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In-Vitro Studies of Bio-Silver Nanoparticles in Cytotoxicity and Anti- Inflammatory

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Keywords: Studies of Bio-Silver; Nanoparticles; Cytotoxicity; Anti- Inflammatory; Amphitheatre; nanotechnology; nanoproducts

Introduction

Nowadays, nanotechnology is a most promising amphitheatre for generating new applications in biotechnology and Nanomedicine [1]. Among several nanoproducts, a most prominent nanoproduct is nano silver. Silver nanoparticles (AgNPs) have become increasingly popular as an antibiotic agent in textiles and wound dressings, medical devices and appliances, such as refrigerators and washing machines [2]. AgNPs have been used for antimicrobial, antifungal, antioxidant, anticancer, and anti-inflammatory effects [3].

Green chemistry/Green synthesis is the design of chemical products or process that minimize the use hazardous chemical products and eliminate the environmental pollution. Therefore, green synthesis of metallic nanoparticles from biological sources instead of any other chemical products and from different methodologies is the promising and challenging field. Due to this reason, biological method has been prepared not only that it's simple but cost effective too.

In several reports it is reported biological synthesis of silver nanoparticles by plants and their antimicrobial studies, but here the divergent attitude of mycosynthesis of silver nanoparticles by mushrooms, especially medicinal mushroom such as *Ganoderma lucidum* and their anti-inflammatory and cytotoxicity studies.

Several diversity of naturally occurring mushrooms is found to have promising antioxidant and anticancer properties and prolong the longevity [4]. Mushrooms are mostly known to have anti-inflammatory, cardiovascular, antitumor, antiviral, antibacterial, hepatoprotective and hypotensive activities in biological systems [5-7]. Studies in the edible and medicinal mushroom has revealed lots of beneficial therapeutic properties of them. In our study *Ganoderma lucidum* (Fr.) Karst. (Ganodermataceae),basidiomycetous fungi, has been widely used for the general promotion of health and longevity in Asian countries for centuries [8]. This edible mushroom was considered to preserve the human vitality and to promote longevity not only that the dried powder of mushroom was used as a cancer chemotherapy agent in ancient China [9]. In addition, *Ganoderma lucidum* has been used to treat various other disorders such as allergy, arthritis, bronchitis,gastric ulcer, hyperglycemia, hypertension, chronic hepatitis, hepatopathy, insomnia, nephritis, neurasthenia,scleroderma, inflammation, and cancer [10-14].

Materials and Methods

Extraction of mushroom extract

Ganoderma lucidum mushroom obtained was washed several times with deionized water.

68g of finely blended sample was boiled for 2-5min in 300mL water and filtered. The filtrate is cooled to room temperature and used as reducing agent and stabilizer [15].

Synthesis of AgNPs

35mg AgNO3 is dissolved in 250mL water. To obtain silver colloids 6ml of mushroom was added in 30ml of AgNO3 solution. The formation of Ag nanoparticles is indicated by light yellow-brown colour and the reduction is completed in 30m. The formation of nanoparticles was examined under UV-visible spectrophotometer [15].

Characterization of the nanoparticles

The particle was characterized by UV-visible studies for preliminary confirmation, and the particles were subjected to HR-TEM studies for their size determination, EDAX to determine the percentage of metals present and XRD was done for determination of size and crystalline nature.

Human ethic clearance

All procedures involving human samples were strictly conducted in accordance with approved guidelines by

the Institutional Human Ethics Committee (Ref No: FLL/IEC/04/2014) by Frontier Lifeline Hospital - Institutional Ethics Committee (Ref: FLLH-IEC, Reg No: ECR/200/INST/TN/2013) and in accordance to the regulatory guidelines prescribed by Ethical Guidelines for Biomedical Research on Human participants, ICMR, 2006; Good Clinical Practice & Guidelines for Clinical Trials on Pharmaceutical products in India, CDSCO, DGHS, MoHFW, Govt of India, including Schedule Y 2005 and its revisions.

In-vitro anti-inflammatory

Membrane Stabilization assay

Preparation of haemoglobin rich red blood cells suspension: The blood was collected from healthy human voluntary who has not taken any NSAIDs (Non steroidal antiinflammatorry drugs) for two weeks prior to the experiment and transferred to the centrifuge tubes.It was centrifuged at 3000rpm for 10 min and were washed three times with equal volume of saline.The volume of the blood was measured and reconstructed with 10% V/V suspension with normal saline [16-18].

