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Effect of *Fusarium graminarum* Silver-Nanoparticles on IL-10 and INF-γ Cytokines Levels in the Mice by *Leishmania donovani in vivo*

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Abstract: Leishmaniasis remains one of the fatal diseases worldwide, and the conventional antileishmanial therapies are toxic and most are expensive. Biological silver nanoparticles possess broad-spectrum antimicrobial activities and could be a future alternative to current antimicrobial agents. In the present study an approach was made to synthesize silver nanoparticles (AgNps) using a *Fusarium graminarum* fungus. The present study it investigates the efficiency of silver nanoparticles against Leishmania donovani compared with pentostam drug *in vivo* by measuring the levels of immune cytokines (IL-10 and IFN-γ) in serum infected mice and treatment with AgNPs (0.1 ml / day) and comparisons with pentostam drug (0.01ml / day) after 21 days of treatment. The results showed that the level of IFN-γ in treating with AgNPs increased significantly in third weeks, compared to the pentostam group. When treated with pentostam/AgNPs together, there is a gradual decrease in the level of IFN-γ, compared with negative control. Also a significant increase occurs in the IL-10 level within 21 days when mice were treated with AgNPs compared with pentostam. It could be conclude that silver nanoparticles induce pro and anti-inflammatory cytokines also it safety, nontoxic and has a good anti-parasitic activity, it can be used as antileishmanial drug or can be used as supportive treatment of visceral leishmaniasis.

Keywords: *F. graminarum*, silver, nanoparticles, IL-10, INF-γ.

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Introduction:

Leishmaniasis is one of the vector-borne diseases caused by obligate protozoan parasites of the genus *Leishmania*, they are transmitted by different species of sand flies belong the genus of *Phlebotomine* as extracellular flagellated promastigotes that replicate as an intracellular parasite (aflagellate amastigotes) in mononuclear cells of mammalian hosts (1). Visceral leishmaniasis is considered as the second cause of mortality and the fourth cause of morbidity after malaria, schistosomiasis and African trypanosomiasis (2). Visceral leishmaniasis affects more than 100 million people worldwide, with 500,000 new cases occur and more than 50,000 year. Over of 90% case reported death from India, Bangladesh, Nepal, Sudan a nd Brazil (3). The Clinical symptoms of visceral leishmaniasis infection include a grossly enlarged abdomen due to associated hepatosplenomegaly and splenomegaly, and general symptoms such as irregular fever, loss of appetite and weight, malaise, chills, wasting, pallor of mucous membranes and hypergammaglobulinemia (4). Pentavalent antimonials are a group of compounds used for the treatment of leishmaniasis. The compounds currently

available for clinical use are sodium stibogluconate and meglumine antimonate. In systemic therapy of leishmaniasis these drugs are used alone or in combination with other compounds. The current drugs is not so much suitable due to resistance reported, high toxicity, various side effects and so forth. So, new therapeutic antileishmanial strategies are urgently required (5). The Role of cytokines such as IFN-γ is to activate macrophages and enhance the microbicidal activity of these cells to kill intracellular pathogens through the generation of reactive oxygen species and reactive nitrogen species. IL-10 promotes intracellular infection, including human visceral leishmaniasis, by disabling Th1 cell type responses and/or deactivating parasitized tissue macrophages (6).

Nanotechnology continues to attract significant attention due to its impact on many currently important areas such as energy, medicine, electronics and the aerospace industry. Nanoparticles that possess one or more dimensions of the order of 100 nm or less continue to attract significant attention due to their unique properties in the realms of chemistry, optics, electronics and magnetism (7). Nano-hydroxyapatite/ poly(l-lacticacid) (nano-HA/PLLA) used for applications in bone tissue engineering and load bearing bone defects repair (8) .

