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Phytogenic fabrication of zinc oxide nanoparticles from the aqueous bark extract of *Acacia nilotica* and evaluation of its bioactivities: An eco-sustainable stratagem

Abstract

The phytonanotechnology stratagem was executed for the genesis of zinc oxide nanoparticles by applying bark aqueous extract of *Acacia nilotica* (AN-ZnO NPs). The AN-ZnO NPs emergence was substantiated through a distinct peak at 350 nm in the ultraviolet-visible spectroscopy. The fourier transform infrared spectroscopy exposed the phyto-organic moieties engaged as reduction and stabilization factors in fabricating nanoparticles. The crystalline trait, elemental proportions, spherical and hexagonal geometry of AN-ZnO NPs was witnessed by X-ray diffraction, energy-dispersive X-ray analysis and scanning electron microscopy. The high-resolution transmission electron microscopic technique provided the average size of 35 nm. AN-ZnO NPs presented larger inhibition zone against *Candida albicans* (22.7 ± 0.32 mm) at 40 μg/mL as an effectual fungicidal agent. Execution of in vitro antiradical scavenging assay of AN-ZnO NPs applying 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO), hydroxyl radical (OH^-), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) divulged radical scavenging trait with the minimum inhibitory concentration (IC_{50}) of 34.54, 38.0, 41.34 and 35.41 μg/mL, respectively. Cytotoxic feature against MCF-7 cell line in a dose-mannerly with IC_{50} value of 37.15 μg/mL in addition to apoptotic proteins expressions were displayed by AN-ZnO NPs. An appreciable larvicidal role of AN-ZnO NPs was rendered against *Anopheles stephensi* with LD_{50} (0.31 ppm) and LD_{90} (0.59 ppm), besides acetylcholinesterase inhibition. Further, biofilm origination was remarkably ceased by the *Staphylococcus aureus* and *Klebsiella pneumoniae*, respectively. All our experimented reports on AN-ZnO NPs attested to the credible applicability of ZnO NPs in biomedical care for health troubles.

Keywords: *Acacia nilotica*, Fungicidal, Antiradical, MCF-7, Larvicidal, Biofilm.

Highlights

- Medicinally worthy *Acacia nilotica* bark assisted fabrication of zinc oxide nanoparticles was achieved and characterized by analytical tools.
- A remarkable antioxidant and antifungal activity against the pathogenic fungi was produced by AN-ZnO NPs.

- AN-ZnO NPs showed effective cytotoxic features on MCF-cells with apoptotic proteins expression.
- AN-ZnO NPs displayed greater toxicity trait on *Anopheles stephensi* mosquito larvae.
- Potent antibiofilm ceasing property was presented by AN-ZnO NPs on both gram-positive and negative strains of bacteria.
- AN-ZnO NPs could function as a novel therapeutic for cancer and eradication of mosquitoes.

1 Introduction

Nanotechnology is stupendously aggrandizing with the urge to fabricate desired nanometric particles (1-100 nm) by shaping multifarious bulk material offering elite imperative physio-chemical traits like exceptional specific greater surface-area, chemical stableness, catalytic and optoelectronic features. Thus, booming its appreciative role in the nano-biomedicine [1]. Distinct physical and chemical (synthetic) methodologies were practiced to speedily tailor the nanoparticles. The need for higher energy, non-biodegradability and adherence of noxious solvents over the nanoparticles makes them unsuitable for the bio-nanomedicine due to aftermath impression on human health. These aforesaid hurdles have engrossed for the alternative eco-sustainable methodologies to fabricate nano-sized particles [2]. Bio-inspired fabrication of nanoparticles is at the current spotlight using natural living entities like microbes (bacteria, algae and fungi) and varying sections of the plant (stem, bark, flower, seed, etc.,). Sterile culture media for microbes were obviated by the adopting plants in the genesis of nanoparticles [3]. The profusely stuffed bio-organic entities in plants like polyphenolic and other active moieties are indulged in reducing and stabilizing nanoparticles by adhering to the metal ions. Size amenability, huge scale fabrication, steadiness, low-budget, safety and eco-sustainability are merits of phyto-inspired fabrication [4]. Already, inorganic metallic forms of nanoparticles such as titanium, silver, gold, zinc, nickel, palladium and copper have been massively produced and adopted in biomedical applications from plant origin [5].

Amid extensive groups of metal or metallic oxide nanoparticles, zinc oxide (ZnO) nanoparticles (NPs) with distinguished and impressive piezoelectric, optical, magnetic features have made its pertinency as a multifaceted metal oxide nanoparticle. Furthermore, the biological safety, chemical steadiness and biocompatible aspects of ZnO NPs have put forward its enormous applicability in medicinal nanoscience to mitigate myriad health maladies. The proposed therapeutic include antimicrobial, anti-diabetic [6], tissue engineering [7], wound healing [8] and drug nanocarrier [9].

The inevitable aerobic mechanism within the cells engenders free radicals as a natural by-product. Milder concentration of free radicals is crucial for immune responses. Reactive free radicals have havocking effect when overwhelmed within the biological system by supporting oxidative stress and fostering deformation of vital bio-organic structures that

includes lipid, nucleic acid and proteins. Consequently, implicating numerous health complaints such as heart problems, cancer, neurodegenerative disorders and diabetes mellitus Antioxidants from the natural platform are supreme to synthetic antioxidants. Medicinal plants with secondary phyto-metabolic active chemicals are stupendous natural antioxidants [10].
In the human race, fungal infections outline the chief burden. Antimycotic agents practiced topically for dermal or other infections can provoke the frequency of toxicity, lesser biological availability, itching and allergic skin responses. Metallic nanoparticles holding diminutive dimensions and modified features are in the task as a novel system of drug carriers with efficient desirability [11].

Globe-wide, cancer is the prime concerned for health complications and evidenced for major mortality in higher and lower-income countries, despite the sophisticated diagnostic and therapeutic tools availabilities. In females, breast cancer is the paramount reason for death. The utility of the chemotherapeutic regimen had substantiated the problem of toxicity to the unrelated tissues [12], especially gastrointestinal and neurotoxicity. Nanoparticles tailored through greener protocol have become the newer viewpoint as nanotherapeutics for neoplastic diseases [13].

Mosquitoes are the extremely deadly arthropod vector that are indulged in transmitting diseases in humans such as yellow fever, filariasis, malaria, dengue and Japanese encephalitis. As scrutinized by WHO, malaria is involved in greater mortality in all age groups. The tremendous resistance to insecticides on mosquitoes, toxicity to the non-related organism in the ecosystem and human illness issues has necessitated the affordable, novel and unfailing strategies for mosquito lysis [14]. The pristine and miscellaneous compounds acquired from plants function as furious bio-reducers and stabilizer agents in the emergence of metallic nanoparticles with an intense larvicidal trait. Plant-inspired metallic nano-larvicides are the non-hazardous and cheapest protocols to eradicate mosquitoes [15].

Biofilms developed by the bacterial communities are a peril to the human as they colonize dramatically over the exterior of the medical instruments rooting lung infections (tuberculosis), dental caries, chronic osteomyelitis, urinary tract infections and chronic wounds [16]. The resistibility of microbial strain to commercially usable synthetic antibiotics is the foremost threat now. There is an urgency for the innovation of newer drugs with utmost efficacy to combat the complications. Phyto-chemicals in curative plants and nano-based metallic particles are alternative origin to exterminate infectious population [17].

Acacia nilotica (*A.nilotica*) in the Fabaceae family is an imperative medicinally valued tree. It is also designated as Babul, Indian gum Arabic or Kikar and dispersed vastly in India, Africa and Middle East countries. The ayurvedic system adopts varying sections of the tree to eradicate human maladies such as earache, microbial infections, bleeding piles, cancer and diabetes [18, 19]. Surplus tannin in the bark has an astringent trait. The bark decoctions are applied to cure mouth cancer, uterus prolapse, leucorrhoea and syphilis diseases. Antioxidant, anti-hypertensive, anti-plasmodial and anti-inflammatory traits are also delineated in *Acacia nilotica* [20].

