

Assessment of Anti-Alzheimer Characterization of Green Synthesized Zinc Nanoparticles Containing Extract of *Cordyceps militaris*

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ABSTRACT

Background: In current research, Zinc Nanoparticles (ZnNPs) were synthesized using green synthesis technology. It is the best method which is eco-friendly and cost-effective. The extract of fungus *Cordyceps militaris* was used for this purpose, which was followed by muffle furnace assisted synthesis of zinc nanoparticles. **Objectives:** The main objective of this research work was to assess the anticholinesterase and antioxidant activity mediated Anti-Alzheimer activity of *Cordyceps militaris* and zinc nanoparticles prepared by green synthesis process. **Materials and Methods:** The zinc nanoparticles were prepared by green synthesis process using extract of fungus *Cordyceps militaris*. Characterization of prepared nanoparticles were done by the Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), X-ray diffraction (XRD), EDX and Fourier Transform Infrared (FT-IR) spectroscopy for shape and size, Surface Plasmon Resonance (SPR), functional groups, crystallinity. It was observed that SPR peak were formed 375 nm, which was determined through UV-visible Spectroscopy. **Results:** SEM and TEM photomicrograph shown particles were formed spherical in the shape with smooth surface having 37.09 nm average particle sizes. SPR for nanoparticles was found at 300 nm. X-ray diffraction analysis confirm crystalline shaped of prepared nanoparticles. Moreover, during the synthesis of nanoparticles, the colour of suspension changed from dark yellow to colourless with development of cloudiness in solution. The different functional groups involved in nanoparticle stabilization were determined using FTIR Spectroscopy. **Conclusion:** Furthermore, the Alzheimer activity of plant extracts and ZnNPs have been carried out using the Ellman technique through cholinesterase with small modification. The IC_{50} value in anticholinesterase inhibitory activity of zinc nanoparticles was found 83.16237 $\mu\text{g/mL}$ while IC_{50} value of donepezil was found 51.38714 $\mu\text{g/mL}$ which was taken as standard. It was concluded that the result indicated the promising inhibitory potential of cholinesterase activity. The antioxidant assay results with percentage inhibition 127.0308 $\mu\text{g/mL}$ showed the most effective antioxidant property source than aqueous extract of *Cordyceps militaris*.

Keywords: Zinc nanoparticles, *Cordyceps militaris*, Anti-cholinesterase, Alzheimer anti-Alzheimer, Green synthesis.

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INTRODUCTION

Alzheimer is a neurodegenerative disease that begins slowly and gets worse over time and it primarily affects old age people. The incidence of this disease in patients has increased in recent years, according to the World Health Organizations from year 2019, across 50 million population are suffering from the dementia,

which have increased upto (10 million) a year. It must be approximated that the number of cases having dementia has raise up to 52-82 in 2030-2050, with people with AD reporting for 60-70% of all citizens. Drugs with Acetyl cholinesterase inhibitory (AChE) effects have shown good results in improving memory problem in Alzheimer's disease patients.¹ As a result, research is focusing on discovering AChE inhibitors as new treatments for Alzheimer's disease. Humans have oxidated stress which shows connections with inflammatory process and further results in causing hypertension, cancer and neurodisorder disease. Reactive Oxygen species known as ROS causes degradation of many bioactive molecules like DNA, proteins and lipids, resulting



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in neurodegenerative disorders and or apoptosis inducing death of neuronal cells. In a paradoxical way, oxidative stress obtained from high production of ROS could be recognized as a biomarker because oxidative stress can affect different molecular receptors, NMDA receptors in neurons, in the brain and/or endothelium have been shown to be highly expressed in response to oxidative stress.² The occurrence of oxidative stress-induced NMDA receptor regulating the expression is also used as a biomarker for treatment of Alzheimer's disease. Because of this, it is used for curing Alzheimer's disease.^{3,4} Recent time, nanotechnology is being used in the growth and enhancement of curing of many diseases. Alzheimer's disease seems to be main neurological problem of the central nervous brain that controls a variety of issues such as people's memories, behavioural patterns and related to cognitive impairments, sometime it causes dementia. Acetylcholinesterase (AChE) suppression has become a significant diagnostic aim for Alzheimer's disease. Lowered level of acetylcholinesterase shows good effects in improving memory. AChE inhibition is frequently prescribed for the treatment, as well as diagnosis of myasthenia gravis, Alzheimer's disease, along with bladder distension diseases and glaucoma. Several nanomaterials and nanoparticles have been used to diagnose neurodegenerative disorder. Nanoscience highlights the rules of material at dimensions range of 1-100 nanometers, which varies from that of bigger particles. They have different properties and unique forms like shape, size, chemical reactivity, distribution and practical benefits in catalytic activity. Since they conjugate phytoconstituents with nanoparticles, green synthesis-based ZnO nanoparticles have been found to have very good therapeutic activity. It has many benefits, such as less time consuming processes, low cost in comparing to others and have non-toxic nature.^{5,6} Zinc oxide nanoparticle is eco-friendly, not toxic, biosafe and biocompatible, used in companies such as piezoelectirc, biodiagnosis, and rubber processing biology, biology labelling, protection of the environment and the pharmaceutical industry.^{7,8} *Cordyceps militaris* is a member of the family (Cordycipitaceae). The genus *Cordyceps* contains approximately 750 identified species that have been found worldwide, but are mainly found in North America, Europe, South Asia and the Himalayan alpine pastures with many phytoconstituents namely Cordycepin, Codymin, Cordycepilic acid, Guanosine and inosine, Myriocin, Inosine, Berberine, Arachidonic acid Linoleyl acetate, Cinnamaldehyde, Deoxyandrographolide, Linoleic acid, Uralene, B-sitosterol.^{9,10} Various *Cordyceps militaris* extracts have shown significant anticancer, anti-inflammatory, antioxidant along with that it also shows antimicrobial and anti-alzheimer properties.^{11,12} present study is aimed to develop ZnNPs utilizing *Codyceps militaris* extracts. The AChE enzyme shows inhibitory activity of of *Codyceps militaris* with methanolic extracts and by green-method of Zinc nanoparticles were then investigated in the present study using donepezil as a standard.¹³ This might benefit well with potential of anti-Alzheimer. The goal