The use of eukaryotic organisms such as fungi, *Fusarium graminarum* and other species offers considerable promise for large-scale metal nanoparticle production since the enzymes that are secreted by the fungi represent an essential ingredient for the biosynthesis of metal silver nanoparticles has attracted high interest due to their unique and excellent properties in addition to its therapeutic potential for the treatment of a variety of diseases that includes retinal neovascularization and acquired immunodeficiency syndrome due to human immunodeficiency virus (9).

Materials and Methods:

Silver nanoparticles preparation:

The mycelia of *F. graminearum* were inoculated in 250mL erlenmeyer flasks, each flask containing 100ml of potato dextrose broth (PDB) medium, then was incubated at 25 ± 2°C for 5 days. Later, mycelia were harvested by filtration through Whatman filter paper No. 42 and washed thrice with sterilized distilled water to remove the traces of the medium on fungal biomass. The washed mycelia were resuspended in 100 ml sterilized distilled water, then incubated at 25°C for 24hours. Again, mycelia were harvested by filtration through Whatman filter paper No. 42. Then, cells filtrate were divided two parts, first one (50 ml) treated with 1mM silver nitrate solution and incubated at room temperature, which change color to brown consider as Positive control, while the second part (50 ml) left without the addition of $AgNO_3$ to the cells filtrate without change in color consider as negative control.

Characterization of nanoparticles:

The detection of AgNPs was primarily carried out by visual observation of color change of the fungal filtrate after adding silver nitrate. The appearance of dark brown color. The exact configuration of the size, concentration, morphology of crystals, aggregation state bioconjugation and was measured by using atomic force

microscopy (AFM) (Angstrom, USA), X-Ray diffraction (XRD) (Shemadzu, Japan), and ultraviolet-visible spectroscopy (UV-VIS) (Shemadzu, Japan).

Parasite strain and culture:

Leishmania donovani was isolated from the bone marrow of an infected child , the strain was obtained from biotechnology center/ Al-Naharin University, it was cultured and maintained by serial passage in (NNN) media each 8 days and incubated at 26°C.

***Leishmania* antigen preparation:**

One milliliter of promastigote culture in stationary phase washed three times with phosphate buffered saline by spin at 4000 rpm for 15 minutes then the concentration was adgusted up to 1×10^7 parasite\ml.

Animals:

Ninety six male *albino* mice aged between 8-12 weeks, weighing 20-28 gm was obtained from The National Center for Drug Control and Research, housed under standard condition in animal house in the Biology Bepartment/College of Science/Al-Mustansrya University.

Seventy eight mice were infected with 1×10^7 parasite/ml of *L. donovani* promastigotes by intraperitonial injection (10). Then the 78 mice divided into the following group:

- Group 1: Inoculated orally by stomach tube (0. 1ml/day) of normal saline considers as control positive group.
- Group 2: Inoculated orally by stomach tube of (0.1ml/day) AgNPs for 21 days considers as an AgNPs treatment group.
- Group 3: Injected with (0.01 ml/day) of pentostam drug by intramuscular each day for 21 days considers as Pentostam treatment group.
- Group 4: Inoculated orally by stomach tube of (0.1ml/day) AgNPs and injected with (0.01ml/day) of pentostam drug by intramuscular for 21 days consider as AgNPs and pentostam treatment group.
- Group 5: Inoculated orally by stomach tube (0.1ml/day) of normal saline considers as negative control without infecting by *L. donovani* parasite..

Determination of cytokine levels:

The IL-10 and INF-γ levels were estimated by ELISA (Human Systems, Germany) using manufacturer's protocol.

Statistical analysis:

The Statistical Analysis System(11) program was used to effect of different factors in study parameters. Least significant difference –LSD test (ANOVA) was used to significant compare between means in this study(12).

