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GREEN SYNTHESIS OF NANOPARTICLES AND THEIR ANTIBACTERIAL ACTIVITY AGAINST PATHOGENIC BACTERIA

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ABSTRACT: The present study investigated the phytochemical analysis of the major bioactive constituents of medicinal important plant of *Punica granatum* peels in its aqueous extract and green synthesis of silver nanoparticles and their evaluation for antibacterial activity. Flavonoid, phenol & tannins, carbohydrates, glycosides and etc were highly concentrated in the peels extract. The synthesis and characterization of silver nanoparticles was confirmed by UV-Visible spectroscopy. UV-Visible spectroscopy of the reaction medium containing silver nanoparticles show the maximum absorbance peaks 430nm (1%), 373nm (3%), 379nm (5%). Green synthesis of silver nanoparticles analyzed against bacterial cultures by using the disc diffusion method. The silver nanoparticles have shown bactericidal effects against *E.coli* (MTCC-40), *Staphylococcus aureus* (MTCC-7443), *Proteus vulgaris* (MTCC-*1771). The peels extract of *Punica granatum* quikely reduces Ag+ to Ago and enhance synthesis of silver nanoparticles with highly antibacterial activity.

INTRODUCTION: Nanotechnology is new emerging as a rapidly growing field with its application in science and technology for the purpose of manufacturing and production of new materials at the nanoscale level [1]. At the present, the synthesized silver and certain other novel metals particles have several important applications in the field of biolabelling sensuous. These nanomaterials exhibit new physic-chemical and biological properties which are not absorbed in the bulk [2]. Biogenic routes to the synthesis of metals particles have been proposed by exploiting bacteria, yeast, fungi, actinomycetes and virus.

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Currently plant-mediated green synthesis of noble nanoparticles is gaining importance due to its eco-friendliness and simplicity [3]. The poteintial therapeutic of *Punica granatum* are wide ranging and include treatment and prevention of cancer, cardiovascular diseases, diabetes, dental conditions and protection from UV radiation. Other potential applications include infant brain ischemia, alzheimer's disease male infertility, arthritis, and obesity [4].

Although biosynthesis of silver nanoparticles by plants such as *Trianthema decandra* [5], *Mulberry* [6], *B. ovalifolitata S. tumbuggaia, S. hyderobadensis* [7], *Ocimum sanctum* [8], *Allium cepa* [9], *Elaeagnus latifolia* [10], *Cassia auriculata* [11], has been reported, the potential of the plants as biological materials for the synthesis of nanoparticles is yet to be fully explored. Nanoparticles can be divided in to two groups: Organic and inorganic particles, due to their shape, size features and advantages over available biological imaging drug agents and drugs, inorganic particles have been examined as potential tools for medical imaging as well as for treating diseases. Recently the researcher from Indian Institute of Technology, Bombay have discovered that the age old complementary medicines of homeopathic pills and ayurvedic bhasmas are having metal nanoparticles such as gold silver, copper, platinum, tin and iron [7].

The silver nanoparticles have formed widespread use for biological assays. The silver nanoparticles have various and important application. Historically, silver has been known to have a disinfecting effect and has been found in application ranging from traditional medicines to culinary items. It has been reported that silver nanoparticles are non toxic to humans and most effective against bacteria, virus, and eukaryotic microorganism at low concentration and without any side effects. Due to the increasing bacterial resistance to classic antibiotics, the investigation on the antibacterial activity of silver nanoparticles has increased [12]. The antibacterial activity of silver species has been well known since ancient times and it has been demonstrated that in low concentration, silver is non toxic to human cells [13].

Researchers support the idea that silver species release Ag+ ions and they intract with thiol groups in bacteria proteins, affecting the replication of DNA. Silver nanoparticles interactions with bacteria are dependent on the size and shape of the nanoparticles. The antibacterial properties of silver nanoparticles increase because of their larger total surface area per-unit volume. The mechanism of the silver nanoparticles bactericidal activity is effectively explained in term of their interaction with cell membranes of bacteria by disturbing its permeability and respiratory function [14]. The plant contains a variety of phytochemical compounds such as Phenols, tannin's, flavonoids, amino acid & protein's, carbohydrates, alkaloids etc. and these presence and there by induce some shape control during metals ion reduction.

