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SYNTHESIS AND CHARACTERIZATION OF GOLD NANOPARTICLES BY *JUSTICIA GENDARUSSA* BURM F. LEAF EXTRACT

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ABSTRACT

The unusual physio and chemical properties of gold nanoparticles are found to have more advantage in the field of medicine, diagnostics and biosensors. In the present document, it is reported that *Justicia gendarussa* leaf extract mediated synthesis of gold nanoparticles by the reduction of gold ions. Three different phytochemical fractions were prepared from methanolic leaf extract by liquid-liquid extraction method using immiscible solvents. The total polyphenols, flavonoids and electron donating capacity (DPPH assay) of each phytochemical fraction was analyzed. The 1mg/ml of diethyl ether phytochemical fraction produced more gold nanoparticles within 15 minutes when exposed to 10ml of 0.5mM chloroauric acid compared to chloroform and ethyl acetate phytochemical fractions. The structural characteristics of diethyl ether phytochemical fraction synthesized gold nanoparticles were characterized by UV-visible spectroscopy, Dynamic light scattering, Transmission electron microscopy, X-ray diffraction and Fourier transform-infrared spectroscopies. These biosynthesized gold nanoparticles showed surface plasmon resonance band at 536nm in UV-visible spectrum. The size of the gold nanoparticles ranged from 20 to 42nm and 62 to 88nm with spherical, triangle, truncated triangle and hexagonal shapes. From the Fourier transform-infrared spectra of diethyl ether phytochemical fraction and synthesized gold nanoparticles, the possible functional group involved in gold ions reduction and capping of gold nanoparticles were identified. The stability of gold nanoparticles for 5 month period and at different pH range (5-10) was analyzed by observing the changes in surface plasmon resonance of gold nanoparticles. Moreover, the diethyl ether phytochemical fraction showed no cytotoxicity up to 100µg/ml in RAW 264.7 cell line .

INTRODUCTION: Gold nanoparticles (AuNPs) are promising metal nanoparticles, utilized in drug delivery [1], biosensing and biolabeling applications [2, 3] due to their unique optical and electronic properties. These properties are size and shape dependent, hence gold nanoparticles with different shapes and sizes are required for each application [4, 5]. In addition, the AuNPs are also having biological activities such as antioxidant, anti-inflammatory, anti-angiogenesis and anticancer properties [6-9]. In recent times, astounding efforts have taken by researchers to develop an efficient methodology for large scale synthesis of

monodispersed gold nanoparticles. In general, AuNPs are synthesized by chemical method in which highly toxic reducing agents are involved. These toxic agents are adsorbed on the surface of gold nanoparticles which may cause undesirable effect in medical field [10-12].

In addition to this, various methods includes electrochemical reduction [13, 14], photochemical reduction [15] and heat evaporation [16, 17] etc., have been used for the synthesis of AuNPs. These methods need sophisticated instruments, toxic reducing agents and expensive stabilizing agents for synthesis of the stable gold nanoparticles. Apart from this, extracellular and intracellular biosynthesis of gold nanoparticles employed with bacteria [18], fungi [19] and yeast [20] etc., are eco-friendly method but cultivation of this biomass and downstream processing of nanoparticles seems to be a problematic task.

The biosynthesis of gold nanoparticles by plant extracts gains importance due to their simplicity, non toxicity, stability and cost effectiveness. Recently biosynthesis of AuNPs by plants leaf extract of Coriander[21], *Cinnamomum zeylanicum* [12], *Sorbus aucuparia* [22], *Rosa rugosa* [23], *Centella asiatica* [24], and *Mangifera indica* [25] have been reported. The plant leaves are important source for antioxidant molecules such as polyphenols and flavonoids which have ability reduce metal ions. These antioxidant molecules produce gold nanoparticles by reduction of gold ions (Au^{3+}) [26]. The potential of *Justicia gendarussa* leaf as source of antioxidant molecules for the synthesis of AuNPs is not yet explored.

