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FACILE GREEN SYNTHESIS OF BIOCOMPATIBLE GOLD NANOPARTICLES WITH *CARDIOSPERMUM HALICACABUM* LEAF EXTRACT UNDER SUNLIGHT IRRADIATION

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ABSTRACT

Gold nanoparticles (GNPs) were synthesized under sunlight irradiation using an aqueous *Cardiospermum halicacabum* leaf extract as reducing agent. Gold nanoparticles formation was confirmed using Ultra Violet–Visible spectroscopy as it exhibited surface plasma resonance band at 534 nm. FESEM and TEM studies confirmed that synthesized GNPs were predominantly spherical in shape. Bright circular rings of SAED pattern correspond to crystal planes of gold and confirmed the crystalline nature of the Ch-GNPs. Particle size analysis revealed that the average size of synthesized gold nanoparticles was about 86.11 nm. Zeta potential was found to be around −9.18 mV. The synthesized GNPs were biocompatible towards red blood cells and fibroblast cells. Zebrafish embryo studies revealed that the synthesized GNPs were safe in *in-vivo*.

Key words: Green synthesis, Gold nanoparticles, *Cardiospermum halicacabum*, Zebrafish, Biocompatibility.

INTRODUCTION

In this decade, metal nanoparticles have received considerable attention in the field of biomedicine [1]. Among metallic nanoparticles, researchers have shown remarkable interest over gold nanoparticles (GNPs) as they exhibit properties such as biocompatibility and unique optical characteristics (strong surface plasmon resonance) [2,3]. Moreover ability of the GNPs to conjugate with different compounds made them more suitable for applications in the field of diagnostics and drug delivery [4]. Various physical and chemical methods are available for synthesis of gold nanoparticles but they have lost their significance due to the employment of toxic chemicals, high temperature and pressure in these methods [5, 6].

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Biocompatibility is the primary requisite for the GNPs to be used for biomedical applications. Utilization of eco-friendly reducing agents for GNPs synthesis is one of the novel ways to achieve the biocompatibility of the GNPs. Considering this fact number of researchers had adopted green nanotechnology to produce biocompatible GNPs using various plant like *Acalypha indica* [7], *Stevia rebaudiana* [8], *Azadirachta indica* [9], *Mangifera indica* [10], *Emblica officinalis* [11], *Punica granatum* [12], *Macrotyloma uniflorum* [13] , polymers like gellan gum [14], starch [15], chitosan [16] and phytoconstituents such as quercetin [17] and apiin [18]. In addition, synthesis of metal nanoparticles under sunlight irradiation is advantageous than conventional chemical and physical methods, as solar energy is renewable and moreover it act as a catalyst which speed up the rate of reduction reaction [19, 20]. *Cardiospermum halicacabum* (Sapindaceae family) was herbaceous climber, commonly found throughout India. *C. halicacabum* is commonly known as balloon vine (England), Jia hu gua (China) and mudakathan keerai (Tamil Nadu, India). Traditionally it was used in the treatment of rheumatism, nerve diseases, and as a demulcent in orchitis and in dropsy. Various pharmacological studies have proved the analgesic activitiy,

antipyretic activity, antimalarial and antioxidant activity of *Cardiospermum halicacabum*. A number of phytoconstituents have been isolated from *Cardiospermum halicacabum* such as arachidic acid, apigenin, apigenin-7-O-glucuronide, chrysoeriol-7-O-glucuronide and luteolin-7-O-glucuronide [21, 22, 23]. It has already been reported that flavonoids has potential to reduce Au^{3+} to Au^{0} and led to the formation of GNPs [24, 25]. In the present study, by considering the phytoconstituents and anti oxidant potential of *C. halicacabum,* the aqueous leaf extract is effectively utilized as a reducing agent for the green synthesis of GNPs under the irradiation of sunlight. Some of the physiochemical and biological properties of the synthesised GNPs were evaluated to understand its potential to be used for wide range of biological applications.

Materials and Methods

Chloroauric acid solution, DMEM with high glucose and MTT dye was purchased from Sigma–Aldrich, USA and used without further purification. NIH-3T3 fibroblasts were procured from National centre for cell sciences (NCCS), Pune, India. E3 medium, a standard medium to work with zebrafish embryos; (34.8 g NaCl, 1.6 g KCl, 5.8 g $CaCl_2.2H_2O$, 9.78 g $MgCl_2.6H_2O$,to prepare a 60 X stock solution. Given amount of salts was dissolved in 2 L of water. pH was adjusted to 7.2 and autoclaved. To prepare 1X medium, dilute 16.5 ml of the stock solution (60 X) to 1 L using sterile water).