The present study was conducted to investigate the antibacterial activity of silver nanoparticles synthesized from the *Punica granatum* peels of aqueous extract by preliminary disc diffusion assay screening. The extracts were tested against the *Staphylococcus aureus*, *Escherichia coli*, and *Proteus vulgaris*.

MATERIAL AND METHODS:

Silver nitrate was purchase from Fisher scientific chemicals Mumbai. All glassware's have been washed with sterile distilled water and dried in an oven before use.

Preparation of *Punica granatum* peels extract:

FIGURE 1: *PUNICA GRANATUM* (A) FRUIT (B) PEELS

Punica granatum (RUBL-211323): freshly peels of *punica granatum* **(Fig.1)** were collected from the Ludhiana local market (Punjab). The peels were washed several times with distilled water to remove the dust particles and then air dried by keeping at room temperature for seven days. Dried peels powdered and 1%, 3% and 5% of aqueous extract was prepared by boiling the powder in distilled water for 10-15min, until the color of the aqueous solution changes from watery to yellow. The extract was cooled to room temperature and filtered. The extract was stored at room temperature in order to be used for further experiment [11].

FIGURE 2: AQUEOUS EXTRACT

Preliminary phytochemical screening: The stock solution were used for preliminary screening of phytochemicals such as carbohydrates (Fehling's test), amino acid & proteins (Millon's test), alkaloid (Mayer's test), flavonoids (NH3 & HCl test), phenol and tannin's (Ferric chloride test), saponin's (Froth & foam test), glycosides (Killer kilani test), steroids, phytosterols, phlobatannin's [15].

Synthesis of silver nanoparticles:
In a typical reaction procedure, 10ml of aqueous peels extract was added to 100ml of 0.01mM $AgNO_3$ aqueous solution in a conical flask at room temperature. The color of solution changing within 15 min. from yellow to greenish brown (1%), dark yellowish to brownish black (3%), dark yellowish to dark brown color (5%) **(Fig.3),** indicating the formation of silver nanoparticles. It is well known that silver nanoparticles exhibit color change in aqueous solution due to excitation of surface Plasmon vibration in silver nanoparticles. Almost all the herbal mediated silver nanoparticles solution after incubation time, were showed the color change from light to dark color.

The UV-Vis spectroscopy of the synthesized nanoparticles were in the range of 300-800nm **(Fig.4).** UV-Vis spectral analysis was done by using UV-Vis spectrophotometer UV-1800 (Shimadzu). A final solution of synthesized nanoparticles was centrifuged at 8,000rpm for 25min. The collected pellets were stored at -4˚C and supernatant was discarded. The results obtained in this investigation were very interesting in terms of identification of potential weeds for synthesizing silver nanoparticles.