2. Experimental sections

2.1. Collection, authentication and processing of bark

The bark of medicinally valued *A.nilotica* (specimen) was collected from Gudiyattam, Vellore district, Tamil Nādu, India. Bark was scrapped with a sterile knife from the trunk section of a healthful disease-free tree. The taxonomy and identity of the selected tree (Voucher No: PARC/2021/4512) was validated at Herbal Plant Anatomy Research Centre (PARC), West Tambaram, Chennai and the voucher was preserved. The bark section collected was surface cleaned recurrently with surplus distilled water to eradicate grimy materials, shade dried to achieve a steady mass and incised into tiny pieces. Subsequently, powdered in a home blender, sieved and placed in an airtight jar at ambient temperature for all experimental investigations.

2.2. Bark extract preparation

The bark extract for the reduction and stabilizing of nanoparticles synthesis was prepared under sterile condition. Exactly, 3 g of powdered bark material was placed into a 250 mL of Erlenmeyer flask containing 100 mL of double distilled water and extraction was accomplished with persistent stirring on a hot plate magnetic stirrer at 60 °C for 20 min. The solution was cooled, double filtration was done using Whatman No.1 filter paper. The obtained clear yellow-brownish coloured aqueous bark extract was taken to synthesize zinc oxide nanoparticles (ZnO NPs).

2.3. Phytochemical profiling of aqueous bark extract of *A.nilotica*

Qualitative screening for the phytochemical active moieties existing within the bark extract of *A.nilotica* was executed as implemented by Pant et al. [21].

2.4. Phyto-based fabrication of zinc oxide nanoparticles

For harnessing ZnO NPs, exactly, 20 mL of bark extract of *A.nilotica* was vigorously stirred with 80 mL of 20mM zinc nitrate hexahydrate [Zn $(NO_3).6H_2O$] on a magnetic stirrer (600 rpm) at 60 °C for 4 h and kept undisturbed, followed by 24 h incubation. The settled muddy-coloured precipitate was centrifuged for 20 min at 10000 rpm. The pellet was triple washed with ethanol and double distilled water to eliminate impurities bound to the nanoparticles. The purified zinc oxide nanoparticles pellet was dried in an oven within 70-80 °C, made into a fine powder and calcinated in a muffle furnace at 400 °C for 2 h. The acquired zinc oxide nanoparticles from *A.nilotica* bark extract were designated as AN-ZnO NPs and retained in a sterile vial. Physiochemical characterization of nanoparticles was executed with advanced analytical and microscopic tools.

2.5. Physicochemical characterization of AN-ZnO NPs

2.5.1. UV-Visible spectroscopy of AN-ZnO NPs

The colour conversion on blending thoroughly the bark extract of *A.nilotica* with zinc nitrate was assessed visually. Through periodic sampling of aliquots (2-3 mL), the biogenic reduction of zinc ions into AN-ZnO NPs in the aqueous reaction solution was checked. The maximum spectral absorption was executed from 200-800 nm in a UV-Visible spectrophotometer (JASCO V-670 PC UV-Visible spectrophotometer).

2.5.2. Fourier Transform Infrared (FTIR) spectral analysis of AN-ZnO NPs

The phytometabolites, their functional groups and chemical bonds that existed in *A.nilotica* to reduce and stabilize the AN-ZnO NPs were verified in the unique spectra formed in the FTIR. Potassium bromide was mingled with AN-ZnO NPs to acquire the pellet under a hydraulic pellet machine, then placed within the sample holder and the scanning wavelength was ranged from 400 cm^{-1} to 4000 cm^{-1} at 4 cm^{-1} (resolution) in Nicolet iS50 (Thermo Fischer Scientific) FTIR.

2.5.3. Powdered X-ray diffraction (P-XRD) spectral assessment of AN-ZnO NPs

The crystallographic fingerprint and purity phase of AN-ZnO NPs was keenly assessed through powdered XRD. The X-ray diffraction was enforced adopting Bruker D8 Advance X-ray diffractometer having Cu Kα radiation, 2 theta range (20°-80° degree) handled at 30 kV with 10 mA (0.5s). The crystallite dimension of the fabricated AN-ZnO NPs was pointed out

using Debye-Scherrer's formula as mentioned, D=0.89λ/βcos Θ.. Here, the crystallographic size (nm) of nanoparticles was denoted to be D, wavelength of the used X-ray as λ (lambda = 1.5406 A°), FWHM (full-width at half maximum) as β and Bragg's angle of diffraction as Θ.

2.5.4. SEM and EDX analysis of AN-ZnO NPs

The surface morphological features of engineered AN-ZnO NPs was assessed in JOEL 6390LA scanning electron microscope (SEM). On a copper grid coated with carbon, a thin layer of AN-ZnO NPs was placed, dried under a mercury lamp and the imaging was done with magnification. The purity of AN-ZnO NPs, qualitatively existed elements were resolved in Energy Dispersive X-ray analysis (EDX) integrated with the scanning electron microscope.

2.5.5. High-resolution transmission electron microscopy (HR-TEM) and Selected area electron diffraction (SAED) of AN-ZnO NPs

The nanostructure, geometry and dimension of the AN-ZnO NPs were reported by FEI-Tecnai G2 20 Twin high-resolution transmission electron (HR-TEM) microscopic technique operated at a voltage of 80 kV. The ethanol sonicated suspension (4 μL) of AN-ZnO NPs was coated over the copper-carbon coated grid and scrutinized for surface morphology under the microscope. Image J Software (1.45s) was used to evaluate the size of the nanocrystals. To further confirm the crystallinity of nanoparticles, SAED (selected area electron diffraction) pattern was also recorded with HR-TEM.

2.6. Antifungal susceptibility of AN-ZnO NPs

A classic well diffusion protocol [22] was applied to check the fungicidal property of AN-ZnO NPs on randomly chosen six pathogenic fungal strains namely, *Aspergillus niger* (MTCC-9652), *Aspergillus flavus* (MTCC-873), *Rhizopus oryzae* (MTCC-9605), *Candida albicans* (MTCC-4748), *Microsporum gypseum* (MTCC-2830) and *Trichophyton rubrum* (MTCC-7859). Appropriately, 100 μL of each fungal strain inoculum was carefully swabbed over the solidified surface of the potato dextrose agar medium taken in a sterile individual petri-plates with a L-shaped glass rod. With the aid of sterile gel borer (6 mm) wells were made within the PDA medium and AN-ZnO NPs with varying concentrations (10 μg/mL, 20 μg/mL, 30 μg/mL and 40 μg/mL) were placed into the wells using a micropipette and incubated for 2-4 days at room temperature. The fungicidal potentiality of the AN-ZnO NPs was visualized by measuring the inhibition zone width (in terms of mm). Amphotericin B with 10 μg/mL was utilized as a drug of positive control.

2.7. Antiradical assay of AN-ZnO NPs

2.7.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

The antiradical potential of phyo-inspired harnessed AN-ZnO NPs was assessed using DPPH (2,2-diphenyl-1-picrylhydrazyl) method as previously documented [23] with minor variations. At first, 1 mL of synthesized AN-ZnO NPs in diverse range of concentrations (20 μg/mL to 60 μg/mL) were individually combined with DPPH of 4 mL prepared as 0.004% solution in methanol and kept undisturbed at the ambient temperature in dark condition for 30 min. The absorbance of the faded purple colour of DPPH to yellow colour was recorded spectrophotometrically at 517 nm. The AN-ZnO NPs reports were compared to the standard reference solution of ascorbic acid. As blank, methanol was used. The radical quenching ability was calculated in percentage (%) as mentioned in the below-provided equation.

$$\text{DPPH radical quenching ability (\%)} = [(C_{(Abs)} - S_{(Abs)})/C_{(Abs)}] \times 100$$

Here, $C_{(Abs)}$ - Control (DPPH in methanol) absorbance, $S_{(Abs)}$ - AN-ZnO NPs /Ascorbic acid (along with DPPH) absorbance. The sample concentration offering 50% antiradical ability (IC_{50}) was also calculated.

