Free radical scavenging activity of zinc oxide nanoparticles biosynthesised using *Aspergillus carneus*

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Zinc oxide nanoparticles (ZnO NPs) were confirmed in this work as a good antioxidant source. They were biosynthesised by the preformed biomass of *Aspergillus carneus* when contacted with 1 mM zinc nitrate solution of pH 9 after 24 h at 150 rpm and 30°C. The biosynthesised NPs were moderately distributed, quasi-spherical in shape with clear edges and the average size of 8–12 nm. The NPs retained full stability at 4°C for at least six months. Their zeta potential was found to be 18.3 mV. Crystalline nature of the ZnO NPs was suggested from diffraction rings appeared as lighted spots on the selected area electron diffraction pattern and confirmed by the X-ray diffraction analysis. High radical scavenging activities (RSA) were found against peroxide (O_2^{2-}) and 2,2–diphenyl-1-picrylhydrazyl (DPPH). Good activities were also recorded against hydroxyl (OH^-) and superoxide (O_2^-) radicals. This potential RSA can be rendered to the adsorbed biomolecules on the NPs surface especially the phenolic compounds detected by Fourier transform infrared analysis.

1. Introduction: Nanotechnology is a multidisciplinary promising area that influences human life and the environment. Nanoparticles (NPs) are being viewed as their fundamental building blocks for diverse applications. The great efforts of many researchers all over the world are devoted to the biosynthesis of metal NPs especially silver (Ag) and/or gold (Au) NPs since the beginning of the new millennium. A relatively few number of workers are interested in the biosynthesis of other metal and metal oxide NPs of which zinc oxide nanoparticles (ZnO NPs) attracted the attention of some workers. ZnO is n-type semiconductor that has a wide bandgap of 3.2 eV [1]. ZnO NPs have drawn interest in the past years due to their good conductivity, chemical stability, catalytic property, photonics and optoelectronics, unique antimicrobial and UV filtering properties [2]. So, they can be utilised in a wide range of applications.

ZnO NPs can be conventionally be produced by different physical and chemical methods. The physical methods involve the use of costly equipment, high temperature and pressure [3] and the chemical methods may lead to the presence of some toxic chemicals adsorbed on the surface causing adverse effects in medical applications [4]. Hence, the emergence of low cost and ecologically benign biological methods as an alternative to the other methods is something logical. Biosynthesis of ZnO NPs is now a major concern of some investigators using either higher plants or microorganisms. The biological methods allow the production of pure ZnO NPs. The plant extracts as well as natural microbial strains secrete some chemicals which act as both reducing and capping agents [5]. Extracts of different plant parts have been utilised for the biosynthesis of ZnO NPs [6–9], but leaf extracts are more pronounced [6].

Limited research works utilised various microorganisms for the biosynthesis of metal oxide NPs especially ZnO NPs. There are some reports on the utilisation of algae and bacteria for the biosynthesis of ZnO NPs but the results are still not encouraging [10–13]. Fungi are greatly desired for the synthesis of different NPs due to their diversity richness and high tolerance under ambient conditions of temperature, pressure and acidity [14]. Some species of *Aspergillus* were recorded as reasonable sources for the biosynthesis of ZnO NPs including *A. fumigates* [15], *A. niger* [16] and *A. terreus* [17].

Numerous pathogenic agents establish their virulence and pathogenicity by virtue of their ability to produce free radicals (FRs) and damage to the cells of the immune system [18]. The FRs are unstable and to restore their stability, they can pick up electrons from other atoms that in turn convert into secondary FRs initiating a chain of reactions leading to cell damage. This oxidative stress is the basis of many serious diseases and can be overcome by either enhancing the body's natural endogenous antioxidant defences or by supplementing with dietary antioxidants. Antioxidants play an important role to neutralise and scavenge the FRs [19]. These substances are produced naturally in healthy cases of the balance between the amounts of FRs and antioxidants. Under conditions of lack of antioxidants or increased production of FRs, antioxidants must be added from outside sources. Unfortunately, many synthetic antioxidants have potentially dangerous side effects [20], so the search for new alternative sources for antioxidants with fewer side effects is an important goal. Nanomaterials represent one of the most promising frontiers in the research for improved antioxidants [21]. Various NPs act as potent FR scavengers and antioxidants but most previous studied concentrated on Ag and/or Au NPs. Metal and metal oxide NPs particularly ZnO NPs are recently examined for their radical scavenging activities (RSA) and antioxidant activities [13]

This study was devoted to study biosynthesis, characterisation and RSA of ZnO NPs using a fungus not studied before in the hope of adding a new safe source of antioxidants.