Preparation of Aqueous leaf extract of *Cardiospermum halicacabum*

Healthy and clean *C. halicacabum* leaves were collected and aqueous extract was prepared by boiling the leaves (25g) with 100 ml of distilled water at 50°C for 15min. Finally the aqueous *C. halicacabum* leaf extract (Ch-LE*)* was filtered and stored at 4°C for further use.

Synthesis of GNPs

Various volumes of Ch-LE (10, 20, 30, 40, 60, 70, 80, 90 and 100 µL) were added to eppendorf tubes containing 200 µL of 1 mM $HAuCl_4$ solution. This reaction mixture was kept under bright sunlight for 15 min. Violet to pink colour was formed indicating the formation of Ch-LE reduced GNPs (Ch-GNPs).The Ch-GNPs was purified by centrifugation (14,000 rpm for 20 min) and the pellet was re-suspended in double distiller water.

UV-Visible spectroscopic analysis

The formation of GNPs was confirmed by UV-visible spectroscopic analysis. Measurements were carried out on using Analytica Jena UV-visible spectrophotometer, Germany.

FESEM analysis

The surface morphology of Ch-GNPs was analysed by *FE-SEM QUANTA* 200 FEG, Netherlands. A drop of Ch-GNPs was dried over aluminum foil and was subjected to FESEM analysis.

DLS measurement and Zeta potential

Ch-GNPs samples were diluted to appropriate concentration by using ultra pure water and analysed using Zetasizer 3000 HSA (Malvern Instruments, UK). Particle sizes were found out based on measuring the time dependent fluctuation of scattering of laser light by the nanoparticles as they diffused through the solvent.

Long term stability studies

To study the stability of Ch-GNPs, the freshly prepared particles were kept at 2–8 °C (refrigerated) for a period of about 3 months. Samples were analyzed spectrophotometrically for stability at the end of 3 months.

Haemolytic assay

Briefly, 30 µl of RBC suspension was added to the eppendorf tubes containing varying concentrations of Ch-GNPs (10, 20, 30, 40 and 50 µM) in heparinised buffer. Samples were incubated at 37°C for about 3 h and finally they were centrifuged at 18000rpm for 10 min at 4°C. Free haemoglobin content in supernatant was analysed by measuring the absorbance of samples at 540 nm. RBC suspension with heparinised buffer and water was considered as negative and positive control respectively.

$$\%\ \text{of Haemolysis} = \frac{O.D\ of\ test\ sample - O.D\ of\ negative\ control}{O.D\ of\ positive\ control - O.D\ of\ negative\ control} \times 100$$

***In-vitro* biocompatibility evaluation using Fibroblasts**

Standard MTT assay using NIH-3T3 fibroblasts was employed to understand the biocompatibility of synthesised Ch-GNPs. Exponential phase cells were seeded into the 96-well plates containing DMEM supplemented with 10 % fetal bovine serum and incubated at 37°C under 5 % CO_2 atmosphere. Known concentrations of Ch-GNPs (25, 50, 100, 150 and 200 µM) were added to the test wells and incubated again for a period of 48 hr. Each concentration was done in triplicate. Similarly control wells were maintained under same conditions without the addition of Ch-GNPs. After 48 h, DMEM medium was removed and 200 µL of 0.5 mg/mL MTT working solution was added and incubated.

At the end of the 4hr , the formed purple formazan crystals were solubilised by adding DMSO and analysed by using Tecan Infinite M 200 plate reader (λ_{max} = 570 nm). Percentage cell viability was calculated as follows.

$$\%\ \text{cell viability} = \frac{Absorbance\ of\ test\ cells}{Absorbance\ of\ control\ cells} \times 100$$

***In vivo* toxicity studies in Zebrafish embryo model**

In vivo toxicity of synthesized Ch-GNPs was evaluated using zebra fish embryo model. Adult zebra fish were allowed for natural mating and the zebra fish eggs were collected and cleaned using E3

medium. 10 healthy embryos were transferred to wells (24 well plate) containing 1 ml of E3 medium. Different concentrations of Ch-GNPs (250, 500, 750 and 1000 ng/mL) was added to the wells containing eggs and incubated at room temperature. The *in-vivo* toxicity was assessed by the measurement of hatching rate and survival rate of embryos. Moreover morphology of the zebrafish embryos at 96hpf was monitored using stereo microscope to understand the toxic effects of Ch-GNPs. All the tests were performed in triplicate.