Results:

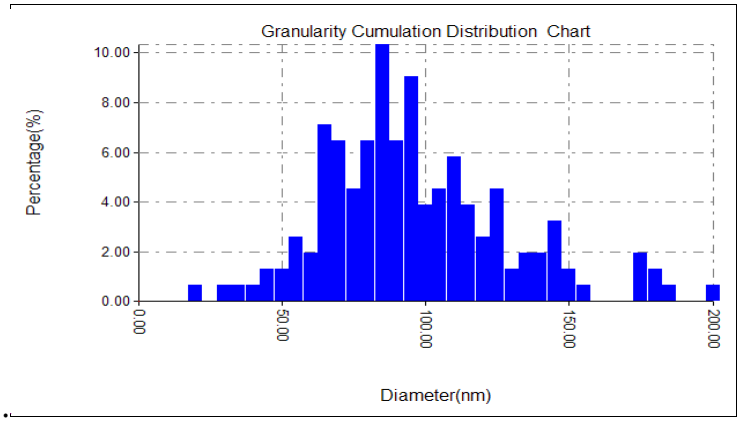
Detection of the existence of *Fusarium* AgNPs:

The synthesis of silver particles by using *Fusarium graminarum* fungi was examined. After adding of silver nitrate ($AgNO_3$) to filtered cell, the color of the mixture changed from colorless to blackish brown compared with negative control remain clear without color (colorless), the changes in color which

confirms the reduction of $AgNO_3$ by *F. graminarum* indicated the presence of AgNPs.

Morphology of *Fusarium*-silver nanoparticles by atomic force microscopy:

Determine *Fusarium* silver nanoparticles (AgNPs) sizes and surface morphology were measured ,using the software of the AFM , the images of AFM for *Fusarium*-AgNPs in (Figure 1) represents particle size distribution, where average diameter is 94 nm. While in (Figure 2A,B) is AFM picture in three dimensions (3D) and two dimensions (2D), it explains structural shape for grains, found that the average roughness (Ra) is 9.33 nm and Root mean square (Sq) is 11.6 nm.



Figure(1): Granularity volume distribution chart of silver nanoparticles produced by *F. graminurum*

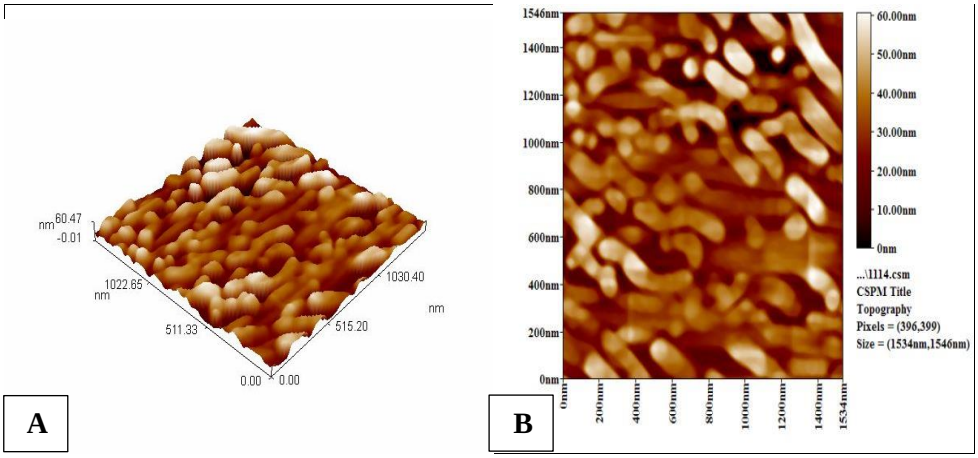


Figure (2): AFM images of silver nanoparticles produced by *F. graminurum* (A) three dimensions 3D and (B) two dimensions 2D.

Characterization of *Fusarium* AgNPs by X-RayDiffraction:

XRD technique is used to identify and characterize compounds based on X-ray diffraction pattern. A typical XRD pattern of *Fusarium* AgNPs as shown in (Figure 3), the diffraction peaks at 38.05°, 44.22°, 64.32 and 77.31° were correspond to the (111), (200), (220) and (311) facets of the face centered cubic crystal structure, therefore the average crystallite size was 28.225 nm.

