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Eco-friendly one pot synthesis of zinc oxide nanoparticles using catkin extract of *Piper longum*: *In-vitro* antibacterial, antioxidant and antibiofilm potential against multi drug resistant enteroaggregative *E. coli* †

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Abstract: Enteroaggregative *Escherichia coli* (EAEC) is a neglected, however emerging bacterial pathogen associated with gastrointestinal illnesses world-wide. Recently, a surprising surge in the multi-drug resistance pattern among EAEC strains has been observed on a global scale; hence the emphasis has been given to adjuvant therapies to combat this nagging public health threat. This study assessed the antibacterial efficacy of ZnO NPs synthesised using the aqueous extract of *Piper longum* catkin against multi-drug resistant (MDR) strains of EAEC. Initially, the synthesis of ZnO NPs was confirmed by UV- Vis spectroscopy and Fourier transform infra- red spectroscopy (FTIR) analysis. The thermal stability of ZnO NPs was evidenced by TGA/DTA, while PXRD analysis revealed a hexagonal wurtzite crystalline structure, which was then confirmed by electron microscopy. The minimum inhibitory concentration as well as minimum bactericidal concentration of biofabricated ZnO NPs determined by microbroth dilution technique against MDR-EAEC (n= 3) strains revealed 125 µg/mL and 250 µg/mL, respectively. In addition, ZnO NPs were tested variably stable and safe. The green synthesised ZnO NPs exhibited a concentration dependent antioxidant activity and inhibited the biofilm forming ability of the tested MDR-EAEC strains. Overall, this study revealed an eco-friendly one-pot synthesis of ZnO NPs, which could be used as a fruitful antimicrobial substitute against MDR-EAEC strains.

Keywords: Antimicrobial resistance; Enteroaggregative *Escherichia coli*; Green synthesis; Nanoparticle; Zinc Oxide

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1. Introduction

Antimicrobial resistance (AMR) has been emerged as a nagging public health menace globally. Failure of conventional antibiotic therapies along with the drastic decline in antibiotic discovery pipeline would jeopardize the socioeconomic development and may even lead to untoward scenario [1]. Of late, an unusual rise of drug resistance among EAEC strains has been recognized worldwide; hence the AMR research paradigm should be shifted towards novel alternative tactics.

Recently, nanotechnology employing ZnO NPs have attained huge recognition due to their unique physicochemical features and tremendous scope for application in the field of biomedicine. The green route of NP synthesis has replaced the conventional physical and chemical methods, as they produce unintended effects such as potential health hazards and environmental pollutions [2].

Piper longum (Family *Piperaceae*), commonly known as "long-pepper" or "Pippali" is a well-known perineal shrub widely distributed in the tropical and subtropical world including India. The extract of *Piper longum* is a rich source of various bio active phytoconstituents including alkaloids, flavonoids steroids and esters and possess excellent antibacterial, antioxidant and anti-inflammatory properties [3]. The synthesis of AgNPs using catkin extract of *P. longum* was previously documented [4] but not ZnO NPs. Considering these facts, the present study was attempted to synthesize ZnO NPs using *P. longum* catkin extract and further to estimate the *in vitro* antimicrobial, antioxidant and antibiofilm activity against MDR-EAEC strains.

2.1. Bacterial strains

The characterized MDR field strains of EAEC (E1; E2; E3) retained in the laboratory repository of College of Veterinary and Animal Sciences, Pookode were reaffirmed using antimicrobial susceptibility testing [5] and PCR assays [6]. The quality control strain used for the antimicrobial susceptibility testing in this study was ***E. coli*** ATCC 25922.

2.2. Preparation of Piper longum catkin extract

The powdered catkin of *P. longum* (10 g) was added to 100 ml of nanopure water which was then heated to 60◦C for 1 h; using Whatman No. 1 filter paper, the solid particulates were removed and the filtrate was stored at 4◦C until the synthesis of ZnO NPs [7].

