Research Article

Anti Diabetic and Anti Cancer Effects of Silver Nanoparticles Synthesised from *Botryodiplodia theobromae*- An Endophytic Fungi isolated from *Euphorbia hirta* – A Weed

Vardhana Janakiraman [*], Mercy Jenifer [1], Sivasankaran Ramarajan[1a]
[1]Department of Biotechnology, SRM Arts and Science College, Chennai, India.
[*& 1a] Assistant Professor, Department of Biotechnology, SRM Arts and Science College, Chennai, India.
***Corresponding author's E-mail:** vardhana88@ymail.com

Received: 28-09-2018; **Revised:** 30-10-2018; **Accepted:** 10-11-2018.

ABSTRACT

Weeds are a plant that is out of place and not intentionally sown.A plant that grows where it is not wanted or welcomed. Endophytes are ubiquitous and have been found in all the species of plants studied to date; however, most of these endophyte/plant relationships are not well understood. In order to extend the observations on the potential inhibition of human colon adenocarcinoma cells, the present study evaluated the effect of silver nanoparticles synthesised by *Botryodiplodia* on HT-29 cells in vitro, and investigated the possible underlying molecular mechanism. The fungal cell filtrate after addition of aqueous $AgNO_3$ (1 mM) was subjected to optical measurements by UV-Vis spectrophotometer; this analysis showed an absorbance of peak at 420 nm. The FT-IR measurements of the cell filtrates, confirmed the existence of free OH and NH groups, aromatic CH stretching, C - C stretching and the existence of –C ≡ C – H: C – H bend. The size of the nanoparticle produced was ranging from 53nm – 79 nm and 48 nm - 68 nm from the cell filtrate and fungal mat respectively. The XRD pattern of the test sample exhibited peaks 38˚, 44°, 64 ° and 77° corresponding to 111, 200, 220 and 311 planes were recorded. The XRD result discloses the formation of pure crystalline. The silver nanoparticles synthesised from fungi *Botryodiplodia theobromae* shows the highest percentage of inhibition for α-amylase (94%). The MTT assay clearly evidenced that on using different concentrations the cytotoxicity was more when 100 µl of the sample was used.

Keywords: Endophytic Fungi, HT-29, Cytotoxicity, α- amylase Assay, Weeds, *Euphorbia Hirta.*

INTRODUCTION

Weeds are a plant that is out of place and not intentionally sown. A plant that grows where it is not wanted or welcomed [1]. A plant that is competitive, persistent, pernicious, and interferes negatively with human activity. Human activities create weed problems since no plant is a weed in nature. Weeds are naturally strong competitors, and those weeds that can best compete always tend to dominate [2].

An endophyte is an endosymbiont (any organism that lives within the body or cells of another organism, i.e. forming an endosymbiosis), often a bacterium or fungus, that lives within a plant for at least part of its life without causing apparent disease [3, 4]. Endophytes are ubiquitous and have been found in all the species of plants studied to date; however, most of these endophyte/plant relationships are not well understood [5, 6].

Nanobiotechnology involves a major role for nanometal studies [7]. The biological synthesis of nanoparticles has been investigated by chemical and physical methods [8, 9]. The recent study of silver nanoparticles reveals many applications, as it is spectrally selected for solar energy absorption, as intercalation material for electrical batteries, as optical receptors, as catalysts in chemical reactions, as antimicrobial and in biotabling [10,11]. Green nanoparticle synthesis and preparation are environmentally needed to grow metal nanoparticles [12]. Nanoparticles do not use toxic chemicals in the synthesis process to avoid effects in medical applications [13].

Cancer is one of the leading causes of death worldwide after cardiovascular diseases. Clinical trials and modern biomedical researchers can be achieved by so many ways for cancer treatment. However, the new therapeutic agents are needed with more active and fewer side effects [14].

In order to extend the observations on the potential inhibition of human colon adenocarcinoma cells, the present study evaluated the effect of silver nanoparticles synthesised by *Botryodiplodia* on HT-29 cells in vitro, and investigated the possible underlying molecular mechanism. The nanoparticle was also used to find out the amylase inhibitory effect to study the Anti Diabetic activity.