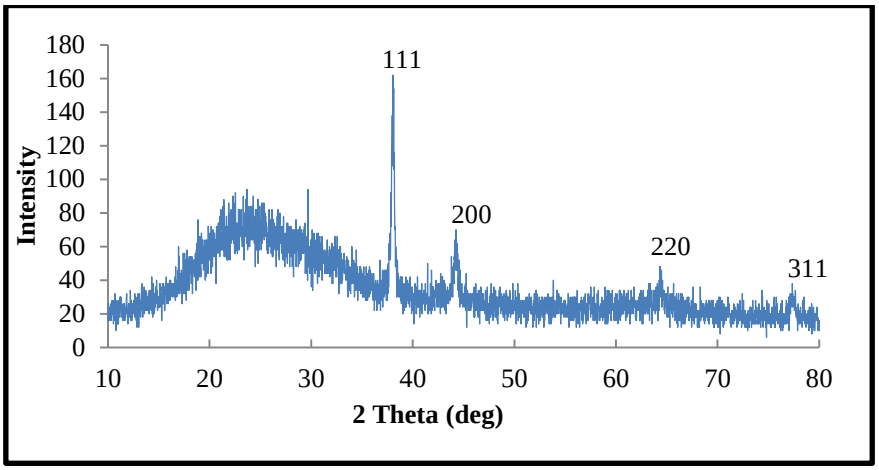


Figure (3): X-Ray pattern of silver nanoparticles produced by *F. graminurum*.

Optical properties of *Fusarium* AgNPs by UV–Visible Spectral:

This technique confirms the presence of *Fusarium* AgNPs by measuring the absorbance of the bioreduced solution at wavelengths between 300 and 800 nm. Extinction spectroscopy of ultraviolet (UV) and visible (Vis) light (UV–Vis spectrum) allows confirming the presence of *Fusarium* AgNPs because of the characteristic plasmon resonance, which showed an absorbance peak at 420 nm, (Figure 4).

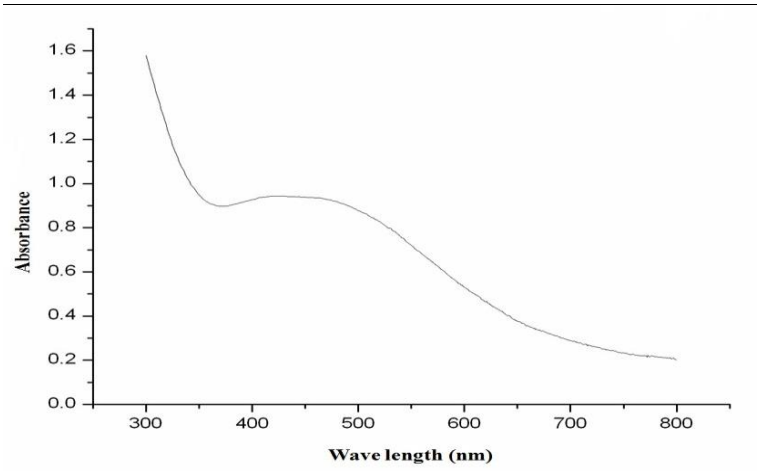


Figure (4): UV-Visible spectroscopy of silver nanoparticles produced by *F. graminurum*.

Estimation of cytokines in serum of mice:

Estimation of Interferon-γ level:

After mice are infected with *leishmania* parasite , the level of INF-γ was increased in the three weeks and after treated of these mice with pentostam and *Fusarium* AgNPs observed that the level of INF-γ has increased in all groups compared with the negative control group, shown in (Table 1) significant differences ($P<0.01$) between all groups compared with control. In control positive group

the level of cytokine was elevated from the first week was (183 ± 13.44 pg/ml) then continued elevated reached in the second and third week (239.17 ± 4.35 and 323.17 ± 14.79 pg/ml). While the pentostam group was observed to increase occurred in the first week (305.17 ± 40.76 pg/ml) then became decreased in the second week (301.83 ± 49.94) and retained increased in the third week (315.67 ± 61.46 pg/ml) . In AgNPs group the level of INF-γ has increased gradually over the three weeks (284.17 ± 42.97Pg/ml , 303.83 ± 51.98 Pg/ml and 410.00 ± 10.52 Pg/ml) respectively. In P/AgNPs group gradual decrease occurred since the first week (284.17 ± 42.97 pg/ml) , and gradually continues to decline in second and third weeks (218 ± 36.63 pg/ml and169.50 ± 8.41 pg/ml) respectively.

