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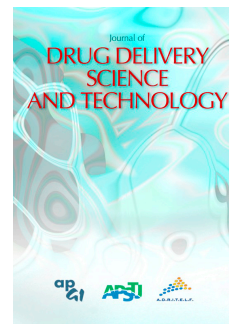
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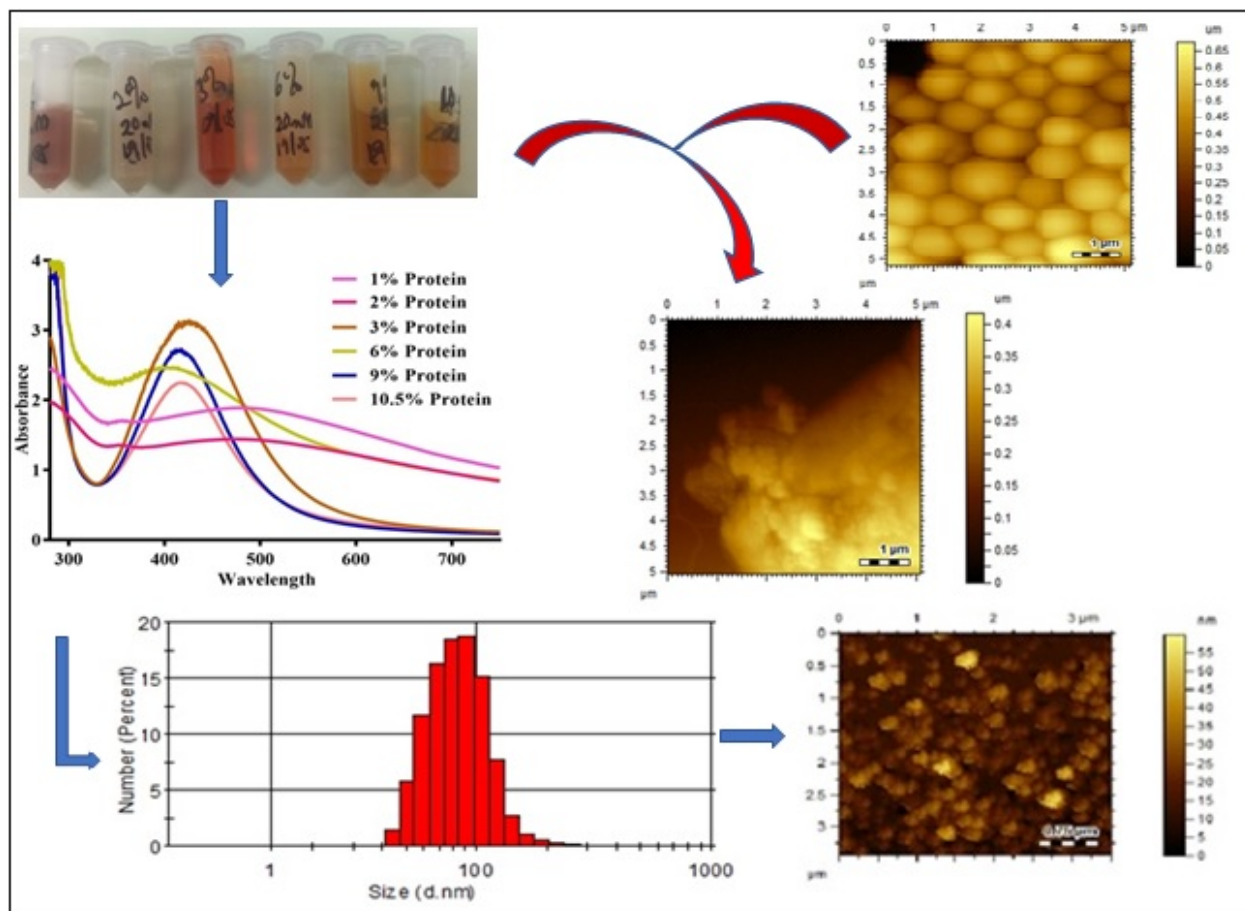
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Egg Proteins Stabilized Green Silver Nanoparticles as Delivery System for Hesperidin**Enhanced Bactericidal Potential against Resistant *S. aureus***

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Abstract

Nano-carriers enhance drugs stability and solubility and prevent their rapid degradation. Silver nanoparticles (AgNPs) are preferable for drug delivery applications due to their unique properties and less toxicity. Protein AgNPs are versatile nano-carriers as they provide the anchoring layer for drug molecules to be transported into cells. Herein, the development of a simple method for the synthesis of egg proteins stabilized AgNPs and their use as nano-carrier for hesperidin is reported. Protein stabilized AgNPs were characterized through UV-Vis spectrophotometer, FT-IR, zetasizer and atomic force microscope (AFM). The bactericidal potentials of hesperidin loaded AgNPs were studied against sensitive and multidrug-resistance bacteria strains via Tetrazolium Microplate Assay. UV-visible spectrum showed peak that corresponds to the plasmon absorbance of AgNPs. FT-IR study reveals bands which indicate effective capping of AgNPs with egg proteins. AgNPs were found spherical in nano-range size with negative charge and loaded increased amount of hesperidin. An increased bactericidal activity of hesperidin was observed against tested bacteria strains upon loading in AgNPs. Current findings suggest a facile approach for proteins stabilized AgNPs and their potential as effective nano-carrier for enhancing the bactericidal activity of hesperidin.

Keywords: Egg proteins, Silver nanoparticles, Nano-carrier, Hesperidin, Bactericidal

1. Introduction

Nanotechnology has emerged as a promising tool that can solve the problems associated with drugs like their nonspecific distribution, lack of targeting specificity, lower aqueous solubility, lower therapeutic index and systemic toxicity [1]. Developments made in the field of nanoparticles (NPs) during last few decades have revolutionized the treatment and diagnosis of various diseases [2]. NPs offer distinct advantages such as increased bioavailability and blood half-life of poor aqueous soluble drugs due to their protection during transit to target sites, on-demand release, loading of multiple drugs and/or diagnostic agents, real-time therapeutic outcomes and improved targeted delivery with their minimum side effects on healthy tissues [3, 4]. Inorganic NPs possess unique physicochemical and biological properties such as optical and magnetic properties, large surface-volume ratio, easy functionalization with many ligands for targeted drug delivery [5]. Silver NPs (AgNPs) have been the subject of greater scientific interest and have been exploited for drug delivery and diagnostic applications. They are less toxic, membrane penetrating, surface enhancers, and have intriguing optical surface properties [6]. Moreover, AgNPs have shown strong broad inhibitory and bactericidal effects [7].

Several approaches have been used to synthesize AgNPs, mostly involved the use of synthetic reducing agents. These hazardous reducing agents are associated with adverse biological effects [8]. Therefore, the use of biological reducing/ stabilizing agents (green chemistry approach) is a benign replacement over other chemical reagents since they are biocompatible, cost effective, environment friendly and renewable. Furthermore, the use of biological agents affords slow kinetics to self-reduced metal precursors and the development of stable coating layers to prevent particle aggregation. For instance, AgNPs have been synthesized using biological capping agents such as microorganisms [9, 10], plant extracts [11, 12], and

natural polymers [13]. Proteins are natural polymers with different functional groups and polypeptide linkages which can facilitate reduction and stabilization of NPs. The use of proteins as stabilizing/ reducing agent is advantageous as it acts as the anchoring layer for drug or genetic materials to be transported into cells [14].

Egg white is transparent liquid within egg composed of proteins that are beneficial to human body. Studies have shown that over 24 different proteins are present in egg white [15], of which the major ones are ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucoid (3.5%), and lysozyme (3.4%) [16]. Ovalbumin, the major protein of chicken egg white, is responsible for most of its multiple functional properties, such as foaming, emulsification, binding (adhesion) and heat-setting in addition to their nutritional significance [17]. The ease of handling, availability, solubility and affinity for metal ions in solution make egg proteins ideal candidate for the synthesis of NPs with interesting properties. The use of egg proteins as reducing/ stabilizing agent for the synthesis of AgNPs would be a simple and innovative concept for designing drug nano-carrier system.

