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Design and development of AuNP-Cys-Flu system by a sustainable route

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Abstract *:* Proteins have a great relevance in bio-modification of metal nanoparticles and enhancing their bioactivity. Nanoparticles intended for drug delivery often fail to fully exploit their clinical potential due to limitations linked to their targeting ability and subsequent bio-distribution issues. In the current study, gold nanoparticles (AuNP) were synthesized by a sustainable method using gelatin as a bio-reducing agent. The AuNPs were produced by modulating temperature and reaction time as input parameters. The optimized AuNP has round shape, particle size of 58 nm, and characteristic surface plasmon resonance wavelength maxima at 530 nm. Functional evaluation of AuNP was performed using a model drug. For this, surface functionalization of AuNP with cysteine (cys) was performed followed by attachment of a model drug fluconazole (Flu) to give AuNP-Cys-Flu and evaluated for drug release.

Index Terms - Gelatin, Gold nanoparticles, Cysteine, AuNP-Cys-Flu, Drug delivery.

1. INTRODUCTION

The development of reliable experimental protocols for the synthesis of nanomaterials over a range of chemical compositions, sizes, and high monodispersity is one of the challenging issues in current nanotechnology. There is a need to develop an environment friendly approach for nanomaterials synthesis that should not use toxic chemicals in the synthesis protocol[1]. There was a school of thought amongst scientists that protein involvement in the nanoparticle formation could alter the properties of nanoparticles giving it a biological identity[2]. Protein adsorption to the nanoparticle surface not only reduces their cytotoxicity but also enhances the biocompatibility and alters the dispersing/agglomerating state[3].

Proteins are remarkably homogeneous, even the components of macromolecules consist of hundreds of protein subunits, which always assemble into the same structure.
This means that proteins are monodispersed and easily form close-packets. These special characteristics will allow protein-mediated nanoparticle to be used in medical and industrial applications[4].

Gelatin is a denatured protein that is obtained either by partial acid or alkaline hydrolysis of animal collagen. Having a long history of safe use in pharmaceuticals, cosmetics, as well as food products, it is considered as GRAS material by the United States Food and Drug Administration (FDA)[5]. Biodegradability, biocompatibility, chemical modification potential and cross-linking possibility make gelatin-based nanoparticles a promising carrier system for drug delivery[6].

Gelatin as a sustainable protein source was used to synthesize gold nanoparticles which do not produce any toxic chemicals during nanomaterial synthesis. Gold nanoparticles (AuNPs) are chemically inert[7]. They are highly biocompatible and exhibit versatility because of their ready functionalization through thiol linkages.

Taking into consideration all above facts, an attempt is made here to synthesize AuNPs using protein sources and assembling a model drug onto AuNPs and evaluation of the assembly.

2. MATERIALS AND METHODS

Chloroauric acid was obtained from Alpha chemika, Mumbai, Gelatin was purchased from Molychem Pvt.Ltd, Mumbai, India. Fluconazole was obtained as a gift sample from FDC Ltd.

UV-VIS spectrophotometer: SHIMADZU 1800, FTIR spectrophotometer: Shimadzu IR Spirit, Particle size analyzer: HORIBA SZ -100 Nanoparticle Analyzer, TEM: FEI Tecnai 12 Transmission Electron Microscopes

2.1. Synthesis of gelatin assisted Gold nanoparticles

A gelatin solution (1% w/v) was prepared in double distilled water. This gelatin solution is mixed with 2mM chloroauric acid solution in the ratio of 1: 1 and pH is made to 7. Then the mixture was stirred at 80°C for 5 hours to get red wine-coloured gold nanoparticles. The product was purified by centrifugation. The purified gold nanoparticles were redispersed in Double Distilled water.

2.2. Surface Functionalization and Fluconazole conjugation of the synthesized Gold Nanoparticles

2.5mM and 14mM Stock solution of cysteine as well fluconazole was prepared respectively. 5ml of prepared cysteine solution was added into the 10ml synthesized gold nanoparticles along 5ml of fluconazole solution and stirred for 4-5 hours. Centrifuged to remove excess cysteine and fluconazole. Characterization of the prepared AuNP-Cys-Flu were performed.