The present study reported rapid, simple and nontoxic route for the synthesis of stable gold nanoparticles from the leaf extract of *Justicia gendarussa.*

MATERIALS AND METHODS:

Plant collection and preparation of Phytochemical Fractions: *Justicia gendarussa* plant was collected from Kanjikode hills, kerala. It was authenticated by Dr. Arumagaswamy, Plant taxonomist of KonguNadu College of Arts and Science, Coimbatore, India. The fresh leaves of *Justicia gendarussa* was washed with distilled water and shade dried for a period of one month. The dried leaves were powdered coarsely and extracted with methanol in soxhlet apparatus for 24h. The methanol extract was concentrated under reduced pressure and added to equal amount of distilled water. After 24h incubation, the chlorophyll was precipitated and removed by centrifugation at 5000 rpm for 15 minutes. The supernatant was concentrated and fractionated by immiscible solvents such as diethyl ether (Et_2O), chloroform ($CHCl_3$) and ethyl acetate (EtOAc) sequentially by liquid-liquid extraction method. Each phytochemical fraction (PF) was dried at 45°C and named as diethyl ether phytochemical fraction (Et_2O-PF), chloroform phytochemical fraction ($CHCl_3$-PF) and ethyl acetate phytochemical fraction (EtOAc-PF).

Estimation of Total Polyphenol Content: The total polyphenol content of PFs was determined according to McDonald *et al*.[27] method using Folin-Ciocalteu reagent. 0.5ml of Folin-Ciocalteu reagent and 5ml of 1M aqueous sodium carbonate was mixed with 0.5ml of each PF (0.3mg/ml). The reaction mixture was incubated for 15 minutes at room temperature. The resulting reaction mixture was diluted with distilled water (1:2) and its absorbance was measured at 765nm using UV-visible spectrophotometer (USB-ISS-UV/VIS spectrophotometer, Ocean optics). Typical procedure was followed for standard catechol in the concentration range of 50-250µg/ml and calibration curve was prepared. The total polyphenol content were expressed in terms of µg/mg PF. The tests were performed in triplicates

Estimation of Total Flavonoid Content: The total flavonoid content of PFs was determined by UV–visible spectrophotometer using aluminium chloride [28]. 0.5ml of each PF (0.3mg/ml) was separately mixed with 1.5ml of methanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. The reaction mixture was incubated for 30 minutes at room temperature. The absorbance of the reaction mixture was recorded at 415nm by using a UV–visible spectrophotometer. The calibration curve was prepared by using methanol solution of quercetin (standard) with concentrations ranging from 20to100µg/ml. The total flavonoid content was expressed in terms µg/mg PF. The test for concentration, of each sample was performed three times.

Free Radical Scavenging Assay: Free radical scavenging activity of each PF was determined by stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) [29]. 1ml of different concentration of each PF (0-50µg/ml) was separately mixed with 1.0ml of DPPH (0.004%, dissolved in methanol). The reaction mixture was kept at room temperature for 15 min. The absorbance of reaction mixture was measured at 517nm by UV-visible spectrophotometer. The percentage of scavenging activity of phytochemical fractions was calculated by using the following formula.

$$\%Scavenged = \left(1 - \frac{A_1}{A_0}\right) X100 \quad \text{---------- (1)}$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

The concentration of phytochemical fraction having 50% radical inhibition activity (IC_{50}) was determined from graph of PF concentration *Vs* Free radical activity (%). Three replicates were performed for each test concentration to get accurate results.

Synthesis of AuNPs: 1mg of each PF was separately mixed with 10ml of aqueous chloroauric acid (0.5mM) to reduce Au^{3+}. The reaction mixture was kept at room temperature for 15 minutes. The maximum production of gold nanoparticles by each PF was determined by UV-visible spectroscopy.

Characterizations of AuNPs: The particle size and distribution were measured in gold suspension, using dynamic light scatting (DLS) equipment (Zetasizer APS, Malvern, UK). Malvern zetasizer equipped with 10mV He-Ne laser (633nm) and operated at an angle of 90^o. The morphology of synthesized AuNPs was identified by Transmission electron microscopy (TEM) analysis. The sample for transmission electron microscopy analysis prepared on carbon coated copper grid. The transmission electron microscopy image was obtained by JEM-1200EX instrument operated at accelerated voltage of 120 kv. The crystalline pattern of powdered AuNPs was recorded by XDL 3000 powder X-ray diffractometer. Fourier transform- infrared spectroscopic (FT-IR) analysis of the dried powder of AuNPs and native diethyl ether phytochemical fraction were performed on FT-IR 8201shimadzu spectrophotometer. The Fourier transform-infrared spectroscopic spectra were recorded in the range of 450–4000cm^{-1} at a resolution of 4 cm^{-1}.

