International Journal of Research in Engineering, Science and Management
Volume-1, Issue-12, December-2018
www.ijresm.com | ISSN (Online): 2581-5792

Development and Evaluation of Hemolytic Efficacy of Guava (*Psidium Guajava*) Based Green Nano Particles

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***Abstract*: Nanotechnology has gained a tremendous impetus in modulating metals into Nano sized shape and to produce pure silver which are used as nanoparticles. In the present study it is reported that a simple, cost effective and environment friendly route of green synthesis of silver nanoparticles using extracts of Psidium guajava due to its biomedical applications such as Hemolysis. Hemolysis affects the red blood cells by destroying them before their normal life span. Hemolysis is caused by blood disorders, toxins produced in the body or due to an infection. Hemolysis is usually diagnosed through analysis of a blood sample. The small size and unique physio- chemical properties of nanoparticles may cause their interaction with erythrocytes. Impact of silver nanoparticles on erythrocytes integrity was assessed by Calorimetric measurement, Transmission Electron Microscopy (TEM) and Fourier Transform Infra-Red Spectroscopy (FTIR).**

***Keywords*: Hemolysis; Psidium guajava; Erythrocytes; Transmission Electron Microscopy; Fourier Transform Infra-Red Spectroscopy**

1. Introduction

Nanotechnology is one of the most active research areas in the modern material science. Based upon their specific characteristics such as size, distribution and morphology, nanoparticles have distinct properties as compared with the bulk form of the same material. It has been known longer due to its medical and therapeutic benefits before the realization that microbes are agents for infections. Silver nanoparticles are attractive as they are non –toxic to the human body at low concentrations and have broad spectrum anti-bacterial actions. Silver nanoparticles are synthesized by physical, chemical and biological methods. Biological methods are more preferred over physical and chemical methods [1]. The three major components involved in the preparation of nanoparticles using biological methods are the solvent medium for synthesis, the environmentally friendly reducing agent, and a nontoxic stabilizing agent [2]. A number of reports prevail in the literature that indicates the synthesis of nanoparticles by chemical and physical approaches are eco- unfriendly and expensive. Thus, there is a growing need to develop environmentally and economically friendly processes, which do not use toxic chemicals in the synthesis protocols. This has conducted researchers to look at the organisms. Psidium guajava is one of the most gregarious of fruits trees. The high presence of tannins give to antidiarrheal properties, also have demonstrated pharmacological activity as antibacterial, antioxidant, antispasmodic, anti-inflammatory, anti-anemic, homeostatic [3]. Hemolysis, also called hematolytic, breakdown or destruction of red blood cells so that the contained oxygen-carrying pigment hemoglobin is freed into the surrounding medium. Silver nanoparticles (AgNPs) are increasingly used in biomedical applications because of their large antimicrobial spectrum. The impact of AgNPs on erythrocyte integrity, platelet function and blood coagulation was studied. Erythrocyte integrity was assessed by calorimetric measurement of hemoglobin release. Platelet adhesion and aggregation was determined by Transmission Electron Microscopy (TEM) [4]. In this study, biosynthesis of silver nanoparticles obtained from guava aqueous leaf extract was reported to study its hemolytic effect.

2. Materials and methods

Preparation of Extract from fruit and leaves of Psidium guajava Psidium guajava fruits were collected from nearby fruit market. The leaves of Psidium guajava were collected from Punjab Agricultural University (PAU), Ludhiana. The fruit and leaves were grinded to form a clear paste. 25 ml of raw fruit and leaf paste was diluted 5 times in double distilled water and was given hot percolation treatment. In hot percolation treatment, paste was diluted in 5 times double distilled water and was kept on hot plate for 2-3 hours till resultant mixture boils completely, then kept undisturbed for 10 minutes. The resultant mixture was then filtered out using Whatman filter paper no. 1 in a conical flask. Filtrate was kept in the water bath at 600 C until reduced volume of raw extract was obtained. This raw extract so obtained from hot percolation treatment was used for the synthesis of silver nanoparticles [5].

A. Biosynthesis of silver nanoparticles

For each time experimental set up, fresh stock of AgNO3 solution was prepared. 1M AgNO3 (MW 169.88) 0.169gm was

dissolved in 1000 ml of double distilled water resulting in 1000ml AgNO3 stock solution. This stock solution was used in further experiment [6]. To set up an experiment, 2.5ml, 3.5ml and 4.5ml concentration of raw fruit and leaves extract were augmented with 50ml AgNO3 solutions in each flask. Each individual flask was incubated at 600 C and same was again repeated at 1000 C .pH 11 was maintained to set up an experiment. The entire procedure was repeated three times and best results out of three readings were used for further experiment to perform hemolysis. Best O.D. at 540nm was observed at 600 C temperature and was selected for further hemolysis.

