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Assessment of antimicrobial potential of green zinc oxide nanoparticles (ZnONPs) of *Bacopa monnieri* (L.) against clinical isolates of *Escherichia coli*

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ABSTRACT

Antimicrobial resistance (AMR) is a major global health issue caused by the overuse of antibiotics across various sectors. It leads to the emergence of resistant microorganisms and could surpass other leading causes of death by 2050. *Escherichia coli*, commonly used as an indicator organism for monitoring AMR in livestock and food products from animals, naturally inhabits the intestines of warm-blooded animals. This study aims to utilize *Bacopa monnieri* (L.) extract as a reducing and stabilizing agent for synthesizing ZnONPs through green synthesis and to test their antimicrobial activity against clinical isolates of multidrug-resistant *E. coli*. The green synthesized ZnONPs were characterized chemically, optically, and morphologically using techniques such as UV-VIS Spectrophotometry, FTIR Spectroscopy, and TEM. Additionally, *E. coli* isolated from mucous samples was characterized morphologically, biochemically, and molecularly using gram-staining, MacConkey agar differential media test, oxidase test, and PCR based molecular confirmation. Later, antimicrobial assay was performed using MIC technique. The presence of the Surface Plasmon Resonance peak in UV-VIS spectra confirmed the successful synthesis of ZnONPs, while FTIR spectra indicated the presence of functional groups associated with phytochemicals involved in the bioreduction process during ZnONPs formation. TEM analysis revealed the development of nanoparticles with small dimensions. Furthermore, green-synthesized ZnONPs of *Bacopa monnieri* (L.) demonstrated promising antimicrobial activity, with a MIC of 316.4 µg/mL against MDR *E. coli*.

Keywords: Antimicrobial activity, *Bacopa monnieri* (L.), *Escherichia coli.* Green synthesis, Zinc oxide nanoparticles

Antimicrobial resistance (AMR) has emerged as a significant public health challenge worldwide in the 21st century (Singh *et al.* 2024). Frequently referred to as the "Silent Pandemic," AMR demands prompt and effective action, instead of being dismissed as a challenge for the future (Steuernagel *et al.* 2024). The World Health Organization (WHO) in February 2017 released pathogens list that require urgent attention for the development of new antibiotics. Among these, the ESKAPE group- comprising *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species – was identified as having "priority status" due to their significant role in antibiotic resistance (WHO 2017). *E. coli*, a member of *Enterobacteriaceae* family, is the most prevalent commensal inhabitant of the gastrointestinal tract of warm–blooded animals and humans (Fakhari *et al.* 2019). Nonetheless, due to its wide range of disease–causing potential, it is also known as one of the most prevalent pathogens for animals and humans (Singh *et al.* 2023) and can cause respiratory illnesses, including pneumonia (Jaureguy *et al.* 2008). Highly pathogenic strains of *E. coli* can significantly impair lung health with aspiration pneumonia, hospital–acquired pneumonia (HAP), ventilator–acquired pneumonia (VAP), and the exacerbation of chronic lung disorders (Malarkodi and Annadurai 2013). *E. coli* is also recognized as a leading cause of bloodstream infections and urinary tract infections (UTIs) with sepsis in both community and hospital settings worldwide. In the most parts of the world's hospitals and emergency departments, *E. coli* is the most commonly detected Gram–negative bacterium in blood and urine samples that have spread globally (Verma *et al.* 2022, Singh *et al.* 2023).