2. Materials and methods

2.1. Chemicals and glassware: All chemicals used in this work were of analytical grade. The glassware was washed with aqua regia (3:1 HCl–HNO_3) and then thoroughly rinsed with deionised water to remove any metal contaminants.

2.2. Organism and cultivation: *Aspergillus carneus*, Blochwitz AUMC 13007 used in this study was isolated from a soil sample, collected from Dakahlia Governorate, Egypt and identified by Assiut University Mycological Centre (AUMC) where it was deposited with its accession number. Such fungus was selected in a preliminary screening programme to access potentiality of the available fungi on the biosynthesis of ZnO NPs. It was routinely grown on Czapek's-agar medium at 30°C and sub-cultured whenever required. The fungus was grown in 250 ml Erlenmeyer flasks containing 50 ml of Sabouraud's medium of the following

composition (g/100 ml):dextrose, 4 and peptone, 1. The flasks were sterilised, left to cool, the medium was initially adjusted to pH 9 and inoculated with 1 ml of spore suspension (~10^6 conidia) obtained from 7-day-old culture. The cultures were then incubated on a rotary shaker adjusted to 150 rpm at 30°C for 72 h. By the end of the incubation period, the biomass was separated from the culture supernatant by filtration through Whatman filter paper No. 1. The fungal biomass was washed thrice with deionised water to remove all possible components of the fermentation medium and used for biosynthesis of ZnO NPs.

2.3. Biosynthesis of ZnO NPs: Typically 10 g of biomass (fresh weight) was brought in contact with 90 ml of 1 mM of zinc nitrate ($Zn(NO_3)_2.6H_2O$), kept on the rotary shaker at 30°C and agitated at a velocity of 150 rpm for 24 h in the dark. Both positive (biomass in deionised water) and negative (1 mM $Zn(NO_3)_2.6H_2O$) controls were run along with the experimental flasks. A schematic diagram of the biosynthesis process of NPs has been presented in Fig. 1.

2.4. Characterisation of the biosynthesised ZnO NPs: The biosynthesised NPs colloidal solution was firstly monitored by visual observation. Change in colour towards turbid yellow (or yellowish) was taken as a preliminary sign of ZnO NPs formation and then confirmed by the UV–Vis spectral analysis after filtration of the coloured solution through 0.22 µm membrane filter (Millex-GS, Millipore, Madrid, Spain). Absorption measurements were carried out at wavelengths from 200 to 800 nm using a double beam spectrophotometer (Metash UV–Vis, model UV-8500) at a resolution of 1 nm. Weekly periodic UV–Vis analysis was carried out for six months to check the NPs stability.

To get rid of any uncoordinated biological molecules, the solution containing the biosynthesised, NPs were then centrifuged at 17,000 rpm for 15 min and the settled ZnO NPs were washed several times with deionised water. The NPs were either air dried or re-suspended in the deionised water by ultrasonication (Chem Tec Ultrasonic Processor UP-500, SN: UH005-0076) and used in the NPs characterisation.

Morphology and size of ZnO NPs were performed by High Resolution-Transmission Electron Microscopy (HR-TEM) in the central lab of the National Research Centre (NRC), Dokki, Giza, Egypt. For this purpose, an aliquot of the aqueous suspension of ZnO NPs was transferred onto a carbon-coated copper grid. Samples were dried and kept under vacuum in desiccators before loading them onto a specimen holder. The grid was then scanned

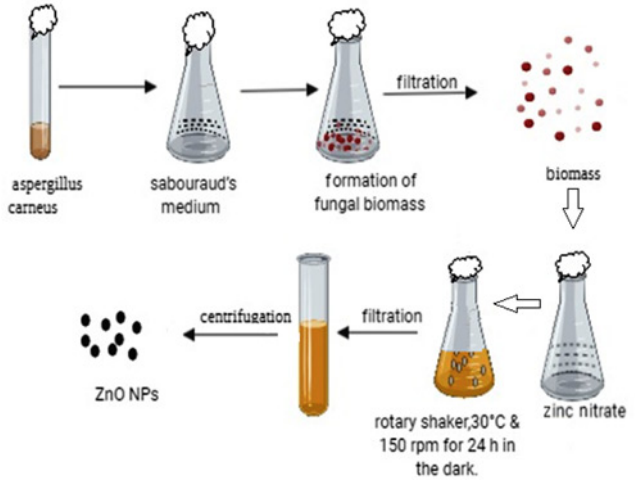

Fig. 1 *Schematic diagram of synthesising ZnO NPs using biomass of A. carneus*

using a Jeol JEM-2100 (Made in Japan Model Year 2000) operated at a voltage of 200 kV.