2.7.2. Nitric oxide (NO) scavenging assay

Griess reaction was adopted to quantify the nitrite ions produced due to the interaction of aqueous solution of sodium nitroprusside with oxygen as earlier prescribed by Sylvie et al. [24] including few alterations. Nitric oxide (NO) scavenging activity was screened for AN-ZnO NPs. About 20 μg/mL to 60 μg/mL concentrations range of AN-ZnO NPs (each 1 mL) were well blended in separate test tubes with 2 mL of 10 mmol/L of sodium nitroprusside solution prepared using 50 mmol/L of phosphate buffer (pH 7.4). The reaction tubes were incubated for nearly 150 min at ambient temperature, subsequently added one mL of 0.33% of sulfanilic acid (diluted in 20% glacial acetic acid) reacted to the reaction mixture (0.5 mL) and left for diazotization for 5 min. Before incubation for 30 min, 1 mL of 0.1 % napthylethylenediamine dihydrochloride was reacted to the test tube contents. Spectrophotometrically, the developed chromophore (pink colour) intensity was recorded at 540 nm. Ascorbic acid served as a positive reference. The blank solution was methanol. The below-provided formula was implemented to calculate the % (percentage) of reduction in nitric oxide radical by AN-ZnO NPs.

$$\text{\% Radical (NO) reduction} = [(C_{(Abs)} - S_{(Abs)} / C_{(Abs)})] \times 100$$

Here, $C_{(Abs)}$ - Control (without AN-ZnO NPs) absorbance, $S_{(Abs)}$ - AN-ZnO NPs /Ascorbic acid absorbance. IC_{50} value was also computed.

2.7.3. Hydroxyl radical scavenging (OH^-) assay

The scavenging potentiality of AN-ZnO NPs on the hydroxyl radical (OH^-) was executed with the protocol implemented by Subramanian et al. [25]. Initially, 0.2 mL of AN-ZnO NPs of varied ranges (20 μg/mL to 60 μg/mL) were added into the independent test tubes along with 1 mL solution of EDTA (0.13 % of anhydrous ferrous ammonium sulphate with 0.26 % of EDTA diluted in 100 mL of distilled water) and completely blended with 0.85 % of DMSO (1 mL) in phosphate buffer of 0.1 M (pH 7.4) to trigger the reaction. Subsequently, 0.2 % of ascorbic acid (0.5 mL) was further added into the reacting mixture. The contents were boiled within the water bath for 15 min at 90 °C. Termination in the reaction was done by pouring 17.5 % of ice-cold trichloroacetic acid (1 mL), 3 mL of Nash reagent (75 % of ammonium acetate, 2 mL of acetylacetone and 3 mL of glacial acetic acid were combined together and volume was brought to 1 L using distilled water) was poured into all reaction tubes, incubated at room condition for 15 min. The yellowish chromophore obtained was spectrophotometrically recorded at 412 nm. Ascorbic acid was utilized as positive reference control and without ascorbic acid, the reaction mixture served as a negative control. By implementing the below-provided formula, the hydroxyl radical scavenging capacity of AN-ZnO NPs was calculated.

$$\text{Percentage of } OH^- \text{ radical scavenged} = [(C_{(Abs)} - S_{(Abs)} / C_{(Abs)})] \times 100$$

Here, $C_{(Abs)}$ – Control (without AN-ZnO NPs) absorbance, $S_{(Abs)}$ – AN-ZnO NPs/ Ascorbic acid absorbance. IC_{50} value was also computed.

2.7.4. ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

The protocol of ABTS scavenging assay of AN-ZnO NPs was executed with slight variations as done by Kuppurangan et al. [26]. By amalgamating an equal proportion of 7mM solution of ABTS with 2.4 mM of potassium persulphate, a working solution was obtained and retained in a dark place for 12 h in room condition. Next, 1 mL of the resulted solution was thoroughly mingled with 1 mL of varying concentrations (20 μg/mL to 60 μg/mL) of AN-ZnO NPs. Six minutes later, the reacted mixture was read spectrophotometrically at 734 nm. The aptness of AN-ZnO NPs to scavenge ABTS was found out in percentage (%) by applying the below indicated formula:

$$\text{Percentage of ABTS inhibition} = [(C_{(Abs)} - S_{(Abs)})/ C_{(Abs)}] \times 100$$

Here, $C_{(Abs)}$ - Control (without AN-ZnO NPs) absorbance, $S_{(Abs)}$ - AN-ZnO NPs /Ascorbic acid absorbance. IC_{50} value was also computed.

2.8. Cytotoxic assay

2.8.1. Culture

The MCF-7 (Breast cancer cell line) was procured from NCCS (National Centre for Cell Science, Pune, India) and cultured in high glucose medium (DMEM) acquired from (Sigma-Aldrich, India), also boosted with 10% of FBS (fetal bovine serum), 20 mL of antibiotic penicillin (1% w/v). The cells were sustained in an atmosphere of CO_2 (5%) with humidification (95%) at 37 °C.

2.8.2. MTT -mediated cytotoxic evaluation of AN-ZnO NPs

MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) assay was executed for AN-ZnO NPs on MCF-7 (Breast cancer cell line) cell line as previously performed by Menon and Shanmugam [27]. Exponentially proliferating MCF-7 cells (10^5 cells/well) were aseptically placed into 96-well cell-culture plate and left undisturbed for 24 h in 5% CO_2 at 37 °C for 1 day to acquire confluency. Diverged concentrations of prepared AN-ZnO NPs (10 μg/mL to 90 μg/mL) was exposed to the MCF-7 cells, re-incubated for 24 h at 37 °C respectively. Next, 100 μL of 0.5% MTT (1mg/mL) diluted in PBS was included in all the wells and re-incubated for 4 h at ambient condition. The supernatant MTT solution used was discarded. Using PBS, cells were washed. The finally procured pink coloured crystals of formazan were diluted in dimethyl sulfoxide (100 μL). The value of absorbance was monitored at 570 nm with the aid of ELISA plate reader for each tried concentration. The inhibition of cell viability percentage (%) was computed using the below-mentioned formula:

Cell viability (%) = [(A_{570} value of tested cells/ A_{570} value of control cells) x 100]

2.8.3. Determination of ROS using DCFH-DA (Dichloro-dihydrofluorescein diacetate method)

The in vivo ROS emergence in the MCF-7 cells on treatment with AN-ZnO NPs was assessed using DCFH-DA staining protocol. The non-fluorescent DCFH-DA dye on interaction with the ROS generates a fluorescent compound DCF (2,7-dichlorofluorescein). In brief, 1 x 10^5 MCF-7 cells/well were carefully laid into a microplate with 6-well. The MCF-7 cells were reacted with IC_{50} concentration of AN-ZnO NPs for 24 h and 48 h within the CO_2 incubator. DCFH-DA stain (10 μM) was exposed to the cultured cells and further incubated for 30 min at room temperature (37 °C). Consequently, the reacted cells were double washed with cold phosphate buffer solution (PBS) and resuspended in PBS. Analysis of cells (reacted

and unreacted) was performed beneath the fluorescence microscopic technique (40 X objective). Doxorubicin (500 μg/mL) was utilized as a positive control [28]. The fluorescence intensity was read with a fluorescence microplate reader at excitation (485 nm) and emission (550 nm) respectively.

