GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM *PUNICA GRANATUM L.* AND STUDY OF ITS ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY

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Abstract: Pomegranate (*Punica granatum* L.), a rich red coloured fruit is found in arid and semiarid parts of the world. Although the fruitoriginated in Iran, it is now widely cultivated. India is one of the leading producers of Pomegranate in the world. Enriched nutritional & medicinal values, exquisite fruit quality has led to a huge demand for this fruit in domestic and international market. In the present investigation, green synthesis of Silver nanoparticles from the seed extract of Pomegranate fruit was performed. The green synthesis method was less expensive, convenient and was monitored by UV-Vis spectroscopy. The antibacterial activity of the nanoparticles was studied by Agar cup method. These biologically synthesized nanoparticles exhibited good antibacterial activity against both Gram positive, Gram negative and yeast test isolates. Antioxidant activity of the extract was checked by ABTS & Reducing power assay. The seed extract exhibited high antioxidant activity. These particles can therefore be further exploited to study their therapeutic applications.

Key words: Green synthesis, Pomegranate extract, Silver nanoparticles, Antimicrobial activity, Antioxidant activity.

I. INTRODUCTION

In the last few decades, there has been a growing amount of research on nanotechnology, particularly involving the green synthesis and characterization of nanoparticles, as nanoparticles less than 100 nm in size are ideal agents for drug delivery and biomedical applications(Dipankar & Murugan.,2012;Banse & Ledwani;2016)

Three methods of nanoparticle production exist today-chemical, physical and “green” routes, with the green route involving the employment of biological reducing agents, including plant extracts and microbial filtrates (Sharma & Dev., 2014; Arokiyaraj & Kim., 2014). The first two methods are often costly and generate toxic by-products (Kesharwani & Rai., 2009; Padalia & Chanda.,2015) but the green nanosynthesis method has been recognized as an inexpensive and eco-friendly process (He & Zhao., 2013; Sharma & Lin., 2009). Metal Oxide nanoparticles have attracted worldwide attention because of their increased use in a variety of fields such as electronic, cosmetic, biomedicine, energy, environment, catalysis and material science (Bar & Misra.,2009;Ahmed & Ikram.,2016). Among the noble metal nanoparticles, AgNPs have received considerable attention due to their attractive physicochemical properties and applications in medicine (Mallikarjuna & Raju., 2011; Chauhan & Rishi., 2011)

Pomegranate can be divided into several anatomical compartments including seed, juice, peel, leaf, flower, bark and root with each possessing interesting pharmacological and toxicological activities (Gnanajobitha & Annadurai., 2013) The edible fruit is a berry, about 5-12 cm in diameter with a rounded hexagonal shape, thick reddish skin and around 600 seeds, each surrounded by a water-laden pulp (aril) ranging in color from white to deep red or purple, aril is the edible part of the fruit. Antioxidants are extensively studied for their capacity to protect cell from damage induced by oxidative stress. A number of synthetic antioxidants like butylated hydroxyl anisole, toluene and gallic acid esters are also available but

they are unstable at elevated temperatures and also cause negative health effects. Hence the objective of present study is to synthesize AgNPs by green mode of synthesis and to determine the antimicrobial and antioxidant activity of the biologically synthesized silver nanoparticles.In the present study we have explored the synthesis of silver nanoparticles using fruit seed extract of *Punica granatum* (Pomegranate). The synthesized nanoparticles were confirmed by color changes and characterized by UV-Visible spectroscopy.

II. MATERIALS & METHODS

2.1 Sample Collection

One kilogram of pomegranate fruits (*Punica granatum*) was purchased from the supermarket. The fruits were washed several times with tap water. After washing, the peel was carefully removed and seeds were separated. The entire seed is made up of Aril,the juicy and edible part of the fruit, and the white seed inside. Ripe pomegranate seeds were used to make the aqueous extract.

2.2 Preparation of Aqueous extract(AE) of the Pomegranateseeds

20 g seeds were grounded in mortar and pestle and added in 100 ml of distilled water. The solution mixture was maintained at 100^{o}C in boiling water bath till solution reduces half of the original volume. The resulting mixture was filtered using a clean muslin cloth to acquire the aqueous extract(Gudikandula & Maringanti., 2016)

2.3 Chemical synthesis of silver nanoparticles (AgNPs)

50 ml of 1mM $AgNO_3$ solution was heated to boiling, then 5ml of 1%trisodium citrate was added drop by drop. Solution was mixed and heated until pale brown color was obtained and then cooled (Nadagouda & Varma.,2014).

