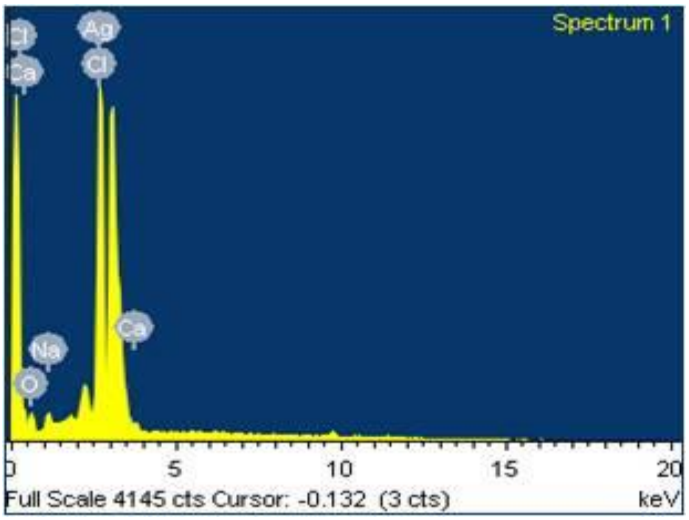

Figure 5: EDAX spectra of silver nanoparticles synthesized by *P.notatum*

Antibacterial activity of Silver Nanoparticles

The antibacterial activity of silver nanoparticles was determined at 5 different concentrations against four bacterial Strains (*Salmonella thyphinurium, Staphylococcus auries, Escherichia coli and Salmonella shigella*) by disk diffusion method and results were summarized in Table-1. The antibacterial activity was found to be increased with increase in concentration of AgNPs. The maximum zone of inhibition of 18mm was exhibited at 0.05µg of AgNPs against *E.coli* followed by *S.auries, Salmonella, Shigella* with zone of inhibition of 14 mm, 17.4 mm 17mm respectively. *Salmonella, Shigella* and *E.coli* were more susceptible than *S.auries*. The effective antibacterial activity is due to the silver nanoparticles which overcome the barriers of bacterial cell wall and therefore inhibits the bacterial growth [16].

Table 1: Antibacterial activity of silver nanoparticles

S.No	Concentration of AgNP (µg)	Zone of Inhibition (mm)			
		Salmonella	*S.auries*	*E.coli*	*Shigella*
1.	0.01	14	12	16	11
2.	0.02	12	12	16.3	12
3.	0.03	15	12.5	16.5	12.5
4.	0.04	17	13	17.1	13
5.	0.05	17.4	14	18	17
6.	Control(Kanamycin)	18	15	15	18

Larvicidal activity of AgNPs

2nd and 3rd instar stage of *Culex quinquefasciatus* larvae were treated with increasing concentration (0.5, 0.7, 0.9, 1.1 and 1.3 ppm) of silver nanoparticles to screen the larval mortality. 76.4% mortality was noted when treated with 0.5 ppm AgNP against 2nd instar larvae; whereas the percentage mortality was found to increase to 89.7% at 1.3ppm of AgNPs treatment (Table 2). The LC_{50} and LC_{90} values for 2nd instar larvae were found to be 0.44ppm and 1.13ppm respectively.

Similarly the mortality rate of 3rd instar larvae was evaluated by treating with higher concentration range (1, 2, 3, 4, and 5 ppm). The range for the 3rd instar larvae was fixed based on the observation in pilot study. AgNP exhibited only 40% mortality against 3rd instar larvae at 1ppm concentration; however the mortality percentage was found to increase in concentration dependant manner (Table 3). The maximum larvicidal activity (92%) was found to be noticed when treated with 5ppm AgNP. The LC_{50} and LC_{90} values of Silver nanoparticles against 3rd instar larvae were noted as 2.3ppm and 4.4 ppm respectively.

From the result obtained, it is very clear that highest concentration is required to induce maximum mortality against 3rd instar larvae when compared to 2nd instar larvae. This could be due to the structural and functional development 2nd instar grows to attain 3rd instar stage. It is concluded that increased silver nanoparticle concentration is suggested to exert consistent toxicity against developed larval stage.

e-ISSN:2322-0139
p-ISSN:2322-0120

Kovendan *et al* demonstrated that 20% mortality rate was exerted upon treatment of 20ppm plant extract against 1st instar larvae. The same extract was found to exhibit 89% mortality at 100ppm concentration [17]. Many studies have proven that percentage mortality in mosquito larvae is directly proportional to the concentration of insecticides that induce toxicity [18]. Potential of silver nanoparticles synthesized by wide range of fungi such as *Diatum capillum*, *Chryosposium tropicum* and *Penicillium notatum* have been published by Christina 2013 and Thangaraj ramasamy 2013. Earlier researchers have clearly demonstrated that 0.9 ppm and 4 ppm AgNPs exhibited 85% mortality against 2nd and 3rd instar larvae respectively. This clearly indicates the dose dependent toxicity of the nanoparticles against two different stages of mosquito larvae [19].