2.8.4. Analysis of mitochondrial membrane potential (MMP) using Rhodamine 123 staining

In brief, MCF-7 cells (1 x 10^5 cells/well) were placed into the 6-well microplate. AN-ZnO NPs at their respective IC_{50} concentration was reacted to the MCF-7 cells and left undisturbed. After 24 h and 48 h, the reacted cells were carefully harvested and using PBS rinsed twice. Then the cells were exposed with Rhodamine-123 dye (Rh-123; 10μg/mL) for 30 min at 37 °C in a dark condition inside the CO_2 incubator and re-rinsed using PBS. At last, the intensity of fluorescence given by the Rh-123 stain was visualized with fluorescent microscope and photographs of the treated cells as well as untreated cells (negative control) along with doxorubicin (500 μg/mL) positive control was captured. The pattern of depolarization in the mitochondrial membrane was seen [29]. The fluorescence intensity was read with a fluorescence microplate reader at excitation (485 nm) and emission (550 nm), respectively.

2.8.5. Morphological assessment of nucleus using DAPI

The condensation of chromosomes in the apoptotic cells was identified by DAPI (4,6-diamidino-2-phenylindole), a fluorescent probe specific to the nuclei. The MCF-7 cells treated with AN-ZnO NPs (IC_{50} concentration) were incubated for 24 h and 48 h. Then using PBS (phosphate buffer solution) the cells were repeatedly washed and fixed with formaldehyde (4%). Using DAPI solution (1μg/mL) the cells were stained and left at 37 °C for 5 min. MCF-7 cells without AN-ZnO NPs exposure were utilized as a negative control whereas doxorubicin (500 μg/mL) exposed cells as a positive control. The cells were finally washed using PBS and the morphology of the nucleus was visualized under a fluorescence microscope with a blue filter (420 nm) [30].

2.8.6. Western blotting technique

To detect the apoptotic (Bax, Caspase-3 and Caspase-9) and antiapoptotic expression of proteins (Bcl-2) along with proliferative protein marker expression (Cyclin D1 and PCNA) in the MCF-7 cells (1 x10^5 density) exposed to AN-ZnO NPs were taken for western blot technique [31]. MCF-7 cells cultured in 6-well tissue culture plates were reacted to AN-ZnO

NPs (IC_{50} concentration) for 24 h and 48 h. In this blot assay, β-actin served as a standard loading control. At the end of incubation time, harvesting of cells was done utilizing RIPA lysis buffer solution and then quantified protein concentration spectrophotometrically. Electrophoresis of protein was executed from the collected sample in 12% SDS-PAGE (sodium dodecyl sulphate -polyacrylamide gel electrophoresis). Transfer of proteins from the gel onto nitrocellulose membrane was carried out and the membrane was blocked with 5% BSA (bovine serum albumin) for 1 h to hinder non-specific binding. The membrane was exposed to primary mAb (monoclonal antibodies) and left for 24 h at 4 °C. Gentle washing of membrane using TBST buffer was done, then secondary antibodies were reacted to the membrane and re-incubated for 1 h at ambient condition. At last, the membrane was thoroughly washed in TBST buffer and the levels of expression of proteins were detected with the aid of a chemiluminescent detection system (Biorad, USA).

2.9. Larvicidal efficacy of AN-ZnO NPs

The larvicidal efficacy of AN-ZnO NPs was accomplished with a standard methodology as applied by the WHO [32] and Rawani et al. [33] with minor changes. For the larvicidal bioassay test, five sets of targeted 3rd instar larvae of *Anopheles stephensi* were taken (100 larvae/set). For each concentration of test sample, one set was used. In a sterile 250 mL glass beaker insertion of 100 mosquito larvae in 200 mL of AN-ZnO NPs solution of desired concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 ppm)) were implemented. Each performed test was incorporated with a control group (distilled water). No feed was given to the larvae during the assay. Mortality was noted after 24 h post-treatment to the AN-ZnO NPs. The total number of dead larvae divided by the number of alive larvae multiplied by 100 was used to calculate individual mortality percentage in each concentration.

2.9.1. Larval whole-body homogenate preparation and acetylcholinesterase assay

The departed larvae of *Anopheles stephensi* (3rd instar) exposed to AN-ZnO NPs for 24 h were retrieved, washed with distilled water to remove adherents and blotted with tissue paper to withdraw moisture content. Then homogenized with ice-cold sodium phosphate buffer solution (20 mM; pH 7.4), centrifuged at 8000 rpm for 15 min and finally the supernatant was taken for enzyme assay [34].

2.9.2. Acetylcholinesterase (AChE) inhibition by AN-ZnO NPs

The protocol of Ellman et al. [35] with certain alterations [36] was applied to assess the AChE inhibition activity of AN-ZnO NPs. Shortly, the solution of AChE (10 μL), AN-ZnO NPs (20 μL) of diverged concentrations and cold-phosphate buffer (150 μL) were placed in the 96-well microtiter plate followed by refrigeration (1-4°C). Then inhibitor of varying concentration diluted in DMSO (0.1%), 0.4 mM acetylthiocholine iodide (20 μL) and DTNB (Ellman's reagent) was poured into each well. The reacting mixture was incubated at 37 °C for 30 min and the colour alteration (yellowish/ colourless) of the solution was recorded at 412 nm by a microplate reader. The inhibition % of acetylcholinesterase enzyme by AN-ZnO NPs was calculated by applying the below-provided equation:

$$\text{Inhibition in } \% = [(C_{(Abs)} - S_{(Abs)})]/\ C_{(A)}] \text{ x } 100$$

Here, $C_{(Abs)}$ = Control absorbance, $S_{(Abs)}$ = Sample absorbance.

2.9.3. Histological assessment of *Anopheles stephensi*

Histological analysis was executed to notify the alteration in the tissues of larvae. Both control larvae along with the AN-ZnO NPs exposed larvae (treated) of *Anopheles stephensi* mosquito were immersed in the fixative solution (10% formaldehyde), dehumidified with ethanol, cleansed with xylene solution, embedded in paraffin and sectioned to get 5 μm thick tissue slices in a rotary ultramicrotome instrument. Paraffin was eradicated from the tissues and stained using Haematoxylin and Eosin stain and abnormalities were examined under an inverted microscope. The photographic images were captured with a digital camera [37].

2.10. Antibiofilm potentiality using Microtiter plate assay

The antibiofilm efficacy of AN-ZnO NPs was executed by acquiring microtiter plate assay (MTP) as applied by Kumar et al. [38] with certain amendments. Shortly, 180 μL of Mueller-Hinton broth and 10 μL of overnight grown biofilm developing pathogenic bacterial cultures of *Staphylococcus aureus* (MTCC-3160) and *Klebsiella pneumoniae* (MTCC-432) were poured in a microtiter well individually and exposed with differing concentrations (1.17µg/mL to 300 μg/mL) of AN-ZnO NPs along with the negative control (without nanoparticles) followed by maintained at 37 °C for 24 h. Using 0.2 mL of PBS (phosphate buffer saline) removed unadhered bacteria. The adherent bacteria in the microtiter plate wall were further fixed by using 2% w/v of sodium acetate and stained using 0.1 % w/v of crystal violet. The extra stain was removed off by complete washing with sterilized distilled water and

left for drying. Then washed with 200 μL of ethanol (95% v/v) and finally, the absorbance was read at 595 nm using a microtiter plate reader. The inhibition % of the biofilm formation was computed by applying the formula:

$$\text{Biofilm inhibition (\%)} = [(Ac- As)]/Ac] \times 100$$

Here, Ac – Control absorbance, As- Sample absorbance.

Statistical analysis

Each experimental study was executed in three trials and the results were provided as mean ± standard deviation (SD). Graphpad Prism 9 software was applied for descriptive statistical analysis. Probit analysis was performed with NCSS 2021, v21.02 software for calculating LC_{50}, LC_{90} values. Data with probability (p) < 0.05 were taken to be statistically significant.

3. Results and discussion

3.1. Phytochemical screening

The chromogenic reactions carried out by adopting standard protocol in bark extracts of *A.nilotica* disclosed the existence of alkaloids, tannins, flavonoids, glycosides, terpenoids, phenol, saponins and proteins (Table 1).

Table 1

Phytochemical profiling of aqueous bark extract of *Acacia nilotica*.

