

Biomedical application of greenly synthesized silver nanoparticles using the filtrate of *Trichoderma viride*: Anticancer and immunomodulatory potentials

Bukola Christianah Adebayo-Tayo^{1,A,C-F}, Gbemisola Elisabeth Ogunleye^{1,B-F}, Omonike Ogbole^{2,C,F}

¹ Department of Microbiology, University of Ibadan, Nigeria

² Department of Pharmacognosy, University of Ibadan, Nigeria

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;

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Address for correspondence

Bukola Christianah Adebayo-Tayo
E-mail: bukola.tayo@gmail.com

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Abstract

Background. Green route biosynthesis of silver nanoparticles using *Trichoderma viride* (*T. viride*) filtrate (TVFSNPs) can serve as an alternative to antibiotics and as an effective drug delivery to combat cancer and act as an immune-stimulator.

Objectives. To biosynthesize silver nanoparticles (SNPs) with *T. viride* filtrate using green route and to characterize and determine the cytotoxic and immunomodulatory potential of nanoparticles.

Material and methods. *Trichoderma viride* filtrate was used for biosynthesizing SNPs. The biosynthesized SNPs were characterized using UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and energy dispersive X-ray (EDX). The cytotoxic properties against Hep-2C and rotavirus and the immunomodulatory potential were evaluated.

Results. *Trichoderma viride* filtrate was able to bio-reduce AgNO_3 to SNPs. The surface plasmon resonance peak was at 450 nm. The presence of aldehydes, amino acids, ethers, esters, carboxylic acids, hydroxyl groups, and phenol among others indicates the capping and stabilization of proteins in the nanoparticles. The nanoparticles were spherical with a size of 0.1–10.0 nm. The EDX analysis revealed a strong signal of silver (Ag). The TVFSNPs had a cytotoxic effect on Hep2C and rotavirus in a dose-dependent manner and increased the production of immunoglobulin (Ig) A (IgA) and IgM.

Conclusions. *Trichoderma viride* filtrate contained some biochemicals that can bio-reduce silver nitrate (AgNO_3) for SNPs biosynthesis. The anticancer and immunostimulatory potential justifies the biomedical application and biotechnological relevance of *T. viride*.

Key words: cytotoxicity, immunomodulation, *Trichoderma* spp, filtrate, biosynthesized silver nanoparticles

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Introduction

Nanobiotechnology is a new research field of biotechnology and engineering which involves which involve investigating nanoparticles synthesis which involve investigating nanoparticles synthesis and regulating the connection at a cellular level between synthetic materials and biological systems.^{1,2} Biosynthesis of metal nanoparticles is of great interest in nanoscience.³ Noble metals such as gold, silver, platinum, and lead are used in the biosynthesis of nanoparticles, in which silver (Ag) is crucial for nanoparticles biosynthesis in biomedicine.

Nanoparticles have various applications in opto-electronics, diagnostic biological probes and catalysis.^{3,4} Nanoparticles can be synthesized chemically, physically and biologically. It is difficult to prepare silver nanoparticles (SNPs) with well-defined size using chemical methods; besides, they are toxic to the environment due to the use of toxic chemicals reducing agents such as borohydride, citrate, or other organic compounds. Physical methods give a low yield of nanoparticles, while the biological methods are eco-friendly, cost-effective have low toxicity, biocompatibility and a better control over size and shape of SNPs.^{5,6}