Stability study of AuNPs: The study of synthesized stable AuNPs was carried out at room temperature. The change in Surface plasmon resonance (SPR) of AuNPs stability dispersion was recorded at regular time intervals (1^{st} day, 1^{st} month, 3^{rd} month and 5^{th} month) and at different pH ranges (pH5-10) using UV-visible spectroscopy. In the pH dependant stability study, change in SPR of AuNPs solution was measured after 24h incubation with different pH solution. The pH of AuNPs solution was adjusted using 0.1N hydrochloric acid for pH 5- 6 and 0.1M sodium hydroxide for pH 7-10 using calibrated pH meter (Elico pH Meter 101, India)[30].

Cell Viability Assay: MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric assay method was used to analyze the effect of phytochemical fractions on Raw 264.7 cell viability, where the cells were cultured in 96-well microtiter plates. The reduction of MTT to blue formazan product was measured by scanning multiwell spectrophotometer (Bio-Rad, Model 680, Japan) and is directly proportional to mitochondrial dehydrogenases present in the viable cells.

Prior to treatment, Raw 264.7 cells were plated at 1×10^4 cells per well and starved in DMEM with 0.5% serum for 5h. To analyze the effect of diethyl ether phytochemical fractions on cell viability, Raw 264.7 cells were treated with test sample (0-100µg/ml) for 24h. At end of the treatment, MTT (10µl/ml) was added up in each well and incubated for 4h (at 37°C). 100µl of dissolving buffer (provided in kit) was added in each well and allowed to stand for 24h and then absorbance was read at 595nm. All measurements were done in triplicate.

Statistic Analysis: All the tests were repeated at least three times and data was expressed as mean±SD. The statistical analysis was carried out using Student's t-test for pair data (GraphPad software, San Diego, CA, USA). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSIONS:

Free Radical Scavenging Activity of PFs: The electron donating capacity of each phytochemical fraction was directly measured by DPPH (free radical) scavenging assay. The stable purple color of DPPH radical decolorized when accepting electron from antioxidant molecules. Free radical scavenging activity of PFs was determined by measuring the absorbance of DPPH free radical at 517nm. The absorbance of DPPH decreased upon reduction by antioxidants. The IC_{50} values of Et_2O, $CHCl_3$ and EtOAc phytochemical fraction are 12.88±1.87, and 16.89±1.6 and 22.25±2.7 µg/ml respectively.

This result concludes that the Et_2O-PF has more scavenging capacity compared to other phytochemical fractions. This increased scavenging capacity was elicit upon presence of high level of polyphenol and flavonoid contents. The results of quantitatively estimated polyphenols and flavonoids in all phytochemical fractions are depicted in **Table 1**. The Et_2O-PF contains 510±9.98 and 213.3±11.9 µg/mg PF of polyphenols and flavonoids respectively, which are higher than $CHCl_3$ and EtOAc phytochemical fractions.

TABLE 1: TOTAL POLYPHENOL AND FLAVONOID CONTENT PRESENT IN PHYTOCHEMICAL FRACTIONS

Phytochemical fractions (PFs)	Polyphenols (µg/mg of PFs)	Flavonoids (µg/mg of PFs)
Et_2O-PF	510±9.98	213±11.9
$CHCl_3$-PF	460±9.53*	157±10.3*
EtOAc-PF	386±13.75*	106±12*

All values are expressed as mean ± SD, n=3. A statistical value was set at *P< 0.05 for the comparison of Et_2O-PF with $CHCl_3$-PF and EtOAc-PF.

Synthesis and characterization of AuNPs: The pink-red color developed after addition of chloroauric acid solution (10ml, 0.5 mM) into corresponding PF; each 1.0mg. **Fig. 1a** shows the color of different reaction mixtures, which confirms the formation of gold nanoparticles. The color of the reaction mixture was elicited upon excitation surface plasmon vibration with AuNPs [31]. UV-visible spectra of each reaction mixtures were recorded after 15 minutes of incubation of chloroauric acid with corresponding PFs.

Results of UV-visible spectra of gold nanoparticles synthesized by different phytochemical fraction are shown in **Fig. 1b.** The maximum absorbance wavelength of gold nanoparticles was seen at 536nm. Usually absorbance value increases with increase in concentration of gold nanoparticles. Therefore measurement of absorbance at 536nm indirectly mentions about the amount of AuNPs present in the reaction mixtures. The Et_2O-PF mediated synthesis of AuNPs shows maximum absorbance within 15 minutes compared to $CHCl_3$-PF and EtOAc-PF mediated synthesis of AuNPs.

