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Original Article

Fusarium oxysporum; its enhanced entomopathogenic activity with acidic silver nanoparticles against *Rhipicephalus microplus* ticks

Fusarium oxysporum e sua atividade entomopatogênica aprimorada com nanopartículas de prata ácida contra carrapatos *Rhipicephalus microplus*

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Abstract

Fusarium oxysporum is an entomopathogenic fungus, and it has anti-biological activity against arthropods. Ticks are blood sucking arthropods which are responsible for transmitting different diseases in humans and animals. The use of chemical insecticides against ticks is not eco-friendly option and results in the development of acaricide resistance. Previously, we had cultured a local isolate of *Fusarium oxysporum* from soil samples which were identified through microscopy and confirmed through molecular technique. In our previous experiments, we have prepared Silver nanoparticles (AgNP) at pH 7 and they had been characterized through X-Ray Diffraction (XRD), UV-visible and zeta-potential. In our current study, the AgNP were prepared at different pH conditions and characterized through Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The protein molecules of *F. oxysporum* were charged with Ag ions. *F. oxysporum* NP were observed to enhance anti-biological activity by killing *Rhipicephalus microplus* and they caused 100% mortality at pH 4 and pH 5 in 24 h in anti-tick biological assay. Our study is the first report to do biological assay against *Rhipicehalus* ticks by using Fusarium AgNP at acidic pH. Biological control using entomopathogenic fungi can be the best alternative of the chemical method to control the tick population.

Keywords: *Fusarium oxysporum*, entomopathogenic fungus, silver nanoparticles, fungal nano-particles, *Rhipicephalus microplus*.

Resumo

Fusarium oxysporum é um fungo entomopatogênico com atividade antibiológica contra artrópodes. Os carrapatos são artrópodes sugadores de sangue responsáveis pela transmissão de diversas doenças em humanos e animais. O uso de inseticidas químicos contra carrapatos não é uma opção ecologicamente correta e resulta no desenvolvimento de resistência acaricida. Anteriormente, havíamos cultivado um isolado local de *Fusarium oxysporum* a partir de amostras de solo que foram identificadas por microscopia e confirmadas por técnica molecular. Em nossos experimentos anteriores, preparamos nanopartículas de Prata (AgNP) em pH 7 e elas foram caracterizadas por Difração de Raios X (XRD), UV-visível e potencial zeta. No presente estudo, os AgNP foram preparados em diferentes condições de pH e caracterizados através de Microscopia Eletrônica de Varredura (MEV) e Microscopia Eletrônica de Transmissão (TEM). As moléculas de proteína de *F. oxysporum* foram carregadas com íons Ag. Assim, observou-se que *F. oxysporum NP* aumenta a atividade antibiológica matando *Rhipicephalus microplus* e causando 100% de mortalidade em pH 4 e pH 5 em 24h no ensaio biológico anticarrapato. Este estudo é o primeiro relato de caso a realizar um ensaio biológico contra carrapatos *Rhipicehalus* usando *Fusarium AgNP* em pH ácido. Nesse sentido, é possível concluir que o controle biológico utilizando fungos entomopatogênicos pode ser a melhor alternativa do método químico para controlar a população de carrapatos.

Palavras-chave: *Fusarium oxysporum*, fungo entomopatogênico, nanopartículas de prata, nanopartículas fúngicas, *Rhipicephalus microplus*.

