ACTIVITY OF EGGSHELL DERIVED CAO NANOPARTICLES AGAINST PATHOGENS ISOLATED FROM HUMAN WOUNDS

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ABSTRACT: Microbes show extreme resistance opposed to multiple drugs which makes the researchers to introduce alternative techniques which enhance healing of infections. The contemporary and revolutionary systems are required to develop modern way of treatment to tackle multidrug resistant properties of bacteria. Nanotechnology is a swift and easy approach to synthesize metallic nanoparticles from various sources. In current research, 50 samples were collected from human wounds without discrimination of age, gender and site of wound injury. The main objectives of the research were to isolate, characterize and identify pathogens from human wounds. These wound pathogens were treated with calcium oxide nanoparticles synthesized from waste hen eggshells. Total 35 samples showed growth on Blood and MacConkey agar with different morphologies. Among 35 positive growth, there were 41 different colonies belonged to 6 common genera named as *Acinetobacter*, *Escherichia coli*, *Klebsiella*, *Proteus*, *Pseudomonas*, and *Staphylococcus*. These bacterial isolates were distinguished by morphological and biochemical identification. Calcium oxide nanoparticles (CaO) were synthesized from waste eggshells while their antimicrobial property was checked by well diffusion and disc diffusion method. These nanoparticles were further characterized by FTIR (Fourier transformed infrared) technique. CaO nanoparticles showed effective results against *Staphylococcus*, *Acinetobacter*, *E.coli*, *Klebsiella*, *Pseudomonas*, and *Proteus* in decreasing order making remarkable steps in the field of research. This research demonstrates that CaO nanoparticles are effective against different bacteria and can be used to treat wound infections.

Key Words: Calcium Oxide, Nanoparticles, Multidrug Resistance, FTIR analysis, Antimicrobial Activity.

INTRODUCTION

Microbes show beneficial role in the environment and many other microorganisms are highly pathogenic to the environment and can cause infectious diseases. These pathogenic microorganisms can be grouped as pathogenic bacteria, viruses, fungi, or algae. Such pathogens are responsible for illness in photosynthetic plants, humans, and animals. They can cause diseases such as hepatitis, AIDS, cholera, dengue, skin infections, allergies, anthrax, tetanus, aspergillosis, blastomycosis, ringworm, malaria, toxoplasmosis, etc. These microbes enter the body of the host to start multiplication and increase number for their survival resulting in illness. Illness can be acute or chronic depending on the pathogenicity of the pathogens while can be cured within days to weeks and months to years respectively (Klemm & Dougan, 2016).

Bacterial infections are most common but different from each other and any living organism such as plants, animals, or humans can be exposed to them. While there are diverse groups and types on the basis of their shape, size, and functions. Bacteria are associated with two different group correspondent to thick and thin cell wall symmetry of peptidoglycan layer named as Gram-positive or Gram-negative respectively. This knowledge gives us a platform to uncover the structural functions of bacteria causing illness. Bacteria survive under severe conditions although they require optimum temperature, pH, oxygen, nutrients, acid tolerance, etc. Bacteria undergo structural modifications to survive in an environment (Poole *et al*., 2018).

Antibiotics are used against bacteria to stop the progression of infections. Antibiotics are classified as bacteriostatic and bactericidal, prevents the bacterial growth and kill the bacteria respectively. All antibiotics are different from each other and show unique mode of action against specific bacteria. For example, vancomycin inhibits the synthesis of cell wall whereas chloramphenicol prevents the protein synthesis by conjugation with ribosomes. But our major problem is the resistance of bacteria towards antibiotics due to the modification in its structure and composition. These modifications are aided by multiple proteins synthesis or gene activation during stress conditions. For example, *E.coli* possess some acid resistance mechanism which induces under specific acidic stress. Outer membrane porins reduce proton influx to adjust cytoplasmic acid tolerance. There are some other systems depend on amino acids are introduced (Li *et al*., 2020).

