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AG-NANOPARTICLES BASED ON POLYSACCHARIDE ISOLATED FROM THE LEAVES OF *MENTHO PIPERITA* AND THEIR STUDY ON MICROBES AND COMPARISON WITH CIPROFLOXACIN

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ABSTRACT

In modern research, the field of science is the most interesting areas for its wide application in chemistry, electronics, ecology, and medicine as well chemists, biologists / microbiologists for their commercial demand as in biological fields. Economically preparation of silver nano particles using green synthesis path having biological entities are gradually increases. Now a day's Ag-nano particles based on synthetic polysaccharide or plant or animal polysaccharide is unique. In the investigation, synthesis of Ag-nano particles were prepared by using polysaccharide, isolated from leaves of *Mentha piperita* and characterized by using UV-VIS spectrum band at 432 nm. Here, the polysaccharide acts as reducing and also capping agent. The synthesized Ag-nano particles were found as very effective against some important human pathogenic bacteria such as *E. coli* ATCC 25922, *K. pneumoniae* ATCC 70063, *S. typhi* MTCC 734 and also effective against *Agrobacterium tumefaciens*, which is responsible for crown gall disease in plants. These Ag-nano particles were very good effective against *E. coli* ATCC 25922, *K. pneumoniae* ATCC 70063, *S. typhi* MTCC 734 compared with antibiotic Ciprofloxacin. These Ag-nano particles have also sporocidal activity. These Ag-nano particles can also reduce the microbial load in sewage water.

INTRODUCTION

Science is a developing interdisciplinary field of research interspersing material science, bio-nanoscience and technology for benefit of life science, health care and industrial biotechnology [1-3]. A reliable and ecofriendly process for synthesis of metallic nano-particles is an important step in the field of nanotechnology. In recent years, novel metal nano-particles have been the subject of focused research due to their unique optical, electronic, mechanical, magnetic and chemical properties that are significantly different from those of bulk materials [4]. There is particular interest in nano-particulate Ag, due to its ability to act as both an electron sink as well as a redox catalyst [5]. Numerous microorganisms and plant extracts have been applied to synthesize inorganic nanostructures either intracellularly or extracellularly. It can be realized by electrostatic interaction between Ag+ and negatively charged carboxylate groups on the cell surface. In a microorganism the reduction of metal ions occurs on its cell surface by enzymes present in the cell wall [6].

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The development of green processes for the synthesis of nano particles in evolving into an important branch of nanotechnology [7,8]. Several plant biomass or plant extracts have been successfully used for extracellular biosynthesis of silver nanoparticles. The metal ion reduction occurs very rapidly, and the reduction of Ag ions will be completed within hours. Rapid synthesis and excellent yield of silver nanoparticles through these plant mediated [9-12] biosynthesis have a time related (2-4 h) advantage in comparison with microbial synthesis (24-120 h) [13-14]. Therefore, green chemistry approaches for nanoparticles synthesis is used in medical applications, targeted drug delivery, imaging, and dye and heavy metal adsorption. The application of nanoparticles as delivery vehicles for bactericidal agents represents a new paradigm in the design of antibacterial therapeutics. In the present investigation, we developed an inexpensive, versatile, and very reproducible method for large-scale synthesis of silver nanoparticles by reduction process using polysaccharide isolated from leaves extract of *Mentha piperita*. This leaves extracted polysaccharide can act both as reducing and capping agent. *M. piperita* is an important leaves which have potential

use in biomedical applications due to its high antimicrobial activity.

MATERIALS AND METHODS

Isolation and purification of Polysaccharide from leaves of *Mentha piperita*

The leaves of *M. piperita* were collected from the nearest home garden and washed with water. The leaves (~300 gm) were cut in small pieces and boiled in 250ml of distilled water for 5 hours. The whole mixture was kept overnight at 4 C and then filtered through linen cloth. The aqueous extract was precipitated in ethanol (1:5, v/v). It was kept overnight at 4 C and then centrifuged at 8000 rpm for 30 min. The precipitated material was dissolved in distilled water and dialyzed through DEAE cellulose bag against distilled water for 2 hours to remove low molecular weight materials. The material was lyophilized (100 mg). 25 mg lyophilized material was weighted and dissolved in minimum distilled water and was purified through a Serulose 6B permeation column (50×1.5 cm) using water as eluant with a flow rate of 0.5 ml/min. A total of 100 test tubes (2 ml each) were collected and monitored spectrophotometrically at 490 nm using phenol-sulphuric acid method [15]. The solution from Test tube (15-30) were collected & freeze dried, the yielding desired Polysaccharide (~ 12 mg).