Selected area electron diffraction (SAED) pattern for NPs is a crystallographic experimental technique that can be performed inside a TEM. This analysis requires a very thin sectioned specimen nearly 100 nm and a high energy electron volt (100–400 keV), so in this case, the electrons will act as a wave and not a particle when interacting with the analysed material. The atoms in the substrate will act as a grating making diffraction to falling electrons, so the diffraction pattern appears as a bright spot [22].

X-ray diffraction (XRD) analysis was conducted on XPERT-PRO-PANalytical Powder Diffractometer (Netherlands) using monochromatic Cu K α radiation ($\theta = 1.5406$ Å) operating at a voltage of at 45 kV and a current of 30 mA at room temperature. The intensity data for the nanopowder were collected over a 2θ range of 4.01°–79.99°.

Fourier transform infrared (FTIR) Spectroscopy was obtained using Berkin-Elmer 293 spectrometer. KBr discs were used as calibrants and all measurements were carried out in the range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}.

Zeta potential of ZnO NPs was evaluated in nanomedicine and tissue engineering lab of NRC using a Malvern Zetasizer Nanoseries Nano ZS (Malvern Instruments Ltd, Malvern, UK). Data obtained were analysed using Zetasizer software.

2.5. RSA of the produced ZnO NPs: The RSA of the synthesised NPs was determined by DPPH, hydroxyl, peroxide and superoxide radicals scavenging assays.

The RSA of the ZnO NPs was examined in vitro using DPPH radical as described by Shimada *et al.* [23]. Ascorbic acid was used as a standard antioxidant agent for the concentration range as considered in the sample. RSA of the DPPH was expressed in percentage of inhibition using the following formula:

$$\text{RSA} = [\text{AC} - \text{AS}]/\text{AC} \times 100$$

where AC is the absorbance of the blank control and AS is the absorbance of the test sample.

Hydroxyl radical OH− was generated by a Fenton reaction system (Fe^{3+}-ascorbate-EDTA-H_2O_2) which attacks the deoxyribose and develops pink chromogen [24]. Gallic acid was used as the standard antioxidant in this assay and the hydroxyl RSA was then estimated from the previous formula.

Peroxide radical (O_2^{2-}) scavenging assay was studied using the slightly modified procedure described by Avani *et al.* [25]. The percentage of inhibition was calculated as above and ascorbic acid was used as a positive control.

Superoxide radical (O^{2-}) scavenging assay was assessed by inhibition of nitro blue tetrazolium (NBT) reduction [26]. Ascorbic acid was used as the positive control. Superoxide RSA was estimated as stated before.

3. Results and discussion: *A. Carneus* used in this work was selected in a preliminary screening of twenty different fungal species isolated from the Egyptian soil as a potential source for the biosynthesis of ZnO NPs. Such an organism was not investigated in this respect before and this is its first report. A turbid yellowish colour was observed after the preformed mycelium of *A. carneus* was contacted with the 1 mM solution of zinc nitrate in a positive sign for ZnO NPs formation. The appearance of this colour was due to surface plasmon resonance phenomenon that occurs in response to the collective oscillations of the conduction electrons confined to the NPs. The highest intensity of this colour was obtained in solution adjusted to pH 9 after 24 h of incubation at 30°C and 150 rpm.

The UV–Vis spectrophotometric analysis for the biosynthesised ZnO NPs revealed an intense peak with maximal absorption at

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310 nm (Fig. 2). No other peaks were recorded indicating the formation of pure ZnO NPs.

The ZnO NPs were firstly examined with HR-TEM where the micrograph image (Fig. 3*a*) revealed that the NPs are quasi-spherical in shape. A less intense layer indicated by arrows is observed at the periphery of the biosynthesised ZnO NPs which could relate to the capping biomolecules indicating their role as reduction and stabilising agents as suggested previously [7]. These spherical NPs have a clear edge of crystal and lattice structure observed in HR-TEM micrograph (Fig. 3*b*) which suggests the crystalline nature of ZnO NPs [15]. The SAED pattern (Fig. 3*c*) showed the diffraction rings which appeared as light spots and this supports the view that the particles were highly crystalline in nature in accord with the previously reported findings [27].

