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ARTICLE TYPE

Moxifloxacin-Capped Noble Metal Nanoparticles as Potential Urease Inhibitors

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Silver-moxifloxacin (Ag-Mox) and gold-moxifloxacin (Au-Mox) nanoparticles were successfully synthesized by a rapid and convenient method which exhibited good stability against variations in NaCl solution, pH and temperature. The structural features of these nanoparticles were ascertained by UV-Vis, AFM, FTIR, SEM and EDX techniques while the EDX analysis revealed the inorganic composition of the synthesized moxifloxacin-capped Ag and Au NPs. Similarly, the stability of nanoparticles complex may be attributed to the binding of amino group to the silver and gold surface respectively. The sliver-moxifloxacin nanoparticles (Ag-Mox) exhibited significant urease enzyme inhibitory activity (0.66 ± 0.042 µg/mL) even 250 times better compared to moxifloxacin (183.25 ± 2.06 µg/mL). On the contrary, the gold nanoparticles (Au-Mox) remained inactive to the same enzyme. Antibacterial assay was also carried out for parent compound as well its noble metal nano-conjugates and ensured encouraging results.

Keywords

Moxifloxacin, Silver and Gold nanoparticles, Atomic Force Microscopy, Urease enzyme inhibition, Antibacterial activity

Introduction

So far various noble metals comprising Ag, Au, Pt and Pd have been used for the synthesis of nanoparticles[1]. These nanoparticles are widely used due to their size, shape and unique optoelectronic properties which have provoked a lot of interest for important biomedical applications. In addition, their applications are gradually increasing in the field of nanomaterial science, photochemistry, photographic chemistry, Raman spectroscopy, physics and life sciences[2–9].

Similarly, on occasions, these nanoparticles have exhibited good antimicrobial efficacy against various pathogens such as bacteria, viruses and other eukaryotic microorganisms and playing a major role in the field of nanomedicine[10].

Now a days, chemical reduction, physical processes and biological methods are considered authentic and popular procedures for the synthesis of noble metal nanoparticles. Among them, the most convenient protocol is the reduction of silver/gold salts with inorganic reducing agents at relatively elevated temperature[12-13]. Due to unique shape and having relatively smaller size, nanoparticles are reasonably susceptible to aggregates. Hence, in order to get rid of this aggregates, various stabilizing agents have been used that include polymers and polyelectrolytes which adhere to the surface of nanoparticles in order to achieve stabilization[14].

In the field of medicine, conjugates of noble metal nanoparticles with antibiotics and antibodies also have been used for selective photo-thermal killing of protozoa and bacteria[15–17]. Metal ions are existing in the active sites of metal containing proteins such as hemocyanin and in ureases enzyme, lactase, tyrosinase and ascorbate oxidase[18-31].

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Urease is a nickel containing enzyme and known to catalyse the hydrolysis of urea into ammonia and carbon dioxide (urea amidohydrolase EC 3.5.15). It allows an organism to use urea as nitrogen source[32].Besides, Urease is coined to be one of the main causes of pathogenesis induced by *Helicobacter pylori*, thus enable them to persist at low pH of the stomach. It plays a key role in the pathogenesis of gastric and pepticulcers[33].

Quinolones include a group of eminent antibacterial agents and the first members have been in clinical practice for more than 40 years[34-35]. Nowadays, clinically they are the most successful synthetic antibacterial drugs[36-37]which successfully inhibited DNA replication and are frequently used for the treatment of numerous infections[37-38]. In contrast to first- (nalidixicb acid, cinoxacin) and second- (norfloxacin, enoxacin, ofloxacin, and ciprofloxacin) generation, the third-generation quinolones such as levofloxacin, sparfloxacin, gatifloxacin, and moxifloxacin have displayed a much broader spectrum of activity providing expanded gram-negative and gram-positive activity against a typical pathogens[39, 40].

