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ANTIFUNGAL ACTIVITY OF ORGANICALLY SYNTHESIZED SILVER NANOPARTICLES AGAINST *Fusarium incarnatum* (Desm.) Sacc., INCITANT OF WILT IN CROSSANDRA

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ABSTRACT

In this study, we synthesized silver nanoparticles (AgNPs) using common weed *Tridax procumbense* and tested its antifungal activity against Crossandra wilt, caused by *Fusarium incarnatum* (Desm.) Sacc. AgNPs were synthesized by the reaction of 1.0 mM Ag NO_3 and 5% *T. procumbense* leaf extract. Synthesized AgNPs were first characterized and then their antifungal activity assessed against the pathogen at various concentrations using poisoned food technique. The particle size analysis using particle size analyzer, ESEM and TEM showed that the particles size ranged 17-40 nm and well dispersed, spherical with zeta potential of around 50 mV. AgNPs inhibited mycelial growth at all the concentrations tested (100 - 800 ppm). Complete mycelial inhibition was observed at 800 ppm followed by 98% at 700 ppm. Ultra-microscopic observations revealed that the nanoparticles caused hyphal abnormality and lysis in *F. incarnatum*. Further, EDX confirmed the presence of silver particles in mycelia indicating that AgNPs penetrated fungal hyphae. These studies confirmed that the method of synthesis is efficient in production of nanoparticles. Antimicrobial activity study revealed a high potential of Tridax leaf extract stabilized AgNPs.

Key words: Ag nanoparticles, antifungal activity, Crossandra, *Fusarium incarnatum, Tridox procumbense*

INTRODUCTION

Crossandra (*Crossandra infundibuliformis* L. Nees), also called 'Fire cracker', is an important commercial flower mainly grown in India. The plant is affected by several fungal diseases, of which wilt disease caused by *Fusarium* spp. is one of the major constraints in its production. Presently wilt of Crossandra is managed through soil application of benzimidazole group of fungicides. However, bulk use of fungicides not only causes environmental pollution but also induces fungicide resistance in pathogens. The development of nanodevices and nanomaterials have opened novel venture in agriculture (Scrinis and Lyon, 2007). The advances in research on nanomaterials have received special attention as a possible antimicrobial agent (Silvers, 2003). Silver and its compounds have strong inhibitory effects as well as show broad spectrum antimicrobial activities against several microbes (Silver, 2003, Cho *et al*., 2005). Compared to other metals, silver has low toxicity to mammalian cells (Zhao and Stevens, 1998). The principle mechanism of antimicrobial properties of silver nanoparticles is through anchoring and penetration of silver nanoparticles into microbial cell wall. Once inside the cell, silver nanoparticles modulate cellular signaling by dephosphorylating

putative key peptide substrates which are critical for cell viability and division (Srivastava *et al.,* 2007). In addition they cause DNA damage, leakage of intercellular substance and eventually cell death. AgNPs have been applied in the form of a wide range of healthcare products such a burn dressings, scaffold, water purification systems and medical devices (Thomus *et al.,* 2007).

Indian greeneries including flowering plants and weeds are the chief and cheap source of medicine extraction since centuries and have extensively been exploited in Ayurveda. Recently many such plants have gained importance due to their unique constituents and their versatile applicability in disease management (Priya *et al.,* 2014). Biological methods have emerged as an alternative to the conventional methods for the synthesis of nanoparticles (NPs). Synthesis of inorganic NPs by biological systems makes them more biocompatible and environmental benign. Biologically synthesized AgNPs have wide range use owing to their remarkable physico-chemical properties; whereas chemical synthesis methods though simple often lead to presence of some toxic residual agents in final product and cause environmental toxicity (Singh *et al.,* 2010). The presence of biochemical compounds in leaf encapsulates the synthesized NPs and the nano size of particles is sustained by prevention of agglomeration even under dry conditions. Green synthesis provides advancement over chemical/ physical methods and is cost-effective, environment friendly and easily scaled up for large scale synthesis. Therefore, the preparation of uniform nanosized silver particles with specific requirements in terms of size, shape, physical and chemical properties are of great significance. The present study was aimed to assess the possibility of using *Tridax procumbense*, a common weed, as bio-reducing agent for the synthesis of silver nanoparticles and then use silver nanoparticle as an antifungal agent against crossandra wilt pathogen *F. incarnatum.*

MATERIALS AND METHODS

The experiment on synthesis of silver nanoparticles and their characterization was carried out at the Department of Nano-Science and Technology, TNAU, Coimbatore (India) while experiment on antifungal activity of nanoparticles was conducted at the Department of Plant Pathology, Agricultural College and Research Institute, TNAU, Madurai (India) during 2013 to 2014. The chemical used for synthesis of silver nanoparticles and silver nitrate ($AgNO_3$) were of analytical grade and procured from Sigma Aldrich, Bangalore, India.