Researchers worked to overcome the spread of bacterial infections by introducing antibiotics against multiple pathogenic bacteria in recent 70 years. These antibiotics worked effectively for elimination of pathogenic bacteria by remarkable reduction in rate of mortality. At the beginning these antibiotics were very helpful but bacteria start to resist them. At this modern age, it cannot be said that antibiotics are working with high efficacy because bacteria start to evolve rapidly. Hence it has become a challenge for researchers to uncover genetic modifications done by bacteria and overcome new outbreaks every year. Many well-known bacteria such as *Acinetobacter, Escherichia coli, Staphylococcus, Pseudomonas, Proteus, Salmonella, Clostridium* etc. showed resistance to the novel antibiotics, while many of them are found in outermost layer of skin. Antibiotic resistance is a world-wide problem that need to be tackled within no time (van Duin & Paterson, 2020).

Researchers are working to eradicate multidrug resistant ability of bacteria so that rate of mortality can be reduced. Contemporary and ingenious approach has introduced the use of metallic nanoparticles as antimicrobial agent. In recent experimental approaches, nanoparticles are widely used against bacteria. These nanoparticles are synthesized from waste organic materials and well admired due to their physiochemical properties like sizes in the range of nanometers. Nanoparticles are successfully used in medical field to enhance drug delivery system, medical biosensors and as therapeutic agents. Their role in medical field has increased their importance and production. (Ma *et al*., 2017).

Metallic nanoparticles especially inorganic oxides are synthesized to reveal their antimicrobial effect against different microbes. Multiple inorganic metal oxide nanoparticles for example, ZnO, MgO, TiO_2, SiO_2, etc showed potential role in therapeutics. These nanoparticles adhere to the cell wall or cell membrane of pathogens and prevent growth of bacteria. Researchers have introduced many experimental techniques for synthesis of ZnO, MgO, TiO_2, SiO_2, Fe_2O_3 etc. due to their potency. In modern era, it is a big need to introduce innovative nanomaterial to overcome multidrug resistance problems. There are only few research projects of calcium oxide (CaO) nanomaterials. The source of organic waste is being utilized for the synthesis of calcium oxide nanoparticles to check their innovative application against pathogens (A. Roy *et al*., 2013).

Natural egg shell is a source of calcium as it is composed of calcium carbonate crystals. It is semipermeable membrane, non-porous and has antimicrobial potency. Egg is being used widely as an important component of daily diet because it is rich in nutritional-components. Eggshell protects its internal content from physical damage and keep embryo safe. Eggshell is a big source of calcium for developing embryo inside it. The bio ceramic property of eggshell provides gaseous exchange and water to the content or embryo inside it. The composition of eggshells plays remarkable role due to its potency against microorganisms isolated from various sources. Research on egg or egg shell is developing day by day to introduce new strategies, applications, and nanomaterial (Sree *et al*., 2020).

Wound pathogens and their sensitivity against different drugs have been studied in this research. *Acinetobacter, Staphylococcus, Pseudomonas, E.coli* etc. have been isolated from wounds which prevent speedy recovery and increase their number making treatment prolonged and difficult Furthermore, current research has focused on antibacterial activity of nanoparticles to uncover efficacy of CaO NPs extracted from hen eggshell.

MATERIALS AND METHOD

Sample Collection: Total 50 samples were collected from the Lahore General Hospital with the help of staff and nurses with sterile swabs.

Isolation and identification of bacteria: Selective media were prepared and all samples were inoculated to the specific media. These petri dishes were then incubated at $37^{o}C$ for 24 hours in incubator to check bacterial growth. After culturing, gram staining was performed to distinguish between gram positive and gram negative bacteria. Moreover, biochemical testing was performed to identify specific bacteria.

Preparation of Calcium oxide nanoparticles: Waste chicken eggshells were collected from cafeteria of Lahore Garrison University, located in Lahore DHA phase VI. Eggshells were first washed and then heated in hot air oven for 3 hours at $150^{o}C$ to remove water. Dried eggshells were ground properly until fine powder and stored in polyethylene bag.