Fungi like *Trichoderma viride* (*T. viride*), *Trichoderma reesei* (*T. reesei*), *Alternaria flavus* (*A. flavus*), *Aspergillus niger* (*A. niger*), *Fusarium oxysporum* (*F. oxysporum*) and *Penicillium* spp. are excellent sources of extracellular enzymes which influence nanoparticles synthesis.^{2,4} Fungi have potential in the production of nanoparticles at a faster rate on a large scale.⁴ *Trichoderma* spp. that frequently colonize soils, decaying wood and vegetable matter. They are the dominant part of the soil microflora in different habitats, have diverse metabolic capabilities and aggressively competitive nature.⁷ *Trichoderma* spp. are highly resistant to biochemicals, chemicals and toxins. Most are strong opportunistic invaders, fast growing, prolific producers of spores and powerful antibiotics.⁸ *Trichoderma* species contain strains of vast economic importance, owing to their production of antibiotics and industrial enzymes and they act as biological control agents against plant pathogens.^{9,10} Some have an antagonistic activity against phytopathogenic fungi by using substrate colonization, antibiosis and mycoparasitism as the main mechanisms. This antagonistic potential is the basis for effective application of different *Trichoderma* strains as an alternative to chemical control against a wide variety of fungal plant pathogens.¹¹ They are prolific producers of extracellular proteins. For instance, different strains produce more than 100 different metabolites that have antibiotic activities.⁸ Based on an eco-friendly approach, low toxicity, biocompatibility and immunomodulation, the potential of greenly synthesized nanoparticles and their applications in various fields cannot be overemphasized. However, nanoparticles can also act as an immunomodulatory agents alone or in combination with established therapeutic immunomodulatory agents. The use of fungi for the biosynthesis of SNPs

provides advantages over chemical and physical methods, as it is cost-effective and environmentally friendly, and fungi can be used on a large scale. This study involves the biosynthesis and characterization of SNPs from *T. viride* and investigates its cytotoxic properties and immunomodulatory activities.

Material and methods

Culture collection

Trichoderma viride, which were previously isolated from soil samples, were obtained from the culture collection of the Microbial Physiology and Biochemistry Laboratory, Department of Microbiology, University of Ibadan, Nigeria. The culture was kept in potato dextrose agar and the stock culture was stored at 4°C and sub-cultured from time to time.

Cancer cell lines (human rhabdomyosarcoma (RD) and laryngeal carcinoma (Hep-2C)) were supplied from the Centre for Disease Control (CDC), Atlanta, Georgia and maintained in WHO Polio Laboratory, Department of Virology, University of Ibadan, Nigeria. Ethical approval for the study was obtained from the University of Ibadan Animal Care and Use Research Ethics Committee.

Production of cell filtrates of *Trichoderma viride*

The cell filtrate of *T. viride* was produced by inoculating pure culture of *T. viride* into a sterile malt extract broth (MEB) and incubated at 25°C for 5 days. The medium was filtered using Whatman filter paper No. 1, the crude filtrates were collected and used for further studies.

Biosynthesis of SNPs using *Trichoderma viride*

The biosynthesis of SNPs using *T. viride* was done using modified method of Devi et al.³ Fifty milliliters of the cell filtrate was mixed with 50 mL of 1 mM aqueous solution of silver nitrate (AgNO₃) prepared freshly in deionized water. The whole mixture was incubated at 25°C in dark place for 2 days. A flask with no addition of Ag⁺ was used as a control. Formation of a brown solution from a colorless solution indicates SNPs biosynthesis.

Characterization of the biosynthesized SNPs

Formation of SNPs was observed visually for color change in comparison to control. The bio-reduction was monitored using UV-visible spectrum Lambda 25 UV/Vis spectrophotometer. UV/V with the resolution of 0.5 nm.¹² Fourier transform infrared spectroscopy (FTIR) was used to characterize the functional groups of SNPs. The dried

SNPs were analyzed using potassium bromide (KBr) pellet (FTIR grade) method in a ratio of 1:100. The spectrum was recorded using JASCO Corporation 2967-5 (Ishikawa-cho, Hachioji-shi Tokyo, Japan) FT/IR-6300 in the range of 500–4000 cm^{-1} at a resolution of 4 cm^{-1} .³ The scanning electron microscopy (SEM) analysis of the gold-coated dried SNPs was done using a coater (JEOL, Akishima-shi, Japan; Model No. JFC-1600) and the images of SNPs were obtained using a scanning electron microscope (ZEISS EVO-MA v. 10; Carl Zeiss AG, Oberkochen, Germany).¹³ The energy dispersive X-ray (EDX) analysis of the SNPs was done at a voltage of 4 keV and current of 350 μA .¹³

MTT assay

The cytotoxicity assay of the samples was determined using MTT (3-(4, 5-dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. The cell filtrate and the SNPs biosynthesized with *T. viride* filtrate (TVFSNPs) were re-dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 10 mg/mL, respectively. The stock (0.1 mL) was added to 0.9 mL of maintenance medium containing antibiotics to obtain a dilution of 1000 $\mu\text{g/mL}$ (neat). Ten-fold serial dilutions of the samples were made from the “neat” using maintenance medium as diluent to obtain different concentrations. Fifty microliters of each diluent was dispensed into 96-well microtiter plates already seeded with monolayer of RD and Hep-2C in triplicates. The plates were incubated at 37°C in a carbon-dioxide environment and the cells were observed under microscope after 72 h.

