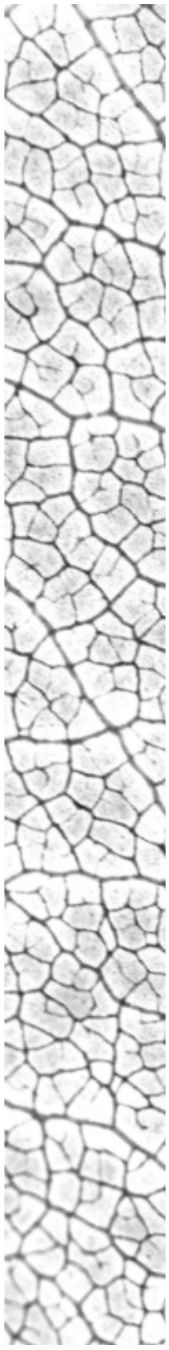

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Hydrothermal assisted biological synthesis of Silver nanoparticles by using honey and gomutra (Cow Urine) for qualitative determination of its antibacterial efficacy against *Pseudomonas sp.*isolated from contact lenses

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Abstract

Background: Biological synthesis of Silver Nanoparticles (AgNPs) by using honey and gomutra shall become biocompatible and cost effective alternative method wherein no extra reducing agents were used which may create problems for living systems and also adds extra cost to the system.
Materials and Methods: The crude gomutra and purified honey (1 mg/mL) in sterile distilled water were used for the synthesis of Ag NPs by hydrothermal method. The characterization of Ag NPs was done by UV-Vis Spectroscopy and Scanning Tunneling Microscopy (STM) to study optical and morphological characteristics, correspondingly. The isolation of bacterial culture was carried out by incubation of swabs taken from contact lenses of healthy volunteers, followed by its identification through microscopic and biochemical studies. Antibacterial activity of Ag NPs was performed against *Pseudomonas* species by anti-well diffusion assay and broth dilution method. Studies were also performed for determination of biofilm reduction by Ag NPs by congo red agar test and tube assay.
Results: Silver Nanoparticles were synthesized by using honey and gomutra by hydrothermal method The absorption spectrum of Ag NPs and band gap was found to be at 350 nm and 3.54 eV respectively, for honey and 275 nm and 4.50 eV, respectively, for gomutra by UV-Vis Spectroscopy. STM image showed Ag NP's cluster morphology on surface at 200 nm for honey and 50 nm for gomutra. Ag NPs was found to be effective antibacterial agent at the concentration of 1mg/mL against *Pseudomonas* predominantly present in contact lenses and therefore have a may have applications in ophthalmology.

Keywords: Ag NPs, contact lens, biofilm, *Pseudomonas*sp, honey, gomutra

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INTRODUCTION

More than 125 million people wear contact lenses globally. Bacterial biofilm formation on contact lenses increases the chances of keratitis which is a serious complication. Approximately 38% people got affected due to Keratitis in India. Introduction of soft lenses increased the chances of microbial keratitis. The reason for this is that dust particles, germs and bacteria are able to enter the eye where they become trapped under the lens. The lens traps them against the eye where they are able to cause damage or infection (Bruinsma et al. 2001, Fleiszig and Evans 2010, Soltys-Robitaille et al. 2001, Tewariet et al. 2012, Willcox et al. 2001).

Three steps involved in the formation of biofilms (Sonkusale and Tale 2015):

1) Initial attachment.
2) Irreversible adhesion, proliferation and maturation of biofilms.
3) Dispersal of planktonic bacteria.

P. aeruginosa forms biofilm on a silicone hydrogel contact lens (lotrafilcon A). The outcome of this is an increased resistance to disinfectants in contact lens multipurpose disinfecting solutions (Szczotka-Flynn et al. 2009). *P. aeruginosa* has been observed in microbial keratitis because of its ability to tightly adhere to the surfaces of contact lenses (Bruinsma et al. 2001). When *P. aeruginosa* interacts and adhere with the

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contact lenses, its production of slime becomes a key factor (Slusher et al. 1987). Infections may occur due to *P. aeruginosa*'s ability to carry out quorum sensing, which allows the biofilm to evade detection of the immune system and the virulence factors are expressed (Willcox 2007). One of the most remarkable mechanisms contributing to antibiotic resistance of *P. aeruginosa* is its tendency to form biofilms which can act as a shield that protected the bacterial cells by the increased production of one or more of three distinct extracellular polysaccharide matrices, designated as Pel, Psl, and alginate (Egamberdieva 2010, Ryder et al. 2007). Additionally, *P. aeruginosa* can mount a defensive response to antibiotic exposure via increased expression of multidrug efflux pumps and beta-lactamases, as well as through the down-regulation of outer membrane porins (Driscoll et al. 2007).

