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Anti-Herpes Simplex Virus (HSV-1 and HSV-2) activity of biogenic gold and silver nanoparticles using seaweed *Sargassum wightii*

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Biogenic gold (Au) and silver (Ag) nanoparticles were synthesized using seaweed *Sargassum wightii* (*Sw*) and their antiviral activity against Herpes Simplex Virus (HSV-1 and HSV-2) was evaluated. Efficacy of *Sw*-Au and *Sw*-Ag nanoparticles against HSV was estimated by the reduction of cytopathic effect (CPE) caused by HSV, which was observed in a dose-dependent manner. *Sw*-Au nanoparticles reduced 70% CPE of HSV-1 and HSV-2 at 10 µL and 25 µL, respectively, whereas 2.5 µL *Sw*-Ag nanoparticles effectively reduced 70% CPE of HSV-1 and HSV-2. Cytotoxicity was estimated in Vero cells by MTT assay. *Sw*-Au nanoparticles were significantly non-toxic in all the concentrations tested, whereas *Sw*-Ag nanoparticles were found to be toxic in higher concentrations. *Sw*-Au nanoparticles showed cell viability of 93.12-85.18 % in the range of 2.5-25 µL, and *Sw*-Ag nanoparticles showed cell viability of 97.21-21.91% in the range of 1-10 µL. *Sw*-Au and *Sw*-Ag nanoparticles effectively reduce the CPE caused by both HSV-1 and HSV-2 in Vero cells and can be used to treat HSV infections.

[**Keywords**: *Sargasum wightii*; Gold nanoparticles; Silver nanoparticles; HSV; Cytopathic effect; Antiviral]

Introduction

Recently, the application of nanoparticles has advanced in therapeutics[1], targeted drug delivery[2,3], bio-imaging[4,5], biosensors[6], fluorescent biological labels[7,8], gene delivery[9,10], tissue engineering[11,12], separation and purification of biological molecules and cells[13], detection of pathogens and proteins[14,15]. Au and Ag nanoparticles have gained considerable attention in the field of bio-molecular sensing, rapid pathogen detection, targeted drug delivery and nanoparticle-based cancer therapy owing to their unique physical and chemical properties[16]. Important biological entities, such as DNA, proteins, antigens, and antibodies which have nanometer-size dimensions, may serve as good candidate targets for interactions with such nanoparticles. Viruses are nano-sized pathogens that can infect and cause various diseases in humans, animals, and plants.

Development of new antiviral drugs that will help target the virus though maintaining host cell viability is challenging[17,18]. Generation of materials with different nanoscales and shapes by utilizing the physico-chemical properties and technological advances available could substantiate the biological applications of nanomaterials[18]. Over the last century, metal nanoparticles have attracted researchers to explore their applications in biomedical sciences[19]. Silver and gold nanoparticles have proved to be potent antiviral agents against human parainfluenza virus[20], H7N3 influenza Avirus[21], human norovirus[22], HIV[23], poliovirus[24], HSV[25], and hepatitis C virus[26].

Herpes Simplex Viruses (HSV-1 and HSV-2) are ubiquitous human pathogens of the alphaherpesviruses family[20]. HSV causes herpes labialis, pharyngitis, herpetic gingivostomatitis, herpes genitalis, keratitis, encephalitis, and neonatal herpes infection[27]. After primary infection, the virus establishes latent infection in sensory and autonomic neural ganglia which persists for lifetime. HSV can result in physical disability, social exclusion, and psychological distress, and can cause even life-threatening infections in newborns and immune compromised individuals[28]. According to the World Health Organization (WHO) report in 2012, globally 3.7 billion and 417 million people have been infected with HSV-1 and HSV-2, respectively[29].

Acyclovir (ACV), penciclovir (PCV), and their pro-drugs are currently being used for treating HSV infections and reactivation[30]. Development of drug resistance among high-risk groups such as immuno-

compromised and transplantation patients, prompted the search of alternative therapeutic agents with a different mechanism of action[28]. Use of nanomaterials in interactions of biological targets (particularly microbes) has gained much attention. The rich diversity of brown seaweeds belonging to the genus *Sargassum* species is an untapped reservoir of their various biological activities[31]. In the present study, an attempt has been made to evaluate the anti-HSV potential of gold and silver nanoparticles synthesized by *Sargassum withtii*.