The MTT colorimetric assay was used to evaluate the reduction of viability of cell cultures in the presence and absence of metabolites. The ability of the SNPs to be cytotoxic was measured using the tetrazolium dye (MTT), which is metabolized by mitochondrial enzymes of viable (surviving) cells to an insoluble, colored formazan product. The level of metabolism that occurs in the individual well of the 96-well microtiter plate is dependent on the number of healthy viable cells present. The plates were placed on a shaker for 15 min, after which absorbance of insoluble formazan salts was assessed at 492 nm wavelength on a multi-well spectrophotometer (Titertek Uniskan, Thermo Scientific™ Multiskan™ GO UV/Vis microplate spectrophotometer).¹⁴

Immunomodulatory activity

This study was conducted using female Swiss albino mice aged 6 weeks, weighing 20 ± 4 g. They were fed with rat pellets and given water ad libitum. The animals were allowed to acclimatize to the laboratory environment for 2 weeks and were later divided into groups for the experiment. Group 1 and 2 served as the control; Group 3 was administered with TVFSNPs and Group 4 was administered *T. viride* fungal filtrate (TVF). All the procedures used in this study conformed to the guidelines for care and use of animals in research and teaching.

Determination of IgG, IgM and IgA

The immunoglobulin (Ig) G (IgG), IgA, IgM of the treated and untreated mice was determined by diluting the blood serum samples and the control samples in 0.9% saline (1:10). Twenty microliters of the diluted samples was added to 900 μL of phosphate buffer and labeled sample A2. The absorbance of sample A1 was taken at 340 nm. One hundred microliters of antibody reagents was added into the prepared samples and mixed properly. The reaction mixture was incubated for 5 min. Absorbance of sample A2 and the control was taken at 340 nm.

Statistical analysis

The analysis of variance (ANOVA) and SPSS v. 25 were used to statistically evaluate the data. Values are represented as the mean \pm standard deviation (SD) of the 3 replicates of each experiment.

Results and discussion

Biosynthesis and characterization of SNPs

The cell filtrate of *T. viride* was used for biosynthesis of SNPs. Figure 1 shows the visual detection of SNPs biosynthesized using filtrate from *T. viride*. Changes in color from yellow to dark brown were observed.

Nanoparticles possess more surface atoms than microparticles, which enhances their functional capabilities. Biocompatible synthesis of metal nanoparticles was encouraged to exploit the biological sources of nanoparticles, because it is cost-effective.¹⁰

The *T. viride* filtrate bio-reduced AgNO_3 for SNPs biosynthesis. The bio-reduction potential of the filtrate from *T. viride* is in accordance with the work by Vahabi and Karimi,¹⁶ who reported that *T. reesei* is an eco-friendly fungus which biosynthesizes SNPs in a large-scale production, in which there was a change in color from yellow to dark brown.

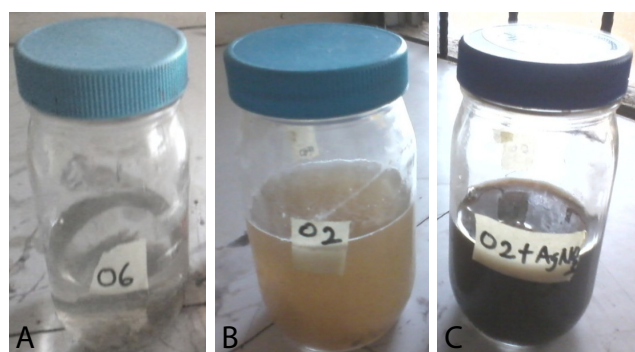


Fig. 1. Visual detection of silver nanoparticles (SNPs) biosynthesized with *Tricoderma viride* filtrate (TVFSNPs)

A – silver nitrate (AgNO_3) solution; B – *T. viride* fungal filtrate (TVF); C – TVFSNPs.