Synthesis of silver nanoparticles

The silver nanoparticles were synthesized by the reduction of $AgNO_3$ in presence of leaf extract of *Tridax procumbense*. Fresh healthy leaves of *T. procumbense* were collected from the fields of TNAU, Coimbatore. Fresh leaves were washed with distilled water 3-4 times to remove dust. The leaves (20 g) were chopped into small pieces, mixed with 100 mL distilled water and then boiled at 65°C for 15-20 min. in a water bath. The extract was allowed to cool, filtered through Whatman filter paper No. 1 and filtrates stored at 4°C before use. Later, 50 mL 1.0 mM $AgNO_3$ boiled at 65ºC for 5-10 min. in 100 mL conical flasks and leaf extract (5-10 mL) added. The mixture was shaken with magnetic stirrer @ 150 rpm at 30ºC. The colour change of solution from greenish yellow to brown indicated the formation of Ag nanoparticles (AGNP). The synthesized AgNPs were extracted from the solution by centrifugation at 12,000 rpm for 15 min. followed by evaporation of moisture.

Characterization of synthesized nanoparticles

UV-VIS spectroscopy analysis: UV-Vis spectral analysis was done by using Elico UV-Vis spectrophotometer. The reduction of pure Ag^+ ions was monitored by measuring UV-Vis spectrum of reaction medium after diluting a small aliquot of sample into distilled water (Mulvaney, 1996).

Particle size analyzer: Particle size and the distribution pattern of synthesized sample suspension were determined using Horiba Scientific Nanopartica SZ-100 (Nanoparticle Analyzer, Japan). A

sample (0.5 mg) was dispersed in 20 mL distilled water, sonicated for 15 min. and suspension analyzed under dynamic light scattering method using 90º or 173º at 25ºC (Brien *et al*., 1995).

Zeta potential: Zeta potential of synthesized organic NPs was determined using particle size analyzer (Horiba, SZ-100). In zeta analyzer, laser light is divided in to two beams as input light and reference light. Scattered light by sample particles and reference light modulated by modular interfere in the prism are detected and detected signals changed in to digital signal are calculated (Melendrez *et al*., 2010).

Environmental scanning electron microscope (ESEM)

The SEM model FEI QUANTA 250 was used to characterize the size and morphology of nanoparticles. The ESEM which works under low vacuum helps to image the fresh unprepared biological sample. The samples of test nanoparticles (0.5-1.0 mg) were dusted on one side of double sided adhesive carbon conducting tape and then mounted on 8 mm dia. aluminium stub. Sample surfaces were observed at different magnification and images recorded (Tuoriniemi *et al.,* 2013).

Transmission electron microscope (TEM)

TEM FEI TECHNAI SPIRIT was used to analyze the samples. Dilute suspensions of nanoparticles (0.05 mg) in pure ethanol (15 mL) were prepared by ultra-sonication. A drop of suspension was placed on 300-mesh lacy carbon coated copper grid, dried and images recorded at various magnification.

Antifungal activity of silver nanoparticles

In vitro assay for antifungal activity of AgNPs was carried against pathogen *Fusarium incarnatum* as per poisoned food technique (Nene and Thapliyal, 1993**)**. PDA medium amended with various concentrations (100, 200, 300, 400, 500, 600, 700 and 800 ppm) of synthesized AGNPs. The PDA medium without nanoparticles served as control. A 9 mm disc of actively growing pathogen *F. incarnatum* from a 5 day old culture was placed in the centre in each of the nanoparticle amended medium as well as in untreated check. Each treatment was replicated five times and the experiment repeated twice. The mycelial growth of pathogen was recorded 10 days after incubation at 28±2ºC. The per cent inhibition of mycelial growth over control was measured to express the antifungal activity. The following formula was used to calculate per cent inhibition:

Inhibition (%) = R-r/R

Where R and r are radial growth of fungal mycelia in control plate and treated with AgNPs.