In this manuscript we present rapid and convenient synthesis of silver and gold nanoparticles which is based on modified Tarkevisch method using sodium tetrahydroborate ($NaBH_4$) as a mild reductant[41-42]. Moxifloxacin, a family of fluoroquinolone antibiotic have been used for capping of silver and gold nanoparticles. Seemingly, amino and carboxylate groups of moxifloxacin are responsible for capping of Ag/Au NPs. As for our understanding, current investigation on the urease inhibitory activity of moxifloxacin conjugated with silver and gold nanoparticles is being reported for the first time.

Experimental

Materials and instruments:

Silver nitrate ($AgNO_3$) was purchased from Sigma-Aldrich while chlorauric acid trihydrate ($HAuCl_4.3H_2O$), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were procured from Merck. Sodium tetrahydroborate ($NaBH_4$) was obtained from Wako Pure Chemical Industries Ltd. Moxifloxacin was gifted by BIOREX pharmaceutical company Islamabad. Deionized water was used throughout the reactions for the synthesis of silver and gold nanoparticles. $NaBH_4$ (50 mM) fresh solution was prepared by dissolving 19 mg in 10 mL triply distilled water. A digital pH meter model 510 (Oakton, Eutech) equipped with a glass working electrode and a reference Ag/AgCl electrode was hired for pH measurements. UV-Vis spectra were recorded with a Shimadzu UV-240, Hitachi U-3200 spectrometer with a path length of 1cm. FTIR spectra were recorded with a Shimadzu IR-460 spectrophotometer. A 1:1 mixture of lyophilized Ag/Au-Mox NPs and KBr was pressed into a pellet. The shape and size of nanomedicine were examined using AFM, Multimode, Nanoscope IIIa, Veeco, (California, USA) in tapping mode and furthermore, confirmed by SEM with EDX (JSM 591 JEOL, Japan).

Synthesis of Ag-Mox and Au-Mox

As a typical procedure, moxifloxacin hydrochloride was first neutralized with equimolar Na_2CO_3, to overcome the problem of precipitation of AgCl in reaction mixture. Ag-Mox and Au-Mox were synthesized using $NaBH_4$ as a moderate reductant. Fresh solutions of metal salts (1 mM $AgNO_3$ and 1 mM HAuCl4) and ligand (1 mM moxifloxacin) were prepared in deionized water**.** Reactions were carried out by mixing different volumes of moxifloxacin and noble metal salt solutions. The reaction mixture was stirred vigorously for about 30 minutes at ambient temperature and then 0.2 ml of 50 mM $NaBH_4$ was added drop wise. Gradual change in colour of solution was used as a clue to formation of desired product i.e., Ag and Au-Mox NPs. After addition of reducing agent, the light yellow solution gradually turned maroon followed by brown and eventually ruby red, depending upon the molar ratio of metal to the ligand as demonstrated in digital photograph (**Figures 3 and 4**). The mixture was stirred robustly for another 30 min. Optimization of reaction conditions was achieved by varying the molar ratio of metal to ligand (Mox) which resulted into appearance of sharpest absorption peak in the proposed UV-Vis region. For Ag-Mox NPs the best optimal ratio observed was 8:1 (metal: ligand) while for Au-Mox NPs the same optimized ratio remained 1:6 (metal: ligand) at ambient temperature (**Figures 2 and 5**). The residual metal salt and ligand were removed by centrifugation through 10 rpm and the supernatants were freeze dried which contained moxifloxacin capped silver and gold nanoparticles. The synthesis of Ag and Au-Mox nano-conjugates were further confirmed by using UV-Vis spectroscopy, FTIR, AFM and SEM. Biological screening of a selected group of Ag-Mox and Au-Mox nano-conjugates possessing suitable size and shape was performed and evaluated accordingly.

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Characterization of moxifloxacin-capped Ag/Au NPs

Synthesized noble metal nanoparticles (Ag-Mox and Au-Mox) were characterized through UV-Vis, FTIR, AFM, SEM and EDX techniques. Absorption maxima of noble metal NPs were recorded as function of retention time in the range of 300 to 700 nm using UV-Vis Spectroscopy. To measure the amount of Mox adsorbed on nanoparticles, Ag and Au NPs were centrifuged out of the colloidal suspensions. The supernatants were lyophilized and the residue weighed. These results reveal that nano-conjugates contained about 6 % by weight of Mox. For FTIR measurements, the freeze dried samples (0.01 g Ag-Mox and Au-Mox NPs) were ground with KBr and transformed into uniform pellets suitable for FTIR analysis. For AFM analysis, the Ag/Au NPs sample were prepared by dissolving thin films in deionized water and dispersing on freshly cleaved sheet of mica. The AFM images were recorded at ambient temperature followed by repeating the experiment with various concentrations of the samples. Surface and size of silver and gold nanoparticles were analysed by SEM.