B. Characterization of silver nanoparticles

UV-Visible spectrophotometry analysis (preliminary test): Absorbance was recorded at 540nm using UV Visible spectrophotometer and a graph using the readings was plotted. Transmission Electron Microscopy (TEM) and Fourier Transform Infra-red Spectroscopy (FTIR) (confirmatory analysis): TEM analysis was carried out to confirm the synthesis of silver nanoparticles [7, 8]. Pellet was prepared by centrifugation at 10,000 rpm for 10 minutes. The pellet formed was carried to the Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh to carry out FTIR analysis and Punjab agricultural University (PAU) for TEM analysis.

C. Hemolysis

The best results of nanoparticles synthesized from Psidium guajava fruit and leaf extract i.e., 2.5ml extract concentration at 600 C was selected for hemolysis.

D. Protocol of hemolysis

Mix all the materials in 1000 ml of distilled water and maintain pH of 7.2 of Phosphate Buffer Saline (PBS) solution.

Table 1
Preparation of Phosphate Buffer Saline (PBS) solution

Chemicals	Quantity(gm)/litre
Sodium dihydrogen orthophosphate	0.437
Dihydrogen sodium hydrogen orthophosphate	1.022
Sodium chloride	8.5
Blood from healthy donor	3ml

Take 1.25 ml of PBS and 0.25 ml of blood sample in 2.0 ml of centrifuge tube. Centrifuge it for 15 minutes at the rate of 2000 rpm. Give at least three successive washings with remained PBS solution. Now discard the supernatant and collect the pellet obtained after three successive washings in a separate centrifuge tube. Collected pellet amount measured was 1.5 ml. If the collected amount of pellet is 1.5 ml then, by 5% of 100 x 1.5 ml is 30 ml, so make final volume 30ml by adding PBS solution. Now take three test tubes for the fruit extract, and add silver nanoparticles, PBS and blood+PBS respectively. To check intensity of hemolysis at 3 different concentrations i.e. 100%, 50% & 25%, add different amount of obtained silver nanoparticles, PBS and blood+ PBS.

Table 2
Intensity of hemolysis at 3 different concentrations

	Silver nanoparticles (µl)	PBS(µl)	Blood +PBS (ml)
100%	100x0.2 = 20	980	2.5
50%	50x0.2 = 10	990	2.5
25%	25x0.2 = 5	995	2.5

Apply a negative control of PBS i.e. 2.5ml of PBS and 2.5ml of blood+PBS. And a positive control of distilled water i.e. 2.5ml of distilled water and 2.5ml of blood+PBS. Incubate the samples for about 2 hours. Record O.D. at 540nm on calorimeter. Calculate the value of % hemolysis.

$$\%\ Hemolysis = \frac{100 \times (\text{sample absorbance} - \text{negative control absorbance})}{(\text{Positive control absorbance} - \text{negative control absorbance})}$$

Table 3
Intensity of hemolysis can be checked by

<2%	Non- hemolysis
2.5%	Slight hemolysis
>5%	Hemolysis

3. Results and discussion

A. UV-Visible spectrophotometric analysis

Fig. 1. 2.5ml of silver nanoparticles extract resulted in change in color augmented with silver nitrate after one hour of incubation at temperature 60^0 C and 100^0 C

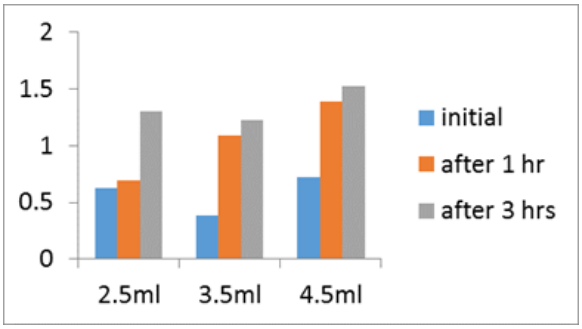

Fig. 2. Absorbance of $AgNO_3$ treated Psidium guajava extract at 540nm at 60^0 C and different extract concentrations.

In the present study, silver nanoparticles were synthesized from the aqueous leaf and fruit extract of *Psidium guajava*. Bio reduction of Ag^+ to Ag^o was observed when the aqueous extract was augmented with $AgNO_3$ and kept at different experimental conditions (temperature-60^0 C and 100^0 C and extract concentration -2.5 ml, 3.5 ml, 4.5 ml). The silver nanoparticles

were preliminary analyzed by change in color and calorimetric readings at 540 nm [9] (Fig. 1, 2). Confirmatory analysis was done by Fourier Transform Infra-Red Spectroscopy (FTIR) and Transmission Electron Microscopy (TEM). 2.5 ml extract concentration with temperature 60^0 C was selected.