2.3. Preparation of topical formulations

The Gel formulation was prepared by using carbopol 934, AuNP-Cys-Flu (0.5%), propylene glycol, glycerol and methyl paraben and the pH was adjusted using triethanolamine. Prepared gel was then stored in a glass container for further use.

The cream was prepared by using beeswax, cetostearyl alcohol, and liquid paraffin, Tween 80 (oil phase) and AuNP-Cys-Flu solution (0.5%), methyl paraben, and deionized water made up the water phase.

Evaluation of prepared topical formulations:

The topical formulations were examined for a number of factors in accordance with official guidelines and contrasted with a commonly used commercial formulation. Evaluation parameters were Drug content, Viscosity, Spreadability, Measurement of pH, Drug release kinetics

Antifungal Assay

The synthesized AuNPs as well as prepared topical formulations were evaluated for antifungal activity; a well diffusion method was applied to screen famous pathogenic microbes. For the antifungal assay, Candida albicans (c. albicans) were spread uniformly on nutrient agar plates. AuNPs normal solution was introduced in the wells under sterilized conditions and incubated at 37°C for 24 h. The zone of inhibition (mm) around well was measured.

3. RESULTS and DISCUSSION

Gelatin assisted AuNP formation:

The purpose of the gelatin here was to reduce the chloroauric acid to produce AuNPs. The gelatin did not form any nanoparticles at room temperature or at melting temperature, but at a temperature more than 70°C or higher. It gave a high rate of nanoparticle production with a shorter reaction time and smaller particle size at 80°C.

According to published research, temperature is a key factor in controlling the pace at which AuNP is formed, as well as the stability and structure of the gelatin coating that forms on the surface of the nanoparticles. Below the gel melting point (~35 °C), gelatin is known to exhibit triple helical areas and an increase in viscosity; above this temperature, the viscosity decreases and the random coil conformation is observed. Furthermore, given that the creation of AuNPs is a result of the electrons moving from the amine groups of gelatin to the Au (III) ions, which is aided at reduced viscosity values. The point to be noted is above 70°C, the triple helical structure of gelatin changes to a single helical structure[8].

3.1 Characterization of nanoparticles

3.1.1 Particle Size Analysis

The particle size measured by particle size analyzer (Horiba) was found to be 58.8 nm for AuNPs. Dynamic light scattering method determines the dynamic diameter particle sizes of AuNPs dispersed in water. The zeta potential for AuNPs was -43.3mV and PI was 0.582. This denotes the stability of prepared Nanoparticles.