Heat induced haemolysis assay: The reaction mixture 2ml (Sample A and sample B) consisted of 1ml of test sample of different concentration (1.4,4.32,8.64,12.9,14.4,21.6 mg/l) and 1ml of 10% hRBCs suspension ,instead of test sample only saline was added to the control test tube.Aspirin (100µg/ml) was used as standard drug.All the test tube containing reaction mixture was incubated in water bath at 56 ^{0}C for 30 minutes.After the incubation,it was cooled for 5min.Then the reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatant was taken at 560nm using UV-Vis spectrophotometer [19,20]. The experiment was performed in triplicates and percentage inhibition of haemolysis was calculated as follows;

$$\text{Percntage inhibition} = (\text{Abs control} - \text{Abs Sample})\ X\ 100 / \text{Abs control}$$

Hypotonicity induced haemolysis assay: The reaction mixture 3ml (Sample A and B) consisted of 0.5ml of different concentration (1.4,4.32,8.64,12.9,14.4,21.6mg/l) and 2ml of hyposaline,0.5ml hRBCs suspension,in control test tube instead of test sample 0.5ml of phosphate buffer was added.Diclofenac sodium (100µg/ml) was used as standard drug. All the test tube were incubated at 37 ^{0}C for 30 minutes and centrifuged at 3000 rpm for 10 min.The absorbance of supernatant was taken at 560nm using UV-Vis spectrophotometer [21]. The percentage inhibition of haemolysis was calculated as follows;

$$\text{Percntage inhibition} = (\text{Abs control} - \text{Abs Sample})\ X\ 100 / \text{Abs control}$$

Protein denaturation

Inhibition of Albumin denaturation: The equal amount of 3ml reaction mixture (0.2ml egg albumin + 2.8ml phosphate buffer saline) was added to different concentration of test sample and make up the 2 ml of distilled water. In control test tube instead of test sample, PBS was added.Aspirin (100µg/ml) was used as standard drug. All the test tube were incubated at 370C for 15 minutes and incubated at 70 ^{0}C for 10 minutes. The samples were cooled for 5 minutes and absorbance was noted at 660nm using UV-Vis spectrophotometer [22]. The percentage inhibition of albumin denaturation was calculated as follows;

$$\text{Percntage inhibition} = (\text{Abs control} - \text{Abs Sample})\ X\ 100 / \text{Abs control}$$

Inhibition of BSA denaturation: The equal amount of 450µl BSA(1mg/ml) was added in different concentration (10,30,60,90,120,150µl/ml) of test samples and made up into 1ml of distilled water. All the rest of steps were followed according to the albumin denaturation [22].

$$\text{Percntage inhibition} = (\text{Abs control} - \text{Abs Sample})\ X\ 100 / \text{Abs control}$$

Proteinase inhibition assay: The test followed according to the modified method of [21]. The reaction mixture (2ml) containing 6µl trypsin, 1ml (20mM) tris HCl and along with different concentration of test samples.The mixture was incubated at 37 ^{o}C for 5 min after that 1 ml of 0.8% (w/v) casein was added. The mixture was incubated again for 20min, 2ml (70%) perchloric acid was added to arrest the reaction. Cloudy suspension was observed,it was centrifuged for 5 min and the supernatant was read at 210 nm using UV-Vis spectrophotometer. Control was run as same procedure but instead of test sample PBS was suspended [21]. The percentage of inhibition of antiprotienase was calculated as follows;

$$\text{Percntage inhibition} = (\text{Abs control} - \text{Abs Sample})\ X\ 100 / \text{Abs control}$$

HET- CAM assay

Pellet preparation: 7.25mg sodium dodecyl sulfate was dissolved with or without concentration of chitosan and sacchachitosan transdermal films in 1ml of agarose solution. 10µl of gelling solution was used for pellet preparation.

Incubation [22,23]: The fertile hen eggs were incubated for 75h at 37 °C and relative humidity of 80%.The eggs were kept in horizontal position and rotated several times. Then open snub end after aspiration of 10ml of albumin from hole. Eggs were traced with scalpel and thereafter the shells were removed with forceps. One pellet per egg was put on newly formed chorio-allanthoic membrane, agarose pellet without SDS acts as negative control, agarose pellet with SDS acts as positive control and agarose pellet with SDS and tested sample acts as treatment. The aperture was covered with parafilm and eggs were returned into the incubator for 24h of incubation.

Interpretation [24]: The inhibition or membrane irritation was observed. Positive control egg, exist if the irritation of membrane induced by SDS. Negative control egg, exist no irritation of membrane. Treated group egg, exist various irritation according to concentrations used.

Three irritation reactions such as hemorrhage, lysis and coagulation were monitored and image was taken with the help of SONY 14.1megapixels video camera at 35cm above CAM. Time was recorded in seconds, from addition of SDS until the

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appearance of three irritation reaction.

Calculation [25,26]: Irritation index was calculated using following e equation.

$$IR = 300\,x(IR) = 5\,x(301-TH)+7\,x\,(301-TL)+9\,x(301-TC)$$

Time (T) of hemorrhage (H), lysis (L), and coagulation (C) during a period of observation of 300 seconds. IR can take values between 0 and 21.