Stability of capped Ag/Au-Mox NPs

Stability of synthesized silver and gold nanoparticles was also tested against brine solution (1 M), temperature (ambient to 60 ºC) respectively. In addition, the stability parameter was also evaluated at variable pH range (pH: 2-13). UV-Visible spectra were also recorded and the variation or persistence of the UV-Vis peak provided rewarding clue about the stability of nanoparticles under investigation.

Biological evaluation

The synthetic nano-conjugates capped with Ag and Au were subjected to enzyme inhibition and microbial activities. Enzyme inhibition was evaluated against urease. The reaction mixture consisting of 25 µL of Jack bean (Canavalia ensiformis) urease, 55 µL of buffer at pH 6.8, 100 mM of urea, and 5 µL of various concentrations of test compounds (from 0.5 to 0.00625 mM) were incubated at 30 °C for 15 min in 96-well plates. In kinetics experiments, various concentrations of both substrates and test compounds were used. Subsequently 45 µL phenol reagents (1% w/v phenol and 0.005% w/v sodium nitroprussside), and 70 µL of alkali reagent (0.5% w/v NaOH and 0.1% w/v NaOCl) were added to each well. After 50 minutes, the shift of absorbance to longer wave length (630 nm) was measured with a microplate reader (SpectraMax M2, Molecular Devices, CA, USA). All reactions were performed in triplicate in a final volume of 200 µL. Indophenol method was used to measure ammonia production as urease activity using thiourea as standard inhibitor[43].Finally, the results were processed by software SoftMax Pro (Molecular Devices, CA, USA), MS-Excel and Ez-fit programs. The % inhibition was calculated from the formula as given below:

% Inhibition = 100-(OD test /OD control) ×100

The antibacterial activity was evaluated by using the well diffusion method with slight modifications. Mueller Hinton agar was used as medium. The cultures were taken in triplicates at incubation temperature of 37 °C for 24 to 72 h. The broth culture (0.6 ml) of the test organism was placed in a sterile Petri-dish and added 20 ml of the sterile molten Mueller Hinton Broth (MHB). Holes were made into the medium using 0.2 ml of parent drug and Ag/Au NPs. Streptomycin was used as the standard anti-microbial agent at a concentration of 2 mg/ml. Inoculation was done for 1 h to make possible the diffusion of the antimicrobial agent into the medium. After incubation for 24 h at 37 °C, the diameters of the zone of inhibition of microbial growth were measured in the plate in millimetre (mm). The bioassays were performed in triplicate.

Results and discussion

Moxifloxacin was used as a capping agent for the synthesis of silver and gold nanoparticles. The main objective behind selection of this drug is reflected due to presence of amino moiety possessing strong ligating potential in the framework (**SI structure 1**). This functionality may be exploited to inhibit agglomeration hence stabilize silver/gold metals during nanoparticles (**Figure 1**) formation[44].

Ag/Au-Mox Nanoassembly

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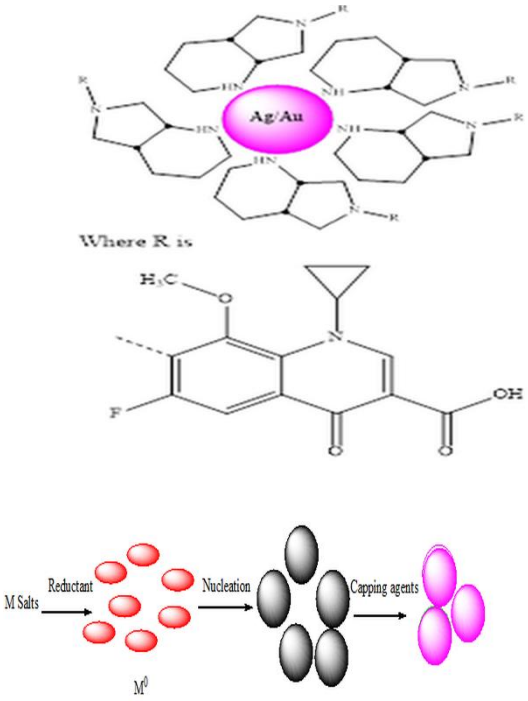