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1. Introduction

Nanoparticles (NP) generally contain 20-15000 atoms and are considered fundamental molecular building blocks for nanotechnology (Zhao et al., 2014). NP synthesis and its potential exploration are of great interest for the use in various applications in optics, electronics, and biomedical sciences (Becker, 1999; Colvin et al., 1994; Crabtree et al., 2003). NP possess the unique properties of optical, chemical, magnetic as well as mechanical nature (Khan et al., 2019). Compared to the large particles, these particles have a relatively high fraction of atoms and a wide surface area to volume ratio (Tang and Zheng, 2018). The NP serves as a link or bridge between the bulk materials and the molecular and atomic structures (Chakraborty and Pradeep, 2017). The nano-technology overlaps different disciplines, so it is easy to rebuild the novel experimental protocols in the synthesis of NP which are safe, reliable, and eco-friendly (Ray, 2010). NP are majorly categorized into two main types, namely organic and inorganic. Organic NP include carbon NP, while, inorganic NP comprise noble metal NP (e.g., Au and Ag), semi-conductor NP (TiO_2 and ZnO_2), and magnetic NP (Komada, 1975; Nagajyothi et al., 2020; Teixeira et al., 2018; Wahid et al., 2020). Because of their adherent functional versatility and material superiority, inorganic NP are widely used in biological sciences (Giner-Casares et al., 2016). The silver nanoparticles (AgNP) are the most promising as they show good antibacterial and antimicrobial properties (Kostadinova et al., 2009; Zhao and Stevens Junior, 1998). During the last two decades, metal NP synthesis and its application emerged as a prime research topic in the modern material sciences (Roco, 2003). These nano-crystals have been employed in sensitive bio-molecular detection, therapy, diagnostic techniques, anti-microbial, and catalysis processes (Colvin et al., 1994; George et al., 2004; Govindarajan et al., 2005; Samish et al., 2001; Wang and Herron, 1991). Because of the anti-microbial properties of silver nanoparticles, they are widely used in the medical industry (Crabtree et al., 2003). Silver-impregnated polymers and AgNP are widely used to prevent bacterial infection in open and burn wounds (Jiang et al., 2004; Rai et al., 2009). Silver embedded fabrics are also used as supporting material in the textile industry (Durán et al., 2007). The earlier synthesis methods involved in the NP were based on physical and chemical processes. These methods have some shortcomings, such as high-temperature requirement causing more expenditure of energy, need for radiations, employment of toxic chemicals that usually result in the liberation of hazardous by-products and (Dolgaev et al., 2002; Evanoff and Chumanov, 2004; Jiang et al., 2004; Kabashin and Meunier, 2003; Komada, 1975). These methods also required specialized apparatus. While, working with biological systems like fungi is simple and the fungi can synthesize NP extracellularly and intracellularly (Zhang et al., 2020). The biological systems used include microorganisms, including bacteria, fungi and plants (Bar et al., 2009; Mishra et al., 2003). In comparison to the bacterially synthesized NP, myco-synthesized AgNP have several merits. These include tolerance for high metal concentration in the medium, reliable and easy large-scale production, better dissemination of NP (Abdel-Aziz et al., 2017). The amount of protein expressed by the fungi is much higher than that of a bacterial system (Dyal et al., 2006). The filtration of fungi is conveniently done by using the simple filtration technique without using any standard equipment which minimizes the investment and energy consumption. (Devi And Joshi, 2015). pH is one of the key factors playing a crucial role in NP synthesis. For silver NP production by *Guignardia mangiferae*, pH 3 to 10 were set to see the development of color (Balakumaran et al., 2015). While, Qian et al. (2013) have shown that alkaline pH favored the AgNP synthesis when 1 mM silver nitrate was challenged with the cell free filtrate of *Epicoccum nigrum*. Among the different concentrations of silver nitrate tested, 1 mM concentration very much facilitated the AgNP synthesis with good monodispersity (Qian et al., 2013). Ticks are a group of arthropods that are notorious for transmitting a wide range of viral and parasitic organisms (Mbanzulu et al., 2020). *Rhipicephalus (Boophilus) microplus* is a common cattle tick which is one of the important specie of ticks responsible for spreading diseases in the cattle globally (Jonsson, 2006) and likewise in Pakistan (Farooqi et al., 2017). It is responsible for blood loss due to its hematophagous behavior and one engorged female tick can engulf 0.5 ml of blood from the host (Urquhart et al., 1996) and it also plays a vital role in transmitting protozoal, bacterial and viral diseases (Teglas et al., 2005). The control of cattle ticks chiefly depends upon pour-on applications of acaricides and systemic inoculation of chemotherapeutic drugs like Ivermectin (Li et al., 2007) but the drug resistance has been developed due to repeated and/or irrational use of these drugs (Klafke et al., 2012; Miller et al., 2007; Olivares-Pérez et al., 2011). The development of resistance and presence of chemical residues in meat, milk and the environment has prompted interest in finding new, less toxic substances to control ticks (Zaman et al., 2012). It also causes bioaccumulation, unbalancing the ecology as inducing bio-magnification in organisms of higher tropic levels in the food chain that affects the non-target animals and mammals (Dalkvist et al., 2009; Schauber et al., 1997; Yadav, 2010). The contamination of the water bodies such as ponds and the environment can indirectly affect human beings through its indirect source (George et al., 2004; Harris et al., 2010; Polson et al., 2011). The biological control of ticks through entomopathogenic fungi is an alternative control strategy using *Beauveria* (Perinotto et al., 2012) and *Metarhizium* (Gindin et al., 2002) against *Rhipicephalus microplus* (Angelo et al., 2010). Keeping in view the development of insecticidal resistance in ticks, the current study has been designed to isolate indigenous *Fusarium oxysporum* fungus for the preparation of NP and to investigate its anti-biological efficacy for the control of ticks.

