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ORIGINAL ARTICLE

Biosynthesis of ZnO Nanoparticles Using the Aqueous Extract of *Mucuna pruriens* (utilis): Structural Characterization, and the Anticancer and Antioxidant Activities

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Abstract
A simple, green, and cost-effective synthesis of ZnO nanoparticles particles (NPs) using an extract of *Mucuna pruriens utilis* is reported. The nanoparticles were characterized by X-ray diffraction, UV–vis spectroscopy, SEM, and TEM measurements. XRD results showed diffraction patterns that are consistent with the hexagonal phase of the wurtzite ZnO structure. Spherical morphology with irregular size and particle distribution was confirmed by the microscopic characterization. The antioxidant activity of the nanoparticles showed a concentration-dependent profile with an IC_{50} of 4.10 µg mL^{-1}, which was quite lower than that of the standard ascorbic acid (4.72 µg mL^{-1}), and indicated a significant free radical scavenging activity of the nanomaterials. The cytotoxicity properties of the nanoparticles were evaluated against human cancer cell lines HeLa and HEK 293 by the MTT assay, and the anticancer drug (5-Fluorouracil) was used as a control. The results showed selective toxicity of the nanoparticles towards cancerous cell lines and non-toxicity to normal cells. The study provides a simple and non-toxic protocol for biosynthesis of ZnO nanoparticles with potential biomedical applications as anticancer and antioxidant agents. However, further studies are necessary to ascertain the biochemical reactions and mechanisms responsible for the antioxidant and anticancer activities.

Keywords Biosynthesis · antioxidant · anticancer · ZnO nanoparticles · *Mucuna pruriens* (utilis)

1 Introduction

Zinc oxide (ZnO) nanoparticles have been explored in several studies [1] and one of the most versatile nanomaterials with their unique electrical and optical properties [2]. They are the most studied metal oxide nanomaterials [3]. Their notable optical, structural, and antimicrobial properties make them potential materials for application in agriculture [4]. Some of their great attributes in agriculture include their potency as bacteriostatic agents for the control of pathogenic infections as well as their enhancement of seed germination, seedling vigor, and radicle elongation [5]. They have

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been conventionally synthesized via several routes including hydrothermal, solvothermal and thermolysis of single source precursor compounds [6]. Compared to these previous methods, the green synthesis approach has recently provided a facile and environmentally benign route to their synthesis. This method involves the use of plant extracts as nanoparticle reducing and capping agents [7, 8]. Multitudes of plants and their anatomical components have been employed in green synthesis of ZnO NPs but very few studies have used extracts from the seeds of mucuna legume [9, 10].

Mucuna [*Mucuna pruriens* (L.) DC var. *utilis* (*aka* velvet bean)] is a tropical leguminous plant and a member of the *Fabaceae* family [11]. It is one of the significant crops widely grown in tropical and subtropical regions as forage and cover crop [12]. It tolerates diverse abiotic stresses such as acidic soils and droughts [13]. The legume possesses a plethora of phytochemical compounds some of which have antioxidant activities that help either in the treatment or reduction of the risk of many diseases [14–16]. In addition to alkaloids, isoflavanones, and lectins with analgesic, anti-inflammatory, antihemolytic, and many other bioactivities [17, 18], mucuna seeds contain L-3,4-dihydroxyphenylalanine (L-DOPA) as its main phytochemical compound that possesses antioxidant properties as demonstrated in their ability to inhibit lipid peroxidation [19]. Also, the plant's seeds contain potent anticancer activities [20]. Hence, it is postulated that the use of the seeds to synthesize nanoparticles would yield nanomaterials with both anticancer and antioxidant attributes that would be of benefit to biological systems. The objective of this study was to biosynthesize ZnO nanoparticles using the aqueous extract of mucuna seeds, characterize them, and evaluate their anticancer and antioxidant activities.

2 Experimentals

2.1 Materials

Zinc acetate $[Zn(CH_3CO_2)_2]\cdot 2H_2O$, dimethyl sulfoxide $(CH_3)_2SO$, and sodium hydroxide (NaOH) were purchased from Merck (Pty) Ltd, South Africa. Dry mucuna seeds were collected from the Faculty of Agriculture, University of Eswatini (UNESWA), Eswatini.