Flavonoids are naturally occurring compounds found abundantly in fruits, vegetables and beverages [18]. These class of compounds exhibit wide range of biological activities and have been of greater pharmacological importance. Hesperidin is a major flavanone glycoside of the citrus flavonoids and is extracted from fruits and peels of citrus species [19]. Recent studies have shown that hesperidin has promising pharmacological properties such as antioxidant [20], anti-inflammatory [21], anti-proliferative [22] and anti-carcinogenic activity [23]. Hesperidin is found abundantly in nature and is highly cost-effective. Its lower aqueous solubility results in its poor absorption, thus limits its clinical utility. It is, therefore, imperative to design a new and efficient drug delivery system to enhance its therapeutic efficiency. Herein, we report the

development of a simple method for the synthesis of egg proteins stabilized AgNPs and their use as nano-carriers for enhancing the solubility and bactericidal potentials of hesperidin.

2. Materials and Methods

2.1. Materials

Fresh chicken eggs were purchased from local mall (Karachi, Pakistan). Silver nitrate, L-ascorbic acid sodium salt (sodium ascorbate), sodium hydroxide (NaOH), hydrochloric acid (HCl), bovine serum albumins (BSA), bicinchoninic acid solution, sodium chloride (NaCl) and hesperidin were purchased from Sigma (Germany). Tryptic soy agar, Soya agar, Mueller-Hinton broth and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Oxoid (UK). Double distilled water was used throughout the research.

2.2. Isolation of Egg Proteins

Egg proteins were isolated from fresh chicken eggs. Chicken egg was carefully broken, and the yolk was separated from the egg white. Egg white stock solution (20% w/v) was prepared by taking egg white in distilled water and was stirred at room temperature for 60 min. The mixture was filtered twice with WhatmanTM filter paper grade 1. The clear filtrate obtained (egg proteins) was stored at 4 °C for further use. Proteins were quantified through bicinchoninic acid (BCA) assay. BSA standard solutions were prepared in a concentration range of 0.025-2 mg/mL. BSA standard solution (20 µL) of each concentration was transferred to microplate well. Each well was added 200 µL BCA solution followed by incubation for 30 min at 37 °C and absorbance was noted at 562 nm. The same procedure was followed for 20% egg white solution after serial dilution to bring its absorbance in the range of BCA assay.

The identification of the obtained egg proteins was carried out with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide gel comprising 0.1% of SDS. A 10 μ l aliquot of sample was applied to SDS-PAGE and run at constant current (150 V) for 55 min in buffer pH 8.3 of tris-glycine comprising 0.5% SDS. The Coomassie B. Blue was used to stain the SDS-PAGE for proteins.

2.3. *Synthesis of Protein Stabilized AgNPs (Pro-AgNPs)*

Pro-AgNPs were synthesized using a simple green method. In a typical synthesis, AgNO_3 (4 mL, 20 mM) was added to egg proteins (9% w/v, 34 mL) under stirring. L-ascorbic acid sodium salt (0.01 M, 2 mL) was added to the mixture to make the final volume 40 mL. The mixture was stirred for 120 min at 39-41 °C. The color of the solution gradually changed from white to brownish yellow indicating the formation of Pro-AgNPs. The resulting NPs suspension was centrifuged at 7000 rpm for 15 min to remove excess reagents. Supernatant was stored at room temperature for further analysis. The formation of Pro-AgNPs was further authenticated with UV-visible Spectrophotometer (UV-240, Shimadzu, Kyoto, Japan). Reaction conditions were optimized through investigating the effects of parameters such as protein concentration (1-10.5%), Ag ion concentration (10-50 mM), and absence of sodium ascorbate on NPs formation.

2.4. *Stability Study*

Stability study was carried out on the optimized Pro-AgNPs by incubating the NPs with human blood plasma or subjecting them to presence of salt, acid or base. Salt stability study was carried out by mixing the NPs solution with different concentrations (0.5-3.0 M) of salt. Typically, different concentrations of NaCl (0.5-3.0 M) were mixed with Pro-AgNPs solution in a 1:1 volume ratio. The mixture of Pro-AgNPs solution with distilled water at same volume ratio

was used as reference. The absorbance of each mixture was read immediately. Acid- base stability study was examined by adjusting the pH of Pro-AgNPs solution (2.0 mL) with conc. HCl or NaOH solution in 1-12 range and absorbance was read immediately. Pro-AgNPs solution without pH modification was used as reference. Plasma stability study was investigated by incubating 1:1 volume ratio mixture of Pro-AgNPs solution and diluted human blood plasma for 26 h. The absorbance of the mixture was read at different time interval (0-26 h).

2.5. *Hesperidin Loading on Pro-AgNPs*

Hesperidin was loaded in Pro-AgNPs by stirring the mixture of hesperidin (10 mg) and Pro-AgNPs solution (5 mL) at moderate speed for 24 h. The resulting mixture was centrifuged at 12000 rpm for 20 min to obtain Hes-Pro-AgNPs pellet. Drug loading efficiency (DLE) was determined using UV-visible spectrophotometer. Hes-Pro-AgNPs suspension containing specific amount of hesperidin was taken and centrifuged at 12000 rpm for 20 min. Supernatant containing free drug was diluted with DMSO and read spectrophotometrically. Hesperidin was detected at 285 nm and was quantified using hesperidin calibration curve in the range of 5-35 µg/mL. DLE was calculated using the equation below:

$$\text{DLE} = \frac{\text{Amount of drug loaded}}{\text{Total amount of drug}} \times 100$$

2.6. *Plasma Stability Study for Hesperidin Loaded Pro-AgNPs*

The stability of Hes-Pro-AgNPs in the presence of human blood plasma was explored by incubating the drug loaded Pro-AgNPs with diluted plasma up to a period of 24 h using a 1:1 volume ratio. Samples were withdrawn at specific time intervals and were processed for

determination of drug retained according to the method mentioned for drug loading efficiency. The stability was determined with reference to the quantity of drug retained.

2.7. Characterization

The Pro-AgNPs and Hes-Pro-AgNPs were characterized with UV-Vis spectroscopy, AFM, FTIR and zetasizer. UV-Vis spectroscopic analysis was carried on UV-Vis spectrophotometer by scanning at a range of 190-900 nm. Fourier Transform Infrared (FT-IR) spectra of protein, Pro-AgNPs, hesperidin and Hes-Pro-AgNPs were recorded in a range of 400-4000 cm^{-1} using spectrometer (IR-470, Shimadzu Kyoto, Japan). The samples were properly mixed with KBr powder at 1% (w/w) and pressed for obtaining self-supporting disks. Inductively coupled plasma mass spectrometer (ICP-MS) was used to determine the concentration of AgNPs in solution. Atomic force microscope (AFM, (5500, Agilent, Santa Clara, USA) was used to determine the shape of Pro-AgNPs and Hes-Pro-AgNPs by placing a drop of the NPs solution on mica slide and air drying at room temperature. Using AFM with tip (Tap300-G; length 125 μm and force constant 40 N/m) the dry slides were visualized and imaged in noncontact mode using. The average hydrodynamic diameter, polydispersity index (PDI) and zeta potential of the Pro-AgNPs and Hes-Pro-AgNPs were performed using Zetasizer (ZS-90 Malvern instruments, Malvern, UK). This was carried out by taken readings of sample solutions directly at scattering angle of 90° at 25 °C without any prior sample preparation.

2.8. Antibacterial study

2.8.1. Bacterial strains

Both sensitive and resistant of Gram-positive *Staphylococcus aureus* (*S. aureus*) were selected for antibacterial assay. Sensitive *S. aureus* ATCC (American Type Culture Collection)

6538 and Methicillin-resistant *S. aureus* ATCC 6538 strains stock cultures were kept on tryptic soya agar (Oxoid, UK) at 4 °C. All the microbial strains were sub-cultured on a fresh appropriate agar plate for 24 h prior to antibacterial test. Inocula were prepared by transferring several single colonies of microbes to a sterile Mueller-Hinton broth. The bacterial cell suspension was mixed to homogeneity to give a final density of 5×10^5 cfu/mL and these were confirmed by viable counts. The infective dose of most microorganisms is 10^5 cfu/mL.

2.8.2. Tetrazolium Microplate antibacterial assay

The minimum inhibitory concentration (MIC) of test samples and reference materials were determined using Tetrazolium Microplate assay [24]. The assay was performed by using 96-well clear microtiter plate. Freshly harvested bacterial cells suspensions of both sensitive and resistant strains *S. aureus* were seeded at 5×10^5 cfu/mL in each well of 96 wells plate. Hesperidin, Pro-AgNPs and Hes-Pro-AgNPs were serially diluted in Muller-Hinton broth in 10-1000 μ g/mL range. Each test sample (200 μ L) was added in triplicate wells and the plates were incubated for 18-24 h at 37 °C \pm 0.5. After incubation, MTT (50 μ L, 0.2 mg/mL) was added to the plate followed by incubation at 37 °C for 30 min. An appropriate solvent blank (DMSO) and bacterial suspensions without any test sample were included as negative control and positive control respectively. The absorbance was measured at 570 nm and a reference wavelength of 650 nm by adding DMSO on a spectrophotometer and the percentage reduction of the dye (indicating the bacterial growth inhibition) was calculated [25] using:

$$\text{IC}_{50} = (\text{O.D. in Control} - \text{O.D. of test} / \text{O.D. in control}) \times 100$$

2.8.3. Statistical analysis

All the experiments were carried out in triplicate form and results were expressed as mean \pm SEM.