The increased production of AuNPs by Et_2O-PF is due to the presence of high level of antioxidant compounds such as polyphenols and flavonoids. Polyphenols and flavonoids are important class of phytochemicals having antioxidant activity. These phytochemicals reduce metal salts like potassium ferricyanide, ferrous sulphate and chloroauric acid by donating electrons. This reducing capacity of PFs on metal ions increased with increase in concentration of polyphenols [26]. Further characterizations were done for Et_2O-PF reduced AuNPs.

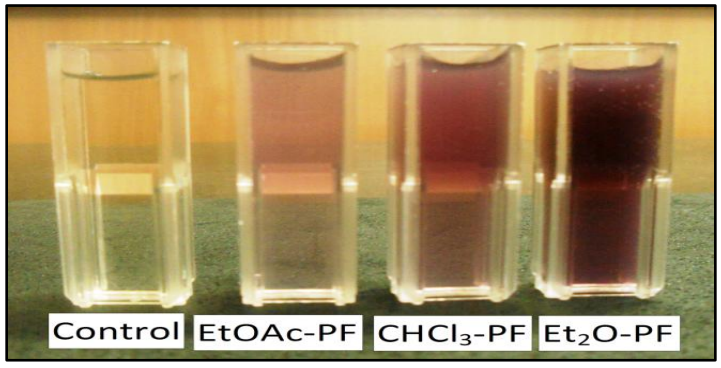


FIG. 1: a) COLOR OF SYNTHESIZED AuNPs AFTER 15 MINUTES INCUBATION OF 0.5mM CHLOROAURIC ACIDS WITH CORRESPONDING PHYTOCHEMICAL FRACTIONS, EACH 1mg

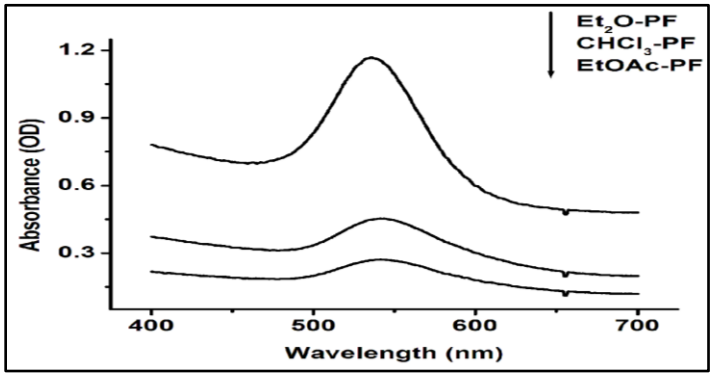


FIG. 1: b) UV-VISIBLE SPECTRUM OF SYNTHESIZED AuNPs AFTER 15 MINUTES INCUBATION OF 0.5mM CHLOROAURIC ACIDS WITH CORRESPONDING PHYTOCHEMICAL FRACTIONS, EACH 1mg. Each reaction mixture shows SPR band at 536 nm**.**

The DLS results of AuNPs suspension is shown in **Fig. 2a**, it shows two peaks. The size of AuNPs are distributed in the range of 20 to 42nm (Peak1) and 62 to 88nm (peak2). The intensity of peak1 is high compared to peak2. Therefore, this concludes that most of the nanoparticles appeared in the range of 20nm to 42nm with average size of 27nm. The TEM image of gold nanoparticles is shown in **Fig. 2b**.

From this image, we identified that the AuNPs had spherical, triangle, truncated triangle and hexagonal morphologies. Such different shaped AuNPs were reported by other plant extracts such as coriander (spherical, triangle, truncated triangles and decahedral) [21], *Coleus amboinicus* Lour (spherical, triangle, truncated triangle, hexagonal and decahedral) [32].

Synthesis of stable AuNPs with various size and shape are important aspect of biomedical applications because the shape of the nanoparticles plays an important role in changing their optical properties [33]. Zhirui Guo *et al*., [34] synthesized gold nanoprism for biosensing application based on sensitive changes in SPR band originated by antigen-antibody recognition events.