Relationship of starting irritation reaction was denoted by H', L', and C'.

$$H' = TH / TH - SDS$$
$$L' = TL / TL - SDS$$
$$C' = TC / TC - SDS$$

Antioxidant assay

DPPH assay: The free radical scavenging activity of the fraction was measured in vitro by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay.About 0.3 \mM solution of DPPH in ethanol was prepared and 1ml of this solution was added to 3 ml of the fraction dissolved in ethanol at different concentration.The mixture was thoroughly mixed,incubated at RT for 30 minutes.Then the reading was taken at 517 nm using UV-Vis spectrophotometer [27]. Ascorbic acid was used as standard drug.The percentage of inhibition of DPPH was calculated as follows;

$$\text{Percntage inhibition} = (\text{Abs control} - \text{Abs Sample})\ X\ 100 / \text{Abs control}$$

Lipid peroxidation assay: Liver was obtained and homogenized using phosphate buffer. Reaction mixture I consists of 1ml liver homogenized(10% W/V),1 ml different concentration of test sample.Lipid peroxidation was induced by 100µl (15mM) ferrous sulphate.Its incubated for 30 minutes at RT.0.1ml reaction mixture II (1% SDS & 0.1% thiobarbutric acid) added to all test tube and made up to 1 ml with distilled water and incubated for 1 hour at 95 °C, After butanol and pyridine was added in the ratio 2:1. The reaction was mixed thoroughly and centrifuged at 3000 rpm for 15 minutes to separate the layers. Organic layer was separated and read at 530nm using UV-Vis spectrophotometer [28]. The percentage of inhibition of lipid peroxidation was calculated as follows;

$$\text{Percntage inhibition} = (\text{Abs control} - \text{Abs Sample})\ X\ 100 / \text{Abs control}$$

Cytotoxicity assay

Cell Line and Culture Conditions: Vero and HeLa cell line were purchased from the virology dept of king's institute,Guindy,chennai . The cancer cell line was maintained in RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 100µg/ml penicillin and 100 µg/ml streptomycin in a 5% carbon dioxide (CO^2) cell incubator at 37 °C.

MTT assay: The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide dye reduction assay was performed to determine the cytotoxic effect of the AgNPs at various concentrations. The assay depends on the reduction of MTT by mitochondrial dehydrogenase, an enzyme present in the mitochondria of viable cells, to a blue formazan product. The cell concentration was adjusted to 1 × 10^5 cells/ml and plated onto 96-well flat bottom culture plates with various concentrations of AgNPs. All cultures were incubated for 24 hours at 37 °C in a humidified incubator. After 24 hours of incubation (37 °C, 5% CO2 in a humid atmosphere), 10ml of MTT (5mg/ml in PBS) was added to each well, and the plate was incubated for a further four hours at 37 °C. The resulting formazan was dissolved in 100 ml of dissolving buffer (provided as part of the kit) and absorbance of the solution was read at 595nm using an Elisa reader. All determinations were carried out in triplicate [29]. Concentrations of AgNPs showing 50% reduction in cell viability (i.e., IC50 values) was then calculated.

Apoptosis Assay - Ethidium Bromide / Acridine Orange Staining: The cells were stained to assess the level of apoptosis [30]. After the treatment period the cells were trypsinized and isolated. 25µl of cell suspension was mixed with 5µl acridine orange and 5µl of ethidium bromide. 10µl of the mixture was added to frosted glass slides and viewed under fluorescent microscope immediately. Cells were analyzed in fluorescent microscopy under 10X objective.

Statistical analysis

Data pertaining to antioxidants and anti-inflammations of silver nanoparticles were expressed as mean ± SEM, n = 6 and the data were analyzed by One-way ANOVA using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA). In all the analysis, $P < 0.05$ was considered as statistically significant.

Results and Discussions

The study on biological synthesis of silver nanoparticles has been previously reported by us [31]. Further applications and cytotoxicity of synthesized silver nanoparticles has been reported in this work.The synthesis of silver nanoparticles from *Ganoderma lucidum* was confirmed and characterized by various analytical techniques such as UV-VIS spectroscopy,HR-TEM,ICP-OES,XRD,EDAX and SAED (Figure 1-5, Table 1 & 2).

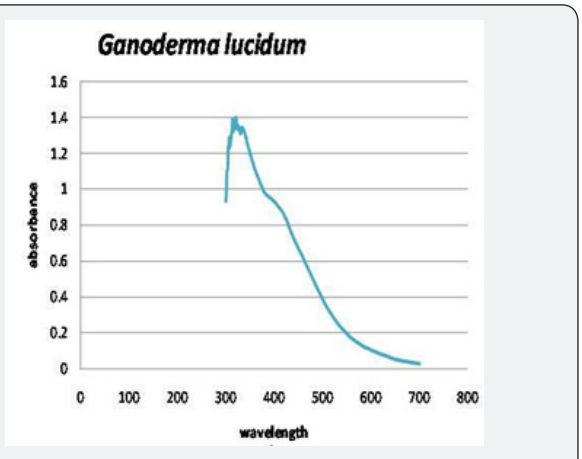

Figure 1: UV-Vis spectra of silver nanoparticles synthesized by *G.lucidum*

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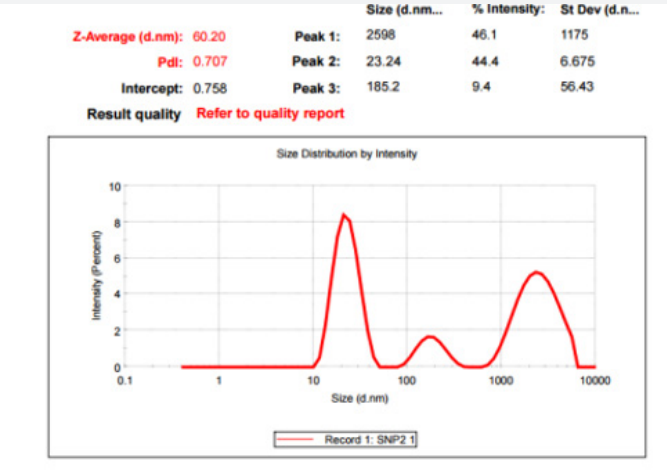

Figure 2: Particle size analysed report of silver nanoparticles.