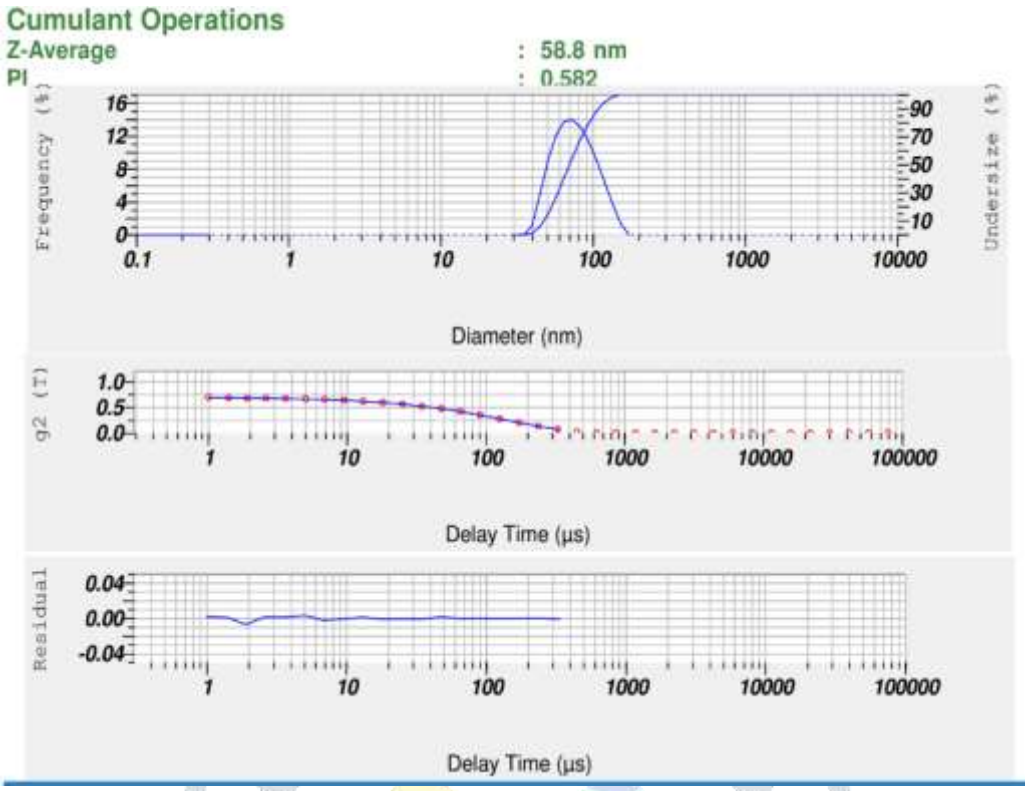

Figure 1: Particle size of AuNPs

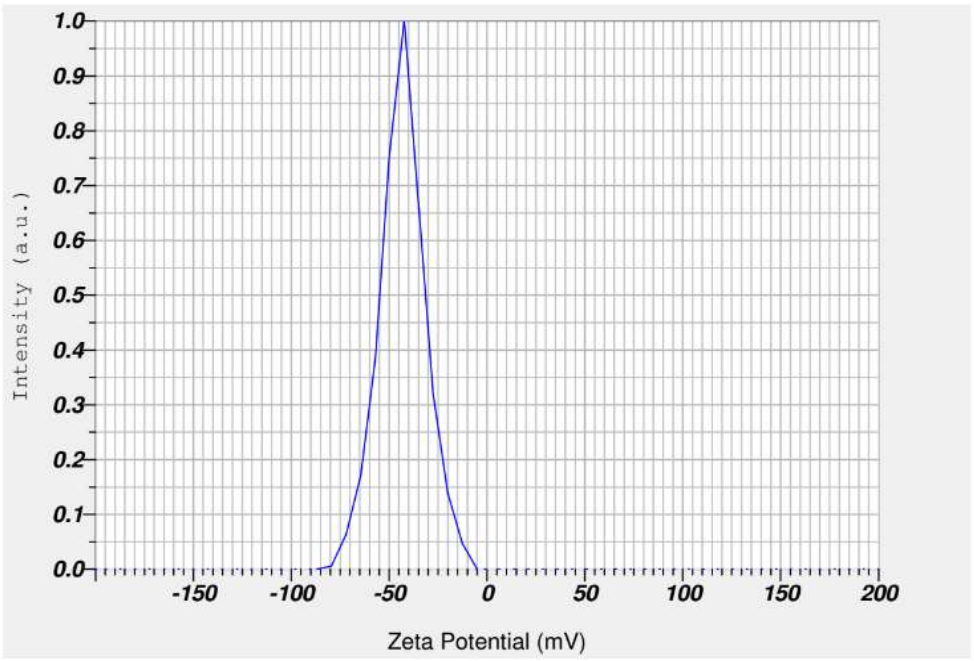

Figure 2: Zeta potential of AuNPs

3.1.2 Fourier Transform spectroscopy studies (FTIR)

The major chemical functional groups present in AuNPs were identified through FTIR studies. The Au nanoparticles showed slight shift in peaks from that of gelatin spectra such as 2967, 1691, 1513, 1242, 1038 cm-1 which represents carboxylic and amide groups of gelatin are responsible for the reduction of Au nanoparticles.