Fig-1: Capping action of moxifloxacin with noble metals (Ag and Au)

Anticipated variation in UV-Visible absorption bands were considered as the preliminary indication of synthesized Ag/Au NPs. The existence of particular peaks in the regions of 400-500 nm and 500-600 nm ensured the formation of silver and gold NPs respectively. The sharpest peak for Ag-Mox NPs was observed for a reaction of 8:1 (metal: ligand) molar ratio while for Au-Mox NPs, the sharp peak was perceived at 1:6 (metal: ligand) molar ratio as shown in the Figures S-2 and S- 4 respectively.

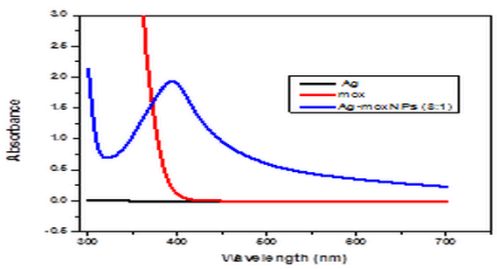

Fig-2: Optimized UV-Vis spectral data of Ag-Mox NPs at molar ratio of 8:1 (metal–ligand)

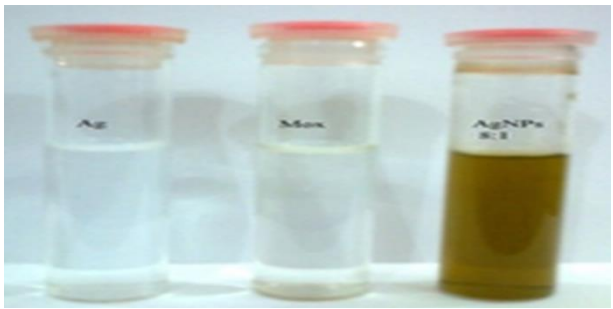

Fig-3: Ag-Mox NPs

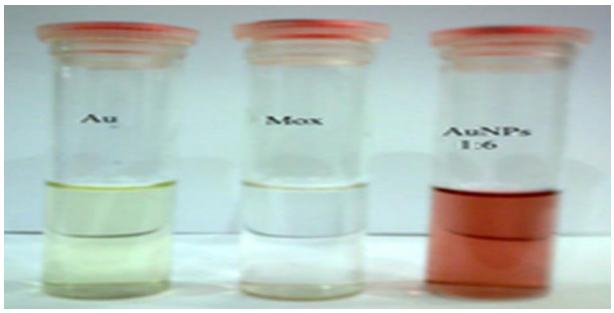

Fig-4: Au-Mox NPs

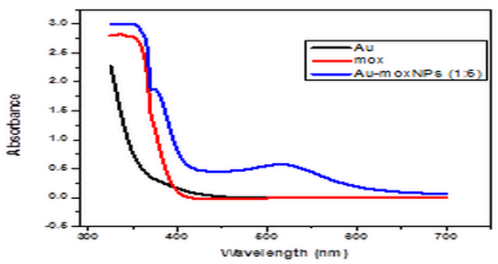

Fig-5: Optimized UV-Vis spectral data of Au-Mox NPs at molar ratio of 1:6 (metal-ligand)

Ag-Mox showed an absorption plasmon band in the region of 410 nm and Au-Mox exhibited absorption peak at 540 nm which revealed the formation of Ag and Au NPs.