The spectra obtained from the biosynthesized TVFSNPs are shown in Fig. 2. A broad-band spectrum between 350 nm and 550 nm was observed for TVFSNPs and the surface plasmon resonance (SPR) peak was at 450 nm, indicating the formation of SNPs.

Strong SPR is very important in the synthesis of nanoparticles and it is characterized by UV-visible absorption spectroscopy. This result is similar to the study by Kanmani and Lim,¹⁷ in which SNPs showed a strong SPR peak at 400–550 nm with a broad band and size, indicating the formation of SNPs.¹⁷ Guangquan et al.⁵ reported that UV-visible spectra of the cell filtrate with AgNO₃ showed a strong broad peak at 440 nm, indicating the presence of SNPs.⁵

The FTIR analysis of TVFSNPs is shown in Fig. 3. 13 bands were present at 3425.69, 2895.25, 2359.02, 1633.76, 1404.22, 1330.93, 1149.61, 1074.39, 968.3, 931.65, 891.14, 738.76 and 597.3 cm⁻¹. The peaks at 3425.69 cm⁻¹ and 2895.25 cm⁻¹ were attributed to O-H stretch of alcohol and C-H symmetrical stretching of aldehydes. The absorption peaks at 2359.02 cm⁻¹ and 1633.76 cm⁻¹ were also attributed to the presence of COOH overtone and the presence of C=O stretch of carboxylates. The absorption peaks at 1404.22 cm⁻¹ and 1330.93 cm⁻¹ corresponded to C-N stretch of primary amide and C-N stretch of secondary amine. The peaks at 1449.61 cm⁻¹ and 1074.39 cm⁻¹ indicated the presence of S=O sulfonic esters and C-N stretch of aliphatic amines. The absorption peaks at 968.3 cm⁻¹, 931.65 cm⁻¹ and 891.14 cm⁻¹ corresponded to C=CH₂ alkenes out-of-plane bend, P-O-P stretch of pyrophosphate and C-O of epoxide. The absorption peaks at 738.76 cm⁻¹ and 597.3 cm⁻¹ indicated the presence of C-H and disulfide. From the obser-

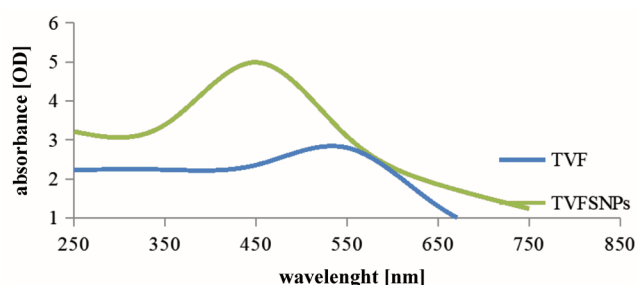


Fig. 2. UV-visible absorption spectra of TVFSNPs and TVF

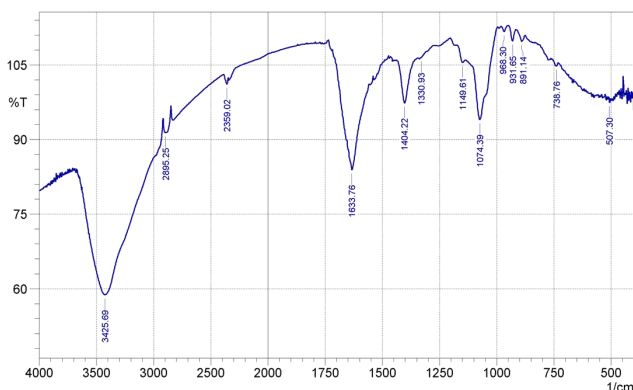


Fig. 3. Fourier transform infrared spectroscopy (FTIR) spectrum of TVFSNPs

vation in the spectrum, the presence of alcohols, aldehydes, carboxylic acids, alkenes in the samples may be responsible for the reduction of AgNO₃ to SNPs.