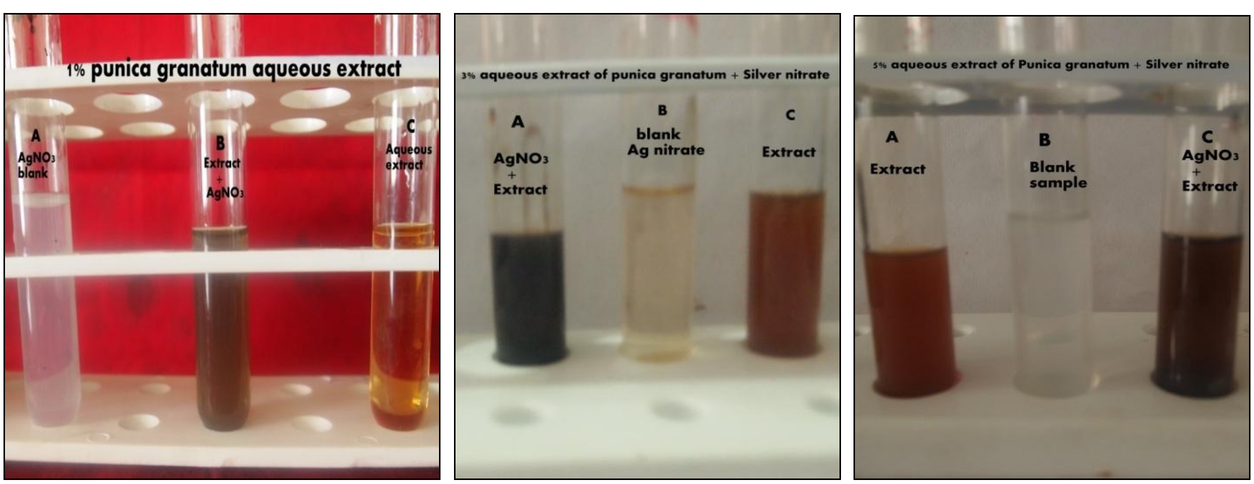


FIGURE 3:COLOR CHANGE SEEN IN PLANT EXTRACT TAKEN FOR SILVER NANOPARTICLE SYNTHESIS (A) 1% *PUNICA GRANATUM* PEELS AQUEOUS EXTRACT (B) 3% *PUNICA GRANATUM* PEELS AQUEOUS EXTRACT (C) 5% *PUNICA GRANATUM* PEELS AQUEOUS EXTRACT

Screening of green synthesized nanoparticles by Disc diffusion method:

Soil cultures of *E.coli* (MTCC-40), *Staphylococcus aureus* (MTCC-7443), and *Proteus vulgaris* (MTCC-*1771) was purchased from the Microbial Type Culture Collection Centre (MTCC) located at the Institute of Microbial Technology Chandigarh, India. A green synthesized silver nanoparticle was screened for their antibacterial activity against *E. coli*, *Staphylococcus aureus*, and *Proteus vulgaris* by disc diffusion method.

Standared size whatman No.1 filter paper discs 6mm in diameter, sterilized by dry heat at 80˚C in an oven for 1hour were used to determine antibacterial activity. Nutrient agar medium for disc diffusion test was prepared. After sterilization, it was poured in to sterilized petri plates and allowed to solidify. The culture suspension of each of the bacterial culture was prepared from 1-2 days old cultures separately. Duplicate plates were swabbed with the fresh cultures of selected pathogenic bacterial cultures. Sterilized filter paper discs were soaked in neat, diluted (100%) concentration of synthesized nanoparticles. A synthesized nanoparticles disc of 100µg/ml per disc was placed on an agar plate containing bacterial cultures suspension. Similarly, solution of standared antibiotic (Streptomycine sulphate) of 100µg/ml per disc concentration of antibacterial activity were prepared and impregnated in the filter- paper discs. These discs were then placed over the plates preceded with respective microorganism. The plates incubated at 37˚C for 24hr. Two replicates were kept in each case and average values were calculated.

The diameter of the inhibition zones was measured in mm and the activity index was calculated on the basis of the size of the inhibition zone. The activity of synthesized nanoparticles was measured by the following formula[16].

Activity index= Inhibition zone of sample/ Inhibition zone of standard.

RESULTS AND DISCUSSION:

Qualitative phytochemical analysis:

The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites [17]. The aqueous extract of peels of *Punica granatum* have revealed the presence of steroid, glycosides, carbohydrates, proteins & amino acid, phytosterol, phenols, tannins, flavonoids, alkaloids, phlobatannins, etc. the results of qualitative phytochemical analysis shown in **(Table 2).**