In order to ascertain the presence of various functionalities available in the drug substrate before and after capping the Ag and Au nanoparticles, the FTIR spectral data was recorded and interpreted accordingly. For instance, as regards the substrate drug (Moxfloxacin), the absorption bands of stretching frequencies for aromatic C-H, secondary N-H, C=O (keto group), O-H besides bending of O-H in case of COOH were observe dat 2949, 3354, 1708,2926and 1457cm^{-1} respectively. However, as anticipated, the N-H stretching band at 3354 cm-^{1} got shifted to 3446 cm-^{1} and slightly broadened in the case of Ag-Mox NPs and

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may be attributed to involvement in conjugate formation. Similarly, as shown in **Figures 6 and 7**, the absorption band at 1323 cm^{-1} due to C-N stretching and carbonyl peak of carboxylic group were seen displaced from 1708 to 1600 cm^{-1}in Ag-Mox conjugate. It was revealed that actually N-H moiety was involved in the capping and stabilization of noble metal nanoparticles.

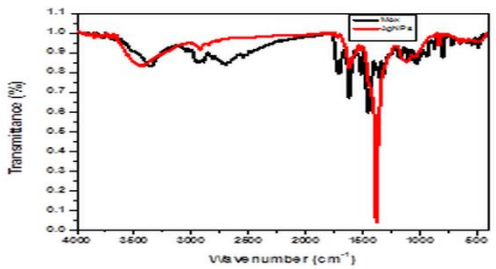

Fig-6: FTIR data for moxifloxacin and Ag-Mox NPs

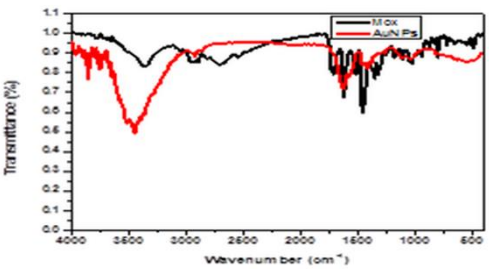

Fig-7: FTIR spectrum of moxifloxacin and Au-Mox NPs

Stability check of silver and gold nanoparticles

The effect of high concentration of brine solution (1 M) on capped silver and gold nanoparticles was also studied. For this purpose, 3 mL of freshly prepared Ag-Mox and Au-Mox NPs were taken in five separate vials. Then 0.2, 0.4, 0.6, 0.8 and 1 mL of 1 M NaCl solution were added to these vials. The resulting solutions were shaken well and then kept at room temperature for 24 h. UV–Vis spectra were recorded for Ag-Mox and Au-Mox NPs. The results showed that higher concentration of brine decreased the λ_{max}. The full width at half maximum (FWHM) also increases and thereby decreasing the stability of noble metal nanoparticles. This rapid decrease in absorbance of Ag/Au NPs containing NaCl may be attributed to the aggregation effect promoted by Cl^{-1} ions. From these clarifications it was concluded that at higher concentration of sodium chloride, however, aggregation turned out to be dominant. As for long term stability, Ag-Mox NPs and Au-Mox NPs are much more stable in neat water than those in brine solution as shown in **Figure 8** and **Figure 10** respectively.

In addition, the stability of synthesized Ag-Mox and Au-Mox nanoparticles against pH variations ranging from 2-13 were also examined. For this study, 3 mL of freshly prepared nano-congugates of Ag-Mox and Au-Mox were taken in six separate vials. The pH of Ag-Mox and Au-Mox was measured and found to be 4.7. The pH of Ag and Au NPs in the range 6–13 was adjusted by using 1 M NaOH solution. Similarly, the pH of Ag-Mox and Au-Mox ranging from 2–3 were maintained by using 1 M HCl. The UV–vis spectra of resulting solutions were recorded after 24 h. Ag NPs were stable in the pH range of 4-7 and completely unstable in highly acidic medium at pH 2- 3 and basic medium 8-13 **(Figure 9)** while the Au NPs showed stability in basic medium (pH=8- 9) and less stable at pH 12-13 **(Figure 11).**

The synthesized silver and gold nanoparticles were also found to be stable up to 60 ^{0}C.