The FTIR spectra of TVFSNPs showed that different functional groups were present. Aldehydes, amino acids, ethers, esters, carboxylic acids, hydroxyl groups, phenol among others are responsible for the synthesis of SNPs. Carbonyl groups from the amino acid residues and peptides of proteins have a strong ability to bind to Ag. These proteins serve as a capping and stabilizing agent. Sonal et al.¹⁷ reported that the biomolecules, especially proteins from the filtrate of *F. oxysporum*, were responsible for synthesizing and stabilizing SNPs.

The TVFSNPs were further characterized by SEM, which showed the morphology and size of the biosynthesized SNPs. The SEM micrograph is shown in Fig. 4. The TVFSNPs were spherical and 0.01–10.0 nm in size.

A scanning electron microscope is an important tool for the characterization of SNPs.^{15–18} The shape of the TVFSNPs is in agreement with the study by Amal and Azzah,² who reported that nanoparticles are spherical with a small percentage of elongated particles with a variation in particle size, 5 nm for *F. oxysporum*, 20 nm for *A. niger* and 25 nm *Alternaria solani* (*A. solani*).

The EDX analysis of biosynthesized TVFSNPs is shown in Fig. 5. Silver had the highest intensity in the range 0.0001–0.2574.

The EDX analysis was used to determine the elemental composition of samples. Strong signals from Ag atoms in the nanoparticles were observed, while there were weaker signals from carbon, oxygen, sulfur, phosphorus, magnesium and sodium atoms. The presence of a strong Ag peak is a result of SPR. The carbon, oxygen, sulfur, phosphorus, magnesium and sodium signals may be due to the X-ray emission from proteins or enzymes present in the cell wall of the organisms. The presence of other EDX peaks for chlorine, sodium and oxygen was as a result of mixed precipitates present in the extract. Pnyabrata et al. had similar report.^{19–20}

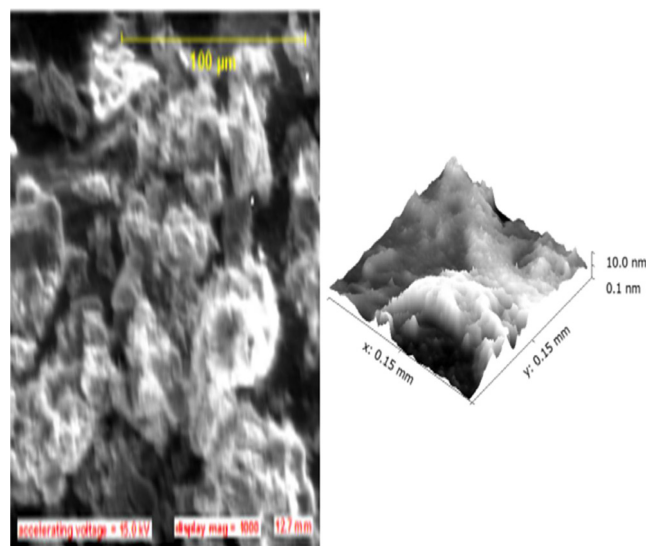


Fig. 4. A scanning electron micrograph of TVFSNPs

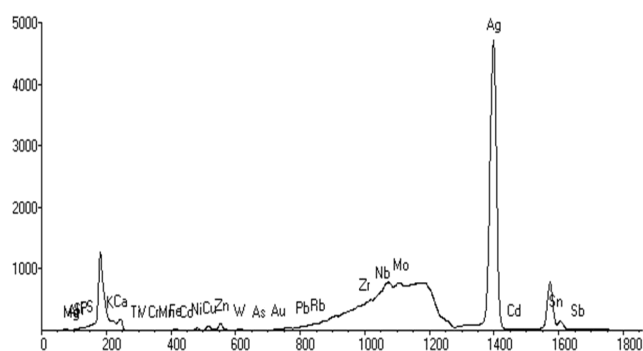


Fig. 5. Energy dispersive X-ray (EDX) analysis of TVFSNPs

Cytotoxicity assay of the TVFSNPs against Hep-2C and rotavirus cell lines

Cytotoxicity activity against hepatitis-2C (Hep-2C) and rotavirus cell lines was evaluated at different concentrations by MTT assay. Table 1 shows the IC_{50} ($\mu\text{g/mL}$) and dose-dependent values for the in vitro cytotoxic activity against Hep-2C cell lines. It was observed that TVFSNPs inhibited the viability of Hep-2C cell lines in dose-dependent manner. Silver nanoparticles were not toxic at lower doses, while mild cytotoxicity was recorded at higher doses.