The treatment of infectious diseases has become an emerging problem due to resistance developed by pathogenic bacteria against many traditional antibiotics (Pawar et al. 2012). Widespread use of broad-spectrum antibiotics has resulted in antibiotic resistance for many human bacterial pathogens. Recently, nanotechnology offers solution to this problem by providing antimicrobial nanoparticles to be used against various types of microorganisms. For instance, CuO NPs used against *Bacillus cereus* (Pawar et al. 2018), ZnO NPs against *Salmonella typhimurium* (Pawar et al. 2017). The nanoparticles obtained from silver are of more interest because of their effective antibacterial activity. Several studies have also shown an important activity of silver nanoparticles against bacterial biofilms (Franci et al. 2015, Hamedi et al. 2017).Silver nanoparticles (AgNPs) are capable of interacting physically with the cell surface of various bacteria. Many studies have shown that AgNPs can damage cell membranes leading to structural changes, which render bacteria more permeable (Lazar 2011, Periasamy 2012). Several studies have reported that activity of AgNPs is strongly dependent on the size (Tamayo et al. 2014, Wu et al. 2014). Small sized nanoparticles seem to have a superior ability to penetrate into bacteria. Actually, the interactions with the membranes and any other damage, which could lead to cell death, are certainly more evident in the case of nanoparticles with smaller diameter and a positive zeta potential (Franci et al. 2015). The AgNPs also damage membranes and stimulate the release of ROS (reactive oxygen species), forming free radicals with a powerful bactericidal action (Wu et al. 2014). Protein synthesis has been shown to be affected by treatment with AgNPs and proteomic data have shown gathering of immature precursors of membrane proteins which results in destabilization of the outer membrane composition (Mirzajani et al. 2011).

Honey has been known to have antimicrobial property as well as wound-healing activity since the ancient times. The antimicrobial activity in most honeys is because of the enzymatic production of hydrogen peroxide. Non-peroxide honey (*e.g.:* manuka honey), shows significant antibacterial effects even when the hydrogen peroxide activity is blocked. In such cases, the low pH level of honey and its elevated sugar content (high osmolarity) are enough to hinder the growth of microbes (Mandal and Mandal 2011).

The biochemical estimation of *gomutra* showed that it contains nitrogen, sodium, sulphur, Vitamin A, B, C, D, E, minerals, manganese, iron, silicon, magnesium, chlorine, citric, succinic, calcium salts, phosphate, lactose, carbolic acid, hormones, creatinine and enzymes. Antimicrobial and germicidal properties are mostly due to the presence of creatinine,urea, swarnkshar (aurum hydroxide), phenols, carbolic acid, calcium and manganese in *gomutra* (Mohanty et al. 2014).

MATERIALS AND METHODS

Sample Collection, Isolation and Biochemical Characterization

The samples were collected from contact lenses of healthy volunteers irrespective of their age, sex, dietary habits and medications. Bacterial cultures were isolated and maintained on Kings B medium. Random colonies were selected and their colony, microscopic and biochemical characteristics were studied. Screening for Pseudomonas: Presence of green fluorescent pigment indicates presence of *Pseudomonas*.

Screening of Isolates for Biofilm Formation

Congo Red Agar test

Congo red agar medium were inoculated with organism and incubated at 37 °C for 48 hours. Biofilm production was indicated by observing black colonies with dry crystalline consistency (Willcox 2007).

Tube assay

The tubes of Trypticase Soy broth were inoculated with bacterial isolates and incubated at 37 °C for 24 hours. Followed by incubation, the medium was decanted and tubes were washed with sterile phosphate buffer saline (PBS). The staining of tubes was done by adding 0.1% crystal violet for 5 minutes. The biofilm formation was confirmed by development of violet film on the wall and bottom of the tubes (Christensen et al. 1982).

Microscopic examination of the biofilm

The Trypticase soy broth (2 mL) was inoculated with 100µL of overnight grown bacterial culture in tissue culture plate and incubated the same at 37 °C for 24 hours. After incubation, the medium was drained off and plates were washed with PBS followed by staining with 0.1% crystal violet. The plates were dried and examined for development of violet color biofilm under microscope.