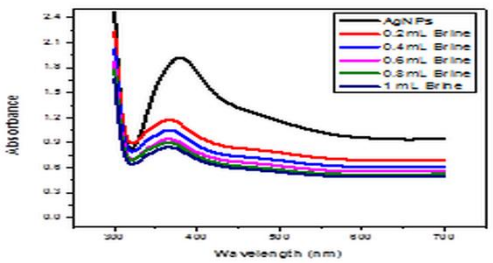

Fig-8: Effect of brine concentration on the stability of Ag-Mox NPs

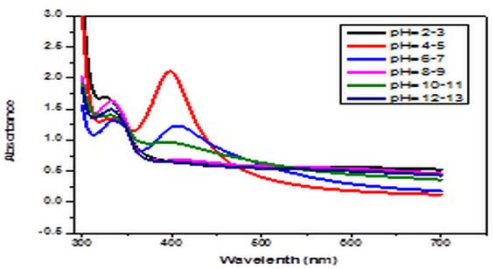

Fig-9: Spectral data for the effect of pH on the stability of moxifloxacin capped Ag NPs

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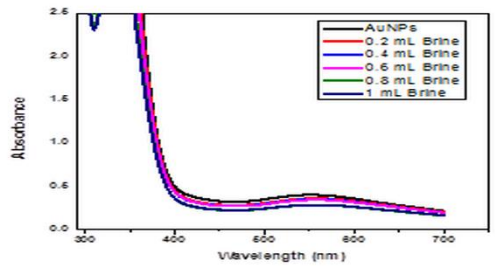

Fig-10: Effect of brine on stability of Au NPs

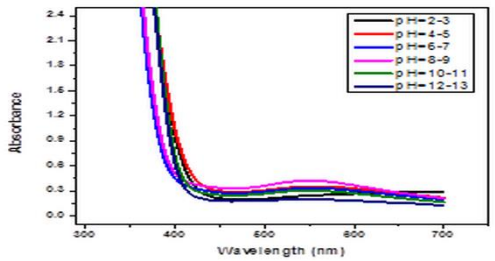

Fig-11: Spectral data for the effect of pH on the stability of moxifloxacin capped Au NPs

Atomic force microscopy (AFM), scanning electron microscope (SEM) and energy dispersive X-ray spectroscopy analysis

Surface topology of the formulated silver and gold nanoparticles was studied by atomic force microscopy (AFM) analysis (**Figures 12 and 13**). The micrographs clearly indicate that the synthesized Ag NPs possess spherical shape and have the calculated sizes in the range of 50 to 60 nm while the Au NPs have slightly spherical shape and have the calculated sizes in the range of 50 to 80 nm. For confirmation of the size and surface morphology of silver and gold nanoparticles, SEM technique was also performed. The SEM image showed spherical Ag-Mox NPs (50-60 nm) with uniform distribution similarly, for Au-Mox nanoparticles the SEM image results were comparable and also witnessed the results of AFM that the Au NPs shape were found slightly spherical in the range of 50 to 80 nm as shown in (**Figures 14 & 15**).