2. Materials and Methods

2.1. Fungal and tick cultures

We have previously isolated and cultured *Fusarium oxysporum* (Sumera et al., 2021). Briefly, the soil samples

were passed through a fine sieve of up to 200µm capacity. Then, the aliquot of the sieved sample was spread over potato dextrose agar (PDA) and broth (PDB) at 27 °C for seven days in a shaker incubator (Komada, 1975). After the incubation, the fungal biomass was sieved through a sterilized cheese-cloth and washed twice using sterile DW to remove the excess medium components. 10 g of fungal biomass (wet weight) was mixed in 100 ml sterile double DW in an Erlenmeyer flask and incubated in a shaker incubator for 48 h at 120 rpm and 28 °C. The aqueous solution components were again filtered with Whatman® filter paper no.1 to get the mycelium-free filtrate. This mycelium-free filtrate was placed in a conical flask and mixed with 1 mM $AgNO_3$ (0.017g/100 ml) as the final concentration for reducing AgNP. The mixer was again incubated in the shaker incubator under light at 28 °C and 120 rpm. The mycelium-free filtrate without $AgNO_3$ was considered to serve as a control and kept under the same conditions (28 °C and 120 rpm). After 24 h, the change in color of the reaction mixture treated with $AgNO_3$ was observed, which was treated with $AgNO_3$. After 120 h of incubation, the AgNP turned into a brownish yellow color solution. The AgNP solution was stored in vials at 4 °C until further characterizations. The above protocol is shown in a flow diagram in Figure 1.

The ticks were collected from the experimental calves reared in the department of Parasitology, UVAS, Lahore. The ticks were identified as *Rhipicephalus microplus* under stereomicroscope by using the key (Foreyt, 2013) as shown in Figure 2. The ticks were cultured in BOD incubator at 28 °C and 80% humidity (Monteiro et al., 2020) as shown in Figure 3.

2.2. SEM analysis

The biosynthesized AgNP were investigated using SEM (Scanning electron microscopy). The morphology and

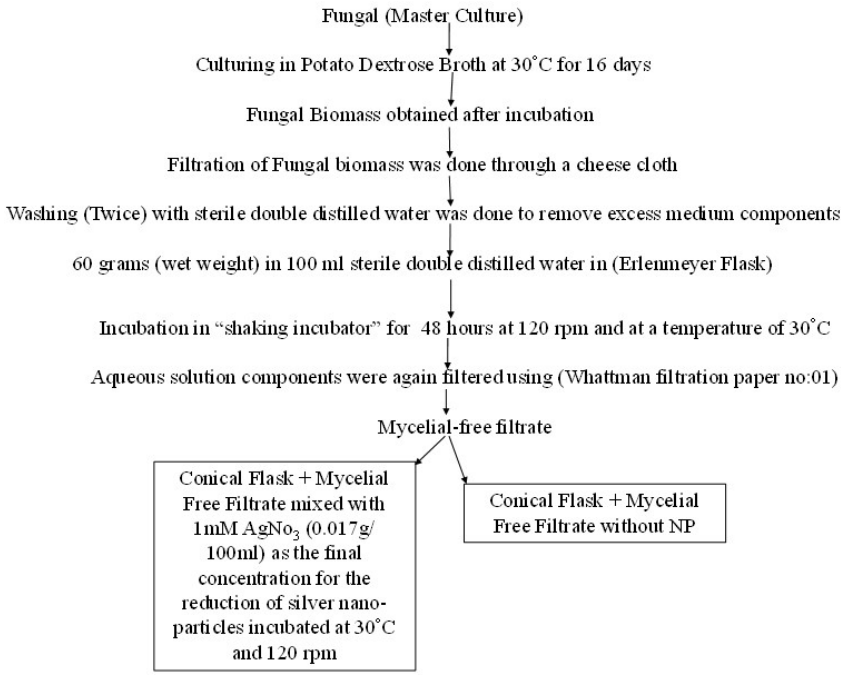

Figure 1. Flow Diagram of Entomopathogenic Fungus *(Fusarium oxysporum)* culture.

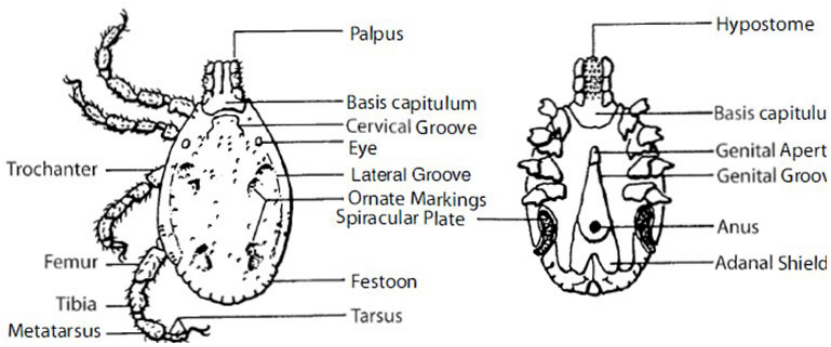

Figure 2. Morphology of ticks (Foreyt, 2013).