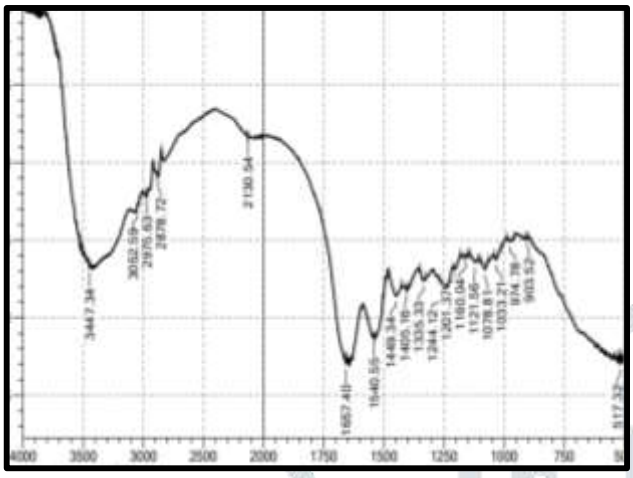

Figure 3: FTIR Spectra of gelatin

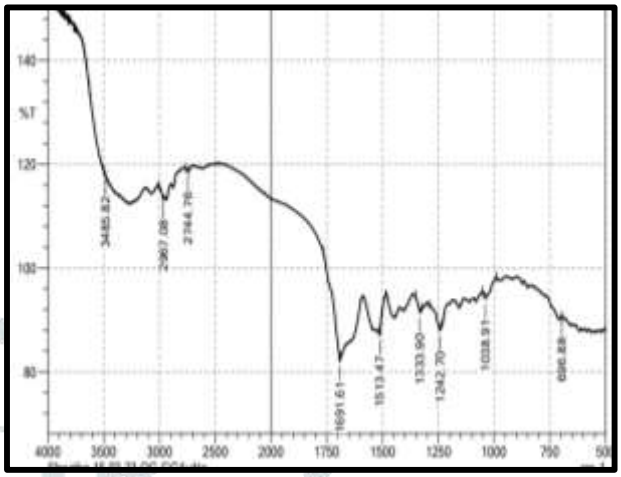

Figure 4: FTIR Spectra of AuNPs

Table 1: FTIR peaks of Gelatin and AuNPs

Gelatin Peak	**AuNPs peak**	**Assignment**
2975	2967	C-H stretching of carboxylic acid group
1657	1691	C=O stretching of amide II group (Beta antiparallel sheet secondary structure of gelatin)
1540	1513	N-H bending of amide III group
1078	1038	C-O stretching of carboxylic group

3.1.3 Transmission Electron Microscopy spectroscopy (TEM)

The transmission electron microscope image is scanned to know the perfect morphology and particle size of the prepared Au nanoparticles. Figure number 05 showed the TEM image of the prepared Au nanoparticle formed by gelatin. The image shows particles are in the nano range, and most of the particles are spherical and also found varied particle sizes.

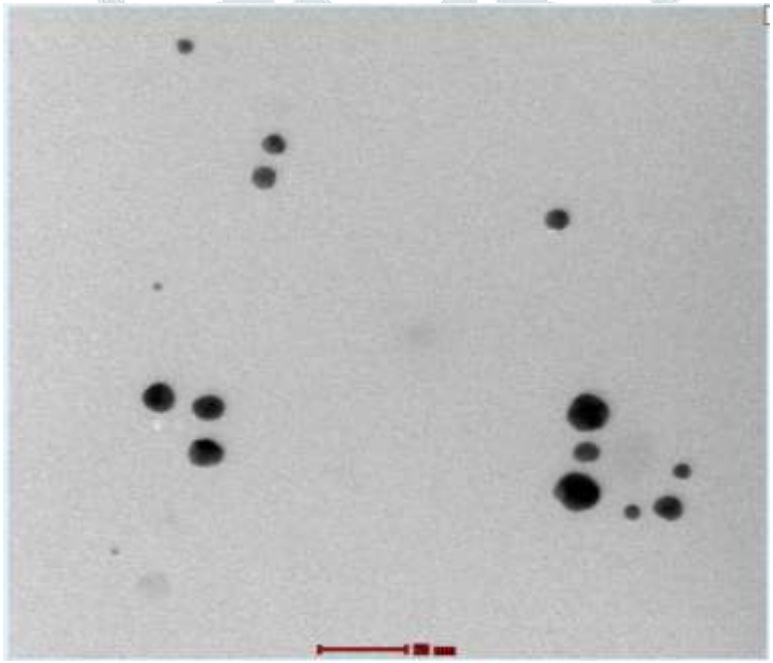

Figure 5: TEM image of AuNPs

3.2 Selection of model drug and conjugation with gold nanoparticles

Fluconazole, a triazole antifungal, is one of the antifungal medications that doctors give the most frequently for Candida infections[9] was selected as a model drug for conjugating AuNPs.