Size of the biosynthesised ZnO NPs calculated from the TEM analysis was found in the range of 4–28 nm with a predominance of NPs of size in the range of 8–12 nm represented about half the total content (Fig. 4).

The crystalline nature of the biosynthesised ZnO NPs using preformed biomass from *A. carneus* was again confirmed by their XRD pattern (Fig. 5). This is documented by the strong and narrow diffraction peaks at 2θ values of 31.62°, 34.29°, 36.12°, 47.42°, 56.48°, 62.75°, 66.37°, 67.85° and 68.96° characteristic to (100), (002), (101), (102), (110), (103), (112), (201) and (202) indexing planes for zinc, respectively. This agrees with the values reported for fcc zinc nanocrystals (JCPDS Card No. 89-7102).

The FTIR spectra were recorded to identify potential biomolecules that contributed to the reduction of the zinc ions and to capping of the biosynthesised ZnO NPs. This technique is a powerful tool for identifying types of chemical bonds in a molecule by producing an IR spectrum that is like a molecular fingerprint. The FTIR spectrum of ZnO NPs biosynthesised by *A. carneus* biomass was obtained in the wavelength range between 400 and 4000 cm^{-1} to identify the possible biomolecules responsible for capping, stabilisation and functions of these NPs (Fig. 6). The broad intense absorption band at 3421.1 cm^{-1}

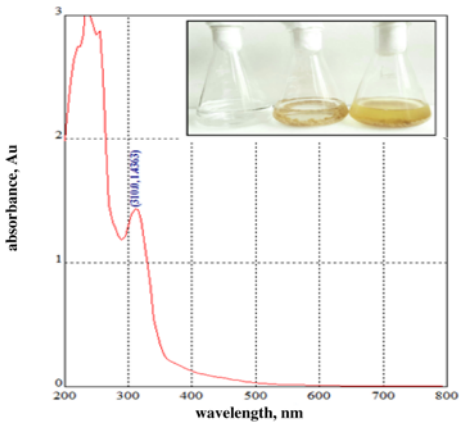

Fig. 2 *UV–Vis absorption spectrum of ZnO NPs formed using biomass of the fungus A. carneus. Inset shows colour change after incubating the biomass in 1 mM zinc nitrate (Right), in comparison with the biomass in deionised water as positive (Middle) and zinc nitrate alone as negative (Left) controls*

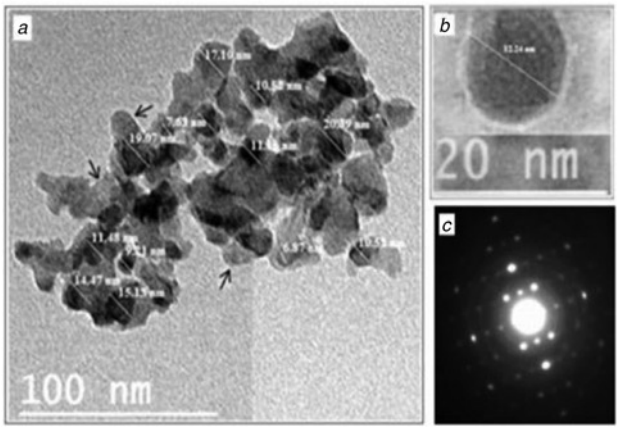

Fig. 3 *TEM micrograph of ZnO NPs biosynthesised by*
a A. carneus biomass
b HR-TEM micrograph of single enlarged ZnO NP and
c SAED pattern for one ZnO NP

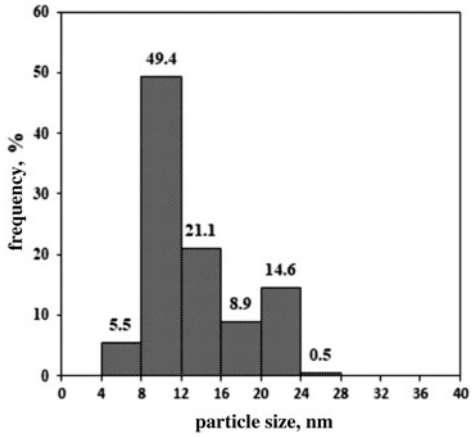

Fig. 4 *Particle size distribution from TEM analysis*

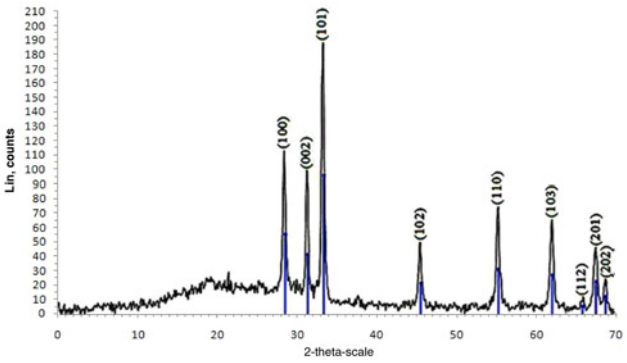

Fig. 5 *XRD patterns of ZnO NPs biosynthesised using biomass of A. carneus. The vertical lines indicate the position and relative intensity of JCPDS diffraction peaks*