3.0. Results and Discussion

3.1. Isolation of egg proteins from Chicken Egg white

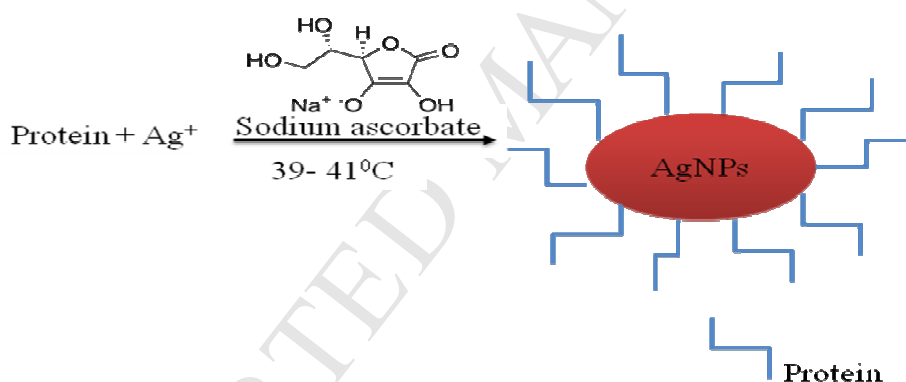
Egg proteins, a mixture of various proteins in egg white, are mostly soluble in distilled water. Egg proteins were obtained from egg white concentrate after processing through dissolution, stirring and filtering. The process of isolation of egg proteins from egg white is depicted in Fig. 1. BCA assay revealed that each mL of 20% egg white solution contains 18.04 ± 0.52 mg of proteins. SDS-PAGE gel electrophoresis was performed for the identification of proteins in the obtained protein mixture. Various pure proteins were observed in 25-150 kD as shown in Fig. 2. Protein contents in the range of 37-75 kD constitute the highest portion of proteins mixture which is attributed to the presence of egg ovalbumin.

Fig. 1 and 2 to be placed here

3.2. Synthesis of Pro-AgNPs

The pathway for the green synthesis of Pro-AgNPs is shown in scheme 1. Synthesis of NPs from the precursor AgNO_3 was mediated with proteins from egg white concentrate and sodium ascorbate, both of which are natural and biocompatible molecules. The protein functions as reducing and stabilizing agent in the synthesis of the AgNPs while sodium ascorbate complements the reducing function of the protein as well as speeds up the reduction process. During the synthesis, the reaction mixture color changed from white through yellow and finally to reddish brown (Fig. 3). The synthesis is envisioned to involve electrostatic complexation of

Ag ions with oppositely charged groups (such as free thiol) present in protein molecules before nucleation of Ag with sodium ascorbate, followed by isotropic growth through attachment of OH or N-H groups and subsequent formation of stable spherical AgNPs. Ovalbumin is major egg protein and its extract consists of 385 amino acids [17] one of which is L-cysteine which has free thiol groups that may act as bonding sites before the nucleation process. The amine, thiol, carboxyl and hydroxyl groups present in the protein and or sodium ascorbate are capable of donating electron for the reduction of Ag^+ to Ag^0 and also stabilize the newly formed NPs. The synthesized Pro-AgNPs showed characteristic surface plasmon resonance peak at 420 nm (Fig. 5) as authenticated by UV-Vis spectral analysis with a total concentration of 21 ppm of silver ions as determined through ICP-MS analysis.



Scheme 1: Green synthesis of egg proteins stabilized AgNPs

Fig. 3 to be placed here

3.2.1. Effect of Proteins Concentration on AgNPs Formation

The synthesis of Pro-AgNPs was optimized through varying parameters such as protein concentration, silver ion concentration and absence of sodium ascorbate while keeping all other reaction conditions constant. Proteins concentration was varied in the range of 0-10.5% to investigate their effect on AgNPs formation. Upon the addition of Ag ion (4 mL, 20 mM) to 1%

protein concentration (34 mL), a dark brown solution (Fig. 4b) with a very broad surface plasmon resonance (SPR) peak was obtained (Fig. 4a) which indicates poorly formed (i.e. stabilized) AgNPs. Similar phenomenon was observed when 2% proteins concentration was used under same reaction condition. However, a sharp surface plasmon resonance (SPR) peak with high absorbance value was obtained with 3% protein concentration under same reaction condition. Proteins concentration of 9% yielded a sharper SPR peak though with decrease in absorbance value. A decrease in absorbance value with slight broadness of SPR was found when proteins concentration was further increased to 10.5%. At 0% proteins concentration, NPs were not formed which indicates the significant contribution of the presence of proteins in the reduction of Ag^+ to Ag^0 and hence NPs formation. Similarly, in absence of sodium ascorbate, no NPs was formed which signifies the involvement of sodium ascorbate in the reduction process. Results of the study suggest that increase in proteins concentration favors NPs formation, however, with significant effect on size distribution which is related to the narrowness of the peak. Proteins concentration of 9% affords absorption peak with good size distribution hence was adopted for further studies.

Fig. 4 to be placed here

3.2.2. *Effect of Ag ion Concentration on AgNPs Formation*

The significance of Ag ion concentration on NPs formation under same reaction condition was exploited in the range of 10-50 mM. Though, increase in Ag ion concentration from 10-30 mM favors NPs formation (Fig. 5a-b), there was an observed shift in the SPR which may be attributed to size distribution as confirmed by the shape of the peak. A red shift was observed when Ag ion concentration was increased from 10-20 mM (i.e. from 415 nm – 420 nm)

while a blue shift was observed from 20-30 mM (420 nm – 418 nm). At 50 mM, AgNPs was not formed under the reaction condition. A concentration of 20 mM Ag ion concentration was adopted for further studies.

Fig. 5 to be placed here

3.3. *Stability Study*

The criteria for the selection of any NPs for drug delivery application is its ability to withstand the acidic or basic biological environment, saturated salt, and the presence of plasma proteins [26, 27]. The optimized Pro-AgNPs was investigated for stability study against plasma, salt and pH changes. Drug delivery systems should be strong enough and should not be destabilized in the presence of various plasma proteins. Plasma stability study of the synthesized NPs was performed by incubating the NPs with diluted human plasma up to 26 h. Incubation of NPs with plasma for a period of 4 h did not cause any instability as their characteristic SPR peak did not change significantly (Fig. 6a). When the incubation time was increased, the characteristic SPR peak of NPs became sharper with increased absorption intensity. The absorption peak was found highly intensified for 26 h incubation period. Results of the study show that the peak intensity of NPs increases upon increasing incubation time but with no significant shift.

The NPs were stable in the presence of different concentration of salt (0.5-3.0 M). There was no significant shift in the SPR peak and the absorbance intensity of the Pro-AgNPs up to 3M concentration of salt (Fig. 6b). pH stability screening was demonstrated in a pH range of 1-12. The NPs were stable at the pH range, however, slight increase in the intensity of absorbance with no significant shift in the SPR peak was observed at pH 5 and 6 (Fig. 6c). Results of the stability

studies indicate that the prepared Pro-AgNPs are stable and can be employed as carriers for biomolecules such as drugs.