Surface functionalization helps to increase the systemic circulation time of colloidal carriers like liposomes, micelles and nanoparticles which include maintenance of optimal therapeutic concentration of drug in the blood after single administration of the drug carrier. It improves drug performance by optimizing pharmacokinetics, increasing bioavailability, decreasing immunogenicity and dosing frequency. preventing opsonin recognition in the reticuloendothelial system (RES) based blood clearance pathway[10]. Most peptides and proteins have cysteine (Cys), an amino acid with a thiol (-SH) group on the side chain, as their primary module. The strong ionic and covalent characteristics of the AuS bond aid in the creation of extremely stable NPs, which makes gold NPs easily bound and capped to sulfur groups. Gold's strong attraction to sulphur atoms results in the formation of nanoparticles with free thiol groups on their surface[11].

Cysteine and fluconazole solutions were mixed with prepared AuNPs and stirred for a specific amount of time. It was observed that cysteine creates a free thiol group on the surface of gold nanoparticles (AuNPs). It provides lipophilic nature to the prepared AuNPs that makes them easily conjugated with fluconazole. The formation of AuNPs-Cys-Flu was confirmed through EDS, FTIR and TEM analysis.

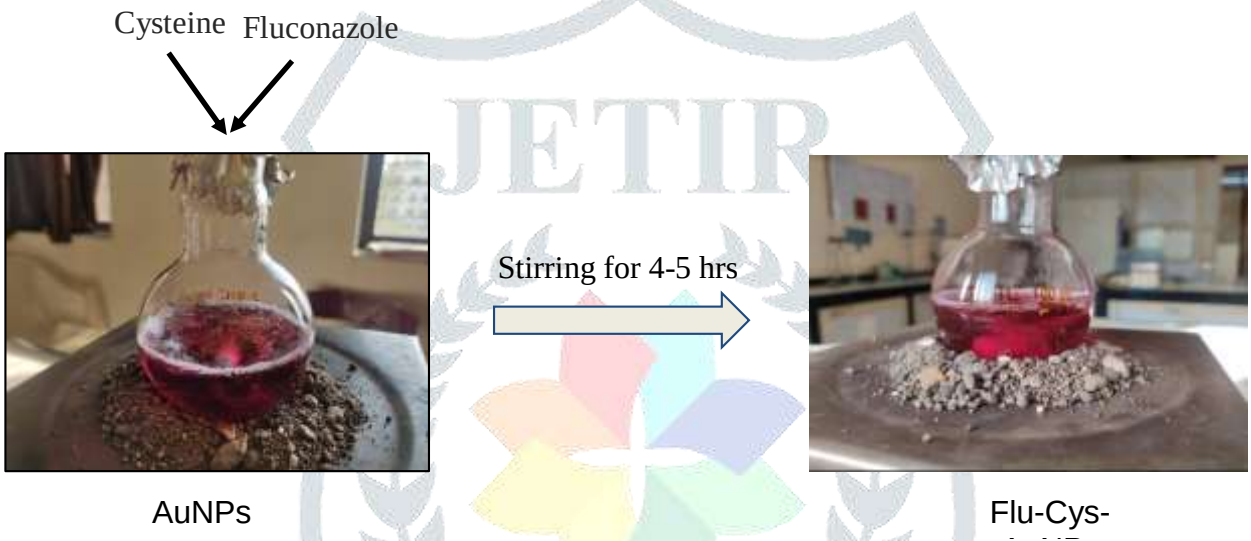