Table 2 shows the IC_{50} ($\mu\text{g/mL}$) and dose-dependent values for the in vitro cytotoxic activity against rotavirus cell lines. The TVFSNPs did not exhibit significant cytotoxicity at their lower concentrations, while cytotoxicity increased at higher concentrations.

Cytotoxicity increased at higher TVFSNPs concentrations. The ability of the TVFSNPs to increase toxicity at a higher concentration may be due to metal nanoparticles overaccumulating inside the cell. It may also be due to the fact that SNPs interfere with the proper functioning of cellular proteins and induce subsequent changes in cellular chemistry. The cytotoxicity impact of SNPs in biological systems depends on their physiochemical properties.²¹

Vimbela et al.²² reported a dose-dependent cytotoxicity effect of nanoparticles against J774 and THPI cell lines, in which there were no cytotoxic effects at low doses (10 μg), whereas mild cytotoxicity effects were observed at high doses of 100–150 μg . Raman et al. reported the dose-dependent cytotoxicity potential of *Melia azedarach* SNPs against HeLa cells.²⁴

The anti-proliferative effect of SNPs on cancer cell line has been reported.^{24,25} Choi et al. reported the cytotoxicity potential of SNPs on A2780 ovarian carcinoma cells and ovarian cancer stem cells at a high concentration. The cells are more sensitive to the treatment with SNPs.²⁵

Immunomodulatory activity of TVSNPs

The immunomodulatory activity of the fungal filtrate and TVFSNPs is shown in Table 3. There was a significant difference in the immunomodulatory activity of the treatments using the biosynthesized TVFSNPs and the TVF on the treated mice.

Group 2, which included mice treated with sheep red blood cells, had the highest IgG. The IgA of the treated mice ranged from 75 to 258 mg/dL. Group 3 (mice treated with TVFSNPs) had the highest IgG, while Group 4 (mice treated with TVF) had the lowest IgG. The IgM of the treated mice ranged from 96 to 24 mg/dL. Group 3 had the highest IgM, while Group 4 had the lowest IgM.

The immunomodulatory potential of TVFSNPs based on in vivo immunological activity was investigated. The TVFSNPs showed significant immunostimulation of IgA and IgM. The ability of TVFSNPs to stimulate IgA and IgM in the immune system of the mice may be due to the easy engulfment of macrophages to the SNPs. Serum glycoproteins are stimulated to produce a subpopulation of white blood cells called lymphocytes. This could be as a result of the nanoparticles stimulating macrophages activity which evolve from immune system to protect

Table 1. IC_{50} ($\mu\text{g/mL}$) and dose-dependent values for the in vitro cytotoxic activity against Hep-2C cell lines

Samples	Concentration [$\mu\text{g/mL}$] \pm SEM						IC_{50} [$\mu\text{g/mL}$]
	0.01	0.1	1	10	100	1000	
AgNO ₃	0.627 \pm 0.02 ^c	25.013 \pm 0.21 ^a	27.729 \pm 0.03 ^b	34.036 \pm 0.02 ^b	38.46 \pm 0.04 ^d	55.793 \pm 0.03 ^c	50.02 \pm 0.02 ^b
TVFSNP	0.623 \pm 0.01 ^c	8.591 \pm 0.02 ^c	20.348 \pm 0.06 ^d	26.823 \pm 0.04 ^d	47.89 \pm 0.21 ^c	51.146 \pm 0.03 ^d	54.27 \pm 0.02 ^a
TVF	1.316 \pm 0.03 ^b	5.406 \pm 0.01 ^d	22.187 \pm 0.06 ^c	27.583 \pm 0.02 ^c	49.756 \pm 0.09 ^b	69.303 \pm 0.04 ^a	37.83 \pm 0.03 ^d
CTX	20.163 \pm 0.05 ^a	22.75 \pm 0.03 ^b	41.093 \pm 0.08 ^a	54.556 \pm 0.09 ^a	73.546 \pm 0.24 ^a	67.946 \pm 0.02 ^b	47.19 \pm 0.02 ^c

SEM – scanning electron microscopy; CTX – anticancer drug. Data presented as mean \pm standard deviation (SD).