Table (1): The serum level of INF-γ in the experimental groups after three weeks (Mean ± SD).

The Groups	Time (day)			LSD value
	7	14	21	
Control(–ve)	115.67 ± 4.80	118.33 ± 5.04	123.33 ± 1.63	18.542 NS
Control(+ve)	183 ± 13.44	239.17 ± 4.35	323.17 ± 14.79	31.602 **
Pentostam	305.17 ± 40.76	301.83 ± 49.94	315.67 ± 61.46	26.524 NS
AgNPs	122.50 ± 8.87	303.83 ± 51.98	410.00 ± 10.52	37.277 **
P/AgNPs	284.17 ± 42.97	218.00 ± 36.63	169.50 ± 8.41	31.842 **
LSD value	42.668 **	47.073 **	61.905 **	---

** **mean a significant difference at P<0.01 , NS: Non-Significant.**
Means having with the different small letters in same column differed significantly.

Estimation of Interleukin–10 level:

The serum levels of IL-10 in treated groups of the present study were high level in groups pentostam and *Fusarium* AgNPs infected with *leshmania* parasite were (338 ± 16.07 and 467.83 ± 34.43) respectively in the third week. While the level of IL-10 in control positive group was lower level (320.67 ± 6.37 pg/ml) in third weeks compared with other control negative group was (97.67 ± 6.62 pg/ml). As well as in the P/AgNPs group also elevated, but less than other treated groups in the third week (336.17 ± 22.06 pg/ml) as illustrated in (Table 2). There was significantly different ($p \leq 0.01$) between all groups compared with positive control.

Table (2): The serum level of IL-10 in the experimental groups after three weeks (Mean ± SD).

Groups	Time (day)			LSD value
	7	14	21	
Control(-ve)	95.17 ± 2.31	96.33 ± 1.97	97.67 ± 6.62	11.547 NS
Control(ve+)	178.50 ± 8.87	232.50 ± 5.89	320.67 ± 6.37	28.761 **
Pentostam	290.83 ± 23.31	451 ±20.53	338.00 ± 16.07	32.804 **
AgNPs	112 ± 8.09	456 ±26.61	467.83 ± 34.43	41.952 **
P/AgNPs	121.83 ± 7.46	141.50 ± 2.88	336.17 ± 22.06	30.776 **
LSD value	27.336 **	42.602 **	41.735 **	---

** **mean significant difference at P<0.01 , NS: Non-Significant.**
Means having with the different small letters in same column differed significantly.

Disscussion:

The synthesis of silver particles by using *Fusarium graminarum* was observed during change the color of the mixture from colorless to blackish brown, the color change confirmed the formation of nanoparticles, these results corresponding with Mahmoud *et al.* (2013); Bawaskar *et al.* (2010)(13,14).

In this study, the results are near to results of Shafiq *et al*. (2016)(15) showed that, the XRD diffraction measured in Ag-NPs resulted in four intense peaks and this further confirms that the Ag-NPs formed in the extracellular filtrate are present in the form silver nanocrystals. The surface morphology of nanoparticles examined by AFM, the AFM is a very good technique for measuring surface morphology and fine structure of nanoparticles (16). Vijayan *et al.* (2016)(17) observed that AFM topology is very helpful in revealing the exact size and shape of silver nanoparticles. The results corroborate those of previous studies such as Birla *et al.* (2013)(18) showed that absorbance peak around 420 nm, which is specific for the SNPs.