Fig. 6 to be placed here

3.4. *Size, Surface Charge and Loading Efficiency*

The morphology, size and surface charge of the prepared NPs were assessed using AFM and zetasizer as described previously. AFM revealed the smooth film of protein capped AgNPs (Fig. 7a). However, change in the surface smoothness was observed upon hesperidin, confirming its presence (Fig. 7b). Furthermore, the mean particle size of empty Pro-AgNPs is smaller (122.9 ± 0.77 nm) as compared to Hes-Pro-AgNPs (257.10 ± 1.48 nm) as shown in Table 2. Nevertheless, both empty and hesperidin loaded NPs are in nano-range and predict better physical stability as well as biological performance of the loaded drug [26]. Hes-Pro-AgNPs were capable of loading increased concentration of hesperidin ($83.29 \pm 1.36\%$). This in turn predicts improved therapeutic efficacy of drug as increased drug loading results in delivery of the drug in higher concentrations to the target sites [28]. Pro-AgNPs were found evenly distributed in size with a PDI value of 0.26 ± 0.01 . Hesperidin loaded NPs demonstrated to be widely distributed as depicted from their PDI value (0.45 ± 0.01). Comparatively larger size distribution of Hes-Pro-AgNPs can be attributed to adsorption of the drug on the NPs surfaces, thus in turn authenticates successful loading of the drug. Moreover, Pro-AgNPs were found negatively charged with -18.52 ± 0.29 mV net zeta potential. The surface negativity of NPs can be attributed to involvement of anionic functional groups in NPs reduction/ stabilization. The surface of NPs further increased upon loading of hesperidin (-20.20 ± 0.43 mV) due its anionic groups. This further authenticates the efficient and successful loading of hesperidin in Pro-AgNPs. Increased

negative surface charge of NPs ascertains a higher physical stability as the particles get repelled and do not fuse, thus remain suspended for longer period of time.

Fig. 7 to be placed here

Table 1 to be placed here

3.5. *Fourier Transform Infrared Spectroscopy (FTIR)*

The functional groups that decorated the surface of the synthesized Pro-AgNPs and Hes-Pro-AgNPs were probed using FTIR spectroscopic analysis. The characteristic band assignments of the spectra of proteins and Pro-AgNPs shown in Fig. 8 are given in Table 2. A reduction in amide band ($C=O_{str}$, 1663 cm^{-1}) of the proteins was observed in the spectrum of Pro-AgNPs ($C=O_{str}$, 1650 cm^{-1}). Similar shift in band was observed for N-H vibrational bending mode and $C-O_{str}$ of the proteins from 1550 cm^{-1} to 1546 cm^{-1} and from 1076 cm^{-1} to 1073 cm^{-1} in Pro-AgNPs spectrum (Table 2) respectively. In addition, the band at 3410 cm^{-1} was red shifted to 3413 cm^{-1} . The observed shift in bands indicates the involvement of these functionalities in the reduction and stabilization of AgNPs.

The spectrum of Hes-Pro-AgNPs (Fig. 8) reveals all the characteristic bands present in the spectrum of hesperidin with no significant shift in bands as shown in Table 2. However, change in intensity was observed for almost all the bands which may suggest change in concentration or composition. This may be linked to non-covalent interaction between the loaded hesperidin molecules and Pro-AgNPs i.e. the mechanism of the drug loading may involve complexation of the drug with the Pro-AgNPs carrier or through encapsulation. The functional groups identified from FTIR analysis corroborate effective capping of AgNPs with proteins and the stability of loaded hesperidin, thus indicates the potential of Pro-AgNPs as a carrier vehicle.

Fig. 8 to be placed here

Table 2 to be placed here

3.6. Plasma Stability study of Hes-Pro-AgNPs

The stability study of the hesperidin loaded NPs against human plasma was investigated by incubating Hes-Pro-AgNPs with diluted human plasma up to 24 h and results are given in Table 3. Stability was evaluated in terms of amount of drug retained by the NPs after incubation with plasma up to 24 h. Results of stability study revealed that NPs were capable of retaining $80.00 \pm 0.55\%$ after 24 h incubation with plasma. Initially, about 15% drug was found to be released at 3rd h of the stability study. This relatively increased release of the drug can be due to the loosely bound drug on the surfaces of NPs. Plasma stability of the drug loaded NPs that they are highly stable upon their contact with plasma and do not release their surface tethered drug molecules abruptly. This authenticates the Pro-AgNPs applicability for designing a highly stable nano-carrier drug delivery system.

Table 3 to be placed here

3.7. Tetrazolium Microplate antibacterial assay

Hesperidin and its loaded Pro-AgNPs were investigated for their antibacterial potential against both sensitive and resistant *S. aureus* using tetrazolium microplate assay. Hesperidin revealed MIC value of $74.33 \pm 2.80 \mu\text{g/mL}$ against sensitive *S. aureus* strain as shown in Fig. 9(a). Its bactericidal potential increased upon its loading in Pro-AgNPs with MIC value of $23.71 \pm 1.93 \mu\text{g/mL}$. Empty Pro-AgNPs revealed MIC of $52.43 \pm 1.53 \mu\text{g/mL}$ against the same *S. aureus* sensitive strain. Hesperidin, Pro-AgNPs and Hes-Pro-AgNPs revealed 588.39 ± 1.65 ,

110.51 \pm 2.83 and 73.22 \pm 0.87 $\mu\text{g/mL}$ IC_{50} values respectively against *S. aureus* sensitive strain as shown in Table 4. When investigated against resistant *S. aureus*, Hesperidin revealed MIC value of 400.31 \pm 3.57 $\mu\text{g/mL}$ as shown in Fig. 9(b). Similarly, its bactericidal potential increased against resistant *S. aureus* upon its loading in Pro-AgNPs, showing MIC value of 53.88 \pm 2.63 $\mu\text{g/mL}$. Empty Pro-AgNPs showed mild bactericidal potential against resistant *S. aureus* with MIC value of 195.76 \pm 2.33 $\mu\text{g/mL}$ as shown in Fig. 9(b). Hesperidin, Pro-AgNPs and Hes-Pro-AgNPs revealed 980.05 \pm 2.80, 410.73 \pm 2.49 and 110.57 \pm 2.41 $\mu\text{g/mL}$ IC_{50} values respectively against *S. aureus* resistant strain as shown in Table 4. The improvement in hesperidin bactericidal potentials upon loading in Pro-AgNPs can be linked to the increase in bacterial cell membrane permeability caused by AgNPs [29]. AgNPs to interact with proteins in bacterial cell wall thus increase its permeability and facilitate the internalization of their surface tethered drugs [30]. Moreover, AgNPs have been inherently bactericidal, thus exerting a synergistic effect for their loaded drug molecules [31].

The unique physic-chemical properties of AgNPs make them versatile systems for drug delivery applications. However, their strong oxidative activity results in various negative effects including immunological responses, genotoxicity, cytotoxicity and even cells death [32, 33]. Surface coating of AgNPs provides protection against cytotoxicity. The nature of coating materials greatly affects the pattern of AgNPs toxicities. AgNPs surface coating with biological molecules like polysaccharides and proteins reduces their associated toxicities [34, 35]. In the current study, Pro-AgNPs revealed lower bactericidal potentials as compared to their hesperidin loaded counterparts. The relative decreased in toxicity of empty AgNPs can be attributed to their coating with biopolymer such as egg proteins. Thus, egg proteins employed as stabilizers or surface coating materials decrease the toxicities related to drug loading nano cargo AgNPs.

Fig. 9 to be placed here

Table 4 to be placed here

4. Conclusion

Egg proteins stabilized AgNPs were successfully synthesized through a simple, cost effective, green chemistry approach using egg white extract as the protein source. The potential of the optimized Pro-AgNPs as effective nano-carrier for hesperidin, a water insoluble bioflavonoid was evaluated. The result obtained FTIR, UV-Vis, AFM and Zeta potential analysis suggest stable, hydrophilic NPs with high loading capacity for hesperidin. The hesperidin loaded NPs were found to exhibit higher bactericidal activity than hesperidin and empty NPs when assessed through Tetrazolium Microplate MIC assay. The study indicates that the use of the prepared Pro-AgNPs as carrier for hesperidin enhance its therapeutic effectiveness via increased bactericidal activity.

Conflict of interests

All the authors declare no conflict of interests

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References

1. He, H., S. Ghosh, and H. Yang, *Nanomedicines for dysfunctional macrophage-associated diseases*. Journal of Controlled Release, 2017. **247**: p. 106-126.
2. Moghimi, S.M., A.C. Hunter, and J.C. Murray, *Nanomedicine: current status and future prospects*. The FASEB journal, 2005. **19**(3): p. 311-330.
3. Chung, B.L., et al., *Nanomedicines for endothelial disorders*. Nano today, 2015. **10**(6): p. 759-776.
4. Sun, T., et al., *Engineered nanoparticles for drug delivery in cancer therapy*. Angewandte Chemie International Edition, 2014. **53**(46): p. 12320-12364.
5. Ojea-Jimenez, I., et al., *Engineered inorganic nanoparticles for drug delivery applications*. Current drug metabolism, 2013. **14**(5): p. 518-530.
6. Brown, P.K., et al., *Silver nanoscale antisense drug delivery system for photoactivated gene silencing*. ACS nano, 2013. **7**(4): p. 2948-2959.
7. Shankar, S.S., et al., *Rapid synthesis of Au, Ag, and bimetallic Au core–Ag shell nanoparticles using Neem (Azadirachta indica) leaf broth*. Journal of colloid and interface science, 2004. **275**(2): p. 496-502.
8. Agasti, N., V.K. Singh, and N. Kaushik, *Synthesis of water soluble glycine capped silver nanoparticles and their surface selective interaction*. Materials Research Bulletin, 2015. **64**: p. 17-21.
9. Raliya, R. and J. Tarafdar, *Novel approach for silver nanoparticle synthesis using Aspergillus terreus CZR-1: mechanism perspective*. Journal of Bionanoscience, 2012. **6**(1): p. 12-16.

