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Earthworm-mediated synthesis of silver nanoparticles: A potent tool against hepatocellular carcinoma, *Plasmodium falciparum* parasites and malaria mosquitoes

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A B S T R A C T

The development of parasites and pathogens resistant to synthetic drugs highlighted the needing of novel, eco-friendly and effective control approaches. Recently, metal nanoparticles have been proposed as highly effective tools towards cancer cells and *Plasmodium* parasites. In this study, we synthesized silver nanoparticles (EW–AgNP) using *Eudrilus eugeniae* earthworms as reducing and stabilizing agents. EW–AgNP showed plasmon resonance reduction in UV–vis spectrophotometry, the functional groups involved in the reduction were studied by FTIR spectroscopy, while particle size and shape was analyzed by FESEM. The effect of EW–AgNP on in vitro HepG2 cell proliferation was measured using MTT assays. Apoptosis assessed by flow cytometry showed diminished endurance of HepG2 cells and cytotoxicity in a dose-dependent manner. EW–AgNP were toxic to *Anopheles stephensi* larvae and pupae, LC_{50} were 4.8 ppm (I), 5.8 ppm (II), 6.9 ppm (III), 8.5 ppm (IV), and 15.5 ppm (pupae). The antiplasmodial activity of EW–AgNP was evaluated against CQ-resistant (CQ-r) and CQ-sensitive (CQ-s) strains of *Plasmodium falciparum*. EW–AgNP IC_{50} were 49.3 μg/ml (CQ-s) and 55.5 μg/ml (CQ-r), while chloroquine IC_{50} were 81.5 μg/ml (CQ-s) and 86.5 μg/ml (CQ-r). EW–AgNP showed a valuable antibiotic potential against important pathogenic bacteria and fungi. Concerning non-target effects of EW–AgNP against mosquito natural enemies, the predation efficiency of the mosquitofish *Gambusia affinis* towards the II and II instar larvae of *A. stephensi* was 68.50% (II) and 47.00% (III), respectively. In EW–AgNP-contaminated environments, predation was boosted to 89.25% (II) and 70.75% (III), respectively. Overall, this research highlighted the EW–AgNP potential against hepatocellular carcinoma, *Plasmodium* parasites and mosquito vectors, with little detrimental effects on mosquito natural enemies.

1. Introduction

For thousands of years, earthworms have been widely used in China, Korea, Vietnam and most of South East Asia, for their therapeutic benefits, and referred as “Earth Dragons”. The coelomic fluid (CF) of the earthworms has agglutinating [41], cytotoxic [31], proteolytic [35], hemolytic [22], antibacterial [5], and antioxidant potential [4], as well as β-1,3-glucan- and lipopolysaccharide-binding and mitogenic activities [26]. Eisenin from earthworms destroys neoplastic cells from several human cancer cell lines [19]. Later on, antitumor activities of earthworm fibrinolytic enzyme on human hepatoma cells were studied by Hong [27]. In addition, the earthworm CF has been recently tested as larvicide against larvae of *Anopheles stephensi* [28].

Mosquitoes (Diptera: Culicidae) represent a key threat for millions of humans and animals worldwide, since they act as vectors for important parasites and pathogens, causing millions of deaths annually [12, 39,56,68]. Malaria is caused by *Plasmodium* parasites, vectored to vertebrates through the bites of infected *Anopheles* mosquitoes, which mainly bite between dusk and dawn. According to the latest estimates, there

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were about 198 million cases of malaria in 2013 and an estimated 584,000 deaths. Most deaths occur among children living in Africa, where a child dies every minute from malaria. Malaria mortality rates among children in Africa have been reduced by an estimated 58% since 2000 [38,71]. Currently, malaria control is challenging, due to insecticide resistance in vector populations, as well as to the development of *Plasmodium* strains resistant to a growing number of antimalarial drugs. In this scenario, novel eco-friendly control tools are urgently needed [12,13]. Besides chemical pesticides, mosquito larval populations can be controlled by a number of aquatic predators, including odonate young instars, tadpoles, fishes, copepods, and water bugs [17,29,45,57]. The use of fishes for controlling mosquitoes was an important tool in the pre-DDT era [23,69]. Typically, fishes have been introduced into many potential mosquito breeding habitats, including rice fields, marshes, dams, canals, and ponds [25,42]. Among them, the mosquitofish, *Gambusia affinis*, is easy to culture in laboratory settings as well as in the field, and has been widely used to evaluate the non-target impacts of mosquitocidals ([14,15,16,52,55,65]).

Primary hepatocellular carcinoma (HCC) is a frequent cause of death among humans. It occurring as a solid tumor with limited therapeutic options. Hence, a thorough understanding of the biological bases of this malignancy might suggest new strategies for effective treatment [2]. The association between malaria and cancer mortality can be possibly explained by the well-established ability of *Plasmodium* to induce suppression of the immune system. A second explanation may be that the *Anopheles* mosquito, the vector of malaria, transmits obscure viruses that initially causes only a mild transitory illness but much later predisposes to cancer [74]. There are few studies of the relationship between cancer and malaria. For instance, Suresh et al. [64] have reported analogies at the cellular level for the two diseases, while Welsh et al. [70] observed no relationship between malaria rates and primary liver cancer. Efferth et al. [21] also reported that anti-malarial artesunate is active against cancer. Notably, Epstein Barr virus-related Burkitt's lymphoma is believed to require cofactors, such as malaria infection, for tumor development [67]. Several uncertainties have risen regarding the absorption and availability of a single drug against malarial vector and tumor. To combat mosquito-borne diseases and cancers, many natural products have been investigated separately for malaria and hepatocellular cancer. However, limited knowledge is available about drugs with multi-potency against both public health concerns [43,48].