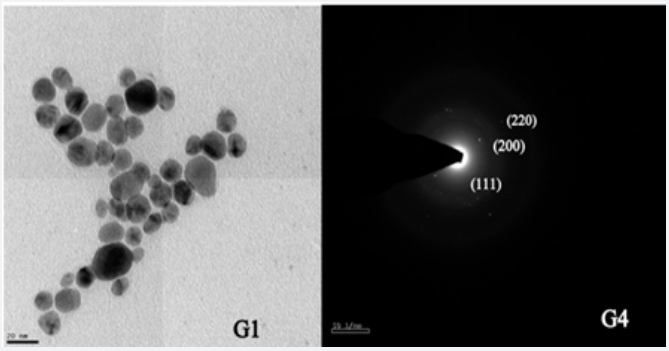

Figure 3: HR-TEM images of SNPs synthesized by G.lucidum.

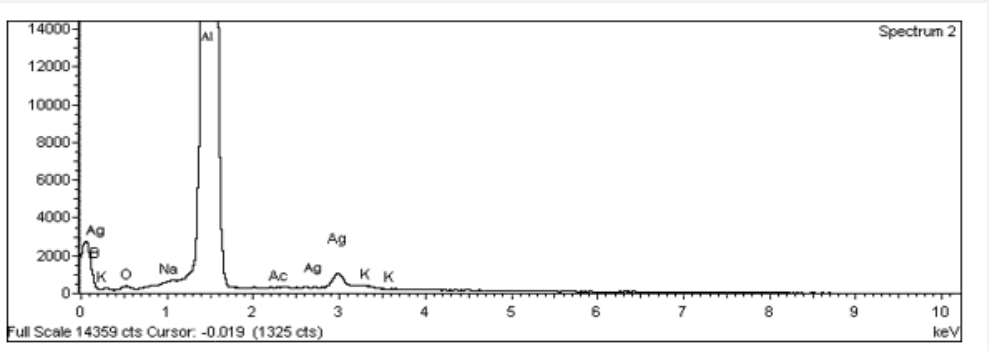

Figure 4: EDAX analysis report.

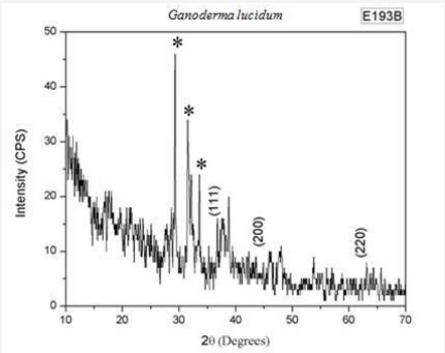

Figure 5: XRD analysis of silver nanoparticles synthesized by *G.lucidum.*

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Table 1: Presence of various components.

Element	Weight%	Atomic%
B K	1.41	3.62
O K	1.37	2.37
Na K	0.15	0.19
Al K	89.45	91.71
K K	0.61	0.43
Ag L	10.11	2.57
Ac M	0.89	0.11
Totals	100	

Table 2: ICP-OES report of silver nanoparticles.

Sample ID	Elements Symbol Wavelength (nm)	Concentration
Sample A	Ag 328.068	144.7 mg/L

Several concentration ranging from 1ssmg to 21.6mg of synthesized silver nanoparticles sample was tested for their antioxidant activity in different *in-vitro* models. It was observed that free radical scavenged by the sample (standard.Ascorbic acid and tested.Silver nanoparticles) in their unique percentage of inhibition in a concentration dependent manner. Figure 6-7 reveals the reductive capacity of tested samples compared to that of standard [32-35].

In the DPPH method, the antioxidants present in the silver nanoparticles reacts with the stable DPPH (deep violet colour) and converts it into 1, 1 diphenyl-2-picrylhydrazine with discoloration. In the present study, the percentage of inhibition of free radicals at different concentrations ranging from 1-21.6 mg for the tested samples was calculated and compared with the standard ascorbic acid and the results are revealed in Figure 6. This is the common and successfully used method for investigating both hydrophilic and lipophilic antioxidant properties [36-38]. The inhibitory effect (IC-50) follows, SNPs (9.0±.001mg/ml) and Std. Ascorbic acid (7.0±.003mg/ml) respectively. The prominent results were observed with the Bio-silver nanoparticles that may be due to synergy effect of components such as *Ganoderma lucidum* and silver nanoparticles comparable to standard ascorbic acid.