of this study is to obtain zinc nanoparticles. by green-synthesis with *Cordyceps militaris* through anticholinesterase potentials by *in vitro* and it can be a first step forward into recognizing the most viable anti-Alzheimer potency that might have less toxicity. Furthermore, antioxidant assay shows more effective result in comparison with aqueous extract.

MATERIALS AND METHODS

Materials

Fungi from *Cordyceps militaris* family (Cordycipitaceae) had been collected from Shree Ram Testing Laboratories Private Limited Mumbai, India. Zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) from the Katyuri Chemicals, Dehradun, Uttarakhand, India were purchased. Reagents and solvents were used are of analytical quality.

Methods

Extraction

To remove dirt particles and parasites, the fungal plants were thoroughly rinsed with distilled water and then it is for two days in the shade to dry at room temperature. Fungal plants were made in powdered form by using a washed electric crusher. The powdered material were filled inside an air-tight, amber glass bottle and kept in hot air oven to complete drying and sterilization. The obtained powder (10 g) was taken in glass container and immersed in 100 mL of purified water which was then warmed on the water bath at temperature set to 50-55°C for about 30 min, then was filtered with the help of Whatman filter paper no.1. Extract was allowed to store in -4°C for future investigations.^{14,15}

Green Synthesis of Nanoparticles

The zinc nanoparticles were produced by the method described by with minor changes in which 10 mL of 10% v/v extract was boiled on water bath for 30 min. When the temperature exceed to 65°C, 1 g of zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) poured and was mixed continuously, till paste is formed. After that, the obtained paste was put in a ceramic crucible cup and placed in a muffle furnace for about 2 hr (400°C). After 2 hr, the crucible was allowed to remove from the muffle furnace containing white colored powder containing zinc nanoparticles. The obtained powder was cooled and placed in tightly packed vessel for the experiment.^{16,17}

Characterization of prepared nanoparticles

UV-Spectrophotometry

The obtained nanoparticles were suspended in methanol and scanned with a UV-spectrophotometer at wavelengths ranging from 200 to 800 nm (Shimadzu UV-1601) for characterizes and monitors Zinc nanoparticles.^{18,19}

Shape and Surface Morphology

Scanning Electron Microscopy (SEM)

A scanning electron microscope had been applied to examine morphological characteristics of the synthesized nanoparticles and X-ray energy dispersive spectroscopy. Nanoparticles that have been prepared in powder were sprinkle on the double adhesive tape which was stucked on aluminium stub followed by gold coating about 300Å by sputter coater.²⁰ The photomicrograph of were taken using Scanning Electron Microscope (EVO-18 Carl Zeiss,).

Transmission Electron Microscopy (TEM)

The green-synthesised ZnNPs were visualized using a transmission electron microscope (Hitachi-H7500) at a 20 kV of accelerate voltage. The nanoparticles suspension was allowed to negatively stain with the 2.0% w/v of phosphotungstic acid solution. A copper grid was put ZnNPs suspension and covered with a carbon supported film for few second. The grid was removed and remaining solvent was cleared with the help of filter paper before placing the grid in the instrument. The image was taken with transmission electron microscope.²¹

XRD Analysis

X-ray diffraction pattern of synthesized ZnNPs was performed using XRD D8 Advance, Bruker and the XRD of prepared ZnNPs was shown in the Figure 4 (A) and (B). By applying copper, K source of radiation ($\lambda=1.5406 \text{ \AA}$) was to analyze absorption spectrum with the correct size and it was determined by calculating with Scherrer's formula.²² All diffracted intensities were measured with 2θ angles from 4° to 90° .

