Eco-friendly Nano Colloids for Enhanced Black gram (Vigna mungo) Seed Viability: Experimental and Computational Analysis

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Abstract

An experiment was designed to fabricate Polyvinylpyrrolidone-coated zein-zipped herbal molecules infused nano colloids (PZCA-NCs) for extending *Vigna mungo* seeds storability. PZCA-NCs was synthesized and characterized in Fourier Transform Infrared Spectroscopy (FTIR), X-Ray diffraction (XRD), Particle size analyser, Zeta Potential, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Energy-dispersive X-ray spectroscopy (EDAX). The bio-efficacy of PZCA-NCs on seed storability was tested under accelerated ageing. The sphere-shaped PZCA-NCs possess a 151nm size with 44.5mV zeta potential at an encapsulation of 73.44 % curcumin and 69.0 % azadirachtin. The spectra of FTIR, UV –Vis, XRD, and TGA confirmed the functionality, composition, and stability of PZCA-NCs. The dialysis diffusion method was utilised to study the maximum cumulative release of biomolecules 6.1ppm (88.4%) azadirachtin and 64.57ppm (88.2%) curcumin at pH 7.4. Density functional theory (DFT) was used to determine the binding mode of molecules and examine ligand interactions in PZCA-NCs. PZCA-NCs treated seeds at 25mL/kg enumerated higher germination, vigour index, α-amylase, dehydrogenase, and catalase and peroxidase activity under ageing. Seeds storage pathogen infection was reduced with an increase in the concentration of PZCA-NCs coating. The bioassay results on insect activity evidenced that PZCA-NCs at 15.76mL/kg killed 50% and 40mL/kg killed 100% of the storage insect *Callosobruchus maculatus*. Toxicity study on *Macrophomina phaseolina* showed that PZCA-NCs at 35mL resulted in 0.8cm mycelia growth with 91.11% inhibition zone, while at 45mL had zero growth of fugal mycelia with 100 % inhibition. The study concludes that PZCA-NCs act as an efficient seed invigoration material to extend the vitality of *Vigna mungo* seeds during ageing.

1. Introduction

Non-toxic nano-based products in agriculture offer a practical solution for managing bioaccumulation potential in the field and minimizing chain transfer within the ecosystem [1, 2]. In agriculture, while research in nanotechnological techniques is fairly extensive, there is comparatively less focus on Seed Science. Nano seed invigoration includes metal [3] and metal oxide nanoparticles seed priming [3, 4], nanoemulsion and nanofiber seed coating [5, 6], and nano-packaging materials has been explored to improve seed quality across various crops. As seed is the vital input in agriculture, the quality of the seeds must be preserved until sowing without deterioration [7]. Seeds deteriorate over time due to a range of biotic and abiotic factors, all of which are directly or indirectly associated with lipid oxidation, leading to the breakdown of cell membranes [8].

The seed industry employs various processing techniques including bio-nano colloidal seed coating, an eco-friendly and affordable technique for enhancing the seed quality [9]. Nano colloidal seed invigoration is an important method for raising seed quality, effectively fortifying them for optimal growth and protection [10, 11]. Nano colloid seed invigoration extends the shelf-life of seeds by reducing deterioration at the early stage. Release rate and pattern of the active biomolecules in nano colloids are controlled through encapsulation whereas the excess release of chemical compounds causes toxicity and ecological imbalance of soil [12]. This study focussed on developing Zein-zipped curcumin and azadirachtin nano-colloids as pre-storage seed invigorate for extending *Vigna mungo* storability. Zein-zipped curcumin and azadirachtin amalgamated before getting injected into an acidic solution containing biosurfactant. The surface-active biomolecules prevent the aggregation of curcumin and azadirachtin-loaded zein nano colloidal solution [13, 14].

Zein, when used as a seed coating agent, is known to delay moisture absorption and germination [15]. Coating seeds with turmeric rhizome powder at a rate of 10 grams per kilogram extended the storability of black gram [16] and chilli [17]. The antioxidant properties of curcumin donate free electrons and pair with unpaired electrons, quenching free radical formation, reducing lipid oxidation, protecting the bilipid cell membrane, and extending seed shelf life [18]. Bio-pesticide molecule azadirachtin acts as an antifeedant affecting chemoreceptors and reducing ecdysone hormone synthesis, resulting in insect larvae death. It also exhibits high insecticidal and antimicrobial activity [19]. Polyvinyl alcohol (PVA) is a water-soluble polymer with varying molecular weights, approved as a good stabilizer and binding agent safe for humans by the Food and Drug Administration. PVA-based nanoformulations for seed coating have shown higher germination and seedling growth in cotton [20]. This study aimed to develop a bio-molecules infused zein colloidal nano system to extend the storability with sustained seed quality of *Vigna mungo* under ageing, besides to investigate the electronic structure, geometry, and chemical reactivity of the colloidal nano system

2. Materials and methods

For the successive synthesis of PZCA-NCs, analytical-grade raw materials like curcumin, azadirachtin, and zein were purchased from Sigma Aldrich, Bangalore, India. The stabilizer PVP is purchased from Merck and used to cap all zipped biomolecules to retain their stability. The genetically and physically pure seeds of *Vigna mungo* (var. VBN 8) were obtained from the Department of Pulses at TNAU, Coimbatore. In order to conduct biosafety tests, cultures of beneficial soil microorganisms such as *Bacillus megaterium*, *Azotobacter chroococcum*, and *Bacillus subtilis* were sourced from the Department of Agricultural Microbiology, TNAU, Coimbatore. Additionally, cultures of *Callosobruchus maculatus* from the Department of Seed Science and Technology, and *Macrophomina phaseolina* from the Department of Plant Pathology, both at TNAU, Coimbatore, were acquired for the bio-efficacy testing of PZCA-NCs.

2.1. Synthesis of PZCA-NCs

Hydrophobic ethanol-solvated bioactive compounds curcumin and azadirachtin at optimized concentrations of 80 and 10 mg respectively was augmented together. The bioactive sited curcumin and azadirachtin zipped in 24ml of 2% zein protein by agitating in the pH range of 5 and 5.2 at controlled temperature 40°C [21–23]. Then it was centrifuged for ten minutes at 4000 rpm, and allowed to settle for 2h and the supernatant was filtered through a 0.2µm filter paper to get zein-zipped curcumin and azadirachtin bio-formulated nano colloidal solution. The colloidal solution was stabilized by adding 0.5% polyvinyl pyrrolidone (PVP) at the ratio of 1:3 (1-part zein solution: 3-part PVP) to get fine-capped high stable PZCA-NCs.

2.2. Characterization studies

Curcumin, azadirachtin, and PZCA-NCs were qualitatively analyzed using UV-visible spectrometry (Model SPECORD plus 210 BU, Analytik Jena AG, Germany) in the spectral range of 200 to 1100 nm using ethanol as a blank. The effectiveness of encapsulated azadirachtin and curcumin in PZCA-NCs was determined with pure curcumin and azadirachtin dissolved in 100% ethanol to provide standards of 20, 40, 60, 80, and 100 ppm for curcumin and 10, 20, 30, 40, and 50 ppm for azadirachtin, respectively. For curcumin and azadirachtin, the absorbance of the generated standards was determined at 404 nm and 215 nm, respectively. Using those same absorbance data, a standard graph with an R^2 value of 0.99 was calculated using the encapsulation efficiency formula [24], where A denotes the amount of herbal molecule loaded initially and B denotes the amount of herbal molecule in the supernatant of formulation during measurement.

