



Zero valent silver nanoparticles capped with capsaicinoids containing *Capsicum annuum* extract, exert potent anti-biofilm effect on food borne pathogen *Staphylococcus aureus* and curtail planktonic growth on a zebrafish infection model

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ABSTRACT

Food plants Hungarian wax pepper (HWP) and Green Bell pepper (GBP), belonging to *Capsicum annuum* were utilized for biogenic fabrication of zero valent, nano-silver (AgNPs) through a photo-mediation procedure. In the bacterial strains evaluated, HWP/GBP AgNPs demonstrated effective bacteriostatic and bactericidal effect against *Staphylococcus aureus*. Time kill results portrayed that HWP/GBP nano-silver exhibited comparable bactericidal potency on *S. aureus*. Anti-biofilm potential of HWP/GBP AgNPs displayed significant effects at sub MIC levels, by triggering 50% biofilm reduction of the food spoilage microbe *S. aureus*, inferring that the anti-biofilm outcome is not dependent on antibacterial result, and this was confirmed by SEM and fluorescence studies. Histopathological analyses of *S. aureus* infected zebrafish liver did not display any abnormality changes such as extensive cell death and degeneration, upon treatment with HWP/GBP AgNPs and the zero-valent silver nanoparticles were comparatively less toxic and more operative in restraining the bioburden in *S. aureus* infected zebrafish model by a > 1.7 log fold. Ability of light reduced HWP/GBP AgNPs to alleviate the *in vitro* and *in vivo* planktonic mode of growth and curb the biofilm formation of *S. aureus* is also demonstrated.

1. Introduction

Microbial infections are the most important cause for the human diseases accounting about millions of death every year. Diseases caused by food borne pathogens are more prevalent even in developed countries. Food borne diseases comprises of a larger account of illnesses and a wide spectrum of bacteria were reported with food borne diseases, worldwide [1,2]. Numerous reports recommends the use of plant extracts [3], purified compounds [4] for combating the pathogens. With the advent of green synthetic methodologies for nanomaterials as promising agents against the microbes [5,6], there is always a question of toxicity/biosafety on nanomaterials as antimicrobials. Due to the vast resource of plant species, search for new source that possess antibacterial and antifungal properties are always in vogue. Plants are also a part of our diet and species like capsicum, cinnamon, cloves, turmeric etc., are an integral part of diet and in such plants, their antimicrobial

compounds ensure a dual role; one as food and other as nutraceutical supplements [7,8].

Chillies/peppers belong to the *Capsicum* genus and have been reported to possess antimicrobial activity [9,10] and these activities have been correlated to the capsaicinoids content [11]. The pungency of the chillies/peppers depends on the content of the capsaicinoids as indicated by the Scoville scale [12]. The Hungarian wax pepper (HWP) and Green bell pepper (GBP) falls in the lowest scale in Scoville classification and they have less capsaicin content. The less spicy varieties like bell pepper also been reported to have anti-microbial activity [13].

In this report we have made an attempt to elucidate the antibacterial activity of AgNPs using less spicy and less capsaicinoids containing chillies/peppers extract. Sunlight mediated, bio functionalization of AgNPs was done, using the HWP/GBP aqueous extracts and in the panel of bacteria tested for antimicrobial activity, the HWP/GBP AgNPs exhibited gratifying antimicrobial/anti-biofilm activity against

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Table 1
Minimum inhibition concentration/minimum bacterial concentration.

	Strain Names	MIC/MBC ($\mu\text{g/mL}$)				
		HWP	GBP	HWP AgNPs	GBP AgNPs	Cefotaxime
1	<i>Enterococcus faecalis</i>	200/400	400/800	8/32	16/32	10/20
2	<i>Bacillus subtilis</i>	200/400	200/400	16/64	16/64	10/40
3	<i>Staphylococcus aureus</i>	100/200	100/200	4/16	8/32	20/40
4	<i>Salmonella</i> Typhi	200/800	400/800	32/64	16/64	20/40
5	<i>Escherichia coli</i>	400/800	400/800	16/64	16/64	20/80
6	<i>Pseudomonas aeruginosa</i>	200/400	400/800	16/32	32/64	20/40
7	<i>Enterobacter cloacae</i>	400/800	400/800	16/64	16/64	10/20
8	<i>Klebsiella pneumoniae</i>	400/800	200/400	32/64	16/64	10/20
9	<i>Proteus mirabilis</i>	400/800	200/400	16/64	16/32	10/20

S. aureus.

2. Materials and methods

2.1. *Capsicum annuum* aqueous extract preparation

The Hungarian wax pepper (HWP) and Green bell pepper (GBP), are varieties of *Capsicum annuum* L, used in this experiment and was purchased from a local market in Thanjavur, India. All other chemicals and reagents used were of analytical grade. Deionized water was used for all the experiments. The collected fruits were copiously washed with tap water and then with deionized water to remove debris, diced into small pieces and shade dried at ambient temperature. About 10 g of diced fruits were transferred and suspended with 100 mL distilled water, and blanched for 30 min on a heating mantle until a pale yellow solution was obtained. Then the extract was cooled, filtered, and refrigerated at 20 °C for further studies.