MATERIALS AND METHODS

Collection of Plant Sample

Weed sample were collected from Kattankulathur, Kancheepuram District,in the month of October,2017. *Euphorbia Hirta* (sometimes called asthma-plant) species belongs to family Euphorbiaceae, synonyms chamaescyehirta (L.) Millsp. It is a pantropical weed, possibly native to India. There are many other species of Euphorbia which are used in traditional medicines.

Isolation of Endophytic Fungus

Leaves Samples were collected washed thoroughly with tap water. Surface sterilized with 75% ethanol (60 sec)

and 1:3 dilution of water and Nacl (5 min).Samples were cut into small segments from 0.5 – 0.6 cm^2.The small pieces of leaf samples transferred to Petridish containing PDA supplemented with Chloromphenicol.Plates were incubated for one week at 20-40°C.From the 7th day growth of the endophytic fungus were observed.

Synthesis of Silver Nanoparticles

The fungus was inoculated (app. 3mm in diameter) in 250 ml Erlenmeyer flasks containing 100 ml potato dextrose broth at 25° C in orbital shaker at 120 rpm for 48 hours. After incubation, washed with sterile distilled water to remove the traces of media components, resuspended in 100 ml distilled water, and incubated at 25°C. After 24 hours, the suspension was filtered through Waatman filter paper. The cell filtrate was treated with AgNO3 solution (1 mM) and incubated at room temperature in dark condition. The wet fungal biomass was mixed with 100 ml aqueous solution of 1mM AgNO3 and was placed in 100rpm rotating shaker for 120 hrs.

In Vitro anti-Diadetic Activity

α -amylase assay

α-amylase was dissolved in phosphate buffer saline (PBS,0.02 mol/L,PH 6.8) at a concentration of 0.1 mg/ml. Various concentration of sample solution (25 to 100 ml) were mixed with α-amylase solution (0.25ml) and incubated at 37^0 C for 5min. Then the reaction was initiated by adding 0.5 ml 1.0% (w/v) starch substrate solution to the incubation medium.After incubating at 37^0 C for 3min, the reaction was stopped by adding 0.5 ml DNS reagent (1% Dinitrosalicylic acid0.05% Na2SO3 and 1% NaOH solution) to the reaction mixture and boiling at 100^0 C for 5 min. After cooling to room temperature, the absorbance (Abs) at 520nm was recorded by spectrophotometer. The inhibition percentage was calculated by following equation:

Inhibition (%) = [(Abs1-Abs2)/Abs1]x100 where,
Abs1=sample and Abs2=control

Cytotoxic Study in HT-29 Cancer Cell Line

The Colon cancer cell line (HT29) were plated separately using 96 well plates with the concentration of 1×104cells/well in DMEM media with 1X Antibiotic Antimycotic Solution and 10% fetal bovine serum (Himedia, India) in CO_2 incubator at 37˚C with 5% CO2. The cells were washed with 200 µL of 1X PBS, then the cells were treated with various test concentration of compound in serum free media and incubated for 24 h. The medium was aspirated from cells at the end of the treatment period. 0.5mg/mL MTT prepared in 1X PBS was added and incubated at 37˚C for 4 h using CO2 incubator. After incubation period, the medium containing MTT was discarded from the cells and washed using 200 µL of PBS. The formed crystals was dissolved with 100 µL of DMSO and thoroughly mixed. The development of color intensity was evaluated at 570nm. The formazan dye turns to purple blue color. The absorbance was measured at 570 nm using microplate reader.

RESULT AND DISCUSSION

Isolation of Endophytic Fungi

From seventh day, observations were made each day for the growth of the most dominant fungi over the study. The most recurring fungi were transferred to a fresh PDA plates by hyphal tipping, and sub-cultured with the media containing plates which was amended with chloromphenicol under sterile conditions, to obtain the pure culture. The plates were incubated at room temperature (27°C) for about 3-4 days. The pure cultures of the isolates were maintained on PDA slants.

Identification of endophytic fungus

Macroscopic appearance of the fungal colonies, their morphological appearance and the mechanism of spore production and characterization of the spores were noted using the standard mycological manuals. The identification of molds was based on the shape, method of production of arrangement of spores (conodial ontogeny). Microscopic appearance was noted following wet mount preparation by lactophenol cotton blue staining and the microscopic slides were mounted observing them under 4X, 10X and 40X objectivities [15, 16].

