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Research Article

Study of the Altered Anisotropy of Erythrocyte Ghost Membrane upon Interaction with Phytoreduced Negatively Charged Gold Nanoparticle from *Celosia cristata and Vigna radiata*

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Abstract

Synthesis of negatively charged gold nanoparticles (GNP) was done from seed extract and seed electrolyte of two locally available plant sources *Celosia cristata* and *Vigna radiata* without using any toxic chemicals and it is a rapid reduction way. Standard techniques of spectroscopy and advanced microscopic study was done in characterizing the particles and simultaneously the phytochemicals present in the electrolyte and extract were also spotted using established process. The effects of nanoparticles on live red blood cells were analyzed by calculating hemolysis percentage. Evaluations of GNP activity when interacted with erythrocyte ghost membrane and their impact on membrane fluidity and anisotropy were also studied as negatively charged GNP are less delineated in previous history.

The results showed that negatively charged gold nanoparticles were reduced by flavonoids and phenols present in seed extract and electrolyte of plants and they are hemocompatible and can help to fluidize the membrane of erythrocyte ghost cells.

The whole experiment suggested a simple, ecofriendly approach of synthesizing negatively charged nanogold which are safe to live cells that can change the fluidity parameter of membrane constituent by decreasing the anisotropy which can further help in curing health problem related with cell membrane issue.

Keywords: Green synthesis; gold nanoparticle; anisotropy; Phytochemicals; erythrocyte cell; fluidity

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Introduction

The ultra small and fine structure currently known as nanoparticles has become popular now a days because of its large surface to volume ratio and their contribution as a bridge between bulk level and atomic or molecular level. Because of the large surface to volume ratio they can able to diffuse easily. Among many of the particles, gold nanoparticles (GNP) has some attracting and distinct properties like thermal conductivity, catalytic activity, antibacterial activity, anti HIV activity, anti arthritis activity, also have important aspects in biolabelling, nano diagnostics, drug delivery, gene delivery [1, 2]. There are many reduction processes which are mainly based on hazardous chemicals some of them are Turkevich method, Brust method, Martio method, sonolysis, citrate reduction by chloride ions [3, 4].

Green approach towards nanotechnology came to focus when hazardous chemicals showed their negative impact when applied to live cells as a diagnostic tool .As GNP often used as tagging agent in drug delivery so it has to be toxic free. Considering all the disadvantages, green nanotechnology came into lime light where plant's secondary metabolite act as a phytoreducer, capping, stabilizing agent and enhance the reduction process and to some extent the particle size can also be manipulated by changing pH, temperature, concentration of the plant extract etc [5] .*Celosia cristata* commonly known as cockscomb grow well in humid and arid condition and are hardy and resistant to many diseases. On the other hand *Vigna radiata* commonly known as mung bean belong to legume plant family cultivated in a hot and dry region. These seeds are enriched with secondary metabolite [6].

The breakdown of intact red blood cells causes the release of hemoglobin refers to as hemolysis which may cause by interaction with foreign material [7]. Erythrocyte cells when remain intact after hemolysis depleted from hemoglobin but they are considered as intact model of cell membrane which are use to study the structural and functional aspects of membrane [8,9].

In the current work an attempt has been made to synthesize the nanoparticle by utilizing bioresource like plant seed electrolyte, plant seed extract which is completely ecofriendly, low cost, and do not need any external helping factors like magnetic field, temperature, pressure, heat etc. and also they need not required sophisticated instruments and long span of time.

Phytochemicals were identified and particle characterizations were done. Fluidization is an important phenomenon with respect to cell function, integrity. Lateral diffusion, cell to cell interaction, entry of food and drug, phagocytosis all has a connection with membrane fluidization [10]. To measure this, 1, 6-diphenyl 1, 3, 5- hexatriene (DPH) a fluorescence amphiphilic dye first interacted with the isolated ghost membrane. They were for interior membrane part which oriented parallel to the lipid acyl chain axis or they resided at center of bilayer parallel to the surface [11]. DPH probe got internalized along with GNP and at constant temperature it provided the information of the internal environment after membrane and GNP interaction.

Result can be interpreted as GNP are safe to live cells and when interacted with ghost cells, negatively charged GNP incorporated and helped to fluidized the membrane as recorded from the change in polarization of the fluorescent probe DPH.