RESULTS AND DISCUSSION

The Ch-LE induced the reduction of $HAuCl_4$ and lead to the formation of GNPs under the irradiation of bright sunlight. The pink colour was observed within 5 minutes and this appearance of characteristic pink colour is due to the surface plasmon resonance phenomena of formed Ch-GNPs in solution [26]. The intensity of the pink colour was increased with time and the reaction mixture attained maximum colour intensity within 15 min. The inset of Figure.1 shows the visual appearance of Ch-GNPs samples. UV-Visible spectrum of the Ch-GNPs was showed in figure 1.In order to study the effect of Ch-LE concentration on GNPs synthesis, 1 mM HAuCl4 (200 µL) was incubated with various concentrations of leaf extracts (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µL). At low concentrations of Ch-LE (10 and 20µL), significant peaks were not observed and this may be due to the fact that at lower volumes, amount of biomolecules present in the extract are not abundant which resulted in the formation of less number of GNPs in the solution. But when the volume of Ch-LE was increased, the intensity of gold SPR band was also steadily increased indicating the formation of more number of GNPs in the solution. Moreover a blue shift in the SPR peak was found with the increasing volume of Ch-LE. The blue shift in the λ_{max} indicates that smaller sized GNPs were formed with the increase in Ch-LE [27]. Gold nanoparticles formed at the concentration of 60µL Ch-LE shown maximum intensity and given SPR peak at 534 nm. Further increase in volume of Ch-LE used did not bring about increase in SPR indicating that 60 µL of Ch-LE was considered to be the optimum volume for the reduction of 200 µL of 1 mM HAuCl4. By considering the above findings, 30 mL of Ch-LE was added to 100 mL of 1mM $HAuCl_4$ solution and exposed to sun light for 15 min and the resulted Ch-GNPs were stored in a refrigerator for further use.

FESEM analysis

A representative FESEM image of the Ch-GNPs was shown as Figure. 2. Ch-GNPs were found to be mostly spherical in shape. The average particle size was found to be around 34.12 ± 5.27 nm.

TEM analysis

TEM images show that most of the particles were spherical in shape. Few triangular shaped particles were also seen in the TEM image Figure 3 (a). The mean size was found to be 35. 87 ± 4.18 nm. Figure 3 (b) shows selected area electron diffraction (SAED) pattern of Ch-GNPs. The pattern corresponds to the (111), (200), (220) and (311) crystal planes of Au nano-crystals [28].

Particle size analysis with Zeta potential

Figure 4(a) shows the size distribution of the Ch-GNPs, as obtained from DLS measurement. The average particle size was found to be 86.11 nm. The Ch-GNPs appear to be considerably larger than the particle size observed by TEM analysis. The biomolecules present over the surface of the GNPs leads to the increase in the hydrodynamic radius of the Ch-GNPs during DLS measurement [29]. The zeta potential of the GNPs synthesized using the Ch-LE was found to be about -9.17 mV (Figure. 4 (b)). Generally colloidal samples with zeta potential above +20 mV or below −20 mV are stable as the nanoparticles will have sufficient electrostatic repulsion between them to remain stable in colloidal solution [30]. Zeta potential of about -9.17 mV indicates that Ch-GNPs will not be stable for a long time. The stability of these Ch-GNPs can be improved by employing suitable stabilising agents (bio-polymers) which will cap the individual particles more firmly and give stability by avoiding aggregation of the GNPs.

Long term stability of Ch-GNPs

After 3 months of incubation period Ch-GNPs were analysed visually and spectrophotometrically. Aggregation and slight change in colour (violet) was found in Ch-GNPs. The stability of Ch-GNPs was evaluated by monitoring the λ_{max} of the SPR peak. It was observed that there were noticeable changes in the SPR peak of the Ch-GNPs samples. Figure. 5 show that the surface plasmon resonance shows a shift of about ~20 nm. Similarly SPR peak intensity of Ch-GNPs was also reduced to a considerable extent as shown in the figure 5. It implies that Ch-GNPs were not having long term stability.