10. Rodrigues, A.G., et al., *Biogenic antimicrobial silver nanoparticles produced by fungi*. Applied microbiology and biotechnology, 2013. **97**(2): p. 775-782.
11. Song, J.Y. and B.S. Kim, *Rapid biological synthesis of silver nanoparticles using plant leaf extracts*. Bioprocess and biosystems engineering, 2009. **32**(1): p. 79.
12. Sadeghi, B. and F. Gholamhoseinpoor, *A study on the stability and green synthesis of silver nanoparticles using Ziziphora tenuior (Zt) extract at room temperature*. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2015. **134**: p. 310-315.
13. Srikar, S.K., et al., *Green synthesis of silver nanoparticles: a review*. Green and Sustainable Chemistry, 2016. **6**(01): p. 34.
14. Hu, C.-M.J., et al., *Erythrocyte membrane-camouflaged polymeric nanoparticles as a biomimetic delivery platform*. Proceedings of the National Academy of Sciences, 2011. **108**(27): p. 10980-10985.
15. Shahidi, F. and Y. Mine, *Nutraceutical proteins and peptides in health and disease*. 2005: CRC Press.
16. Mine, Y., *Recent advances in the understanding of egg white protein functionality*. Trends in Food Science & Technology, 1995. **6**(7): p. 225-232.
17. Pereira, M.M., et al., *Single-step purification of ovalbumin from egg white using aqueous biphasic systems*. Process Biochemistry, 2016. **51**(6): p. 781-791.
18. Cincin, Z., et al., *Hesperidin promotes programmed cell death by downregulation of nongenomic estrogen receptor signalling pathway in endometrial cancer cells*. Biomedicine & Pharmacotherapy, 2018. **103**: p. 336-345.

19. Garg, A., et al., *Chemistry and pharmacology of the citrus bioflavonoid hesperidin*. *Phytotherapy Research*, 2001. **15**(8): p. 655-669.
20. Parhiz, H., et al., *Antioxidant and anti-inflammatory properties of the citrus flavonoids hesperidin and hesperetin: an updated review of their molecular mechanisms and experimental models*. *Phytotherapy Research*, 2015. **29**(3): p. 323-331.
21. Rao, K., et al., *Gum acacia stabilized silver nanoparticles based nano-cargo for enhanced anti-arthritic potentials of hesperidin in adjuvant induced arthritic rats*. *Artificial cells, nanomedicine, and biotechnology*, 2018: p. 1-11.
22. Manthey, J.A. and N. Guthrie, *Antiproliferative activities of citrus flavonoids against six human cancer cell lines*. *Journal of Agricultural and Food Chemistry*, 2002. **50**(21): p. 5837-5843.
23. Hosseinimehr, S.J., et al., *Hesperidin inhibits cyclophosphamide-induced tumor growth delay in mice*. *Integrative cancer therapies*, 2012. **11**(3): p. 251-256.
24. Piaru, S.P., R. Mahmud, and S. Perumal, *Determination of antibacterial activity of essential oil of *Myristica fragrans* Houtt. Using tetrazolium microplate assay and its cytotoxic activity against vero cell line*. *Int J Pharmacol*, 2012. **8**(6): p. 572-576.
25. Sarkar, A., et al., *Antagonistic roles of Rac and Rho in organizing the germ cell microenvironment*. *Current biology*, 2007. **17**(14): p. 1253-1258.
26. Rao, K., et al., *Gum tragacanth stabilized green gold nanoparticles as cargos for Naringin loading: A morphological investigation through AFM*. *Carbohydrate polymers*, 2017. **174**: p. 243-252.

27. Khandekar, S.V., M. Kulkarni, and P.V. Devarajan, *Polyaspartic acid functionalized gold nanoparticles for tumor targeted doxorubicin delivery*. Journal of biomedical nanotechnology, 2014. **10**(1): p. 143-153.
28. Imran, M., et al., *Double-tailed acyl glycoside niosomal nanocarrier for enhanced oral bioavailability of Cefixime*. Artificial cells, nanomedicine, and biotechnology, 2017. **45**(7): p. 1440-1451.
29. López-Heras, M., et al., *Towards understanding the antibacterial activity of Ag nanoparticles: electron microscopy in the analysis of the materials-biology interface in the lung*. Environmental Science: Nano, 2015. **2**(4): p. 312-326.
30. Chen, X. and H.J. Schluesener, *Nanosilver: a nanoproduct in medical application*. Toxicology letters, 2008. **176**(1): p. 1-12.
31. Slavin, Y.N., et al., *Metal nanoparticles: understanding the mechanisms behind antibacterial activity*. Journal of nanobiotechnology, 2017. **15**(1): p. 65.
32. Cho, J.-G., et al., *Stepwise embryonic toxicity of silver nanoparticles on *Oryzias latipes**. BioMed research international, 2013. **2013**.
33. Chernousova, S. and M. Epple, *Silver as antibacterial agent: ion, nanoparticle, and metal*. Angewandte Chemie International Edition, 2013. **52**(6): p. 1636-1653.
34. Travan, A., et al., *Non-cytotoxic silver nanoparticle-polysaccharide nanocomposites with antimicrobial activity*. Biomacromolecules, 2009. **10**(6): p. 1429-1435.
35. Akter, M., et al., *A systematic review on silver nanoparticles-induced cytotoxicity: Physicochemical properties and perspectives*. Journal of advanced research, 2017.

Table 1: Characteristics of drug loaded Pro-AgNPs (Composition, particle size, PDI, Zeta Potential and drug loading efficiency)

Sample	Drug loading efficiency (%)	Average size (nm)	PDI	Zeta Potential (mV)
Hes-Pro-AgNPs	83.29 \pm 1.36	257.10 \pm 1.48	0.45 \pm 0.01	-20.20 \pm 0.43
Pro-AgNPs	-----	122.9 \pm 0.77	0.26 \pm 0.01	-18.52 \pm 0.29

Table 2: Assignment of Infrared bands in Protein, Pro-AgNPs, Hesperidin and Hes-Pro-AgNPs

S/N	Study (cm ⁻¹)					
	Protein	Pro-AgNPs	Assignment	Hesperidin	Hes-Pro-AgNPs	Assignment
1.	3700	-	O-H _{Str} of free alcohol	3477	3477	O-H _{Str} of alcohol
2.	3661	-	O-H _{Str} of free alcohol	3424	3424	O-H _{Str} of alcohol
3	3410	3413	N-H / O-H _{Str} of 2° amide / hydrogen bonded alcohol.	2980		C-H _{Str} of arene
4	2963	2931	Methyl / Methylene (CH ₃) _{sym str}	2918	2918	Methyl/ Methylene C-H _{Str}
5.	1811		C-O-O- <i>str</i> of carboxylic esters (arising from	1647	1648	C=O _{Str} of ketone

			protein side chain residue)				
6	1663	1650	C=O <i>str</i> of 2° amide (amide I band)	1609	1610	C=C <i>sym str</i> of aromatic ring	
7	1549	1546	N-H <i>bend</i> of 2° amide (amide II band)	1519	1519	C=C <i>asym str</i> of aromatic ring	
8	1443.7	1443.5	C-H <i>bend</i>	1443.9	1444.2	C-H <i>bend</i>	
9	1240		C-N <i>bend</i> of amide (amide band III)	1205	1206	C-O-C <i>sym str</i> of aryl ether	
10	1076	1073	C-O <i>stretch</i> of 1° alcohol	1183	1184	C-O <i>str</i> of phenol	
11.	669	669	N-H wagging of 2° amide (671)	1132	1132	C-O-C <i>str</i> of alkyl ether	
12.				1096	1097	C-O <i>str</i> of 2° alcohol	
13.				1070	1071	C-O-C <i>asym str</i> of aryl ether	
14.				912	912	C-H <i>out</i> of plane <i>bend</i> of aromatic ring	