Experimental

Materials:

Chemicals were purchased from Merck specialties private limited and used without further purification. Plant seeds were collected from local market of India. Double distilled water (DDW) was used throughout the experiment. Blood samples were collected from eukaryotic system.

Preparation of seed extract and electrolyte:

Celosia cristata and *Vigna radiata* are locally available plants in India.80-90 seeds of *Vigna radiata* were immersed in double distilled water (DDW) overnight and after that the seed electrolyte which came out from the seeds were collected and filtered through Whatman filter paper. *Celosia cristata* seeds were crushed with mortar pestle and then boiled with distilled water for a certain time. Extracts were filtered and further used.

Synthesis of gold nanoparticle:

100ul of $HAuCl_4$ diluted in 1ml DDW were added individually to each of the two conical containing 1000ul of seed extract and seed electrolyte. The change of color of the solutions from dark purple black to wine red conformed that the reaction got completed. It took 4-5 hrs at room temperature (37 ℃).

Different characterization methods such as UV-VIS spectrophotometer, Fourier transformed infrared spectroscopy, X-ray diffraction. Zeta potential, Field emission scanning electron microscopy, Energy-dispersive X- ray spectroscopy etc were used for analyzing GNP size, morphology and surface charge distribution. For the study of UV-VIS spectra and zeta potential, samples were in solution form then were dried after centrifugation and heated at 60 ℃ for further characterization.

Perusalize the content of seed extracts and electrolyte, phytochemical assays for flavonoids, phenols, starch, ascorbic acid, reducing sugar have been done and phytoreducers were identified among sugar and secondary metabolites present in extract and electrolyte. For the same, standard techniques were followed [12]. To check the biocompatibility of the GNP, hemolysis percentage were calculated where erythrocytic cells were separated from the whole blood sample (5ml) through repeated washing and centrifuged at 700g and RBC suspensions were treated with GNP (0.005mg) and calculation was done by taking optical density at 540nm with respect to positive and negative control [13]. Erythrocyte ghost cells were prepared following a standard protocol [14]. The intact membranes were collected and solution of DPH in tetrahydrofuran (THF 0.1%) were added to the erythrocyte suspension to final concentration of 2μM. 100μg/ml GNP were added in ghost suspension and then at 37ºC samples were incubated for 30min.Fluorescence anisotropy were calculated by using a software FL Win Lab at 360nm and 435nm of excitation and emission wavelength [15].

Results and discussion

The synthesis of the GNP were primarily conformed by change of the color of the solution from dark purple black to wine red color shown in Fig 1.

Secondly taking the absorbance of the sample with respect to the raw plant seed extracts and electrolyte; we can see that there are 4 graphs with Surface Plasmon Resonance at different positions. In Fig 2(A&B) A that is *Vigna Radiata* seed electrolyte where we can see two prominent peaks at270nm and 340nm were obtained. But in case of B which was *Celosia cristata* seed extract only single peak could be identified which was at 270nm. Peaks at 340nm and 270nm were obtained mainly due to the presence of phytochemicals like flavonoids and phenols in seed extract and electrolyte. Fig 2 C&D showed UV-Vis absorbance after the synthesis of GNP where both the graph's absorbance were highlighted in between the range of 535nm-540nm.In case of graph C, which was for GNP derived from *Vigna radiata* showed minor peaks at 270nm and 340nm as they were derived from *Vigna radiata* and there was an additional major peak at 535 which conformed the synthesis of GNP. On the other hand graph D was the absorbance for GNP derived from *Celosia cristata* which showed a major peak at 540nm for GNP [16-17].