The chemicals used for this purpose were hydrochloric acid (HCl) and Sodium hydroxide (NaOH). 250ml of 1M HCl solution was mixed with 12.5gm of eggshell powder (ESP) to prepare calcium chloride solution ($CaCl_2$). Took another autoclaved flask and 250ml distilled water was added in it. 10gm NaOH pallets were added to 250ml distilled water. Mixed it by stirring slowly which resulted in turbidity and heat production. Then solution of NaOH was mixed with the solution of HCl and ESP slowly and drop by drop. After mixing of both solutions, placed the flask at room temperature for further 24 hours and allowed it for condensation. After 24 hours, filtration of solution was done and saved the obtained precipitates. Then obtained precipitates were washed with distilled water for 2 times and placed in hot air oven for 24 hours at $60^{o}C$ to remove water properly. Then dried powder was kept in muffle furnace at 900^{o}C for 1 hour (Habte *et al*., 2019).

Characterization: For the characterization of CaO nanoparticles, Fourier transform infrared (FTIR) determined different functional groups present in obtained nanoparticles. While it can identify chemical bond in a molecule by infrared absorption spectrum. Sample was set on the crystal to check its absorbance. Egg shell powder (ESP) was also used to compare it with CaO NPs to check their functional groups. The analysis was performed on Omnic software whereas Thermo Scientific machine was used (Yeh *et al*., 2018).

Antimicrobial activity assay: Antimicrobial activity has been checked by Kirby-Bauer method on Muller Hinton agar (MHA). There were different antibiotics used named as, AK (Amikacin), TZP (Tazobactam) FEP (Cefepime), MEM (Meropenem), SXT (Cotrimoxazole), CAZ (Ceftazidime), and IPM (Imipenem), for *Acinetobacter*, *Klebsiella*, *E. coli*, *Pseudomonas*, and *Proteus.* Whereas, E (Erythromycin), P (Penicillin) and DA (Clindamycin) were used as addition to these antibiotics against *Staphylococcus* as it was gram-positive bacterium. Then all the plates were kept in incubator for 24-48 hours at 37^{o}C to check maximum results. After incubation, readings were measured according to CLSI criteria (Booq *et al*., 2021).

While on the other side, antibacterial activity of CaO nanoparticles was tested against isolated bacteria from wound pathogens. For this purpose, MHA media was autoclaved and cooled down at room temperature for pouring in sterilized petri plates which were labelled with the names of isolates. Media was poured and allowed to solidify under room temperature. The antibacterial activity of CaO nanoparticles was tested by disc diffusion method and well diffusion method.

For well diffusion method, lawn was prepared on MHA plates with the help of sterile cotton swab with wooden stick. Then a sterile cork borer was used to make three holes on Muller Hinton agar's plate at specific distance and each well was filled with diluted CaO nanoparticles. Nanoparticles were added in distilled water, ethanol and methanol in different concentrations to evaluate maximum antimicrobial activity. All test tubes with nanoparticles were stirred for 30 seconds then wells were filled with CaO nanoparticles and labelled the wells according to dilution. Plates were incubated for 24 hours at 37^{o}C to check antimicrobial activity.

For disc diffusion method, lawn was prepared on MHA plates with the help of sterile cotton swab with wooden stick. On the other hand, filter paper discs were prepared and autoclaved then these discs were dipped in ethanol, methanol and distilled water dilutions of CaO nanoparticles separately. These discs were placed on the surface of Muller Hinton Agar (MHA) with the help of sterile forcep at specific distance and labelled the plates accordingly. These plates were incubated for 24 hours at 37^{o}C to check antimicrobial activity.