Ultra-microscopic changes on Fusarium incarnatum induced by nanoparticles

Structural abnormality, hyphal lysis and inhibition of conidial formation induced by AgNPs were examined under SEM (Model FEI Quanta 250) at various resolutions (2500 - 6000 X). The stub of SEM fixed with double side adhesive carbon tape was gently placed over mycelial mat of petri-dishes having hampered growth and removed immediately upon visual confirming for the presence of hyphae. This was fixed in appropriate location of SEM and observed for hyphal characters under low vacuum condition.

Observation of silver nanoparticles in mycelium (EDX)

The energy-dispersive X-ray (EDX) analysis is an analytical technique used for elemental analysis or chemical characterization of a sample. It was conducted by using SEM instrument to confirm the presence of Ag element in mycelium and to detect the composition of elements in nanoparticle treated mycelium (Dipankar and Murugan, 2012).

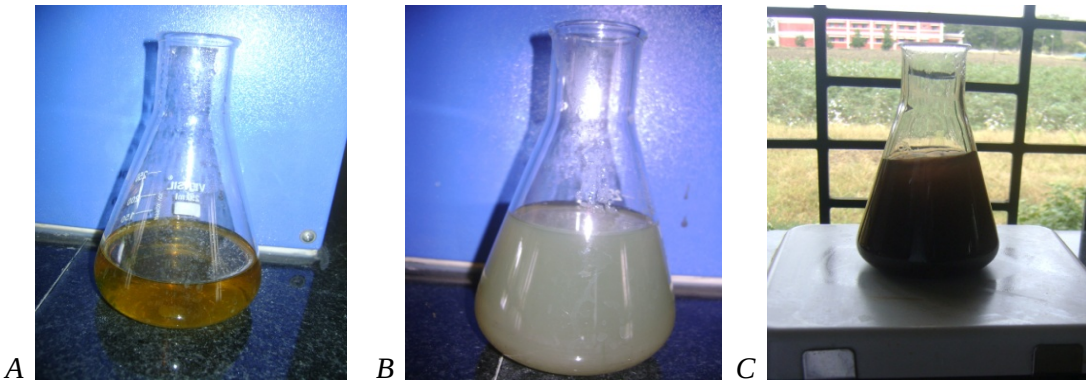
Statistical analysis

The data was analyzed by using the standard method as described by Gomez and Gomez (1984). Software OPSTAT was used for analysis purpose.

RESULTS AND DISCUSSION

Synthesis of silver nanoparticles

The organic AgNPs were successfully synthesized using standard set of protocol. The formation of AgNPs by reduction of $AgNO_3$ during exposure to Tridax leaf extract can easily be monitored from the change in colour of reaction mixture. AgNPs bear a characteristic yellow brown colour due to the excitation of surface plasmon vibration. The change in colour of reaction mixture indicated the formation of AgNPs (Fig. 1). The silver ions in reaction medium have been converted to elemental silver having the size in nanometric range. The findings are in conformity with Rajasekhara Reddy *et al*. (2010). Different compounds such as caffeine and theophylline also cause reduction leading to the synthesis of silver nanoparticles. Natural antioxidants reportedly possess strong reducing ability Rajasekhara Reddy *et al*. (2010). Dhanalakshimi *et al.* (2012) reported the synthesis of silver nanoparticles using *T. procumbense.* AgNPs can be part of novel drug delivery system in treatment of diseases but further investigation for clinical applications is necessary.



A *B* *C*

Fig. 1: Flasks showing A) Tridax leaf extract; B) $AgNO_3$ solution at 65°C; C) Change of colour to brownish yellow indicating silver nanoparticle formation

The formation of AgNPs was confirmed by UV-Vis spectrograph which showed surface plasmon resonance peak at around 417 nm indicating the presence of spherical and roughly spherical AgNPs (Fig. 2A). The absorbance peak around 400-500 nm with the use of plant leaf extracts has previously been reported in case of *Trienthema decandra* and *T. procumbense* (Dhanalakshimi *et al.*, 2012). The particle size determination by dynamic light scattering study revealed that particle size obtained from DLS were monodisperse in nature and lied in the mean range of 23.3 nm (Fig. 2B). Zeta potential analysis indicated the dispersion stability of synthesized nanaoparticle with zeta potential value in the range of 50 mV (Fig. 2C).

The surface morphology of nanoparticle examined under SEM revealed that the particles were spherical in shape with size ranging from 32 to 44 nm (Fig. 3). To confirm the results of SEM, the same particles were characterized under TEM which revealed that AgNPs were spherical and not in physical contact with each other. Their size ranged from 17 to 30 nm with mean size 24 nm (Fig. 4). The lower magnification images revealed that the nanoparticles were embedded in a dense matrix which may be organic stabilizing components of Tridax leaf extract. Similar observations have been reported by Charusheela *et al.* (2013) in case of tulsi (*Ocimum sanctum*) leaf extract.