Plant metabolites	**Identification test**	**Colour visualized**
Alkaloids	Mayer's test	Creamy coloured precipitate formed
Tannins	Ferric chloride test	Bluish-black colouration developed
Flavonoids	Shinoda test	Crimson red colouration appeared
Glycosides	Keller-Killiani test	Brownish-ring deposited at the interface of two liquids.
Terpenoids	Salkowski's test	Reddish-brown precipitation formed
Phenol	Ferric chloride test	Deep bluish-green colouration appeared
Saponins	Foam test	Tenacious bubbles formation
Proteins	Biuret test	Violet colouration appeared

3.2. Visual observation and predictable mechanism in the genesis of AN-ZnO NPs

As a greener and eco-benefited avenue, ZnO NPs availing bark aqueous extract of *A.nilotica* was produced. The mild yellowish bark extract was turned into darkish-brown on complete blending with zinc nitrate in 24 h (Fig. 1). This predicted the productive fabrication of ZnO NPs. Surface plasmon resonance (SPR) excitation attributed to the colour-shifting demonstrated AN-ZnO NPs genesis. The existed chemical groups electrons of plant bark extract were accountable for the biological reduction of zinc ion (Zn^{2+}) in the zinc salt to nano zinc oxide [39]. Therefore, concluded the function of bark extract as bio-reducer and stabilizing medium for nanoscale zinc oxide formation. Similarly, phyto-extract based colour shifting was detected in the study by Aminuzzaman et al. [40] in ZnO NPs fabrication.

Fig. 1. Photograph revealing the visual colour change from mild yellowish to darkish-brown and fabrication of AN-ZnO NPs by blending bark (aqueous) extract of *A.nilotica* and zinc nitrate solution.

3.3. UV-Visible (UV-Vis) spectroscopy of AN-ZnO NPs

UV-Vis spectral analytical study is the most extensively applied optical technique to substantiate ZnO NPs formation via bioreduction. The synthesized AN-ZnO NPs were taken for UV-Vis spectral analysis. The optical spectra of formed AN-ZnO NPs were noted periodically. An intensified peak at 350 nm substantiated the nanocrystal zinc oxide formation (Fig. 2). In conformity to Gupta et al. [41], a systematic shift of absorption spectra wavelength to higher or lower happens with the diminution of nanoparticle size. Surface plasmon resonance sways the shape and the nano dimension of the zinc oxide particles. Thus, a bold absorption spectra shift (blue shift) is confined to smaller size nanoparticles than an exciton Bohr radius of ZnO [42]. UV-Visible spectrum reported in our findings also correlated as experimented by already mentioned findings where phyto-inspired genesis of ZnO nanoparticles has been accomplished utilizing *Kalopanax septemlobus* and *Carica papaya* bark extract [43, 44]. The acquired AN-ZnO NPs were further characterized.

Fig. 2. UV-Vis absorption spectrum of phytofabricated AN-ZnO NPs implementing *A.nilotica* bark extract.

3.4. Fourier Transform Infrared (FTIR) spectral analysis of AN-ZnO NPs

The responsible chief chemical groups (minor/major) engaged to reduce zinc ions and capped zinc oxide nanoparticles in the bark of *A.nilotica* were unfolded in the FTIR spectroscopic analysis (Fig. 3). AN-ZnO NPs obtained a broad strong band at 3467 cm^{-1} indicated for O-H stretching owned to phenolic and alcoholic compounds. Two weak bands at 2923 cm^{-1} and 2850 cm^{-1} were implied for alkane and carboxylic groups (C-H vibrational asymmetric/symmetric stretching) [45]. Alkene group (C=C) was verified at 1623 cm^{-1}. The C-N vibration stretching of the aromatic ring in aromatic amine was represented at 1325 cm^{-1}. A band represented at 1130 cm^{-1} (C-O) for carboxylic and an ester group. Then at 620-440 cm^{-1} band (metal oxide stretching) was assigned to Zn-O stretching mode [46]. A band achieved at 440 cm^{-1} further evinced the generation of AN-ZnO NPs by the display of vibrational Zn-O stretching trait. This was in parallelism to the before documented data of experimental ground [47]. The FTIR data reinforced the miscellaneous biochemical entities (proteins, alkaloids, polyphenols, flavonoids, terpenoids and carboxylic acids) that have been implicated in the plant-mediated greener route for synthesizing AN-ZnO NPs as tested in phytochemical profiling.

Fig. 3. Fourier Transform Infrared (FTIR) spectral analysis of AN-ZnO NPs.

3.5. Powdered X-ray diffraction (P-XRD) spectral assessment of AN-ZnO NPs

The acquired diffraction patterns added knowledge on the existing phase chemistry, pureness and crystalline trait of the AN-ZnO NPs. The XRD peak pattern of AN-ZnO NPs conveyed peak (2 theta) at 31.97°, 34.65°, 36.45°,47.63°,56.66°,62.87° and 67.84° that cognate to the crystalline phase (hkl) of 100, 002, 101, 102, 110, 103 and 112 respectively and indexed to be the wurtzite or hexagonal feature of AN-ZnO NPs (Fig. 4). The crystallite dimension was computed and the average grain size was calculated to be 39.01 nm. The achieved sharp peaks matched with the standard JCPDS file number 89-0510. The AN-ZnO NPs size executed in the XRD was in correlation to the data obtained in TEM characterization [48].

Fig. 4. Powdered-XRD spectral pattern of AN-ZnO NPs.

3.6. SEM and EDX analysis of AN-ZnO NPs

Assessment of surface structure of AN-ZnO NPs was done using scanning electron microscopy. Spherically and hexagonally shaped nano zinc oxide particles with non-uniformity, randomly arranged were pictured in the SEM micrographs presented in Fig. 5A. Aggregation of nanoparticles was spotted. The aggregation may be due to the occurrence of

Van der Waals force between the nanoparticles. This sort of aggregation does not have any impression on particle stability. The obtained AN-ZnO NPs structures were in accordance as previously documented by Agarwal et al. [49] and Nithya et al. [50]. The elemental composition yield of the AN-ZnO NPs was 63.07 % of zinc and 36.93 % of oxygen in EDX (Fig. 5B). The lack of other elemental peaks justified the purity of AN-ZnO NPs. Already similar EDX elemental composition has been published in the work of Agarwal et al. [49] and Fakhari et al. [51].

Fig. 5. (A) Scanning electron microscopic photographs of AN-ZnO NPs **(B)** EDX graph with elemental composition of phytofabricated AN-ZnO NPs.

3.7 High-resolution transmission electron microscopy (HR-TEM) and Selected area electron diffraction (SAED) of AN-ZnO NPs

The surface morphology and size of the AN-ZnO NPs were proffered by high-resolution transmission electron microscopic (TEM). The micrograph of TEM manifested varied morphology like hexagonal and spherical for the AN-ZnO NPs. The average width size was calculated to be 35 nm respectively (Fig. 6A). Vijaykumar et al. [52], produced ZnO NPs from *Atalantia monophyla* leaf extract also illustrated similar morphology in TEM. As the synthesis was achieved in an aqueous medium, the particles owned higher surface energy which probably led to ZnO NPs agglomeration and even densification might also have trigger agglomeration causing confined space among the nanoparticles. This was displayed in the TEM micrograph [53]. The calculated nanoparticle size in TEM complements with XRD results. Particularly, the hexagonal shape (Wurtzite shape) congruent to the pattern in XRD. High-resolution transmission electron microscopy (HR-TEM) micrograph with selected area diffraction pattern was depicted having the concentric ring with bright coloured spots authenticated for the nanocrystalline form of AN-ZnO NPs in Fig. 6B. Previously, biogenically prepared ZnO NPs displayed a similar SAED pattern [54].

Fig. 6 (A) Transmission electron microscopy photograph of phyofabricated AN-ZnO NPs **(B)** Selected area electron diffraction (SAED) pattern of AN-ZnO NPs.