The results of cytokine indicated an increase occurs in serum level of IL-10 and INF-γ during three weeks post-infection as showed by Kamil *et al.* (2013)(19) reported a significantly increased serum level of IFN-γ and IL-10 in VL patients compared to healthy controls, also confirmed by Khoshdel *et al*. (20) reported that the levels of the serum cytokines, IL-10, IL12, and IFN-γ were higher in patients than in family members and control individuals.

Also, in a previous study demonstrate the elevation of both IFN-γ and IL-10 mRNA levels in the lesional environment of the bone marrow in patients with kala-azar before therapy, findings which may be of importance in understanding how this organism is able to avoid immune-mediated destruction by its host macrophages (21). Inactive visceral leishmaniasis, Al-Autabbi *et al.* (22) reported that the immune system is highly activated and produce both the macrophage-activating cytokines IFN-γ and the macrophage-deactivating cytokines IL-10.

In a typical Th1 and Th2 responses, besides IFN- γ, other cytokines such as IL-10, IL-12 and IL-13 have been shown to be important factors in the regulation of immune responses (23).

IL-10 has been systematically linked to the VL pathogenesis, this cytokine has been considered as a key regulatory cytokine in VL due to its pleiotropic effects associated with suppression of microbicidal functions in infected macrophages (24). IL-10 is able to inhibit Th1 cell and macrophage activation; therefore, higher levels in the sera of VL might be expected, IL-10 also plays an important role in regulation of inflammatory response, and is important for the survival and persistence of the parasite inside macrophages (25). In other reports showed that, the data favor a role for IL-10 in conditioning the host cells so that they become poorly responsive to even high levels of IFN-γ for intracellular killing (26). The current results showed that with active VL produced increased levels of IL-10, which is in agreement with study Gatta *et al.* (27) reported that, the levels of IL-10 detected pre-treatment were associated with the expression of toll like receptor2 (TLR2).

IFN-γ plays an essential role in macrophage-mediated antileishmanial

activity, contributing to parasite elimination and the subsequent resolution of infection (28). Also, IFN-γ cytokine is the main factor in inducing the transcription of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) (29). IFN-γ causes tyrosine kinase phosphorylation, Janus kinase (JAK1 and JAK2), and subsequently the phosphorylation and dimerization of signal transducer and activator of transcription (STAT), activated STAT migrates to the nucleus and binds to iNOS promoter sequences finally resulting in NO production (30). Bhowmick *et al.* (31) showed that silver nanoparticles loaded quaternized PVA hydrogel acts as a reservoir of silver nanoparticles, which helps in maintaining a sterile environment for longer time duration by releasing Ag nanocrystallite in a sustained manner. gallium nanoparticles exhibited better antibacterial properties against *Pseudomonas aeruginosa* and lower *in vitro* cytotoxicity for human lung fibroblasts IMR-90 and mouse fibroblasts L929 (32) .

Nanoparticles can undergo a series of processes, including binding and reacting with proteins, phagocytosis, deposition, clearance, and translocation. At the same time, nanoparticles can elicit a spectrum of tissue responses, such as cell activation, generation of reactive oxygen species, inflammation, and cell death (33). After treating infected mice with *Fusarium* AgNPs and compartion with pentostam drug for three weeks, showed increase occur in the level of IL-10 and INF-γ as marker to enhance humoral and cellular immune response of mice against infection when treated with *Fusarium* AgNPs and pentostam drug. A significant increase in the level of IFN-γ was detected in the serum of patients during treatment with pentostam when compared to its level before treatment, this explains that a successful drug therapy were restored T-cell proliferation and IL-2, IFN-γ production in response to *Leishmania* antigen (21).

Conclusion:

Silver nanoparticles synthesis from *Fusarium graminarum* is safety, nontoxic and it can be considered as a new antileishmanial agent. Also *Fusarium* AgNPs lead to induce pro and anti-inflammatory cytokines.

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