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Antibacterial activity of silver and zinc nanoparticles against *Vibrio cholerae* and enterotoxic *Escherichia coli*

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ABSTRACT

Vibrio cholerae and enterotoxic *Escherichia coli* (ETEC) remain two dominant bacterial causes of severe secretory diarrhea and still a significant cause of death, especially in developing countries. In order to investigate new effective and inexpensive therapeutic approaches, we analyzed nanoparticles synthesized by a green approach using corresponding salt (silver or zinc nitrate) with aqueous extract of *Caltropis procera* fruit or leaves. We characterized the quantity and quality of nanoparticles by UV–visible wavelength scans and nanoparticle tracking analysis. Nanoparticles could be synthesized in reproducible yields of approximately 10^8 particles/ml with mode particles sizes of approx. 90–100 nm. Antibacterial activity against two pathogens was assessed by minimal inhibitory concentration assays and survival curves. Both pathogens exhibited similar resistance profiles with minimal inhibitory concentrations ranging between 5×10^5 and 10^7 particles/ml. Interestingly, zinc nanoparticles showed a slightly higher efficacy, but sublethal concentrations caused adverse effects and resulted in increased biofilm formation of *V. cholerae*. Using the expression levels of the outer membrane porin OmpT as an indicator for cAMP levels, our results suggest that zinc nanoparticles inhibit adenylyl cyclase activity. This consequently deceases the levels of this second messenger, which is a known inhibitor of biofilm formation. Finally, we demonstrated that a single oral administration of silver nanoparticles to infant mice colonized with *V. cholerae* or ETEC significantly reduces the colonization rates of the pathogens by 75- or 100-fold, respectively.

Introduction

Recently, nanotechnology has become increasingly important in the biomedical and pharmaceutical areas as alternative antimicrobial strategy due to re-emergence infectious diseases and the appearance of antibiotic-resistant strains especially within Gram-negative microorganisms (Desselberger, 2000). Biosynthesis of green nanoparticles using plant extracts is an interesting area in the field of nanotechnology, which has economic and eco-friendly benefits over chemical and physical methods of synthesis (Suzan et al., 2014). Nanoparticles (NPs) are typically no greater than 100 nm in size and their biocidal effectiveness is suggested to be owing to a combination of their small size and high surface-to-volume ratio, which enable intimate interactions with microbial membranes (Allaker, 2010; Morones et al., 2005). In addition, inorganic antibacterial agents such as metal and metal oxides are advantageous compared to organic compound due to their stability (Sawai, 2003; Sondi and Sondi, 2004). Among these metal oxides, ZnO has attracted a special attention as antibacterial agent. For instance, ZnO inhibits the adhesion and internalization of enterotoxigenic *E. coli* (ETEC) into enterocytes (Roselli et al., 2003). In addition, ZnO nanoparticles (ZnO-NPs) exhibit antibacterial activity and can reduce the attachment and viability of microbes on biomedical surfaces (Brayner et al., 2006; Yamamoto, 2001). Interestingly, several results suggest a selective toxicity of ZnO-NPs preferentially targeting prokaryotic systems, although killing of cancer cells has also been demonstrated (Hanley et al., 2008; Reddy et al., 2007; Taccola et al., 2011). Several mechanisms have been reported for the antibacterial activity of ZnO-NPs. For example ZnO-NPs can interact with membrane lipids and disorganize the membrane structure, which leads to loss of membrane integrity, malfunction, and finally

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to bacterial death (Krishnamoorthy et al., 2012; Zhang et al., 2007). ZnO may also penetrate into bacterial cells at a nanoscale level and result in the production of toxic oxygen radicals, which damage DNA, cell membranes or cell proteins, and may finally lead to the inhibition of bacterial growth and eventually to bacterial death (Apperlot et al., 2009; Irzh et al., 2010; Makhulf et al., 2005; Moody and Hassan, 1982; Zhang et al., 2007).

Furthermore, Ag^+ ions and Ag-based compounds are highly toxic to several microorganisms, which make them interesting candidates for multiple applications in the medical field (Furno et al., 2004; Prakash et al., 2013). Ag is generally used as nitrate salt, but in the form of Ag nanoparticles (Ag-NPs) the surface area is increased and thereby antimicrobial efficacy is greatly enhanced. Though Ag-NPs find use in many antibacterial applications, the action of this metal on microbes is not fully known. It has been hypothesized that silver nanoparticles can cause cell lysis or growth inhibition via various mechanisms (Kim et al., 2007; Prabhu and Poulose, 2012). The lethality of silver for bacteria can also be in part explained by thiol-group reactions that inactivate enzymes (Chen and Schluesener, 2008; Feng et al., 2000). Also, Steuber and colleagues suggested a mechanism for Ag^+ action in *Vibrio alginolyticus* involving the direct displacement of FAD from the holo-enzyme Na^+-NQR, which results in loss of enzyme activity (Steuber et al., 1997). In summary, silver treatment inhibits DNA replication, expression of ribosomal and other cellular proteins, and interferes with the bacterial electron transport chain (Bragg and Rainnie, 1974; Feng et al., 2000; Yamanaka et al., 2005).

Several reports demonstrated the synthesis of ZnO- and Ag-NPs from natural sources like plants or microorganisms by green chemistry approaches (Babu and Prabu, 2011). The use of plant extracts for nanoparticles synthesis may be advantageous over other biological processes, because it drops the elaborate process of maintaining cell cultures and can also be used for large-scale NPs synthesis (Jeeva et al., 2014). Additionally, the green chemistry approach for the synthesis of NPs using plants avoids the generation of toxic byproducts. Among the various known synthesis methods, plant mediated NPs synthesis is preferred as it is cost-effective, eco-friendly and safe for human therapeutic use (Kumar and Yadav, 2009).