2.2 Preparation of Mucuna Extract

Mucuna seeds were collected and rinsed with distilled water before air-drying in the laboratory for 3–5 days [21]. Thereafter, they were pulverized into fine powders and sieved using a 40 mm mesh. About 8 g of the powdered seed was mixed with distilled water (400 mL) and heated at 80 ℃ for 1 h. This was followed with the cooling of the product to room temperature and filtering (Whatman No. 1). The filtrate was then kept in a reduced temperature.

2.3 Synthesis of ZnO Nanoparticles

About 20 mL solution of the seed extract in a 100 mL flask, sodium hydroxide solution was added drop wise to adjust the pH to approximately 10. Another 20 mL solution of 0.05 M $[Zn(CH_3CO_2)_2]\cdot H_2O$ was prepared and heated up to 80 ℃ while stirring steadily on a magnetic stirrer to ensure a homogeneous solution. Thereafter, the 20 mL solution of *mucuna* extract was introduced drop-wise to the $[Zn(CH_3CO_2)_2]\cdot H_2O$ and the resulting solution heated for 2 h at 85 ℃ [22]. During heating the process, a change in solution color from black to brown occurred, signifying the end of the reaction. The solution was cooled to room temperature and centrifuged at 5000 rpm for 30 min, followed by washing 3 times with a solution of ethanol and water to isolate the product. The product was dried in the oven at 50 ℃ for 3 h, and thereafter calcinated at 350 ℃ for 2 h to afford ZnO NPs (Fig. 1).

2.4 Characterization of ZnO Nanoparticles

Crystallinity and phase identification of the nanoparticles was ascertained using a Bruker D8 Advance X-ray diffractometer over 2θ range of 20–80º. A Cary 30 UV–visible spectrophotometer by Agilent was used in the wavelength range of 200 to 900 nm to evaluate the UV–Vis spectrum. The morphological characterization was by TECNAI G2 (ACI) electronic microscope and a Quanta FEG 250 (FEI Inc., USA) scanning electron microscope (SEM) for the measurement of the internal and external morphologies respectively. The particle size measurement as well as their distribution was obtained from the TEM micrographs using Image J software. Energy-dispersive X-ray spectroscopy (EDS) attached to the SEM machine was used to examine the atomic-level compositions of the nanoparticles.

2.5 Cytotoxicity Analysis of ZnO Nanoparticles

The cytotoxicity assay was carried out following the methods previously reported with slight modifications [23]. The assay utilized the human embryonic kidney (HEK293) and cervical carcinoma (HeLa) cells lines purchased from ATCC, Manassas, USA. The cell culturing was done using Dulbecco's Modified Eagle's Medium (DMEM) in a 25 cm^2 tissue culture flasks and 10% fetal bovine serum. Other reagents include penicillin (100 U/mL) and streptomycin (100 µg/mL). The MTT assay was conducted using a 96-well plate that contained 2.5×10^3 cells/well in DMEM (100 µL).