3.8. Antifungal susceptibility of AN-ZnO NPs

AN-ZnO NPs were analysed on the chosen pathogenic strains of fungi for fungicidal role using a well-diffusion method in the PDA media. The inhibition zone was achieved for each fungal strain on the desired varied concentrations of AN-ZnO NPs. There was a dose-

mannered rise in the width of the inhibition zone on each tested fungal pathogen. The greatest inhibitory zone was achieved for *Candia albicans* (22.7 ± 0.32 mm) at 40 μg/mL followed by *Aspergillus flavus* (21.2 ± 0.22 mm), *Rhizopus oryzae* (18.3 ± 0.51 mm), *Aspergillus niger* (18.1 ± 0.76 mm) and *Trichophyton rubrum* (17.6 ± 0.30 mm). Least width of inhibition zone was formed for *Microsporum gypseum* (15.3 ± 0.50 mm) at 40 μg/mL, respectively. The outcome of the tested AN-ZnO NPs at pathogenic fungal strains was revealed in Fig. 7 and Table 2, respectively. Amphotericin B was used as a comparative standard drug in this assay. Dobrucka et al. [55], also displayed a similar type of antifungal trait of *Chelidonium majus* extract bio-formed ZnO NPs on the tested yeast, filamentous and dermatophytes as achieved in our test. Contact of zinc oxide nanoparticles with the fungal cell membrane causes intracellular upsurge of reactive oxygen species (ROS), peroxidation of lipid content, distortion of protein and nucleic acid molecules leading to entirely collapse of fungal membrane integrity and killing fungal cells via apoptotic mechanism thus leakage of mitochondrial cytochrome c [56]. Also, it has been elucidated that phyto-involved ZnO NPs were able to arbitrate the swelling of fungal hyphae or expansion of its vacuoles thus deforming the fungal architecture and ultimately fungal lysis [57].

Table 2

Fungicidal activity of AN-ZnO NPs on diverged range of pathogenic strains of fungi.

Pathogenic fungal strains	Inhibition zone size in mm				
	10 μg/mL	20 μg/mL	30 μg/mL	40 μg/mL	Amphotericin B (10 μg/mL)
Aspergillus niger	12.0 ± 0.00	14.2 ± 0.80	17.0 ± 0.27	18.1 ± 0.76	20.3 ± 0.52
Aspergillus flavus	13.1± 0.72	16.3 ± 0.57	19.4 ± 0.59	21.2 ± 0.22	28.3 ± 0.73
Rhizopus oryzae	10.2 ± 0.51	12.0 ± 0.44	16.3 ± 0.29	18.3 ± 0.51	19.0 ± 0.86
Candida albicans	14.3 ± 0.60	17.5 ± 0.30	19.1 ± 0.87	22.7 ± 0.32	27.0 ± 0.90
Microsporum gypseum	9.0 ± 0.32	11.5 ± 0.73	13.1 ± 0.00	15.3 ± 0.50	22.1 ± 0.56
Trichophyton rubrum	10.4 ± 0.51	12.2 ± 0.48	14.4 ± 0.63	17.6 ± 0.30	24.3 ± 0.49

The numerical reports (n=3 trials) are given as mean ± standard deviation.

Fig. 7. Antifungal assay images of *A.nilotica* bioengineered AN-ZnO NPs against the randomly chosen pathogenic fungal strains by agar well diffusion protocol.

3.9. Antioxidant assessment of AN-ZnO NPs

3.9.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity

The free radical scavenging trait of AN-ZnO NPs was executed out using standard assay protocol and the acquired data were illustrated in Fig. 8A. The report acquired provided that with the elevation in the concentrations of AN-ZnO NPs, an impressive increased antiradical activity was achieved in DPPH test. The radical scavenging efficacy of AN-ZnO NPs was from 19.0% to 80.21%. Also, the IC_{50} level for AN-ZnO NPs was 34.54 μg/mL. Ascorbic acid reference conveyed IC_{50} value of 30.55 μg/mL, respectively. *Ruta graveolens* (L.) [58] extract inspired genesis of ZnO NPs, also showed a dose mediated elevation in radical scavenging trait. Discolouration of the purplish chromophore of DPPH to yellowish chromophore by adopting electrons from the antioxidants is the signature trait in this assay. The diminutive size and transfer of electrons from the densified layer of an oxygen atom to the odd electrons in the residing nitrogen atom of DPPH are liable for the antioxidant trait [59].

3.9.2. NO (Nitric oxide) radical scavenging activity

In vivo NO (nitric oxide) genesis occurs consistently in diverse biological functions. The superfluous nitric oxide along with oxygen forms highly reactive free radical species that include nitrite and peroxyl nitrite anions. An escalation of the nitric oxide, free radical species is toxic and directly creates tissue damage attributing to the threat of cancer, arthritis and Alzheimer's disease [60]. Varied concentrations of AN-ZnO NPs were experimented for nitric oxide scavenging capacity. With the escalation of concentrations of AN-ZnO NPs, the radical scavenging trait were also elevated which was 13.74% (at 20 μg/mL) and 80.61% (at 60 μg/mL) as provided in Fig.8B. The acquired results on the NO assay offered utmost activity for AN-ZnO NPs with IC_{50} of 38.0 μg/mL. Ascorbic acid, IC_{50} was calculated as 35.54 μg/mL. The biogenically formed AN-ZnO NPs directly competed with the oxygen and inhibited the concentration of reactive radical species thereby bearing a trait of counteracting the deleterious nitric oxide formation and thus concluded that they may possess activity to hinder the ill-effects of in vivo generated nitric oxide. A remarkable antioxidant property of ZnO NPs designed using *Curcuma longa* was also witnessed [61].

3.9.3. Hydroxyl radical (OH^-) scavenging activity

Hydroxyl radical are an immensely reactive form of free radical generated within the living entities and bring about a deleterious impression on the pivotal bio-organic molecules

like lipids, proteins and nucleic acid molecules [62]. The inhibitory trait on hydroxyl radial by the formed AN-ZnO NPs was perceived by using varying amounts and the results are presented in Fig. 8C. Ascorbic acid was utilized as a comparative reference standard. At the lowest amount (20 μg/mL) the radical scavenging capacity of AN-ZnO NPs was 14.13% and at the highest used concentration (60 μg/mL) was 91.39%. The IC_{50} value of 41.34 μg/mL was for AN-ZnO NPs whereas, 37.10 μg/mL was for ascorbic acid. The tested AN-ZnO NPs presented a dose-mannerly scavenging activity. Dhandapani et al. [63], fabricated ZnO NPs from *Melia azedarach* and presented hydroxyl radical scavenging property on a dose-related basis (14.57% to 54.97%).

3.9.4. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

The accomplishment of ABTS radical scavenging assay was done with diverged concentrations of AN-ZnO NPs. Ascorbic acid was utilized as a comparative standard against the test AN-ZnO NPs for the antiradical trait. A dose-proportional antiradical activity was presented by the AN-ZnO NPs. The radical scavenging activity of 14.69% to 88.78% was achieved with the raised AN-ZnO NPs concentrations. Results were presented in Fig. 8D. The IC_{50} value were calculated for both AN-ZnO NPs and ascorbic acid which was 35.41 μg/mL and 30.39 μg/mL. Chemically provoked ABTS free radicals and their scavenging assay is a specific and highly sensitive test. Our results are in concurrence with currently available report [64].

Fig. 8. Antioxidant role of AN-ZnO NPs executed in three trials for each assay. The results are given as mean ± standard deviation (SD) at $p < 0.05$ **(A)** DPPH radical quenching efficacy of AN-ZnO NPs **(B)** Nitric oxide (NO) scavenging efficacy of AN-ZnO NPs **(C)** Hydroxyl radical scavenging efficacy of AN-ZnO NPs **(D)** ABTS radical scavenging efficacy of AN-ZnO NPs.