Scherrer formula:

$$D = \frac{K\lambda}{\beta \cos\theta}$$

Where,

D denotes the size of particles;

K s Scherrer constant ($k=0.9$);

λ =wavelength of light used for diffraction ($\lambda=1.54 \text{ \AA}$), β is the full width at half maximum of the diffraction peak;

nd θ is known as angle of reflection.

Infra-Red (FT-IR) spectroscopy

Jasco, Japan, FTIR-6100 spectrometer was used to determine spectral of ZnNPs to find the functional group attached on the ZnNPs. All components were scanned and recorded at 400-4000 cm at normal temperature with 1 resolution at 1 spectral range.²³

Differential Scanning Calorimetry (DSC)

ZnNPs sample was examine to determine isothermal oxidation behaviour.²⁴

In vitro Anti-Alzheimer Study

Anticholinesterase activity in selected *Cordyceps* species

Anticholinesterase activity of prepared zinc nanoparticles was examined in a 96 wellled plate by Ellman method assay kit through Enzo Pak. 10 mg extract with 10 mL methanol solution in separate wells, which will be used for inhibition (100%). After which, tested extracts of 20 μL at various concentrations 10-10000 $\mu\text{g mL}^{-1}$, 1 mL with AChE, in left out wells 15 min at 37°C for incubation have been added to sample. In the same way, different concentrations of donepezil and test extracts are been prepared. At 412 nm absorbance was analysed at 10-1000. (IC_{50}) needed inhibit the enzyme reaction (acetylcholine hydrolysis) through 50% was determined.^{25,26}

Using the excel program regression analysis concentration versus inhibition percentage IC_{50} values were examined by performing a linear approximation.

$$\text{Activity in V/L} = \frac{\Delta\text{Abs}}{30\text{sec} \times \text{factor}}$$

Factor=TV $\times$ 1000 $\times$ 2 $\div$ 14.64 $\times$ SV $\times$ 1;

Where TV=Total reaction volume in mL;

SV=Sample volume in mL;

and 14.64=millimolar absorption coefficient of 5-into-2-nitrobenzoic acid;

P=Cuvette pathlength in cm;

2=Abs/30 sec to Abs/min conversion.

Antioxidant Activity

Fungal antioxidant activities were examined using a 2,2-diphenyl-1-picrylhydrazyl. Then for this study, 0.1 mM methanolic solutions of DPPH of 200-800 μL were used. For 30 min, it was kept for incubation at 37°C and 0.714 nm, absorbance was taken.^{27,28} To compare the antioxidant properties of prepared nanoparticle for standard ascorbic acid was taken. By using the following formula, the % inhibition for oxidation were recorded

$$\text{Percent radical scavenging activity} = \frac{[(\text{Abs control} - \text{Abs sample})]}{\text{Abs control} \times 100}$$

$\text{Abs}_{\text{control}}$ =DPPH radical absorbance with methanol.

$\text{Abs}_{\text{sample}}$ =DPPH radical absorbance with sample extract/standard.

Statistical Analysis

Throughout this study, triplicate experiments and analyses were conducted and Microsoft Excel 2012 was used for graphs.

RESULTS

The synthesized Zinc nanoparticles prepared with *Cordyceps* species analysed using UV spectrophotometer. Green-synthesised zinc nanoparticles have such an absorbance peak in the 300-550 nm range. All samples had a high adsorption band from 354-368 nm, Zinc nanoparticles with their high bands with normal and Surface Plasmon Resonance (SPR) 375 nm.¹⁸ Place of a Surface plasmon resonance is because of the amount of charge getting transfers surrounded by the medium and relationship of particles along with particulate shape and size. Furthermore, towards the absorption peak at 500-550 nm, an excitonic absorption peak at 260-280 nm was found. The motion of the electronic cloud on the Zinc nanoparticles can cause the greater wavelength absorption band. These small variations in absorption intensity values are owing to particle size changes. Zinc nanoparticles capture a lot of light in the UV spectrum. The size and shape of a nanoparticle had been determined by using Malvern zeta sizer and scanning electron microscopy. SEM photomicrographs revealed that the nanoparticles surfaces were smooth and spherical in shape (Figure 1). The appearance of spherical nanoparticles with a diameter of approximately of below 75 nm is clearly illustrated. The existence of metallic ZnNPs is been confirmed through EDAX analysis (Figure 2). It shows the composition of Zinc (25.4%), Oxygen (42.6%) and Carbon (25.9%).

SEM EDAX analysis

The morphological characteristics and proportions of the green synthesized ZnNPs have been mainly shown in TEM images given in Figure 3.

Transmission electron microscopy image showed consisted with tiny spherical shape particles. The TEM image of ZnNPs shows spherical shape varying in size from 20 to 100 nm.

X-ray Diffraction analysis of prepared nanoparticles was found that the intensities observed from 2000 to 8000 are considered as diffraction intensities. Moreover, Bragg reflections with (2θ value) at different angles 31.7100, 34.3200, 36.1700, 47.44°, 56.48°, 67.84°, 68.97°, 72.4500 shows correspondence with these intensities.²⁵ The green synthesized ZnNPs forms crystalline nature and exhibit higher purity as shown in Figure 4 (A) and (B). Using Scherrer equation, peaks forming at 36.17, 34.32 and 36.17 nm with high-intensity peaks were discovered.