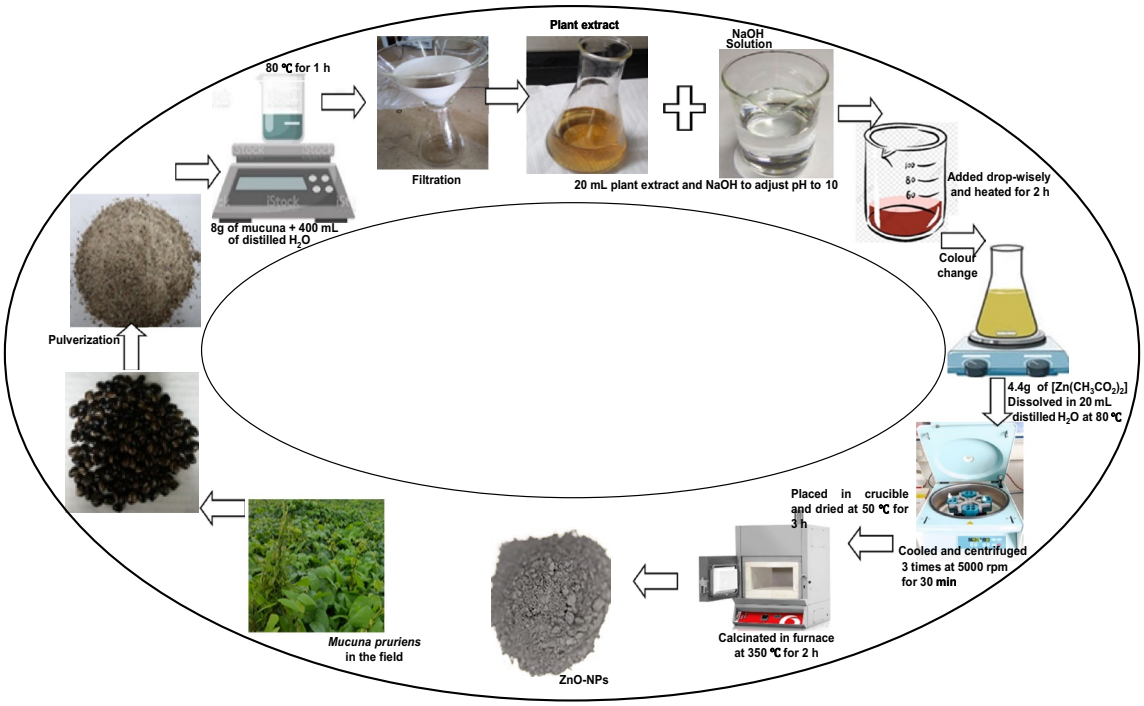

Fig. 1 Schematic illustration of the steps for the biosynthesis of ZnO NPs using *Mucuna pruriens* seed extract

The cells were incubated overnight at 37 ℃ before treatment with the samples, and ZnO nanoparticle solutions of 10, 25, 50, and 100 µg/mL were added to the cells, which were then incubated further for 48 h at 37 ℃, and then followed by the MTT assay. 5-fluorouracil (5-FU) was used as standard to compare the efficiency of the nanoparticles, and the fresh medium containing 10% MTT reagent was used to replace media in the wells for 4 h incubation at 37 ℃. After incubation, crystals of formazan were dissolved in 100 µL of dimethyl sulfoxide (DMSO), and absorbance recorded using a Mindray MR-96 A microplate reader (Vacutec, Hamburg, Germany) at 570 nm in DMSO solution used as a blank. The analysis was done in triplicate and absorbance values obtained by calculating the average of the measurements. The calculation of cell viability was done using the Eq. (1) as reported by Mthana et al. [22].

$$\% \text{ Cell viability} = \frac{Treated\ cells}{Untreated\ cells} \times 100 \quad (1)$$

2.6 Evaluation of Antioxidants Activity

The antioxidant analysis followed a similar procedure with previously reported studies [22, 24, 25] with modifications. The standard in vitro protocol for investigating the radical scavenging potential of ZnO nanoparticles was adopted. The deep violet coloured 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), which is considered one of the most utilized compounds for free radicals assay, was used to measure the antioxidant activity molecules [26]. The positive control used was ascorbic acid. In a volumetric flack, 12.8 mg of DPPH was mixed with 50 mL DMSO and the mixture properly shaken, wrapped in aluminum foil paper and kept in the dark for 30 min. About 10 mg of the ZnO nanoparticles and ascorbic acid were mixed with 10 mL DMSO and thoroughly sonicated. The scavenging activity was studied using different concentrations of 3.13, 6.25, 12.5, 25, and 50 µg/mL to assess the scavenging trend. The control sample was established by not adding the test samples (i.e., ZnO NPs). All the samples were prepared in triplicate. The absorbance was recorded at 540 nm in an AMR-Microplate. The IC_{50} values that represent effective concentration (for scavenging at 50%) for both standard and ZnO NPs were measured from the sigmoidal curve in the graph of percentage inhibition versus concentration, using linear regression analysis [27]. The inhibition percentage was calculated by using the Eq. (2).

$$\% \text{ Inhibition } = \frac{(AC - AS)}{AC} \times 100 \quad (2)$$

where Ac is the absorbance of the control and As is the absorbance of the test sample (ZnO NPs and standard) [28, 29].

3 Results and Discussion

3.1 Synthesis and Characterization

The properties of nanoparticles are dependent on their shape, size, structure, and surface area dispersity. The observed colour change when the mucuna extract was added to the zinc acetate solution indicated the initial stage in the formation of ZnO NPs [30]. This reaction was observed because of the phytochemicals from the seed extract and their interaction with the Zn(II) ions in solution, which was responsible for the reduction of the precursor compound into the ZnO NPs [31].