Table 3: Drug retaining capability of Hes-Pro-AgNPs upon incubation with plasma

Formulation	Time interval (h)	Drug retained (%)
Hes-Pro-AgNPs	3	85.04 ±2.32
	6	84.56 ±0.91
	12	82.54 ±1.54
	24	80.00 ±0.55

Table 4: IC₅₀ (µg/mL) values of Hesperidin, Pro-AgNPs and Hes-Pro-AgNPs tested against both sensitive and resistant *S. aureus*

Bacterial Strain	Hesperidin	Pro-AgNPs	Hes-Pro-AgNPs
<i>S. aureus</i> (Sensitive)	588.39 ±1.65	110.51 ±2.83	73.22 ±0.87
<i>S. aureus</i> (Resistant)	980.05 ±2.80	410.73 ±2.49	110.57 ±2.41

Captions for Figures:

Fig. 1: Isolation of egg proteins from egg white concentrate, (a) shows egg white concentrate, (b) shows solution containing soluble ovalbumin and the globulins precipitate and (c) shows Egg proteins

Fig. 2: SDS-PAGE analysis of various proteins present in 20% egg white

Fig. 3: Color of mixture of egg proteins, AgNO₃ and sodium ascorbate solution (a), intermediate color change (b) and Pro-AgNPs solution (c)

Fig. 4: Effect of proteins concentration on AgNPs formation (a) and color of AgNPs solution at different protein concentrations (b)

Fig. 5: Effect of Ag ion concentration on AgNPs formation (a) and color of AgNPs solution at different Ag ion concentrations (b)

Fig. 6: Plasma stability study of synthesized NPs against human plasma for a period of 26 h (a), Salt stability study of synthesized NPs against different concentration of NaCl (b), pH stability study of synthesized Pro-AgNPs (c) and UV-Vis Spectrum of optimized Pro-AgNPs (d)

Fig. 7: AFM image of (a) Pro-AgNPs and (b) Hes-Pro-AgNPs

Fig. 8: FTIR spectra of egg proteins (blue), Pro-AgNPs (red), Hesperidin (Hes, violet) and Hesperidin loaded AgNPs (Hes-Pro-AgNPs, olive)

Fig. 9: MIC values of Hesperidin, Pro-AgNPs and Hes-Pro-AgNPs tested against (A) sensitive *S. aureus* and (B) resistant *S. aureus*



Fig. 1.

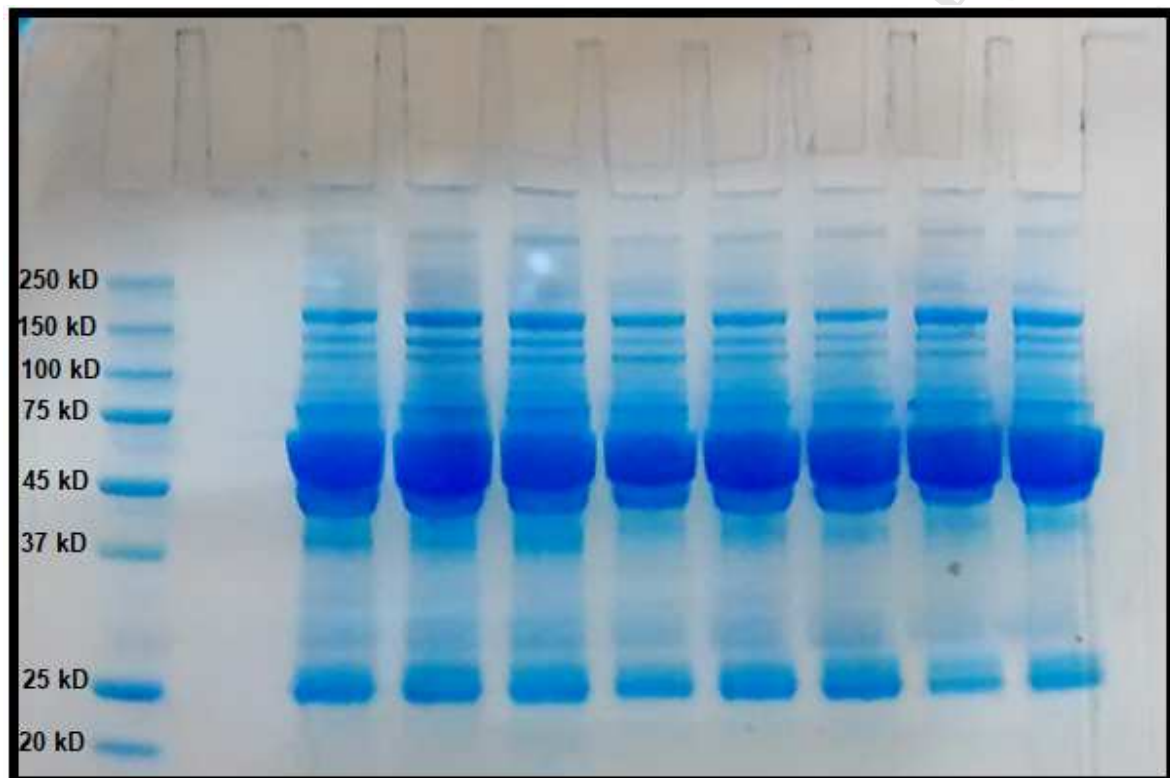


Fig. 2.

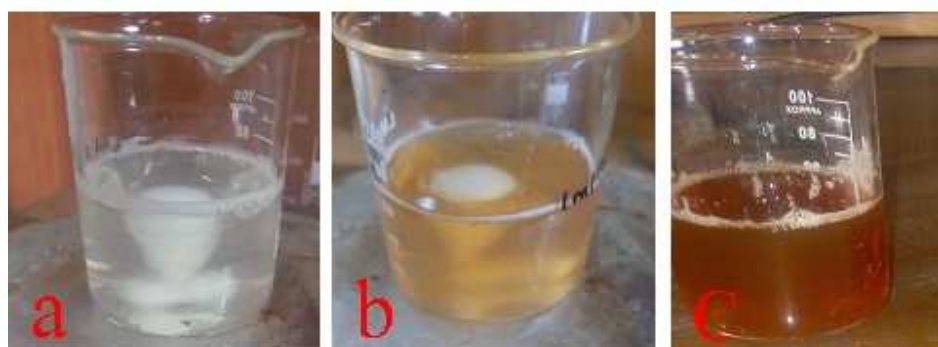


Fig. 3.

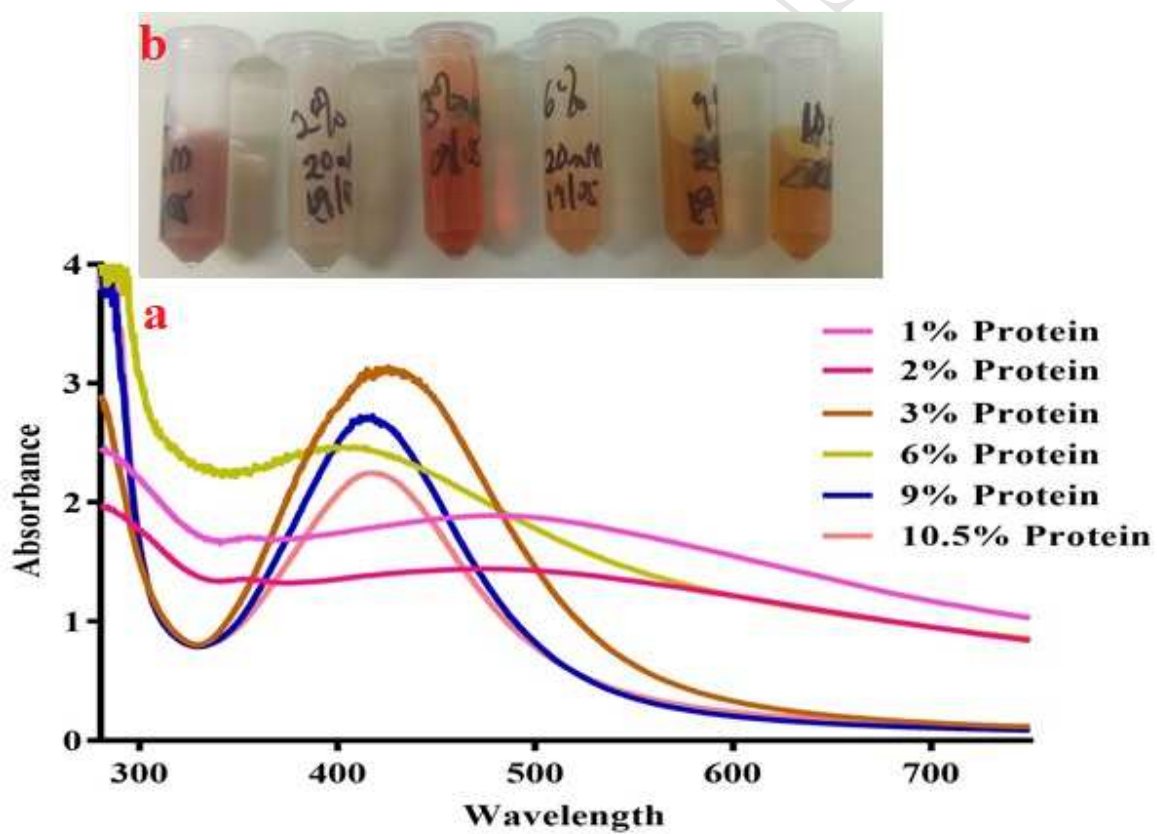


Fig. 4.