Synthesis of Ag-nanoparticles

The silver nanoparticles were prepared by using the polysaccharide as reducing agent. The process performed by adding 20 mL of 0.5 mg/ml of the polysaccharide into 20 ml of aqueous solution of 1 mM AgNO for reduction of Ag ions into Ag. The total mixture was stirred with magnetic bar for 14 hours at room temperature. The reddish brown color of the solution indicated the presence of Ag- nanoparticles which was confirmed by UV-Visible spectrophotometer (Lamda 35).

Preparation of standard nanoparticles

20 ml of aqueous solution of 1 mM $AgNO_3$ was reduced with $NaBH_4$ at cold condition until black ppt was appeared.

Analysis of effect of polysaccharide based Ag-nanoparticles on microorganisms

The antibacterial activities of Ag-nanoparticles

The antibacterial activities of polysaccharide based Ag-nanoparticles was done on human pathogenic *E. coli* ATCC 25922*, K. pneumonia* ATCC 70063*, Salmonella typhi* MTCC 734, by standard disk method. The Nutrient broth media was used for overnight culture of pathogenic strain of bacteria at 37 C under shaking condition (150 rpm). The log phase cultures of each bacterium were spread in nutrient agar plates. Now in the agar media four disks were placed with diameter of 0.6 cm. Among them, one disk was absorbed with 200µl sample of concentrate polysaccharide based Ag-nano particle (450µg/ml concentration), one disk was absorbed with 100µl sample of diluted polysaccharide based Ag-nano particle (225µg/ml, and one disk was absorbed with 200µl of standard Ag-nano particle (450µg/ml concentration) respectively. The plates were incubated at 37 C for overnight.

Assay on bacterial growth

The effect of polysaccharide based silver nanoparticles on growth of different pathogenic bacterial strain (*E. coli* ATCC 25922*, K. pneumonia* ATCC 70063, *Salmonella typhi* MTCC 734*)* were done in nephelometric flask containing Nutrient broth at 37 C under shaking condition (150 rpm) using polysaccharide based-SNPs (final concentration 1500 µg/ml) at different time intervals (0 to 6 hours). The growth of *E. coli* ATCC 25922*, K. pneumonia* ATCC 70063*, Salmonella typhi* MTCC 734, in broth media was indexed by measuring the optical density (at λ=600 nm) at regular intervals using colorimeter . Whereas control does not contain any exposure of silver nanoparticles synthesized from polysaccharide extract of *Mentha piperita.*

Effect on endospore

Bacterial endospore is dormant structure that is formed in unfavorable condition by certain group of bacteria (e.g. Bacillus sp.) and are resistance to most of the antibacterial agent. So destruction of endospore in now a challenging field in biological world by natural way. The spores of bacteria were purified by a method as demonstrated by TjakkoAbeeetal with few modifications. For example spores of the *Bacillus subtilis* were prepared on a nutrient-rich, chemically defined sporulation medium designated Y1 medium, which contained the following components (final concentrations): D-glucose (10 mM), L-glutamic acid (20 mM), L-leucine (6 mM), L-valine (2.6 mM), L-threonine (1.4 mM), L-methionine (0.47 mM), L-histidine (0.32 mM), sodium-dl-lactate (5 mM), acetic acid (1 mM), $FeCl_3$ (50 µM), $CuCl_2$ (2.5 µM), $ZnCl_2$ (12.5 µM), $MnSO_4$ (66 µM), $MgCl_2$ (1 mM), $(NH_4)_2SO_4$ (5 mM), Na_2MoO_4 (2.5 µM), $CoCl_2$ (2.5 µM), and $Ca(NO_3)_2$ (1 mM). The medium was buffered at pH 7.2 with 100 mM potassium phosphate buffer. Furthermore, spores were prepared on modified G medium; the medium contained 0.2% yeast extract, $CaCl_2$ (0.17 mM), K_2HPO_4 (2.87 mM), $MgSO_4$ (0.81 mM), $MnSO_4$ (0.24 mM), $ZnCl_2$ (17 µM), $CuSO_4$ (20 µM), $FeCl_3$ (1.8 µM), and (NH_4) $2SO_4$ (15.5 mM) and was adjusted to a pH of 7.2.