Similarly, Yao-Chen Chuang *et al*., [35] estimated protease activity based on optical properties of gold nano-triangles. Ankamwar *et al*., [36] synthesized gold nano-triangles for vapor sensing applications. In recent report spherical gold nanoparticles functionalized with acetylated Dendrimers is utilized for detecting cancer cells by Computer topography imaging [37].

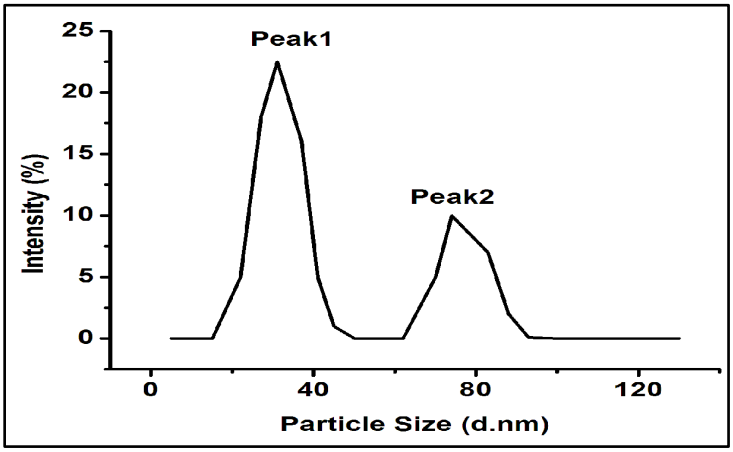
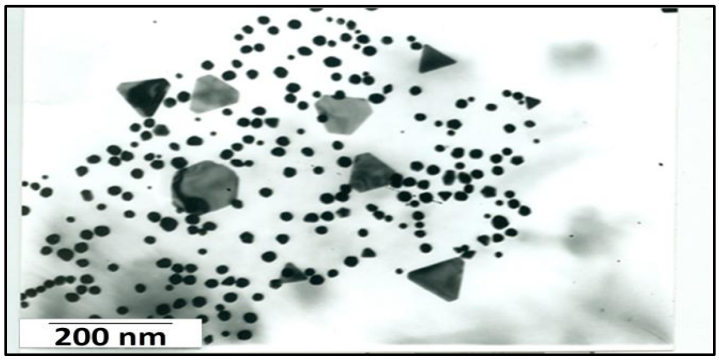


FIG. 2: a) SIZE DISTRIBUTION OF DIETHYL ETHER PHYTOCHEMICAL FRACTION REDUCED GOLD NANOPARTICLES MEASURED BY DLS SYSTEM



b) TEM MICROGRAPH OF DIETHYL ETHER PHYTOCHEMICAL FRACTION REDUCED GOLD NANOPARTICLES SHOWING SPHERICAL, TRIANGLE, TRUNCATED TRIANGLE AND HEXAGONAL SHAPES

XRD analysis of powdered AuNPs showed clear peaks of cubic phases (JCPDSNo. 03-0921) at 38.2 (1 1 1), 44.3 (2 0 0), 64.9 (2 2 0), 77.5 (3 1 1) and 81.5 (222), which confirms the crystalline nature of AuNPs. The broad bottom width of the peaks indirectly represents formation of smaller size AuNPs. The results of XRD pattern of AuNPs is showed in **Fig. 3a**. The FT-IR spectrum of AuNPs and native Et_2O–PF showed in **Fig. 3b**. The FT-IR spectrum of AuNPs and native Et_2O–PF resembles each other.

Both FT-IR spectrum showed characteristic bands for alcohol (917 and 3350cm^{-1}), C-N stretching vibration for aliphatic amines (1015 cm^{-1}), phenols (1146 cm^{-1}), C-N stretching for aromatic amines (1372cm^{-1}), germinal methyl (1442 cm^{-1}), carboxyl (1691cm^{-1}), and C-H (2849 cm^{-1}) functional groups. These bands originated from the functional groups present in the various phytochemicals of the leaf extract. From the FT-IR results, we conclude that the phytochemicals of Et_2O–PF gets adsorbed on the surface of AuNPs through free amino (-NH_2) and carboxylic (-COOH) groups makes highly stable nanoparticles [24].