Materials and Methods

Collection of marine alga and nanoparticle synthesis

Marine brown alga *S. wightii* Greville was collected from Mandapam camp, Rameshwaram, Tamil Nadu, India, during April 2016. The collected algal samples were washed with deionized water to remove other debris and shade-dried. Dried algal samples were powdered using an electronic blender. *Sw*-Au and *Sw*-Ag nanoparticles were synthesized as reported earlier[32,33] (Fig. 1). Briefly, for Au nanoparticles, 1 g of seaweed powder was soaked in 10 mL 10^{-3}M concentration of chloroauric acid ($HAuCl_4$) solution for 12 h at stirring condition. After 12 h, the color turned into transparent ruby red, which indicates the formation of Au nanoparticles. For Ag nanoparticles, 1 g of seaweed powder was soaked in 100 mL double distilled water for 24 h and filtered using Whatman no.1 filter paper. The filtrates (10 mL) react with 90 mL of 10^{-3} M silver nitrate solution for 15 h under stirring condition. After 15 h, the color turned to dark brown which indicated the formation of Ag nanoparticles.

Cells and viruses

Vero cells were procured from the National Centre for Cell Science (NCCS), Pune, India and maintained in minimal essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution (penicillin and streptomycin). Cells were then grown in T25 tissue culture flask and after reaching 70-80% confluence, the cells were infected with stock HSV and incubated for three days. After observing 100% cytopathic effect (CPE), the flask was freeze-thawed repeatedly. The content was transferred to a new 15 mL tube and centrifuged for 10 min. The viral suspension containing the supernatant was aliquoted into sterile screw-capped cryovials and stored at -80 ^{o}C until use. The infectivity of the viruses was estimated by following the Spearman and Karber method[34,35].

Cytotoxic assay

Cytotoxic activity of *Sw*-Au and *Sw*-Ag nanoparticles was estimated using Vero cells as described by Mosmann *et al.*[36] Briefly, 5×10^4 cells/well were seeded in 96 well tissue culture plates and incubated for 24 h at 37 °C with 5% CO_2. After 24 h, the complete medium was removed and the cells were treated with varying concentrations of *Sw*-Au and *Sw*-Ag nanoparticles in triplicate and the plates were incubated for 48 h. After incubation, the medium was removed and 100 µL of MTT reagent (MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) was added and the plates were incubated again at 37 °C for 4 h. After incubation, the MTT was completely removed, 100 µL of dimethyl sulfoxide (DMSO) was added, the plates were gently shaken and readings were taken at 570 nm in an ELISA

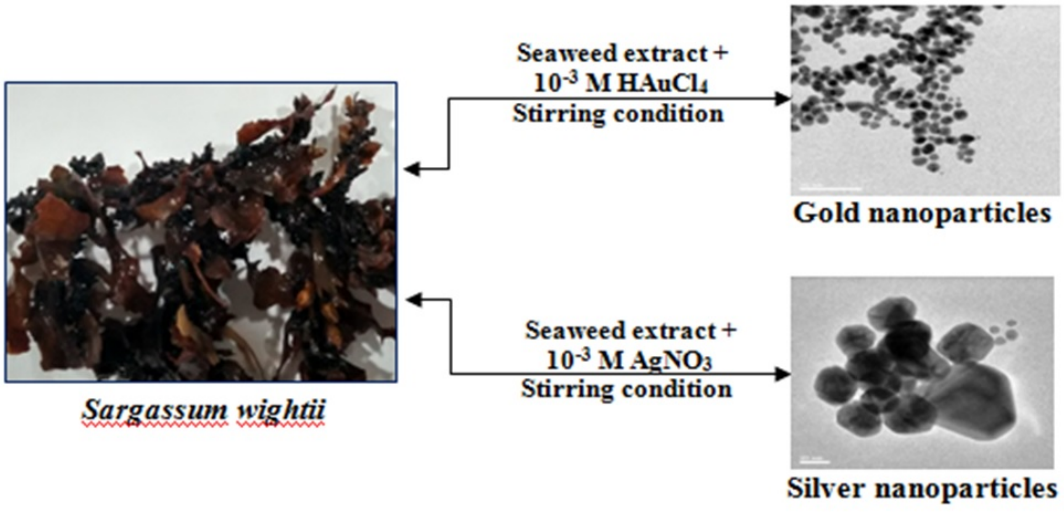

Fig. 1 — Schematic representation of gold and silver nanoparticles using seaweed *Sargassum wightii*

reader (BioTek, USA). The percentage cell viability was calculated using the following formula:

$$\% \text{ Cell viability} = \frac{\text{OD of the sample}}{\text{OD of the control}} \times 100$$