Figure 6: Synthesis of AuNP-Cys-Flu

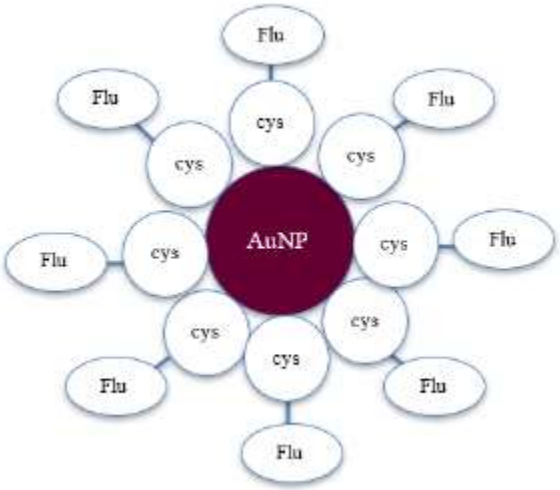

Figure 7: Structure of AuNP-Cys-Flu

FTIR analysis

The Fourier transform infrared (FTIR) analysis of the AuNP-Cys-Flu shows a very slight shift in the bands in comparison with those in the reported literature. The spectra show a distinctive peak at 3233 cm^{-1} corresponds to O-H stretching vibration of carboxylic group, whereas the band at 2881 cm^{-1} corresponds to N-H stretching of amine group C=C. The band at 1626 cm^{-1} is attributed to C = N stretching imine group. The peaks at 1278.32 cm^{-1} are assigned to the C-F stretching fluoro compound. This confirms the formation of AuNP-Cys-Flu. FTIR spectra of cysteine showed a distinct peak at 2586 cm^{-1} indicating the presence of S-H stretching bond. This peak disappears in AuNP-Cys-Flu due to S-H bond breakage. Cysteine was found to have C-S stretching modes at 698 cm^{-1} from the sulfur-containing residues. This demonstrates the development of a link between gold nanoparticles from the S head group of cysteine[12-13].

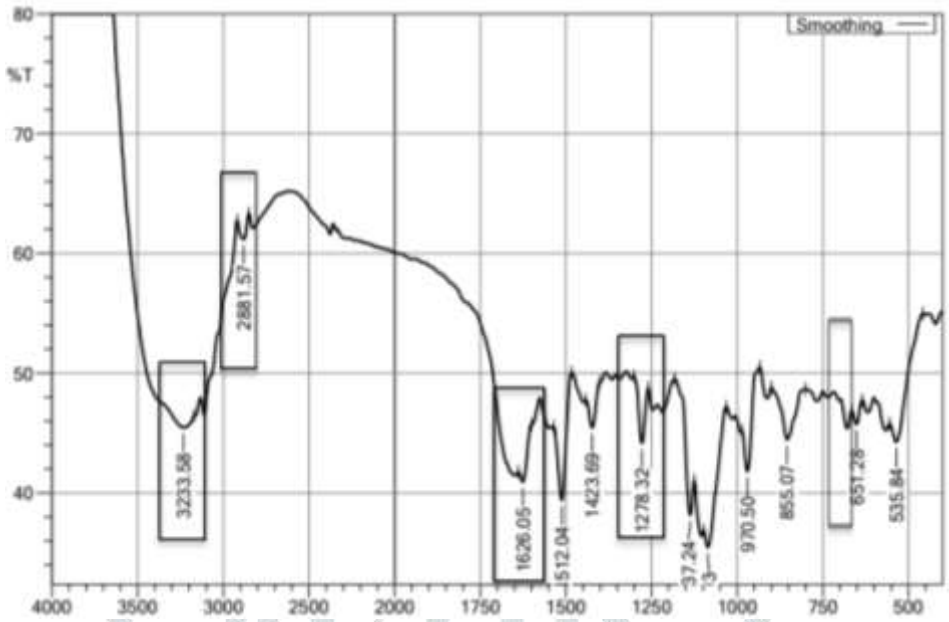

Figure 8: FTIR spectra of AuNP-Cys-Flu

TEM

The TEM analysis was performed for the confirmation of the morphology and size of AuNP-Cys-Flu. The formation and growth of AuNP-Cys-Flu were confirmed. Spherical shape of AuNP-Cys-Flu was confirmed by TEM analysis.