This medium was expected to contain approximately 14 mM amino acids, based on a 70% protein content of the yeast extract. Cultures were incubated at 30 C with shaking at 225 rpm, which resulted in >99% free spores in both media, after incubation for 48 h followed by incubation at 55 C for 1 hour. The presence of endospore was confirmed by endospore staining using malachite green and counted by hemocytometer. The spores were then harvested, °washed repeatedly and treated with polysaccharide based silver nanoparticles to analyze the sporocidal activity. For example the 2 mL of spore culture (1.37 x107 CFU/ml) were treated with 1000 µg of polysaccharide based silver nanoparticles and spreaded on neutrient agar media incubated for 24 hours. The treated spore solutions were plated in nutrient agar medium and incubated at 37°C for 24 hours. Again 2 ml of spore culture was spreaded on nutrient agar media incubated under inverted condition to count the colonies number.

Effect on endospore with respect to different concentration of Ag-nano particles

To measure the inhibition zone of endospore culture by disk diffusion method 0.1ml endospore culture was spread over the nutrient agar plate, then the different concentrated

(1500µg/ml,1000µg/ml,750µg/ml) Ag-nano particles prepared from *Mentha piperita*, were added by disk (which absorbed 200µl sample and diameter of 0.5cm). The plates were incubated at 37°c for overnight.

Effect on Sewage water with respect to different concentration

4 ml of sewage water was mixed with 400µl Ag-nanoparticle solution with respect to different concentrations (200µg/ml, 100µg/ml, 25µg/ml, 12.5µg/ml, and 6.25µg/ml). And again 4 ml sewage water was mixed with 400µl sterilized (autoclaved) water in different sterilized test tubes. Now, 0.1 ml sample taking from each test tube, and spread over the nutrient agar plate, and incubated at 37° C for 24 hours under inverted condition.

inverted condition. These steps are repeated for 4 times at the interval of 30 mins (0h-2h 30 min), and count the colony.

Effect on *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is the causal agent of crown gall disease (the formation of tumours) in the plants. *Agrobacterium tumefaciens* is a member of the family Rhizobiaceae. These bacteria are gram negative and grow aerobically, without forming endospore. The cells are rod-shaped and motile, having one to six peritrichous flagella. Tumor producing Agrobacterium species are pathogenic and do not beneficial for the plant. So destruction of these bacteria is now a challenging field in biological world by natural way. These bacteria were isolated from gall tissue, produce in mango tree.

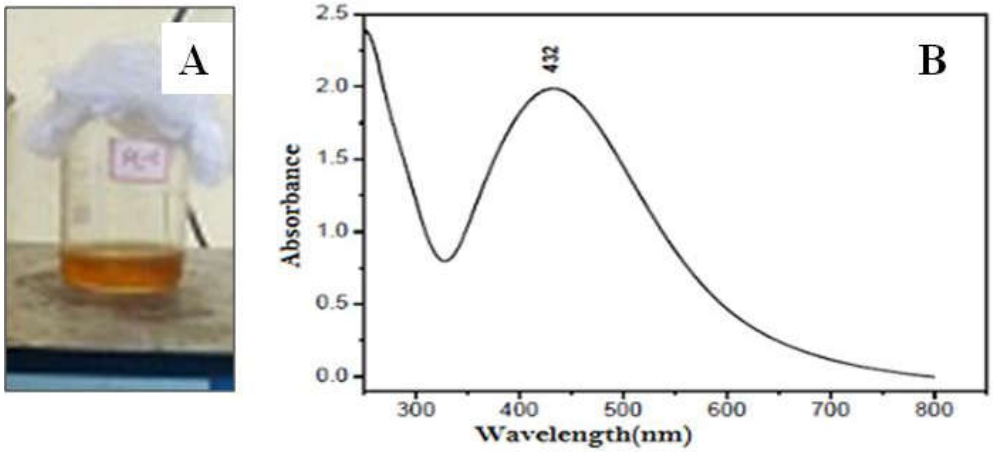

Figure 1. Ag-nanoparticle synthesized from purified polysaccharide *Mentha piperita* (A), UV-VIS spectrum of the Ag- nanoparticles (B)