3.10. Cytotoxic assay

3.10.1. MTT-mediated cytotoxic evaluation of AN-ZnO NPs

The biocompatible and cytotoxic trait of AN-ZnO NPs was executed by MTT assay on MCF-7 cell line. The reduction of yellow coloured MTT to purple-blue coloured formazan by the enzyme succinate dehydrogenase of mitochondria was measured. The impact of AN-ZnO NPs on viability of MCF-7 cell line was performed with varied concentrations (10 μg/mL to 90 μg/mL) and the values were provided in terms of IC_{50}. The percentage rate of survival of the breast cancer cells (MCF-7 cell line) was repressed with the raised concentration of AN-ZnO NPs (Fig.9). Thus, proving that the raised concentration of fabricated AN-ZnO NPs

depicted inhibition in the cancerous cell growth and division. The minimum inhibitory concentration (IC_{50}) to cause 50% cellular death in breast cancer cells on AN-ZnO NPs exposure to 24 h was observed as 37.15 μg/mL. Furthermore, it can be concluded that the phytofabricated AN-ZnO NPs could profoundly serve as a breast cancer controlling factor. Loganathan et al. [65], enlightened the antiproliferative potentiality of *Knoxia sumatrensis* based harnessed ZnO NPs on MCF-7 cell line with an IC_{50} value of 58.87 μg/mL. Spontaneous development of ROS by the ZnO NPs with the diminutive feature is a critical factor in exposing out the cytotoxic impression on the cancerous cells [66].

Fig. 9. Impact of AN-ZnO NPs on the MCF-7 (breast cancer cell) cells viability. Results are provided as mean ± standard deviation (SD) of three separate trails for each concentration ($p < 0.05$).

3.10.2. Determination of ROS using DCFH-DA (Dichloro-dihydrofluorescein diacetate method)

The impact of phytofabricated AN-ZnO NPs on the evolution of ROS in the MCF-7 exposed cells was scrutinized using DCFH-DA stain. Intense greenish coloured fluorescence in the AN-ZnO NPs treated MCF-7 cells were visualized at IC_{50} range of 37.15 μg/mL on 24 h and 48 h treatment, respectively. The photographic images captured delineated for the enhanced *in vivo* generation of ROS in the AN-ZnO NPs treated cells. There was a lack in the fluorescence intensity within the control (negative) cells implying diminutive ROS formation. But in the standard (Doxorubicin) drug-treated cells greenish fluorescence was high concluding more ROS release (Fig. 10A-E). The intensity of fluorescence obtained by the spectrofluorimetric tool has direct proportionality to the ROS quantity. In vivo copious production of reactive oxygen species (ROS) provokes oxidative stress which is the key inducer in apoptotic reactions [67] and proposing oxidative-based obliteration of mitochondria and other integral molecules [68]. Experimental outcomes have illustrated the active involvement of inorganic nanoparticles (ZnO NPs) in contributing cellular toxicity straight through the genesis of ROS conciliated oxidative stress [69].

Fig. 10 Fluorescence microscopic photograph of MCF-7 breast cancer cells on DCFH-DA staining for ROS genesis identification **(A)** Negative control **(B)** AN-ZnO NPs (37.15 μg/mL) exposed cells for 24 h **(C)** AN-ZnO NPs (37.15 μg/mL) exposed cells for 48 h **(D)** Doxorubicin (500 μg/mL), a positive control exposed MCF-7 cells **(E)** Bar chart illustrating the fluorescence

intensity of DCF related to ROS origin in negative control, AN-ZnO NPs exposed and Doxorubicin reacted MCF-7 cells.

3.10.3. Analysis of mitochondrial membrane potential (MMP) using Rhodamine 123 staining

The outcome of AN-ZnO NPs at IC_{50} value (37.15μg/mL) on the potentiality of the mitochondrial membrane was assessed by utilizing Rhodamine 123 dye. Damaged mitochondrial membrane morphology can be excellently notified using Rhodamine123 dye in the cancerous cells. In the image captured by fluorescence microscope, the untreated (negative control) cells displayed a higher range of green coloured fluorescence in contrary to the AN-ZnO NPs treated MCF-7 cells with mildest greenish fluorescence. Thus, clearly illustrating the loss in the intactness of mitochondrial membrane after AN-ZnO NPs exposure in MCF-7 cells. Even the doxorubicin (500 μg/mL) utilized as positive control exposed lessened fluorescence. Further, the spectrofluorimetric data validated the fact that AN-ZnO NPs would have brought loss in the potentiality of mitochondrial membrane. Illustration of report were given in Fig. 11(A-E). The intactness trait is pivotal for the effectual ATP generation by mitochondria for cellular survival. The damage to the mitochondrial membrane leads to drastic falls in the ATP thus mediating apoptotic-induced cellular killing [70,71].

Fig. 11. Fluorescence microscopic photograph of MCF-7 breast cancer cells on Rhodamine 123 staining illustrating MMP **(A)** Negative control **(B)** AN-ZnO NPs (37.15 μg/mL) exposed cells for 24 h **(C)** AN-ZnO NPs (37.15 μg/mL) exposed cells for 48 h **(D)** Doxorubicin (500 μg/mL), a positive control exposed MCF-7 cells **(E)** Bar chart illustrating the fluorescence intensity of Rhodamine-123 in negative control, AN-ZnO NPs exposed and Doxorubicin reacted MCF-7 cells.

3.10.4. Morphological assessment of nucleus using DAPI

The morphological alterations of the nucleus induced on MCF-7 cells by phytofabricated AN-ZnO NPs at IC_{50} of 37.15 μg/mL were shown in Fig. 12(A-D). Clear nucleus damage was visualized in the AN-ZnO NPs treated MCF-7 cells. There was a greater intensity in the blue coloured fluorescence in AN-ZnO NPs exposed MCF-7 cells, contrary to the control (untreated) cells thus depicting damage in the nucleus of the MCF-7 cells. The acquired results were compared to Doxorubicin (positive control). Oxidative damage triggered by ROS upsurges DNA strand cleavage effectuating apoptotic mechanism [72].

Fig. 12. DAPI staining depicting fragmentation of nucleus in MCF-7 cells **(A)** Negative control **(B)** AN-ZnO NPs (37.15 μg/mL) exposed cells for 24 h **(C)** AN-ZnO NPs (37.15 μg/mL) exposed cells for 48 h **(D)** Doxorubicin (500 μg/mL), a positive control exposed MCF-7 cell.

3.10.5. Western blotting technique

Verification of expression of the anti-apoptotic, proapoptotic gene and proliferative protein markers in the phytofabricated AN-ZnO NPs on exposure to MCF-7 breast cancer cells was executed by applying the western blot analytical technique. When exposed to the AN-ZnO NPs, there appeared a significant upregulation of certain proteins like Bax, Caspase-3 and Caspase- 9, whereas Bcl-2, Cyclin-D1 and PCNA were downregulated in the breast cancer cells (MCF-7). The results were compared to β-actin (standard control) (Fig. 13A & B). The performed experiment confirmed the potentiality of phytofabricated AN-ZnO NPs to inhibit the expression of cancer proliferative proteins and regulate the expression of apoptotic-related proteins. Kavithaa et al. [73], in her experimentation reported for the cytotoxicity feature of *Santalum album* leaves fabricated ZnO nanorods in dose-basis on MCF-7 cells. Even the overexpression of Bax and Bcl-2 decreased expression occurred on treatment with 10 μg/mL and 15 μg/mL concentration of ZnO nanorods.

Diminutive Bcl-2 and excessive expression of proapoptotic protein Bax, upgrade apoptotic programmed cell death [74]. In the apoptotic mechanism, caspase-9 and caspase-3 are crucial as the first one serves as the initiator of caspase cascade whereas, the caspase-3 is engaged in the activation of apoptosis-based mechanism [75]. Studies have proclaimed that raised Bax and dropped Bcl-2 levels have escorted caspase-3 and caspase-9 activation rendering irreversible apoptotic mediated cancer cell lysis [76]. Over-presentation of cyclin D1 has been shown for raising the risk of breast cancer [77]. PCNA ((proliferating cell nuclear antigen) is a crucial protein playing a significant function in the replication of DNA, remodelling of chromatin and repair of DNA. As it over occurs in the cancerous cell, it serves as a proliferative marker for cancer [78]. PCNA is a potent biomarker for breast cancer diagnosis as its level is found to be excessive within the cancerous cells [79]. Furthermore, it insinuated the probable usage of zinc oxide nanoparticles in cancer therapeutics in the future.