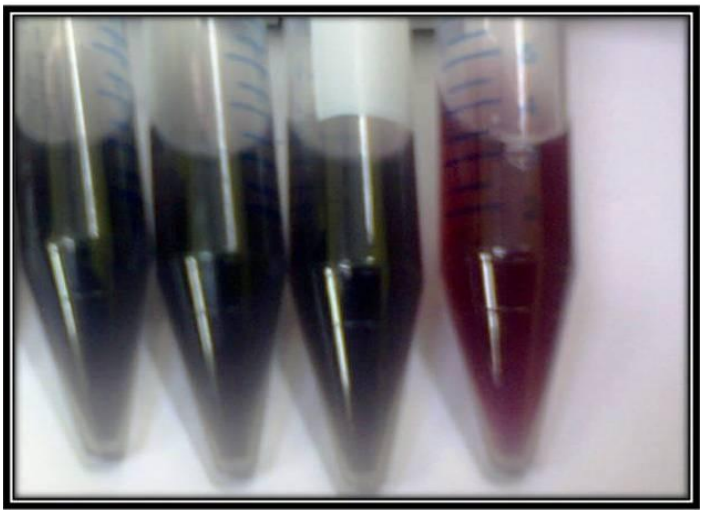

Figure1 Synthesis of GNP shown in wine red color

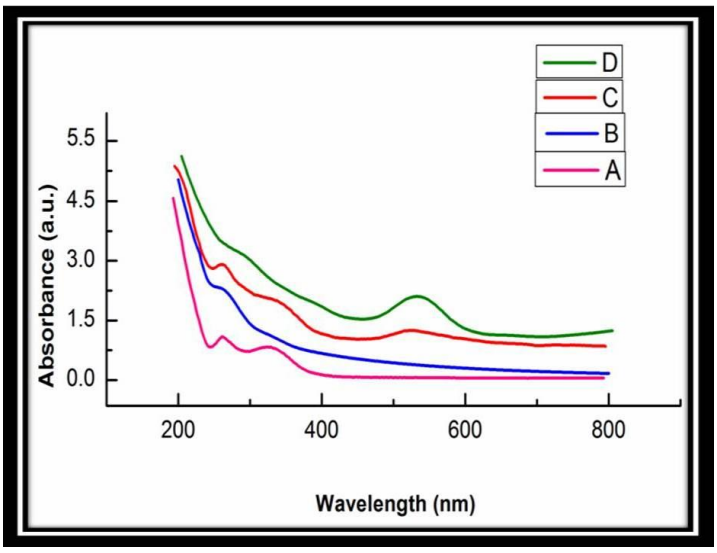

Figure2 A:- *Vigna radiata* seed electrolyte B:-*Celosia cristata* seed extract C:- GNP derived from *Vigna radiata* D:- GNP derived from *Celosia cristata*

Presence of secondary metabolites was confirmed through phytochemical assays.

Phytochemical analysis:

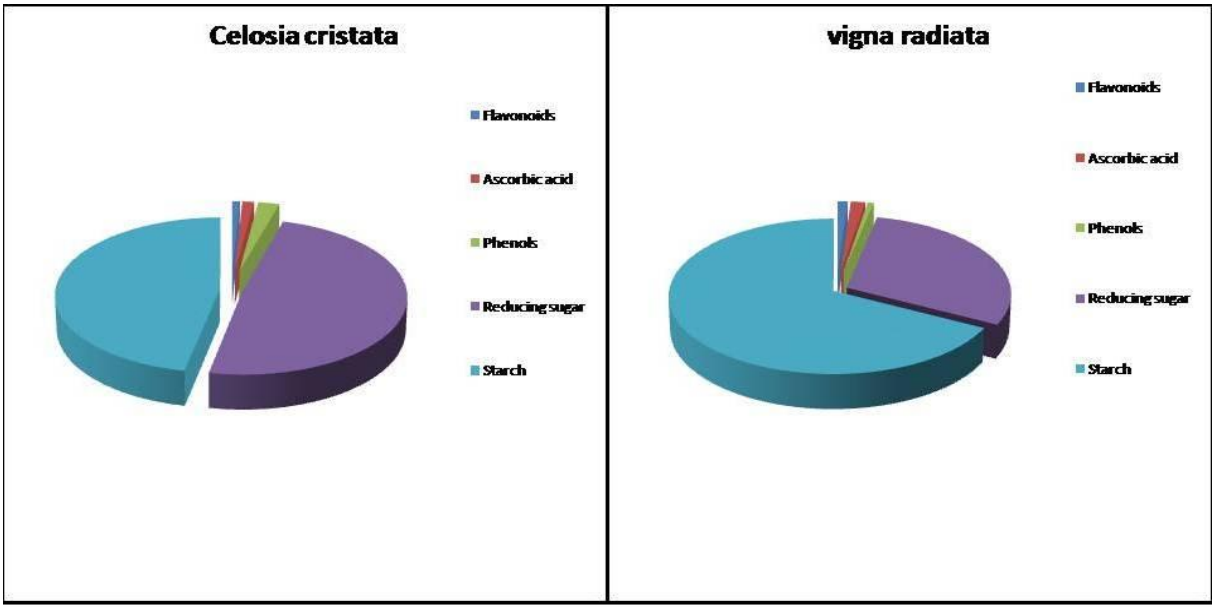

Figure 3 A statistical graph which depicts the amount of phytochemicals expressed in µg/ml present in individual plant extract and electrolyte.

