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Genotyping of insulin plant *Costus igneus* using trnH-psbA using intergenic spacer gene trnH-psbA (PTIGS) and Biogenic gold nanoparticles synthesis

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Abstract : The aim of the study was to identify the genotype of plant species *Costus igneus* (*Costus igneus*) using *psbA-trnH* gene sequences followed by phylogenetic analysis and to evolve an alternative ecofriendly method to synthesis gold nanoparticles for biological application. Optimized gold nanoparticles were synthesized using different concentrations of Chloroauric acid ($HAuCl_4$), plant extract, temperature and pH. The same were characterized for surface plasmon bands, surface morphology, size. The nanoparticles obtained at 1mM concentration of $HAuCl_4$ at the temperature of about 70° C and at the pH 7. These nanoparticles showed an absorption peak at 538 nm in Ultraviolet-Visible spectrum (Uv-Vis) corresponding to the Plasmon resonance of gold nanoparticles. The size of gold nanoparticles ranged from 17 to 24 nm. The nanoparticles also showed long term stability in terms of aggregation for about 15 days in distilled water under room temperature. In lyophilized form it is showing long term stability for about 3 months under room temperature. Our results demonstrate the synthesis of new genre green gold nanoparticles by using *C.igneus* will provide opportunities towards development of nanoparticles biologically. This green chemistry approach is amenable to large scale production and this renewable plant material offers enormous benefits towards nanoparticles synthesis.
Keywords: *Costus igneus*, Gold nanoparticles, Phytochemically, Plant extract, Temperature.

Introduction

Plants have been used throughout human history for food and medicine[1]. Normally, the use of medicinal plants varies from species to species as diseases vary from one form to another in various locations. However, due to the physical similarities of plant parts in the market, resulting in a loss of efficacy and a danger of toxicity[2]. Therefore, the correct identification of medicinal plants is urgently needed to ensure the safety and quality of these natural health products. Conventionally, botanists tend to describe medicinal plants by their appearance and morphological identification[3]. However, this morphological technique can cause confusion, particularly when identifying unstructured plant parts[4]. Therefore, the developments of DNA-based markers has been important for the authentication of medicinal plants[5]. DNA barcoding is a novel technique of identifying biological specimens, which uses short DNA sequences from either nuclear or organelle genomes[6]. This technique has been successful in animal identification at the species level, and has been used in determining species boundaries, identifying new species, and species delimitation[7,8]. However, the standard DNA barcode is composed of a portion of the mitochondrial gene COI, which evolves too slowly in plants to serve as a useful

DNA barcode and has led to the search for a suitable DNA barcode for plants. Although this is growing area of scientific interest, our study has been conducted on species identification using trnH-psbA in *Costus igneus*.

Nanotechnology is a branch of scientific technology in which structures that have excellent properties creates by controlling atoms and molecules, functional materials, devices and systems on the nanometer scale by involving précis placement of individual atoms of the size around 0.1 – 100 nm[9]. The ideas and concepts behind nanoscience and nanotechnology were given by Physicist Richard Feynman also known as Father of Nanotechnology. He described the idea of creating things out of tiny pieces, instead of making things smaller in his lecture- " There's Plenty of Room at the Bottom" at an annual American Physical Society meeting in Pasadena, California on Dec. 29, 1959[10]. The major goals in designing nanoparticles are to control particle size, surface properties and release of pharmacologically active agents in order to achieve the site specific action of the drug at the therapeutically optimal rate and dose regimen with high volume ratio[11, 12, 13]. In recent years, synthesis of metal nanoparticles using plant leaf extract has attracted attention of many researchers because of availability of materials, inexpensive and process is easy to carry out in any laboratory use of non-toxic reagent[14, 15, 16].

Nanobiotechnology is a sprouting interdisciplinary field of research interlacing material science, bionanoscience and technology. Although different techniques such as, ultraviolet irradiation, aerosol technology, lithography, laser ablation, ultrasonic fields, and photochemical reduction of metals have been used to produce gold nanoparticles[17]. With the development of new protocols based on chemical or physical methods results in tedious downstream processing and environmental contamination provoke a release of large amount of hazardous by-products. Thus, there is a need for "green chemistry" that includes a clean, nontoxic, and environment-friendly method of nanoparticles synthesis[18].

