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Proceeding Paper

Antioxidant, Antibacterial and Antibiofilm Potential of Green Synthesized Silver-Zinc Oxide Nanocomposites from *Curcuma longa* Extract against Multi-Drug Resistant Enteroaggregative *E. coli* †

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Abstract: Enteroaggregative *Escherichia coli* (EAEC) has widely been recognized as one of the leading causes of infantile diarrhoea and nutrient malabsorption in developing as well as developed countries. In recent times, drug resistance, particularly multi-drug resistance (MDR) among the EAEC strains has widely been documented and would result in a therapeutic stumble; antimicrobial alternatives are employed widely to curb this emerging public health menace. In the present study, a facile one-pot synthesis of silver/zinc oxide nanocomposites (Ag/ZnO NCs) using methanolic extract of stem and leaves of *Curcuma longa* was performed. The synthesis of Ag/ZnO NCs was confirmed using UV-Vis spectroscopy and Fourier transform infrared spectroscopy, while the thermal stability was ascertained by thermogravimetric analysis with differential thermogravimetric analysis, whereas crystallinity was determined using Powder X-ray diffraction. The shape and size of the green synthesized Ag/ZnO NCs determined using field emission-scanning electron microscopy and transmission electron microscopy revealed an irregular polycrystalline morphology with a size of 31.34 ± 1.27 nm. Later, the antibacterial potential of the green synthesized Ag/ZnO NCs evaluated against MDR- EAEC strains revealed a minimum inhibitory concentration of 31.25 μg/mL and a minimum bactericidal concentration ranging from 62.50 to 125 μg/mL. Moreover, the green synthesized Ag/ZnO NCs inhibited the biofilm-forming ability of the tested strains of MDR-EAEC. Besides, concentration-dependent antioxidant activity was exhibited by the green synthesized Ag/ZnO NCs as evidenced by ABTS assay and reducing power assay. Overall, this study demonstrated the antibacterial potential of Ag/ZnO NCs synthesized using *C. longa* extracts with antifouling as well as antioxidant properties, which could therefore be used as an alternative therapeutic candidate.

Keywords: Ag/ZnO NCs; Antimicrobial resistance; *Curcuma longa*; Enteroaggregative *Escherichiacoli*; Green synthesis

1. Introduction

Antimicrobial resistance (AMR) is a significant global public health threat in recent times [1]. Of late, enteroaggregative *E. coli* (EAEC) has recently been recognized as one of the major emerging enteric pathogens owing to its increased recovery from diarrhoeal episodes around the world [2]. Furthermore, the dissemination of multi-drug resistant (MDR)-EAEC pathotypes has closely been linked to morbidity, case fatality and healthcare expenses [3]. In this context, there is an urgent need to look into alternatives to traditional antibiotics since they are becoming less effective in curing drug-resistant infections [4].

Recently, nanotechnology using metal or metal oxide nanoparticles (NPs) has been promoted as a viable alternative strategy for combating AMR [5]. In general, the antibacterial properties of silver (Ag) NPs against a wide range of drug-resistant pathogens have been well-known in recent years [6]. Similarly, zinc oxide (ZnO) NPs have received recent attention, since it has been designated as 'generally regarded as safe' (GRAS) by U.S. Food and Drug Administration (US-FDA) and are known to possess characteristic anti-inflammatory, antibacterial, antifungal, antidiabetic, photocatalytic properties [7]. A combination of Ag and ZnO forming Ag/ZnO nanocomposites (NCs) heterostructure with the synergistic generation of reactive oxygen species (ROS), has recently been recognized as a broad-spectrum antibacterial agent in tackling drug-resistant infections [8].

Green synthesis of NPs has currently received a lot of attention since these NPs are easier to produce, biodegradable, economical and eco-friendly when compared to the NPs synthesized by conventional physical and chemical methods [9]. Since time immemorial, *C. longa*, a perennial rhizomatous plant belonging to the Zingiberaceae family has been reported as an integral component of Asian and has widely been used as a food preservative and colouring agent in culinary dishes [10]. In this regard, the present study is envisaged with an objective to evaluate the antibacterial and anti-biofilm potential of green synthesized Ag/ZnO NCs using methanolic extract of stem and leaves of *C. longa* against MDR-EAEC pathogens and to explore the antioxidant property.

2. Materials and Methods

2.1. Bacterial Strains

Antimicrobial susceptibility testing [11] and PCR assays [12] were used to revalidate the MDR field strains of EAEC (E1; E2; E3). *E. coli* ATCC 25922 served as quality control strain.