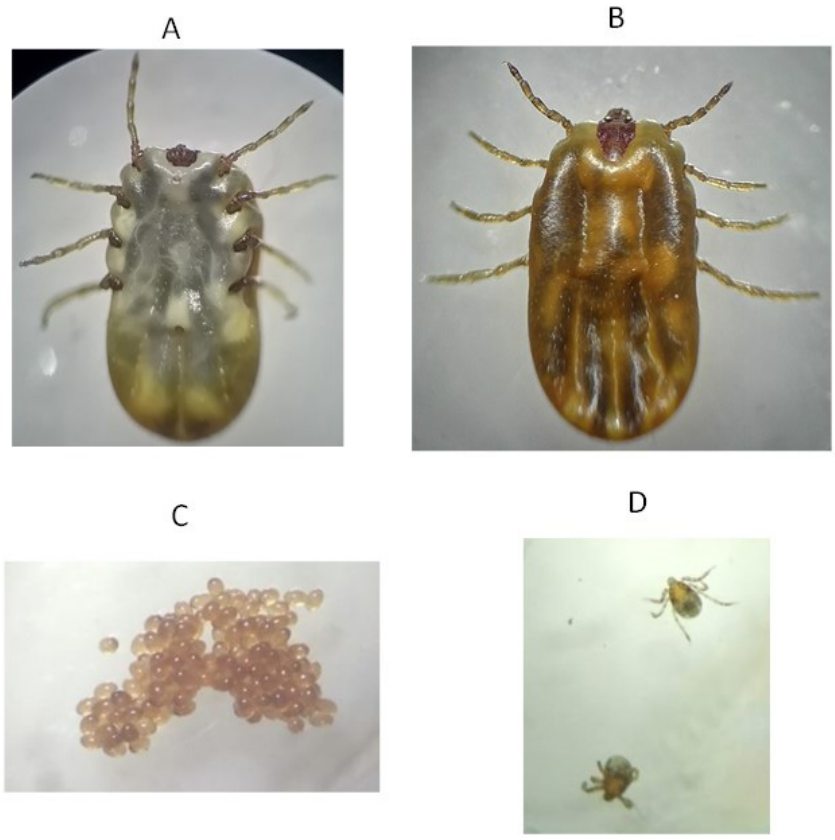

Figure 3. Culture of *Rhipicephalus* ticks. Adult tick with its ventral view (A), Adult tick with its dorsal view (B), Eggs of the tick (C), Larvae of the ticks (D).

nanostructure of AgNP were analyzed with Nova Nano SEM 650 at 10 KV. The SEM micrographs were taken at 80,000x magnification. For SEM imaging, a thin film of AgNP was prepared by drop coating of purified AgNP from prepared solution onto carbon-coated copper SEM grid. The grid was allowed to evaporate for 5 min. The surplus sample was removed using a blotting paper.

2.3. TEM analysis

TEM measurements were taken to determine the shape and morphology of AgNP. TEM was performed on Hitachi HT7800 instrument with an accelerating voltage of 120 kV. After the synthesis of AgNP, a drop of the sample containing AgNP was placed on a carbon-coated copper grid and was kept under Infrared lamp for 6 minutes to dry the sample. Extra solution was removed using a blotting paper before holding the grid onto the specimen holder. Then the grid was scanned at different magnifications for the observation of AgNP.

2.4. Anti-tick Assay

A total of 9 groups were formed. There were 3 control groups and 6 experimental groups of pH: 04 to pH: 09. There were 2 replicates in each experimental group containing 6 ticks per group/subgroup with fungal culture alone or in combination with NP as shown in Figure 4.

3. Results

3.1. Culture and myco-synthesis of Fusarium NP

Crystal white growth of *F. oxysporum* was observed on the broth of the medium. The filtered fungal residues were apparent (without NP and dark brown (with NP) after incubation at 28 °C for 48 h.

After incubation of 72 hours, the Nano-particles were used to check the lethal effect on the ticks. Optical density values were taken after every 24 h for 96 h at different pH as shown in Figure 5.

3.2. SEM analysis

The SEM micrographs of biosynthesized AgNP synthesized at different pH reaction conditions show well defined spherical nanoparticles which are smooth, isotropic (i.e with low aspect ratio) and monodispersed. It is observed from the micrographs that pH of filtrate effects the size of AgNP. The observed size of AgNP decreases with increasing initial pH of fungal filtrate. More uniformity was observed in size and shape of nanoparticles with increasing pH. The micrographs show no agglomeration of nanoparticles but the particles are well dispersed in the solution showed intense stabilization of AgNP. The average particle size and its size ranges obtained at different pH

reaction conditions were calculated by SEM micrographs with the help of Image J software. At pH 4, 5, 6 and 7, the average sizes of the AgNP were 88 nm, 52 nm, 38 nm and 13 nm, respectively. The particle size histogram of AgNP is shown in Figure 6.