2.3. Synthesis of ZnO NPs

The aqueous extract of *P. longum* catkin and zinc acetate dihydrate (0.10 M) solution was used for the green synthesis of ZnO NPs. In short, *P. longum* catkin filtrate (20 ml) was mixed with zinc acetate dihydrate solution (80 ml) for 2 at 60◦C using a magnetic stirrer (300 rpm). The colour of the reaction mixture changed from colorless to light brown, demonstrating the formation of ZnO NPs. To remove impurities, the ZnO NPs were washed three times with methanol, followed by nanopure water. Finally, the ZnO NPs were air dried overnight at 80 ◦C and stored at 4 ◦C until further use [7].

2.4. Characterization of green synthesized ZnO NPs

The characterization of green synthesized ZnO NPs was accomplished by UV-Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), Thermogravimetric analysis (TGA) and differential thermal analysis (DTA), powder X-ray diffraction (PXRD), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). To begin with, the ZnO NPs dissolved in ultrapure water (1 mg/ml) was scanned using a UV- Vis spectrophotometer (ThermoFisher Scientific, USA) within a range of 250 to 450 nm. In order to assess the functional groups present on the green synthesized ZnO NPs, FTIR analysis was carried out at a resolution of 4 cm^{-1} within the range of 4000 to 400 cm^{-1} (Perkin Elmer C94012, USA). Approximately 12.473 mg of samples were heated at a rate of 40 ◦C per min in a nitrogen atmosphere between 40 and 1300 ◦C to accomplish the TGA- DTA of ZnO NPs (Perkin Elmer STA 600, USA).

The structural properties of the green synthesized ZnO NPs was investigated by means of PXRD (Bruker D8 Advance, USA) operated with a scanning step size of 0.02λ (λ= 1.54060 Å) at CuKα radiation using 40 KeV and 40 mA. The high-resolution TEM (HR-TEM; JEM 2100, Jeol, Japan) and SEM (Jeol 6390LV, Japan) analysis of the samples were used to perform the morphological analysis of ZnO NPs [7].

2.5. In vitro antibacterial efficacy of ZnO NPs

The antimicrobial potency of green synthesized ZnO NPs against the field strains of MDR-EAEC was assessed *in vitro* by evaluating the MIC and MBC values employing micro broth dilution method [4].

2.6. In vitro stability assays of ZnO NPs

The stability of green synthesized ZnO NPs using the catkin extract of *P. longum* was evaluated by subjecting them to high-end temperatures (70◦C and 90◦C), proteases (trypsin, lysozyme, and proteinase- K), the physiological concentration of cationic salts (150 mM NaCl and 2 mM MgCl2) as well as varying pH (4.0, 6.0, and 8.0) [8].

2.7. In vitro safety assays of ZnO NPs

A haemolytic assay employing chicken erythrocytes as well as cytotoxicity assay using human epithelial embryonic kidney (HEK) cell lines, and effect on beneficial gut lactobacilli (*L. acidophilus* MTCC 10,307 and *L. plantarum* MTCC 5690) was determined to investigate the *in vitro* safety of green synthesized ZnO NPs [8].

2.8. In vitro antioxidant activity of ZnO NPs

By using the reducing power assay and the 2,2′- azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+)- free radical scavenging test, the *in vitro* antioxidant property of green synthesized ZnO NPs was evaluated [9].

2.9. In vitro antibiofilm efficacy of ZnO NPs

By using the crystal violet staining technique in 96-well microtiter plates at 24 and 48 h, the ***in vitro*** antibiofilm efficacy of green synthesized ZnO NPs against the investigated strains of MDR-EAEC was evaluated [10].

3. Results and discussion

3.1. Green synthesis of ZnO NPs

Currently, the physical and chemical methods of nanoparticle synthesis are gradually being swapped by green routes employing natural and eco-friendly resources owing to the release of toxic chemicals and complexities in synthesis conditions [2]. Being a rich source of pharmacologically active secondary compounds, *P. longum* has been widely used in ayurvedic preparations for various ailments [11].