$$Encapsulation\ efficiency\,(\%) = \left(\frac{A-B}{A}\right) \times \ 100$$

Fourier transform infrared spectral pattern demonstrates how a sample absorbs light at various wavelengths, leading to various chemical arrangements indicating the colloidal solution contains encapsulated biomolecules. The FTIR spectra of the sample were examined using a JASCO FTIP/6800 Spectrometer (JASCO Japan) equipped with an Attenuated Total Reflectance Unit (ATR) sensor acquainting spectral data between 400 and 4000 cm^{-1}. The PZCA-NCs calcination temperature was confirmed through TG/DTA-EXSTAR/6300 (Thermo Gravimetric Analyzer, which determines the precise compound thermal stability, formation, and decomposition temperature. Here, the PZCA-NCs was lyophilized by taking 100ml and freeze-dried at -80^{o}C for 32 hours under a vacuum pressure range of 4 to 40 Pa [25]. Samples were heated in an aluminum pan at a rate of 10● C per minute from 25^{o} to 1000● C [26]. Particle size and distribution pattern of zein colloidal nanosystem were determined using Nanopartica SZ-100, Horiba Scientific (Nanoparticle analyzer), Japan. PZCA-NCs 1 ml was diluted in water at a ratio of 1:2 and sonicated in a water bath sonicator for 10 minutes. Then the formulation was analyzed under the dynamic light scattering method using 90^{o} or 173^{o} at 25^{o}C. The zeta potential expresses the surface charges of PZCA-NCs in the zeta analyzer varied from −200 mV to 200 mV and the data acquisition time was less than one minute. Using an X-ray diffractometer (Model Ultima IV, RIGAKU, Japan), the structure of the PZCA-NCs was analyzed at 40kV, 30mA, and Cu-K radiation (0.154nm). The range of 0 to 100 degrees was scanned at a rate of 5 degrees per minute, at room temperature (25^{o}C). The surface morphology of the sample was characterized using a scanning electron microscope with EDAX (Quanta 250, FEI, and Netherlands) scanning with a beam of high-energy electrons in a raster pattern. EDAX is an analytical method for determining elemental composition. The interior morphology of the PZCA-NCs was studied using the TEM FEI Technai Sprit to predict the high-resolution images. The peak height in NMR provides detailed structural and dynamic information of PZCA-NCs using DMSO as the solvent and 1,3,5-trimethoxybenzene as the internal standard. A 600 MHz spectrometer was used for the quantitative analysis which averaged 64 scans.

2.3. Computational studies

DFT calculations have been carried out using the Gaussian 09 package at B3LYP/6-31G (d,p) level in the gas phase [27]. Optimization followed by frequency calculations is performed to confirm their ground state stability as evidenced by the absence of the imaginary frequencies. To gain a better understanding of the chemical reactivity and stability, Frontier molecular orbital (FMO) and electrostatic potential (ESP) analyses are performed. Molecular docking studies have become increasingly important in understanding the chemical and biological aspects of a molecule [28]. In this context, it is important to understand how these molecules interact with proteins. The protein structure of Zein was modelled using the FASTA sequence Q9SYT3 from EMBL-EBI [29] using AlphaFold an AI system [30]. This modelled protein is later refined using the Maestro software. Molecular docking is performed using Maestro to predict the binding modes [31]. Protein Preparation Wizard of Schrödinger suite of the program is used to prepare the protein for molecular docking. Then, Glide's receptor grid generation was used to generate a grid with a maximal size of 20 x 20 x 20 Å and 0.5 Å spacing. The molecular docking was performed using glide to predict the binding modes. The results of the docking were analysed using Maestro.

2.4. In vitro control release of biomolecules from PZCA-NCs

The release pattern of PZCA-NCs at pH 7.4 was studied using the dialysis diffusion technique. 15 mL of PZCA-NCs was put into a 12.4 cm long dialysis bag. The sample-containing dialysis bag was placed in a beaker with 50mL of phosphate buffer and agitated in a motorized shaker at 100 to 150 rpm min^{-1} at room temperature. The release pattern is periodically analysed by removing 3 mL and maintained to a constant volume with 3 mL of phosphate buffer and examined with a UV-Vis scanner. The release pattern of biomolecules was derived by using different kinetic models like the Korsmeyer-Peppas model, First Order model, Zero Order, Higuchi model, and regression coefficient (R^2) was computed from the appropriate plots based on the release percentage of biomolecules from PZCA-NCs.

2.5. Bio-efficacy test of PZCA-NCs on seed storability of *Vigna mungo* under ageing

2.5.1. PZCA-NCs on physiological seed quality parameters

Genetically pure black gram seeds of 100 g were coated with PZCA-NCs at different doses as 5, 10, 15, 20, 25, 30, 35, and 40 ml/kg along with control untreated seeds. The coated seeds along with the control were placed for accelerated ageing for 10 days. The seed quality changes like germination percentage, root length, shoot length, dry matter production, and Vigour index -I & II were tested for different dosed black gram compared to the control [3, 32].

2.5.2. PZCA-NCs on biochemical seed quality parameters

The electrical conductivity was measured in a digital model conductivity meter (Elicotype Cm-82) positioning electrode at a cell constant of 1.1 with calibration on electrical current mode [33]. The bio-enzymatic actions like α-amylase [34], dehydrogenase [35], catalase [36], peroxidase [37], and lipid peroxide [38] were experimentally analysed using a standard procedure to test the seed quality parameters.

2.5.3. Seed health: storage pathogen infection

On the third, sixth, and ninth days, 25 seeds from each replication of the accelerated seeds were drawn and evenly distributed on three layers of sterile blotter paper moistened with 0.2% 2, 4-D solution in sterile Petri plates. The seeds were then incubated at 20 ± 2°C for seven days with alternate cycles of 12 hours in the near ultraviolet light (NUV) range and the remaining 12 hours in darkness [39]. The seeds were tested for fungal infestation on the eighth day. The number of infected seeds was counted, and pathogen infection was reported as a percentage.

2.6. Bioassay Test of PZCA-NCs on *Callosobruchus maculatus* and *Macrophomina phaseolina*

2.6.1. PZCA-NCs on *Callosobruchus maculatus*

A toxicity test was carried out in a small plastic container containing PZCA-NCs treated seeds at different doses as 5, 10, 15, 20, 25, 30, 35, and 40 ml/kg along with untreated seeds. 100 g of seeds from each treatment were placed in a plastic container with fifteen pairs of adult *Callosobruchus maculatus*, and the container was covered with a muslin cloth. The containers were kept at 27 ± 3°C and 70% RH. For seven days, insect mortality (lack of movement and/or responsiveness to repeated probing) was recorded every 24 hours. The Abbotts formula was used to calculate adjusted mortality rates by LD_{50} value [40] where x is the percentage mortality of *Callosobruchus maculatus* in PZCA-NCs treated seeds and y is the percentage mortality in the untreated seeds.

$$\varvec{I}\varvec{n}\varvec{s}\varvec{e}\varvec{c}\varvec{t}\ \varvec{m}\varvec{o}\varvec{r}\varvec{t}\varvec{a}\varvec{l}\varvec{i}\varvec{t}\varvec{y}\ \varvec{$$

2.6.2. PZCA-NCs on *Macrophomina phaseolina*

By following the reported work by Athira. 2017, PZCA-NCs at different concentrations were mixed with 20 mL of autoclaved Potato dextrose agar (PDA) medium, and inoculated with *Macrophomina phaseolina* fungal disc (9mm in diameter) in the centre of a sterilised Petri plate [41]. The zone of inhibited fungal mycelia growth was measured on the third day of incubation and represented in percentage as detailed below where I denote the radial mycelial growth inhibition percentage, C is the radial mycelial growth in control, and T is the radial mycelial growth in treated.

$$\varvec{I}\ \varvec{\%} = \frac{\varvec{C} - \varvec{T}}{\varvec{C}} \times 100$$

2.7. Biosafety test of PZCA-NCs on beneficial microbes

Biosafety of different concentrated PZCA-NCs combined with 20 mL of autoclaved nutrient agar medium, and transferred to Petri plates having 60 mL culture of *Bacillus megaterium, Azotobacter chroococcum, and Bacillus subtilis* with 10^{-6} dilution maintained at 37 ± 10°C for three days. After three days of incubation, the bacterial colonies were counted and the results represented the number of CFU [42]. After counting the number of colonies, the significant difference between the number of colonies in control and the number of colonies in various treatments was analysed and the safety of the nano colloid was assessed. All the experiments were designed in Factorial Completely Randomized Block Design (FCRD) with four replications and statistically analysed and reported as per the procedure [43].