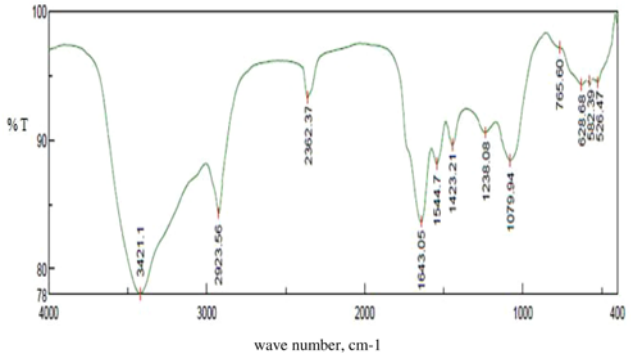

Fig. 6 *FTIR spectrum of ZnO NPs biosynthesised by A. carneus biomass*

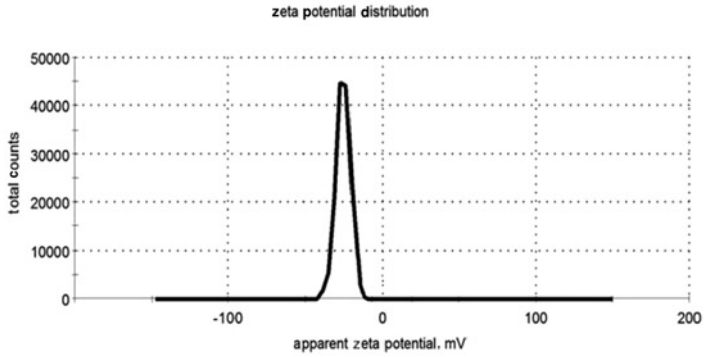

Fig. 7 *Zeta potential measurements of the biosynthesised ZnO NPs using A. carneus biomass*

corresponds to the hydroxyl (OH) functional group. Such group indicates the presence of polyphenols attached to the NPs surface [28]. The band appeared at 2923.59 cm^{-1} represents the C–H stretching of an aromatic compound of phenol group [28]. Different compounds of polyphenols are well known to have marked antioxidant activities depending on their molecular structures [29]. The characteristic bands recorded at 1643.05 and 1544.70 cm^{-1} were those of the bending vibrations of amide-I and amide-II, respectively [30]. Amides I and II are the major bands in the protein IR spectrum and this suggested that proteins are interacted with the biosynthesised ZnO NPs [31] as capping and stabilising agents. The band detected at 2857.99 cm^{-1} was described [32] as C–H stretching corresponding to aromatic mode arises from aromatic amino acids. Other distinct peaks were recorded 2362.37, 1423.21 and 1238.08 cm^{-1} corresponding to –COO [33], $–CH_3$ bending bonds [34] and C–C stretching vibration of alkanes [26], respectively. In addition, the weak peak with a wavelength 765.60 cm^{-1} was suggested as –CH by [35]. The band observed at 1079.04 cm^{-1} refers to the presence of sulphur-containing amino acids in proteins of *A. carneus* [36] that act as antioxidants under oxidative stress conditions [37]. The band detected at 765.60 cm^{-1} was assigned to aromatic groups [16]. Moreover, the bands in the range between 500 and 650 cm^{-1} are allotted to stretching modes of the Zn–O bond [17, 33]. These results confirm the attachment of different biomolecules to the surface of the biosynthesised ZnO NPs by *A. carneus* involved in biosynthesis, stabilisation and functions of these NPs.

The nanofluid was found stable for at least six months through a repeated examination of its characteristic colour and analysis of the UV–Vis spectrum of the biosynthesised ZnO NPs. No change in colour or in the absorption spectrum was detected indicating the stability of these NPs. The correlated or bounded proteins and other chemical compounds to the NPs surfaces as shown in the FTIR pattern can partially explain this stability. Further insight into the stability of ZnO NPs was obtained from zeta potential distribution analysis (Fig. 7). It was carried out to detect the surface charges acquired by the NPs and found to be −18.3 mV. This negative value confirms the repulsion among the NPs and thereby increases stability and prevents their aggregation.