The identified fungi was found to be *Botryodiplodia theobromae (Fig 1)*, blongs to coelomycetes group. *Botryodiplodia theobromae* (Pat. Ponnappa, 1970., Tandon & Verma, 1964., Petrak 1923) Mycelium immersed or superficial, branched, septate. Initially white, when mature it becomes dark chocolate brown. Conidiomata pycnidial, usually papillate with prominent ostioles, 160-185 µm, globose. The conidiogenous cells holoblastic, annellidic. Conidia hyaline when young, ellipsoid to oblong, thick walled with granular contents. Later, the conidia become two celled and cinnamon to dark brown. 23-27 x 10-13 µm with longitudinally running striations.

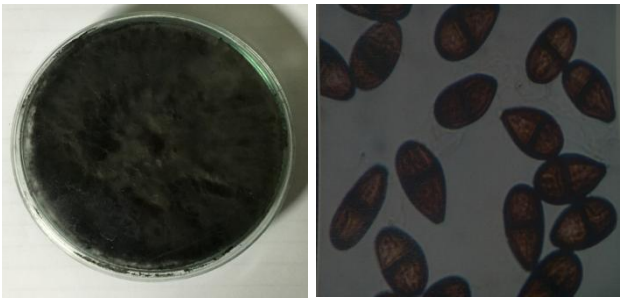

Figure 1: Culture and Conidia of *Botryodiplodia theobromae*

Synthesis of silver nanoparticles by *B.theobromae*

Both the fungal mat and the cell filtrate were treated with 1 mM AgNO3, and it was incubated in room temperature in dark. The change in colour was observed in both the fungal mat and the cell filtrate of the test fungi (Fig 2).

The colour change from pale yellow to dark brown indicates the preliminary synthesis of silver nanoparticles.

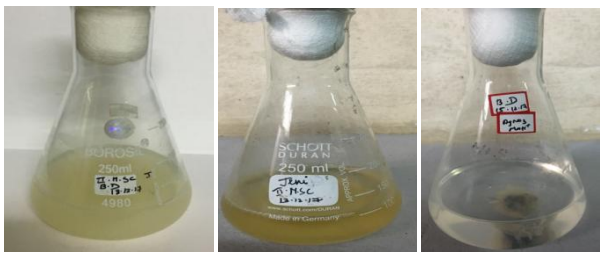

Figure 2: Synthesis of Silver nanoparticles from *B.theobromae*

UV- VIS SPECTRA

The fungal cell filtrate after addition of aqueous AgNO3 (1 mM) was subjected to optical measurements by UV-Vis spectrophotometer; this analysis showed an absorbance of peak at 420 nm (Fig 3) which was specific for the silver nanoparticles.

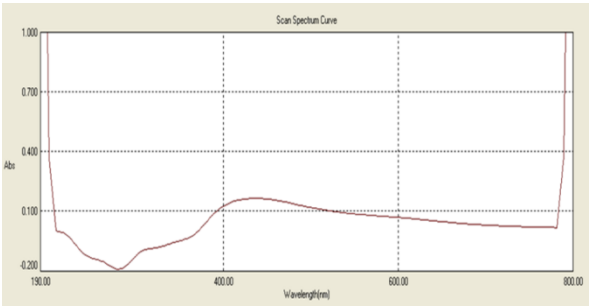

Figure 3: UV- VIS Spectroscopy

FT-IR Spectra

FT-IR measurements of the cell filtrates were carried out to identify the possible interactions between silver and bioactive molecules, which may be responsible for synthesis and stabilization (capping material) of silver nanoparticles. The FT-IR measurements of the cell filtrates, showed (Fig 4) the presence of peaks at 3327, confirms the existence of free OH and NH groups. The peaks at 2109, indicates the presence of aromatic CH stretching. The peaks at 1636 indicates the presence of C - C stretching. The presence of peak at 623 indicates the existence of –C ≡ C – H: C – H bend.