Antifungal activity of silver nanoparticles

The antifungal activity of synthesized AgNPs against *F. incarnatum* showed inhibition in mycelial growth of pathogen at all the concentrations tested (Table 1). The various concentrations of AgNP's tested revealed maximum inhibition zone at highest concentration of AgNP. Complete inhibition was observed at 800 ppm AgNP, followed by 98%t at 700 ppm AgNP (Fig. 6). The results showed

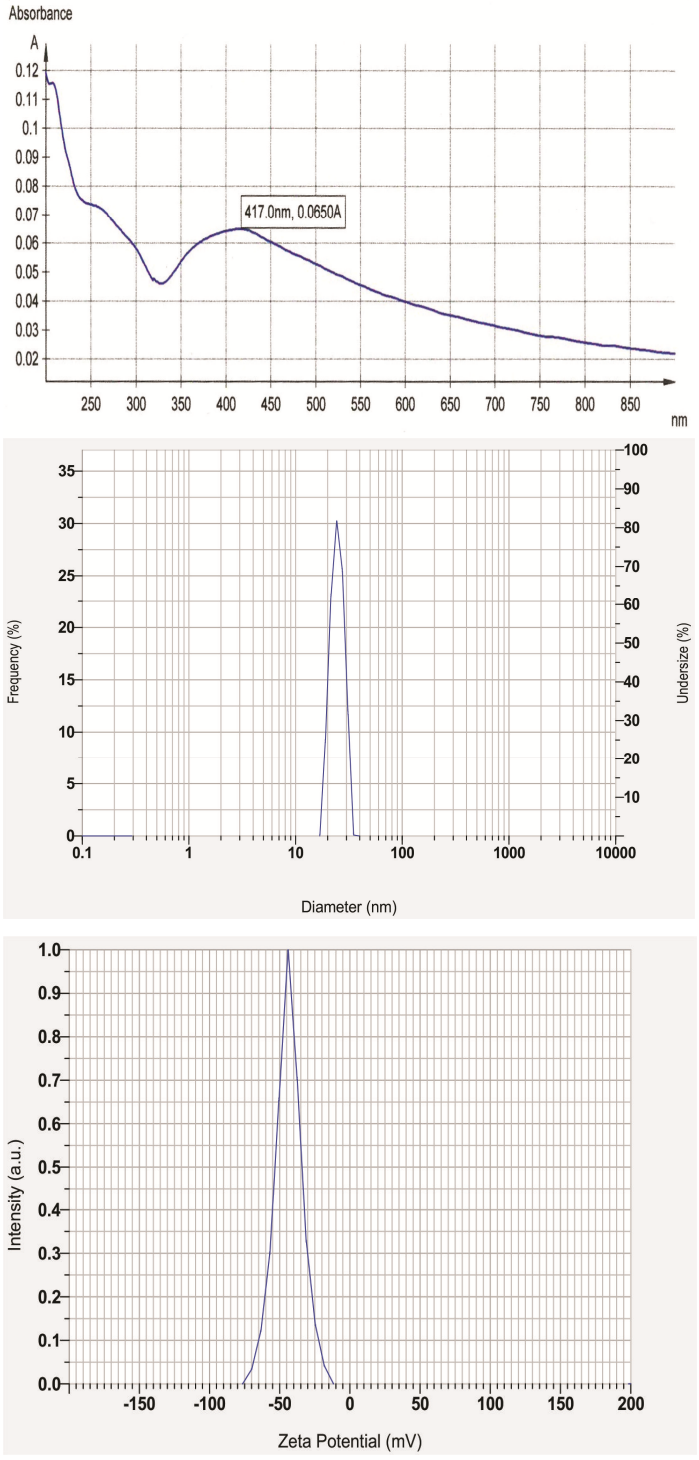


Fig. 2: Above) UV-Visible absorption spectrum; Middle) Particle size analysis graph; Bottom) Zeta potential of synthesized silver nanoparticles