Biological Synthesis and Characterization of Ag NPs

Biosynthesis of Ag NPs from honey

In typical experimental procedure, crude honey (10gms) was dissolved in 80mL of warm sterile distilled water. 0.1M $AgNO_3$solution was prepared in 15 mL distilled water. Both solutions were mixed under constant stirring for 5 minutes to get homogenous reaction mixture which was then incubated at 40^0C for 1 hour. Similarly, in another set 10 mL of Cow urine was mixed with 15 mL sterile distilled water, which was then added into 15 mL of 0.1 M $AgNO_3$solution and provided the same incubation conditions as mentioned above. The resultant precipitate was washed by centrifugation at 5000 rpm for 15 minutes each with DI water. The final product was dried in hot air oven at 70^0C for 8 hours and then calcination at 400 ^{0}C for 4 hours to obtain powder of Ag NPs.

Characterization of Ag NPs

The structural and morphological features of resultant Ag NPs was characterized by UV-Vis spectroscopy, STM and SEM for identifying their morphological and optical information.

Qualitative Determination of Antibacterial Potential of Ag NPs

The antibacterial activities of Ag NPs synthesized from honey and gomutra were tested on bacterial cultures isolated from contact lenses. The antibacterial activity of Ag NPs was carried out by agar well diffusion assay (AWDA) (Pawar et al. 2017). The Muller-Hinton (MH) agar plates were spread inoculated with overnight grown cultures of *Pseudomonas spp.* The synthesized AgNPs were dispersed in sterile DI water by ultra-sonication to make colloidal solution of nanoparticles. On the surface of agar plates wells of 5 mm in diameter and of 18 µL in capacity were formed by using sterile gel borer. The 15 µL of AgNPs suspension were placed in each well and incubate all plates at 37^0C for 24 hours. The zone of inhibition was measured and susceptibility of the nanoparticles was estimated as compared to control (crude honey and gomutra). Additionally, the test cultures of final cell density of $1X10^8$ CFU/ mL were spread inoculated on MH agar plate along with 100 µg/mL concentration of AgNPs. The plates were incubated at 37^0C for 24 hours and subsequent growth inhibition of bacterial cultures was determined. All experiments were performed in triplicates.

Determination of AgNPs Potential for Biofilm Reduction

The Trypticase soy broth (2 mL) was inoculated with 100 µL of overnight grown bacterial culture and 100 µL of Ag NPs (0.1 mg/mL) in tissue culture plate and incubated the same at 37 °C for 24 hours. After incubation, the medium was drained off and plates were washed with PBS followed by staining with 0.1 % crystal violet. The plates were dried and examined for development of violet color biofilm under microscope.

Table 1. Biochemical test for identification of isolates

Sr. No.	Biochemical test		Result
1	Sugar Fermentation	Lactose	Negative
		Dextrose	Negative
		Mannitol	Positive
2	Glycerol Fermentation		Negative
3	Urea test		Negative
4	EMB agar test		Negative
5	Citrate Utilization test		Positive

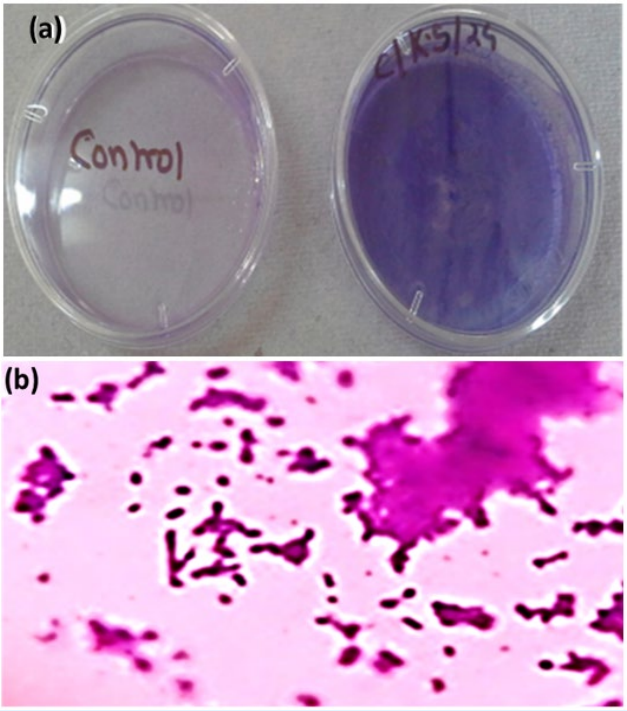

Fig. 1. The biofilm formation is evident by (a) tube assay and (b) microscopic observation

RESULTS AND DISCUSSIONS

Isolation and Biochemical Characterization

All the isolates were tentatively identified as Gram negative *Pseudomonas* sp. based on microscopic and biochemical characterization (**Table 1**).