Beginning works on nanoparticle synthesis using plant extracts have been carried out by many authors who reported that nanoparticles can be synthesized using plant extracts at rates equivalent to those of chemical methods[19]. In recent years, plant mediatedsynthesis of nanoparticles is important due to itssimplicity and eco-friendliness[20]. Studies elsewhere used the extracts of *Achillea wilhelmsii*[21], *Azadirachta indica*[22], *Sesbania drummondii*[23], *Cochlospermum gossypium*[24], *Camellia sinensis*[25] to synthesize gold nanoparticles. However, the homogeneity with regard to size and shape, shelf life of the nanoparticles scanty.

In this present study, the synthesis of gold nanoparticles was done by using single step reaction of the $HAuCl_4$ with the leaf extract of *C.igneus*. This plant is also known as *Fiery Costus* or spiral flag or insulin plant is used in India to control diabetes and its known that diabetic people eat one leaf daily to keep their blood glucose level low[26]. Leaves of *C.igneus* were one among the plants known to be effectively used for treating diabetes by the tribal people of Kolli hills of Namakkal district, Tamilnadu[27].

Taxonomic classification

Botanical name: *C.igneus*, Domain: Eucaryota, Kingdom: Plantae, Subkingdom: Viridaeplantae, Phyllum: Tracheophyta, Subphyllum: Euphylophitina, Infraphyllum: Radiotopses, Class: Liliopsida, Subcalss: Commelinidae, Superorder: Zingiberane, Order: Zingiberales, Family: Costaceae, Subfamily: Asteroideae, Tribe: Coriopsidae, Genus: *Costus*, Specific epithet: *igneus*[28].

The plant is famous for its high medicinal values andreported for antidiabetic property and non-toxicity nature motivated us to carry out this work[29]. In this paper, we reported the green synthesis of stable gold nanoparticles (AuNPs) by the direct reduction of $HAuCl_4$ via *C.igneus* extracts without using conventional stabilising agents. The advantages of using this approach include: (1) the leaf extract acts as both reducing agent and stabilising agent during the synthesis process; (2) the aqueous synthesis process is environmental friendly and produces no toxic waste products; and (3) the technique is simple, straight forward and does not require specialised equipment.

Experimental

Chemicals

Chloroauric acid trihydrate ($HAuCl_4.3H_2O$), Cysteine, BSA, Phosphate buffer saline was purchased from Sigma Aldrich, Chennai, India. All the chemicals were of analytical grade and used as such without any further purification.

Sample collection

Fresh leaves of *C. igneus* plants were identified and collected from the Horticultural Department, Hulimau, Bangalore, Karnataka, India in February, 2013.

Preparation of aqueous leaf extract

The fresh leaf of *C.igneus* were washed thrice with distilled water to remove dirt and excess water was blotted with tissue paper and shade dried at room temperature for 3 weeks. The leaves were cut into small pieces and powdered in a blender and sieved to get uniform size range and stored at 4°C for further studies. 5.0 g of leaf powder was added to 100 mL of sterile distilled water taken in 500mL Erlenmeyer flask and soaked for 2 hours and filtered usingWhatman No 1 filter paper. The filtrate was used for the synthesis of gold nanoparticles. The extract was prepared fresh as per the need.

Isolation of genomic DNA

The genomic DNA was isolated from plant sample by following the method of Ibane[30]. Polymerase Chain Reaction (PCR) amplification was performed using a 50µL reaction mixture containing 100ng of template DNA, 20µmol of psbA-trnH primers, 200µM of deoxy nucleotides (dNTPs), 1.5mM of $MgCl_2$, 1U of *Tag* DNA polymerase (MBI Fermentas) and 10µL of 10x *Taq* polymerase buffer. The sequences of psbA-trnH primers used were as follows.

psbA3_f: (5'-GTTATGCATGAACGTAATGCTC-3') – Forward

trnHf_05: (5'-CGCGCATGGTGGATTCACAATCC-3') - Reverse

Amplification was carried out with an initial denaturation at 98°C for 45 sec followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 64°C for 30 sec, extension at 72°C for 40 sec and final extension at 72°C for 10 min using a thermocycler (iCycler; Bio-Rad Laboratories, CA).PCR products were analyzed on 1% agarose gel for psbA-trnH amplicons in 1x TBE buffer at 100 V.