An imbalance between antioxidants and ROS leads to oxidative stress and results in cellular damage. An important application of the NPs is their use as antioxidants and FR scavenging [18] to protect cells against the damaging effects of ROS. The RSA of ZnO NPs biosynthesised using *A. carneus* biomass was examined in vitro using different FR scavenging assays (Fig. 8). Scavenging assay of the stable DPPH is widely used to study the radical scavenging property of materials.

ZnO NPs of this study had significant DPPH scavenging activity reaching 81.92% when used at a concentration of 100 µg/ml in comparison with 88.13% for the same concentration of the standard antioxidant ascorbic acid. The half-maximal inhibitory

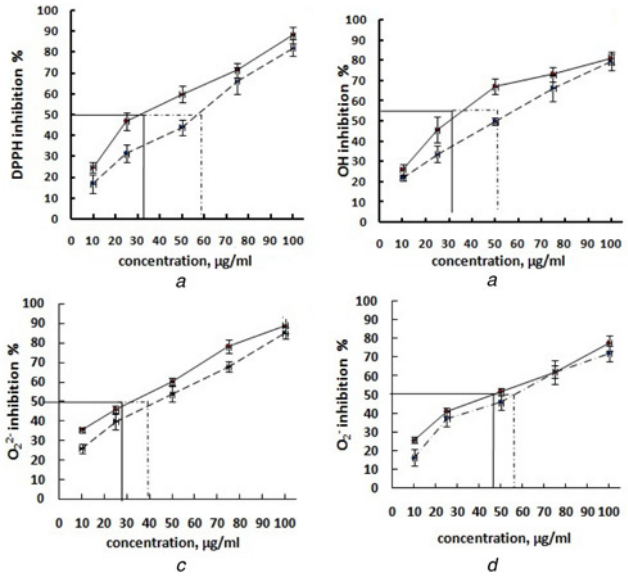

Fig. 8 *FR scavenging activity of the biosynthesised ZnO NPs (...) in comparison with the standard antioxidant (—)*
a DPPH scavenging assay
b OH^- scavenging assay
c O_2^{2-} scavenging assay and
d O_2^- scavenging assay

concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. It was calculated to be 57 and 31 µg/ml for the NPs and the standard, respectively. The positive DPPH test suggests that these NPs are FR scavengers exhibiting the potentiality to quench such radical. Although DPPH scavenging assay is the most popular method of studying the antioxidant activity, it is not enough to claim the antioxidant potential of the investigated NPs [13], hence additional assays were performed.

The hydroxyl radical (OH^-) is the most powerful ROS in causing biological damage [38]. It is able to react unselectively and instantaneously with the surrounding biomolecules [39] so it plays a role in several pathological conditions. At the highest investigated concentration, the ZnO NPs could scavenge 79.22%. This was very near from that of the standard antioxidant gallic acid that selected in this case due to the involvement of ascorbic acid in the reaction mixture of the assay procedure. IC_{50} was estimated to be 50 for the NPs and 30 µg/ml for the standard antioxidant.

Peroxide (O_2^{2-}) scavenging assay is another useful method for evaluating the RSA of materials. The inhibition of this was increased with increasing concentration of the ZnO NPs reaching its maximum at 100 µg/ml amounting to 84.95% in comparison

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with 88.93% in case of the standard antioxidant. The IC_{50} was found to be 43 and 32 μg/ml for the NPs and the reference antioxidant, respectively.

Superoxide anion (O_2^-) is also very harmful to cellular components. It is comparatively stable radical generated in living systems. The superoxide RSA of *A. carneus* ZnO NPs was relatively increased with increasing concentrations. The highest activity amounting to 72.04% with IC_{50} 56 μg/ml was recorded at the highest studied concentration compared to 77.62% with IC_{50} 46 μg/ml for the reference antioxidant.

4. Conclusions: Generation of FRs has been suggested as the main reason for the pathogenicity of many pathogens. To overcome the damaging effect of these radicals for cell wall and cell membranes of the hosts, it is required to neutralise off them. Some NPs can scavenge these FRs and prevent their deleterious effect. ZnO NPs biosynthesised using the fungus *A. carneus* were confirmed in this work to have radical scavenging activity against some important FRs. The NPs were biosynthesised by this fungus for the first time and completely characterised.

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