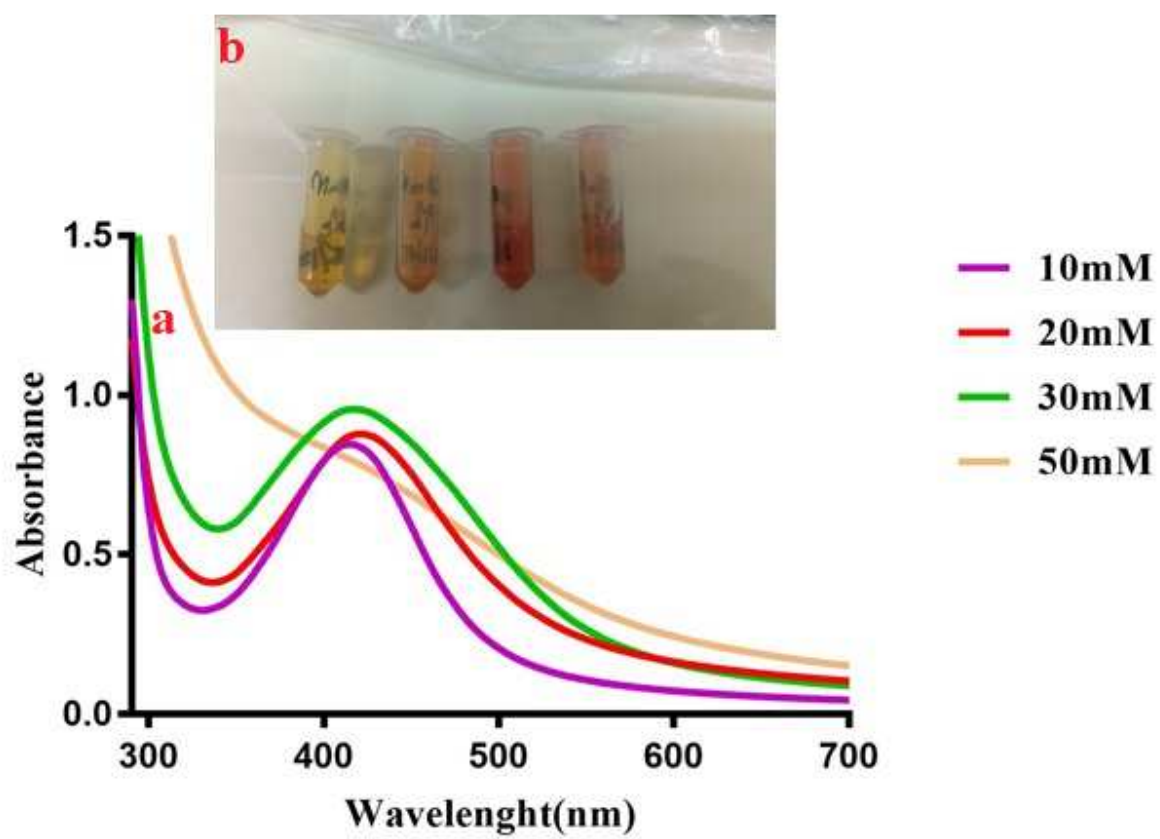


Fig. 5.

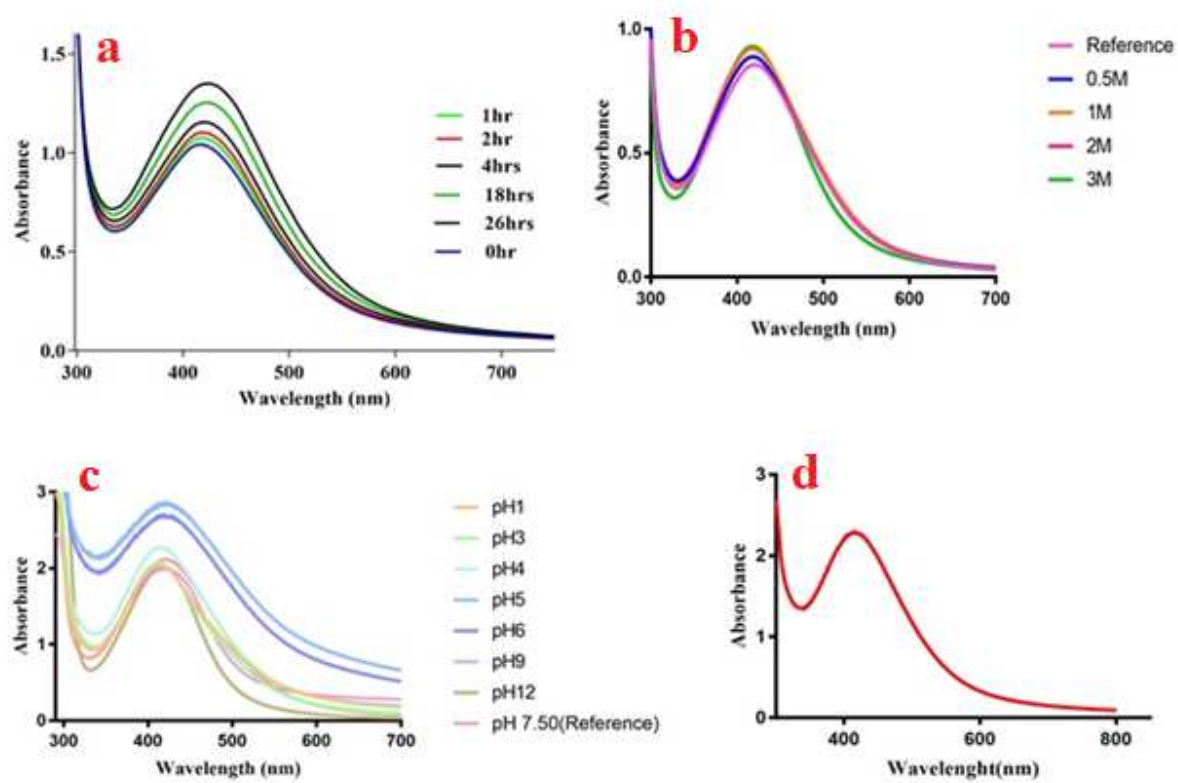


Fig. 6.