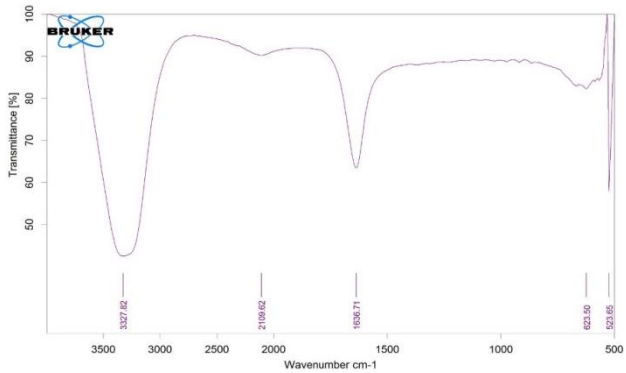

Figure 4: FT-IR spectra of the cell filtrate of *B. theobromae*

XRD Spectral Analysis

The XRD pattern of the test sample exhibited peaks 38˚, 44°, 64 ° and 77° corresponding to 111, 200, 220 and 311 planes were recorded. The XRD result discloses the formation of pure crystalline (Fig 5).

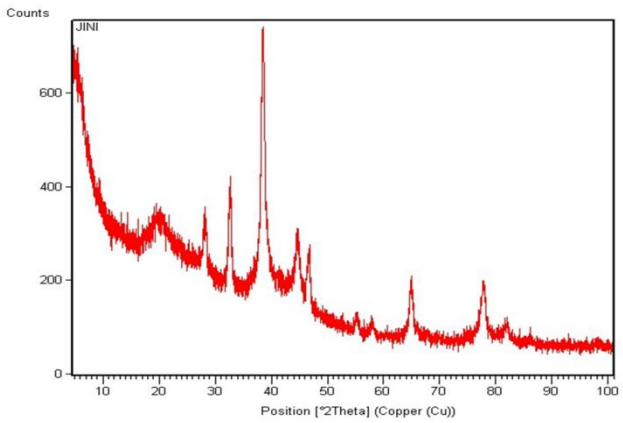

Figure 5: XRD PATTERN OF AgNP'S

Scanning Electron Microscope (SEM)

It was found that, the cell filtrate and the fungal mat of both the selected fungi were able to synthesize Silver nanoparticles. The size of the nanoparticle produced was ranging from 53nm – 79 nm and 48 nm - 68 nm respectively (Fig 6). SEM confirms the synthesis of effective silver nanoparticles from the Endophytic fungi.

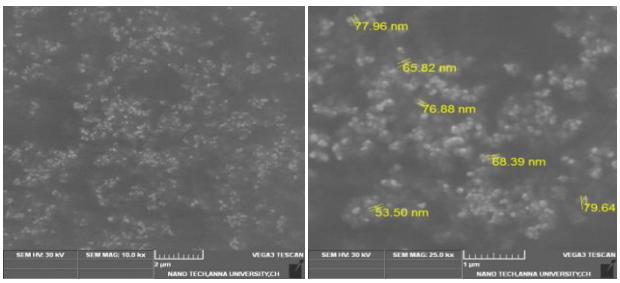

Figure 6(a): Silver Nanoparticles produced by the cell filtrate of *B.theobromae*

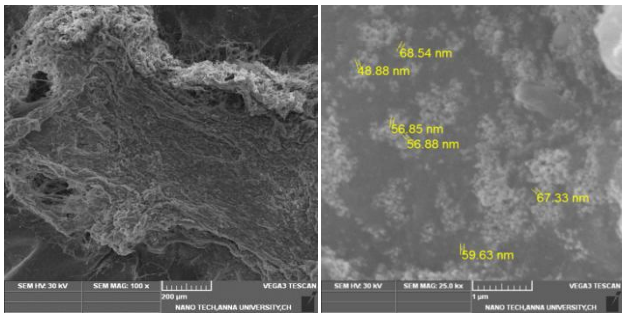

Figure 6(b): Silver Nanoparticles produced by the fungal mat of *B.theobromae*

Anti-Diabetic Activity

α- amylase assay

Percentage of inhibition by *Botryodiplodiatheobromae* cell filtrate in α- amylase assay was carried out. Different concentrations were used (25µl, 50µl, 75µl, 100µl) and

absorbance was measured at 520nm. It was found that, at the concentration of 100 µl the percentage of inhibition was 94 % which was highest and the lowest inhibition percentage was 88 % at 25 µl concentration (Table 1).From this it can be concluded that the AgNP's showed potent effect on anti diabetic activity at higher concentration.