Anti HSV activity determination by CPE reduction method

An antiviral assay was performed as described by Tang *et al*.[37], with some modifications. Briefly, 96 well plates were seeded with Vero cells at a density of 5×10^4 cells/well. After reaching 80% confluence, the medium was removed and 100µL of $10^{7.5}$ $TCID_{50}$/mL virus particles of HSV-1 and $10^{6.5}$ $TCID_{50}$/mL HSV-2 particles were added and incubated for 1 h for viral adsorption. Unadsorbed viral particles were removed by washing the cell monolayer with serum-free medium. The cells were treated with different concentrations of *Sw*-Au (2.5, 5, 10 and 25 µL) and *Sw*-Ag (0.5, 1, 2.5 and 5 µL) nanoparticles. Cells treated with ACV (100 µg/mL) served as drug control, cells infected with virus alone served as virus control and Vero cells alone served as cell control. Then, the plates were incubated for three days at 37 ºC in 5% CO_2. The plates were examined microscopically for CPE every day. Anti-viral activity of *Sw*-Au and *Sw*-Ag nanoparticles was assessed using the grading system described by Kudi *et al.*[38] The degree of CPE inhibition upon treatment was marked as follows: '++++' total inhibition, '+++' 75% inhibition, '++' 50% inhibition, and 'Nil' as no inhibition.

Results and Discussion

The pharmaceutical potential of marine algae (seaweeds) has been recognized for several years. Seaweeds contain various biologically active substances such as polysaccharides and phenolic compounds with well-recognized biological and pharmacological potential in therapeutic applications for human diseases[39,40]. Several physical and chemical protocols for synthesis of metallic nanoparticles are followed by the material scientists currently. In earlier studies, synthesis of gold, silver and palladium nanoparticles using seaweed *Sargassum* species, such as *S. myriocystum,*[41] *S. swartzii,*[42] *S. polycystum,*[43] *S. plagiophyllum,*[44] and *S. wightii*[45] has been successfully carried out. The application of functionalized nanomaterials in biomedical fields in recent years is derived from physical and chemical properties of nanomaterials with the combination of surface-bound ligands (natural or synthetic). As surface-bound ligands, these biomolecules or their synthetic analogues are spatially directed and render their carrier nanomaterials into multivalent biologically effective compounds. Bio-functionalization of nanomaterials can be extended to the development of antivirals that act by interfering with viral infections, particularly during entry and attachment.

Cytotoxicity analysis of *Sw*-Au nanoparticles at 2.5, 5, 10 and 25 µL showed 93.12 %, 88.40 %, 85.40 % and 85.18 % cell viability, respectively (Table 1). *Sw*-Ag nanoparticles at 5 and 10 µL showed toxicity but the lower concentrations, i.e., 1 and 2.5 µL tested were non-toxic and showed 97.21 % and 84.58 % cell viability, respectively (Table 1). *Sw*-Au nanoparticles were found to be non-toxic to Vero cells at the four different concentrations tested.

The antiviral efficacy of *Sw*-Au and *Sw*-Ag nanoparticles against HSV was estimated by the reduction of CPE caused by the virus. *Sw*-Au nanoparticles (2.5, 5, 10 and 25 µL) and *Sw*-Ag nanoparticles (0.5, 1, 2.5 and 5 µL) were tested for anti-HSV activity; both *Sw*-Au and *Sw*-Ag nanoparticles (Figs. 2&3) effectively reduced CPEs caused by HSV. Reduction of CPE was observed in a dose-dependent manner. *Sw*-Au nanoparticles reduced 70% CPE of HSV-1 and HSV-2 at 10 and 25 µL, whereas at 1 µL *Sw*-Ag nanoparticles effectively reduced 70% and 50% CPE of HSV-1 and HSV-2, respectively (Table 1). About 2.5 µL was found to reduce 70% CPE for both HSV-1 and HSV-2. *Sw*-Ag nanoparticles at 5 µL had better anti-viral activity but morphological changes were observed in Vero cells owing to toxicity (Table 2).