3.2 X-ray Diffraction Patterns of ZnO Nanoparticles

The XRD patterns of the biosynthesized ZnO NPs (Fig. 2) showed eight diffraction peaks corresponding to (100), (002), (101), (102), (110), (103), (200) and (112) lattice planes [30]. The sharp peaks clearly indicated the crystalline nature of the biosynthesized ZnO NPs and corresponded to the reference pattern (JCPDS Card No: 00-036-1451) confirming the successful preparation of ZnO NPs [2]. The nanoparticle crystallite size was evaluated using the Scherrer's Eq. $(0.9\lambda)/(\beta \cos\theta)$ [32] and found to be 46.5 nm.

3.3 3 SEM, TEM Images and EDS Analysis Of ZnO Nanoparticles

The morphology and size of ZnO nanoparticles were studied using SEM and TEM techniques. The SEM image (Fig. 3a) showed spherically shaped nanoparticles with some level of agglomeration [33]. The agglomeration may be attributed to the polarity and electrostatic attraction between the ZnO NPs [8]. The TEM images also confirmed the spherical shape of the ZnO NPs (Fig. 3b), and the particle size distribution histogram (Fig. 3c) showed a size range from 21.60 to 47.16 nm, with an average of 30.50 nm, which is within the estimated range of the crystalline size from the XRD pattern. The EDS spectra (Fig. 3d) showed that the nanoparticle was composed of Zn and O, and confirmed the high purity for the synthesized ZnO NPs, with the carbon arising from the presence of the mucuna plant or the carbon tape on the surface of the sample holder [34].

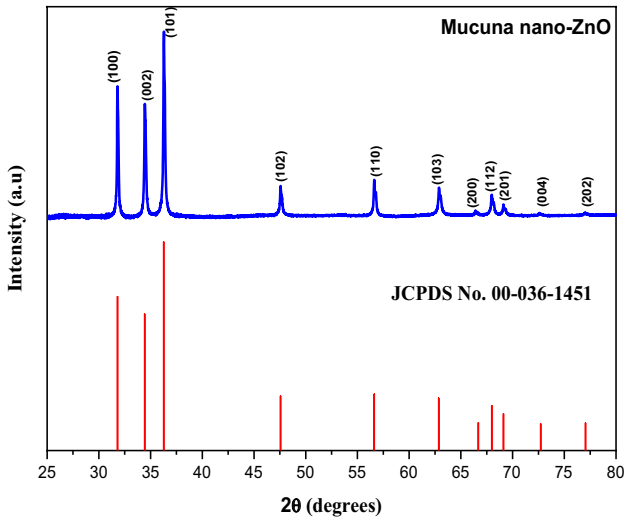

Fig. 2 XRD diffraction patterns of ZnO NPs biosynthesized using *M. pruriens utilis* seed extract

3.4 4 UV–Vis Characterization

UV–vis spectra were measured using the ethanol solution of ZnO. The UV absorption spectrum presented in Fig. 4 shows a maximum absorption peak at 350 nm (3.54 eV), which indicates a blue shift with respect to the absorption maximum of bulk ZnO (~ 376 nm; 3.3 eV) and this could be ascribed to quantum confinement effects [35, 36]. This is confirmation of the successful synthesis of ZnO NPs. The direct band gap of the ZnO NPs was estimated from the Tauc graph of $h\nu$ versus $(\alpha h\nu)^2$. Here, α is the absorption coefficient, and it is related to the band gap Eg as $(\alpha h\nu)^2 = k(h\nu - Eg)$, where $h\nu$ denotes the incident light energy and k is a constant. From this graph, the extrapolation of a straight line to the point where $(\alpha h\nu)^2 = 0$ is the estimated value of the band gap energy E_g. In the current study, the optical band gap (E_g) was estimated to be 3.75 eV, which denotes an increase in the band gap of the ZnO nanoparticles and a confirmation of reduction in particle size with respect to bulk ZnO. Similar studies have reported band gap energies of ZnO NPs of 4.52 and 3.40 eV using extracts of *Ocimum tenuiflorum* and *Camellia sinensis* [37, 38], and the variation in the band gap energy may be attributed to the difference in temperature of synthesis, volume of plant extract used, the concentration of precursors as well as type of extract used. Jayachandran et al. [39]. reported that different conditions of synthesis have various influences on the properties of ZnO NPs.