Sequence analysis of PCR products

The psbA-trnH amplified fragments were purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) from the agarose gel and sequencing using automated DNA sequencer (Model 3730, Applied Biosystems, USA). The sequences were analyzed using the option Basic Local Alignment Search Tool (BLAST) software available in NCBI National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast).

Phylogenetic analysis

The sequences of these psbA-trnH genes were compared against the sequences available from GenBank using the BLASTN program[31] and were aligned using CLUSTAL W software[32]. Phylogenetic trees were constructed using the UPGMA method[33]. Bootstrap analysis was done based on 1000 replications.The MEGA4 package[34] was used for all analysis.

Synthesis of gold nanoparticles from *C. igneus* leaf extract

Plant extract were prepared by mixing 5g of plant powder in 100 mL of distilled water, soaked for 2 hours and filtered using Whatman No. 1 filter paper. The obtained filtrate was used as reducing and stabilizing agent for the synthesis gold nanoparticles. 1 mM $HAuCl_4$ solution was prepared using deionised water and from this 20 mL of solution was mixed with 10 mL of plant extract and kept in waterbath at 70^0C for 2 minutes.

Optimization for the synthesis of gold nanoparticles

Optimization of gold nanoparticles synthesis was done by changing the parameters like plant extracts concentration, temperature followed by synthesizing procedures and checked for its Plasmon resonance band. Gold chloride solution (5 mL) was treated with different volumes of plant extract (1 mL, 2 mL, 3 mL, 4 mL and 5 mL) and kept at an elevated temperature (60-80°C)[35].

Characterization of phytochemically synthesized gold nanoparticles

UV-Visible spectroscopy analysis

The colour change in reaction mixture (metal ion solution + trisodium citrate dihydrate solution/leaf extract) was recorded through visual observation. The samples were diluted with 2 mL of deionized water and measured for UV-Vis spectrum at regular time intervals. The deionized water was used as a blank for background correction of all UV-Vis spectra. All samples were loaded into a 1 cm path length quartz cuvette for UV-Vis spectrophotometric readings and scanned form 300-800 nm at a scanning speed of 0.5nm interval[36].

X- Ray Diffraction analysis (XRD)

The crystallite domain size was calculated from the width of the XRD peaks by assuming that they were free from non-uniform strains using the following Scherer formula,

$$D = 0.94\lambda/\beta \cos \theta$$

Where, D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the FWHM and θ is the diffraction angle expels the added instrumental broadening[37].

FTIR spectroscopy analysis

The synthesized gold nanoparticles solution was centrifuged at 8000 rpm for 15 minutes and the obtained pellet was dried, lyophilized and mixed with KBr pellets, and then subjected to a wide range of FTIR spectral analysis (Schimadzu, Japan) various peaks were obtained for the test samples *viz.,* plant extract and gold nanoparticles.

Energy dispersive X-Ray analysis (EDAX*)*

EDAX analysis was carried out to confirm the presence of elemental gold.

Transmission electron microscopy (TEM)

The size and morphology of the AuNPs was investigated using TEM. A drop of solution containing as synthesized gold nanoparticles was placed on the carbon coated copper grids and kept under vaccum desiccation for overnight before loading them onto a specimen holder. TEM micrographs were taken by analyzing the prepared grids on TEM instrument (TECHNAI10-Philphs) operated at 80 Kev.

***In-vitro* Stability studies of synthesized gold nanoparticles**

In-vitro stability study of optimized *C.igneus* stabilized gold nanoparticles was tested in the presence of 0.5% BSA (Bovine Serum Albumin), 0.2M Cysteine, PBS 5.8, PBS 7.2, PBS 8. Typically, 1mL of gold nanoparticles solution was added to 24 well plates containing 0.5mL of 0.5% BSA, 0.2M Cysteine, PBS (pH 5.8,, pH 7.2, PBS 8) solution, respectively and incubated for 30 min. The stability and the identity of gold nanoparticles were measured by recording UV absorbance after 30 min[38].

Results and Discussion

Isolation of genomic DNA and PCR amplification

The DNA was isolated by following the method of Ibane[30] and the extracted DNA of *C.igneus* followed by PCR amplification using the primer for DNA barcode region psbA-trnH (300bp). The details of A. *Costus igneus* plant, B. Extracted DNA, C. PCR product of psbA-trnH gene (Figure 1)given below.