B. Transmission electron microscopy analysis

For the confirmatory analysis of synthesized silver nanoparticles from Psidium guajava TEM was performed. The shape and size of the resultant nanoparticles were elucidated with the help of TEM [7]. Aliquots of silver nanoparticles solution were placed on a carbon-coated copper grid and allowed to dry under ambient conditions and TEM images were recorded. The TEM micrographs suggest that the sizes of the particles were around 30nm with an average size of 18.0 nm. The particles were of spherical shape. (Fig. 3)

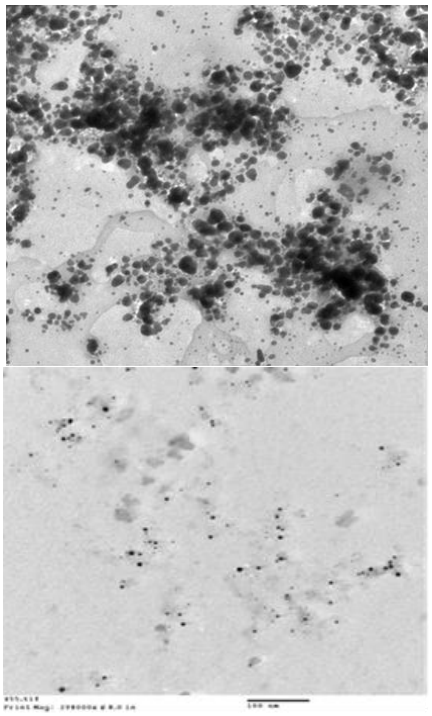

Fig. 3. Confirmatory analysis of synthesized silver nanoparticles with the help of TEM images

C. Fourier transform infrared spectroscopy analysis

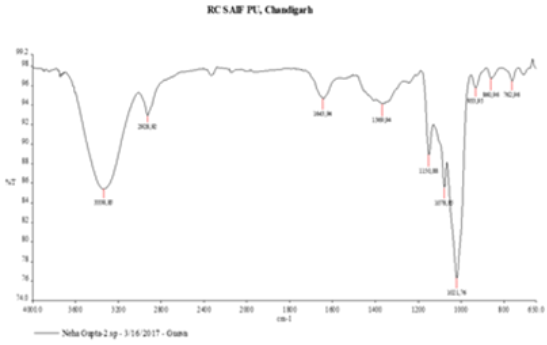

Fig. 4. C Phytochemical Analysis using FTIR spectra

FTIR measurement was carried out to identify the possible biomolecules in the Psidium guajava extract [10]. FTIR spectra of dried aqueous extract and synthesized silver nanoparticles are shown in Fig. 4. The phytochemical analysis of Psidium guajava reveals the presence of flavanoids, alkaloids, steroids, saponins and proteins. The peaks are observed at 540 nm.

D. Hemolysis

After mixing PBS with blood, centrifugation was given (Fig. 5, 6&7) at 2000rpm for 15 minutes. To check intensity of hemolysis at three different concentrations i.e. 100%, 50% & 25%, add different amount of obtained silver nanoparticles to each of the three test tubes and results were measured by calorimetric reading at 540nm. (Fig. 5).

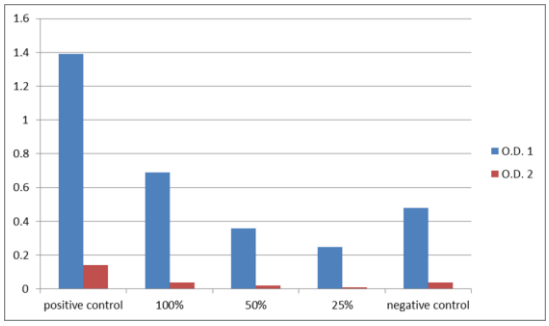

Fig. 5. Intensity of hemolysis at different concentrations at O.D. 540nm

4. Conclusion

Psidium guajava can be used as an excellent source for biosynthesis for the silver nanoparticles. The reduction of the metal ions through fruit and leaf extracts leading to the formation of silver nanoparticles of fairly well-defined dimensions. Silver nanoparticles were preliminary analyzed by change in color and calorimetric readings. Confirmatory analysis was done by TEM and FTIR. The major advantage of synthesizing silver nanoparticle using Psidium guajava is that they are easily available, safe, and nontoxic. They have a broad spectrum of metabolites that can aid reduction of silver ions, and are quicker than microbe assisted synthesis. Silver nanoparticles are increasingly used in biomedical applications such as Hemolysis.

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