HAEMOCOMPATABILITY

Haemocompatibility is one of the important criteria that GNPs should possess for their utility in drug delivery applications [31]. Ch-GNPs (10- 50 µM) caused haemolysis to extent of about 2% only (Figure 6). It was reported that the biological materials can be considered safe if the haemolysis rate is less than 5% [32].Hence, it can be said that Ch-GNPs are haemocompatible in nature.

Evaluation of *in-vitro* biocompatibility

Standard MTT assay was employed to assess the *in-vitro* biocompatibility of the Ch-GNPs over NIH-3T3 cell line. The percentage cell viability was found to be above 95% in Ch-GNPs treated cells (Figure. 7). There is no significant difference in

cell viability between the Ch-GNPs treated cell lines and control cells. The non toxic behaviour of the Ch-GNPs can be ascribed to the biomolecules that coated the surface of the GNPs. Similar finding was reported by Nripen Chanda *et al* [33] regarding the *in-vitro* cytcompatibility of the GNPs synthesized using cinnamon phytochemicals. MTT assay results demonstrate that Ch-GNPs are highly cytocompatible in nature.

In-vivo Zebrafish toxicity studies

The *in-vivo* toxicity of synthesized Ch-GNPs was evaluated using zebrafish embryos and the results were shown in Figure 8. Percentage hatching rate, percentage survival rate was assessed to understand the *in-vivo* safety of Ch-GNPs in zebra fish embryos. It was found that Ch-GNPs did not affect the hatching rate and survival rate of the zebra fish embryos. Further morphology of zebrafish embryos were observed using stereomicroscope for the presence of any morphological changes. Body shapes, heart, tail of the larvae were found to be normal after the exposure to Ch-GNPs of concentration about 1000 ng/mL indicating that Ch-GNPs were safe in *in-vivo* (Figure 8).

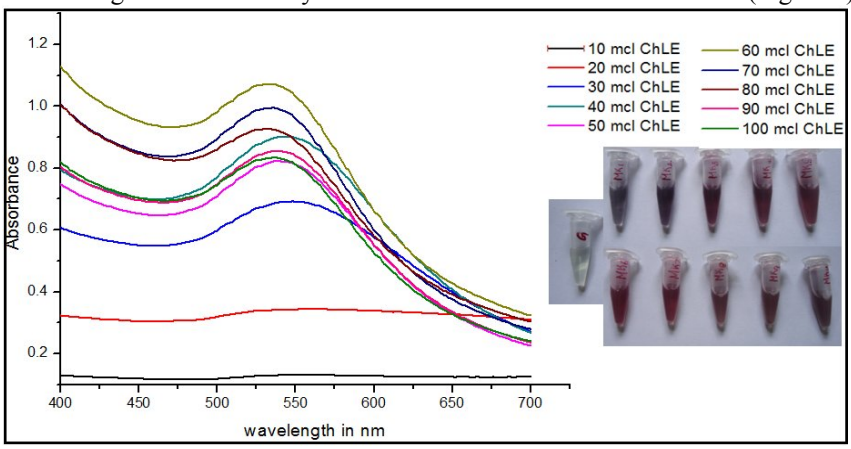

Figure 1.UV-Visible spectroscopic analysis of *Cardiospermum halicacabum* leaf extract reduced GNPs.