The synthesis of ZnO NPs was accomplished by employing the *P. longum* catkin extract which reduced the aqueous solution of zinc acetate dihydrate (0.10 M; 1:4 ratio) to ZnO NPs under vigorous stirring at 60 ◦C. After 2 h, the colour of the solution changed from colorless to brown with the appearance of a brown-colored precipitate at the bottom clearly indicating the formation of ZnO NPs.

The occurrence of various functional groups as well as the stabilizing and capping properties of the aqueous *P. longum* catkin extract plays a crucial role in the synthesis of ZnO NPs. In addition, the excitation of surface plasmon resonance (SPR) and reduction of Zn ion by the extract might have led to the colour change during ZnO NP synthesis [7].

3.2. Characterization of green synthesized ZnO NPs

To begin with, the synthesis of ZnO NPs was confirmed by performing UV−Vis spectroscopy (Figure 1). The obtained green synthesised ZnO NPs shown a progressive SPR band flanked by 320 nm to 350 nm with a maximum absorption peak at 340 nm which was in accordance with previous literatures [12,13]. In this study, FTIR peaks were observed at 3640 cm^{-1}, 2850 cm^{-1}, 2100 cm^{-1}, 1739 cm^{-1}, 1490 cm^{-1}, 870 cm^{-1}, 915 cm^{-1} and 620 cm^{-1}. The presence of phytochemical components associated to the biosynthesized ZnO NPs in the extract was confirmed by all of these detected peaks. Moreover, the TGA data demonstrated an initial weight loss of about 6% from 40°C to 100°C, which was corroborated by the DTG graph, with an exothermic peak seen at 200°C (Figure 1). The weight of the green synthesized ZnO NPs had been steadily decreasing as a result of this rise in annealing temperature. Additionally, annealing temperatures between 900°C and 1300°C showed satisfactory thermal stability.

The PXRD graph obtained in the present study was juxtaposed with the Joint Committee on Powder Diffraction Standards (JCPDS) standard powder diffraction card of ZnO (File No. 36-1451). As shown in Fig. 1, the lattice planes (100), (002), (101), (102), (110), (103), (200), and (202) corresponded to the 2θ values of 31.85◦, 34.50◦, 37.1◦, 51◦, 59◦, 64◦, 67.1◦ and 77◦, respectively [14]. The intense and sharp peaks revealed the hexagonal wurtzite crystalline structure of the green synthesized ZnO NPs [7].