With the increasing resistance against almost all the recently developed antibiotics, the search for an alternate therapy is going on, including herbal extracts. The present study involved *Bacopa monnieri* (L.), widely known as "Brahmi", a creeping herb in the *Scrophulariaceae* family reported with bioactive molecules against uropathogens (Mehta *et al.* 2022). It has been used for ages in the Ayurvedic medicinal system as a brain tonic to improve memory, concentration, irritable bowel syndrome, asthma,

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bronchitis, gastric ulcers and to provide comfort to people suffering from epilepsy and anxiety (Dwivedi and Gopal 2010). With the advancement of nanotechnology, the development of nanoparticles has significantly contributed to combating AMR by utilizing materials engineered at the nanometre scale, which enables the assembly of numerous molecules to form an active interface. These nanomaterials primarily serve as delivery vehicles, with their design centred on controlling the timing and location of antibiotic release (Kurul *et al.* 2025) and display fundamentally new or enhanced qualities (Agarwal *et al.* 2017). With the growing demand for environmentally benign nanoparticles, scientists are synthesizing different metal nanoparticles for deployment in therapeutic uses by sustainable approaches (Albarakaty *et al.* 2023). More focus has been directed towards metal nanoparticles because of their unique characteristics, like catalytic, magnetic, optical, and electrical capabilities (Awwad *et al.* 2012). However, ZnONPs are adaptable substances for their antibacterial and antimicrobial activities (Barcella *et al.* 2016). Numerous researchers have reported the process of biosynthesis of ZnONPs and their utilization in various disciplines (Deepa *et al.* 2023). Based on these, the present study explains the process of green synthesis of ZnONPs using *B. monnieri* (L.), characterization of nanoparticles, and their antimicrobial assessment against *E. coli*, isolated from clinical samples.

MATERIALS AND METHODS

The present study involved collection of *Bacopa monnieri* (L.) plant leaves, green synthesis of ZnONPs using *B. monnieri* (L.), characterization of nanoparticles, and their antimicrobial assessment against *E. coli* isolated from clinical samples. The clinical samples submitted to pathology laboratory for the antibiotic sensitivity were used to isolate *E. coli.*

Green synthesis of ZnONPs: *B. monnieri* (L.) was collected from the herbal garden situated at SRM Instutite Science and Technology, Delhi NCR Campus, Ghaziabad, U.P., India. These were shade–dried for about 25–30 days and ground to a fine powder. About 10 gm of powder was soaked in 100 mL distilled water for 48 h and subsequently, filtered through Whatman filter paper no.1 and stored at 4°C (Messika *et al.* 2012). ZnONPs were prepared by dissolving 8.8 gm of zinc nitrate hexahydrate into 10 mL of plant extract and 60 mL of distilled water. This solution was stirred for 1 hour on a magnetic stirrer at 75°C. After one hour, the solution was titrated with 50 mL of 2M NaOH solution, causing a change in color of the solution from greenish to white and further, allowed to stir magnetically for 2 h. Then, the solution was centrifuged for 5 min at 7000 rpm, washed twice with 70% ethanol followed by distilled water (Osinska *et al.* 2023) to remove all the impurities from the synthesized nanoparticles. The precipitated nanoparticles were dried in hot air oven overnight at 100°C. After drying, the nanoparticles were meticulously ground into a fine powder and securely stored in an airtight container for subsequent characterization and utilization.

ZnONPs characterization: These phytogenic ZnONPs were characterized optically using a UV–VIS double beam spectrophotometer (Shields and Cathcart 2010). FTIR (Thermo Scientific Nicolet Summit X) was used for chemical characterization (Singh *et al.* 2011), and Transmission Electron Microscopy (TEM) was used for morphology study of synthesized nanoparticles (Parvekar *et al.* 2020).

E. coli isolation and characterization: 25 mucus samples were collected from the pathology laboratory and inoculated into the nutrient broth and incubated at 37°C for a duration of 24 h. After 24 h, the cultures were streaked onto Nutrient Agar Media (NAM) to obtain discrete colonies. The isolated colonies were subjected to Gram staining for morphological characterization and biochemical differentiation using MacConkey Agar differential test (Rajendra *et al.* 2010) and oxidase test (Trochimchuk *et al.* 2003) to confirm the *E. coli* isolates. Subsequently, DNA was isolated from all the isolates appearing to be *E. coli* using the PCI method and subjected to *E. coli* specific Uid A primer-based amplification (Russo and Johnson 2000).