Table 1: Inhibitory effect of AgNP's on α- amylase

Endophytic fungal extract	% of amylase inhibition			
	25µl	50µl	75µl	100µl
B. theobromae	88	90	91	94

Cytotoxicity of the samples against HT-29 Cancer cell line

The samples were treated on HT-29 cancer cell line, at various concentrations from 6 µl, 12 µl, 25 µl, 50 µl and 100 µl (Fig 9). The assay clearly evidenced that on using different concentrations of the samples ranging from 6 µl – 100 µl, cytotoxicity was more when 100 µl of the sample was used (Fig 7).

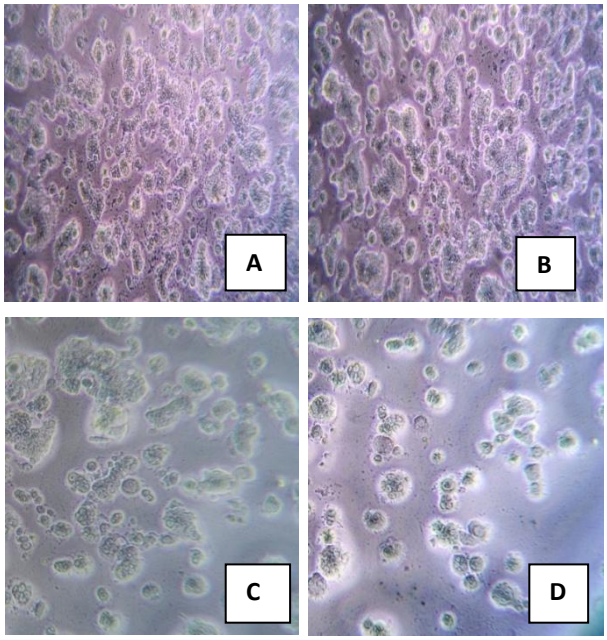

Figure 7: Cytotoxicity Of The Sample In Ht-29 Cell Line

A) Control – HT-29 cell line B) 50µl of the test sample treated with cell line C) 75 µl of the test sample treated D)100 µl of the test sample treated

The percentage of cell viability based on IC50 value was observed using graph pad prism 5 software, for 24 hrs in triplets at various concentrations from 10 µl, 25 µl, 50 µl, 75 µl and 100 µl (Table 2) in HT-29 Cell lines (Fig. 8). The assay clearly evidenced that on using different concentrations of the samples ranging from 10 µl – 100 µl, cytotoxicity was more when 100 µl of the sample was used.

Table 2: % of cell viability at different concentrations of the sample.

Tested concentration(µg/ml)	% of cell viability (triplicate values)		
10	84.19	85.91	91.07
25	84.54	80.76	78.35
50	69.76	70.45	72.16
75	53.26	54.3	55.67
100	41.92	44.67	42.27
Control	100	100	100