ZnNPs FT-IR spectra of prepared zinc nanoparticles was shown in Figure 5 that shows exact bands and various significant peaks locations shows correlation with synthesized ZnNPs. OH, stretching vibrations has been revealed by the values 3409.65 cm^{-1} shows wide peak. C-O-H bending peaks were formed at 1383.83, cm^{-1} . A zinc oxide nanoparticle was examined at Absorption 400 cm^{-1} to 600 cm^{-1} . Zn-O stretching and deformation vibrations have shown peaks at 3409.65 and 1383.83 cm^{-1} and 1116.43 and 447.23 cm^{-1} , respectively. Through above findings,²⁵ it has been revealed that zinc oxide nanoparticles shows formation with extract of *Cordyceps militaris*.

The DSC endothermic peak observed at 140.80°C shows low temperature (Figure 6). During the process of synthesis because loss of surfactant molecule which was previously adsorbs on the surface of zinc oxide nanoparticles formed peak on high temperature known as endothermic peak at 144.09°C shows zinc nanoparticles as compared with a temperature with slightly high

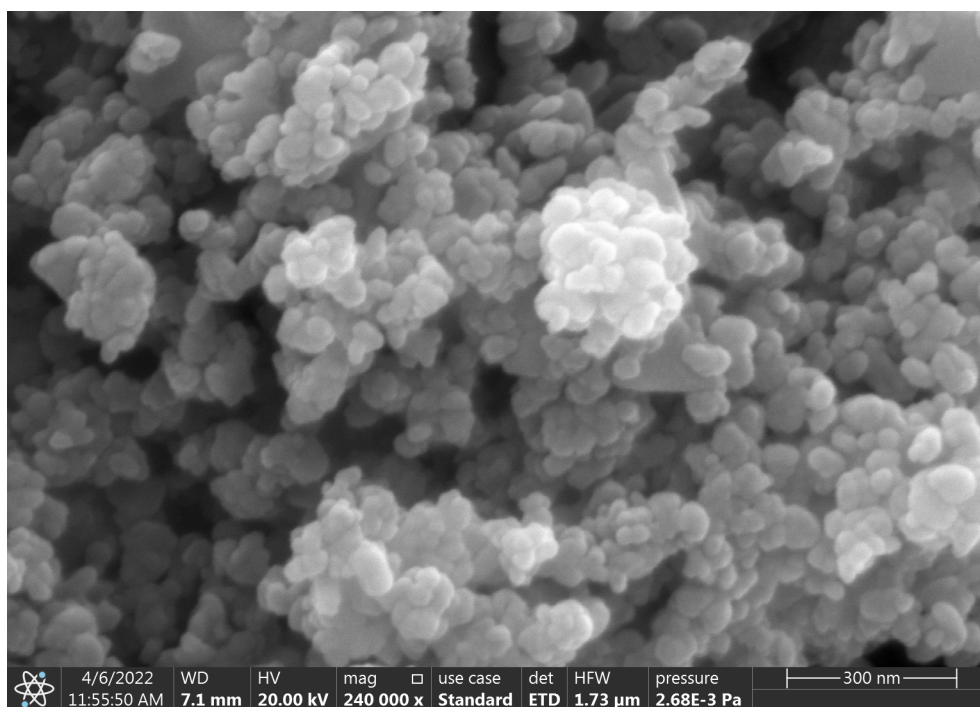


Figure 1: SEM photomicrograph of the Synthesized ZnNPs using *Cordyceps* species.

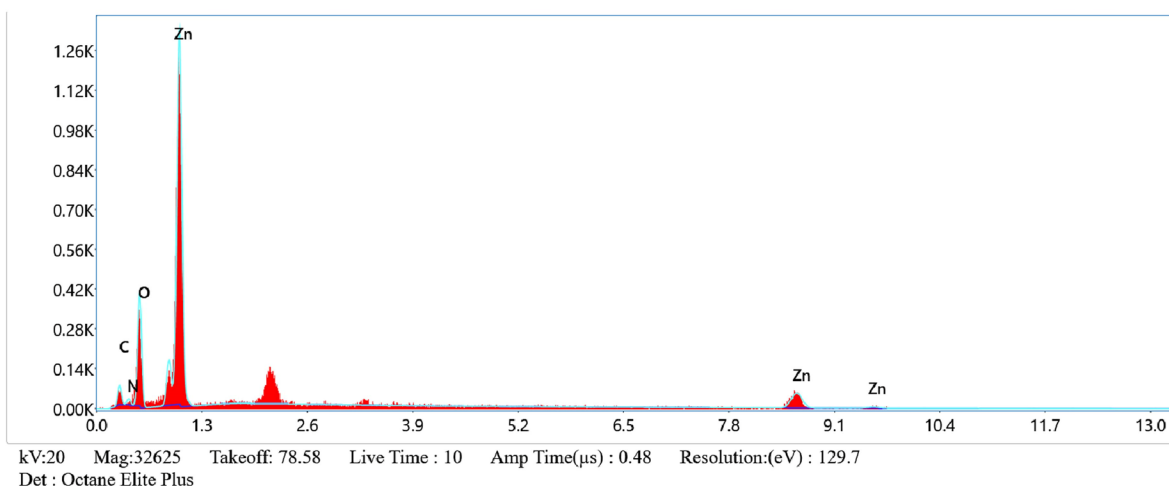


Figure 2: EDAX analysis of the ZnNPs.