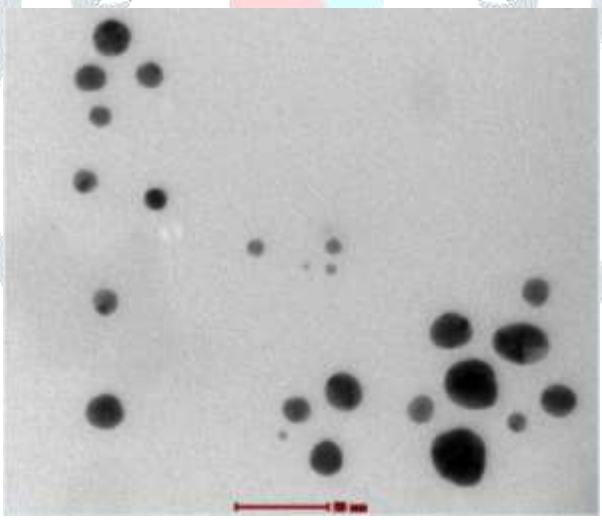

Figure 9: TEM image of AuNP-Cys-Flu

EDX analysis

The EDX data is presented as a graph with KeV on the x-axis and peak intensity on the y-axis. The peak location on the x-axis is converted into the atoms that the energy changes represent by a computer program. Energy dispersive X-Ray (EDX) spectroscopy was carried out to obtain an elemental spectrum in the AuNP-Cys-Flu sample. SEM–EDS, which revealed the presence of peaks for Au, S, O, N & F atoms. These characteristic absorption peaks of Au, S, O, N & F atoms confirm the formation of AuNP-Cys-Flu.

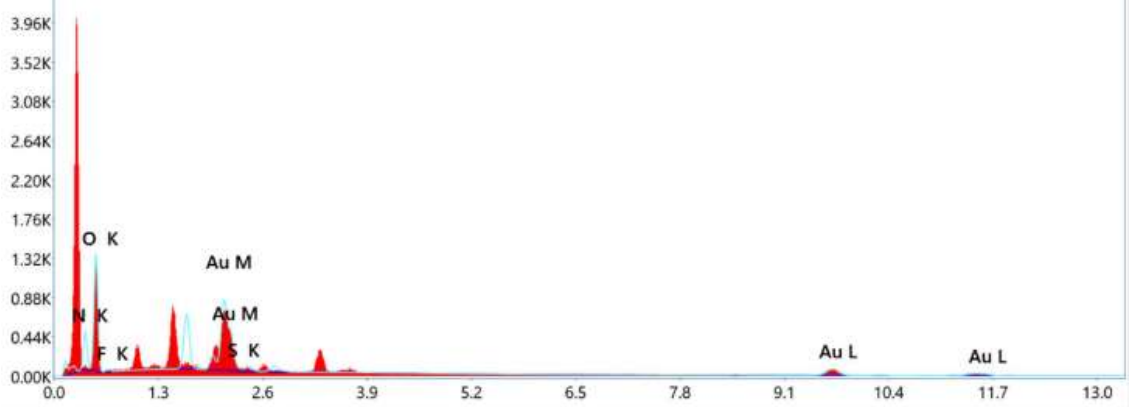

Figure10: EDX analysis of AuNP-Cys-Flu

EVALUATION OF PREPARED TOPICAL FORMULATIONS

Drug content

The high drug content supports the authenticity of the used method to prepare topical formulations. The drug content of gel and cream was found to be 97.45% and 96.35% respectively, indicating minimum loss of drug during manufacturing.

Rheological Studies

The viscosity of gel and cream formulations plays a vital role in regulating drug permeation. In general, viscosity of gel & cream affects its consistency, which is particularly an essential factor as it must be applied on the skin in the form of thin layers. The viscosity of gel and cream was found to be 41500 cps and 82000 cps respectively.

Spreadability

A good spreadability of topical formulations helps in the consistent application of the gel & cream formulations and takes less time to spread. The spreadability of gel & cream formulations were found to be 38.63 g.cm/s & 22.36 g.cm/s respectively

pH

The pH value of the gel & cream formulations were found to be 6.8 & 6.4 respectively, which is considered within the normal range to avoid skin irritation, and so the developed formulations were suitable for dermal application.