RESULTS

Isolation of bacteria: All wound samples were inoculated on agar plates and after incubation time, only 35 samples showed growth on media. There were 28 male subjects and 22 female subjects with different age ranges between 6 to 82 years. There were 41 colonies identified on the basis of morphology, microscopy, and biochemical tests. The common isolates were *Acinetobacter, E. coli, Klebsiella, Pseudomonas, Proteus, and Staphylococcus*. There were 2 isolates of *Proteus*, 8 *Staphylococcus*, 5 *Pseudomonas*, 8 *Acinetobacter*, 8 *Escherichia coli*, and 10 *Klebsiella* as shown in table.

Table 1: Possible isolates from patients sample.

Sample ID	Isolates
F3150-1, M50-3, M1630-1, F50-1, M50-4, F1630-2, F3150-3, F3150-7	*Acinetobacter*
M1630-3, M1630-8, F50-4, M50-6, F1630-3, M3150-3, M115-2, M1630-5	*E.coli*
F1630-1, M1630-2, F50-2, F50-3, M1630-4, M1630-5, M1630-6, M1630-9, M3150-8, M1630-10	*Klebsiella*
M50-2, M50-7	*Proteus*
M50-1, M3150-2, M50-5, F1630-4, F3150-6	*Pseudomonas*
M3150-1, M115-1, F115-1, M1630-7, F3150-2, F50-5, F3150-4, F3150-5	*Staphylococcus*

Antimicrobial susceptibility test: Antimicrobial testing was performed according to the criteria of CLSI by the disc diffusion method. According to the isolate, antibiotic discs were selected to check their efficacy. There were multiple drugs such as Tazobactam (TZP), Imipenem (IPM), Meropenem (MEM), Amikacin (AK), Cefepime (FEP), and Ceftazidime (CAZ) used against gram-negative bacteria. These antibiotics were checked against 8 isolates of *Acinetobacter*. Among these, SXT showed 100% efficacy, TZP, IPM, MEM, AK and FEP showed 88.8% efficacy and CAZ showed 77.7% efficacy. These antibiotics were also checked against 5 isolates of *Pseudomonas*. AK showed 100% efficacy, CAZ, FEP, TZP, IPM showed 80% efficacy, and MEM showed 60% efficacy. *E. coli.* showed 100% sensitivity for TZP, IPM, MEM, and AK whereas CAZ showed 75% efficacy and

FEP, SXT showed 62% efficacy. There were 10 isolates of *Klebsiella* which are gram-negative bacteria. IPM, MEM, AK showed 90% efficacy, TZP showed 80% efficacy, CAZ, FEP showed 70% efficacy and SXT showed 50% efficacy. These antibiotics were checked against 2 isolates of *Proteus*. CAZ, FEP, TZP, IPM, MEM, AK showed 100% efficacy and SXT showed 0% efficacy. Cotrimoxazole (SXT), Penicillin (P), Erythromycin (E), and Clindamycin (DA) were used against *Staphylococcus*. Among them P, E showed 75% efficacy and SXT, DA showed 62% efficacy.