3. Result and Discussion

3.1. Absorbance and Stability of PZCA-NCs

Figure 1a shows the broad peak of the curcumin standard forms ranges from 400 to 430 nm. Figure 1b shows the spectrum of azadirachtin with peaks at 217 and 298 nm. The large peaks at 298 nm, 404 nm, and 430 nm in the UV-Vis spectra of PZCA-NCs confirm the presence of azadirachtin and curcumin shown in Fig. 1c. With the optical density value, the encapsulation efficiency of curcumin and azadirachtin percent in PZCA-NCs was calculated as 73.44 and 69%. Figure 1d shows the particle size of PZCA-NCs which was 151 nm and Fig. 1e exposes the maximum zeta potential of PZCA-NCs at 44.5 mV and its stability was shown to be stable with a zeta potential of 43.8 mV after one month and 38.4 mV after two months. The zeta potential from 0 to ± 30 shows incipient instability, and from ± 31 to ± 60 shows good stability when it is more than ± 61 it shows excellent stability. The reduced particle size with good stability of zein zipped curcumin and azadirachtin is due to increased concentration of PVP and also the pH of 5-5.2. This might also be attributed to adding low concentration curcumin and azadirachtin. Addling high concentration of curcumin in zein colloidal system resulted bigger aggregates with higher particle size with maximum poly dispersity index and poor stability [23].

Zein includes a high concentration of non-polar amino acids, particularly alanine, leucine, and proline, making it insoluble in water but dissolves in aqueous solutions that are highly alkaline, including urea, alcohol, or anionic surfactants. Zein has various solubility properties, which can be exploited to make colloidal particles that are good candidates for encasing bioactive substances [44]. The biomolecules like curcumin and azadirachtin were filamented in molecular planes of zein molecules stacked through glutamine interactions with intramolecular and intermolecular hydrogen bonds and van der Waals interactions. The interaction with neighbouring helices permits distributed polar and hydrophobic residues along the helical surfaces to account for the dense, membrane-encapsulated deposits of the proteins in maize seeds [45]. The zein proteins display significant hydrophobic properties and bind the curcumin and azadirachtin.

3.2. Functionality and diffraction pattern of PZCA-NCs

FT-IR pattern in Fig. 2a helps to confirm the zipped curcumin and azadirachtin in PZCA-NCs compared with pure biomolecules. Miao et al. investigated the bio functionality of Poly (L-lysine) modified zein nanofibrous membranes which are analogous to the pure zein transmittance peaks at 3284, 1368, 1636,

1229, and 1125 cm^{-1} which is almost similar to PZCA-NCs [46]. The transmittance peaks of curcumin spectra at 2970, 1992, 1370, 1626, 807, and 465 cm^{-1} depend on its functional group which occupies the same position in PZCA-NCs. The wavenumbers 2969, 1737, 1271, 1230, 1043, and 874cm^{-1} is shown for pure azadirachtin and PZCA-NCs had similar peaks at 2969 and 1271cm^{-1} and have strong bonding interaction in wavenumbers 1739, 1229, 1047, 880 cm^{-1}. The result was evident that the prepared PZCA-NCs hold similar bio functional groups present in pure zein, curcumin, and azadirachtin which shows the presence of all molecules in it. PZCA-NCs were analysed using XRD in the 2θ range of 10–100° and shown in Fig. 2b. Patel et al. investigated the XRD patterns of curcumin-loaded zein nano colloids [23]. Curcumin shows diffraction peaks at 12.20°, 15.15°, 15.79°, 18.23°, 21.22°, 23.81°, 29.04°, 34.92°, and 44.57°, with the largest peaks seen at 2θ = 18.23° and matching d spacing of 2.46. The azadirachtin analysis revealed that it is amorphous in form and lacks pronounced peaks. The XRD result of PZCA-NCs has a wavy pattern, an amorphous nature, a tiny peak at 19.76°, and a corresponding d spacing of 2.27 Å. Badr-Eldin et al. exposed their results on particle size, stability and morphology which is reliable due to the crystalline nature of curcumin changed to an amorphous state indicating that the curcumin zipped in zein solution [47].

3.3. Differential temperature change in PZCA-NCs

The weight of the PZCA-NCs decreases as the temperature increases by 100°C steps from 0 to 1000 ^{o}C stable up to 300^{o}C as shown in Fig. 3. The PZCA-NCs weight was first decreased gradually, but when the temperature reached 300 ^{o}C, a large drop in weight loss was noticed. The stability of PZCA-NCs was stable up to a temperature of 290 ^{o}C and started to continually degrade with a fast weight loss (24.2%) from 300 to 380 ^{o}C. The outcome of differential thermal analysis (DTA) demonstrated the exothermic reaction of PZCA-NCs (Fig. 3). The initial weight loss in PZCA-NCs is caused by the evaporation of remnants ethanol and water.

3.4. Surface morphology and size of PZCA-NCs

The SEM image in Fig. 4a shows that the PZCA-NCs contains spherical shapes of colloidal particles and size ranged from 233.1nm to 349.7nm. The majority of the spherical particles were similar in size and distribution. Under TEM, PZCA-NCs were detected and its interior morphology was examined and displayed in Fig. 4c, d. The encased core shell-like structure is confirmed by the TEM with a distinct contrast in the micrographs. Nano-colloidal particles in TEM ranged in size from 69 nm to 281 nm. Patel et al. studied the curcumin loaded zein colloidal nano system under TEM and the result illustrated the spherical shaped colloidal particles in size ranged from 100–150 nm with smooth surface [23]. Figure 4b shows the EDAX describing the elemental composition of the PZCA-NCs indicating the existence of herbal compounds due to the high composition of carbon (69.75% by weight and 80.3% by atoms) and oxygen (20.3% by weight and 17.52% by atoms). The significant nitrogen content in Fig. 4e suggests that there is a protein in the PZCA-NCs.

3.5. NMR spectra of PZCA-NCs

Figure 5a shows the ^{1}H NMR spectrum of azadirachtin showed a pair of coupled doublets [τ 3.6 (1H) and 4.95 (1H) (J 2.5 Hz)], indicating low value of the coupling constant for dihydro-furan. Data on dihydrofuran ring systems are rare; however, comparison with chemical shifts was given for sterigmatocystin, Rodin, aflatoxins, and 2,3-dihydrofuran. The coupling observed between the 3- and 4-protons and between the 2- and 3-protons in a 2,3 dihydrofuran was confirmed by the identification of the NMR absorption at 4–36 (lH, s). It was unaffected when azadirachtin was converted into many derivatives and was not shifted when in (2H) dimethyl sulphoxide. However, in dihydroazadirachtin the signal was shifted to τ 4.7, remaining as a singlet. The lack of coupling confirmed that the 3-position is fully substituted. The chemical shift of the 2-proton in dihydroazadirachtin (τ 4.7) corresponds to that of the 21-proton (τ 4.62) in the hemiacetal system of me1ianone [48].

Figure 5b shows the ^{1}H NMR spectra of curcumin in DMSO, three reference peaks were seen: 7.165 (d, 2H, J141.8 Hz, H6 and H06), 6.662 (s, 1H, H-1), and 3.881 (OCH3, 6H). At 600 MHz, all of the proton peaks were distinct. A distinct single signal was seen at 9.675 ppm, which was identified as the hydroxyl groups of curcumin [49]. In NMR studies, the Curcumin, and azadirachtin biomolecules exist distinct peaks depending on their proton intensity and chemical shift. The peaks obtained for zipped zein curcumin-azadirachtin did not match more with curcumin and azadirachtin shown in Fig. 5c. This mismatching peak pattern may be due to deprotonation occurs in two molecules to bind on zein molecules, and formation of hydrogen bonds, van der Waals forces, or π-π interactions with the amino acid residues in zein.