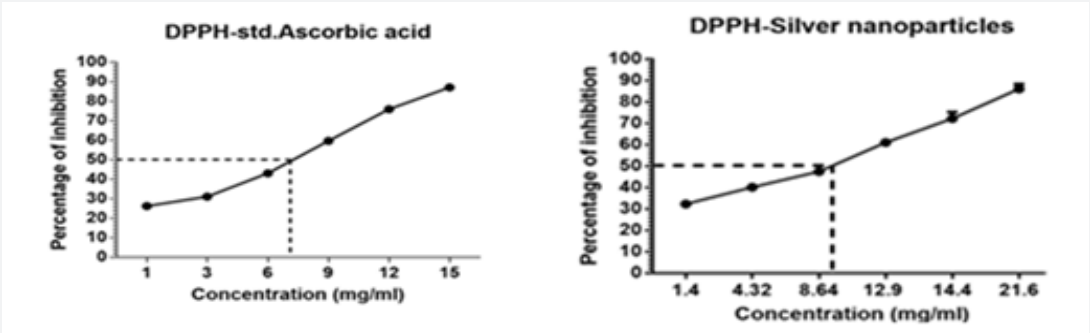

Figure 6: DPPH radical scavenging assay.

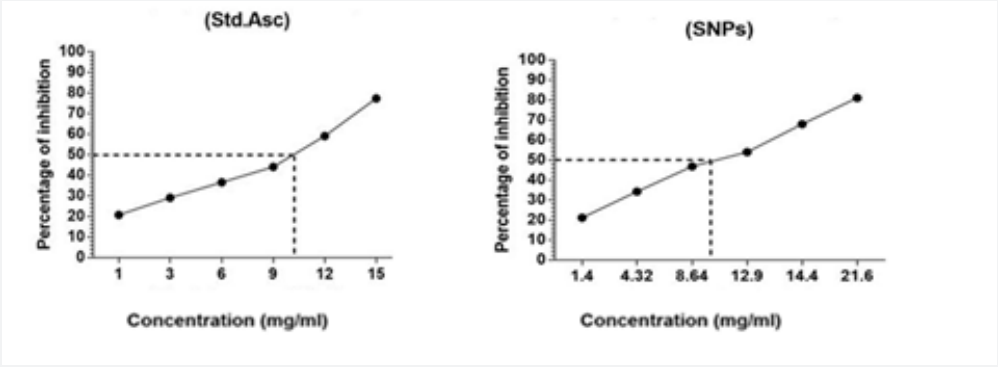

Figure 7: Lipid Peroxidase scavenging assay.

Lipid peroxidation is a critical procedure in free radical pathology as it is cause damage to cells. The liver of hen was utilized as a source of polyunsaturated unsaturated fats for determining the degree of lipid peroxidation [39]. Malondialdehyde is lipid peroxidation product is a pointer of receptive oxygen species (ROS) generation in the tissue

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restraint of lipid peroxide development by tested sample (silver nanoparticles) and standard (ascorbic acid) appeared in Figure 7.

Anti-inflammatory assays

The HRBC membrane stabilization is one of the techniques followed to study the anti-inflammatory activity. Erythrocytes membrane is closely resemblance of lysosomal membrane [40,41]. Furthermore, stabilization of the lysosomal membrane depends on the sample and the concentrate, stabilization of lysosomal membrane is vital in constraining the inflammation response by preventing the release of initiated neutrophil, for example, bacterial proteins and proteases, which creates additional tissue irritation and damage upon extracellular discharge. The enzyme released during response results in various disorder especially with chronic and acute inflammation. The role of drug administrated showed the inhibition and stabilization of lysosomal membrane.

The inhibition of hypotonicity induced HRBC membrane lysis and the stabilization of the membrane by tested sample (silver nanoparticles) and standard sample (Diclofenac sodium) was taken as the measure of anti-inflammatory activity. The percentage of inhibiting lysis of membrane depends on tested sample,it was depended on concentration gradient the tested sample (Silver nanoparticles) were significant ($P<0.05$) to that of standard drug and has been illustrated at Figure 8. Silver nanoparticles showed the maximum protection of 58% at the concentration 21.6mg, whereas diclofenac sodium showed 69% at the concentration range of 15mg.