2.2. Preparation of C. longa Methanolic Extract

The *C. longa* leaves and stems obtained from a turmeric plantation near the university campus (11º33′02.8″N; 76º01′46.1″E) were thoroughly washed and finely ground using a mortar and pestle. The ground material (100 g) was filtered using Whatman No. 1 filter paper after being soaked in methanol (Loba Chemie Pvt. Ltd., India) overnight. The extract thus obtained (100 mL) was kept at 4°C and employed for the green synthesis of Ag/ZnO NCs.

2.3. Green Synthesis of Ag/ZnO NCs

A 2:3 mixture of 4 parts of silver nitrate solution (0.1 M) and zinc nitrate dihydrate solution (0.1 M) and one part of the methanolic extract of *C. longa* were for the green synthesis of Ag/ZnO NCs and was further subjected to characterization.

2.4. In Vitro Antibacterial Activity of Ag/ZnO NCs

In order to assess the in vitro antibacterial efficacy of green synthesized Ag/ZnO NCs against the MDR field isolates of EAEC, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by micro broth dilution technique [11].

2.5. In vitro Antioxidant Activity of Ag/ZnO NCs

The in vitro antioxidant activity of green synthesized Ag/ZnO NCs were compared using the reducing power assay and an ABTS•+ (2,2′- azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-based free radical scavenging assay [13].

2.6. In Vitro Antibiofilm Efficacy

The in vitro antibiofilm efficacy of green synthesised Ag/ZnO NCs was determined by performing a crystal violet assay against the MDR-EAEC strains in 96-well microtiter plates at 24 and 48 h [14].

3. Results and Discussion

3.1. Green Synthesis of Ag/ZnO NCs

The methanolic extract of C. longa was used in the present study to successfully synthesize Ag/ZnO NCs under continual stirring at 25 ºC for 12 *h*. A brown precipitate formed at the bottom of the conical flask indicated that Ag/ZnO NCs had been synthesized, and UV-Vis spectroscopy confirmed the synthesis

3.2. Characterization of Green Synthesised Ag/ZnO NCs

Ag/ZnO NCs exhibited surface plasmon resonance (SPR) bands at 293 nm, 340 nm, and 450 nm (Figure 1). These SPR peaks could be the result of interphase interactions between nearby colloidal NPs caused by electromagnetic coupling between Ag and ZnO [15]. When compared to the reference chart, the FTIR spectra of the greenly synthesised Ag/ZnO NCs showed distinctive peaks at 520 cm^{-1}, 730 cm^{-1}, 1020 cm^{-1}, 1380 cm^{-1}, 1620 cm^{-1}, 2920 cm^{-1}, and 3420 cm^{-1} (Figure 1). An initial weight loss of around 4% from 40°C to 100°C was exhibited by the green synthesised Ag/ZnO NCs, which was further confirmed by the DTG graph (Figure 1). This weight loss may have been caused by the loss of water molecules linked to the green synthesised Ag/ZnO NCs [4] with an exothermic peak observed at 97°C. Furthermore, progressive thermal degradation of green synthesised Ag/ZnO NCs between 200 °C and 450 °C with an exothermic peak at 320 °C was noted. The weight of the green-synthesised Ag/ZnO NCs has steadily decreased as the annealing temperature has increased, leaving 65% of the sample at the endpoint temperature and undermining the thermal stability.

The three diffraction peaks exhibited by the green synthesised Ag/ZnO NCs on PXRD analysis at 32.4°, 46.4°, and 57.5° exactly matched with (100), (102), and (110) planes of ZnO, while the standard diffraction peaks shown in the PXRD at 38.1°, 44.3°, 64.8°, and 77.7° belonged to (111), (200), (220), and (311) planes of pure Ag, respectively (Figure 1). Thus, the investigated sample validated the face-centred cubic structure of Ag NPs (JCPDS Card No. 89-3722) and hexagonal wurtzite structure with $P6_3$ mc symmetry of ZnO NPs (JCPDS Card No. 00-036-1451) [16]. Additionally, using the Debye-Scherrer formula, the Ag/ZnO NCs′ average crystallite size was estimated to be 15.525 nm [17].