3.6. Molecular docking of PZCA-NCs

Understanding the action of molecules on protein requires a complete understanding of the molecule-protein interactions [50–52]. The optimized geometry of molecules has been given in Fig. 6a. From the geometries one can see that the curcumin structure is almost planar and there is delocalization throughout the molecule. The azadirachtin is not planar and is highly twisted. Figure 6b gives an electrostatic potential map. In the ESP distribution red represents more electron-rich areas with negative potential, blue shows electron-poor parts with positive potential, and green shows neutral regions with zero potential value. One can see that in curcumin the oxygen atoms are electron-rich and the other regions are mostly neutral. The same is observed in azadirachtin. In azadirachtin, the hue of red on the oxygen atoms is greater than the hue of red on the oxygen atoms in curcumin which signifies that the oxygen atoms on the azadirachtin have greater negative potential. Figure 6c gives the frontier molecular orbitals (HOMO and LUMO). These frontier molecular orbitals indicate the curcumin molecule shows π ◊ π* interactions from HOMO to LUMO whereas the azadirachtin molecule shows intra-molecular charge transfer character. The optimized geometry has been used for molecular docking analysis.

The modelled protein which is further refined with maestro is given in Fig. 7a. The active sites of the modelled zein protein have been found using Maestro [31]. Five active sites have been found. The active site with the highest site score of 1.150 is usually taken for docking. The curcumin molecule has been docked in this active site whereas the azadirachtin could not be docked at this active site because its size was 168 Å. For azadirachtin, the active site with the second-best site score of 1.088 with a size of 177Å has been taken for docking. The 3D binding of the molecules has been given in Fig. 7b. The glide score for curcumin is -7.626 whereas it is -5.275 for azadirachtin. This suggests that the curcumin molecule binds better to the zein protein than the azadirachtin molecule. Figure 7c gives the 2D interaction images between the ligand and the protein. From the one can see that the binding site in the case

of curcumin has the following amino acids: Leu164, Pro163, Ser162, Ser160, Leu159, Gln156, Tyr214, Gln217, Arg218, Leu221, Leu185, Phe203, Leu200, Ile181, Tyr196, Leu178, Leu177, Gln174, Leu173, and Ala170. In the case of curcumin, one can see that there are two interactions between the molecule and the protein. One interaction is the hydrogen bond between the OH group of curcumin and the SER160 amino acid of the protein. The other interaction is a π-π stacking between one of the benzene moieties in curcumin and the Phe203 amino acid of the protein. In azadirachtin the binding pocket consists of the amino acids Pro202, Phe203, Gln205, Leu206, Asp207, Leu164, Pro163, Ser162, Ser161, Ser160, Leu159, Ala170, Leu173, Gln174, Leu177 and Gln130 amino acids. There are two hydrogen bond interactions between azadirachtin and the protein. One is between the amino acid Asp207 and C = O of the azadirachtin molecule. The other is between the OH group of the azadirachtin molecule and the Ser162 amino acid of the protein.

3.7. Control release of biomolecules from PZCA-NCs

The release pattern of PZCA-NCs at pH 7.4 was examined using the dialysis diffusion method, and the results revealed that sustained and controlled release of azadirachtin and curcumin from PZCA-NCs was noted at 72 h. The maximum cumulative release of 6.1ppm (88.4%) in azadirachtin and 64.57ppm (88.2%) in curcumin was obtained at 72 h from the total of 6.9 ppm azadirachtin and 73.44 ppm of curcumin zipped in zein nano colloids. Zero-order, First-order, Higuchi, and Korsmeyer-Peppas models R^2 values 0.9932, 0.8467, 0.9396, and 0.8467 were matched with azadirachtin and Curcumin release pattern R^2 values are 0.9896, 0.7924, 0.9277, and 0.9897, respectively. In supplementary information Fig.S1 and S2 show PZCA-NCs release kinetic models, the zero-order and Higuchi models suit well for curcumin and the zero-order, Higuchi, and Korsmeyer-Peppas model fit well for azadirachtin. The ion exchange between the interlayer curcumin and azadirachtin from the zipped PZCA-NCs in the environment led to the release of biomolecules. The regulated diffusion shows distinct phenotypic and enzymatic action initially by curcumin release and later azadirachtin coated in the seeds gets release supports in storage pathogen growth, inhibition percentage, insecticidal toxicity, and biosafety testing. The controlled release formulations of zipped biomolecules on accurate time duration provide positive results on black gram seeds as a control release mechanism of ions from 2,4-D@HTlc nanosheets [53].

3.8. Phenotypic changes on *Vigna mungo* by PZCA-NCs

Green nano chemistry infuses the emergence of a nano colloidal formulation with herbal compounds to boost germination and improve their storage capacity [54]. Nano-colloids fabricated using polysaccharides, lipids, and proteins are biocompatible to react on seed membranes [55]. Seeds coated with PZCA-NCs at 25 mL/kg initially responded higher than other doses and control in registering higher germination (93%), root (18.1cm), and shoot length (20 cm), seedling dry matter production (0.328g/10 seedlings), seedling vigor index-I (3328) and vigor index-II (29). The control seeds recorded the minimum germination (75%), root (16.2 cm) and shoot (17.9 cm) length, dry matter production (0.276 g/10 seedlings), Vigor index -I (2616), and Vigor index-II (22). On increasing the ageing duration (10th day), seeds coated with PZCA-NCs at 25 mL/kg showed 75% germination, root (14.2 cm), shoot length (15.2 cm), dry matter production (0.209g/10 seedlings), seedling Vigor index-I (2205) and Vigor index-II (16) were found to be reduced due to the additive effect of zein, curcumin, and azadirachtin. Table 1 shows the phenotypic changes like germination percentage, root length, shoot length, Dry matter production, and Vigor index -I & II in different treatments of PZCA-NCs primed on black gram compared to control. Overall variation in the seedling's growth by physiological and enzymatic changes was tabulated and given in the Supplementary information as Table.S1 to S12. In Table. S13 impact of PZCA-NCs treatment on blackgram var. VBN 8 bio efficacy was noted under accelerated ageing. Insecticidal activity and toxicity pattern of PZCA-NCs against *C. maculatus* on blackgram var. VBN 8 was resulted in table. S14.

Table 1
Effect of PZCA-NCs on black gram showing phenotypic changes var. VBN 8 under accelerated ageing

Treatments (T)	Germination %		Root Length		Shoot Length		DMP		Vigour Index - I		Vigour Index - II	
PZCA-NCs	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Control (T_0)	93(74.66)	56(48.45)	19.5	12.2	21.4	13.6	0.395	0.149	3804	1445	37	8
5mL/kg (T_1)	94(75.82)	67(54.94)	19.9	13	21.8	14.5	0.403	0.182	3920	1843	38	12
10mL/kg (T_2)	94(75.82)	68(55.55)	20.2	13.2	22.1	14.6	0.405	0.187	3976	1890	38	13
15mL/kg (T_3)	95(77.08)	69(56.16)	20.3	13.5	22.5	14.8	0.408	0.193	4066	1953	39	13
20mL/kg (T_4)	95(77.08)	71(57.41)	20.6	13.8	22.8	15	0.411	0.2	4123	2045	39	14
25mL/kg (T_5)	96(78.47)	75(60.00)	20.8	14.2	23	15.2	0.417	0.209	4205	2205	40	16
30mL/kg (T_6)	95(77.08)	73(58.69)	20.5	13.9	22.9	14.9	0.412	0.201	4123	2102	39	15
35mL/kg (T_7)	94(75.82)	70(56.79)	20.1	13.7	22.5	14.6	0.408	0.192	4004	1981	38	13
40mL/kg (T_8)	93(74.66)	68(55.55)	19.8	13.4	22.1	14.2	0.402	0.185	3897	1877	37	13
Mean	**94(75.82)**	**69(56.16)**	**20.19**	**13.43**	**22.34**	**14.60**	**0.41**	**0.19**	**4013.11**	**1926.78**	**38.33**	**13.00**
SEd	**0.595**	**0.623**	**0.41**	**0.59**	**0.54**	**0.48**	**0.01**	**0.02**	**128.02**	**214.67**	**1.00**	**2.24**
Final- 10th day of accelerated ageing												