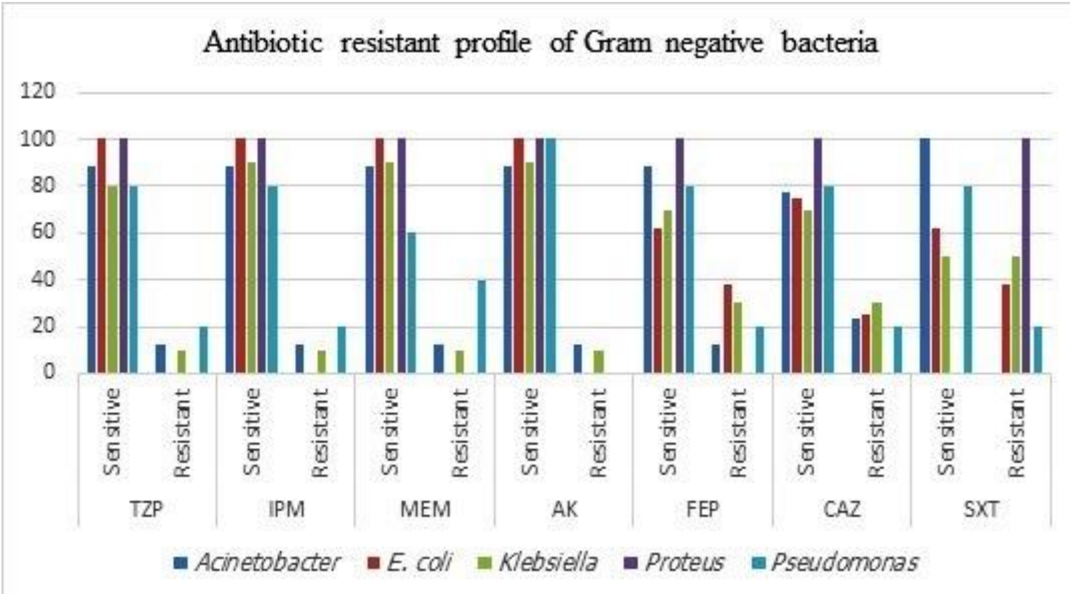

Figure 1: Antibiotics resistant profile of Gram-negative bacteria

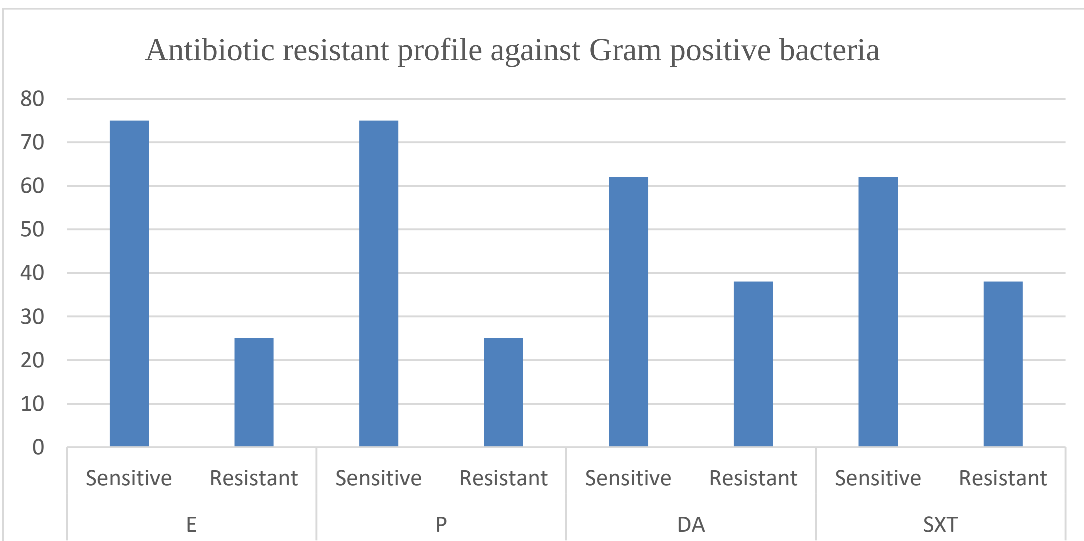

Figure 2: Antibiotics-resistant profile of Gram-positive bacteria

Characterization of synthesized CaO nanoparticles: Chicken eggshell derived nanoparticles were characterized by FTIR. According to the results of FTIR. Calcium oxide nanoparticles (CaO NPs) represented the peaks at wavenumber 1397.53 cm^{-1}, 871.41 cm^{-1}, 711.72 cm^{-1}, 588.15 cm^{-1}, 574.70 cm^{-1}, 559.13 cm^{-1}, 536.16 cm^{-1} and 531.68cm^{-1}. Whereas main peaks as shown in figure were 1397.53 cm^{-1}, 871.41 cm^{-1}, and 711.72 cm^{-1} which represent different bonds.