Table 2. IC_{50} ($\mu\text{g/mL}$) and dose-dependent values for the in vitro cytotoxic activity of against rotavirus cell lines

Samples	Concentration [$\mu\text{g/mL}$] \pm SEM						IC_{50} [$\mu\text{g/mL}$]
	0.01	0.1	1	10	100	1000	
AgNO ₃	2.083 \pm 0.02 ^c	17.951 \pm 0.02 ^b	22.386 \pm 0.02 ^c	24.113 \pm 0.02 ^c	25.163 \pm 0.03 ^c	96.606 \pm 0.02 ^a	31.00 \pm 0.06 ^c
TVFSNP	2.976 \pm 0.02 ^b	19.956 \pm 0.02 ^a	27.786 \pm 0.03 ^b	32.016 \pm 0.03 ^b	35.206 \pm 0.03 ^b	82.256 \pm 0.03 ^d	38.33 \pm 0.04 ^b
TVF	3.883 \pm 0.03 ^a	12.689 \pm 0.03 ^c	18.156 \pm 0.04 ^d	20.653 \pm 0.03 ^d	22.662 \pm 0.02 ^d	86.146 \pm 0.02 ^b	28.54 \pm 0.03 ^d
CTX	3.612 \pm 0.02 ^a	9.475 \pm 0.03 ^d	34.533 \pm 0.02 ^a	55.303 \pm 0.02 ^a	73.173 \pm 0.02 ^a	84.596 \pm 0.03 ^c	49.05 \pm 0.02 ^a

Table 3. Immunomodulatory activity of TVFSNPs

S/N	Group	SRBC	SNPs	FF	IgG [mg/dL]	IgA [mg/dL]	IgM [mg/dL]
1	GRP1a	-	-	-	0.000	0.000	0.000
2	GRP1b	-	-	-	79 ±0.65 ^b	171 ±0.97 ^c	38 ±0.12 ^c
3	GRP2	+	-	-	118 ±0.23 ^a	230 ±0.45 ^b	73 ±0.26 ^b
4	GRP3	-	+	-	48 ±0.27 ^d	258 ±0.73 ^a	96 ±0.22 ^a
5	GRP4	-	-	+	63 ±0.39 ^c	75 ±0.81 ^d	24 ±0.17 ^d

n = 6; p < 0.05 – significant difference; GRP1a – mice not exposed to cigarette smoke and not treated; GRP1b – mice exposed to cigarette smoke and not treated; GRP2 – mice administered sheep red blood cells; GRP3 – mice administered TVFSNPs; GRP4 – mice administered TVF; SNPs – silver nanoparticles; FF – fungal filtrate; SN – serial number, SRBC – sheep red blood cell.


the host from potentially pathogenic agents, eliminate neoplastic cells and to reject non-self-components. Swarnakar et al. reported that the chemically synthesized nanoparticles act as an immunomodulatory agent alone or in combination with established therapeutic immunomodulatory agents, and can be a targeted drug/vaccine delivery vehicle to macrophages.²⁷


Conclusions

The filtrate from *T. viride* mediated the biosynthesis of SNPs, which were spherical in shape and nontoxic at a lower concentration. The TVSNPs exhibited cytotoxicity against Hep-2C cell line and RD cell line in a dose-dependent manner and had immune-stimulation potential by increasing the production of IgA and IgM. The anticancer and immunomodulatory potential of TVSNPs justifies its biomedical application and showcases the biotechnological relevance of the fungus.

ORCID iDs

Bukola Christianah Adebayo-Tayo

 <https://orcid.org/0000-0003-2404-1686>

Gbemisola Elizabeth Ogunleye  <https://orcid.org/0000-0003-2344-8177>

Omonike Ogbole  <https://orcid.org/0000-0002-6487-9494>

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