3.5 Anticancer Evaluation

Nanoparticles possess enormous potential as anticancer agents. Many studies have reported the substantial cytotoxicity property of ZnO NPs against various types of cancer cells compared to their macro-sized counterpart materials [40]. In the current study, ZnO NPs showed selective toxicity towards the cancerous cell lines and proved non-toxic to

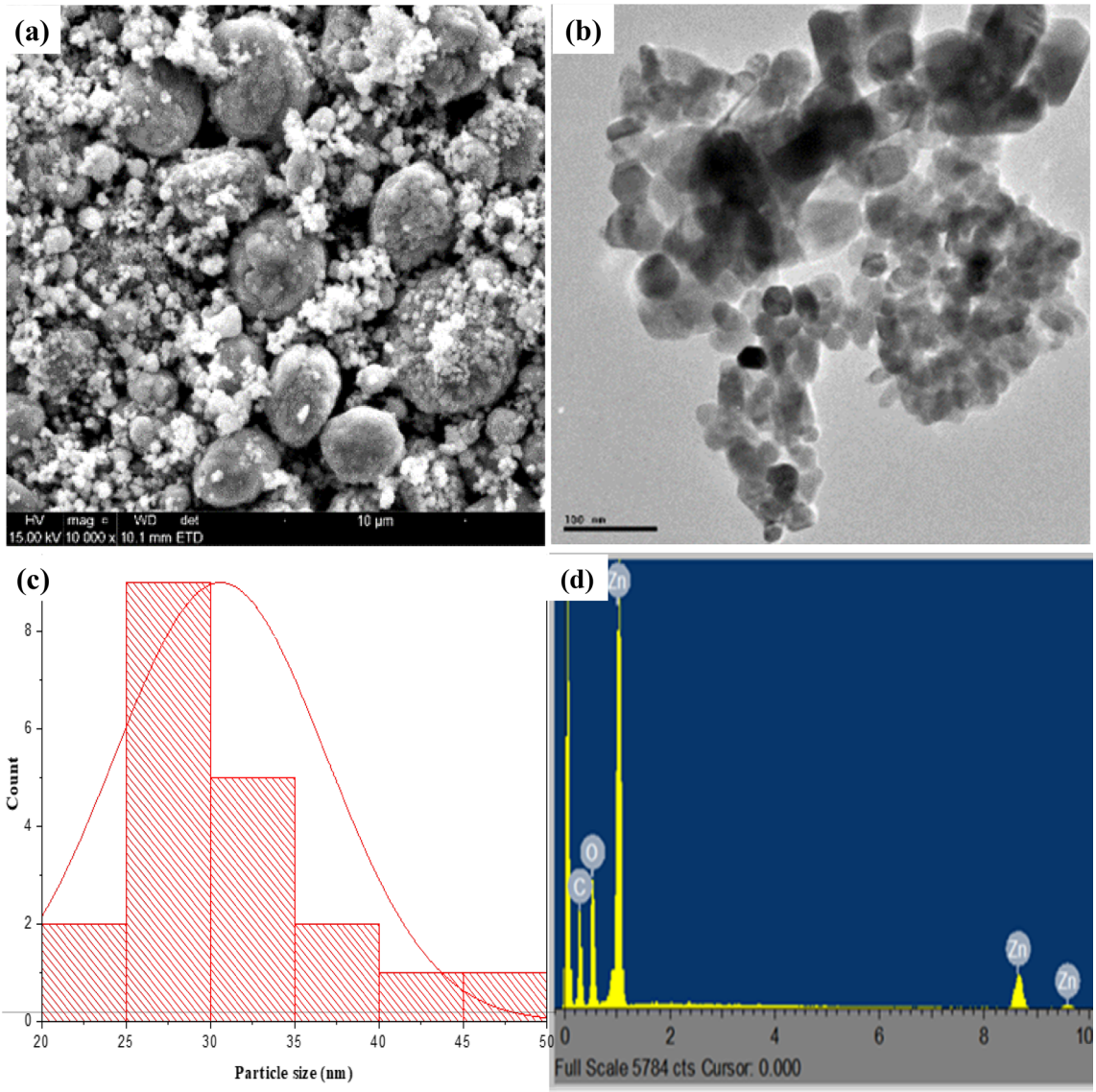

Fig. 3 **a** SEM, **b** TEM, **c** particle size distribution histogram, and the **d** EDS spectrum of the ZnO NPs synthesized using the extract of *M. pruriens utilis* seed