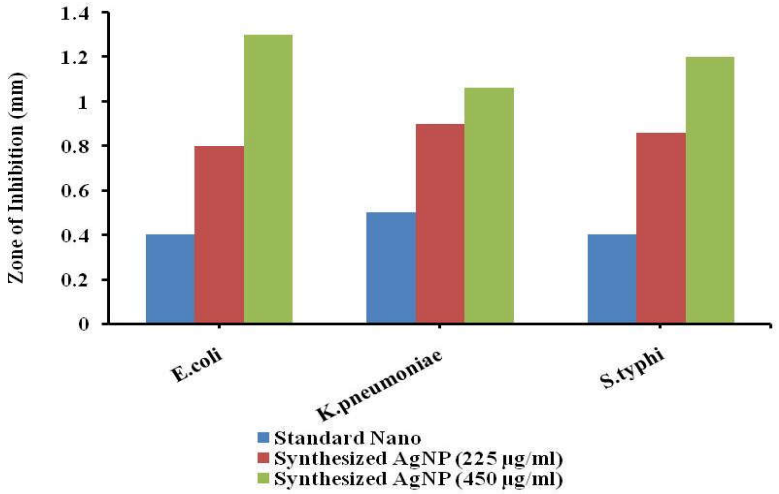

Figure 2. Graphically presentation of antimicrobial activity of Ag-nanoparticles on E. coli ATCC 25922, K. pneumonia ATCC 70063 & Salmonella typhi MTCC 734

Effect on Sewage water with respect to time variation

4ml of sewage water was mixed with 400µl Ag-nano particle solution, in sterilized test tube. Now, 0.1ml sample was spread over the nutrient agar plate and kept in incubator at 37°C for 24 h, under inverted condition, for count the colony. After, 30 mins, 0.1ml sample taken from test tube and spread over nutrient agar plate and incubated at 37°C for 24 h, under

Then the tissue were cutted into small spices and vortexed for 20 min. now, the mixture were grown in YEP media (bactopeptone 10g, Nacl 5g, Yeast extract 10g, Agar 15g, K2HPO4 0.5g, MgSO4.7H2O 0.2g, distilled water 1lit, pH 6.8), and incubated at 30 c for 24 h, under shaking condition(200 rpm). Presence of *Agrobacterium tumefaciens* was confirmed by gram staining. After these, 0.1ml sample from the YEP media were spreaded over the YEP Agar media plate.

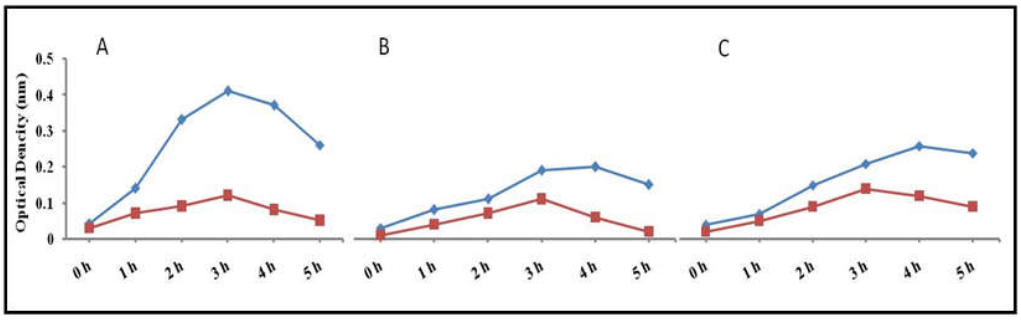

Figure 3:- The bacterial Growth curve (at 600 nm) [x axis- Time and Y axis-O.D.] A. *E. Coli* B. *K. pneumoniae* and C. *S. typhi*