Antimicrobial assay: The micro dilution method of minimum inhibitory concentration (MIC) was performed to assess the antimicrobial activity of synthesised ZnONPs against isolated *E. coli* bacteria. Precisely, the *E. coli* culture from the F_2 generation was suspended in saline (0.9%), where the turbidity was adjusted to 0.5 McFarland standard tubes. However, for preparing an antibiotic dilution for *E. coli* bacteria, the suspension was diluted to 1:100, containing 1×10^6 CFU/mL. A 96-well microtiter plate was used for analyzing the antimicrobial activity of synthesized ZnONPs (Sangeetha *et al.* 2011). Chloramphenicol was used as a standard antibiotic in the study with 2-fold dilution of concentrations in the first row (100 µg/mL to 0.19 µg/mL). The other rows of the plate contained a 3/4th fold dilution of ZnONPs ranging from 1000 µg/mL to 75 µg/mL. After 24 h of incubation of the microtiter plate, MIC values of ZnONPs were recorded using ELISA reader (Meril, India). Three sets of tests were performed to finalize the MIC of synthesized ZnONPs against *E. coli*.

RESULTS AND DISCUSSION

Characterization of ZnONPs: The synthesis of proper nanoparticles is key for its application. Hence, the synthesised zinc nanoparticles were characterized by three different methods. The phytogenically synthesized ZnONPs of *B. monnieri* (L.) showed a broad Surface Plasmon Resonance peak (SPR peak) at 323.25 nm due to the transition of electrons from the valence band to the conduction band, validating the synthesis and stability of nanoparticles (Bekele *et al.* 2024) [Fig. 1(a)]. The FTIR spectroscopy technique was used to identify the biomolecules responsible for reducing Zn^{++} to zinc oxide. The spectra were recorded at a wave number range of 4000–500 cm^{-1} [Fig. 1 (b)]. The bands at 3429.54 and 2345.28

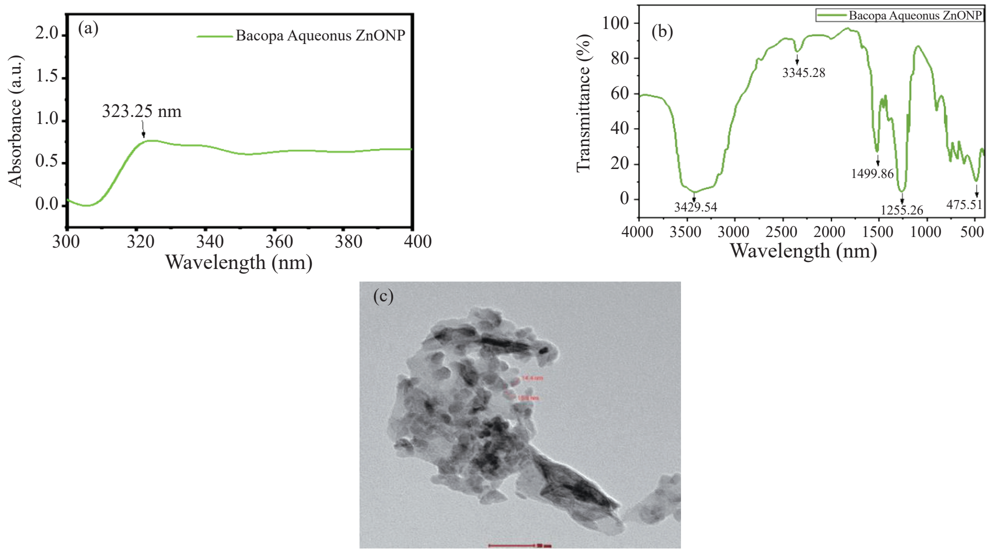

Fig. 1(a) Surface plasmon resonance peak of UV–VIS spectra, (b) FTIR spectra, and (c) TEM monograph of synthesized ZnONPs