3.9. Biochemical changes in *Vigna mungo* by PZCA-NCs

In biochemical seed quality, the results of the present study revealed that seeds coated with PZCA-NCs at 25 mL/kg were found to be superior over other treatments and control under accelerated ageing. Electrical conductivity (0.072dSm^{-1}) and lipid peroxidation (0.097 OD value) were less in seeds coated with 25mL/kg. However, the same seeds registered higher α-amylase activity (1.417mg maltose min^{-1}), dehydrogenase activity (2.273OD value), catalase activity (3.838 µmol H_2O_2 reduced min^{-1}g^{-1} of protein), and peroxidase activity (2.253 U mg^{-1}protein min^{-1}) over 10 days accelerated ageing. Electrical conductivity and lipid peroxidation values were increased with an increase in the ageing period whereas α-amylase activity, dehydrogenase activity, catalase activity, and peroxidase activity were decreased with an increase in the ageing period. Concerning interaction effect, seeds invigorated with PZCA-NCs at the rate of 25ml/kg recorded the minimum electrical conductivity (0.097dSm^{-1}) & lipid peroxidation (0.111 OD value) and a maximum of α-amylase activity (1.120mg maltose min^{-1}), dehydrogenase activity (1.833 OD value), catalase activity (3.310µmol H_2O_2 reduced min^{-1}g^{-1} of protein) and peroxidase activity (1.830 U mg^{-1}protein min^{-1}) at 10th day of accelerated ageing as compared to the control which recorded maximum electrical conductivity (0.110 dSm^{-1}) & lipid peroxidation (0.122 OD value), and minimum α-amylase activity (0.970 mg maltose min^{-1}), dehydrogenase activity (1.617OD value), catalase activity (2.930 µmol H_2O_2 reduced min^{-1}g^{-1} of protein), and peroxidase activity (1.510 U mg^{-1}protein min^{-1}). The enhanced enzymatic action was analysed on varied treatments on treated seeds and the result is shown in Table 2.

Table 2
Effect of PZCA-NCs treated black gram seeds on enzymatic action var. VBN 8 under accelerated ageing

Treatments (T) PZCA-NCs	Electrical Conductivity dSm^{-1}		α-amylase activity mg maltose min^{-1}		dehydrogenase activity		catalase activity µmol min^{-1}g^{-1}		peroxidase activity U mg^{-1}protein min^{-1}		lipid peroxidation	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Control (T_0)	0.052	0.11	1.45	0.97	2.466	1.617	3.93	2.93	2.21	1.51	0.087	0.122
5mL/kg (T_1)	0.05	0.107	1.48	1.01	2.512	1.638	3.97	3.01	2.29	1.57	0.086	0.121
10mL/kg (T_2)	0.05	0.105	1.52	1.04	2.554	1.682	4.07	3.08	2.38	1.64	0.084	0.119
15mL/kg (T_3)	0.047	0.104	1.57	1.07	2.607	1.733	4.16	3.15	2.49	1.71	0.082	0.116
20mL/kg (T_4)	0.046	0.1	1.61	1.1	2.648	1.778	4.25	3.23	2.57	1.77	0.082	0.114
25mL/kg (T_5)	0.045	0.097	1.64	1.12	2.713	1.833	4.34	3.31	2.69	1.83	0.081	0.111
30mL/kg (T_6)	0.047	0.101	1.62	1.09	2.681	1.791	4.28	3.25	2.61	1.78	0.082	0.113
35mL/kg (T_7)	0.049	0.105	1.57	1.04	2.61	1.716	4.17	3.17	2.49	1.69	0.084	0.115
40mL/kg (T_8)	0.05	0.107	1.49	0.99	2.551	1.652	4.01	3.02	2.37	1.58	0.085	0.107
Mean	**0.05**	**0.10**	**1.55**	**1.05**	**2.59**	**1.72**	**4.13**	**3.13**	**2.46**	**1.68**	**0.08**	**0.12**
SEd	**0.00**	**0.00**	**0.07**	**0.05**	**0.08**	**0.07**	**0.14**	**0.13**	**0.16**	**0.11**	**0.02**	**0.07**
Final- 10th day of accelerated ageing												

The protected viability under accelerated ageing is attributed to the combined effects of zein, curcumin, and azadirachtin. The hydrophobic nature of zein, with its high prolamin content (over 70%), forms a moisture-resistant barrier over the seeds during storage. Additionally, the antioxidant properties of curcumin and azadirachtin, when used at optimal concentrations, help maintain cell membrane integrity by protecting against auto-oxidation. This results in the extended storability of blackgram seeds under accelerated ageing conditions [56, 57]. Botanical molecules act as catalysts for producing reactive oxygen species (ROS) in quantities within the oxidative window, thereby preserving cell membrane integrity and extending the viability of groundnut seeds under ageing [18]. Furthermore, higher germination rates under ageing in custard apple leaf and fenugreek seed nano powder-coated seeds may be due to cell activation and enhanced mitochondrial activity, which increases energy production for the growing seed tissues during the early phase of germination in cluster bean [58].

The minimum electrical conductivity and lipid peroxidation of PZCA-NCs coated seeds is due to quenching of free radical's formation leading to cell membrane integrity. The decreased activity of hydrolytic and antioxidant enzymes is mainly due to the ageing process [59]. The decreasing antioxidant and dehydrogenase enzyme activity under storage is due to the decomposition of unsaturated fatty acid into alkoxyl free radicals during the lipid auto-oxidation process [60]. Reduced biochemical seed quality attributes and higher values of electrical conductivity and lipid peroxidation in control and vice versa in PZCA-NCs coated seeds might have indicated the capacity of curcumin and azadirachtin in maintaining the cell endowments triggering the germination process [61].

3.10. Effect of PZCA-NCs on Storage Pathogen Infection

The storage pathogen infection in PZCA-NCs invigorated black gram seeds stored under accelerated ageing was studied and storage pathogen (*Aspergillus flavus*, *Aspergillus niger*, *Rhizopus microspores* and *Fusarium* sp.) infection was found to be low in PZCA-NCs coated seeds compared to control. The percentage of infection was the minimum in seeds coated with PZCA-NCs @ 40 mL/kg (3%) whereas, in the case of untreated seeds, about 24% infection

was observed. The total storage pathogen infection was increased from 2–20% over 10 days of accelerated ageing. In interaction, seeds coated with 40 mL/kg had the lowest (7%) pathogen infection, while control registered the highest (43%) pathogen infection on the 10th day of accelerated ageing. Table 3 shows the bio-efficacy and pest control results noted on the different treatments of PZCA-NCs on primed seeds. Overall, the results indicated that the percentage of total storage pathogen infection in black gram seeds was reduced with an increase in the concentration of PZCA-NCs coating due to the antimicrobial activities of curcumin and azadirachtin. The reduced storage pathogen infection might be due to antimicrobial activities of curcumin and azadirachtin [62]. According to Kandhare, blackgram seeds treated with leaf powder of *Azadirachta indica*, *Ocimum basilicum*, and rhizome powder of *Cyperus rotundus* showed a seed mycoflora infection rate of approximately 42%, in contrast to 60% in untreated seeds [63].

3.11. Bio-efficacy assessment of PZCA-NCs against *Callosobruchus maculatus* and *Macrophomina phaseolina*

The toxicity studies of PZCA-NCs on *Callosobruchus maculatus* using probit regression analysis showed LD_{50} and LD_{90} values as 15.76 and 149.10 respectively. It is evident that 15.76 mL of azadirachtin was able to kill 50% of the adult population and 100 percent mortality was observed in 40 ml/kg while the control registered 13.33% insect mortality. The results demonstrated the superior entomotoxic activity of PZCA-NCs adhered over the surface of the insect body and disturb*ed* the contact between male and female insects. Moreover, a maximum number of insects died within five days due to physical toxicity and desiccation caused by PZCA-NCs adhered to the body of the insect [64, 65].