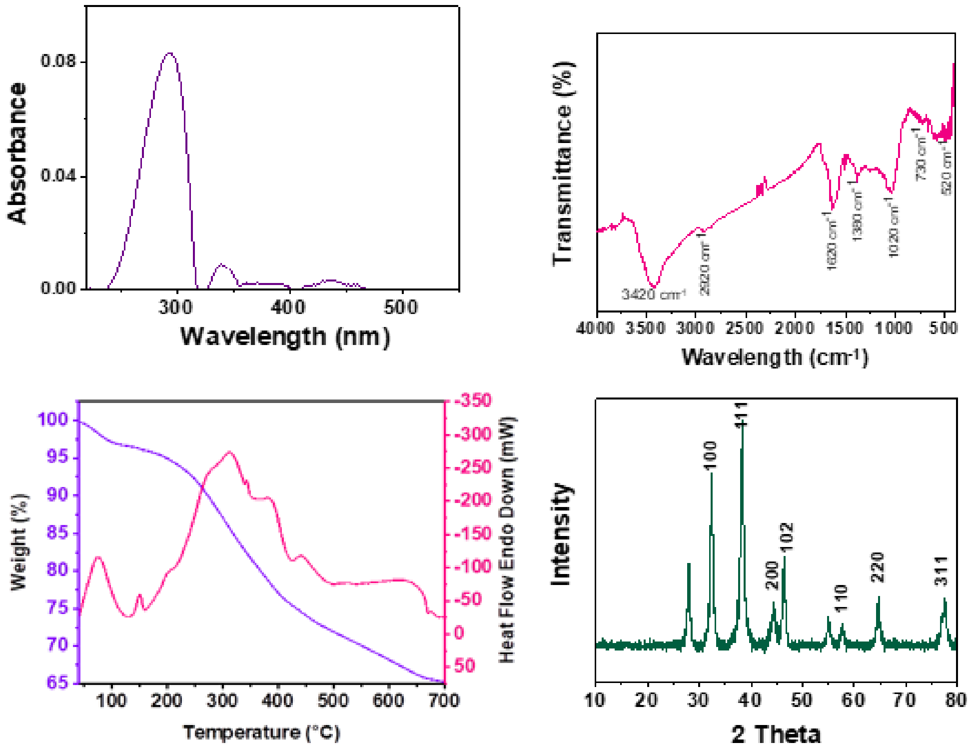

Figure 1. Physico-chemical characterization of Ag/ZnO NCs.

On FE-SEM evaluation, Ag/ZnO NCs exhibited an agglomerated poly-crystalline morphology (Figure 2a,b). In addition, the TEM images depicted the typical spherical shape of Ag/ZnO NCs (Figure 2e), with a mean diameter of 31.34 ± 1.27 nm (Figure 2c) with lattice fringe spacing of around 0.22 nm (Figure 2e). Interestingly, the SAED pattern of Ag/ZnO NCs (Figure 2f) complemented our PXRD findings, which in turn proved the poly-crystalline structure.