Bio-functionalization of nanoparticles enables higher dose drug delivery to target cells or tissue[46]. Hence, in the present study, Au and Ag nanoparticles were functionalized with *S. wightii* extract and tested against HSV. It was observed that 10 and 25 µL *Sw*-Au nanoparticles reduced 70% CPE of HSV-1 and HSV-2, whereas 2.5 µL of *Sw*-Ag nanoparticles reduced 70% CPE of both HSV-1 and HSV-2. Also, all these concentrations were non-toxic to Vero cells.

Table 1 — Dose-dependent cytotoxicity of Ag and Au nanoparticles in Vero cells

Ag nanoparticles treated (µl)	Cell viability (%)	Au nanoparticles treated (µl)	Cell viability (%)
1	97.21 ± 0.74	2.5	93.12 ± 1.79
2.5	84.58 ± 1.17	5	88.40 ± 1.67
5	43.50 ± 0.63	10	85.40 ± 1.77
10	21.91 ± 0.28	25	85.18 ± 1.54
Control	100 ± 0.00	Control	100 ± 0.00

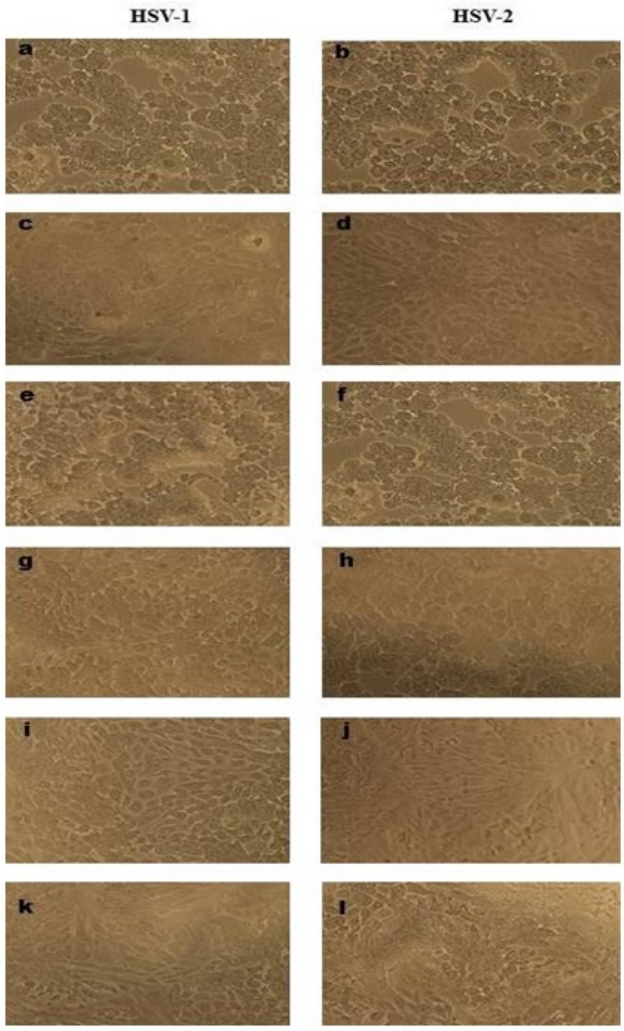

Fig. 2 — Photographs exhibiting anti-HSV 1 and 2 activity of Sw-Au nanoparticles: (a and b) virus control; (c and d) ACV drug treated (positive control); (e and f) 2.5 µL; g and h) 5 µL; (i and j) 10 µL; and (k and l) 25 µL of Sw-Au nanoparticles treated