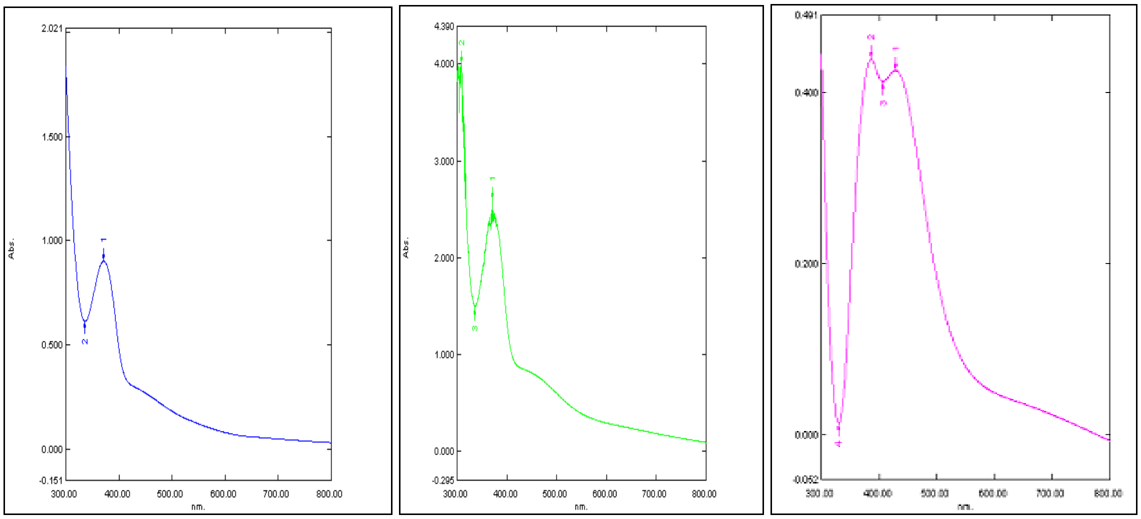
TABLE 2: PHYTOCHEMICAL SCREENING OF *PUNICA GRANATUM* PEELS AQUEOUS EXTRACT

S.No.	Name of the test	Aqueous extract
1	Carbohydrates	+
2	Proteins and amino acid	+
3	Alkaloids	+
4	Flavonoids	+
5	Steroids	+
6	Phlobatannins	+
7	Glycosides	+
8	Phenols and tannins	+
9	Saponins	+

The preliminary phytochemical screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development. Further, these tests facilities their qualitative separation of pharmacologically active chemical compounds [18]. The phytochemical screening present study has revealed the presence of phenols & tannins, carbohydrates, glycosides, proteins & amino acid, flavonoids, saponins, phytosterol, phlobataninns, alkaloids, and steroids in the peels extract. These extract may be responsible for the therapeutic properties of *Punica granatum.* Phenolic, saponnins and tannins are major group of compounds.

For example, saponins have a hypotensive and cardiodepressant properties glycosides are naturally cardioactive drugs used in the treatment of congestive heart failure and cardiac arrhythmia. Stability and formation of silver nanoparticles in sterile distilled water is conformed using UV-Vis spectrophotometer in a range of wavelength from 300 to 800nm. As soon as *Punica granatum* peels extract was mixed in aqueous solution of silver ions complex, the reduction of pure Ag^+ ions to Ag^o was monitored by measuring UV-Vis spectra of the reaction media at regular interval **(Fig 4)**.

We observe there is highest peak showing sign for the synthesis of silver nanoparticles the surface Plasmon resonance of silver occur at 430nm (1%), 373nm (3%), 379nm (5%) **(Table.1)** is clearly visible and is attributed to electronic excitation in tryptophan and tyrosine residues in proteins. This indicates the release of extracellular proteins in the colloidal solution and their possible mechanism in bioreduction process.