Fourier transformed infrared spectroscopy (FTIR) analysis was done where four transmittance graph having different bands were obtained and which was scanned from 400-4000cm^{-1} observed in Figure 4.These bands signified the participating groups found in or existing in seed extracts and electrolyte and also in GNP. In *Celosia cristata* seed extract there were five prominent bands were found at 3409,2917,1635,1458,1116 cm^{-1} which was due to the presence of hydroxy group H bonded OH stretch,C-H asym/sym, alkenyl C=C and methane C-H stretch. Alkyl substitute ether, C-O stretch. In *Vigna radiata* seed electrolyte there were 4 distinct peaks were obtained at 3449,1609,1322,1062 for hydroxyl group H-band OH stretch, open chain azo (-N=N-),primary or secondary OH in plain bend., skeletal C-C vibration. The next peak was formed after GNP synthesis derived from *Celosia cristata* where four exact transmittance points were observed which was at 3368,1009,1599,1448. Due to normal polymeric OH stretch and aliphatic phosphate stretch cyclo hexane ring vibration 3368, 1009cm^{-1} peak were observed .1599 and 1448 cm^{-1} were due to formation of GNP. Lastly transmittance for GNP derived from *Vigna radiata* had 6 peaks at 3367, 2917, 1599, 1448, 1295, 1022 They were due to the presence of normal polymeric OH stretch, methylene C-H aym/sym stretch for 3367 and 2917.Next 1448 and 1599 were due to synthesis of GNP as explained in earlier case, 1295and 1022 were due to presence of vinylidene C-H in plane bend, OH in plane bend and cyclohexane ring vibrations [18-19].