Debnath et al. and Arumugam et al. confirmed the physical effects and abrasion of nano structured silica on the cuticle in *C. maculatus* in pulses [66, 62]. Choupanian et al. found that 1% azadirachtin caused higher mortality of *Sitophilus oryzae* and *Tribolium confusum* in rice only after two days of exposure [67]. The poisoned food technique was employed to assess the impact of PZCA-NCs against the dry root fungus *Macrophomina phaseolina* noted on the 7th day of incubation under a room environment. The control plate without PZCA-NCs was almost covered with the growth of fungal mycelia growth (9 cm) with zero inhibition zone. The plate added with 35 mL of PZCA-NCs had 0.8 cm mycelia growth with a 91.11 percent inhibition zone whereas in case of plate added with 40mL had zero growth of fungal mycelia with 100 percent inhibition. Latha et al. assessed the effectiveness of 750 ppm tebuconazole-infused nanofibers against *Macrophomina phaseolina* using the poisoned food technique and reported complete inhibition [5]. The effective control of fungus mycelia growth is due to diffusion and complete spread of curcumin and azadirachtin in the media as such inhibition was obtained in *Macrophomina phaseolina* [65, 41]. Radwan et al. found that turmeric powder demonstrated significant bio efficacy against Colletotrichum species effectively controlling the fungus's mycelial growth when evaluated using the poisoned food technique [68]. The treatment PZCA-NCs and untreated control were pictured and aligned in Fig. 8 showing seedling growth, pathogen infection, fungal inhibition, insect mortality, and growth of beneficial bacteria.

3.12. Biosafety Assessment of PZCA-NCs on beneficial microbe

The spread plate technique was used to examine the compatibility of PZCA-NCs with bacteria like *Bacillus megaterium*, *Azotobacter chroococcum*, and *Bacillus subtilis*. Figure 8 demonstrated that there was no statistically significant difference in the number of *Bacillus megaterium* colonies found on the control and PZCA-NCs-added plates. Here, 5, 10, 15, 20, 25, 30, 35, and 40 ml/kg of the nano colloidal solutions were added to a liter of NA medium, and the *Bacillus megaterium* colonies of 1859, 1872, 1865, 1846, 1835, 1869, 1861, 1854, and 1891x 10^6 CFU/mL were found. The result of the other two bacterial pathogens shown in the table demonstrates that PZCA-NCs had compatibility with beneficial microorganisms.

Table 3
Bio efficacy of PZCA-NCs on storage pathogen growth, Inhibition percentage, storage pathogen infection, Insecticidal activity

		Mp		*Cm*			
Treatments (T) PZCA-NCs	**Storge pathogen**	**Mycelial growth (cm)**	**Inhibition %**	**Total mortality %**	**No. of colonies of bacteria grown (10^6 CFU/mL)**		
					Bm	**Ac**	**Bs**
Control (T_0)	24(29.33)	9	0	6(14.18)	1859	2548	2712
5mL/kg (T_1)	17(24.35)	8.2	9(17.46)	22(27.97)	1872	2562	2731
10mL/kg (T_2)	14(21.97)	7.4	18(25.10)	33(35.06)	1865	2571	2698
15mL/kg (T_3)	11(19.37)	6.5	28(31.95)	36(36.87)	1846	2532	2689
20mL/kg (T_4)	9(17.46)	5.2	42(40.39)	43(40.98)	1835	2580	2722
25mL/kg (T_5)	7(15.34)	4.1	54(47.30)	47(43.28)	1869	2547	2715
30mL/kg (T_6)	6(14.18)	3.2	64(53.13)	51(45.57)	1861	2521	2703
35mL/kg (T_7)	4(11.54)	1.8	80(63.44)	56(48.45)	1854	2515	2741
40mL/kg (T_8)	3(9.97)	1	89(70.63)	65(53.73)	1891	2567	2693
Mean	11(19.37)	**5.2**	**43(40.98)**	**40(39.23)**	**1861**	**2549**	**2712**
SEd		**0.133**	**1.853**	**0.938**	**47.844**	**58.625**	**54.674**
CD (P = 0.05)	0.347	**0.282**	**3.922**	**1.856**	**NS**	**NS**	**NS**

Mp- Macrophomina phaseolina, Cm- Callosobruchus maculatus, Bm -Bacillus megaterium, Ac-Azotobacter chroococcum, Bs-Bacillus subtilis

4. Conclusion

Hydrophobic ethanol-solvated curcumin and azadirachtin biomolecules zipped in zein achieves the optimal stabilized PZCA-NCs. Molecular docking studies of PZCA-NCs helped in understanding the binding affinity of the biomolecules with protein and stabilize them by hydrogen bonding interactions along with π-π stacking interactions. Results revealed that curcumin binds more readily than azadirachtin toward the protein. The controlled release and storability effect of PZCA-NCs at 25 mL/kg showed the best physiological parameters and biochemical enzymatic action with reduced storage pathogen infection compared to control and other dosages. Bio assay study results revealed the efficacy of PZCA-NCs in controlling the storage pest *Callosobruchus maculatus* and fungus on *Macrophomina phaseolina*. The PZCA-NCs showed compatible results on beneficial microbes *Bacillus megaterium*, *Azotobacter chroococcum*, and *Bacillus subtilis*. The study demonstrated the successful infusion of PZCA-NCs and its bio-efficacy in extending the life of black gram seeds. This is a preliminary nanotechnological involvement to evolve an innovative seed invigoration technique for prolonging the storability of black gram seeds. Hence, eco-friendly PZCA-NCs on large-scale testing of black gram before reaching the farm gate support the development of green biosafety fertilizers and pesticides.

Abbreviations

Not applicable

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Data will be made available on request from the corresponding author.

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

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All the authors have contributed to the structural formation of this work.

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Figures

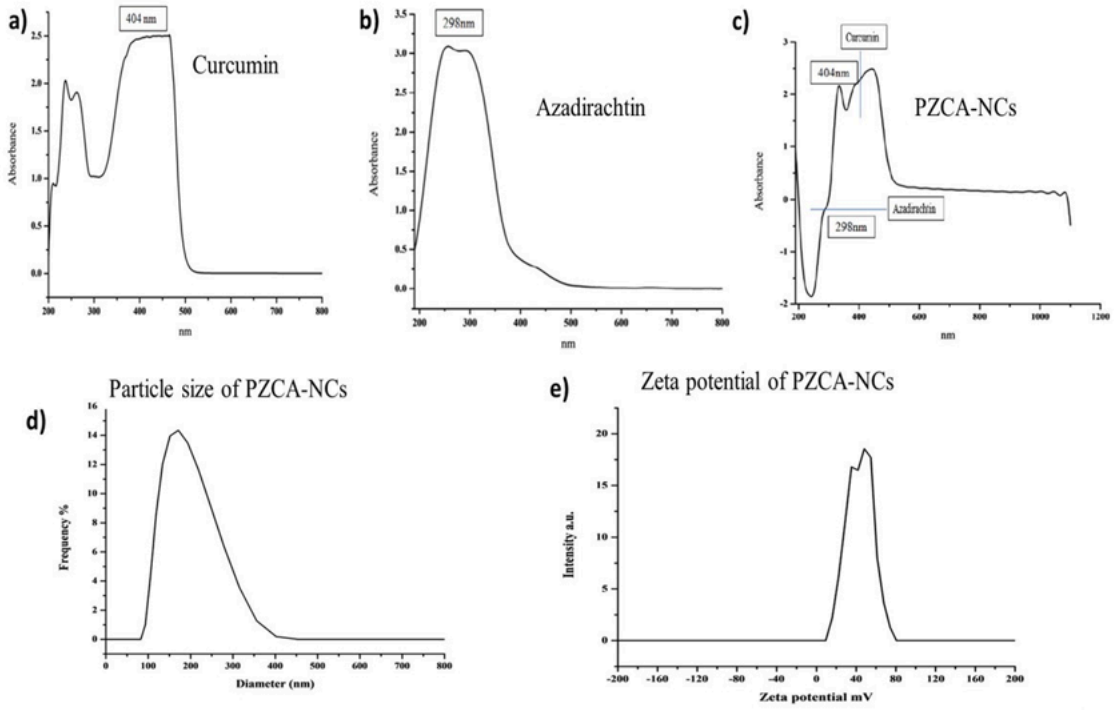

Figure 1

a) UV spectrum of Curcumin b) UV spectrum of Azadirachtin c) UV spectrum of PZCA-NCs, d) Particle diameter of PZCA-NCs e) Zeta potential of PZCA-NCs