normal cells. Both cells treated with ZnO NPs showed > 50% decrease (Fig. 5) in cell viability at 100 µg mL^{-1} concentration. These results showed the ability of ZnO NPs to impede the proliferation of cancer cells, and this effect was concentration-dependent. Similar results showing anticancer effects of bio-synthesized ZnO nanoparticles have previously been reported [41, 42]. The IC_{50} value of 50.44 µg mL^{-1} (Table 1), for the obtained ZnO NPs exhibited a lower toxic effect than the 5-FU drug (17.48 µg mL^{-1}). Notwithstanding, other studies have demonstrated higher cytotoxic potential of green ZnO NPs in HeLa cells than that reported in our study. For example, ZnO NPs synthesized using the extract of *Tagetes erecta* flower displayed 50% cell viability at 26.53 µg mL^{-1} [43]. In contrast to our results, the percentage cell viability was recorded at 88.6% at the highest conc. (100 µg mL^{-1}) when ZnO NPs were biosynthesized using *A. arabica* leaf extract [44]. In another study, the IC_{50} concentration required for *Tradescantia pallida* mediated ZnO NPs was 62.5 µg mL^{-1} [45]. The discrepancies could be ascribed to differences in the concentrations of nanoparticles used. It could also be due to the variations in cellular density and the differences in the morphology and particle size of the NPs

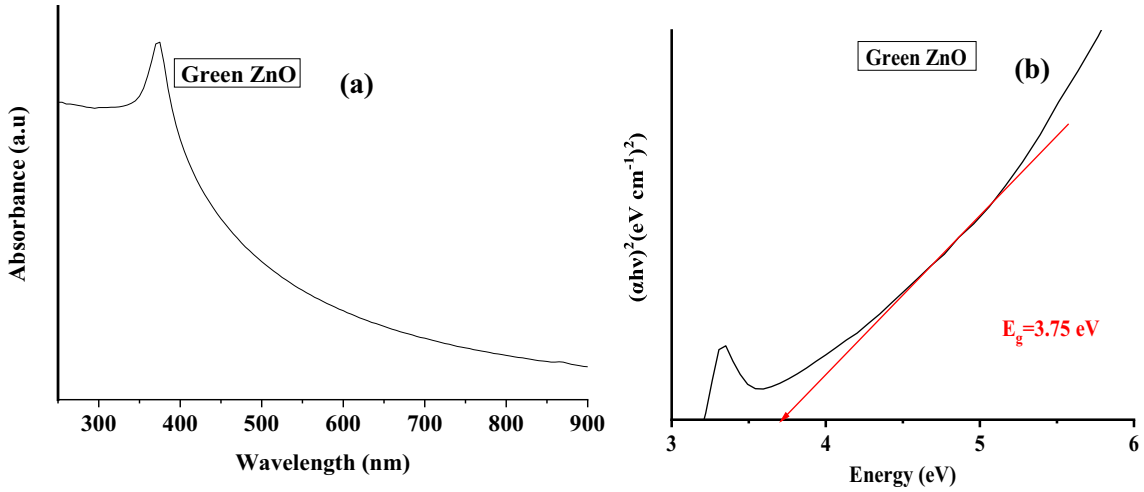

Fig. 4 **a** Absorption spectrum, and **b** Tauc plot of synthesized ZnO NPs using *M. pruriens utilis* seed extract

Table 1 The IC_{50} concentration of 5-FU versus that of ZnO NPs in HeLa and HEK 293 cells

Cell lines	Samples	Concentrations (μg mL^{-1})				IC_{50} μg mL^{-1}
		10	25	50	100	
HeLa	5-FU	78.40 ± 0.034	58.89 ± 0.037	50.47 ± 0.015	38.00 ± 0.017	17.48
	ZnO	76.56 ± 0.025	63.67 ± 0.046	46.57 ± 0.012	40.57 ± 0.055	50.44
HEK 293	5-FU	77.36 ± 0.048	51.92 ± 0.003	35.38 ± 0.010	11.33 ± 0.017	6.05
	ZnO	99.87 ± 0.040	74.36 ± 0.114	59.92 ± 0.049	35.75 ± 0.107	63.60