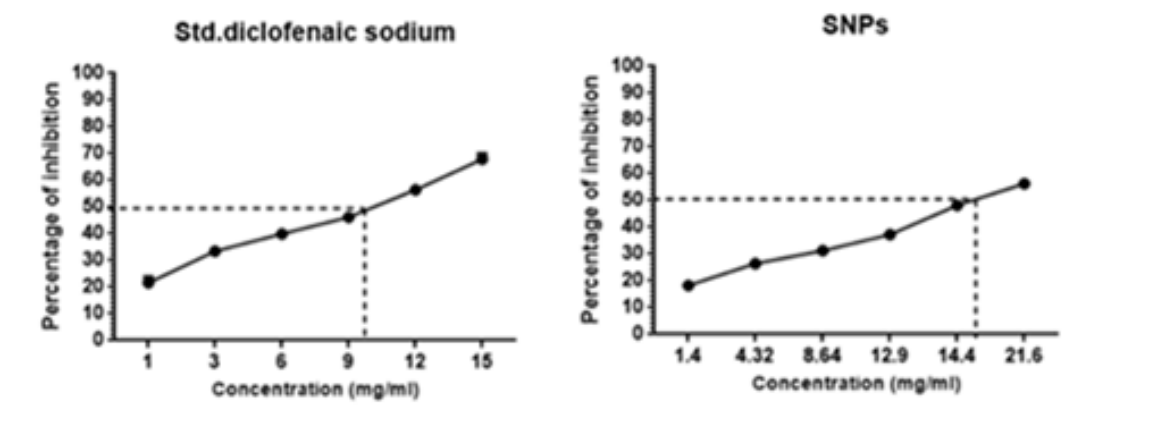

Figure 8: Membrane stabilization (Hypotonic induced).

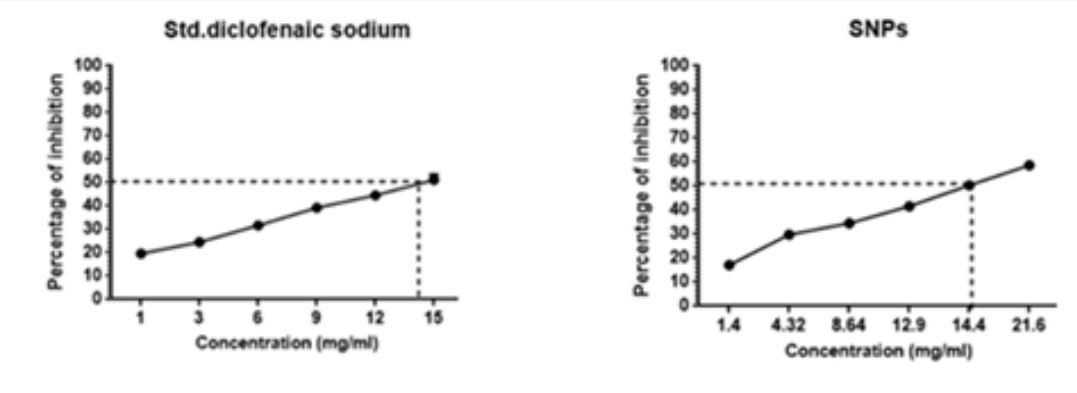

Figure 9: Membrane stabilization (Heat induced).

The tested samples were analysed to study the inhibiting reaction against the membrane lysis. From the results observed all the samples along with standard showed a good and statistically significant ($P<0.05$) protection of erythrocyte membrane from damage. The detailed report has been analysed and reported at Figure 9.

Protein denaturation is technique in which the protein loses their original form by the external stress or any other chemical compounds [42]. It is one of main cause during the inflammation process. As part of our investigation on the anti-inflammatory mechanism, different concentration of test sample along with standard were checked for the ability of inhibiting protein denaturation. It was effective in inhibiting the protein denaturation; checked with egg albumin and maximum protection was seen in tested sample (sacchachitosan transdermal film) 79.71% & 72.3% at the concentration range

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of 36.7mg/L, whereas 50% of inhibition was seen at range of 1-2mg/L, other samples shows the maximum inhibition activity such as follows GL;50.1 & 49.86% (15mg/ml), SNPs; 70.06% & 72.54% (21.6mg/ml), C; 64.32% & 67.63% (15mg/ml), Sc; 61.8 & 65.16% (15mg/ml) and CF; 73.24 & 73.23% (32.5mg/l) compared with std. diclofenac sodium; 61.78 & 74.32% (15mg/ml). Each value represented (Figure 10 &11) the average mean ± SEM; N=6. All the samples were statistically compared with the standard ****P<0.001 considered extremely significant (one-way ANOVA followed by ordinary test was performed using GraphPad Prism version 6).

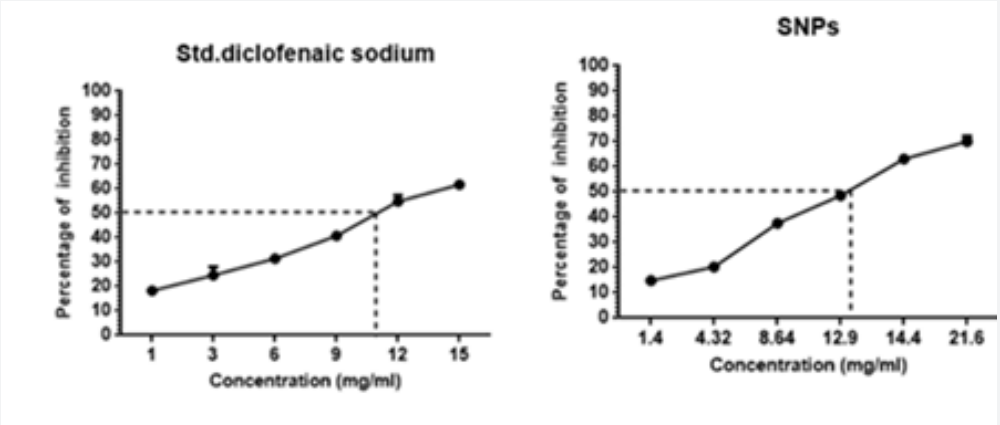

Figure 10: Protein denaturation (Egg Albumin).