2.4 Green synthesis of silver nanoparticles(AgNPs)

5ml of aqueous pomegranate seed extract was added to 100 ml of Silver nitrate solution ($AgNO_3$; 0.1 mM). For uniform mixing, the solution was kept on shakerfor 5 min. Control flask without the extract was also maintained. The reactionmixture was found to change its color from a colorless solutionto a brown-colored solution within 5 min, indicatingthe reduction of the silver ions into silver nanoparticles.The solution was incubated at refrigerator for 24hr and then the nanoparticle solution was centrifuged at15,000 rpm for 15 min. Finally, purified AgNPs were collected, and furtherassays were performed to analyze the characteristics andantimicrobial activities of the synthesized NPs(Humbarwadi & Patel., 2018)

2.5 Characterization of synthesizednanoparticles

The chemically and biologically synthesized nanoparticleswas characterized using a UV-Vis spectrophotometerby recording its absorbance atwavelength 300 to 700 nm(Lediga & Vuuren., 2018)

2.6 Antimicrobial activity of synthesized nanoparticles

2.6.1 Bacterial strains

The antibacterial activity of synthesized nanoparticles was determined against standard MTCC strains of *Escherichia coli* MTCC 1885,*Pseudomonas aeruginosa* MTCC 1688,*Staphylococcus aureus* MTCC 3160,*Streptococcus mutans* MTCC 801 (Kutty & Deshmukh.,2015)

2.6.2 Yeast strains

The antifungal activity of enzyme was determined against standard MTCC strains of *Saccharomyces cerevisiae* MTCC 170 and Candida *albicans* MTCC 3017.

2.6.3 Agar well diffusion technique

Bacterial and Yeast standard MTCC strains were inoculated in sterile Nutrient and Sabouraud's broth respectively and incubated at 37ºC and RT respectively for 24 hrs. Antibacterial and Antifungal activity was assayed using the agar well diffusion technique. Muller Hinton agar medium (MH) plates were prepared of pH 7.4 for bacteria and pH 5.4 for yeast test cultures.

A sterile cotton swab was used for spreading the test microorganism from the 24 hours inoculated broth evenly on separate plates and left for few minutes to allow complete absorption of the inoculum. In each of these plates four wells of 6mm diameter were made using sterilized cork-borer. Nanoparticles prepared by biological and chemical meanswere added to the respective wells on the plates. For better comparative study aqueous crude pomegranate extract and antibiotic solutions were also tested. The test sample loaded plates were kept for incubation at 37ºC (for bacteria) and RT (for yeast) respectively for 24 hrs. After incubation, plates were observed for clearance around the wellindicating antibacterial and antifungal activity against the test cultures. Diameters of the zone of inhibition were measured in mm (Rajguru & Nabar.,2015)

2.7 Study of Anti-oxidant potential

2.7.1 Reducing Power Assay

The in vitro antioxidant activity of the extract and the biologically synthesized nanoparticle solution was estimated using Reducing Power Assay (Bursal and Köksal, 2011). In this assay, the sample was mixed with 2.5 ml potassium ferricyanide [$K_3Fe(CN_6)$] (1%)and incubated at 50 ºC for 20minutes. To this, 2.5ml of Trichloroacetic acid (10%) was added and centrifuged at 3000rpm for 10 minutes. Finally, 2.5 ml of the solution was mixed with 2.5ml of distilled water and 0.5ml $FeCl_3$ (0.1%) and the absorbance (A) was measured at 700nm. Ascorbic acid (20-100µg/ml) was used as a standard. A blank was prepared without adding the sample. Increased absorbance of the reaction mixture indicates stronger reducing power. The reducing power was calculated using following formula.

% increase in Reducing Power = A_{Test}/A_{Blank}-1x100

Where, A_{Test} is absorbance of test sample and A_{Blank} is absorbance of blank

2.7.2 ABTS Assay

The in vitro antioxidant activity of extract and the biologically synthesized nanoparticle solutionwas studied by 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay (Thiapong & Byrne.,2006).The photometric assay was conducted on 0.9ml of $ABTS^+$ solution and 0.1ml of tested samples and mixed for 45 sec; measurements were taken immediately at 734nm after 15 min. Ascorbic acid (20-100µg/ml) was used as a standard. A blank was prepared without adding the sample. The antioxidant activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the following equation:E = [(Ac-At)/Ac] x 100, Where;

At and Ac are the respective absorbance of tested samples and $ABTS^+$, was expressed as µmol.