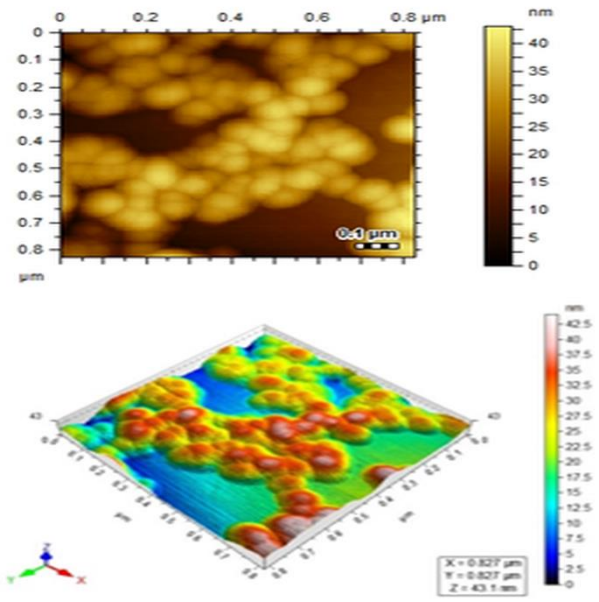

Fig-12: Atomic force images of moxifloxacin stabilized Ag NPs

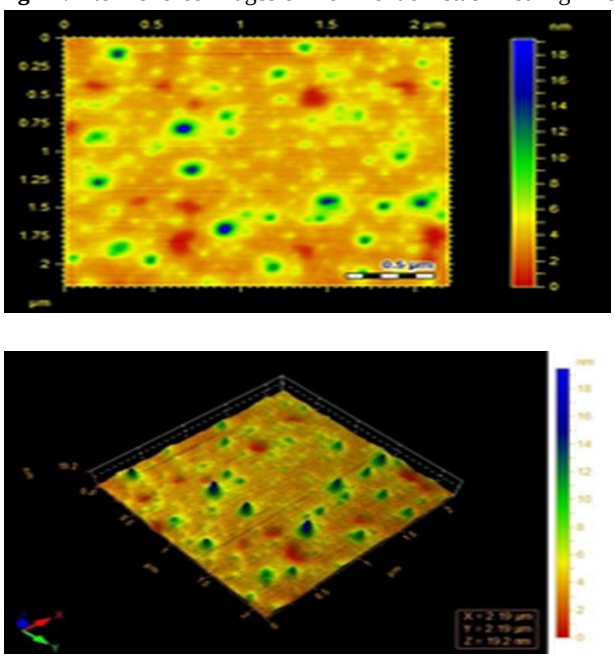

Fig-13: AFM images of moxifloxacin-capped with Au NPs

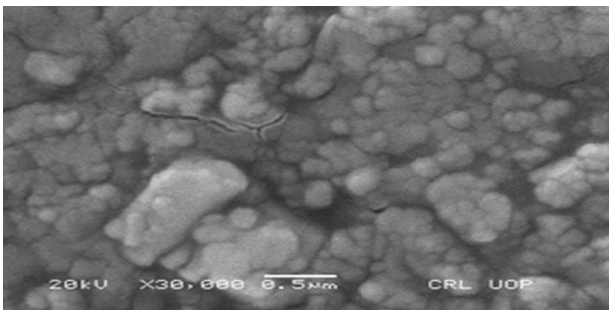

Fig-14: SEM image of Ag-Mox

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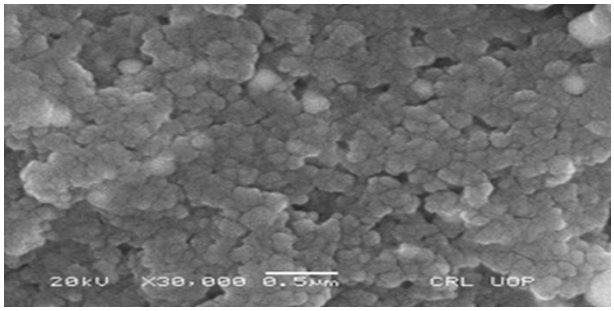

Fig-15: SEM image of Au-Mox

Energy-dispersive X-ray spectroscopy (EDX) (**Figures 16 and 17**) illustrated the chemical nature of synthesized silver and gold nanoparticles. The energy dispersive X-ray analysis (EDX) shows strong signal in the silver region and confirms the formation of silver nanoparticles. Metallic silver nano crystals generally display optical absorption peak approximately at 3 keV due to surface plasmon resonance[45]. The peak was obtained at the energy of 3 keV for silver, and also some of the weak peaks for C, O, Cl, N, Mg, Ca and Na were found. For gold nanoparticles, the EDX spectrum also reveals the presence of peaks characteristic of gold at 2.12 and 9.71 keV and few of the weak signals for Cl, Na, C, O, Mg and Ca were observed**.**