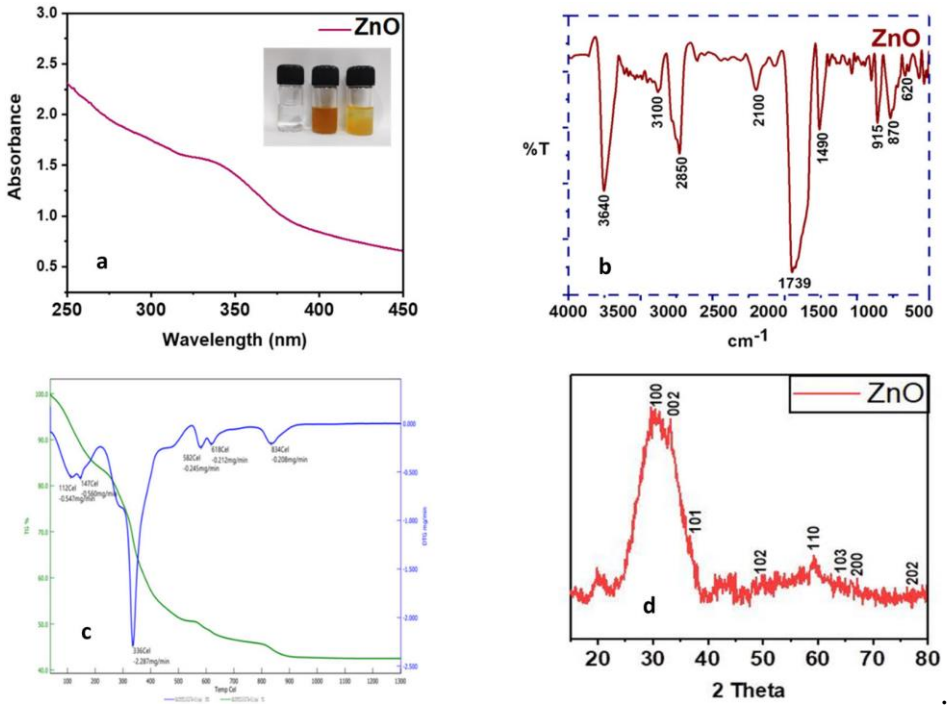


Figure 1. Characterization of green synthesised ZnO NPs [7].

Moreover, the SEM image showed primarily hexagonal shaped ZnO NPs and few in the form of cubes. Additionally, the TEM images confirmed the hexagonal shaped ZnO NPs thereby supporting the results of SEM (Figure 3). The presence of reducing agents in the catkin extract could be the mian reason for the agglomeration and morphological differences of the biosynthesized ZnO NPs [2].

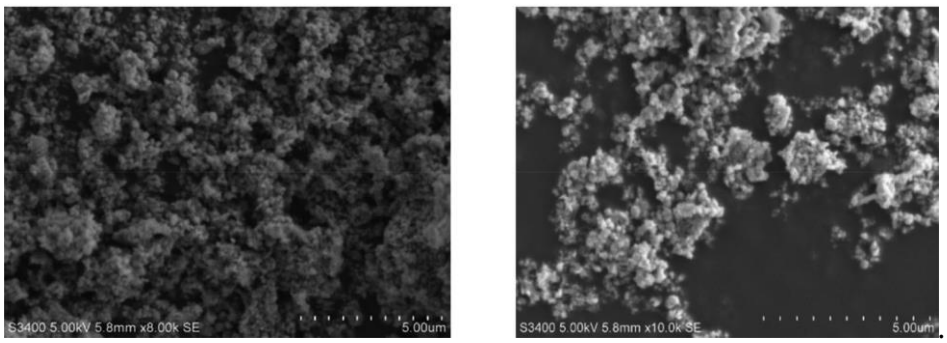


Figure 2. Scanning Electron microscopy of green synthesized ZnO NPs [7].

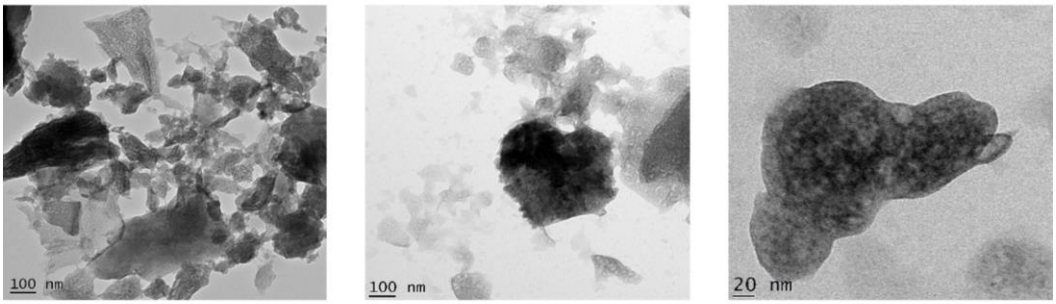


Figure 3. Transmission Electron microscopy of green synthesized ZnO NPs [7].

3.3. In vitro antibacterial activity of ZnO NPs

In this study, the anti-bacterial efficacy (MIC and MBC values) of the green synthesized ZnO NPs against the MDR- EAEC test strains were found to be 125 µg/ml and 250 µg/ml, correspondingly (Table 1). A relatively lower MIC and MBC values of ZnO NPs in this study might be due to the lipopolysaccharide present in the Gram-negative bacterial cell wall which exerted the resistance and subsequent aversion towards NPs [7].