Screening of Isolates for Biofilm Formation

After 48 hours of incubation, *Pseudomonas sp.,* black colonies on the streaking lines of congo red agar medium indicated their biofilm forming ability. Results of biofilm formation were apparently confirmed by attachment of biofilm on walls of the test tube found in tube assay (**Fig. 1a**). It was also evident that through the staining and observation of tissue culture a plate under microscope which has revealed the architecture of biofilm and bacterial attachment (**Fig. 1b**).

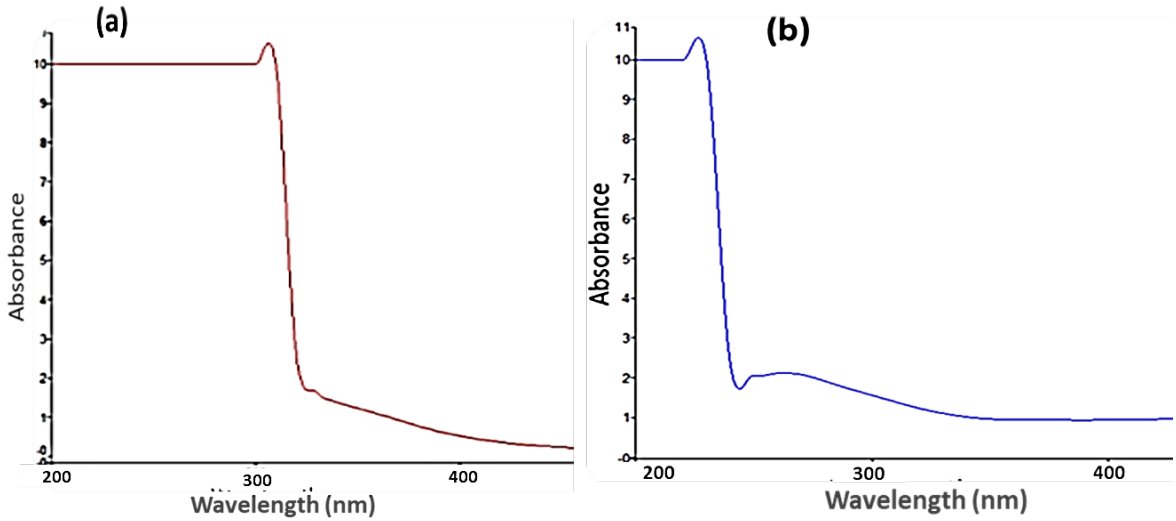

Fig. 2. Spectroscopic results of Ag NPs synthesized from (a) gomutra and (b) honey