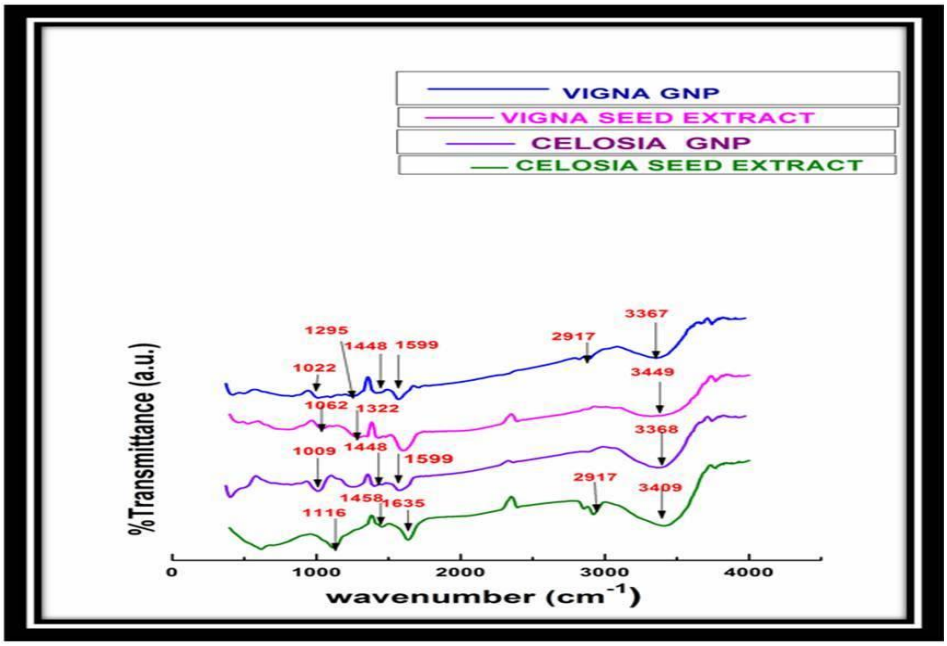

Figure4 FTIR spectral analysis of plant extract and electrolyte and GNP

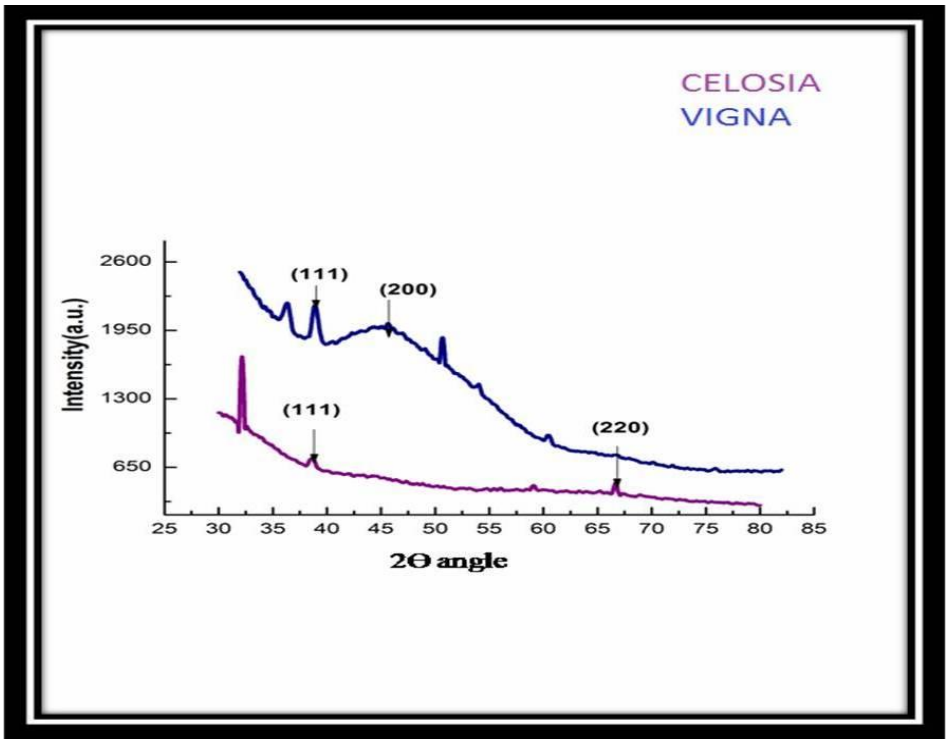

Figure 5 Absorbance peak of XRD for *Celosia cristata* and *Vigna radiata*

Figure 5 showed a crystalline phase of synthesized GNP where *Vigna radiata* has 2 distinct diffraction

peaks at 38.26 º, 44.48 º and *Celosia cristata* also had 2 peaks at 38.26 º and 66.29 º.These peaks namely 38.26 º, 44.48 º,66.29 º were indexed with the planes (111),(200),(220) for the cubic face centered gold. The lattice plane relevant to Bragg reflection were obtained which were indexed according to face centered cubic gold .They were confirmed by standard JCPDSNo.04-0784 database values [20].

Zeta potential were taken for verification of total surface charge present at the outer most surface of synthesized GNP. The value was -7.36mV in case of *Celosia cristata* derived nanoparticle and- 18.0mV for *Vigna radiata* derived nanoparticle. Electrokinetic is a very vital parameter for stability purpose. Smaller the GNP size means higher zeta potential which finally conclude with particle stability. The more negative value means there are less chances of particle aggregation [12]. So from the above mentioned data *Vigna radiata* derived GNP had more negative values of zeta potential as compared to *Celosia cristata* derived GNP hence we can say that may be *Vigna radiata* derived GNP were more stable than *Celosia cristata* derived GNP.