The mean viability (%) ± Standard deviation (n = 3)

[39]. Further, it was noted in the HEK 293 cell lines that the cytotoxic impact of ZnO NPs increased with the increase in their concentration. The ZnO NPs used in this study exhibited higher cytotoxicity (Table 1) than those synthesized using *Acantholimon serotinum* extract that had an IC_{50} value of 60 μg mL^{-1} [46]. Mkhize et al. [47]. reported 56 μg mL^{-1} for *Pleurotus ostreatus* mushroom-mediated ZnO NPs. Also, ZnO NPs prepared from extract of *Hertia intermedia* had IC_{50} of 240 μg mL^{-1} [48]. However, *Holigarna grahamii* mediated ZnO NPs displayed a higher cytotoxicity with an IC_{50} value of 9.060 μg mL^{-1}, which showed higher activity than the NPs reported in this study [49]. The variations in these reports for the HEK 293 cell lines may be attributed to properties including size of particles, surface area, and surface reactivity. Smaller-sized particles are reported to be more toxic than the larger particles [47], since they are able to penetrate the cellular walls.

3.6 Antioxidant Activity Analysis

The antioxidant activity of the green-synthesized ZnO NPs was assessed by evaluating their ability to scavenge free radicals generated by DPPH by measuring the reduction in the DPPH absorbance at 504 nm wavelength. The deep violet colour of the DPPH solution gradually changed to pale orange in the presence of ZnO nanoparticles and also with ascorbic acid (used as reference), which confirmed the antioxidant capacity of the nanoparticles, as previously observed [50]. Notably, the ZnO nanoparticles displayed better antioxidant potential (Table 2) compared to ascorbic acid, which may be attributed to the size of the nanoparticles and the phytochemicals in the mucuna plant extract used for nanoparticle synthesis [51]. These results agree with similar studies that reported higher antioxidant properties of ZnO NPs synthesized from the leaf extract of *Tecoma castanifolia* [52].

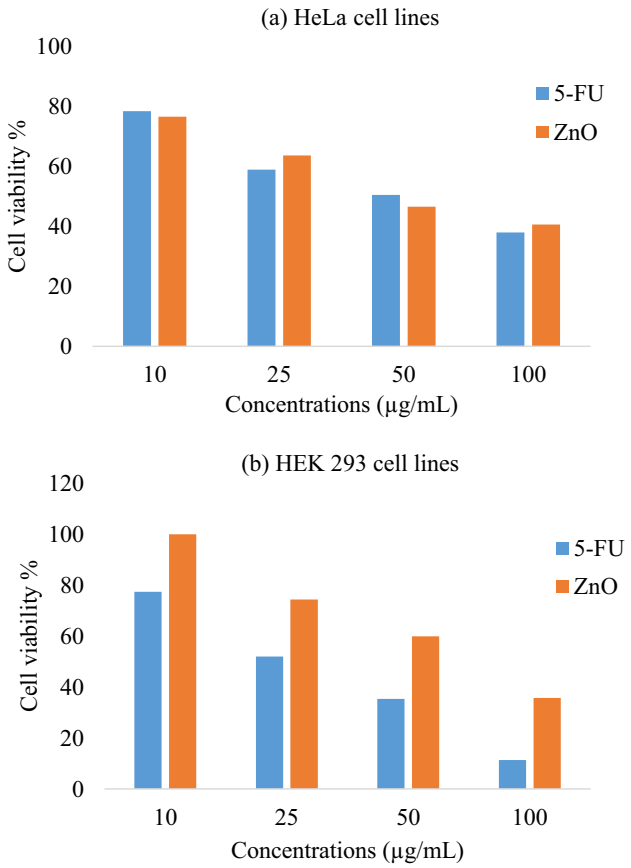

Fig. 5 The percentage viability of **a** HeLa and **b** HEK 293 cells treated with different concentrations of 5-FU and ZnO NPs. Each bar indicated mean ± standard deviations of three replications