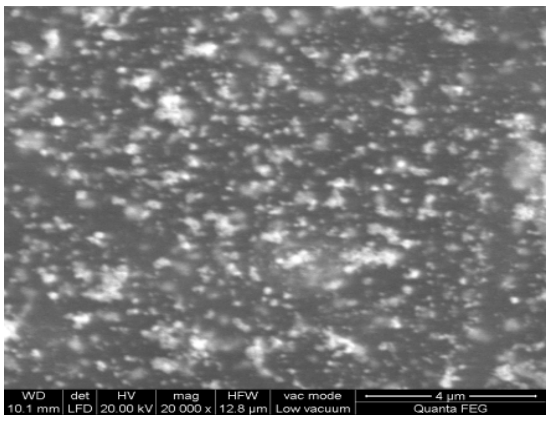

Figure 2. FESEM image of Ch-GNPs

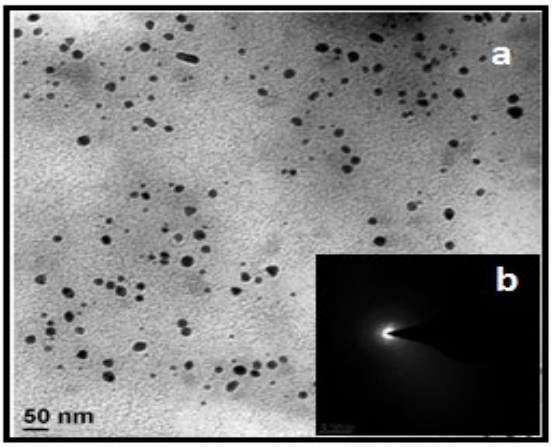

Figure 3 TEM images of CH-GNPs

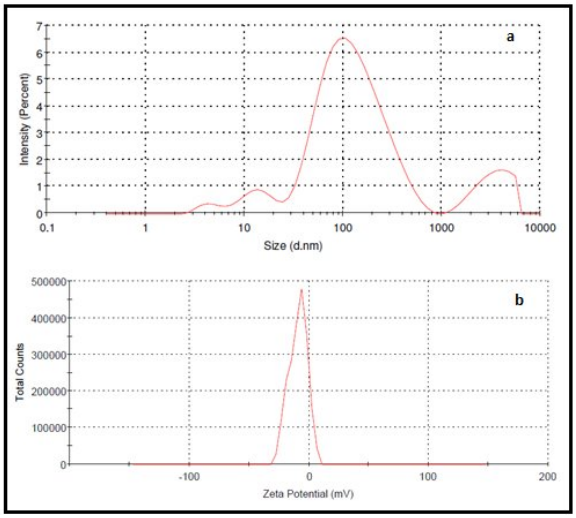

Figure 4. Particle size (a) and zeta potential (b) by Zeta sizer

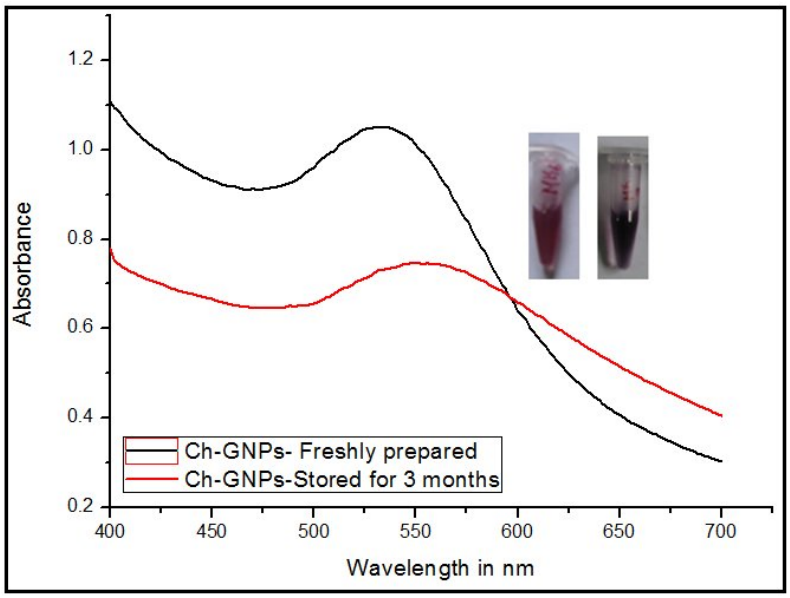

Figure 5. Long term stability analysis by UV-Visible spectroscopic analysis

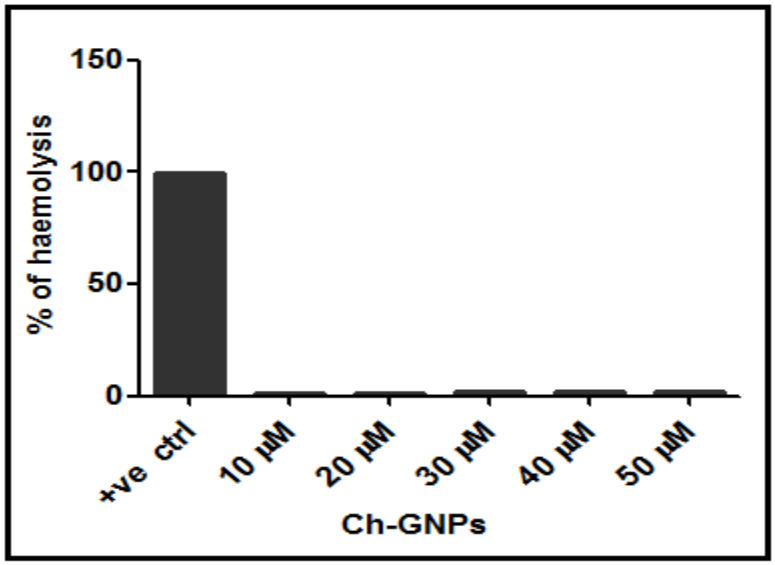

Figure 6. Haemolytic assay of Ch-GNPs

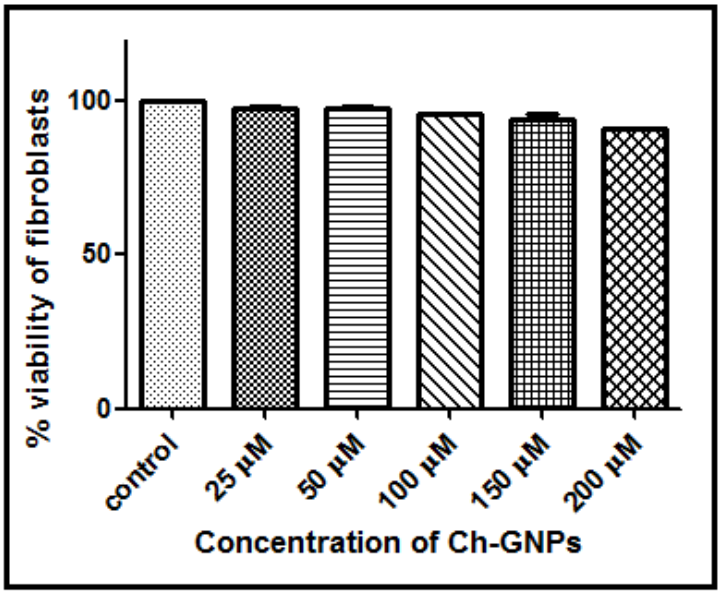

Figure 7. Biocompatibility analysis using NIH-3T3 fibroblast cell line by MTT assay

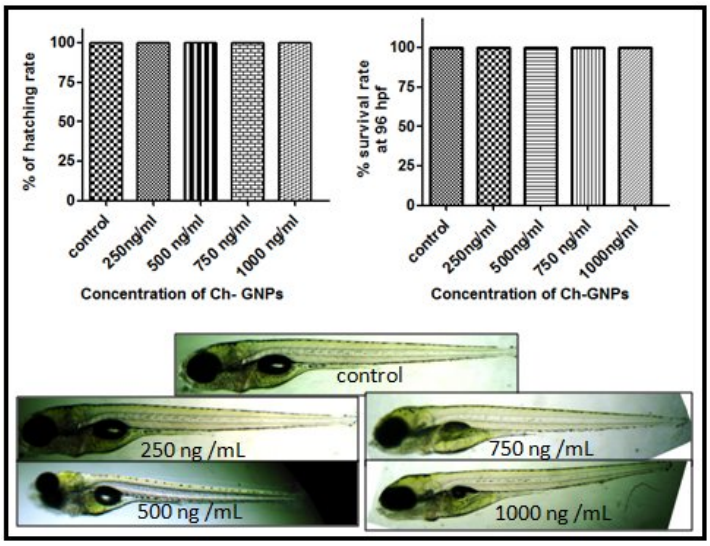

Figure 8. *In-Vivo* Zebra fish toxicity studies

CONCLUSION

The biomolecules in the *Cardiospermum halicacabum* leaf extract leads to the formation of gold nanoparticles under the irradiation of sunlight. Preliminary characterisation of gold nanoparticles was carried out by UV-Visible spectral analysis, FESEM and TEM. DLS measurement showed that the average particle size was about 86.11 nm. At this stage it can be presumed that flavonoids present in the Ch leaf extract such as apigenin having the ability to act as reducing agent was responsible for the reduction of Au^{3+} to GNPs. Moreover we found that obtained Ch-GNPs were haemocompatible and biocompatible in nature. *In-vivo* safety was ensured using zebra fish embryo model. Accordingly, it can be said that Ch-GNPs will be more suitable for variety of drug delivery and biological applications if the stability of the Ch-GNPs was improved by employing suitable biopolymers as stabilising agents.

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