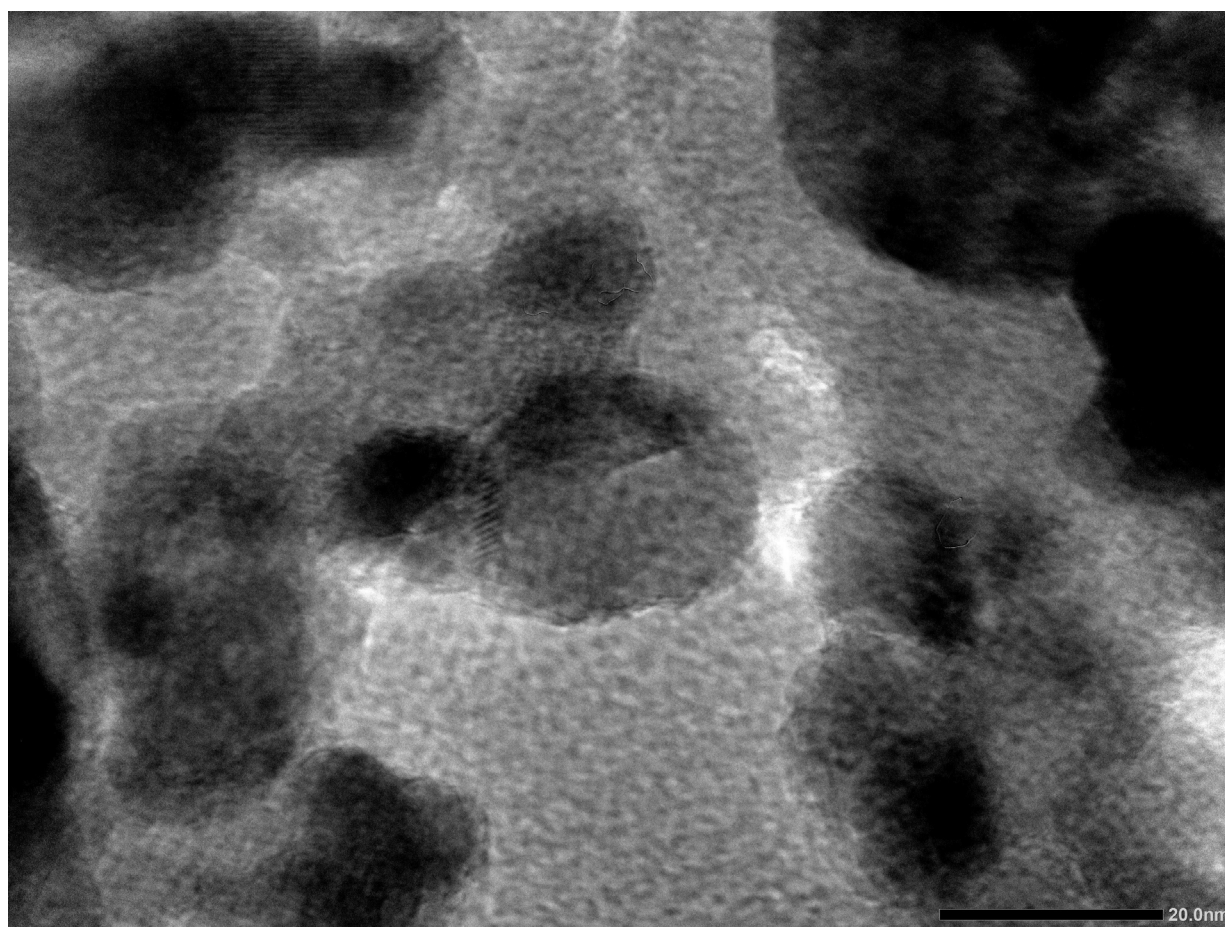


Figure 3: TEM photomicrograph of the synthesized ZnNPs of the selected *Cordyceps* species.

at 147.31°C which clearly shows the formation of zinc oxide into the ZnNPs.²⁴

The inhibitory activity for (Alzheimer) was determined by performing anticholinesterase study. In this study, methanolic extracts of *Cordyceps militaris* with ZnNPs was assayed (Table 1). Moreover, the anticholinesterase activity of ZnNPs was

compared with donepezil as a standard. It is well known fact that our brain consists of two different cholinergic and noncholinergic neurons. Anticholinesterase (AChE) inhibition increases levels of acetylcholine which helps in curing Alzheimer. Green synthesized ZnNPs prepared with crude extract showing IC_{50} value 83.16237 $\mu\text{g/mL}$. The % inhibition of cholinesterase