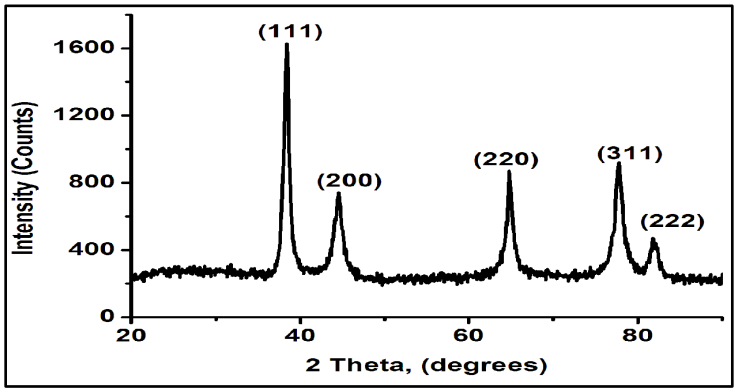
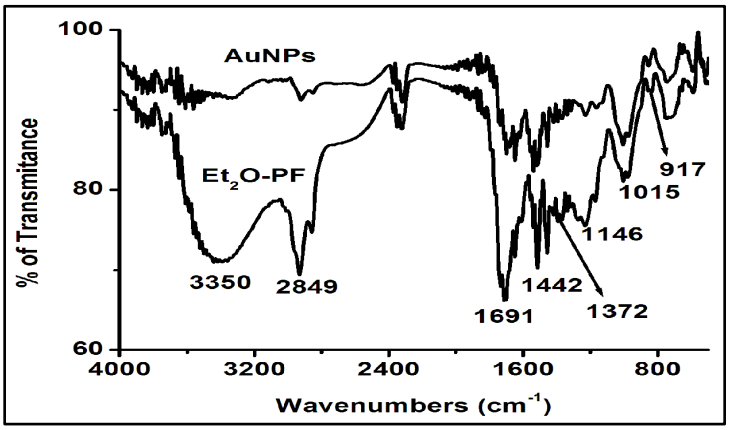


FIG. 3: a) XRD PATTERN OF GOLD NANOPARTICLES SYNTHESIZED BY DIETHYL ETHER PHYTOCHEMICAL FRACTION



b) COMPARISON OF FT-IR SPECTRUM OF DIETHYL ETHER PHYTOCHEMICAL FRACTION AND DIETHYL ETHER PHYTOCHEMICAL FRACTION REDUCED GOLD NANOPARTICLES

Stability of AuNPs: For various therapeutic and biomedical applications, we performed stability study of Et_2O-PF reduced AuNPs for different time period (at pH 7 and 37°C) and under different pH (at 37°C) by monitoring SPR of AuNPs. The change in red shift in UV/Vis spectra of AuNPs should be associated with either an increase in the average size of the nanoparticles or aggregation of nanoparticles or a combination of both [38].

The UV-visible spectrum of Et_2O-PF reduced AuNPs was not changed over 5 months stability period which reveals that synthesized AuNPs are highly stable over reasonable period of time (**Fig. 4a**).

Further, the AuNPs did not show any changes in SPR over the pH range of 7-10 but ≈10nm SPR shift found in the pH range of 6-5. Thus, the significant change of SPR of AuNPs at pH 6-5 indicates aggregation of nanoparticles by minimization of negative repulsive force between nanoparticles in acidic environment [39]. The Et_2O-PF reduced AuNPs have better stability compared to borohydrate or citrate reduced AuNPs aggregate [40] **(Fig. 4b).**

Therefore, the chemically synthesized gold nanoparticles are needed to be stabilized by capping agents such as polymers and surfactants, which are expensive [39].

These drawbacks are significantly overcome by plant mediated synthesis of nanoparticles.