3.3. TEM analysis

TEM micrographs of synthesized AgNP by using 1 mM AgNO3 with different initial pH conditions has been shown in Figure 7. The AgNP formed were spherical in shape.

3.4. Anti-tick biological activity

The results of biological activity of Fusarium NP are shown in Table 1 and Figure 8. All the ticks were dead in the groups treated with AgNP prepared in acidic media and the control positive group treated with cypermethrin. Dead ticks were also found to have fungal growth on them.

Experiment of Ag NPs on Ticks of *Rhipicephalus anatolicum*:
Control Group:

1. Cipermethrin
2. Distilled water
3. Ticks only

Experimental Group:

pH: 4	***Negative (without AgNPs)***
	AgNPs Particles
pH: 5	***Negative (without AgNPs)***
	AgNPs Particles
pH: 6	***Negative (without AgNPs)***
	AgNPs Particles
pH: 7	***Negative (without AgNPs)***
	AgNPs Particles
pH:8	***Negative (without AgNPs)***
	AgNPs Particles
pH:9	***Negative (without AgNPs)***
	AgNPs Particles

Figure 4. Set-up of anti-tick assay.

4. Discussion

Myco-synthesis of the intercellular or extracellular synthesis of silver nanoparticles is represented by trapping of Ag+ ions on the surface of entomopathogenic fungal cells and subsequent reduction of silver ions by the enzymes present in the fungal biomass (Mukherjee et al., 2001b) of *Fusarium oxysporum* (Kumar et al., 2015; Mohanpuria et al., 2008). Organic molecules such as enzymes and acids are responsible for the formation of spherical crystalline AgNP (Balaji et al., 2009) that enhanced biological activity of the fungus against ectoparasites like ticks.

Previously, *Fusarium oxysporum* derived AgNP have enhanced entomopathogenic activity against *Culex* mosquito larvae. We have isolated a fungus from the soil in Lahore, Pakistan through PCR, which has phylogenetic similarity with the strain of *Fusarium oxysporum* isolated from the body of *Aedes aegypti.* Moreover, we have characterized our NP through UV-Vis, SEM, DLS, ZP and FTIR. We have observed 100% mortality of Aedes mosquitoes larvae at 17 h and 24 h with AgNP and without AgNP by using 1 ppm concentration, respectively (Sumera et al., 2021).

In anti-tick biological assay with Fusarium AgNP, we achieved 100% mortality with conditions of pH 4 and pH 5 in 24 h in comparison with control positive but we did not achieve any mortality of ticks with the fungal derived AgNP prepared in pH 7 even though the size of the nanoparticles were smaller at pH 6 and 7 than at pH 4 and 5. The size of the nanoparticles were found <100 nm at pH 4, 5, 6 and 7. The acidic pH (4 and 5) was found significant in killing ticks in our experiments in 24 h duration. All ticks were dead in the experimental groups except control negative after 3 days with or without Fusarium AgNP. So, AgNP enhanced the killing of *Rhipicephalus* ticks in our experiments. Most of researchers prepared Fusarium AgNP from pH range 6 to 9 or above (Birla et al., 2013; Husseiny et al., 2015). Birla et al.,

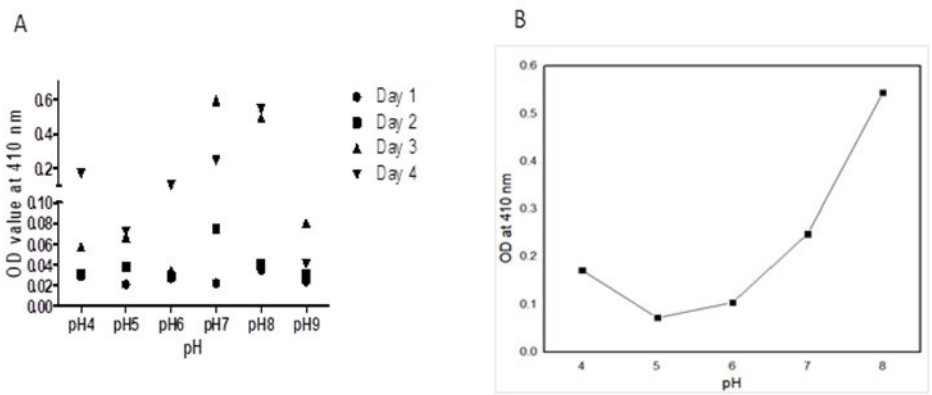

Figure 5. Values of optical density at 410 nm at different pH. OD value after every 24 h up to 96 h (A), Average OD value (B).