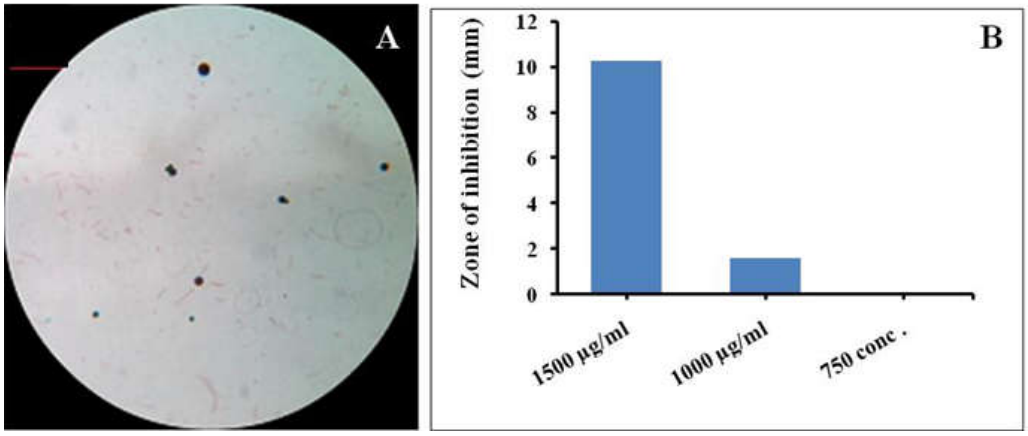

Figure 4: *B. subtilis* spore by staining with malachite green (A), graphical presentation of Sporocidal activity of different con^{c} of Ag-nano particle solution (B).

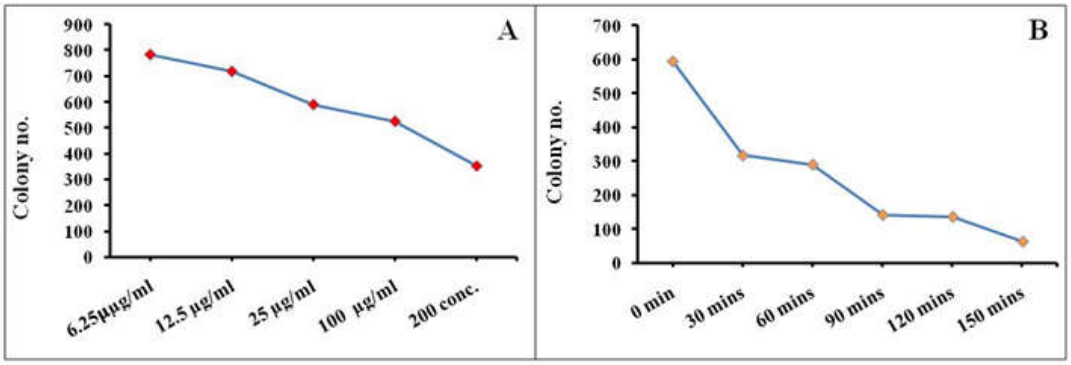

Figure 5. Result of effect of different con^{c} of Ag-nano particle solution on sewage water (A). Effect of Ag-nano particles on sewage water with respect to different time (B)

Then, 0.2ml (750µg/ml) Ag-nano particle solution was added by pore (0.7cm) method, and the plate was kept in incubator at 30°C for 24h.

Compare the effectiveness of Ag-nano particles and antibiotic solution against different bacteria

It is the major way to judgment the effectiveness of the destruction of pathogenic microorganisms through polysaccharide based nano particles comparing with antibiotics. At first lyophilized Ag-nano particles (10mg) were dissolved in 5ml distilled water, thus 2000µg/ml Ag-nano particle solution was prepared. Repeatedly 1500µg/ml, 1000µg/ml, 200µg/ml Ag-nano particle concentration were prepared through serial dilution. Similarly effective antibiotic (ciprofloxacin) concentration (2000µg/ml, 1500µg/ml, 1000µg/ml, 200µg/ml) were prepared thoroughly. Each bacterium (*E.coli, S.typhi, K. pneumoniae*) was spreaded over nutrient agar media plate (4 plates respectively). Then same concentrartion of antibiotic and Ag-nano particle solution (2000µg/ml, 1500µg/ml, 1000µg/ml, and 200µg/ml) were added respectively into four respective plates by disk diffusion method. Then the plates were incubated at 37°C for 24h.

RESULT AND DISCUSSION

Isolation and purification of Polysaccharide from leaves of *Mentha piperita*

The leaves of *Mentha piperita* (~300 gm) was extracted with distilled water for 5 hours and precipitated in ethanol followed by centrifugation. The precipited material dissolved in distilled water and dialyzed through DEAE cellulose bag for 2 hours. The material was lyophilized, yielded 100 mg crude polysaccharide. The crude polysaccharide (25 mg) was purified through a serulose 6B permeation column (50×1.5

cm) using water as eluant with a flow rate of 0.5 ml/min and one pure fraction was obtained (12 mg).