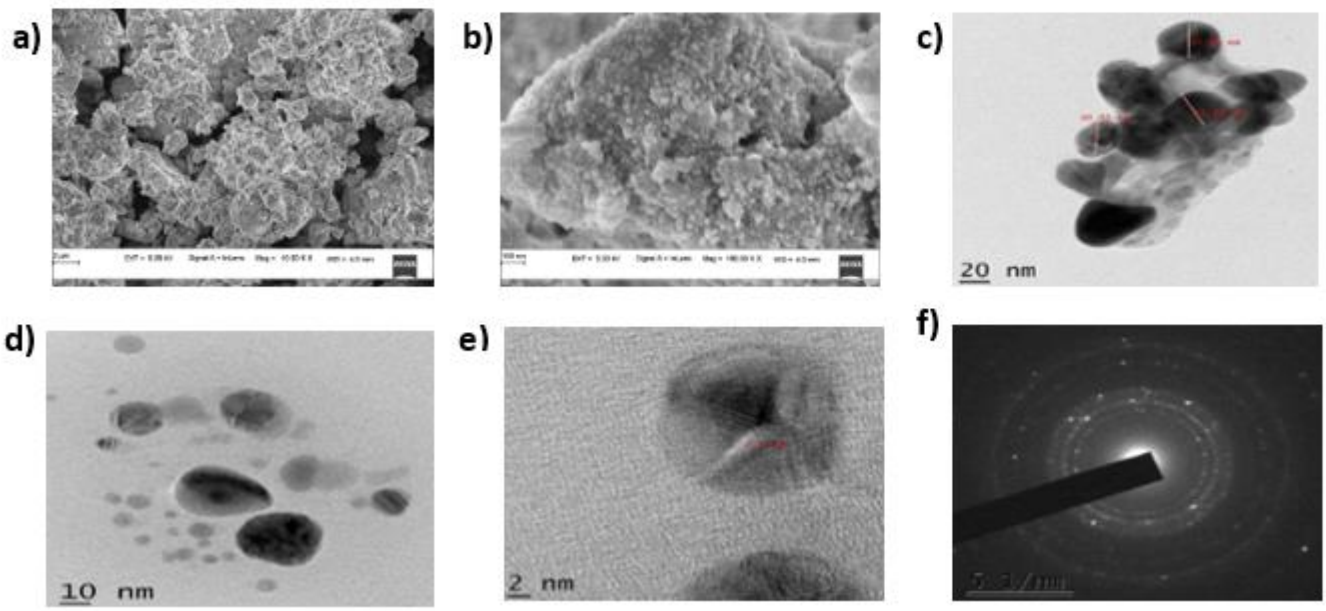

Figure 2. FE-SEM and TEM imaging of Ag/ZnO NC.

3.3. In Vitro Antibacterial Activity of Green Synthesised Ag/ZnO NCs

The green synthesised Ag/ZnO NCs demonstrated MIC of 31.25 µg/mL and MBC values ranging from 62.50 µg/mL to 125 µg/mL against all of the tested MDR- EAEC strains using the micro broth dilution technique (Table 1). The antibacterial activity of Ag/ZnO NCs might be due to the generation of ROS, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and hypochlorous acid (HOCl), leading to oxidative stress and thereby inducing the desired bacterial cell toxicity [8]. Furthermore, the Ag NPs coated on the surface of the ZnO NPs contain electrons produced by the photocatalytic reactions of the ZnO NPs resulting in strong interaction between the semiconductor zinc oxide and the metallic silver ruptures the cell membrane and boosts the antibacterial activity [18].

Table 1. MIC and MBC values of green synthesised Ag/ZnO NCs against MDR-EAEC isolates.

Isolate ID	MIC (µg/mL)	MBC (µg/mL)
E1	31.25	62.50
E2	31.25	125
E3	31.25	125

3.4. In Vitro Antioxidant Activity of Green Synthesised Ag/ZnO NCs

Antioxidants are substances that shield cells from the harmful effects of reactive oxygen species (ROS) [19]. The in vitro antioxidant-scavenging capacity of green synthesised Ag/ZnO NCs was evaluated by employing ABTS and reducing power assays with ascorbic acid as the reference standard. In this study, a dose-dependent increase in their antioxidant capabilities, indicating an improved capacity to scavenge free radicals, was exhibited by the green synthesised Ag/ZnO NCs in ABTS (Figure 3a) and reducing power assays (Figure 3b). However, compared to the conventional antioxidant, ascorbic acid, the antioxidant activity of greenly synthesised Ag/ZnO NCs was lower. Furthermore, *C. longa* has been regarded as one of the most promising sources of natural antioxidants due to the high concentrations of polyphenols, flavonoids, tannins, and ascorbic acid contained in it [20]. Hence, a combination of Ag and ZnO together forming plant-based nanomaterials have been found to exhibit an increased antioxidant capacity and anti-proliferative action, which eliminates free radicals [21].

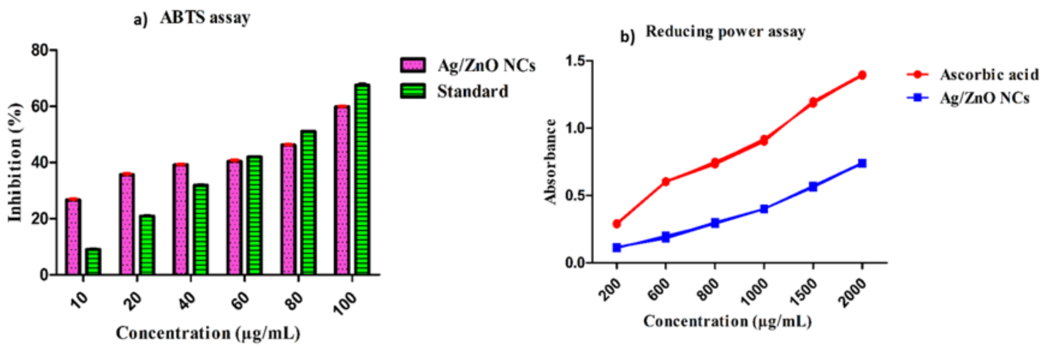

Figure 3. In vitro antioxidant activity of green synthesied Ag/ZnO NCs (**a**) ABTS assay (**b**) Reducing power assay.