(a) (b) (c)

FIGURE 4: UV-VIS SPECTRA OF SILVER NANOPARTICLES AT THE FOLLOWING CONCENTRATION OF PEELS EXTRACT (A) 3% (B) 5% (C) 1%

The reduction of green synthesized silver nanoparticles during exposure to the plant extracts could be followed by color change. Silver nanoparticles exhibit dark colour in aqueous solution due to the surface Plasmon resonance absorption band, due to the combined vibration of electrons of metals nanoparticles in resonance with light wave. The sharp bands of silver colloids were observed best peaks. These UV-Visible spectroscopy characteristics color variation is due to the excitation of the surface Plasmon resonance in the synthesized nanoparticles. The mechanism behind the activity of nano silver on bacteria are not yet fully elucidated the most common mechanism of toxicity proposed up to now are: (a) Direct damage to cell membrane (b) Formation of Reactive Oxygen Species (ROS) (c) Uptake to free silver ions followed by disruption of ATP production and DNA replication. The bactericidal activity of nanoparticles depends on the stability in the cultured medium too.

TABLE 1: UV-VIS SPECTROSCOPY OF THE SYNTHESIZED NANOPARTICLES AT 1%, 3% AND 5% CONCEMTRATION OF AQUEOUS EXTRACT FROM PEELS OF *PUNICA GRANATUM*

S.No.	Wavelength (5%)	Observation	Wavelength (3%)	Observation	Wavelength (1%)	Observation
1	379nm	2.186	373nm	0.902	430nm	0.426
2	315nm	4.000	336nm	0.613	386nm	0.440
3	339nm	1.486			406nm	0.413
4					351nm	0.014

Antibacterial assay: Antibacterial activity of the green synthesized silver nanoparticles was examined against selected bacterial strains was investigated and represented in **(Table. 3).** It reveals that the green synthesized silver nanoparticles antibacterial activity against *Staphylococcus aureus* (MTCC-7443), *Escherichia coli* (MTCC-40), *Proteus vulgaris* (MTCC-*1771) showed the maximum inhibition of bacterial zone. Silver nnoparticles exerted highest toxicity against *Proteus vulgaris* and lowest effect on *Staphylococcus aureus* (1%, 3%, 5% green synthesized AgNPs). Silver nanoparticles green synthesized at 5% concentration were found better on all the bacteria tested followed by 3%, 1%.

The maximum toxicity was observed in silver nanoparticles synthesized from 3% and 5% of leaf extract. The reason could be that the smaller size of the particles which leads to increased membrane permeability and cell destruction. Our results are in agreement with those of found in *Cassia auriculata*. Finally these reports suggest that silver nanoparticles synthesized from even green plant sources. Some report shows that many microorganism like algae, bacteria and fungi.

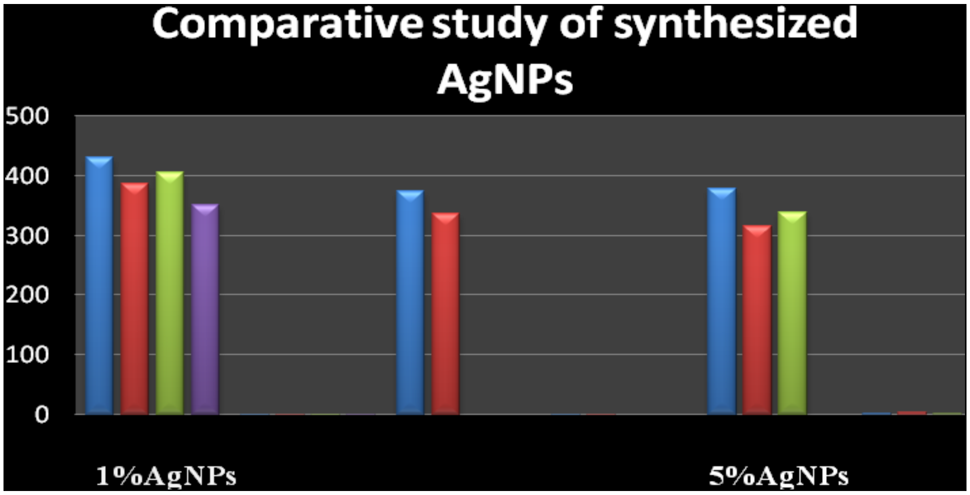


FIGURE 5: GRAPHICAL REPRESENTATION OF SYNTHESIZED SILVER NANOPARTICLES AT THE FOLLOWING CONCENTRATION OF PEELS AQUEOUS EXTRACT