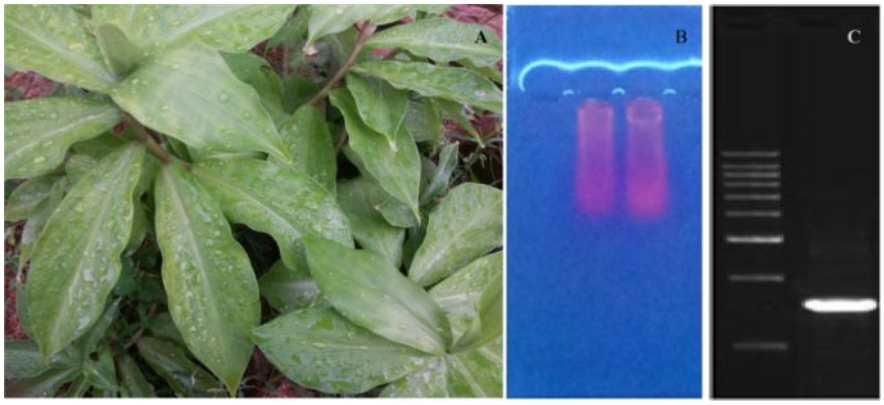

Figure 1: A. *Costus igneus* plant, B. Extracted DNA,

C. PCR product of psbA-trnH gene (PTIGS).

>S_trnH_S6613_D11_080.ab1

AGAGCTGCTGTTGAAGTTCCATCTATCAATGAGATAAGATTTCGGTCTTAATTATATACGAGCTTC
TAAAAAGGTAAAGGAGCAATAACTAATTTCTTGTTTTCTCAAGAGGATTGATATTGCTCCTTAATT
ATGAATAAATTTTACATAAGTTTTTTTCTAATTTCTACTTATACATAGTTAGTTATAGGGTAGGGGG
GCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAATCCACCATGCGCGT

Phylogenetic tree analysis of the sample

The evolutionary history was inferred using the unweighted Pair Group Method with Arithmetic Mean (UPGMA) method by Nakkala[39]. The optimal tree with the sum of branch length= 0.133965832 is shown. The percentage of replicate trees from the taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches[33]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA4[34]. Based on the (BLAST)and phylogeny analysis it was clearly revealed that the given plant sample was belongs to the taxa *C. igneus*. (Figure 2)