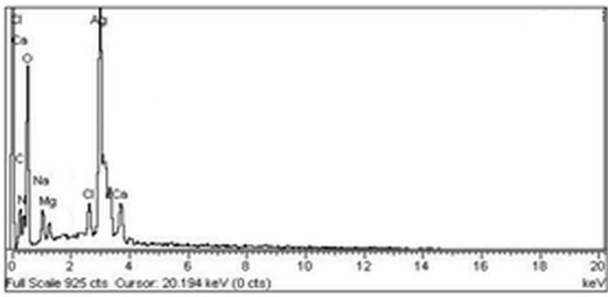

Fig-16: The EDX spectrum for silver nanoparticles

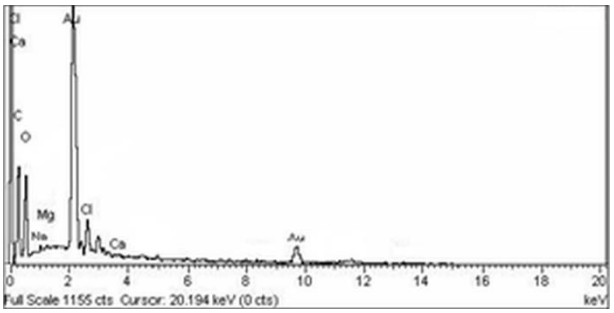

Fig-17: The EDX spectrum for gold nanoparticles

Biological evaluation

The synthesized nanoparticles Ag-Mox and Au-Mox along with the capping ligand (Moxifloxacin) and the silver metal (Ag) were independently screened for jack bean urease enzymes inhibition potentials. These studies led us to conclude that the Ag-Mox nanoparticles exhibited significantly higher level of enzyme inhibition activity of 93% at 0.2 mg/ mL and IC_{50} value of 0.66 ± 0.042 µg/mL. Only 6 % of the ligand made nanoparticle, therefore the activities of Ag-Mox may be better than the given values. According to 6 % ligands attachment, the activity of Ag-Mox was 0.039 ± 0.0025 µg/mL (39 ± 2.5 ng/mL). The Ag metal alone did not show any inhibition activity while the ligand (Moxifloxacin) revealed weak inhibition with IC_{50} value of 183.25 ± 2.06 µg/mL. The Au-Mox was found inactive as compared to the parent compound (Mox) having IC_{50} = 183.25 ± 2.06 µg/mL. The results deduced that after conjugation moxifloxacin with Ag, the activity of moxifloxacin was significantly increase even more than 250 times. Our findings are in good agreement with the literature in terms of biological activities enhancement of silver capped nanoparticles[46-48]. Interestingly, after conjugation with gold the activity of moxifloxacin was significantly decreased. The urease inhibition effect produced by Ag-mox in comparison to Mox was greater than standard drug (thiourea) **SI-table S-1**. It means conjugation of Mox to Ag had a robust inhibition effect in comparison to pure Ag and Mox.

The moxifloxacin and its capped noble metal nano-conjugates (Ag-Mox and Au-Mox) were tested against three bacterial strains *Staphylococcus aureus*, *Bacillus subtilis* and *Klebsiella pneumonia*. The antibacterial activities of these nanoparticles are summarized in **SI-table S-2**. The silver and gold nanoparticles displayed good antibacterial activity against all three stains *Staphylococcus aureus*, *Bacillus subtilis* and *Klebsiella pneumonia*. The Ag-Mox exhibited significant antibacterial activity against *S. aureus*, *B. subtilis* and *K. pneumonia* with zone of inhibition ranging from 15-18 mm at 3 mg/ mL, which were comparable to the parent compound (Mox) with inhibitory zone range from 16-20 mm. The Au-Mox also showed good bactericidal activity against *S. aureus*, *B. subtilis* and *K. pneumonia* with zone of inhibition ranging from 13-17 mm. Streptomycin was used as standard drug.

Conclusion

Employing sodium borohydride (NaBH4) as a mild reductant, a convenient protocol to produce silver and gold nanoparticles capped with moxifloxacin, was developed. The other mild reducing agents such as triethylamine and quinol were also tested

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but failed to show anticipated results. The spectroscopic study revealed that amine moiety of the substrate drug is responsible to promote capping of these silver and gold nanoparticles. The energy-dispersive X-ray (EDX) analysis demonstrated the inorganic composition of the synthesized silver/gold nanoparticles. The synthesized nano-medicines of noble metals are freely water soluble and nontoxic. Furthermore, the moxifloxacin-capped noble metal nanoparticles (Ag-Mox and Au-Mox NPs) showed inhibition against urease enzyme and also exhibited significant antibacterial activity.

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