TABLE 3: ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED USING *PUNICA GRANATUM* PEELS EXTRACT

S.No.	Bacterial name	Zone of inhibition			
		Silver nanoparticles (100µg/ml)			Streptomycine sulphate (Standard Antibiotics) (100µg/ml)
		1%	3%	5%	
1	*Staphylococcus aureus*	22mm	23mm	26mm	35mm
2	*Escherichia coli*	30mm	31mm	33mm	30mm
3	*Proteus vulgaris*	35mm	39mm	43mm	45mm

SEM Analysis: Scanning electron microscopy image has shown individual silver nanoparticles as well as a number of aggregates. The surface deposited silver nanoparticles are clearly seen at high magnification (X 10,000) in the micrograph. The morphology of the silver nanoparticles was equally spherical (1%) with interparticles distance, spherical shape (3%) and predominately spherical and aggregated in to smaller irregular (5%) shape with no well defined morphology observed in the micrograph. It was clear from the SEM pictures that control silver nanoparticles were more than 1000nm size, where as silver nanoparticles in the bioreduced colloidal suspensions measured 100-200nm (1%), 50-200nm (3%) and 50-100nm (5%) in size (Fig.6) is the SEM of bioreduced silver nitrate.

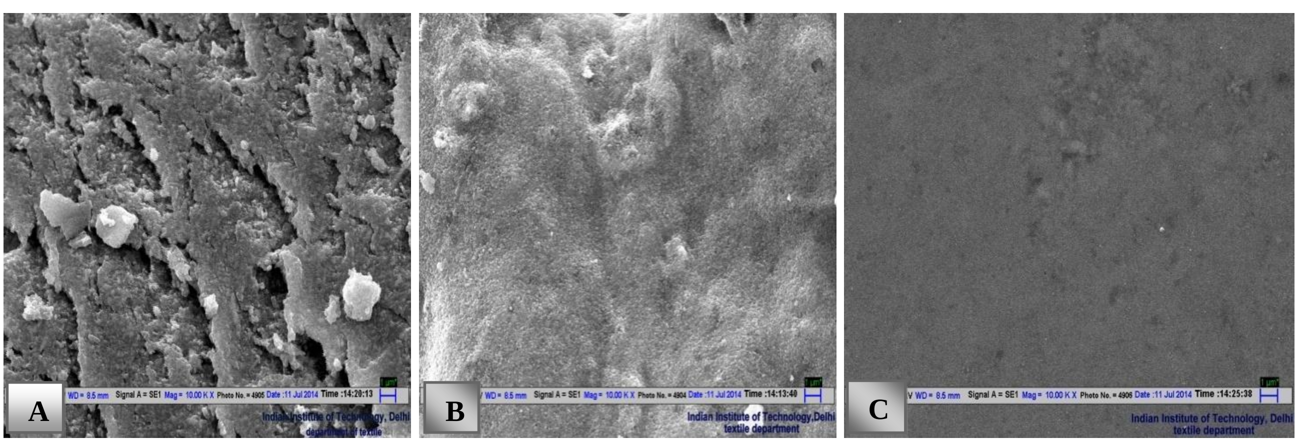


FIG. 6: SEM ANLAYSIS IMAGE OF SILVER NANOPARTICLES FORMED BY *PUNICA GRANATUM* LEAVES AQUEOUS EXTRACT (A) 1%, (B) 3% AND (C) 5%.