2.2. Synthesis of silver nanoparticles

To 700 μL of Hungarian wax pepper (HWP) aqueous extract, was added 5 mL of 1 mM silver nitrate solution and this solution mixture was vortexed, exposed to sunlight periodically (5, 10, 15, 20, 25 and 30 min) and observed for color changes. The transformation of the colorless solution mixture before the exposure to pale yellow and finally to deep yellowish-brown color, indicated the formation of silver nanoparticles (HWP AgNPs). After every set time, the sample aliquots were taken and UV–Vis Absorption spectra was observed. Likewise, the same protocol was repeated for Green Bell Pepper (GBP), to produce the GBP stabilized AgNPs.

2.3. Microbial strains

The pathogens engaged in the current study were Methicillin Resistant *Staphylococcus aureus* ATCC43300, *Bacillus subtilis* MTCC441, *Enterococcus faecalis* MCC2409, *Salmonella enterica* serovar Typhi MTCC 733, *Proteus mirabilis* MTCC 425, *Escherichia coli* MG1655, *Klebsiella pneumoniae* MTCC432, *Enterobacter cloacae* MCC2072 and *Pseudomonas aeruginosa* MTCC1688. All cultures were sustained in $-80\text{ }^{\circ}\text{C}$ as

glycerol stocks. For experiments, the microbes were sub cultured on to LBA/TSA plates and inoculated into MH/LB broth.

2.4. Anti-bacterial assays and biofilm inhibition with HWP/GBP and HWP/GBP AgNPs

Minimum Inhibitory Concentration (MIC) of HWP/GBP and its equivalent nano-size silver were assessed by two fold reduction methods as reported [14]. Ability of HWP/GBP extracts and HWP/GBP AgNPs, to thwart the biofilm formation against food borne micro-organisms were evaluated with the crystal violet (CV) bio-film staining method, as described in literature [15]. The Crystal violet was removed with 30% HOAc for 10–20 min and OD was documented at 595 nm with a microplate reader (Infinite F50, TECAN, Denmark).

2.5. Bacterial kill curves

Staphylococcus aureus is a highly adaptive pathogen, for *Staphylococcal* food poisoning. By virtue of its potent anti-biofilm effects, the, HWP/GBP extracts and HWP/GBP AgNPs were chosen for further studies, and the protocol described elsewhere were used [16]. The time kill experiments were executed three times.

2.6. Fluorescence biofilm imaging

For live/dead imaging, biofilms were allowed to form on a glass slide, which was housed in falcon tubes with BHI media and exposed with HWP and GBP AgNPs, and the protocol for live/dead staining was carried out as reported in literature [17]. All viable cells exhibited green fluorescence and all non-viable cells displayed red fluorescence, when imaged with fluorescence microscopy.

2.7. SEM imaging

Overnight developed cultures of *S. aureus* was diluted to 0.05 absorbance and was spotted on to a cover glass, kept inside a 24 well micro titer plate, and after bacterial accumulation, was incubated for 12–14 h. Following incubation media was gently aspirated; cover glass was gently washed with PBS, and to remove unbound cells, the biofilm on the coverglass was fixed with 2% glutaraldehyde. Later the fixative was removed, and biofilms were dehydrated with a sequence of ethanol washes (50% till 100%) for 10 min, each. Then, the biofilms were dried at ambient temperature, sputter coated and imaged using FESEM at 1000 to 10000 $\times$ magnifications to determine the biofilm architecture formed with HWP/GBP and/or HWP/GBP AgNPs [18].

2.8. In vivo zebrafish toxicity and antibacterial studies

Adult zebra fish (*Danio rerio*, both sex, 2 months old), 4–5 cm in length, and weighing 300–400 mg, were procured from an aquarium in Vallam, India. Animal acclimatization was performed with well-known zoological protocols [19]. To find the influence of HWP/GBP AgNPs on zebra fish liver enzyme data, 4 fish each were treated to 1 $\times$ minimum bacterial concentration of HWP/GBP AgNPs and standard drug (Cefotaxime) for 48 h. At the end of treatment, anesthesia was administered with ms-222, on all fish, and they were euthanized. Liver tissue(s) were combined from fish in each pool and homogenized at pH 7 with 0.1 M, Tris-HCl ice-cold buffer. The homogenate was centrifuged and the supernatant was used for liver α - and β -carboxy esterase enzyme profiles and protein estimation was done by known protocols [20,21].

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