The “green synthesis” of silver nanoparticles (AgNP) is considered an eco-friendly technology leading to a reduction in the employment or generation of hazardous substances [8,14,46]. Silver ions and silver-based compounds are highly toxic to microorganisms, including important species of pathogen bacteria [73]. Therefore, AgNP have emerged with diverse medical applications including silver-based dressings and silver-coated medicinal devices, such as nano-gels and nano-lotions [53]. In this research, earthworm-synthesized silver nanoparticles (EW–AgNP) were characterized using UV–vis spectrophotometry, Fourier transform infrared spectroscopy (FTIR) and Field Emission Scanning Electron Microscopy (FESEM). Then, we evaluated their antimicrobial potential against pathogen bacteria (*Bacillus subtilis*, *Bacillus thuringiensis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Staphylococcus aureus*) and fungi (*Candida albicans*, *Fusarium solani* and *Aspergillus niger*). We examined in vitro cytotoxic effect on HepG2 cell lines and apoptosis determination to prove its pharmacological potential against hepatocellular carcinoma. Furthermore, the larvicidal and pupicidal potential of EW–AgNP were assessed against the malaria vector *A. stephensi*. Concerning non-target effects, the predation efficiency of the western mosquitofish, *G. affinis*, was studied against *A. stephensi* larvae under both standard conditions and in an aquatic environment contaminated with ultra-low doses of EW–AgNP. Lastly, EW–AgNP were investigated for their growth inhibition potential against chloroquine-sensitive (CQ-s) and chloroquine-resistant (CQ-r) strains of *Plasmodium falciparum* parasites.

2. Materials and methods

2.1. Chemicals

Silver nitrate trypsin-ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT), saline (PBS), phosphate–citrate buffer, propidium iodine, Triton X-100, sodium bicarbonate, albumax, hypoxanthine, gentamycin and chloroquine were purchased from Sigma-Aldrich, USA. Nutrient agar, Potato dextrose agar and DNase-free RNase A were purchased from Hi-media, India. Millipore double-distilled water has been used in all the experiments.

2.2. Earthworm-mediated synthesis of silver nanoparticles

Living earthworms, *Eudrilus eugeniae*, was collected from Aarthi farms, Kondegoundan Palayam village, Pollachi Taluk, Coimbatore District, Tamil Nadu, India. Earthworms were gently washed with tap water to remove any dirt from their bodies. The earthworms were kept in 0.5% NaCl at room temperature for 3 h with at least five solution changes until their digestive systems were clean. Cleaned earthworms were exposed to −20 °C for 10 min, then finely minced with scissors and homogenized using distilled water. The extract was prepared adding 10 g of the homogenate in a 300-ml Erlenmeyer flask filled with 100 ml of sterilized double distilled water and then boiling the mixture for 5 min. The extract was filtered using Whatman filter paper n. 1. The extract was then treated with aqueous 1 mM $AgNO_3$ solution and incubated at room temperature. The reduction of silver ions is visualized by the change in color of the solution from yellowish to dark brown.

Following Panneerselvam et al. [51], the synthesis of EW–AgNP was confirmed by sampling the reaction mixture at regular intervals and the absorption maxima was scanned by UV–vis, at the wavelength of 300–800 nm in a UV-3600 Shimadzu spectrophotometer at 1-nm resolution. Furthermore, the reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min, and the resulting pellet was dissolved in deionized water and filtered through a Millipore filter (0.45 μm). 25 μl of sample was sputter coated on a copper stub, and the morphology of EW–AgNP was investigated using a FEI QUANTA-250 FESEM. Surface groups of the nanoparticles were qualitatively confirmed by using FTIR spectroscopy [59], with spectra recorded by a Perkin Elmer Spectrum 2000 FTIR spectrophotometer.

2.3. Antimicrobial potential

Six bacteria species, *B. subtilis*, *B. thuringiensis*, *E. coli*, *K. pneumoniae*, *P. vulgaris* and *S. aureus*, and three fungal species, *C. albicans*, *F. solani* and *A. niger*, were used in this study. All were provided by the Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology Sector 39-A, Chandigarh-160,036 (India).

Antimicrobial activity of EW–AgNP was tested against the selected Gram-positive and Gram-negative bacteria and fungal strains using the disk diffusion method [10,20]. Target species were incubated in the nutrient broth and incubated at 28 ± 2 °C for 24 h. Bacteria and fungi were grown on their respective media, nutrient agar and potato dextrose agar, respectively. 20 ml of medium was poured into the plates to obtain uniform depth and allowed to solidify. The standard inoculum suspension (10^6 CFU/ml) was streaked over the surface of the media using sterile cotton swab to ensure confluent growth of the organisms. 6 mm diameter disks were prepared with Whatman n.1 paper. 10 μl of EW–AgNP was diluted with two volumes of 5% dimethyl sulfoxide (DMSO), impregnated on the filter paper disks, placed on the surface of the plates with sterile forceps, and gently pressed to ensure contact with the inoculated agar surface. Petri plates were kept for incubation at room temperature (27 °C ± 2) for 24 h. After incubation, plates were observed and zones of inhibition (mm) were measured using a