First main peak at 1397.53 cm^{-1} represent COO^- group in class of carboxylic acid salts. Furthermore, there were two sharp bands at 871.41 cm^{-1} and 711.72 cm^{-1} showing C-O bond. Egg shell powder (ESP) was also used to compare it with CaO NPs to check their functional groups.

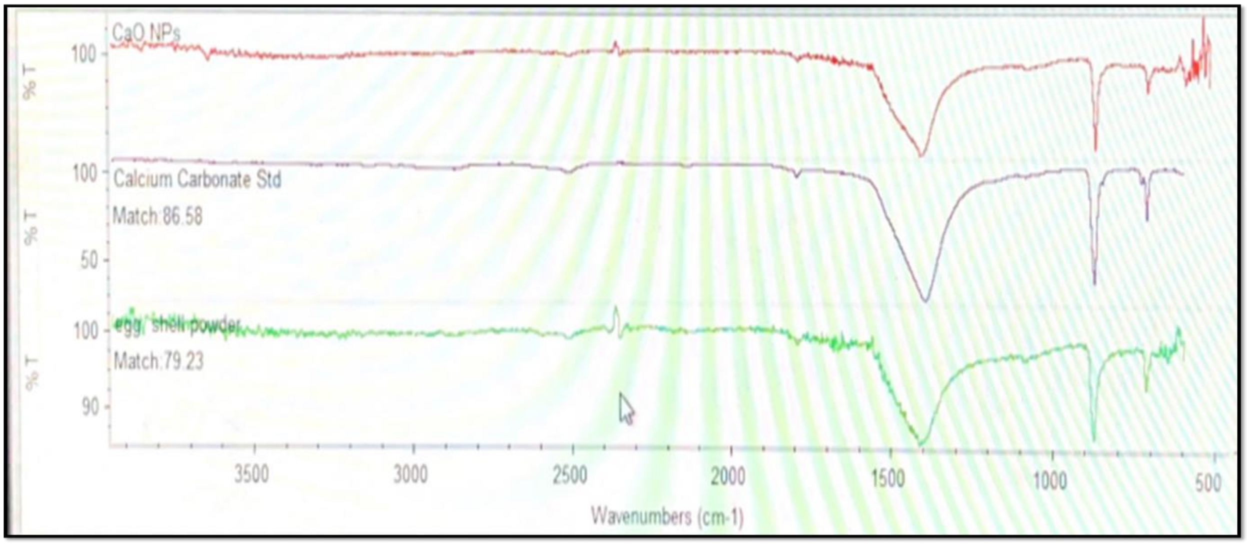

Figure 3: FTIR of CaO NPs, Raw eggshell powder and Calcium Carbonate Std.

Antibacterial activity of CaO NPs: CaO nanoparticles showed efficacy against wound pathogens by well diffusion method and disc diffusion method. *Staphylococcus* showed maximum susceptibility against CaO nanoparticles and 30mm zone of inhibition was observed. *Acinetobacter* showed 29mm zone of inhibition, *Pseudomonas* appeared after 13mm zone of inhibition *E.coli* gave 19mm zone of inhibition and *Klebsiella* gave 16mm zone of inhibition. Rest of all possible isolates *Proteus* showed minimum susceptibility against nanoparticles. All best results were observed with dilution of nanoparticles in ethanol, methanol and distilled water.

DISCUSSION

Multidrug resistance is a major problem for biologists to eradicate the burden of disease globally because it is considered as alarming situation. The basic purpose of current research was to introduce an innovative approach to overcome wound infections caused by different gram negative and gram positive bacteria under the benefits of nanoparticles synthesized from waste material. The ratio of sample collection according to the gender was more in female samples as compared to male samples. But the number of isolates was higher in male samples.

Overall 8 *Acinetobacter* were found from sample F3150-1, M50-3, M1630-1, F50-1, M50-4, F1630-2, F3150-3, and F3150-7. Total 8 *Escherichia coli* were found from the sample M1630-3, M1630-8, F50-4, M50-6, F1630-3, M3150-3, M115-2, and, M1630-5. Whereas, F1630-1, M1630-2, F50-2, F50-3, M1630-4, M1630-5, M1630-6, M1630-9, M3150-8, and M1630-10 were *Klebsiella* isolates. M50-2 and M50-7 were *Proteus* whereas M50-1, M3150-2, M50-5, F1630-4, and F3150-6 were *Pseudomonas*.