represented the OH bend of water and the hydroxyl group. The band confirmed aromatic group C=C bonds at 1499.86 cm^{-1}. The intensive band at 1255.26 cm^{-1} represented the N–O stretch because of the presence of nitrate compound, while the absorption band at 475.51 cm^{-1} represented the 0=Zn=0 stretch (Shields and Cathcart 2010). The size and shape of these phytogenic ZnONPs were analyzed using the TEM and the result is shown in Fig. 1(c). It clearly displayed the spherical structure of ZnONPs with sizes ranging between 14.4 nm to 50 nm. The majority of the examined particles exhibited a nearly uniform size distribution. The use of plant extract in place of chemical agents like gelatin, ethylene glycol, and polyvinyl derivatives have been well established to reduce metal ions like gold, silver, copper etc (Ying *et al.* 2022).

The TEM analysis revealed the reducing and capping potential of synthesized ZnONPs (Singh *et al.* 2011). The green synthesis of ZnONPs reduces the cost of production, decreased pollution and improved the environmental, human and animal health safety and can be used safely in all cases (Singh *et al.* 2011, Ying *et al.* 2022).

Isolation and characterization of E. coli isolates: The identification of *E. coli is* usually done based on cultural, morphological, and biochemical tests. It is further confirmed by molecular detection. Gram staining was used for morphological examination and the bacteria appeared as small, straight, pink colour rods under the microscope as depicted in Fig. 2(a). These are in accordance with previously published report (Singhal *et al.* 2011). The MacConkey Agar (MLA) differential test used to detect the presence of *E. coli* revealed red or bright pink color colonies suggestive of lactose fermenting bacteria (Rajendra *et al.* 2010) [Fig. 2(b)]. Oxidase test was also utilized to determine the presence of cytochrome oxidase enzyme, that catalyzes and transports electrons between electron donors and the redox dye tetramethyl-p-phenylene-diamine (Trochimchuk

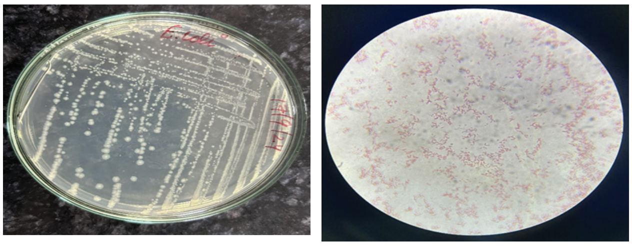

Fig. 2(a) Cultural and morphological characterization of *E. coli*

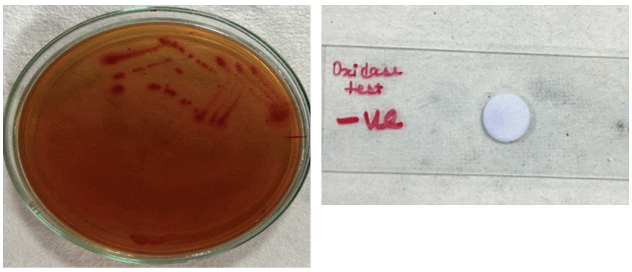

Fig. 2(b) Biochemical characterization of *E. coli*

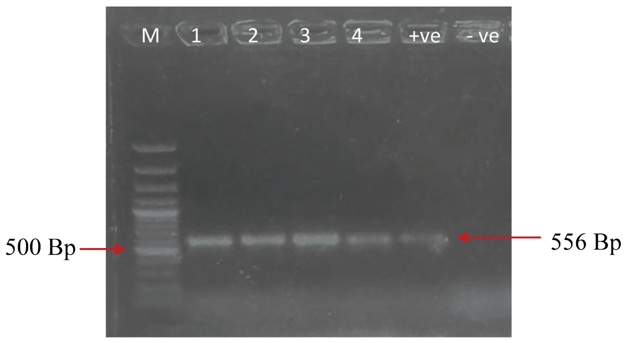

Fig. 2(c) Molecular characterization of *E. coli.* Electrophoretic bands of the amplified products of the four samples by a PCR using *uidA* gene primer in a 1% agarose gel. Lane 1: 100 bp ladder, Lane 2: sample 1, Lane 3: sample 2, Lane 4: sample 3, Lane 5: sample 4, Lane 6: Positive sample, Lane 7: Negative sample.