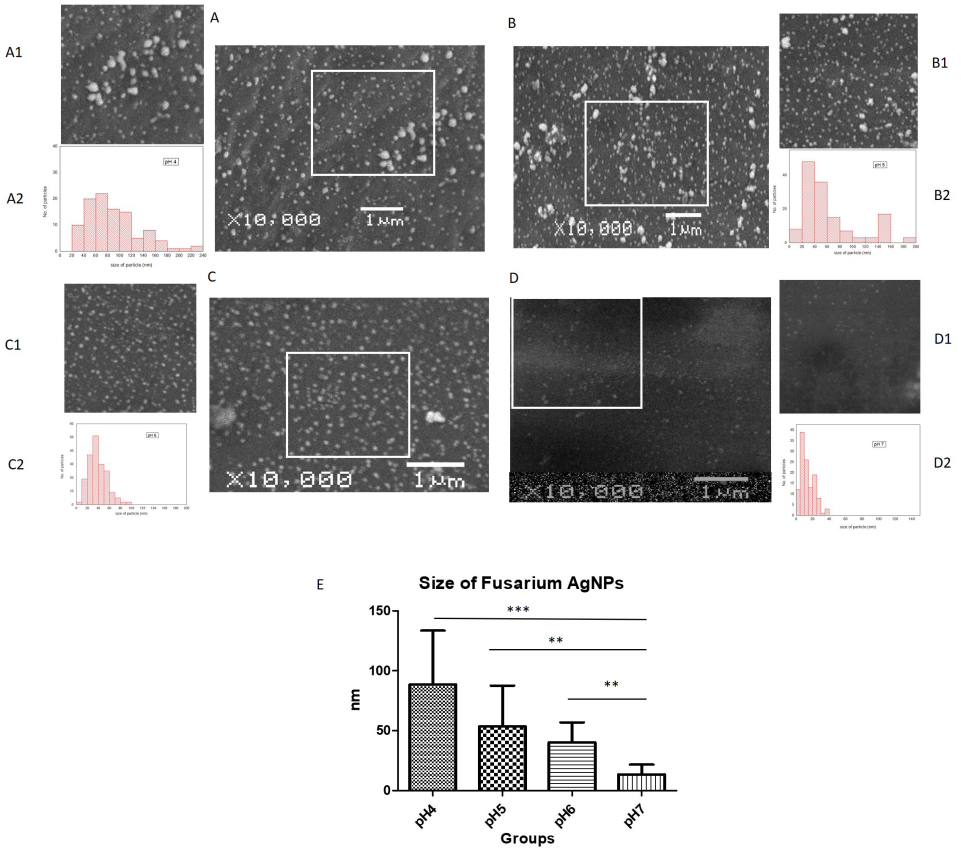

Figure 6. SEM Analyses and size of AgNP at pH 4 (A) with the area selected for particle estimation (A1) and size of particles (A2), pH 5 (B) with the area selected for particle estimation (B1) and size of particles (B2), pH 6 (C) with the area selected for particle estimation (C1) and size of particles (C2) and pH 7 (D) with the area selected for particle estimation (D1) and size of particles (D2), Size of Fusarium AgNP (E). ** indicates moderate significance and *** indicates highly significance.

Table 1. Results of anti-biological activity of Fusarium NP at different pH against ticks.

Control Group			pH: 4		pH: 5		pH: 6	
Distilled Water	**Cipermethrin**	**Ticks only**	**Negative (only filtrate)**	**Myco-AgNPs**	**Negative (only filtrate)**	**Myco-AgNPs**	**Negative (only filtrate)**	**Myco-AgNPs**
All alive. No change in movement	All dead within a few minutes	All alive. No change in movement	The fungal filtrate added/ sprayed had no effect alone on the ticks.	The nano-particle containing filtrate had lethal effect. The ticks died within a few minutes.	The fungal filtrate added/ sprayed had no effect alone on the ticks.	The nano-particle containing filtrate had lethal effect. The ticks died within a few minutes.	The fungal filtrate added/ sprayed had no effect alone on the ticks.	The sprayed solution had some effect killing some ticks.
6/6 ticks were alive.	6/6 ticks were dead.	6/6 ticks were alive.	6/6 ticks were alive.	6/6 ticks were dead.	6/6 ticks were alive.	6/6 ticks were dead.	6/6 ticks were alive.	2/5 ticks were dead.
Control Group			**pH: 7**		**pH: 8**		**pH: 9**	
Distilled Water	**Cipermethrin**	**Ticks only**	**Negative (only filtrate)**	**Myco-AgNPs**	**Negative (only filtrate)**	**Myco-AgNPs**	**Negative (only filtrate)**	**Myco-AgNPs**
All alive. No change in movement	All dead within a few minutes	All alive. No change in movement	The fungal filtrate added/ sprayed had no effect alone on the ticks.	The nano-particle containing filtrate had no lethal effect. The ticks were alive.	The fungal filtrate added/ sprayed had no effect alone on the ticks.	The nano-particle containing filtrate had no lethal effect. The ticks were alive.	The fungal filtrate added/ sprayed had no effect alone on the ticks.	The nano-particle containing filtrate had no lethal effect. The ticks were alive.
6/6 ticks were alive.	6/6 ticks were dead.	6/6 ticks were alive.	6/6 ticks were alive.	6/6 ticks were alive.	6/6 ticks were alive.	6/6 ticks were alive.	6/6 ticks were alive.	6/6 ticks were alive.