the inhibitory potential of AgNP against *F. incarnatum*. The mycelial fragments from nanoparticle-treated and untreated plates on ESEM examination revealed that AgNPs damaged the hyphae (Fig. 5). In comparison the hyphae in control was normal and intact. The treated mycelia were sunken, wrinkled and damaged with deformities in mycelial growth and the shape of hyphal walls, which could have been caused due to the release of internal cellular materials resulting in shrinkage of hyphae. In nanoparticle treated plates less number of conidia those too showing deformities were produced in comparison to controlled plates. The layers of hyphal walls also tore off and many hyphae were collapsed in AgNP treatment. Mycelia in control was turbid, soft and without shrivel or damage. The observations are in agreement with Kabir *et al.* (2011) in case of *Colletotrichum* sp. in pepper. The elemental composition of AgNP treated mycelia employing EDX showed the presence of weak signal from silver atoms (0.48%) (Fig. 6) and optical absorption peak in the range of 3 to 4 keV which is a typical

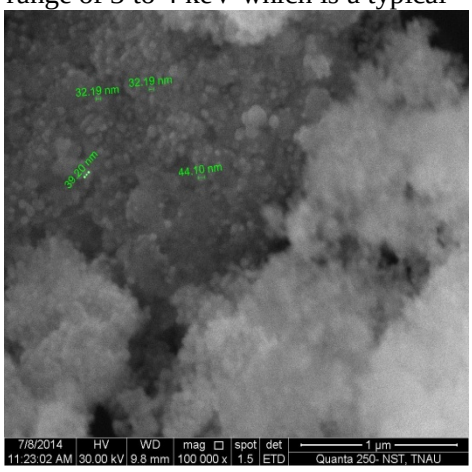


Fig. 3. SEM image of Ag-nanoparticle

for metallic silver, indicating that silver nanoparticles penetrated fungal hypha and caused hyphal abnormalities even at lower concentrations. Similar findings have been observed by Saha *et al.* (2010).

Table 1: Efficacy of silver nanoparticles against the mycelial growth of *F. incarnatum in vitro*

Ag nanoparticle conc. (ppm)	Mycelial growth at 14 DAI (cm)*	Growth reduction over control (%)
100	7.40	17.8
200	6.84	24.0
400	5.32	40.9
500	2.14	76.2
600	1.72	80.9
700	0.26	97.1
800	0.00	100
Control (0 ppm)	9.00	-
Carbendazim 500 ppm	0.00	100
$CD_{0.05}$	0.27	-
SE(m)	0.085	-

*Mean of five replications; DAI – Days after inoculation

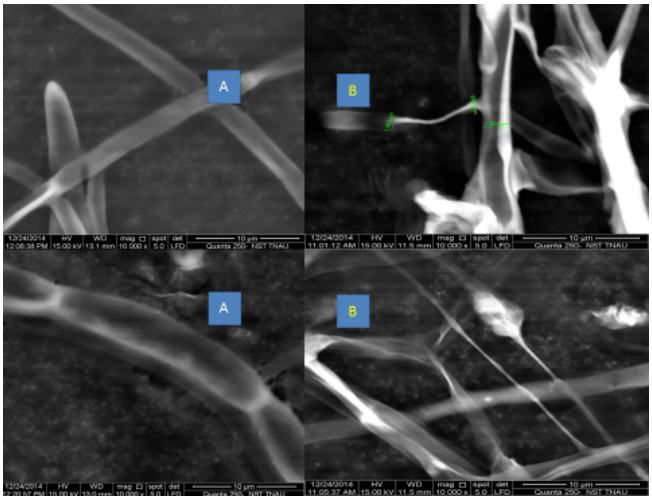


Fig. 5: Scanning electron microscopy of hyphae of *F. incarnatum;* A) Growth on PDA (water treated) B) Growth on PDA treated with Ag-nanoparticles