Table 1. MIC and MBC of ZnO NPs synthesized from the extract of *P. longum* catkin against MDR-EAEC strains.

Isolates.		**MIC/MBC (µg/mL)**
EAEC	E1	125/250
	E2	125/250
	E3	125/250

3.4. In vitro stability assays of ZnO NPs

The green synthesized ZnO NPs were investigated for their stability to high-end temperatures (70 °C and 90 °C), proteases (trypsin, lysozyme and proteinase-K), the physiological concentration of cationic salts (150 mM NaCl and 2 mM $MgCl_2$) and pH (4.0, 6.0, and 8.0). All the strains exhibited a constant MIC value when incubated at 70◦C and 90◦C. The MBC values remained unchanged until 5 min at both temperatures; nevertheless, a 2-fold rise in MBC value was observed thereafter (Table 2). Overall, the green synthesized ZnO NPs were found to be variably stable and capable of withstanding high-end temperatures [15].

Table 2. Effect of temperatures on the *in vitro* stability of green synthesized ZnO NPs.

	MIC/MBC (µg/mL)					
Isolates	**70◦C**			**90◦C**		
	5 min	**15 min**	**30 min**	**5 min**	**15 min**	**30 min**
E1	125/250	125/500	125/500	125/250	125/500	125/500
E2	125/250	125/500	125/500	125/250	125/750	125/500
E3	125/250	125/500	125/500	125/250	125/500	125/500

The residual antimicrobial activity of green synthesized ZnO NPs on exposure to protease enzymes (trypsin, lysozyme and proteinase-K) at different incubation intervals is green synthesized ZnO NPs were also investigated. Remarkably, the MIC values of green synthesized ZnO NPs were halved on exposure to protease enzymes, and MBC

values remained constant throughout the exposure period, except certain conditions where it reduced to half (Table 3)

Table 3. *In vitro* stability of green synthesized ZnO NPs on exposure to trypsin, lysozyme, and proteinase- K.

Isolates	MIC/MBC (µg/mL)											
	Proteinase-k				Lysozyme				Trypsin			
	30 sec	5 min	15 min	30 min	30 sec	5 min	15 min	30 min	30 sec	5 min	15 min	30 min
E1	62.5/250	62.5/250	62.5/250	62.5/250	62.5/250	62.5/250	62.5/250	62.5/125	62.5/250	62.5/125	62.5/125	62.5/250
E2	62.5/250	62.5/250	62.5/250	62.5/250	62.5/125	62.5/250	62.5/250	62.5/125	62.5/250	62.5/250	62.5/125	62.5/250
E3	62.5/250	62.5/250	62.5/250	62.5/250	62.5/250	62.5/250	62.5/250	62.5/125	62.5/250	62.5/125	62.5/125	62.5/250

The fate of NPs is greatly affected by their interaction with the biological fluids. Hence, in the present study, the stability of green synthesized ZnO NPs against physiological concentration of cationic salts were also evaluated. The green synthesized ZnO NPs maintained their antibacterial activity (MIC and MBC values) throughout the incubation period irrespective of the cationic salts (Table 4)

Table 4. *In vitro* stability of green synthesized ZnO NPs on exposure to the physiological concentration of cat ionic salts (150 m*M* NaCl and 2m*M* MgCl2).

Isolates	MIC/MBC (µg/mL)	
	NaCl (150 mM)	$MgCl_2$ (2 m*M*)
E1	125/250	125/250
E2	125/250	125/250
E3	125/250	125/250

An modification in the physicochemical conditions like pH and ionic strength of the solution can affect the intrinsic properties of NPs, such as their size, stability, zeta potential, morphology, and shape of the synthesized NPs [16]. Therefore, the stability of green synthesized ZnO NPs at varying pH (4,6,8) was estimated by determining their antimicrobial activity against the MDR-EAEC strains. The green synthesized ZnO NPs tested were found to be stable at different pH, as they retained their antimicrobial activity (MIC and MBC values). Notably, the MIC value of ZnO NPs was reduced to half at pH 8 as demonstrated by their MIC and MBC values (Table 5)

Table 5. *In vitro* stability of green synthesized ZnO NPs on exposure to different pH (four, six, eight).