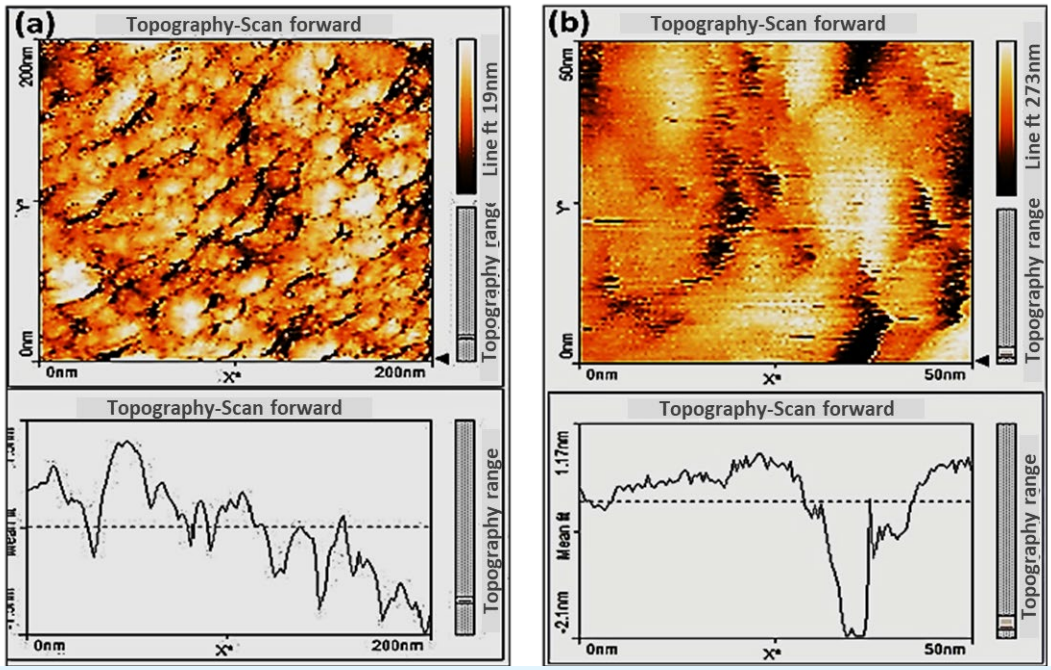

Fig. 3. STM images of Ag NPs synthesized by (a) honey and (b) gomutra

Synthesis of Silver Nanoparticles from Honey and *Gomutra* and its Characterization using U.V. Visible Spectroscopy

Silver nanoparticles were synthesized from crude honey preparation and *gomutra*. Color change observed due to reduction of silver nitrate to silver nanoparticles.UV- visible spectroscopy (Thermo Scientific UV-10) was used to study an optical property of Ag NPs. Spectroscopic results clearly indicated the production of silver nanoparticles. Absorption spectra of both Ag NPs samples have shown in **Fig. 2**. The gaussian peak observed in the range of 200-300nm for AgNP's synthesized from gomutra and between 300-400 nm for AgNP's synthesized from honey.The maximum absorption of synthesized Ag NPs synthesized from gomutra and honey was found to be at 275 nm and 350 nm, respectively. The bang gap was calculated to be 4.50 eV for Ag NPs of gomutra and 3.54 eV for Ag NPs of honey.

The STM images of Ag NPs show the clustered surface morphology at 200 nm (for honey) and 50 nm distance (for gomutra) (**Fig. 3**). The line graph of both the samples indicates the preparation sample, positioning of tip and approach was properly managed and had good tunneling contact.

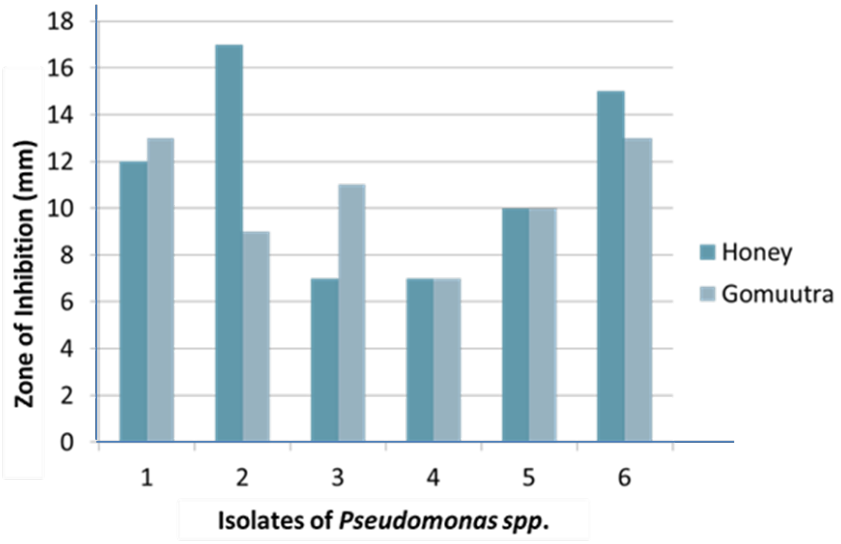

Fig. 4. Antimicrobial activity of AgNPs synthesized from honey and gomutra against isolates of *Pseudomonas spp.*