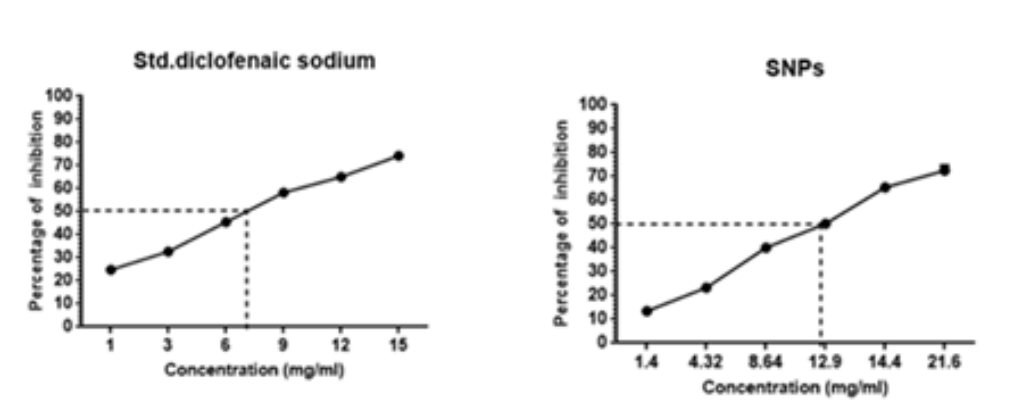

Figure 11: Protein denaturation (BSA).

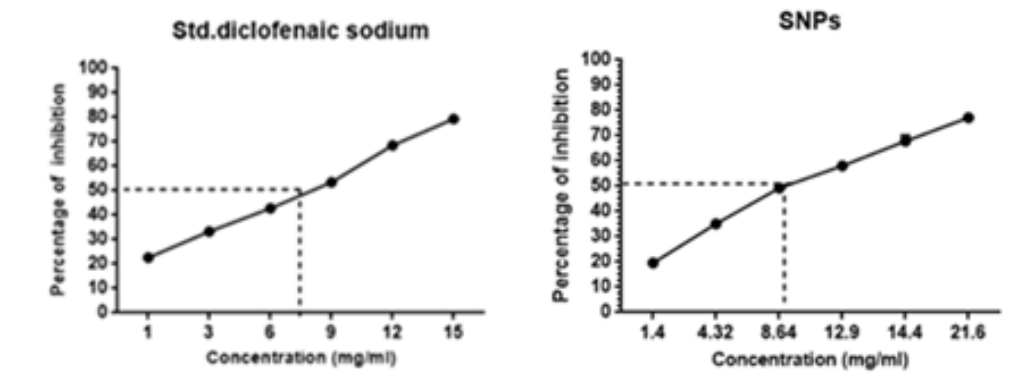

Figure 12: Proteinase inhibiting assay.

Serine proteinase is one of major enzyme released from neutrophils and plays a vital role in the development of tissue damage during inflammation response [43] and significant protection was given by proteinase inhibitors. Samples such as SNPs and standard diclofenac sodium exhibited significant anti-proteinase activity at different concentrations as illustrated in

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Figure 12. Samples showed maximum inhibition of SNPs; 77.18% (21.6mg/ml) and Diclofenac sodium; 74.36% (15mg/ml). It's significant ****P<0.001 with standard (diclofenac sodium).

HET-CAM assay is a unique model to analyse the anti-inflammatory role. The two concentrations such as low and high concentration of silver nanoparticles was taken to analyze the role of anti-inflammatory and the membrane irritation resistant. Haemorrhage, lysis and coagulation are the different parameters to be observed during this study.

The inhibitions of irritant reaction in the group of negative control (Induced-SDS) and in the treatment group (silver nanoparticles-SDS) are subjected to chorioallantoic membrane and the changes in CAM was observed and shown at Figure 13. The mean time for the initial irritation reaction in the membrane for each one from tested to control has been calculated and shown (Table 3). From the results; it clearly shows that low concentration gave less irritation compared to higher concentration. There was significant statistical difference between concentrations. Intensive studies of anti-inflammatory analyses are carried by different researchers [44-46] in context with different application. Accordingly, this study has revealed the scientific justification that silver nanoparticles posses anti-inflammatory property. Hereby may be taken further for *in-vivo* wound healing activity Figure 14.

Table 3: Scoring index.