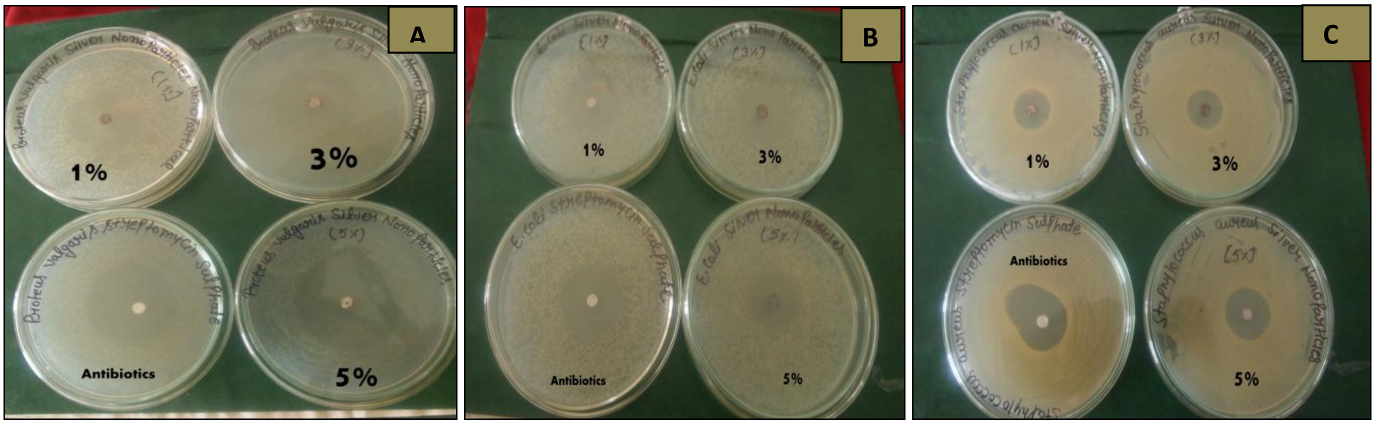


FIGURE 6: ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES (1) 1%, (2) 3%, (3) 5%, (4) ANTIBOITIC (A) *PROTEUS VULGARIS* (B) *ESCHERICHIA COLI* (C) *STAPHYLOCOCCUS AUREUS*.

That silver nanoparticles exhibit a high antibacterial effect due to their well developed surface which provides tha maximum contact with the environment. Furthermore, toxicity is presumed to be size and shape dependent because small size of nanoparticles may pass through cell membranes. Insides a bacterium, nanoparticles can interact with DNA, thus losing its ability to replicate which may lead to the cell death. Green synthesized nanoparticles also have the more effective antimicrobial zone inhibition of the pathogens.

The main problem is that bacteria have developed resistance towards many antibacterial agents. The silver nanoparticles produced here shows significant inhibitory activity against *E.coli* (MTCC-40), *Proteus vulgaris* (MTCC-*1771) and *Staphylococcus aureus* (MTCC-7443).

There are alarming reports of opportunistic bacterial infections. The infections caused by apportunistic bacterial are included under new spectrum of bacterial pathogens. The results suggest silver nanoparticles may have excreted

antibacterial activity by disrupting the structure of cell membrane integrity. The present study indicates silver nanoparticles have considerable antibacterial activity deserves further investigation for chemical application.

CONCLUSION: Most of the biologically active phytochemical were present in the aqueous extract of *Punica granatum* peels. Since the aqueous extract of contains more constituents it can be considered beneficial for the further investigation.

We have green synthesis nanoparticles from the *Punica granatum* peels extract which act as an excellent source for the reducing agent. The primary conformatory for the silver nanoparticles was color changes and UV-Visible spectroscopy of silver nanoparticles formed the highest peaks at 430nm (1%), 373nm (3%), 379nm (5%). The green synthesized nanoparticles have more effective of antibacterial activity to the pathogens. Synthesized nanoparticles was analyzed against bacterial strains *Staphylococcus aureus*, *E.coli* and *Proteus vulgaris* and show that silver nanoparticles show greater antibacterial effect as the concentration increases. So green synthesis of nanoparticles can be ecofreindly involved in tmany application of clinical electronics and etc.

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