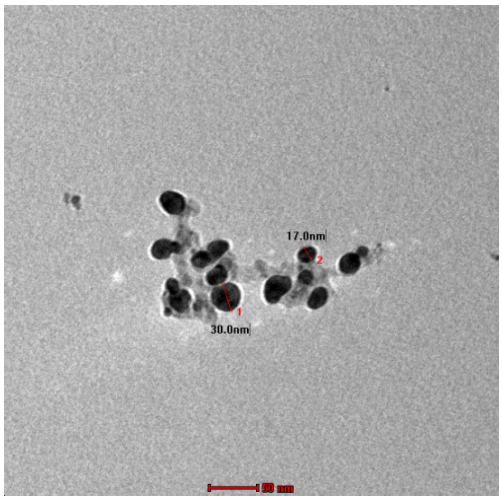


Fig. 4. TEM image of silver nanoparticles

The results suggest AgNPs to be most promising as they showed remarkable antimicrobial properties due to their large surface area to volume ratio and reduced particle size which increased the contact surface, an important condition for the effectiveness of silver (Holister, 2003). Another reason for the superiority of AgNPs is their broad antimicrobial activity. AgNP were highly reactive and generate Ag^{o} ions while metallic silver is relatively unreactive (Spacciapoli, 2001). In our study the AgNPs efficiently penetrated into microbial cells so a better attribute, especially for the organisms that are less sensitive to fungicides due to poor penetration (Thurman and Gerba, 1999). Morenes *et al.* (2005) have reported that AgNPs disrupt transport systems including ion efflux. The dysfunction of ion efflux can cause rapid accumulation of Ag ions, interrupt cellular processes at their lower concentrations such as metabolism and respiration by reacting with certain molecules. Nanoparticles believably inactivate microbial enzymes, facilitate reactive oxygen species production that causes damage to protein, lipids, and nucleic acid and ultimately leads to microbial cell death (Hwang *et al*., 2008; Allahverdiyev, 2011).

Energy-dispersive X-ray spectroscopy (*EDX) analysis*

The EDX analysis is an analytical technique used to determine elemental composition or chemical characterization of a particular material based on the signal peaks observed in graph. It relies on an interaction of some source of X-ray excitation and a sample. Its characterization capabilities are due in large part to the fundamental principle that each element has a unique atomic structure allowing unique set of peaks on its X-ray emission spectrum. As represented in graph (Fig. 6), the major elements observed in the study included carbon and oxygen which contributed to 99.52% of mycelia composition by weight and 99.98 % by atomic percentage. A very weak signal of silver (Ag) was observed which contributed 0.48% by weight and 0.02 % by atomic (Fig. 6). This study

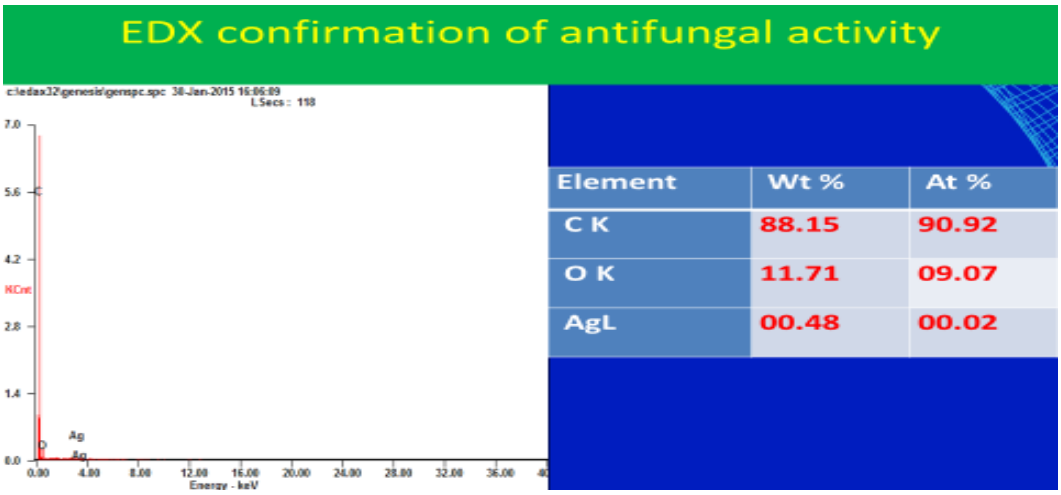


Fig. 6: EDX spectrum and elemental composition of silver nanoparticle treated mycelium of *Fusarium incarnatum;* C = carbon, O = oxygen, Ag = silver, At % = atomic percentage, Wt % = weight percentage, K,L are the cells or orbits of elements from which electron dispersion was measured in super computer of electron microscope.; Ex: C K indicates carbon concentration at K cell (orbit), each element has particular cell to measure electron dispersion during x-ray beam hit. For silver it is L (Ag L).

confirmed the penetration of Ag nanoparticles into the fungal body, because Ag is not the part of fungal body elemental composition, and caused deformities in fungal hyphae.

Conclusion: The present study confirmed that highly monodisperse silver nanoparticles (AgNPs) can be synthesized by using leaf extract of *T. procumbense*. The method of AgNP synthesis is efficient with size range of 17-40 nm. Antimicrobial activity study against *F. incarnatum* revealed high potential of Tridax leaf extract stabilized AgNPs.

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