Groups	Induced	Low concentration	High concentration
Irritation Index	19.06±1.234	4.24±1.0222	15.66±0.874

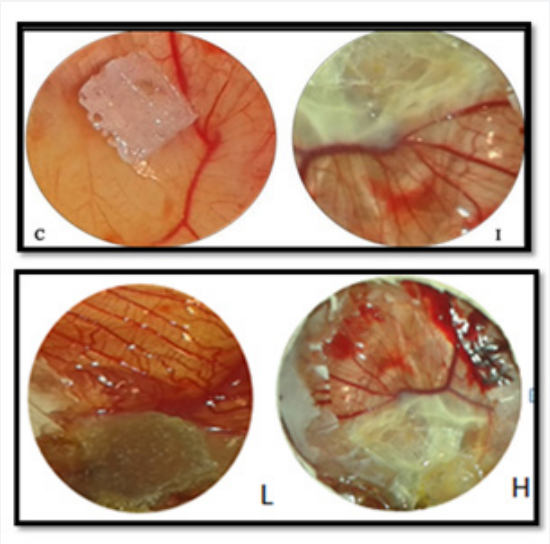

Figure 13: HET-CAM assay.

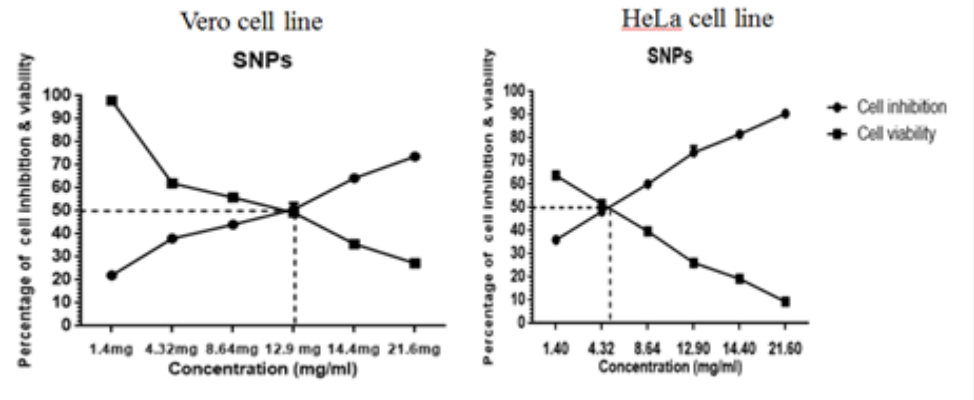

Figure 14: Showing cell viability and cell inhibition in vero and HeLa cell line.

Cytotoxicity assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay was performed to evaluate the cytotoxicity effect of silver nanoparticles against the African green monkey kidney cell (vero) and cervical cancer cell (HeLa). The different concentrations of samples were used to determine the cell viability after 24hrs incubation of cells with test samples. The

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cytotoxicity was dependent fully on concentration gradient as the concentration increased mild toxicity was observed at vero but in HeLa cytotoxicity level was higher and viability of cells are lesser (14). The data was analysed by two-way ANOVA followed by paired T tail test to determine significance difference and correlation between samples. From the results, we have observed that significant ($P<0.05$) difference and correlation ($P>0.05$) between cell viability and cell inhibition. Similar kind of studies was portrayed by [47], with different extract in to compare toxicity.Further AO-EB staining was performed to analysis the apotosis of cells, IC50 value (5.10±0.41mg/ml) of silver nanoparticles in HeLa cells reveals almost 60% Figure 15.

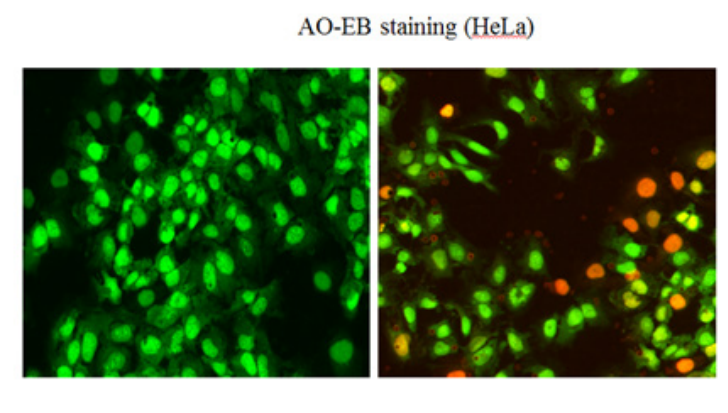

Figure 15: Acridine orange-ethidium bromide dual staining of AgNPs treated HeLa cells.

The apototosis and necrosis property of silver nanoparticles was assesd by IC 50 value against HeLa cells using acridine orange and ethidium bromide dual staining.It was incubated for 24h and picture was taken with fluorensce microscope.The treated cells showed early apoptotic cells with the appearance of fluorensce green nuclei due to nuclear fragmentation and chromatin condensation and necrotic cells indicated by orange coloured cells due to the condensation of nuclei; nuclear shrinkage and blebbing.Thus confirming apoptotic activity (see fig). Similiar kind of results were followed with different cancereous cells [48-50]. On comparison, the untreated (control) cells showed green color when stained with AO-EB, indicating their viability nature.

Conclusion

The outcomes of our research work demonstrated that the biological synthesized silver nanoparticles have indicated less toxicity impact on normal cell line than cancerous cell line and have advocated the ramifications of silver nanoparticles in curing inflammations and tumour suspected afflictions. Additionally this investigation is a bench top model and may be explored further for the anti-inflammatory and wound recuperating application.

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