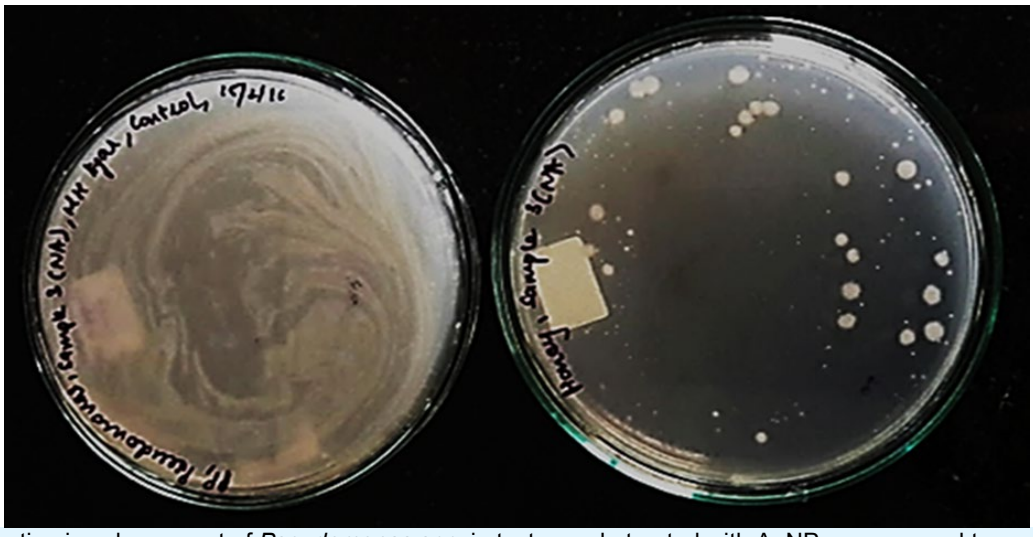

Fig. 5. Reduction in colony count of *Pseudomonas spp.* in test sample treated with AgNP as compared to control

Antibacterial Activity of Ag NPs by Anti-well Diffusion Assay

The antimicrobial activity of Ag NPs suspension of 1000 µg/mL concentration were tested on test microorganisms and found to have antibacterial activity. It has been found in this study that by increasing the concentration of Ag NPs in wells, the growth inhibition has also been increased consistently because of proper diffusion of nanoparticles in the agar medium. In our study, silver nanoparticles synthesized from honey showed a greater significant zone of inhibition against all the isolates of *Pseudomonas spp.*as compared to gomutra (**Fig. 4**). The spread plate with nanoparticles showed significant reduction in number of colonies compared to control plate, which showed confluent growth (**Fig. 5**).

Determination of AgNPs Potential for Biofilm Reduction

Microscopic observation of biofilm was carried out using tissue culture plate indicated significant reduction in adherent biofilm in bacterial culture treated with various concentrations (0.1mg/ml and 0.2mg/ml) of Gomutra AgNP as compared to test sample.

DISCUSSION

In this study, the antibacterial activity of Silver nanoparticles synthesized from cow urine and honey against *Pseudomonas* was studied. Gomutra and honey have been reported to be beneficial for eye related infection and are used various Ayurvedic medicines. (Mohanty et al. 2014). Silver nanoparticles are reported to have very good antibacterial activity against the pathogenic bacteria. (Tamayo et al. 2014, Wu et al.

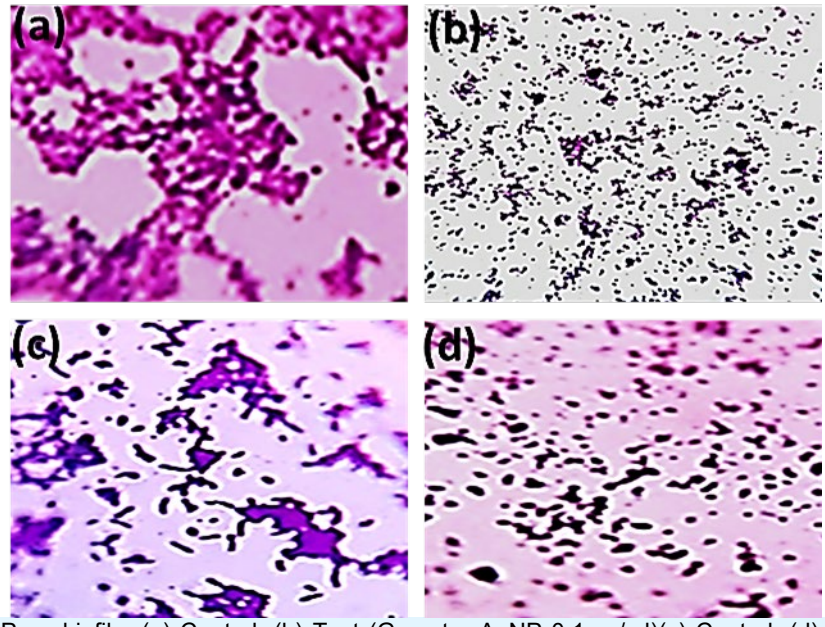

Fig. 6. Effect of AgNP on biofilm (a) Control, (b) Test (Gomutra AgNP 0.1mg/ml)(c) Control, (d) Test (Gomutra AgNP 0.2mg/ml)