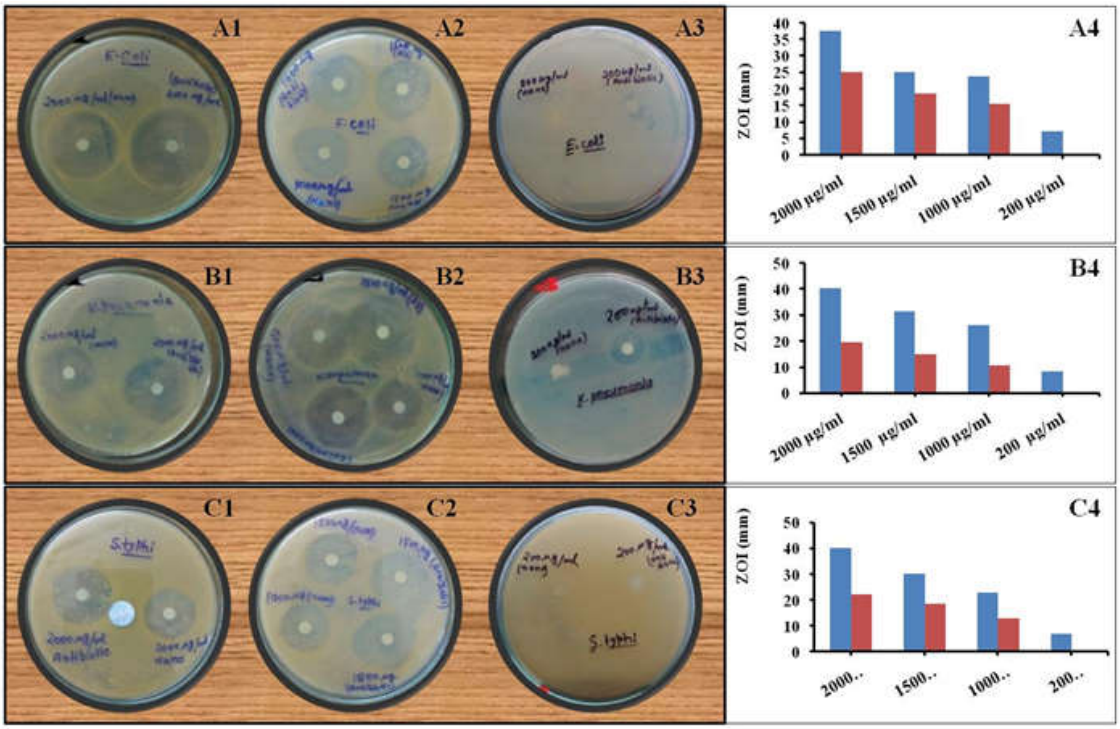

Figure 6. Comparison of diameter of inhibition zone (DIZ) between Ag-nano particle solution and antibiotic solution

Synthesis of Ag-nanoparticles

It is well known that silver nanoparticles exhibit reddish/brownish/yellowish color in water; this color arises due to combined vibration of free electrons of silver nanoparticles in resonance with light wave, which give rise to a surface plasmon resonance (SPR) absorption band in the visible region of electromagnetic radiation. The synthesis of Silver nanoparticles by reduction of the aqueous metal ions during exposure of 20 mL of 0.5mg/mL of polysaccharide extract from *Mentha piperita* into 20 mL of aqueous solution of 1 mM AgNO detected by the development of brown color (Figure 1.A) and confirmed by UV-VIS spectra. UV-VIS absorption spectrum of silver nanoparticles in the presence of polysaccharide extract is shown in Figure 1.B. The Surface Plasmon band in the silver nanoparticles solution remains close to 432 nm throughout the reaction period, suggesting that the nanoparticles were dispersed in the aqueous solution with no evidence for aggregation in UV-VIS absorption spectrum.