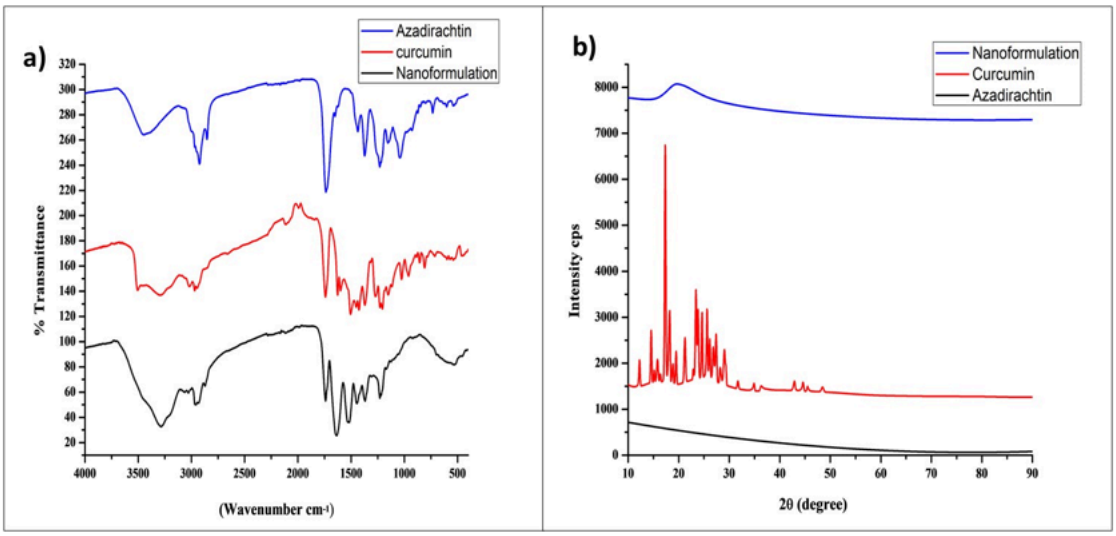

Figure 2

a) FT-IR of Azadirachtin, Curcumin, Nano formulation (PZCA-NCs) b) XRD pattern of Azadirachtin, Curcumin, Nano formulation (PZCA-NCs)

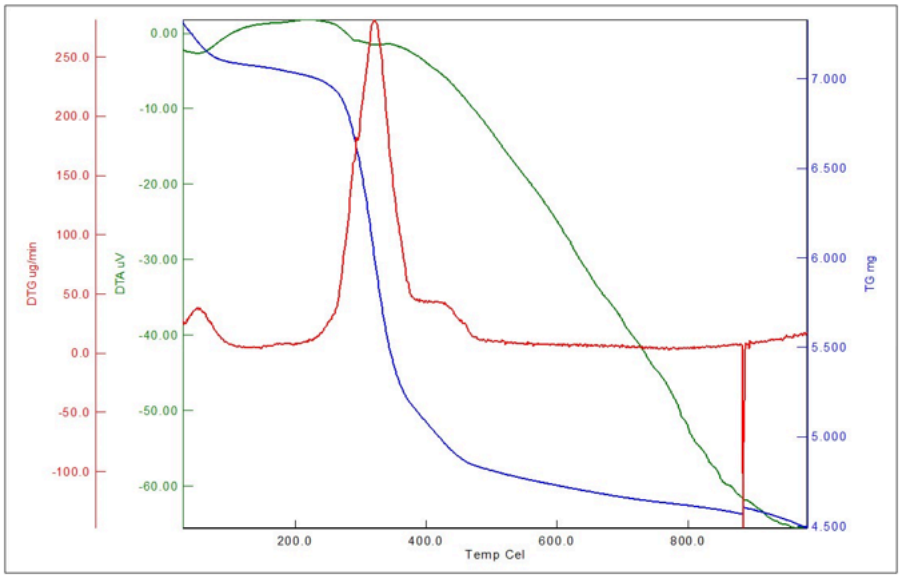

Figure 3

Colour representation (blue) TG for PZCA-NCs; (red) DTG for PZCA-NCs; (green) DTA for PZCA-NCs

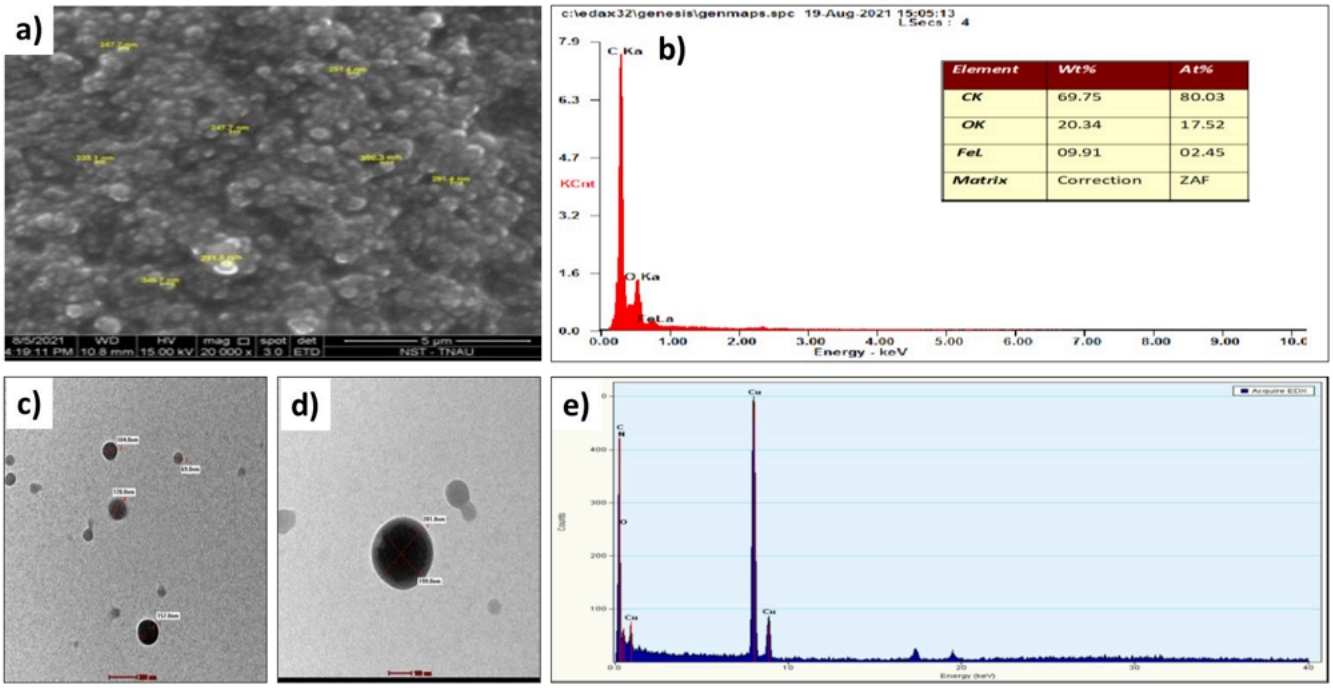

Figure 4

a) SEM image of PZCA-NCs b) SEM with EDAX spectra of PZCA-NCs c) TEM images of PZCA-NCs, d) TEM image of PZCA-NCs showing the capping molecules e) TEM with EDAX of PZCA-NCs