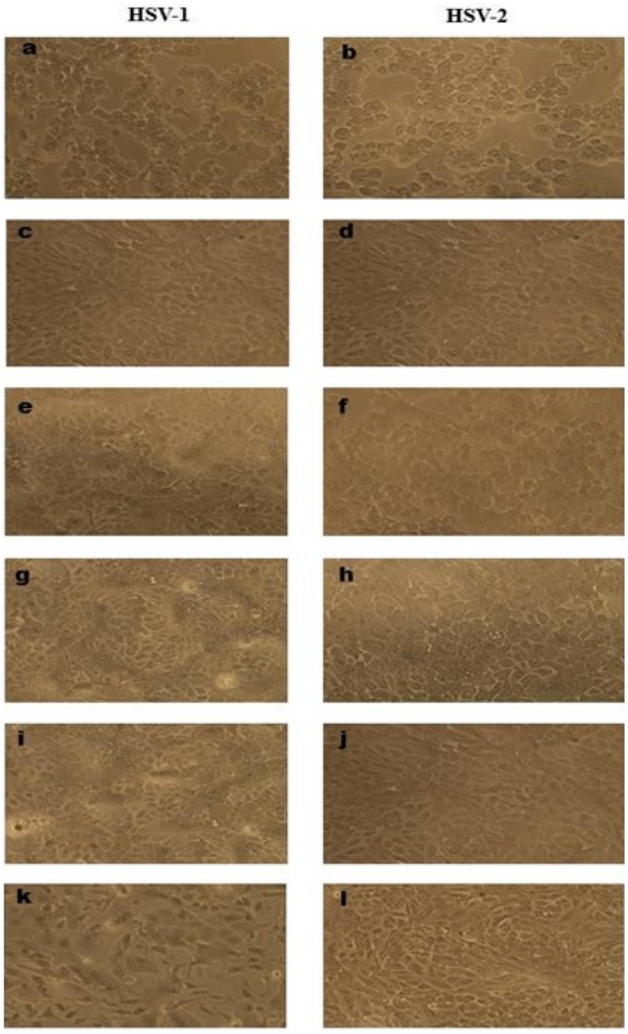

Fig. 3 — Photographs exhibiting anti-HSV 1 and 2 activity of Sw-Ag nanoparticles: (a & b) virus control; (c and d) ACV drug treated (positive control); (e and f) 0.5 µL; g and h) 1 µL; (i and j) 2.5 µL; and (k and l) 5 µL of Sw-Ag nanoparticles treated

Table 2 — Anti-HSV 1 and 2 action of Sw-Au and Sw-Ag nanoparticles at different concentrations

Conc. of nanoparticles (µL)	Viruses	
	HSV-1	HSV-2
Sw-Au nanoparticles		
2.5	Nil	Nil
5	++	++
10	+++	+++
25	+++	+++
Sw-Ag nanoparticles		
0.5	++	++
1	+++	++
2.5	+++	+++
5	+++	+++
Acyclovir (100 µg/mL)	++++	++++

Nil, no inhibition; ++, 50 % inhibition; +++ , 70% inhibition; ++++ , 100% inhibition

Mercapto-ethanesulfonate capped silver[47] and gold[48] nanoparticles inhibit HSV activity and their ability to mimic cell surface receptor heparin sulphate. Size-dependant tannic acid-modified silver nanoparticles (13 nm, 33 nm and 46 nm) are capable of reducing HSV-2 infectivity both *in vitro* and *in vivo*[49]. Through the literature, it is inferred that functionalized gold and silver nanoparticles possess size-dependent interaction and the ability to block virus attachment and entry, and this is responsible for the observed antiviral activity.

Conclusion

The study revealed that *Sw*-Au and *Sw*-Ag nanoparticles inhibit HSV-1 and HSV-2 infection to

Vero cells in a dose-dependent manner. The dose response inhibition showed that *Sw*-Au nanoparticles reduced 70% CPE of HSV-1 and HSV-2 at 10 and 25 µL, respectively. *Sw*-Ag nanoparticles effectively reduced 70% CPE of both HSV-1 and HSV-2 at 2.5 µL. Cytotoxicity analysis revealed that *Sw*-Au nanoparticles were significantly non-toxic in higher concentration of 25 µL tested and showed 85.18% of cell viability. *Sw*-Ag nanoparticles were found to be toxic in higher concentrations; up to 2.5 µL it was nontoxic and showed 84.58% cell viability. On the basis of these observations, this study concludes that bio-based preparation of metal nanoparticles could be used as novel antiviral agents.

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