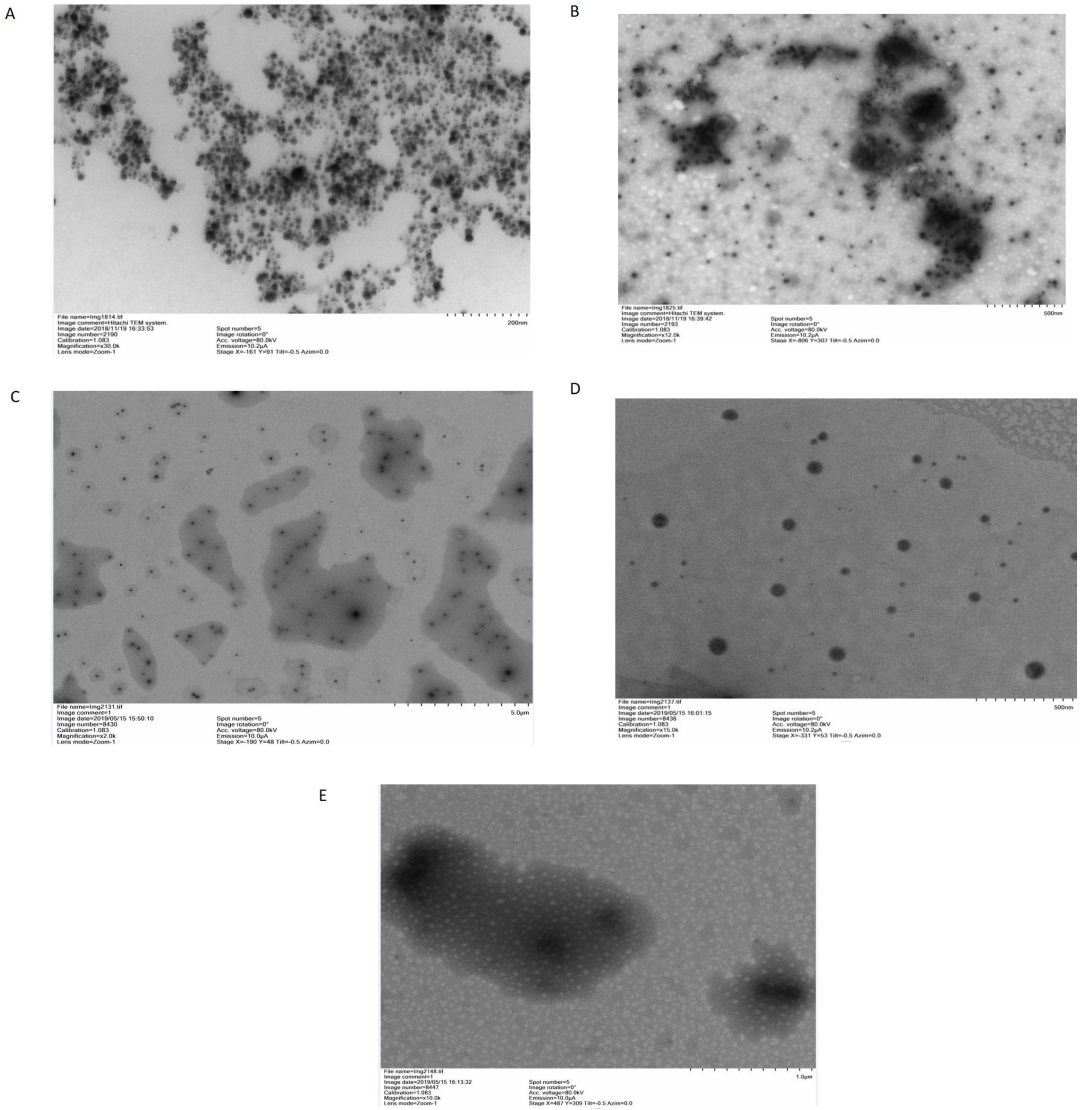

Figure 7. TEM results of Fusarium AgNP at pH 4 (A), at pH 5 (B), at pH 6 (C), at pH 7 (D) and without NP at pH 7 (E).