Table 2: Cream evaluation parameters

pH	6.4
Viscosity	82000 cP
Spreadability	22.36 g.cm/s
Drug Content	96.35%

Table 3: Gel evaluation parameters

pH	6.8
Viscosity	41500 cP
Spreadability	38.63 g.cm/s
Drug Content	97.45%

In-vitro Release study

Fluconazole conjugated AuNPs gel, cream and marketed formulation was subjected to in-vitro diffusion study using Franz diffusion cell. The cell had a 18 ml receptor compartment. The dialysis membrane was soaked in a pH 7.4 Phosphate buffer for 24 hrs before activation. The dialysis membrane was mounted between the donor and receptor compartments. The receptor compartment was filled with 18 ml of phosphate buffer pH 7.4 maintained at 37+0.5°C. Calculated quantity of gel/cream was placed in the donor compartment over a dialysis membrane and the compartments were clamped together. The hydrodynamics in the receptor compartments were maintained by stirring with a magnetic bead at 150 rpm. At time intervals of 1, 2, 3, 4, 5, 6 hrs., aliquot of 1 ml was withdrawn and the sink conditions were maintained. The samples were analyzed spectrophotometrically at 260 nm. The in vitro drug release study for gel, cream and marketed formulation were found to be 62.36 ± 3.96, 47.35 ± 2.3 and 82.31 ± 7.4 respectively.

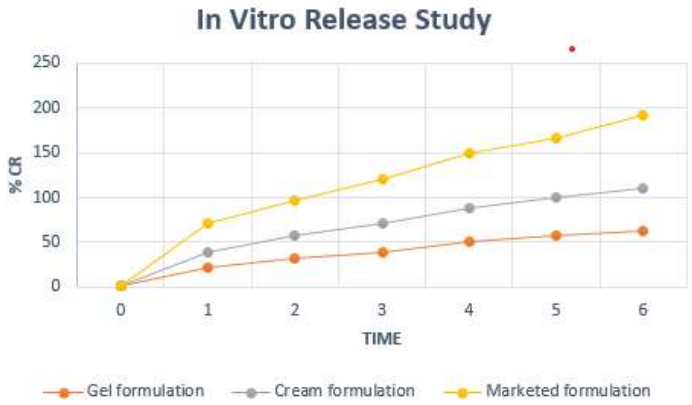

Fig. No.11 Graphical representation of In-vitro release study

ANTIFUNGAL ASSAY

Table No.04 Zone of inhibition for C. Albicans species

P1		P2	
Sample	**Zone of inhibition**	**Sample**	**Zone of inhibition**
Sample Gel (F)	15mm	Sample cream (F)	11mm
		AuNPs-Cys-Flu	12mm
Standard (S)	19mm	Standard (S)	10mm

The synthesized topical formulations & nanoparticles show remarkable activity against Candida albicans.

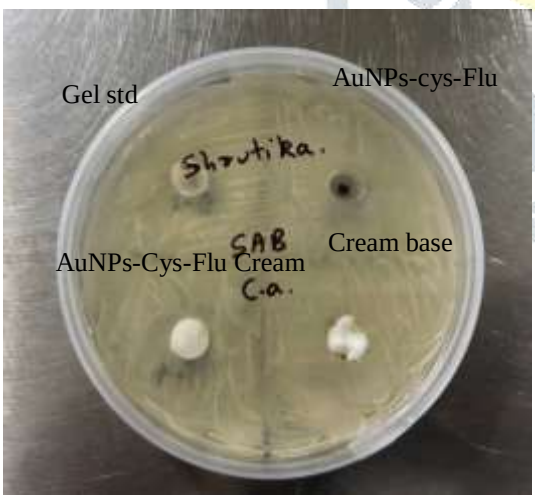

Fig. No.12 Antifungal activity of cream (P1)

Fig. No.13 Antifungal activity of Gel (P2)

CONCLUSION

Synthesis of gelatin assisted gold nanoparticles by sustainable source was successfully carried out, here, the bottom-up approach is used for the synthesis of metal nanoparticles. AuNPs were successfully surface functionalized using cysteine, where a model drug fluconazole could get attached. The AuNP-cysteine-Fluconazole system could be effectively incorporated into gel and cream formulations and gave good antifungal activity.

This facile strategy of sustainable synthesis of AuNP, surface functionalization could be tried with different agents for the effective delivery approaches.

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5. ACKNOWLEDGEMENT

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