2014). Hence Ag NPs from such sources could prove effective in preventing microbial keratis. During the study, Kings B agar was used for the isolation of bacteria. Isolates were characterized and identified as *Pseudomonas sp.* which is predominant in causing microbial keratitis due to biofilm formation. Biofilm formation indicated significant biofilm formation by *Pseudomonas sp*in accordance with reports with the reports by Tewari et al. (2012) nanoparticles synthesized from biological sources are found to less toxic as compared to chemical synthesis. Studies on nanoparticle characterization indicated sharp Gaussian peak at 200-300nm for AgNP's synthesized from gomutra and between 300- 400 nm for AgNP's synthesized from honey.

AgNP were found to be effective against *Pseudomonas sp* with significant reduction in colony count in accordance with reports by Mirzajani et al. (2011). These nanoparticles have also shown significant reduction in biofilm formation by bacteria. Hence such biologically synthesized Ag NP can prove effective for further application related microbial keratitis.

CONCLUSION

Pseudomonas species were isolated from contact lenses of 6 individuals. Silver nanoparticles were synthesized in crude honey preparation and *Gomutra* and characterized by using U.V. visible spectroscopy and further characterized by STM. It was found that the silver nanoparticles have significantly reduced the growth of biofilm forming *Pseudomonas sp*. Silver nanoparticles from *Gomutra* showed significant antibacterial activity against *Pseudomonas sp.* as compared to honey.

REFERENCES

Bruinsma GM, Van der Mei HC, Busscher HJ (2001) Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. Biomaterials 22(24): 3217-24. https://doi.org/10.1016/S0142-9612(01)00159-4

Christensen GD, Simpson WA, Bisno AL, Beachey EH (1982) Adherence of slime-producing strains of Staphylococcus epidermidis to smooth surfaces. Infection and immunity 37(1): 318-26.

Driscoll JA, Brody SL, Kollef MH (2007) The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. Drugs 67: 351–368. https://doi.org/10.2165/00003495-200767030-00003

Egamberdieva D (2010) Colonization of tomato roots by some potentially human-pathogenic bacteria and their plant-beneficial properties. Euro. Asia J. Biol. Sci. 4: 112-118. https://doi.org/10.5053/ejobios.2010.4.0.14

Fleiszig SM, Evans DJ (2010) The pathogenesis of contact lens-associated microbial keratitis. Optometry and vision science: official publication of the American Academy of Optometry 87(4): 225.

Franci G, Falanga A, Galdiero S, Palomba L, Rai M, Morelli G, Galdiero M (2015) Silver nanoparticles as potential antibacterial agents. Molecules 20(5): 8856-74. https://doi.org/10.3390/molecules20058856

Hamedi S, Shojaosadati SA and Mohammadi A (2017) Evaluation of the catalytic, antibacterial and anti-biofilm activities of the Convolvulus arvensis extract functionalized silver nanoparticles. Journal of Photochemistry and Photobiology B: Biology 167: 36-44. https://doi.org/10.1016/j.jphotobiol.2016.12.025

Lazar V (2011) Quorum sensing in biofilms–how to destroy the bacterial citadels or their cohesion/power?. Anaerobe 17(6): 280-5. https://doi.org/10.1016/j.anaerobe.2011.03.023

Mandal MD, Mandal S (2011) Honey: its medicinal property and antibacterial activity. Asian Pacific Journal of Tropical Biomedicine 1(2): 154–160. https://doi.org/10.1016/S2221-1691(11)60016-6

Mirzajani F, Ghassempour A, Aliahmadi A, Esmaeili MA (2011) Antibacterial effect of silver nanoparticles on Staphylococcus aureus. Research in microbiology 162(5): 542-9. https://doi.org/10.1016/j.resmic.2011.04.009

Mohanty I, Senapati MR, Jena D, Palai S (2014) Diversified uses of cow urine. International Journal of Pharmacy and Pharmaceutical Sciences 6(3): 20-22.