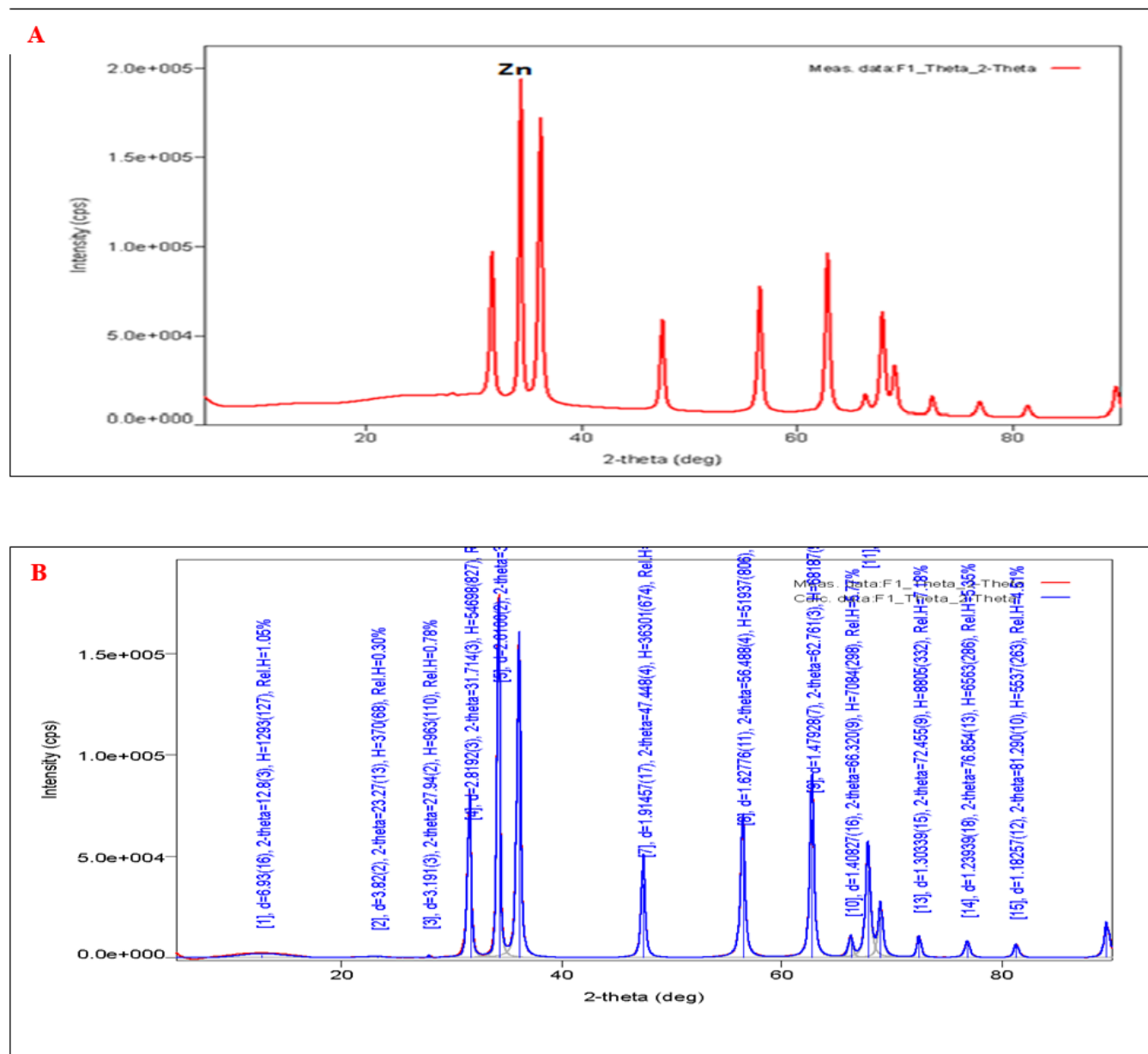


Figure 4: X-ray Diffraction analysis of ZnNPs.