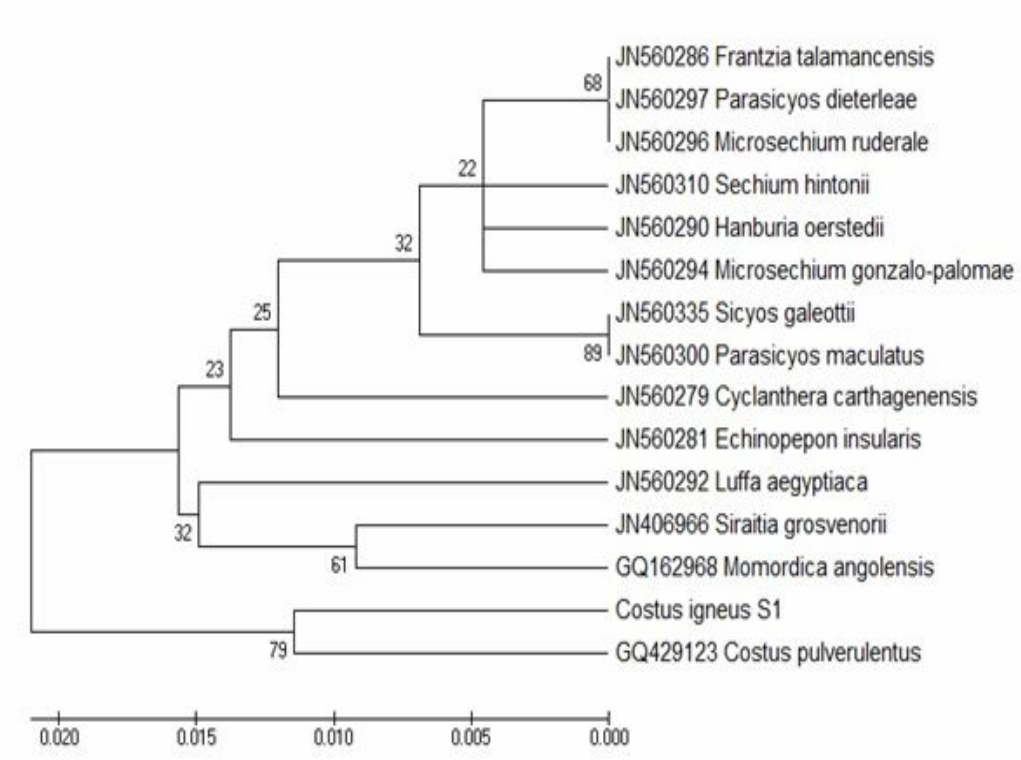

Figure 2: Phylogenetic analysis using MEGA4.

In the present study, the biogenesis of gold nanoparticles was derived by using aqueous leaf extract of *C.igneus* followed by characterization. The UV- visible absorption spectra showed its highest peak at 538 nm which resembles the surface plasmon resonance band (SPR) of gold nanoparticles and the spectrum of aqueous leaf extract of *C. igneus* was found to be 150 nm. The results of the Ultraviolet-visible spectroscopic analysis of gold nanoparticles from the *C. igneus* plant are shown in Figure 3.

Different concentrations of aqueous solution of leaf extract of *C. igneus* was treated with 1ml of 1mM Chloroauric acid solution in the ratio (1:1, 2:1, 3:1, 4:1 and 5:1) at room temperature and at elevated temperature (60, 70 and 80ºC). The synthesis of gold nanoparticles gets completed (yellow to wine red) in 3 minutes at higher temperature whereas in room temperature it is taking time upto 2 hours[35].

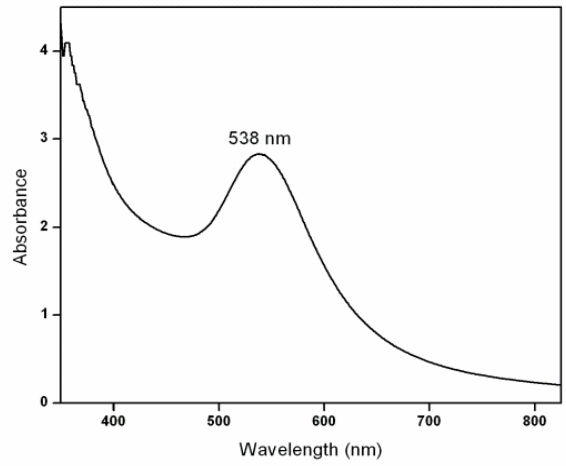

Figure 3: SPR band of *C. igneus* synthesized AuNPs.

While noticing the reactions at different concentration of plant extracts shown the decreases the spectrum as the volume of the aqueous solution of leaf extract of *C.igneus* increases. This may be due to the presence of more reducing phytoconstituents in higher concentration of extract which results in an additional interaction between the surface capping molecules and secondary reduction process obtained through high dispersion in higher temperature.

The Au crystalline phases present were found to be reliable with standard phases incorporated in the ICDD (International Centre for Diffraction Data). The XRD patterns showed an intense and sharp peak at 2θ = 38.2°, 44.26° and 66.13° and 77.58°, which may be indexed to the planes (111), (200), (220) and (222) respectively and it was shown in the figure 4. The presence of four prominent Braggs reflection, corresponding to the (111), (200), (220) and (222) orientations agree with those reported for gold nanoparticles and interestingly the broadness of these reflection demonstrates that the formed particles are in the nanoscale dimension.