Isolates	MIC/MBC (µg/mL)		
	pH : 4	pH : 6	pH : 8
E1	125/250	125/250	62.5/250
E2	125/250	125/250	62.5/250
E3	125/250	125/250	62.5/250

3.4. In vitro safety assays of ZnO NPs

The in vitro haemolytic assay is often employed as a versatile tool for carrying out the initial toxicity assessment of a therapeutic compound [17]. In the present study, minimal haemolysis (less than 2% was noticed for the green synthesized ZnO NPs at 2X, 5Xand 10X MIC levels (Figure 4, Table 6). Similarly, the in vitro cytotoxicity effect of green synthesized ZnO NPs on the viability of HEK cell lines was evaluated using the MTT assay. Overall, the green synthesized ZnO NPs did not exhibit any cytopathic effect at 1X, 5X and 10X MIC levels.

Table 6. In vitro haemolytic activity of green synthesized ZnO NPs on poultry RBCs.

Concentration of ZnO NPs.	Haemolysis (%)
MIC (1X)	1.04
MIC (5X)	1.23
MIC (10X)	1.34

Figure 4. In vitro haemolytic activity of green synthesized ZnO NPs on poultry RBCs.

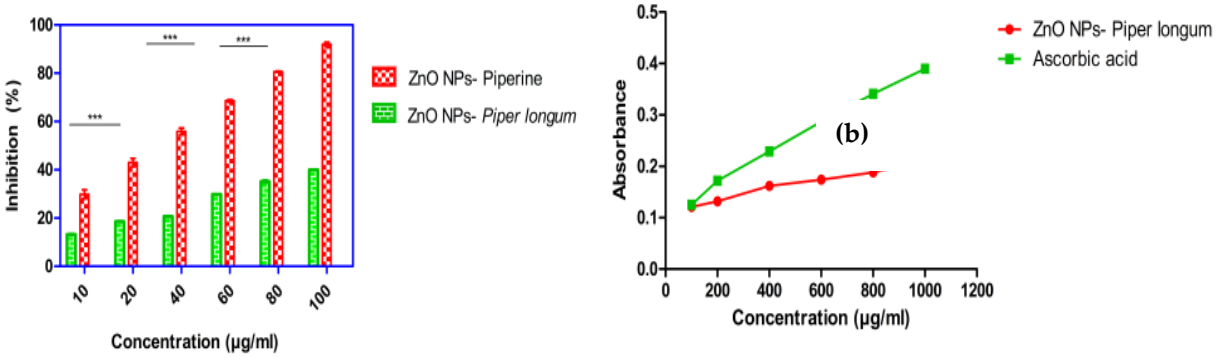
Furthermore, the investigation of its effect on beneficial gut microflora is also important as they form an vital part of the body's innate immune system [18]. In this study, L. acidophilus and L. plantarum revealed similar growth patterns in both treatment control (treated with green synthesized ZnO NPs) as well as the untreated control. Moreover, a non-significant (P> 0.05) antimicrobial efficacy was observed for the green synthesized ZnO NPs against the tested strains of L. acidophilus and L. plantarum (Figure 5).

Figure 5. In vitro efficacy of green synthesized ZnO NPs on *L. acidophilus* MTCC 10,307 (a); *L. plantarum* MTCC 5690 (b); PC: Positive control, NC: Negative control.