III. RESULTS & DISCUSSION

3.1 Sample collection and preparation of Aqueous extract (AE) of the Pomegranate seeds

The preparation of the pomegranate aqueous extract (AE) was performed as mentioned above.

3.2 Synthesis of silver nanoparticles

In this study, Ag NPs were synthesized by chemical method (Fig.3) and biological method using an aqueous extracts of pomegranate seeds (Fig.2). The colour change was noted by virtual observation in pomegranate fruit seed extract incubated with aqueous solution of AgNO3 within 5min. The color from pale to yellowish brown was observed due to the bioreduction of silver ions, this exhibit the formation of silver nanoparticles (Fig. 2). The silver nitrate solution (control) without seed extract did not show any change in color. The color intensity of the synthesized nanoparticles increased with duration of time and color of extract changes to brownish black after 24 hrs. of incubation (Fig.4). A similar report was observed by Selvaraj *et al.*2014.

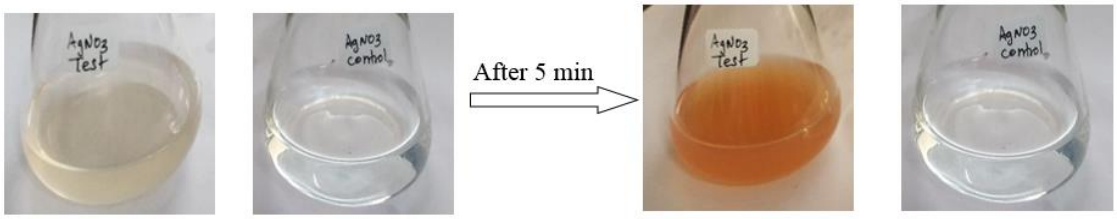

Figure 1: Biological synthesis of silver nanoparticles

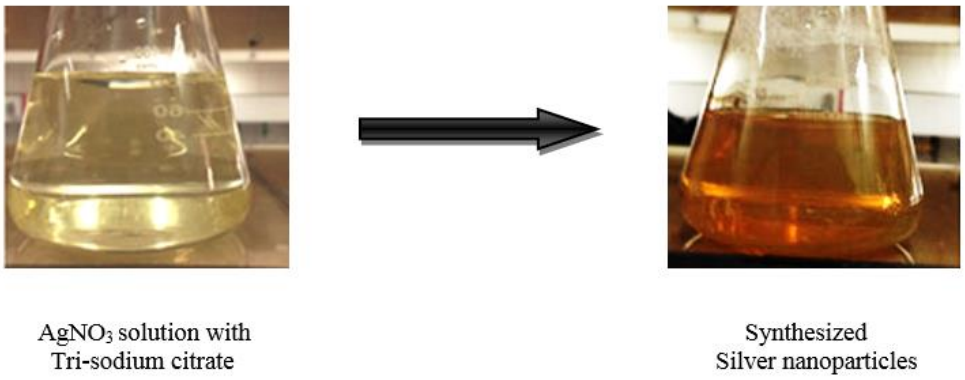

Figure 2: Chemical synthesis of silver nanoparticles

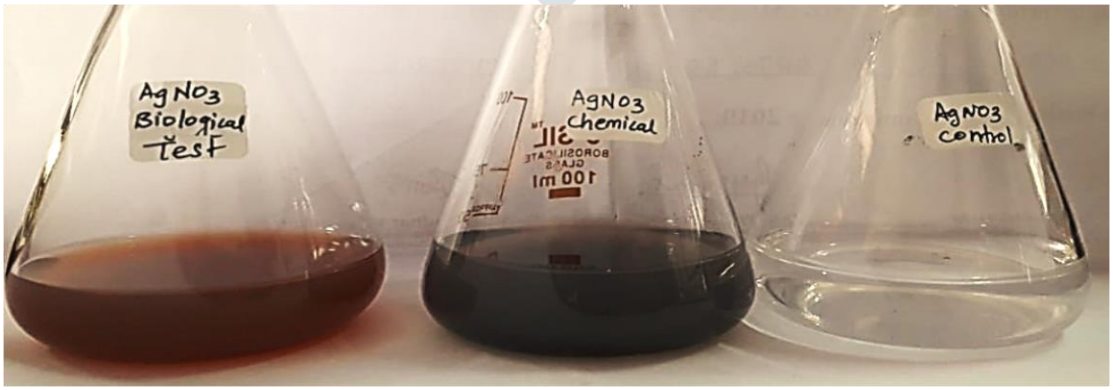

Figure 3: Synthesized nanoparticles after 24hr

3.3 UV-Vis analysis of synthesized nanoparticles

The formation of Ag NPs in low level concentration was confirmed by one of the imperative technique UV–vis spectroscopy analysis. Absorption spectrum at different wavelengths ranging from 400-700 nm revealed a peak of λ_{max}at 430 nm for chemically synthesized nanoparticle and 425nm for biologically synthesized nanoparticle (Fig.5).