The results also indicated that as the concentration of DPPH increased, the maximum DPPH radical scavenging activities increased to 55.25% (Fig. 6). This implies that the free radical scavenging ability of the ZnO nanoparticles is dose-dependent. The good antioxidant potential of ZnO NPs relates to their lower IC_{50} value (Table 2) of 4.10 µg mL^{-1} IC_{50}, which indicated quite a lower value than that of standard 4.72 µg mL^{-1}. Hence, the results of our study showed that the ZnO nanoparticles displayed higher antioxidant potency than the standard ascorbic acid which is in contrast to other reports by Chandra, Patel [27]. This could be attributed to the effect of size and shape of ZnO nanoparticles [53], as well as the effect of phytochemicals in mucuna seeds [54–56]. These results suggest that ZnO NPs could be used as a source of antioxidants in antioxidant-based therapies.

4 Conclusion

Zinc oxide nanoparticles, with average of size of 46.5 nm, were successfully synthesized by an environmentally friendly route using *M. pruriens utilis* seed extract. The crystallinity, morphological and optical properties, and composition of the ZnO NPs were determined by the different analytical techniques. The synthesized ZnO NPs showed good cytotoxic and antioxidant activities as they displayed significant percentage inhibition with IC_{50} values better than the reference compound. The results demonstrate the potential utility of the green synthesized ZnO NPs in different biological applications especially in tumor cells. Further research

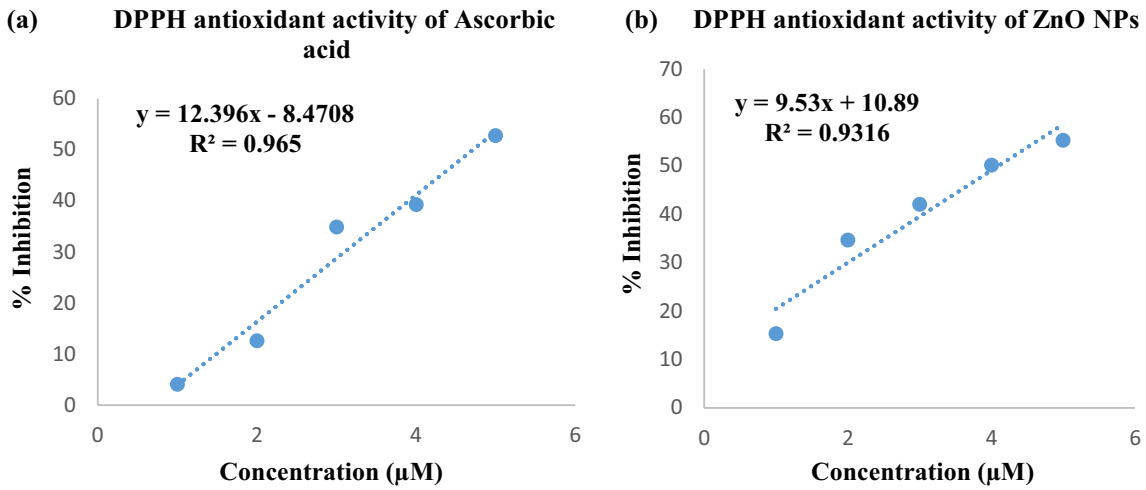

Fig. 6 The plot of percentage inhibition versus concentrations using **a** Ascorbic acid and **b** ZnO NPs

Table 2 DPPH antioxidant activity (%) of ascorbic acid and ZnO NPs

Test sample	Sample concentrations (μg mL^{-1})					IC_{50} (μg mL^{-1})
	3.13	6.25	12.50	25.00	50.00	
Ascorbic acid	4.08 ± 0.01	12.63 ± 0.004	34.88 ± 0.02	39.25 ± 0.01	52.75 ± 0.02	4.72
ZnO NPs	15.33 ± 0.02	34.66 ± 0.1	42.04 ± 0.02	50.11 ± 0.02	55.25 ± 0.01	4.10

The values are expressed as mean inhibition (%) ± standard deviation (n = 3). Ascorbic acid is the positive standard

is needed to explore their applications at the in vivo level, and to ascertain the biochemical reactions and mechanisms responsible for the antioxidant and anticancer activities.

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Declarations

Conflict of Interest On behalf of all authors, the corresponding author declares that there is no conflict of interest.

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