Table 1. MIC values for the antibiotic and ZnONPs against *E. coli*

Compound	Concentration (µg/mL)	Mean OD values		
		E. coli isolates	+ve control	–ve control
Standard antibiotic chloramphenicol (100–0.19 µg/mL)	100 µg/mL	0.0416±.002	0.6797	0.0312
	50 µg/mL	0.0394±.001		
	25 µg/mL	0.0442±.003		
	12.5 µg/mL	0.0471±0.007		
	6.25 µg/mL	0.0458±0.006		
	3.12 µg/mL	0.2077±0.013		
	1.56 µg/mL	0.3001±0.097		
	0.78 µg/mL	0.4387±0.101		
	0.39 µg/mL	0.5382±0.135		
	0.19 µg/mL	0.6191±0.107		
Bacopa monnieri (L.) ZnONPs (1000–75 µg/mL)	1000 µg/mL	1.9726±0.125	0.6989	0.057
	750 µg/mL	1.0363±0.098		
	562.5 µg/mL	0.6091±0.036		
	421.8 µg/mL	0.1758±0.083		
	316.4 µg/mL	0.1328±0.075		
	237.3 µg/mL	0.335±0.107		
	177.97 µg/mL	0.6216±0.132		
	133.4 µg/mL	0.7083±0.156		
	100 µg/mL	0.6289±0.141		
	75 µg/mL	0.6591±0.170		

et al. 2003). This test revealed negative results suggesting absence of cytochrome oxidase enzyme, a characteristic of Gram–negative bacteria [Fig. 2(b)]. All the isolates suggestive of *E. coli* based on cultural, morphological and biochemical examination were further confirmed by PCR using *E. coli* specific Uid primer as Uid A gene is a marker gene for bacteria and encodes beta–glucuronidase enzyme (Zhang *et al.* 2010). The PCR product was then run through an agarose gel by gel electrophoresis using a 100 bp ladder. Out of 25 mucous samples, 4 isolates were confirmed as *E. coli* as shown in Fig. 2(c).

Antimicrobial activity of green nanoparticles: The mean MIC for chloramphenicol antibiotic was observed to be 6.25 µg/mL, while for *B. monnieri* (L.) ZnONPs, it was 316.4 µg/mL (0.316 mg/mL) against clinical isolates of *E. coli*. The MIC values for the antibiotic and ZnONPs against *E. coli* at different concentrations are shown in Table 1, Fig. 3. This study attempted to explore the antimicrobial properties of ZnONPs produced through a green synthesis procedure utilizing *B. monnieri* (L.) extract, particularly in the context of *E. coli* isolated from clinical samples. The MIC of 316.4 µg/mL for green synthesized ZnONPs suggested a moderate but promising antimicrobial effect. The MIC of any nanoparticle also depends upon the metal and plant source, type of bacteria, size and shape of nanoparticles (Rezk *et al.* 2022). Different plants have different level of antibacterial activities depending upon their phytoconstituents (Sharma *et al.* 2014). Moreover, source of bacteria and its drug resistance pattern also determines the sensitivity of any bacterial isolate (Malik *et al.* 2013, Anita *et al.* 2014). Even though this MIC value is comparatively higher, even the green nanoparticles offer significant benefits regarding biocompatibility, eco-friendliness, and functional surface modifications derived from plant–based phytochemicals.