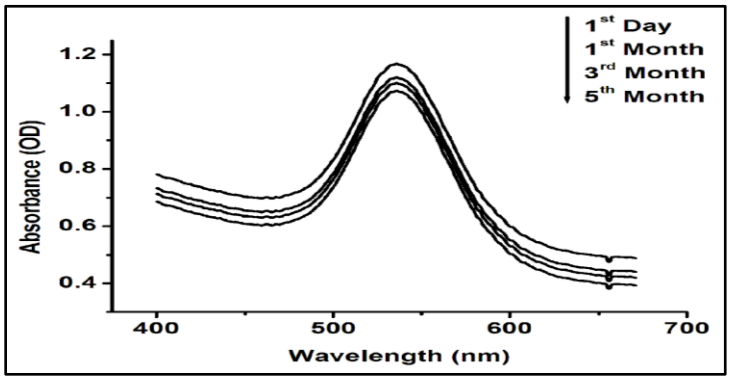
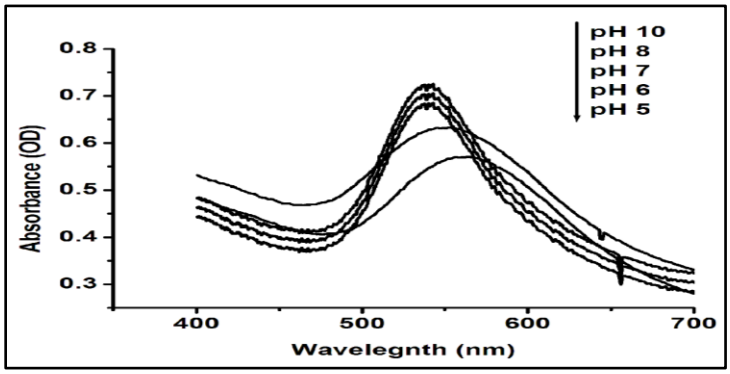


FIG. 4: a) SURFACE PLASMON RESONANCE OF DIETHYL ETHER PHYTOCHEMICAL FRACTION REDUCED GOLD NANOPARTICLES AT DIFFERENT TIME PERIOD



b) SURFACE PLASMON RESONANCE OF DIETHYL ETHER PHYTOCHEMICAL FRACTION REDUCED GOLD NANOPARTICLES AT DIFFERENT pH RANGE (5-10)

Cytotoxicity of Et_2O-PF: We performed cytotoxicity of various concentration of Et_2O-PF tested in Raw264.7 cell line. Et_2O-PF did not decrease Raw 264.7 cell viability at any of the tested concentrations (10-100 µg/ml) significantly (**Fig. 5**). Therefore, Et_2O-PF reduced gold nanoparticles may not show any adverse effect when used in biomedical applications. This advantage is not significant to chemically reduced AuNPs which involves highly toxic chemical species [10-12].

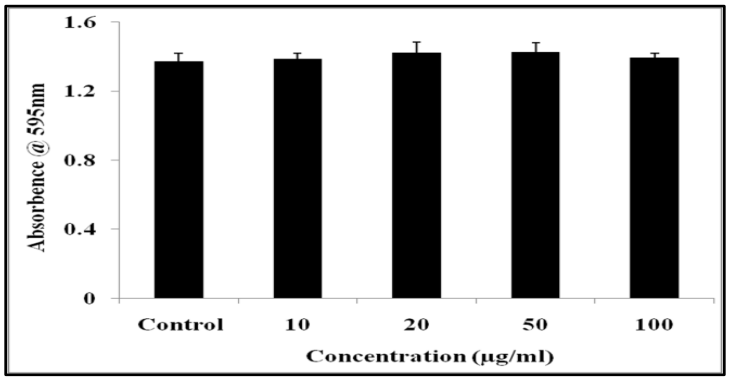


FIG. 5: EFFECT OF DIETHYL ETHER PHYTOCHEMICAL FRACTION ON RAW 264.7 CELL VIABILITY

Amount of viable Raw 264.7 cells were determined by MTT assay after 24h incubation with different concentration (10-100 µg/ml)

of phytochemical fraction. Values are expressed in mean ± SD, with each test performed in triplicate (n=3, *p<0.05 Vs control)

CONCLUSION: The reduction of gold ions by *Justicia gendarussa* leaves extract has been reported here. The diethyl ether phytochemical fraction have more polyphenol and flavonoid contents which produce maximum gold nanoparticles within 15 minutes compared to the chloroform and ethyl acetate phytochemical fractions of *Justicia gendarussa* leaves. The diethyl ether phytochemical fraction reduced gold nanoparticles shows maximum absorbance wavelength at 536 nm with different morphologies such as spherical, triangle, truncated triangle and hexagonal shapes.

The average size of diethyl ether phytochemical fraction reduced AuNPs is 27 nm and stable for reasonable period of 5 months and at pH above the 7. The functional groups such as alcohols, phenols, aromatic amines, aliphatic amines, carboxyl groups present in Et_2O-PF are involved in reduction and capping of AuNPs. This method proved that nontoxic plant material has tremendous benefits and is eco-friendly and compatible for biomedical applications. Biosynthesis of AuNPs by *Justicia gendarussa* leaf is simple, rapid, nontoxic and scalable for large scale synthesis.

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