Diarrheal diseases are still a common worldwide cause of morbidity and mortality especially in the developing world. Within these areas, *V. cholerae* (~25%) followed by ETEC (~15%) are most prevalent bacterial pathogens causing diarrheal diseases (Chowdhury et al., 2011; Walker et al., 2007). *V. cholerae* is the causative agent of cholera, a life-threatening secretory diarrheal disease. According to Southeastern and Central Asia reports the annual acute diarrheal cases for *V. cholerae* infection were estimated more than 1 million (WHO, 2013). ETEC is a common cause of traveler's diarrhea, being responsible for up to one-half of diarrheal episodes in travelers to Asia, Africa and Latin America (Gupta et al., 2008; Qadri et al., 2005; Sanchez and Holmgren, 2005; Tobias et al., 2011). Particularly children show a high mortality rate in developing countries where diarrheal diseases remain the second most common cause of death (Levine, 2006). Even today treatment of these diarrheal diseases relies on a simple rehydration therapy, sometimes in combination with antimicrobial agents (Sack et al., 2004). The rehydration therapy is highly effective, but appropriate sterile solutions, antibiotics and medical expertise are not always available and during the explosive outbreaks medical facilities cannot cope with the massive numbers of incoming patients. Thus, alternative strategies should be investigated.

Calotropis procera is a shrub (F: Asclepiadaceae) distributed in West Africa, Asia and other parts of the tropics. The plant is erect, tall, large, branched and perennial with milky latex throughout (Irvine, 1961). Interestingly, Babu and Prabu recently described the Ag-NPs synthesis using aqueous extract of *Calotropis procera* flower, while the reduction was considered to occur due to the phenolics, terpenoids, polysaccharides and flavonoids present in the extract (Babu and Prabu, 2011).

In the present study, we synthesized metallic ZnO- and Ag-NPs using leaf and fruit extract of *Calotropis procera* and characterized their antibacterial activity against *V. cholerae* and ETEC. Especially Ag-NPs synthesized from leaf extracts showed the most robust antibacterial efficacy against both pathogens throughout the study. Furthermore, these Ag-NPs reduced fitness of the bacteria in biofilms as well as in vivo.

Materials and methods

Bacterial strains, culture conditions and supplements. *V. cholerae* AC53 and ETEC H10407, spontaneous streptomycin-resistant (Sm^R) derivatives of the clinical isolates O1 El Tor Ogawa E7946 (Miller et al., 1989); (Schild et al., 2007) or ETEC O78:H11:K80 (Evans and Evans, 1973), were used in this study. Unless stated otherwise strains were grown in LB broth with aeration at 37 °C or for biofilm formation under static conditions at room temperature (RT). If required, streptomycin was used with a final concentration of 100 μg/ml.

Plant materials and preparation of the extracts

Healthy leaves and fruits of *Caltropis procera* were collected from South Valley University campus at Qena city (Egypt), washed thoroughly with tap water followed by distilled water, and air dried on a paper towel for 4–6 days. Dry leaves were shredded and ground in a tissue grinder (IKA A10, Germany) to fine powder. Ten grams of the powder were dissolved in 100 ml sterile double distilled water and heated for 1 h at 80 °C. The obtained extract was filtered through Rotilabo® Typ 601P filter paper; the filtrate was collected in a 250 ml Erlenmeyer flask and then stored at 4 °C for further use (modified from (Verastegui et al., 1996).

Green synthesis of silver and zinc oxide nanoparticles (Ag-NPs and ZnO-NPs)

Ag-NPs and ZnO-NPs were essentially synthesized as previously described (Babu and Prabu, 2011; Prakash et al., 2013; Sangeetha et al., 2011, 2012; Sun et al., 2014; Suzan et al., 2014; Vimala et al., 2014) using leaves (L) or fruits (F) extracts from *C. procera* resulting in the four different types of nanoparticles Ag-NPs-L, Ag-NPs-F, ZnO-NPs-L and ZnO-NPs-F. Solutions with silver nitrate or zinc nitrate (without *C. procera* extract) were also incubated at the same conditions and served as a negative control (Kumar et al., 2011).

To obtain Ag-NPs, 20 ml of a 1 mM $AgNO_3$ (Sigma–Aldrich) solution were added drop-wise to 20 ml of the respective aqueous plant extract of *C. procera* under constant stirring at 80 °C within 30–45 min, for the reduction of Ag^+ ions. This material was incubated in the dark (to minimize the photoactivation of silver nitrate) at 37 °C. The synthesis of ZnO-NPs was performed as previously described with some modifications. Briefly, 2 g of zinc nitrate (Sigma–Aldrich) was dissolved in 100 ml aqueous leaf or fruit extracts solution of *C. procera* under constant stirring. After complete dissolution of the mixture, the solution was kept under vigorous stirring at 80 °C for 2 h, subsequently allowed to cool at room temperature and the supernatant was discarded. Obtained NPs solutions were centrifuged at 4,500 rpm for 15 min after thorough washing and dried at 80 °C for 7–8 h. Crude pellets were then resuspended in sterile double distilled water, filtered through 0.2 μm filter and stored at 4 °C in the dark prior to their use.