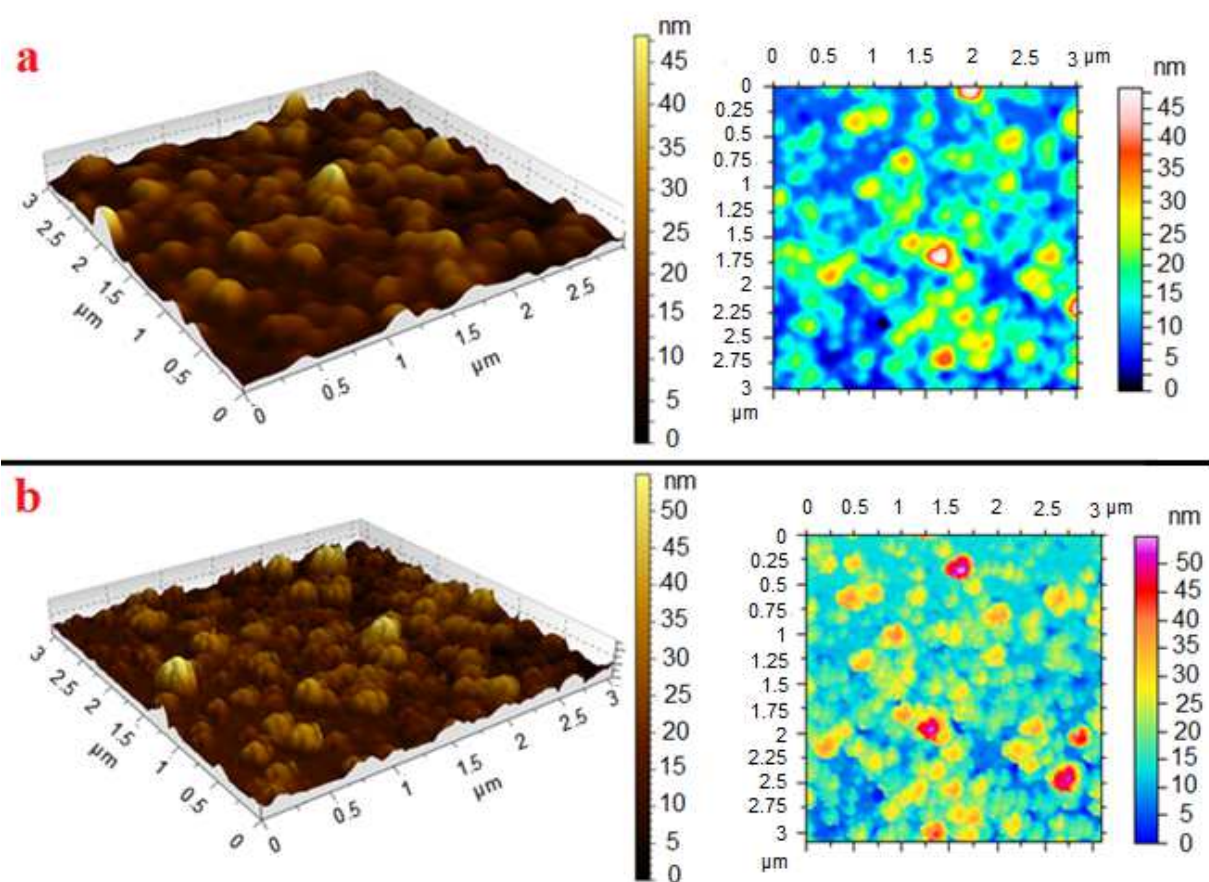


Fig. 7.

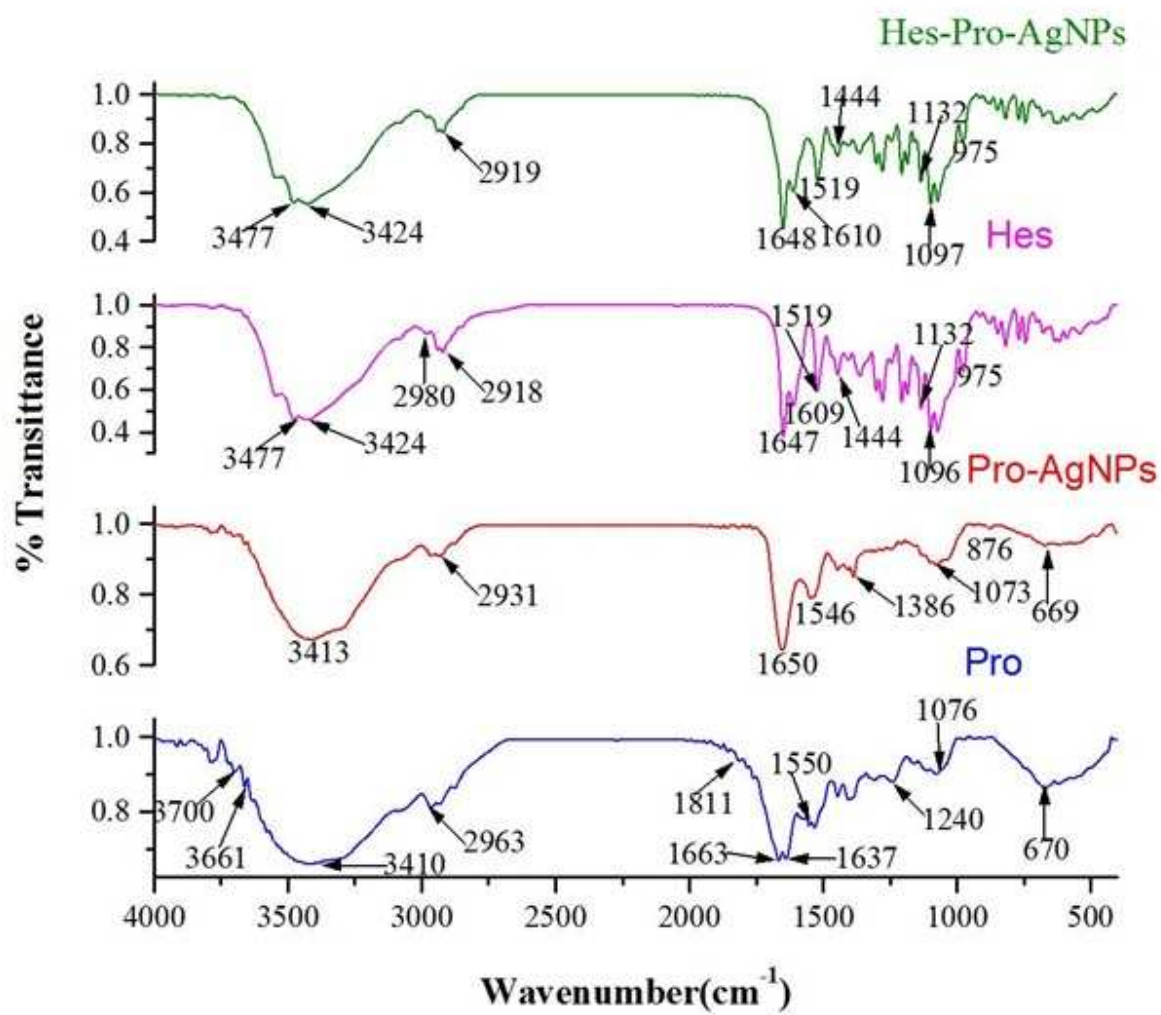


Fig. 8.

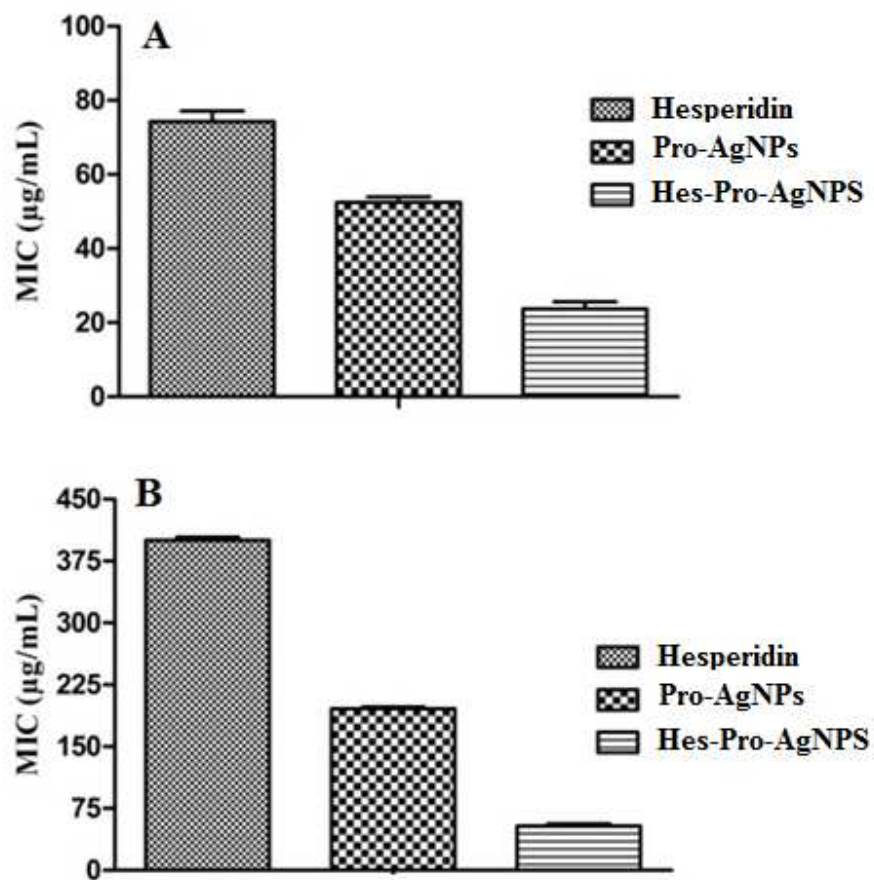


Fig. 9.