AgNP induced changes in Biochemical parameters.

Carbohydrate content in the 3rd instar larval tissue was found to increase from 13.3 mg/g (control) to 19.33 mg/g after treating with 5ppm AgNPs. Similarly, the level of carbohydrate in 2nd instar larval tissues was observed to be 14.67 mg/g when treated with 1.3ppm AgNP (Table 2 and 3). It was noticed that carbohydrate content was relatively high in the treated larvae than in the untreated larvae. These observations signify that level of carbohydrate in the AgNP treated larval tissues increased in both 2nd and 3rd instar larvae of mosquitoes when treated with increasing concentration of silver nanoparticles. The larvae might be unable to assimilate the food thereby increasing the level of carbohydrate in their tissues [20]. The larvicidal stress induced by AgNP might have enhanced glycogenolysis leading to the hyperglycemia [21].

After treatment with silver nanoparticles, lipid level was found to be 90% and 73% in 3rd and 2nd instar larval tissues when compared with control larvae. The significant reduction in the lipid content indicates the negative effect of the AgNP on the lipid metabolism and lipid peroxidation. Stress induced in the mosquito larvae might be responsible for the altered energy metabolism leading to increased lipid catabolism and declined lipid level [22, 23]. The similar negative effect was observed in malathion treated insects in which lipid depletion of oocytes, fat bodies and haemolymph was majorly noticed [24].

Table 2: Percentage mortality and biochemical changes in treated II instar larvae

Concentration of AgNP (ppm)	Larval Mortality % (IInd instar larvae)	Biochemical estimations (mg/g)		
		Total protein	Carbohydrate	Lipid
Control	0	4	7.99	0.76
0.5	76.4	3.5	9.33	0.28
0.7	85.3	2.9	10.67	0.32
0.9	86.8	2.2	11.99	0.36
1.1	87.1	1.1	13.33	0.44
1.3	89.7	0.11	14.67	0.21

* Values are the average of triplicates

Table 3: Percentage mortality and biochemical changes in treated III instar larvae

Concentration of AgNP (ppm)	Larval Mortality % (IIInd instar larvae)	Biochemical estimations (mg/g)		
		Total protein	Carbohydrate	Lipid
Control	0	5.0	13.3	2
1	45.9	4.4	13.9	1.6
2	63.7	3.6	16.67	1.1
3	66.4	2.7	17.3	0.8
4	85.5	2.1	18.67	0.4
5	92.4	1.4	19.33	0.12

* Values are the average of triplicates

Total protein content was found to be decreased in both 2nd and 3rd instar mosquito larvae when treated with different doses of AgNP. However, the reduction in the level of protein was comparatively lesser in 3rd stage larvae of *Culex quinquefasciatus.* Body wall of larvae and adult mosquitoes is made up of chitin and other proteins which are the structural and functional features. In our study, we observed that treatment with AgNP caused damage and rupture in the dead larvae. The structural deformities observed might be due to the diminished protein profile in the AgNPs treated larvae when compared with untreated control mosquito larvae [25-26]. Insecticidal interference in the hormones which regulate the protein synthesis might lead to the disturbance in the normal protein metabolism, rupture and destruction in the larval body [23]. Nanoparticles induced intoxication and growth retardation in larval stage of mosquitoes

were found to be correlated with biochemical changes particularly in decrease or increase of the total protein, lipid and carbohydrates to ascertain functional and physiological interactions [27- 31].

CONCLUSION

P. notatum based silver nanoparticles induced consistent mortality against 2nd and 3rd stage of *Culix quinquefasciatus* larvae. Biochemical parameters such as lipid, protein and carbohydrate level were also foundto be altered in the larval tissues upon intoxication with silver nanoparticles. The synthesized silver nanoparticles were also found to possess considerable inhibitory effect against bacterial growth. In conclusion, the study indicates that *P.notatum* synthesized silver nanoparticles can be used to control the vector and vector borne diseases.

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