The antibacterial activities of Ag-nanoparticles

E. coli ATCC 25922is more sensitive than, *K. pneumonia* ATCC 70063, *Salmonella typhi* MTCC 734. The antibacterial activity of polysaccharide based SNPs were done on human pathogenic *E. coli* ATCC 25922*, K. pneumonia* ATCC 70063, *Salmonella typhi* MTCC 734, by standard disk method, depicted in figure-2.The antibacterial activity of silver nanoparticles was compared for various microorganisms using the diameter of inhibition zone. The zone of inhibition (ZOI) reflects magnitude of susceptibility of the microorganisms. The strain's susceptible disinfectants exhibit larger (ZOI), whereas resistant strains exhibit smaller (ZOI). The data support the sensitivity of bacterial strain towards polysaccharide based nanoparticles in comparison to polysaccharide and standard nano. Again the data show that *E. coli* ATCC 25922is more sensitive than, *K. pneumoniae* ATCC 70063, *Salmonella typhi* MTCC 734.

Assay on bacterial growth

The growth curves of *E. coli* ATCC 25922*, K. pneumonia* ATCC 70063*, Salmonella typhi* MTCC 734 treated with SNPs were shown in figure-3A, 3B, and 3C respectively by measuring optical density at 600 nm. In presence of 1500 µg/ml of polysaccharide based SNPs, the growth curves of each of the bacterium decreased (lag phase, exponential phase, and stationary phase), however decline phases in each growth curve could not be revealed because we only assayed the total numbers of bacteria, including live and dead ones, based on the value of O.D. 600. In absence of nanoparticles the growth curve reached exponential phage quickly but treatment with polysaccharide based SNPs decrease the growth rate of each bacterium and most immersed effect are found in case of *E. coli* ATCC 25922 and *K. pneumonia* ATCC 70063, *Salmonella typhi* MTCC 734. The experiment proved that silver nanoparticles generated by treatment of polysaccharide from *E. coli* ATCC 25922 exhibits strong antibacterial activity due to their well developed surface which provides maximum contact with the environment.

Effect on endospore

Spore are resistant form of bacteria that create a threat in medical world. We first prepared the concentrated culture of *B. subtilis* spore and confirmed by staining with malachite green (Fig 4A). To analyze the sporocidal activity of silver nanoparticles we first time demonstrate that polysaccharide based SNPs responsible immensely decreased spore.

Effect on endospore with respect to different concentration of Ag-nano particles

The Ag-nano particle solution, isolate from *Mentha piperita* was very much effective at high concentration against

endospore . But in lower concentration it can not killed the spore. At the con^c of 1000µg/ml, 1500µg/ml and above it works but the con^c below 1000µg/ml (750µg/ml, 500µg/ml, 250µg/ml) it can't work (fig:-4B).

Effect on sewage water with respect to different concentration of Ag-nano particle solution

The plates which contain sewage water with high con^c of Ag-nano particles is much effective than the plates which contain low con^c of SNP_s .The number of colony in plate contain 200µg/ml nano-particles is low than the 100µg/ml and below it (Fig 5A). From the above experiment it can be concluded that the high con^c of SNP_s reduce more microbial load in sewage water.

Effect on sewage water with respect to time

The plate which spreaded first (initial time 0 h) contain huge amount of bacterial colony. But the plate which spreaded last (2 hour and 30 mins) contain very low amount of bacterial colony.(fig-5B).So, the bacterial colony decreased, when time of exposure of mixture (sewage water+nano solution) was increased. From the above experiment it can be concluded that the, increased in time decreased the bacterial load of sewage water.

Compare the effectiveness of Ag-nano particles and antibiotic solution against different bacteria

Antibacterial activity of antibiotic at high con^c (2000µg/ml, 1500µg/ml, 1000µg/ml) was too much high, but also antibacterial activity of Ag-nano particles at high concentration is good (Fig 6). Inhibition zone of both SNP_s and antibiotic at high con^c was near about same. But the activity at low con^c of antibiotic is good but, SNP_S has no activity to inhibit the bacterial growth (Fig 6).

Conclusion

The green synthesis of silver nanoparticles using polysaccharide extracted from *Mentha piperita* is an eco-friendly and simple process and also economic one. The polysaccharide based SNPs responsible for destruction of different multi drug resistant (MDR) human pathogenic bacteria. Our research also proved that the green synthesis SNPs is responsible for sporocidal activity and to inhibit the growth of Agrobacterium tumefaciens. Antibacterial activity of antibiotic Ciprofloxacin at high con^c (2000µg/ml, 1500µg/ml, 1000µg/ml) was too much high, but also antibacterial activity of Ag-nano particles at high concentration is good.

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