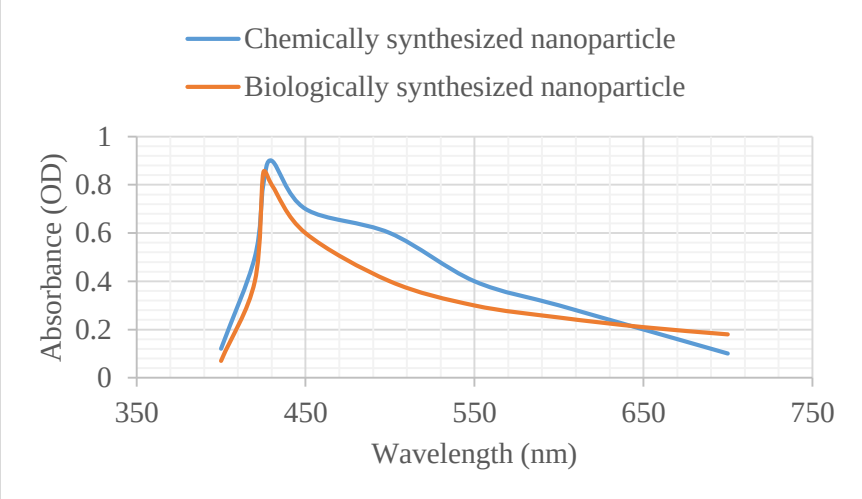

Figure 4: UV-Vis Spectra of the synthesized nanoparticle

3.4 Antibacterial and Anti-fungal activity

The nanoparticles synthesized were tested against human pathogenslike Escherichia *coli* MTCC 1885, *Pseudomonas aeruginosa* MTCC 1688, *Staphylococcus aureus* MTCC 3160, *Streptococcus mutans* MTCC 801 by agar well diffusion method and was found to possess anti-bacterial and antifungal activity (Fig .5). The zone was compared with inhibition zone of two standard antibioticschloramphenicol (30 mcg) and penicillin (10 U) (Fig. 6). The antifungal activity of silver nanoparticles was also tested against *Saccharomyces cerevisiae* MTCC 170, *Candida albicans* MTCC 3017 along with antibiotic nystatin (100 U).

Table 1 compares the zone of inhibition obtained with chemical and biological nanoparticles. The zones were compared with the crude pomegranate extract and respective sensitive antibiotic as positive control. The crude pomegranate extract also shows antimicrobial activity. The activity of chemically synthesized nanoparticles is found to be less than biologically synthesized nanoparticles. Thus the green synthesis method was most efficient as compared to the chemical method. The activity of biologically synthesized nanoparticles was similar to the sensitive antibiotic, hence it can be used instead of the antibiotic for drug resistant strains.

Table 1: Antibacterial and anti-fungal activity

Sr.No.	Compounds	ZONE OF INHIBITION (mm)					
		S.aureus	***E.coli***	***P.aeruginosa***	***S.pyogenes***	***C.albicans***	***S.cerevisiae***
1.	Crude extract	14	15	10	25	10	12

2.	Biologically synthesized nanoparticles	27	31	32	38	32	31
3.	Chemically synthesized nanoparticles	17	23	27	35	28	26
4.	Standard antibiotic	28	29	30	40	31	30