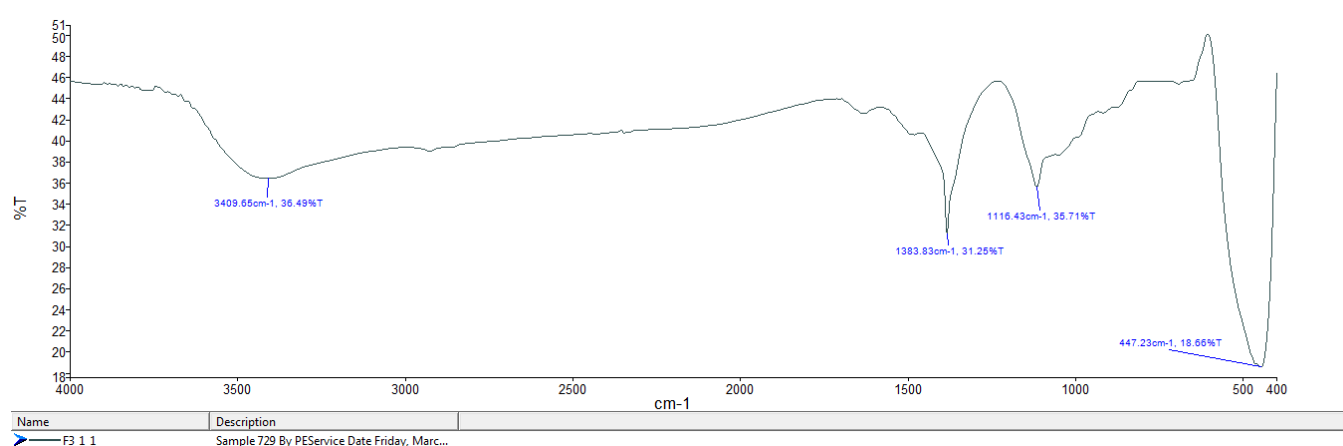


Figure 5: Fourier Transform Infrared Spectroscopy (FTIR) ZnNPs.

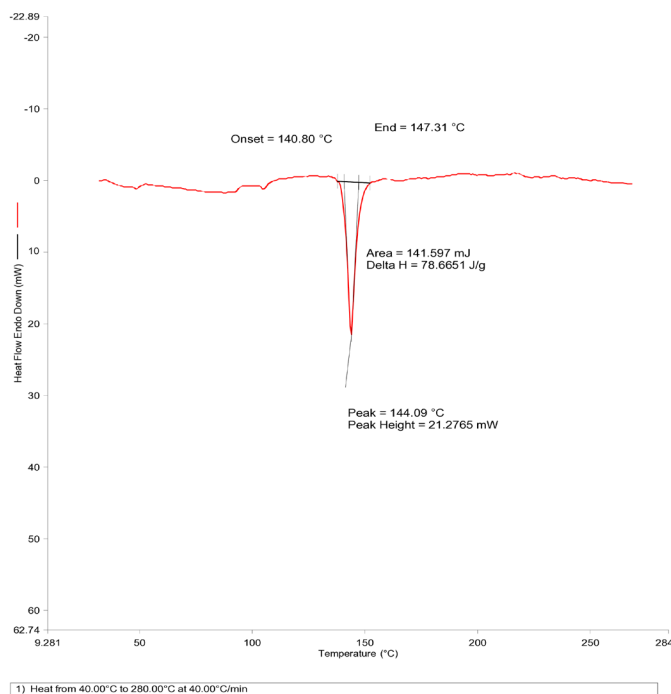


Figure 6: DSC thermograph of ZnNPs.

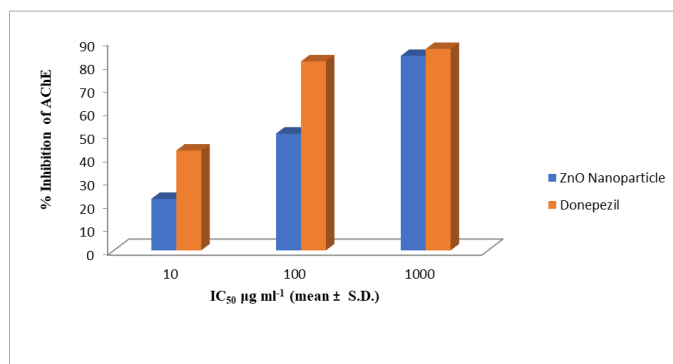


Figure 7: Percent inhibition of cholinesterase enzyme activity.

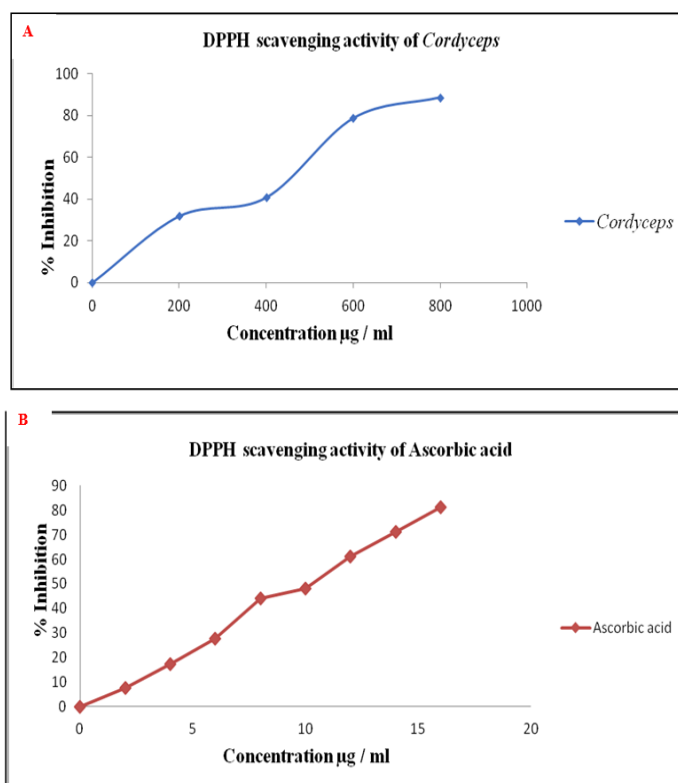


Figure 8: % inhibition (DPPH scavenging) of *Cordyceps militaris* and ascorbic acid.