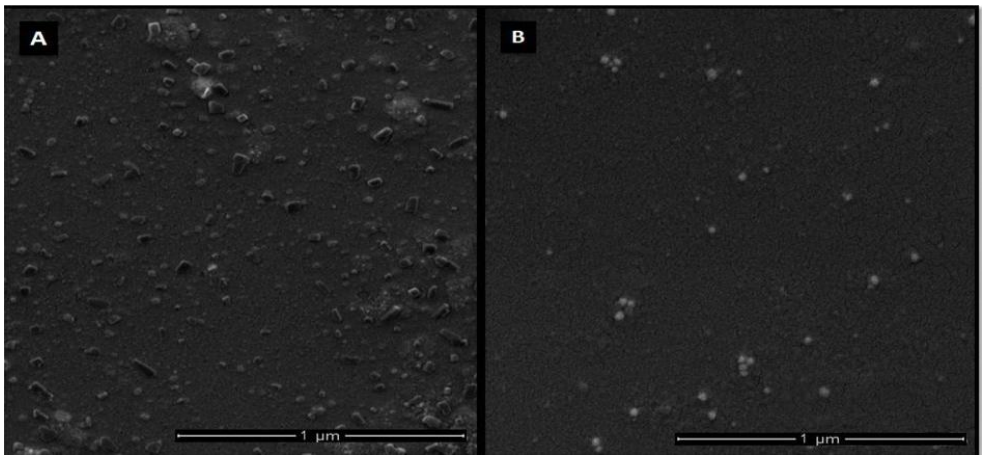

Figure6 FESEM IMAGES:A. Celosia derived GNP, B-Vigna radiata derived GNP

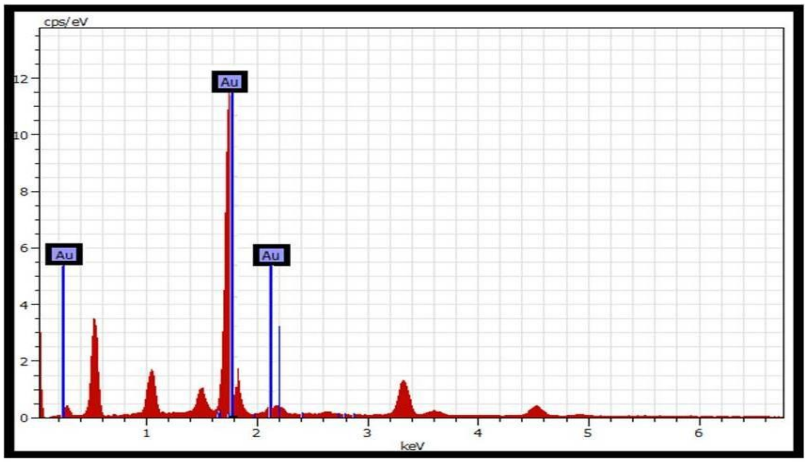

Figure 7 EDX of GNP of *Vigna radiate*

Nextly the size, shape, morphology of the synthesized particles were analyzed by Field Emission Scanning Electron Microscope(FESEM) as shown in Figure 6 and probability of occurrence of gold nanoparticle was quite high as evidenced by Energy- dispersive X-ray spectroscopy(EDX) graph where

three different sized peaks for GNP derived from *Vigna radiata* was highlighted as shown in Figure 7.

Figure 6 A showed the picture of GNP derived from *Celosia cristata* where size ranged from ~25-60nm having a varied morphology say pentagonal, square, hexagonal, spherical. But in case of Figure 6 B the GNP which was derived from *Vigna radiata* showed size range from ~20-60nm and almost all the particles were observed as spherical in shape.

For hemolysis percentage calculation, four sets of blood samples were prepared and treated with positive control, negative control and two types of GNP derived from two different plant sources. For positive control Triton-X 100 was used where almost all the erythrocyte cells were expected to get rupture.0.9 % saline was used for negative control where erythrocytes were expected to stand intact without rupture and lastly GNP from *Celosia cristata* and *Vigna radiata* were interacted with erythrocyte cells and total hemolysis percentage was calculated by using the formula :

$$\frac{\text{SAMPLE} - \text{NEGATIVE CONTROL}}{\text{POSITIVE CONTROL} - \text{NEGATIVE CONTROL}} \text{X } 100$$

After the treatment, total disruption of RBC lysate by Triton –X 100 was observed and cells were intact in presence of 0.9% saline.

The erythrocyte cells after interacted with GNP showed a percentage of hemolysis where *Celosia cristata* derived GNP showed 2.95% and *Vigna radiata* showed 2.3% . It has been reported that up to 5% hemolysis is permissible [21] and our result was quite satisfactory as that they were less than 5% so we can say that these synthesized GNP can be used as a tagging agent with any drug as they are safe for live cells.

Isolated erythrocyte ghost membranes were interacted with 20-60nm sized separately derived GNP and result was interpreted by change in anisotropy of the membrane. As fluorescence anisotropy is proportional to the order of molecular packing and inversely proportional to membrane fluidity. So actually fluidity is measured through considering the change in anisotropy. Crossing the cell membrane is inherently challenging due to the nature of the lipid bilayer and from an evolutionary point of view to protect the cellular functions. It was observed that neutral and negatively charged nanoparticles entered much less on the negatively charged cell membrane surface and consequently show lower level of interaction as compared to the positively charged particle. Internalization of negatively charge nanoparticle is believed to occur through nonspecific binding and clustering of the particle on cationic sites on the plasma membrane (that are relatively lesser than negatively charged domains and their subsequent endocytosis.)[22]

Our synthesized GNP are negatively charged conformed through zeta potential as explained above. When they interacted with the erythrocyte ghost membrane their target was to penetrate the membrane which was confirmed by fluorescence study. Among different probes like 8-Anilinonapthalene-1-sulfonic acid (ANS),(1-(4-trimethyle)-6-phenyl-1,3,5-Hexatriene p-toluenesulfonate (TMA-DPH),DPH was chosen because it has maximum chances to penetrate the inner leaflet of the membrane as compared to others.DPH is always a popular probe of membrane interior [15].