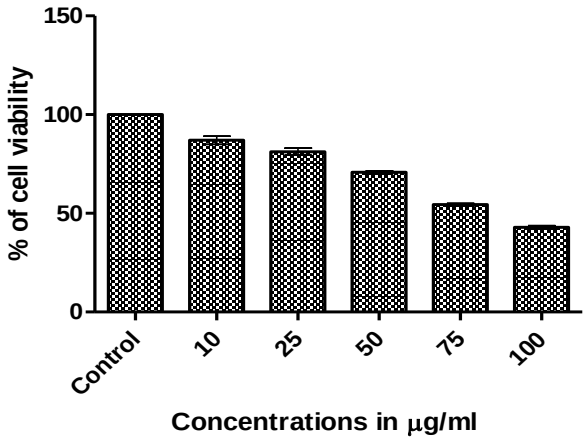

Figure 8: Graphical representation of % of cell viability

CONCLUSION

Endophytes are considered plant mutualists as they receive nutrition and protection from the host plants while the host plant may benefit from enhanced competitive ability. Evidence suggests that plants infected with endophytic fungi have distinguishable advantage against stress (biotic and abiotic) over non-endophytic counterparts [17]. Among the endophytes isolated from the weed *Euphorbia hirta*, the colonies of *Botryodiplodia theobromae* grew well up to 7cm diameter after five days and good sporulations were obtained in about 7 to 10 days incubation. The fungi were treated with 1mM AgNO3 for the synthesis of silver nanoparticles. The production of silver nanoparticles was confirmed using the change in the colour of the cell filtrate and the mat from colourless to dark brown. The silver nanoparticles synthesised from fungi *Botryodiplodia theobromae* showes the highest percentage of inhibition for α-amylase (94%). The MTT assay clearly evidenced that on using different concentrations of the samples ranging from 6 µl – 100 µl, cytotoxicity was more when 100 µl of the sample was used.

Thus the study proves that silver nanoparticles can be synthesized biologically which is cost effective method. It also proves that the synthesized nanoparticles are toxic to cancer cells selected for the study.

REFERENCES

1. Cruttwell M.R.E. (2000) Successes in Biological Control of Weeds Proceedings of the X International Symposium on Biological Control of Weeds, Montana State University, Bozeman, Montana, USA Neal R. Spencer [ed.]: 3 4, 14 July 1999, 3-14.
2. Dwight D.L. and Nathan L.H. Introduction to Weeds and HerbicidesThe Pennsylvania State University, 328 Boucke Building, University Park, PA 2013, 16802-5901.
3. Clay K, and Schardl C . Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *The American Naturalist.* 160 (4), 2002, S99–S127.
4. Carroll GC . The biology of endophytism in plants with particular reference to woody perennials". In Fokkema, N. J.; Van den Heuvel, J.*Microbiology of the phyllosphere*. Cambridge: Cambridge University Press. 1986, 205–22.
5. Faeth SH. Asexual fungal symbionts alter reproductive allocation and herbivory over time in their native perennial grass hosts. *The American Naturalist.* 173 (5), 2009, 554–65.
6. Faeth SH and Fagan WF . Fungal endophytes: common host plant symbionts but uncommon mutualists. *Integrative and Comparative Biology*. 42 (2), 2002, 360-368.
7. Gericke M, Pinches A. Biological synthesis of metal nanoparticles. Hydrometallury 83, 2006, 132-40.
8. Henglein, A, Electronics of coloidall nanometer Bunseng Ber Phys Chem 99, 1995, 903-13.
9. Daniel MC, Astruc D. Gold nanoparticles: Assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. Chem Rev 104(1), 2004, 293-346.
10. Dimitrijevic NM, Bartels DM, Jonah CD, Takahashi K, Rajh T. Radiolytically induced formation and optical absorption spectra of colloidal silver nanoparticles in supercritical ethane. J Physchem B 105, 2010, 954-9.
11. Mandal D, Bolander ME, Mukhopadhyay D, Sarkar G, Mukherjee P. The use of microorganisms for the formation of metal nanoparticles and their application. Appl Microbiol Biotechnol 69(5), 2006, 485-92.
12. Shah ZA, Vohora SB. Antioxidant/restorative effects of calcined gold preparations used in Indian systems of medicine against global and focal models of Ischemia. Pharmacol Toxicol 90(5), 2002, 254-9.
13. Shah ZA, Gilani RA, Sharma P, Vohora SB. Attenuation of stress - Elicited brain catecholamines, serotonin and plasma corticosterone levels by calcined gold preparations used in Indian system of medicine. Basic Clin Pharmacol Toxicol 96, 2005, 469-4.
14. Arvindganth Rajasekar, Vardhana Janakiraman, Kathiravan Govindarajan. *In Vitro* Cytotoxic Study Of Green Synthesized Gold And Silver Nanoparticles Using *Eclipta Prostrata* (L.) Against Ht-29 Cell LINE. *Asian J Pharm Clin Res,* 9(5), 2016, 189-193.
15. Ellis MB. Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, UK. 1971.
16. Sutton BC. The coelomycetes. Fungi imperfecti with pycnidia, acervuli and stromata. Commonwealth Mycological Institute, Kew, UK. 1980.
17. Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM. (2002). Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science*. 298, 1581.

Source of Support: Nil, **Conflict of Interest:** None.