3.4. In vitro antioxidant activity of ZnO NPs

Antioxidants play a vital role in the functioning of all the biological systems due to their importance in scavenging the noxious free radicals produced in the body, and thus pre- venting oxidative stress. Hence the antioxidant scavenging activity of green synthesized ZnO NPs were evaluated by employing ABTS and reducing power assay, keeping ascor- bic acid as standard. In this study, a dose-dependent increase in their antioxidant properties was revealed by ABTS (Figure 6a) and reducing power assays (Figure 6b) of green synthesized ZnO NPs suggesting an enhanced ability to scavenge free radicals.

Figure 6. *In vitro* antioxidant activity of green synthesized ZnO NPs (a) ABTS assay (b) Reducing power assay**.**



3.4. In vitro antibiofilm activity of ZnO NPs

Owing to the potential antibacterial property of ZnO NPs, it could be employed as a versatile tool for the treatment of bacterial biofilms. In this study, we investigated the abiity of green synthesized ZnO NPs to inhibit the biofilm forming ability of MDR-EAEC isolates both at 24 and 48 h employing crystal violet staining assay. All the MDR-EAEC isolates treated with green synthesized ZnO NPs exhibited a significant antibiofilm effect after 24 h and 48 h ($P< 0.001$) relative to their respective controls (Figure 7).

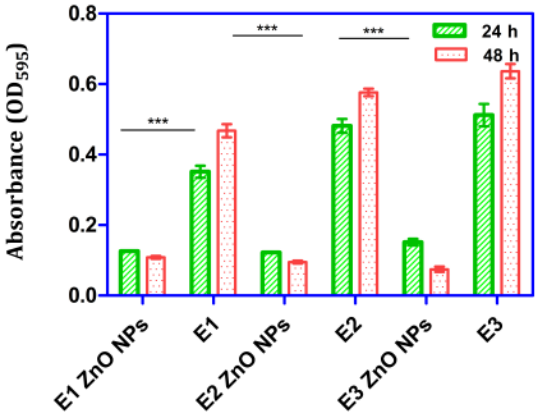


Figure 7. Inhibition of MDR-EAEC biofilm at 24 h and 48 h when treated with green synthesized ZnO NPs.

4. Conclusion

In short, we successfully synthesized ZnO NPs employing the aqueous extract of *Piper longum* catkin. The green synthesized ZnO NPs were characterized by spectroscopy, XRD, TGA, and electron microscopy. Antibacterial activity of the ZnO NPs were exhibited by the micro broth dilution technique against MDR -EAEC strains. The *in vitro* assays revealed that the synthesized NPS were variably stable, safe and possess excellent antioxidant and antibiofilm activity. Overall, the study demonstrated an eco-friendly and safe approach for NP synthesis which further highlights the significance of these particles as a suitable candidate for treating drug resistant pathogens. However, in vivo clinical studies should also be performed to validate the application of ZnO NPs in suitable target hosts.

Author Contributions: Conceptualization: [Jess Vergis; Deepak Bhiwa Rawool; Nitin VKurkure; Sukhadeo B Barbuddhe]; Methodology: [Varsha Unni;Abishad Padikkamannil; Jess Vergis]; Formal analysis, visualization,and investigation: [Varsha Unni; Abishad Padikkamannil; Lijo John;Prejit Nambiar; Sanis Juliet; C. Latha; Bibin Mohan; Jess Vergis]; Validation: [VarshaUnni; Abishad Padikkamannil; Vemula Prasastha Ram; Niveditha Pollumahanti; Jyothsana Yasur]; Writing - original draft preparation:[Varsha Unni; Abishad Padikkamannil; Vemula Prasastha Ram;Niveditha Pollumahanti; Jyothsana Yasur]; Writing - review and editing:[Jess Vergis; Deepak Bhiwa Rawool; Sukhadeo B Barbuddhe];Funding acquisition: [Deepak Bhiwa Rawool; Jess Vergis; NitinV Kurkure; Sukhadeo B Barbuddhe]; Resources: [Jess Vergis; Deepak bhiwa Rawool]; Supervision: [Jess Vergis; Deepak Bhiwa Rawool].

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