(2013) synthesized AgNP derived from *F. oxysporum* on malt glucose yeast extract peptone medium at pH 9–11. They found maximum synthesis of nanoparticles while, in acidic pH 3 and pH 5 aggregates were observed. At pH 7, there was less synthesis of SNPs as compared to alkaline pH (Birla et al., 2013). Husseiny et al., (2015) prepared *F. oxysporum* in Erlenmeyer flasks containing 100 ml PDA broth medium in incubator at 28 °C. After 5 days of incubation, the biomass was separated from the medium by filtration through Whatman filter paper no.1 and washed three times in sterile DW. They found pH to be an important parameter affecting AgNP synthesis in *F. oxysporum*. It was also proved that smallest size occurred at pH 6 and very less synthesis at alkaline pH (Husseiny et al., 2015). But our study is unique to report the activity at pH 4 and 5. The plausible explanation might be due to increase of the protonation in acidic condition that may influence the H-bonding (Pal et al., 2013; Yeh et al., 2020) or biomass of fungus synthesizes NP intracellularly on exposure of AgNO3 (Mukherjee et al., 2001a).

Fernandes et al. (2012) reviewed the biological activity and the mode of actions of entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* (Fernandes et al., 2012). The ability of entomopathogenic fungi to penetrate the cuticle of arthropods, make them good candidates as biocontrol agents but they are slow in killing their host, they need high humidity to germinate and sporulate (Gindin et al., 2001). Thus, the fungi can take several days to kill ticks. For instance, *M. anisopliae* takes up to a week time to kill *Rhipicephalus annulatus*, *Hyalomma excavatum* and *Rhipicephalus sanguineus* (Gindin et al., 2002). The nanoparticles formulated at acidic pH might speed up the killing process of *Rhipicephalus microplus* ticks by *Fusarium oxysporum.*

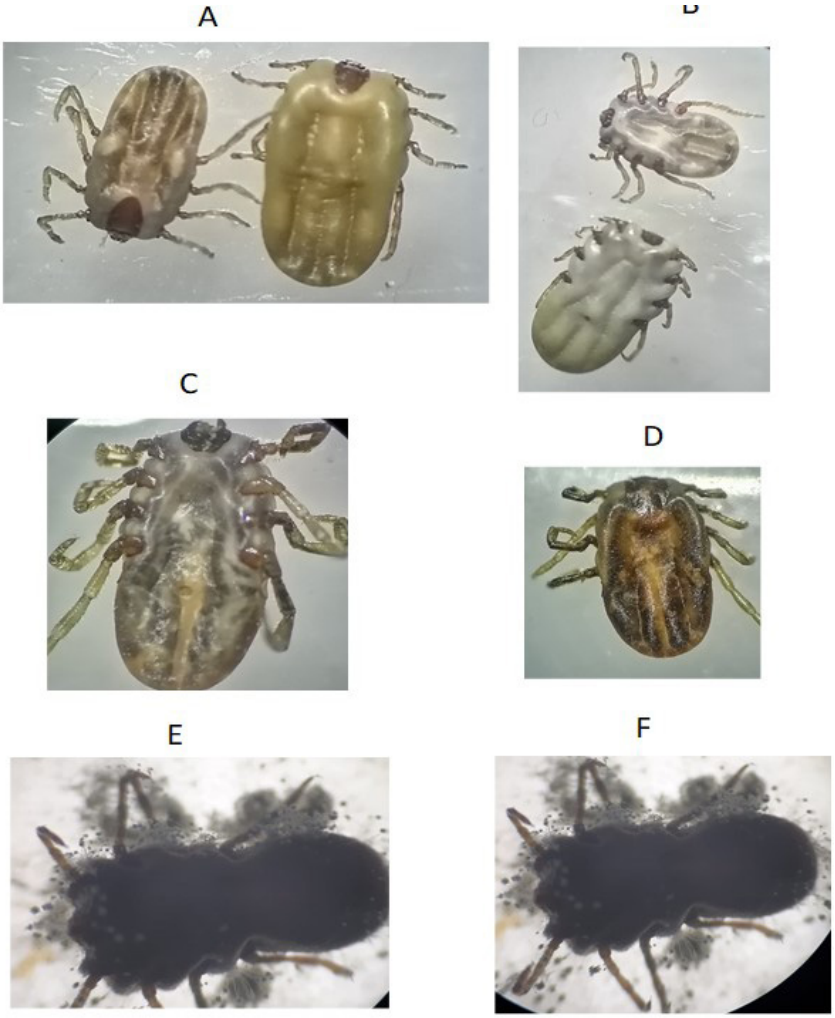

Figure 8. *Rhipicephalus microplus* ticks after the application of Fusarium NP. Live adult ticks with their dorsal view (A), Live adult tick with its ventral view (B), Dead tick with its ventral view (C), Ded tick with its dorsal view (D), Dead tick with the growth of the fungus (E and F).

5. Conclusion

It is concluded that *Fusarium oxysporum* NP cause 100% mortality with conditions of pH 4 and pH 5 in 24 h in anti-tick biological assay. Our study is the first report to do biological assay against *Rhipicehalus* ticks by using Fusarium AgNP at acidic pH.

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