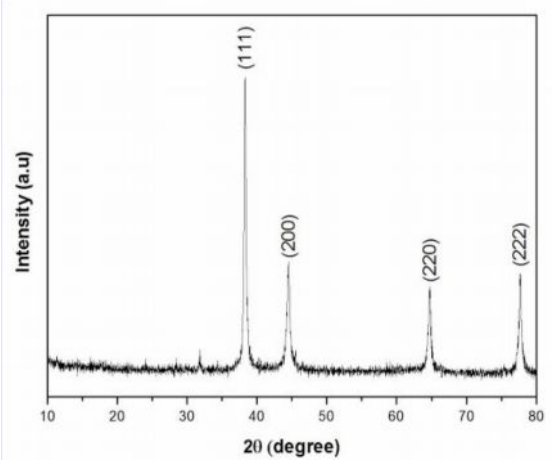

Figure 4: XRD spectra of AuNPs using *C. igneus.*

FTIR spectra of *C. igneus* extract and AuNPs. The spectrum of leaf extract showed peaks at 3878, 3767, 3687, 3614, 3203, 2953, 2882, 2355, 1711, 1581, 1021, 656 cm^{-1}. Characteristic peaks of gold nanoparticles synthesized by the reduction of $HAuCl_4$ ions by *C. igneus* leaves extract was found to be 3847, 3732, 3611, 2905, 2838, 2341, 1746, 1929, 1686, 1537, 1010, 899, 807, 737, 613. The absorption peaks at 3614 cm^{-1} may be due to stretching vibration mode of O-H group. The corresponding peak centered at 2882 cm^{-1} shows the presence of C-H bend likewise for 1581(C=C), 1711(C=O), 1686 (N-H bending) which is illustrated in the figure 5. FTIR spectra revealed the presence of possible reducing groups responsible for AuNPs synthesis.

The EDAX analysis of gold nanoparticles shows strong signals for gold atoms along with weak signals from sodium, magnesium and potassium. These weak signals could have arisen due to the presence of phytochemical constituents of *C.igneus* were illustrated in the figure 6. TEM showed biosynthesized gold nanoparticles were found to be spherical in shape with prominent size ranging from 17 to 24 nm. The results obtained from the TEM study givesclear indications regarding the shape, size and size distribution ofthe nanoparticles (Figure 7).

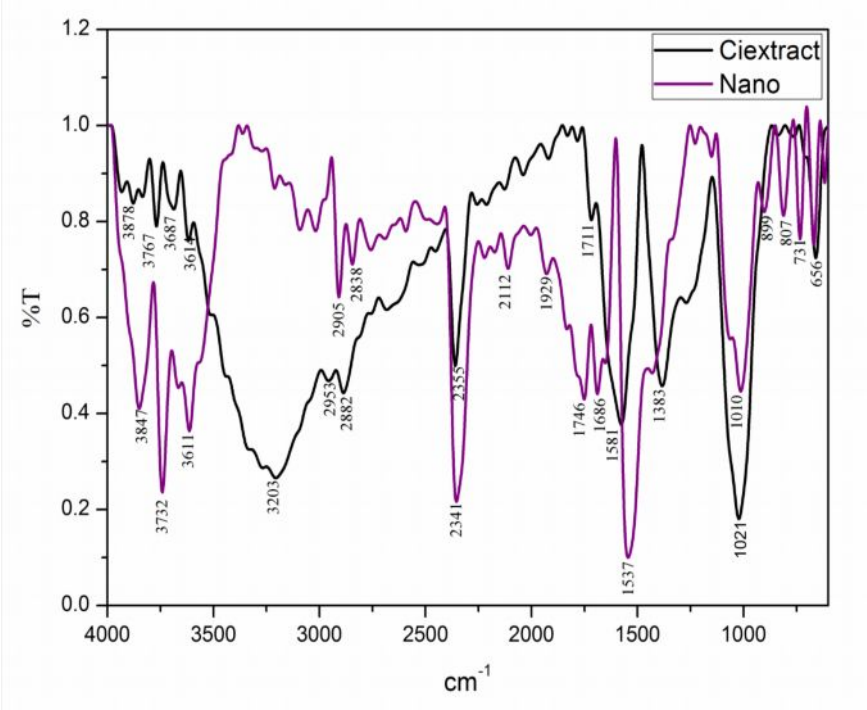

Figure 5: FTIR spectrum of aqueous plant extract of *C. igneus*

Stability testing

The stability of *C. igneus* stabilized gold nanoparticles were evaluated by monitoring its Plasmon band by using 0.2 M cysteine, 0.5% BSA, phosphate buffer solution at pH 5.8, 7.2 and 8. The plasmon wavelength was found to be 565nm in cysteine, 552nm in BSA, 537nm in 7.2, 524 nm in 5.8 and 516nm in 8, in all above shows shift of ~1 – 10 nm. Our result from the *invitro* stability studies has confirmed that gold nanoparticles were stable in biological fluids at physiological pH. The synthesized nanoparticles are found to be more stable in distilled water (7.2).