While rest of the samples M3150-1, M115-1, F115-1, M1630-7, F3150-2, F50-5, F3150-4, and F3150-5 were *Staphylococcus*. *Acinetobacter* was least in male isolates as compared to the female samples. *Escherichia coli* were found in 2 female samples and 6 male samples. There were 3 female samples of *Klebsiella* and 7 positives for male samples. While *Proteus* was identified only in male samples. 3 Male samples and 2 female samples were positive for *Pseudomonas*. The ratio of *Staphylococcus* was more in female isolates as compared male isolates. There were 3 male samples and 5 female positive isolates.

There was different ratio of different bacteria found on wounds such as 19% *Acinetobacter*, 20% *Escherichia coli*, 5% *Proteus*, 12% *Pseudomonas*, 20% *Staphylococcus*, and 24% *Klebsiella*. All bacteria were identified by their morphological characterization, gram staining and biochemical testing. In contrast to recent study, CaO nanoparticles were tested against *Pseudomonas*, *Staphylococcus*, and *Candida* (Arup Roy *et al*., 2013).

There are multiple ways to synthesize CaO nanoparticles such as eggshells, seashells, veld grape, and pomegranate peels etc. In 2018, CaO nanoparticles have been synthesized from aqueous extract of broccoli by various concentrations of its aqueous extract. The purpose of this research was to degrade bromocresol green by photo irradiation method. While synthesis of CaO nanoparticles were calcined under high temperature treatment to get desired results (Osuntokun *et al*., 2018). Another research revealed the synthesis of CaO nanoparticles from limestone using honey by thermal decomposition method (Arul *et al*., 2018).

In very recent study, calcium oxide nanoparticles were synthesized by sol-gel method using room temperature which costs very low, least time consuming and no additives. In sol-gel method nanoparticles are calcinated under high temperature in muffle furnace for short time which enhance their efficiency (Chebil *et al*., 2019). In present research, sol-gel method was selected and utilized for low cost synthesis of calcium oxide nanoparticles (CaO NPs).