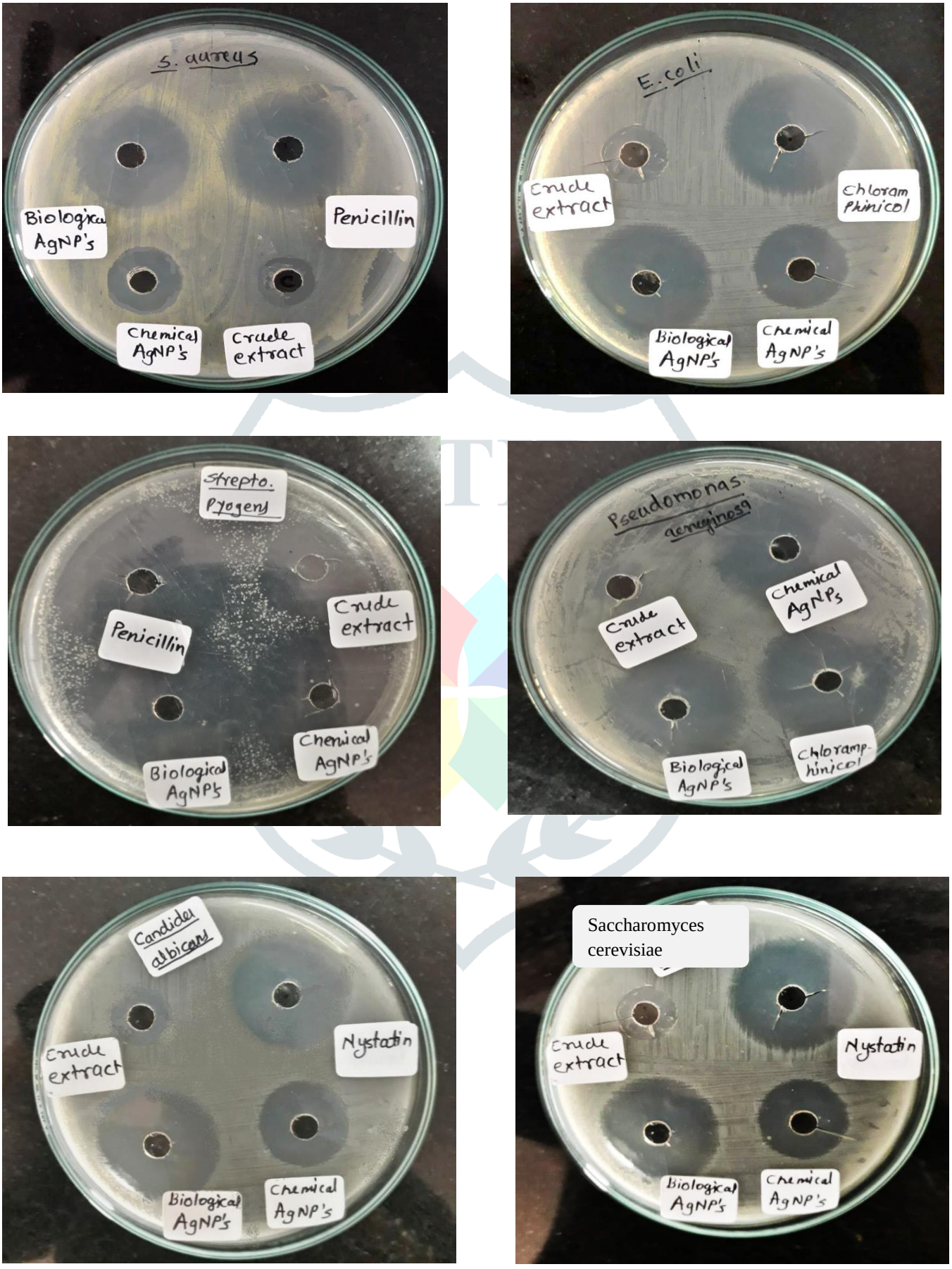

Figure 5: Antibacterial and antifungal activity obtained by Agar cup diffusion method on MH agar plate

3.5 Antioxidant activity

The antioxidant activity of the aqueous extract and the biologically synthesized nanoparticleswas checked by reducing power assay and ABTS assay. The best antioxidant activity is observed for biologically synthesized AgNPs as compared with crude extract. Detailed results of the samples are represented in Table 2.

Table 2: Antioxidant activity of extracts

Sr.No.	Method	Extracts	Minimum effective concentration (µg/ml)
1.	Reducing Power assay	Crude Extract	50
		Bio-NPs	75
2.	ABTS assay	Crude Extract	65
		Bio-NPs	80

IV. CONCLUSION

AgNPs were successfully synthesized using seed extract of *Punica granatum* L. The results recorded from UV–visible spectrum support the biosynthesis of silver nanoparticles. The Antimicrobial activity is observed against Gram positive, Gram negative and also yeast strains. Hence, it is therefore suggested that the nanoparticles has broad spectrum activity. It can be employed for its various application in cosmetics and pharmaceutical industry.

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VI. CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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