Table 1: Percent inhibition of cholinesterase enzyme activity.

Concentration (µg/mL)	Percent inhibition of cholinesterase enzyme activity	
	ZnNPs	Donepezil
10	22.08	42.84
100	50.10	81.08
1000	83.43	86.40

Table 2: % Inhibition (antioxidant) of *Cordyceps*.

Concentration (µg / mL)	200	400	600	800
Cordyceps	31.86	40.79	78.72	88.64

enzyme activity was found (22.08, 51.10 and 83.43 respectively with different concentrations of 10, 100, 1000 µg/mL of extract while Donepezil shown (42.84, 81.08 and 86.40% inhibition respectively with concentrations of 10, 100, 1000, µg/mL with IC_{50} value 51.38714 µg/mL is displayed in (Figure 7). Apart from that, Donepezil demonstrated one of most effective and powerful acetylcholinesterase inhibitor for this activity.²⁵

Antioxidant activity of prepared zinc nanoparticles was performed which shown that the aqueous extract shows IC_{50} value 127.0308 µg/mL, respectively in comparison with IC_{50} standard antioxidant ascorbic acid value 10.3622 µg/mL (Table 2). Figure 8 (A) and (B) depicts the extract's DPPH radical scavenging effect respectively of *cordyceps* extract and ascorbic acid (B). In this research, *Cordyceps militaris* extract exhibited greater radical scavenging activity against DPPH than aqueous extract.²⁷

CONCLUSION

Green nanoparticle synthesis is an excellent method for connecting nanotechnology with plants. The main advantage of using extract for nanoparticle biosynthesis is that it contains a large number of metabolites that can contribute to the reduction of different metallic ions (gold, silver, zinc). The prepared nanoparticles are sub nanometric in size offering an excellent avenue for targeting to brain and other cellular organelle. The metallic nanoparticles have good adsorption capacity towards both lipophilic and hydrophilic therapeutic molecules. In this study, we illustrated *Cordyceps militaris* with green synthesized nanoparticles along with anti-Alzheimer activity. Besides this, the FTIR analysis indicates many functional groups with ZnNPs offer wide variety of functionalization with different targeting and therapeutic molecules. Green-synthesised ZnNPs using *Cordyceps militaris* extract was determined to be extremely potent and showing significant inhibitory effects of Acetylcholinesterase which represent the potent anti-Alzheimer effect. In contrast to aqueous extract, the DPPH assay with extract has been found to be more effective for future predictions for the effective management of anti-Alzheimer effect. The study can bring new beginnings for Alzheimer which will guide future researchers in their research work.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ZnO: Zinc oxide; **ZnNPs:** Zinc nanoparticles; **SEM:** Scanning Electron Microscopy; **SEM-EDAX:** Scanning Electron Microscopy-Energy Dispersive X-ray Analysis; **TEM:** Transmission Electron Microscopy; **SPR:** Surface Plasmon Resonance; **XRD:** X-ray Diffraction; **DSC:** Differential Scanning Calorimetry; **FT-IR:** Fourier Transform Infrared Spectroscopy; **AChE:** Acetylcholinesterase; **DPPH:** 2,2-Diphenylpicrylhydrazyl.

SUMMARY

Zinc nanoparticles (ZnNPs) were synthesized using green synthesis using extract of fungus *Cordyceps militaris*. The fungus *Cordyceps militaris* extract have a strong anticholinesterase and antioxidant properties which has beneficial effect to manage the Alzheimer disease. Electron microscope photomicrograph revealed that prepared ZnNPs were found round in the shape

and smooth surface with optimum sub nanometric average particle size. The SPR for nanoparticles was found different from its salt confirm the nanoparticles formation. X-ray diffraction analysis confirm crystalline shaped of prepared nanoparticles. The functionality of nanoparticle was confirmed by FTIR Spectroscopy. Alzheimer activity of plant extracts and ZnNPs have been carried out using the Ellman technique through cholinesterase with small modification. The IC₅₀ value in anticholinesterase inhibitory activity of zinc nanoparticles was found similar as compared to standard. It was shown promising inhibitory potential for cholinesterase enzyme and has potential antioxidant properties. The strong anti cholinesterase and antioxidant study from the mushroom fungus *Cordyceps militaris* extract and prepared zinc nanoparticles using the extract represent potential anti-Alzheimer activity.

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