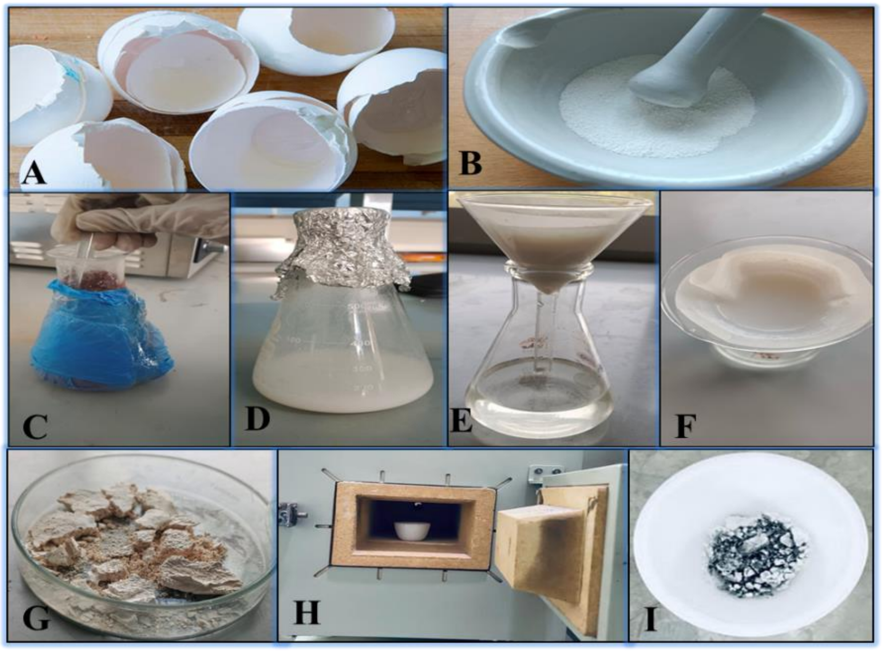

Figure 4: Synthesis of CaO nanoparticles (A) Raw hen eggshells, (B) Eggshells powder, (C) Mixture of HCl and NaOH containing eggshell powder (D) Condensation reaction after 24 hours (E) Filtration process (F) Washing of obtained product with distilled water (G) oven drying of filtered gel (H) Muffle furnace calcination of dried gel (I) Calcinated gel powder (CaO Nanoparticles)

CaO nanoparticles were characterized by FTIR technique to determine chemical group present in compound. Hence, Calcium oxide nanoparticles (CaO NPs) represented the peaks at wavenumber 1397.53 cm^{-1}, 871.41 cm^{-1}, 711.72 cm^{-1}, 588.15 cm^{-1}, 574.70 cm^{-1}, 559.13 cm^{-1}, 536.16 cm^{-1} and 531.68cm^{-1}. Whereas main peaks as shown in figure were 1397.53 cm^{-1}, 871.41 cm^{-1}, and 711.72 cm^{-1} which represent different bonds. First main peak at 1397.53 cm^{-1} represent COO^- group in class of carboxylic acid salts. Furthermore, there were two sharp bands at 871.41 cm^{-1} and 711.72 cm^{-1} showing C-O bond. Egg shell powder (ESP) was also used to compare it with CaO NPs to check their functional groups. The results of FTIR were almost similar to the results represented in research work of Habte *et al*., 2019.

Roy *et al*., only worked for the synthesis and characterization of CaO nanoparticles but in current research, antimicrobial efficacy of CaO nanoparticles was also performed. Marquis highlighted the palnt mediated synthesis of nanoparticles and their use against *E.coli, Staphylococcus, Vibrio, Klebsiella, Shigella*, and *Pseudomonas*. The best results of inhibition were observed against *E.coli* (Marquis *et al*., 2016). In a previous study CaO NPs were synthesized from waste hen egg shells same as in current study and used against heavy metal which was lead (Pb(II)) extracted from aqueous solution. CaO NPs were used for remediation of toxic heavy metal isolated from industrial waste (Jalu *et al*., 2021). In present study, hen eggshell derived CaO nanoparticles were used against gram positive and gram negative bacteria isolated from wounds.

In present study, *Staphylococcus* showed maximum susceptibility against CaO nanoparticles and 30mm zone of inhibition was observed. *Acinetobacter* showed 29mm zone of inhibition, *Pseudomonas* appeared after 13mm zone of inhibition *E.coli* gave 19mm zone of inhibition and *Klebsiella* gave 16mm zone of inhibition. Rest of all possible isolates *Proteus* showed minimum susceptibility against nanoparticles. All best results were observed with dilution of nanoparticles in ethanol, methanol and distilled water.

Conclusion: Microbes are showing great resistance against therapeutic drugs which make their survival easier and it enhances the virulence and potential risk of disease. In this study, antimicrobial susceptibility was demonstrated to eradicate life threatening problem of multidrug resistance mechanism of microorganisms. Moreover, it can be concluded that waste products such as eggshells used to synthesize calcium oxide nanoparticles (CaO NPs) which are cheap to synthesize by most eco-friendly mechanism, sol-gel method. CaO nanoparticles showed the ability to combat resistant bacteria i.e. *Acinetobacter, Pseudomonas, E.coli, Klebsiella*, *Staphylococcus,* and *Proteus* in decreasing order making remarkable steps in the field of research. If we make transformations insignificantly towards standard protocols by using solvents affecting microbial growth and acknowledge other biologists to compare results can give desired solutions and consequences.

Conflicts of interest: There is no conflict of interest.

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