DPH penetrate the inner layer and gave information about the movement of polar and nonpolar region.

But there are many factors which help in fluidization like temperature, pressure, composition of the membrane. Lipids with shorter chains are less stiff and less viscous. So to establish a confirmatory result that GNP was the only reason for fluidity; the parameters were kept constant under consideration. Temperature was constant at 37℃ and comparison of change in anisotropy was done using a control set data whose membrane composition was exactly same as treated one.

So the total anisotropy was calculated and result showed (Fig 8) that as compared to control (Black bar) both the GNP ie, derived from *Celosia cristata* and *Vigna radiata* penetrated the inner leaflet of the membrane and graph also showed a prominent decrease in anisotropy with respect to time. We can see declined image of anisotropy of membrane has been started after 18min.when interacted with *Celosia cristata* derived GNP (Red bar) .And it was after 30min.in case of *Vigna radiata* derived GNP when interacted with membrane (Blue bar).Among the two GNP *Celosia cristata* derived GNP was more potent in detoriating the membrane anisotropy, making the membrane more fluidized than *Vigna radiata*. And major advantage of fluidization can be utilized in case of cardiovascular diseases(CVD) where previous report said that in case of cardiovascular disease deeper layer of erythrocyte membrane were became more rigid as compared with normal patients [23]. So if gold nanoparticles were used along with drug for treating CDV then it will help in curing the problem.

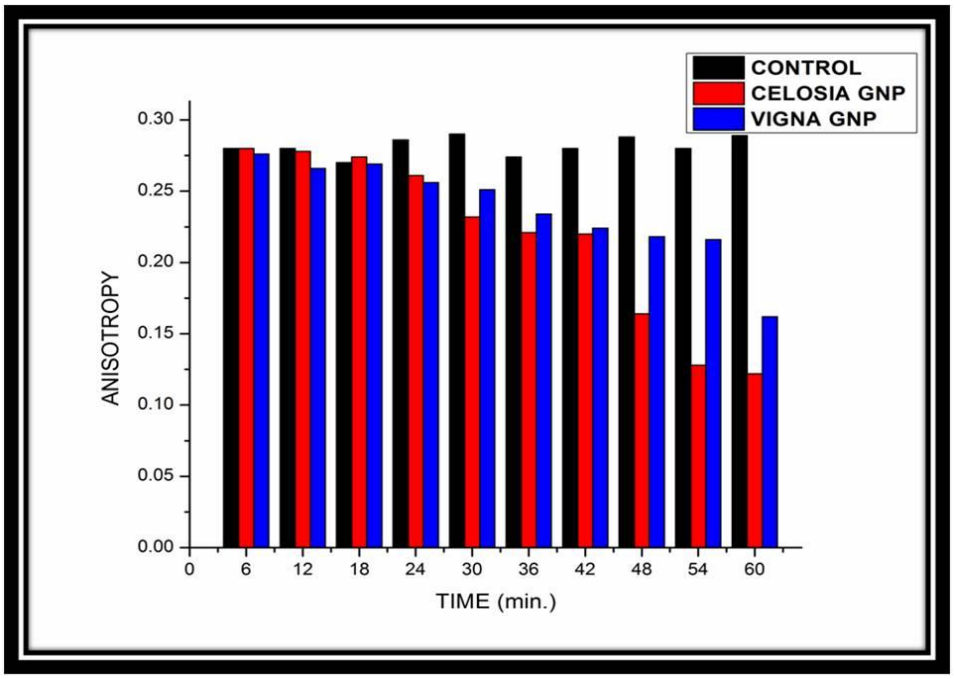

Figure 8 Anisotropic graph of GNP of different source (BLUE, RED) and untreated control. (BLACK)

Conclusion

The above experiment showed a pure, rapid, ecofriendly method of synthesizing gold

nanoparticles without using harmful chemicals which when interacted with live cells, they protect the cells from rupturing it means they are safe to live cells and also helped the erythrocyte cells to get fluidized by decreasing the membrane anisotropy.

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