Pawar J, Bipinraj NK, Singh EA, Nikam N (2012) Determination and Partial Characterization of Antimicrobial Material of Pseudomonas aeruginosa Isolated from Milk. A Journal of Food Science & Technology 1(2): 16-23.

Pawar J, Henry R, Viswanathan P, Patwardhan A, Singh EA (2018) Testing of antibacterial efficacy of CuO nanoparticles by methylene blue reduction test against Bacillus cereus responsible for food spoilage and poisoning. Indian Chemical Engineer 27: 1-6. https://doi.org/10.1080/00194506.2018.1548948

Pawar J, Shinde M, Chaudhari R, Singh EA (2017) Semi-Solvo Thermal Synthesis and Characterization of Zinc Oxide Nanostructures Against Food Borne Pathogens. Int J Pharm Bio Sci 8(2): 311-5. https://doi.org/10.22376/ijpbs.2017.8.2.b311-315

Pawar J, Viswanathan P, Vinchurkar P, Shinde M, Henry R, Mandke Y, Patwardhan A, Singh EA (2017) Application of semi-solvo thermally synthesized zinc oxide (ZnO) nanoparticles in food technology and their characterization. International Journal of Nanotechnology and Applications 11(1): 75-80.

Periasamy S, Joo HS, Duong AC, Bach TH, Tan VY, Chatterjee SS, Cheung GY, Otto M (2012) How Staphylococcus aureus biofilms develop their characteristic structure. Proceedings of the National Academy of Sciences 109(4): 1281-1286. https://doi.org/10.1073/pnas.1115006109

Ryder C, Byrd M, Wozniak DJ (2007). Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Current opinion in microbiology 10(6): 644–648. https://doi.org/10.1016/j.mib.2007.09.010

Slusher MM, Myrvik QN, Lewis JC, Gristina AG (1987) Extended-wear lenses, biofilm, and bacterial adhesion. Archives of Ophthalmology 105(1): 110-5. https://doi.org/10.1001/archopht.1987.01060010116042

Soltys-Robitaille CE, Ammon Jr DM, Valint Jr PL, Grobe Iii GL (2001) The relationship between contact lens surface charge and in-vitro protein deposition levels. Biomaterials 22(24): 3257-60. https://doi.org/10.1016/S0142-9612(01)00163-6

Sonkusale KD, Tale VS (2015) Isolation and Characterization of Biofilm Forming Bacteria from Oral Microflora. International Journal of Current Microbiology and Applied Science 2: 118-27.

Szczotka-Flynn LB, Imamura Y, Chandra J, Yu C, Mukherjee PK, Pearlman E, Ghannoum MA (2009) Increased resistance of contact lens related bacterial biofilms to antimicrobial activity of soft contact lens care solutions. Cornea 28(8): 918. https://doi.org/10.1097/ICO.0b013e3181a81835

Tamayo LA, Zapata PA, Vejar ND, Azócar MI, Gulppi MA, Zhou X, Thompson GE, Rabagliati FM, Páez MA (2014) Release of silver and copper nanoparticles from polyethylene nanocomposites and their penetration into Listeria monocytogenes. Materials Science and Engineering: C40: 24-31. https://doi.org/10.1016/j.msec.2014.03.037

Tewari A, Sood N, Vegad MM, Mehta DC (2012) Epidemiological and microbiological profile of infective keratitis in Ahmedabad. Indian journal of ophthalmology 60(4): 267. https://doi.org/10.4103/0301-4738.98702

Willcox MD, Harmis N, Cowell BA, Williams T, Holden BA (2001) Bacterial interactions with contact lenses; effects of lens material, lens wear and microbial physiology. Biomaterials 22(24): 3235-3247. https://doi.org/10.1016/S0142-9612(01)00161-2

Willcox, MD (2007) Pseudomonas aeruginosa infection and inflammation during contact lens wear: A review. Optometry and Vision Science 84(4): 273-278. https://doi.org/10.1097/OPX.0b013e3180439c3e

Wu D, Fan W, Kishen A, Gutmann JL, Fan B (2014) Evaluation of the antibacterial efficacy of silver nanoparticles against Enterococcus faecalis biofilm. Journal of endodontics 40(2): 285-90. https://doi.org/10.1016/j.joen.2013.08.022