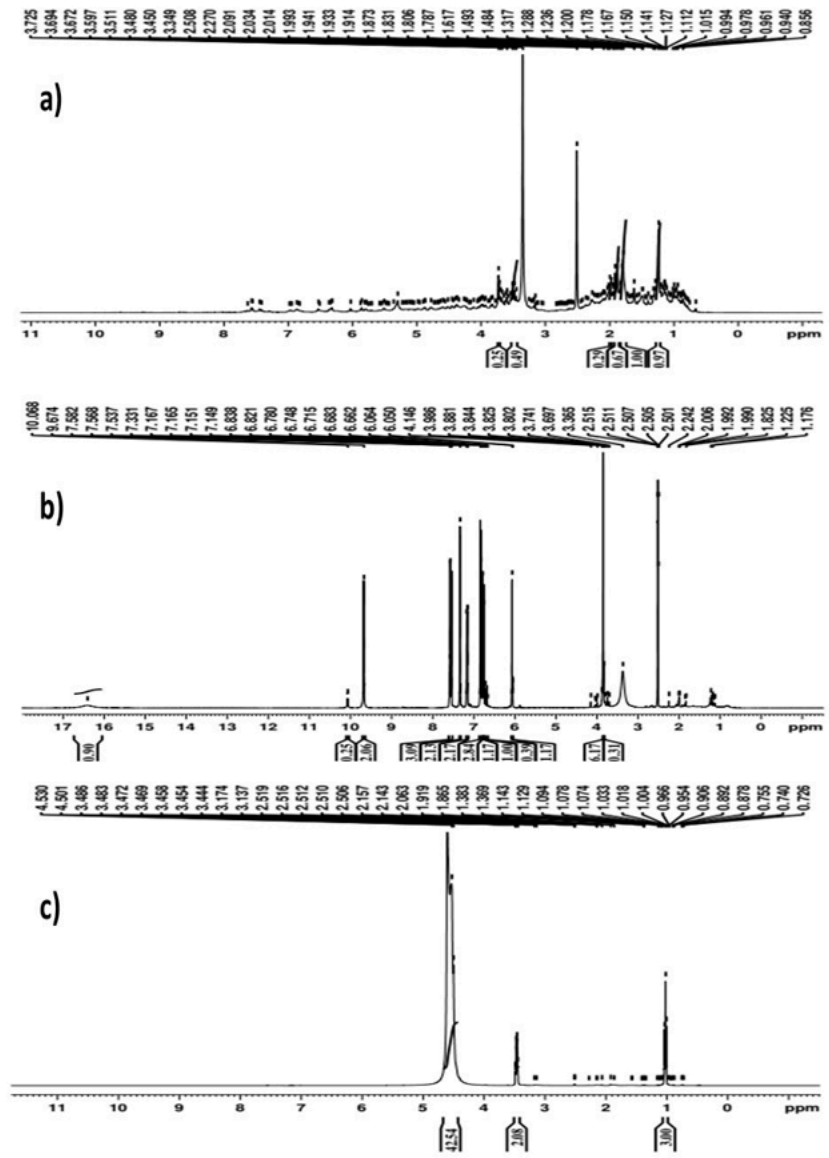

Figure 5

NMR spectra a) Azadirachtin b) Curcumin c) PZCA-NCs

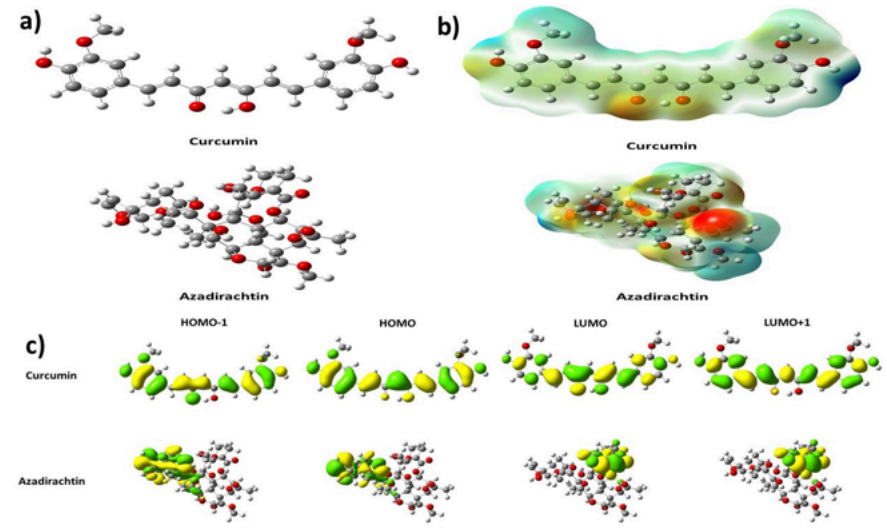

Figure 6

a) Optimized geometries of the molecules, b) Electrostatic potential map of the molecules c) Frontier molecular orbitals of the molecules

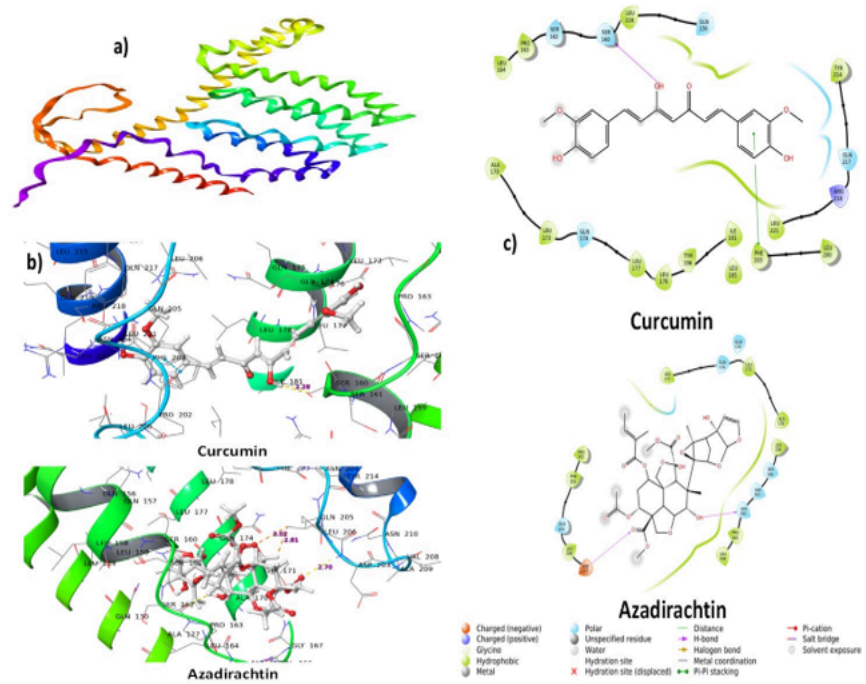

Figure 7

a) Modelled Zien protein using AlphaFold and further refined using Maestro, b) 3D view of the binding conformations of the curcumin and azadirachtin molecules with the highest binding energy at the active site of the protein c) LigPlot of the binding conformations of the molecule with the highest binding energy at the active site of the protein where the interacting residues inside the active site are portrayed for the curcumin and azadirachtin molecules.

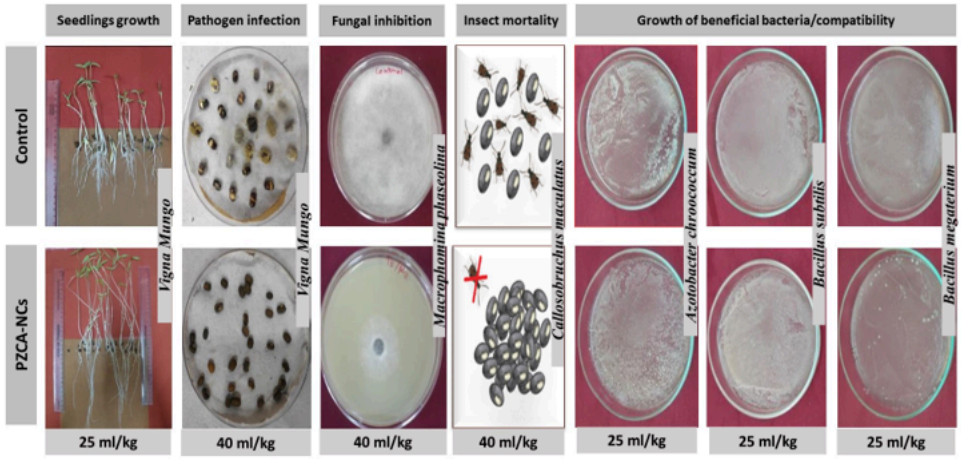

Figure 8

Observation of seedlings growth, pathogen infection, Fungal inhibition, Insect mortality, Growth of beneficial bacteria and its compatibility for control and PZCA-NCs

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.doc