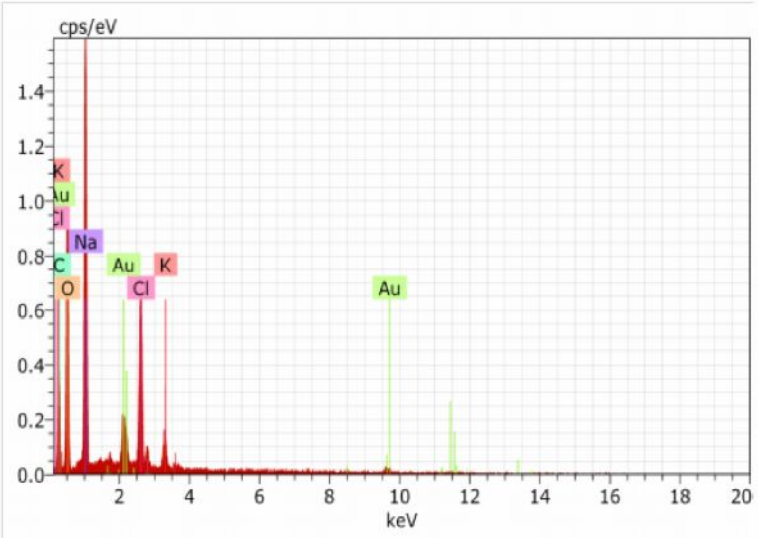

Figure 6: EDAX pattern of AuNPs.

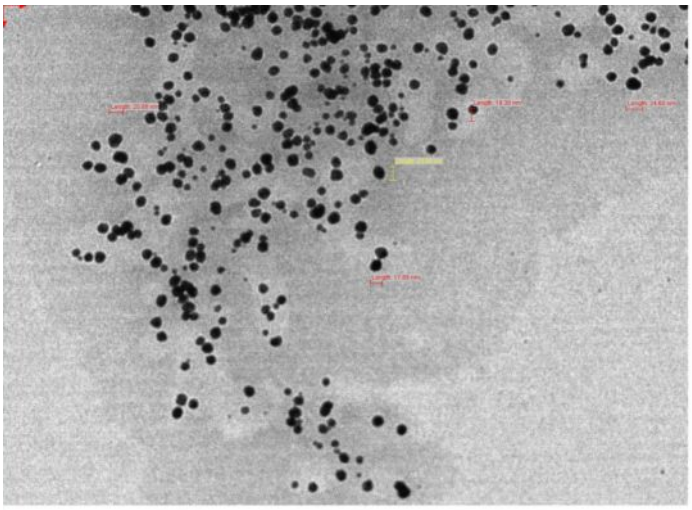

Figure 7: TEM image of AuNPs.

Based on the BLAST analysis and phylogeny analysis revealed that the isolated genomic DNA of the plant sample belongs to the taxa *C. igneus*. Further the plant extract itself acts as a reducing and stabilizing agent to synthesize gold nanoparticles extracellularly. The reduction of gold ions by *C. igneus* leaves extract resulted in the formation of stable nanoparticles at p^H 7.2) and at the optimum temperature of about 70°C The stability of nanoparticles was confirmed by using plasmon absorption and by means of aggregation[40]. Based upon the investigation, lyophilized nanoparticles are showing maximum lifespan of about 3 months and in aqueous stage for 7 days in room temperature.

The reduction time of $HAuCl_4$ using *C.igneus* (3 minutes)was found to be more rapid than *Mirabilis jalapa* mediated synthesis (1.0 h)[41] and *Coriander* leaf mediated synthesis (12 h)[42]. The concentrations of leaves extract and metal ions are playing an important role in the green synthesis of AuNPs. This work may fortify the screening of a new plant as a potential source of reducing agent for the synthesis of gold nanoparticles.

FT-IR spectra revealed the presence of possible reducing groups in the plant extract responsible for AuNP[43]. The synthesized gold nanoparticles were nearly spherical in shape. The rapid reduction, relatively smaller-sized and spherical-shaped particles of Au ions using *C. igneus* provides several advantages in the direction of biogenic process and also denotes the superiority over the chemical synthesis in providing green, environmentally safer method of nanoparticle production.

Conclusion

The green synthesized and characterized gold nanoparticles were crystalline in nature with uniform size and shape so in future it may acts an alternative source for the production of gold nanoparticles by avoiding chemical methods.

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