3.5. In Vitro Antibiofilm Efficacy of Green Synthesised Ag/ZnO NCs

The present scenario necessitates the research for an antibacterial agent against biofilms since these are known to be closely linked to numerous microbial diseases [22]. Ag/ZnO NCs might be regarded as a potential therapeutic alternative against bacterial biofilms owing to their intrinsic antibacterial activity. In this study, the in vitro anti-biofilm efficacy of green synthesised Ag/ZnO NCs against the MDR-EAEC pathotypes was evaluated by employing a crystal violet staining assay at 24 and 48 h. The green synthesised Ag/ZnO NCs (1X MIC) have demonstrated a highly significant ($p < 0.001$) biofilm inhibition after 24 h and 48 h since all of the tested MDR-EAEC isolates exhibited a drop in biomass compared to their respective controls (untreated bacterial cultures) (Figure 4). The antibiofilm activity of green synthesised Ag/ZnO NCs might be due to the increased generation of ROS along with the suppression of exopolysaccharides of MDR-EAEC, which are a crucial component of bacterial biofilms [4,23]. Hence, the green synthesised Ag/ZnO NCs can be thought of as an effective choice to prevent and inhibit the formation of bacterial biofilms since the MDR-EAEC cultures were highly susceptible to them.

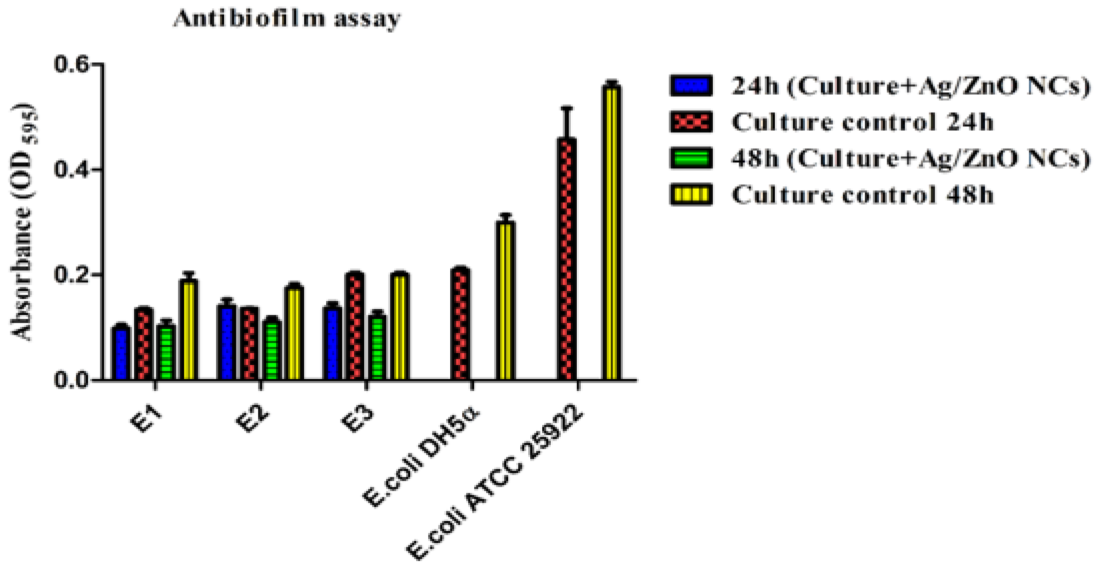

Figure 4. In vitro antibiofilm efficacy of green synthesised Ag/ZnO NCs.

3. Conclusion

Using the methanolic extract of *C. longa* leaves and stem, we successfully synthesised Ag/ZnO NCs in this study. While the green synthesised Ag/ZnO NCs exhibited absorbance peaks (340 nm and 450 nm) that corresponded to ZnO NPs and Ag NPs in UV-Vis spectroscopy, a progressive thermal deterioration was seen between 200 °C and 450 °C that reflected a 21% weight loss in TGA/DTA plots. The poly-crystalline morphology of Ag/ZnO NCs with homogeneous Ag distribution on the surface of ZnO was further supported by the PXRD, SEM, and TEM. In addition, the promising antibacterial efficacy of green synthesised Ag/ZnO NCs against MDR pathogens was also discovered by using the micro broth dilution technique. Furthermore, the green synthesised Ag/ZnO NCs exhibited significant anti-biofilm as well as concentration-dependent antioxidant activity against the tested MDR-EAEC strains. Overall, the study demonstrated an environmentally benign way to make Ag/ZnO NCs from *C. longa* extract, which might be used as a promising antibacterial candidate with potential antioxidant and antibiofilm action. However, the effectiveness of Ag/ZnO NCs must be further validated by conducting in vivo clinical trials in appropriate laboratory models.

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