The higher MIC value could also be attributed to a variety

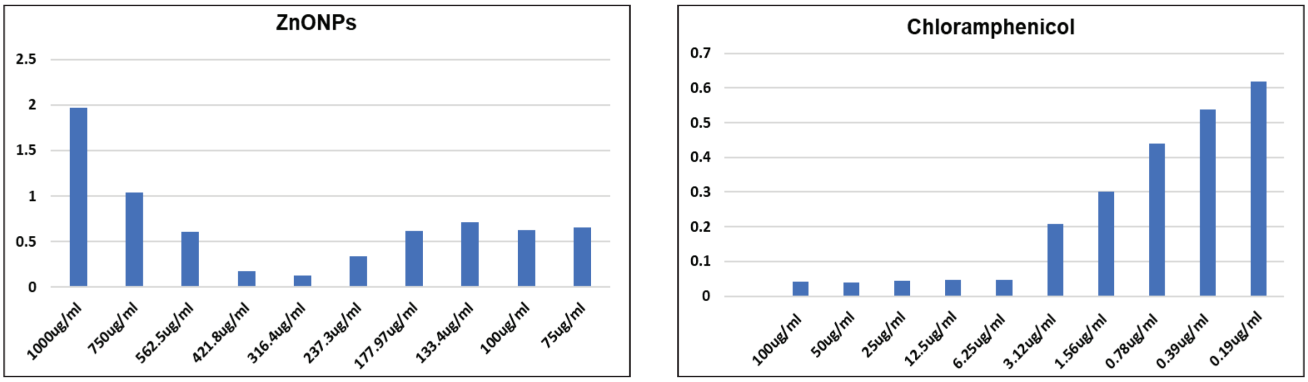

Fig. 3. Mean MIC values of antibiotic (chloramphenicol) and ZnONPs against *E. coli*

of factors, including the particle size, surface chemistry, and crystallinity of ZnONPs, which are influenced by the composition of *B. monnieri* (L.) extract (Raghupathi *et al.* 2011).The existence of phytochemicals like terpenoids, bacosides, and flavonoids could have played a role in the nucleation and growth of nanoparticles, potentially impacting their surface charge and bioavailability (Paut *et al.* 2024). Despite this MIC being comparatively higher, the biosynthesized ZnONPs demonstrated a distinct dose–dependent antimicrobial activity, which is promising for applications required for a desired gradual and sustained antimicrobial effect. In comparison, chemically synthesized ZnONPs have been noted to demonstrate lower MIC values as low as 50 µg/mL – 100 µg/mL (Raghupathi *et al.* 2011) against *E. coli* bacteria. However, the process frequently utilizes toxic reagents or stabilizers that are not compatible with food–related and biomedical applications. Conversely, employing *B. monnieri* (L.) extract offers a naturally sourced capping and reducing environment, which diminishes environmental toxicity while preserving antimicrobial effectiveness. The mechanism by which ZnONPs exhibit antimicrobial properties is mainly linked to the generation of reactive oxygen species (ROS), alteration of membrane permeability, and liberation of Zn^{++} ions, which disrupt bacterial nucleic acids and enzyme systems. These zinc nanoparticles' environmentally friendly synthesis and their dose dependent antimicrobial potency highlight their potential as biocompatible antimicrobial agents. Further, optimizing synthesis parameters like nanoparticle size, morphology, and surface functionality could potentially lower MIC values while boosting their applications in biomedical science.

This study investigated the reducing and stabilizing capabilities of *Bacopa monnieri* (L.) aqueous extract to synthesize ZnONPs. A green approach was used in the biogenesis of ZnONPs, which is an eco–friendly and cost–effective method that provides biocompatible zinc nanoparticles. The biogenesis of ZnONPs was confirmed by UV–VIS spectrophotometry, FTIR spectroscopy, and TEM. The antimicrobial potential of phytogenically synthesised biogenic ZnONPs of *B. monnieri* (L.) was tested against *E. coli*, isolated from clinical samples. The dose dependent antibacterial activity was confirmed with 316.4 µg/mL as the effective bactericidal concentration of *B. monnieri* (L.) ZnONPs against *E. coli* isolates. Based on the findings, it can be concluded that *B. monnieri* (L.) ZnONPs has moderate antibacterial activities. However, optimization of synthesis method for improvement of its dose along with *in vitro* as well as *in vivo* toxicity studies on those doses are further required to recommend it as novel therapeutic agent in the treatment of various bacterial diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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