Fig. 13. Western blot technique displaying **(a)** The level of expression of apoptotic protein expression in the control, AN-ZnO NPs (24 h & 48 h) exposed and Doxorubicin (24 h) in MCF-

7 cells **(b)** Inhibition of proliferative marker protein expression in control, AN-ZnO NPs (24 h & 48 h) exposed, Doxorubicin (24 h) in the MCF-7 cells.

3.11. Larvicidal efficacy of AN-ZnO NPs

The larvicidal efficacy was observed by applying varied concentrations (0.1- 0.5 ppm) of AN-ZnO NPs on *Anopheles stephensi* mosquito larvae after 24 h treatment. The obtained results inferred 75.66% mortality at 0.5 ppm (higher concentration) and 15.33% mortality at 0.1 ppm (lowest concentration). The lethality dosage values (LC_{50} and LC_{90}) determined from mortality percentage of *Anopheles stephensi* mosquito larvae was 0.31ppm and 0.59 ppm, respectively (Fig. 14). The AN-ZnO NPs displayed excellent dosed-mannered larvicidal trait. Penetration and accrual of ZnO NPs in the cytosolic section of larval cells and genesis of ROS by the released cytotoxic zinc ions distorts vital bio-organic moieties such as nucleic acid, lipids, enzymes and proteins. Thus, forming the pivotal apoptotic mechanism for the larval lysis by ZnO NPs [80]. A constructive type of larvicidal trait was accounted for a study on *Momordica charantia* implemented ZnO NPs fabrication at *Anopheles stephensi* and *Culex quinquefasciatus* with the IC_{50} values of 5.42 mg/L and 4.87 mg/L [81].

Fig. 14. Probit graph for the larvicidal activity of AN-ZnO NPs on *Anopheles stephensi larvae*.

3.11.1. Acetylcholinesterase inhibition by AN-ZnO NPs

AN-ZnO NPs with six varying concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 μg/mL) were scrutinized for the inhibition activity on acetylcholinesterase enzyme acquired from the dead larval homogenate solution of *Anopheles stephensi*. A stupendous inhibition of acetylcholinesterase enzyme activity occurred with the elevation in the concentration of AN-ZnO NPs. The results were provided in Fig. 15. The IC_{50} level for the acetylcholinesterase inhibition by AN-ZnO NPs was computed to be 0.77 μg/mL The green fabricated ZnO NPs contained acetylcholinesterase inhibition trait which may be owned to its huge-surface area [82]. Dhavan and Jadhav [83] found that *A.aeypti* larvae on exposure to *Lumnitzera racemose* wrapped ZnO NPs displayed paralysis of respiratory muscles leading to rigidity and tremors. This happened due to the inhibition of AChE enzyme. Thus, the undertook study pinpointed the neurotoxicity trait of AN-ZnO NPs.

Fig. 15. Decline in the activity of acetylcholineasterase enzyme with the increase in the concentration of AN-ZnO NPs. Results are provided as mean ± standard (SD) of three separate trails for each concentration ($p < 0.05$).

3.11.2. Histological assessment of *Anopheles stephensi*

Visualization of histological slides of the control and AN-ZnO NPs exposured mosquito larvae of *Anopheles stephensi* were provided in Fig. 16 (A & B). AN-ZnO NPs reacted larvae of *Anopheles stephensi* presented disorganized layer of columnar epithelial cells, swelling along with the exuded cellular material in the anterior section of the gut. In the lumen of the gut, the epithelial cells appeared to be vacuolated and expanded with a huge cytoplasmic compartment due to AN-ZnO NPs aggregation. Degeneration in the ganglia cells was also validated for neurotoxic damage. All these morphological distortions appeared in the larvae of the mosquito confessed for excellent nanolarvicidal trait of AN-ZnO NPs. In the control larvae of *Anopheles stephensi*, the morphology was normal. Likewise, morphological distortion was presented by Kalpana et al. [84], on *Lagenaria sceraria* extract and ZnO NPs produced from the plant extract on *Anopheles stephensi*.

Fig. 16. Histological images of larvae of *Anopheles stephensi* **(a)** Control (without AN-ZnO NPs exposure) **(b)** AN-ZnO NPs exposed larvae showing morphological distortions.

3.12 Antibiofilm efficacy of AN-ZnO NPs

The efficacy of AN-ZnO NPs on the formed biofilm by pathogenic *Staphylococcus aureus* and *Klebsiella pneumoniae* was noted by executing a microtiter plate assay with a crystal violet stain. The data obtained by this assay proclaimed for the antibiofilm trait of AN-ZnO NPs in a dose-basis on tested gram-positive and gram-negative strains of bacteria. With the rise in the concentration of AN-ZnO NPs, the inhibition trait on biofilm development was noticed in both the selected strains of bacteria. The greater inhibition was 91.94% against *Staphylococcus aureus* with IC_{50} level of 23.80 μg/mL, whereas, raised inhibition of 72.13 % was achieved for *Klebsiella pneumoniae* with IC_{50} level of 23.31 μg/mL at a maximum concentration (300 μg/mL) (Fig. 17). The biofilm genesis inhibition was chiefly due to bacterial cell suppression by the AN-ZnO NPs. *Laursu nobilis* leaf extract wrapped ZnO NPs, displayed good biofilm inhibition activity against *S.aureus* and *P.aeruginosa* in dose-mannered as achieved in synchroneity to our findings. Rupturing of the cellular membrane of bacteria in responsive to the electrostatic interactive trait between the nanoparticles and bacterial exterior lyse the cells. ROS further imposes lysis of bacteria by distortion of proteins of the bacterial membrane [85].

Fig. 17. Biofilm inhibition in percentage (%) by phytofabricated AN-ZnO NPs on *Staphylococcus aureus* and *Klebsiella pneumoniae*. Results are provided as mean ± standard (SD) of three separate trails for each bacterial strain ($p < 0.05$).

4 Conclusion

Phytonanotechnology has superiority to the intricate physicochemical methodologies for nanoparticles fabrication. Herein, zinc oxide nanoparticles were harnessed extracellularly implementing the medicinally worthy *Acacia nilotica* bark aqueous extract in a lower-budget, eco-sustainable way without the applications of noxious chemicals. As disclosed in FTIR report, the plant bioactive natural chemical groups provided multiple traits as bio-reducer, stabilizer and capping factor in nanoparticles fabrication. The advanced characterization tools, pinpointed the crystalline trait, spherical and hexagonal morphological features of AN-ZnO NPs with average size of 35 nm. The fabricated AN-ZnO NPs presented astounding antifungal activity towards the tested fungal pathogenic strains. Antioxidant property of AN-ZnO NPs were clearly concentration-based. On MCF-7 cell line, AN-ZnO NPs presented cytotoxic trait tested by MTT assay. ROS origination, membrane distortion in mitochondria and nuclear fragmentation were the chief factors for the cytotoxic trait of AN-ZnO NPs in a dose-mannered. Inhibitory role on acetylcholinesterase (AChE) enzyme was achieved on AN-ZnO NPs exposure to the *Anopheles stephensi* larvae on dose-credential. Additionally, biofilm development hindered when AN-ZnO NPs were reacted on pathogenic biofilm generating bacterial species such as *Staphylococcus aureus* and *Klebsiella pneumoniae*. Comprehensively, the bioactivities displayed in our report, conclude that AN-ZnO NPs may be practiced in the bionanomedicine province as an amendatory for health illness. Also, paving a new route for eradicating mosquitoes with nanolarvicidal trait. To relate and understand the in-depth of bioactivities of AN-ZnO NPs, in vivo experimentation has to be executed in future.

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