Tuning and Evaluating The Synergistic Effect of Silver Nanoparticles-Quercetin With Conventional Antibiotics

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Abstract- *Identification of effective drug combination and their synergy is critical to address the emergence of antimicrobial resistance. In the present study, we have described an investigation on drug combination effects using silver nanoparticles-quercetin comparing that with and without conventional antibiotics. Due to selective pressure conferred by conventional antibiotic treatment, incidence of pathogenic antibiotic resistance continues to present as increasing problem. Many flavonoids have been found to have antibacterial effects. Synergies between silver nanoparticles-quercetin and between quercetin-antibiotics, silver nanoparticles-antibiotics and combination of all three have been found to enhance the inhibition in some bacterial groups. Quercetin along with silver nanoparticles, was tested for activity against the bacterial strains i) E.coli ii) S. aureus iii) Klebsiella pneumoniae. Using the disc diffusion method, minimum inhibitory concentration (MIC) values for individual and combinations of silver nanoparticles-quercetin, quercetin-antibiotics, silver nanoparticles-antibiotics and combination of three were established against all the chosen bacteria respectively. FIC index (FICI) values were calculated in order to determine synergistic or additive effects of the different combinations. Strong additivity effect (FICI > 4) was found for E.Coli, Klebsiella pneumoniae and S. aureus. Our results indicated potential synergistic effects with silver nanoparticles-quercetin combinations in gram positive bacteria. Combinations between quercetin-conventional antibiotics showed enhanced inhibition which could be useful in multidrug resistant bacteria.*

Keywords- Silver nanoparticles; Quercetin; Synergistic activity; Conventional antibiotics; MIC, FIC index.

I. INTRODUCTION

Antibiotic-resistant bacteria may be tougher to cure than previously thought as the microorganism have adapted defenses against antibiotics and continue to develop new resistance. In recent years, much attention has been given to find a strategy to mitigate antibiotic resistance. As more microbial species and strains become resistant, diseases have become difficult to treat, a phenomenon frequently described to both indiscriminate and inappropriate use of antibiotics in human medicine (1). Nanotechnology provides a strategic platform with alter physio-chemical properties of different materials compared to their bulk counterpart that can be harnessed for biological applications.

Use of nanoparticles has increased over the years as they exhibit new or improved properties based on their specific characteristics such as size, distribution and morphology. The most important feature of nanoparticles is their surface area to volume ratio that allows them to interact with other particles. Nobel metal nanoparticles such as gold and silver have received great interest due to their optical properties. Chemical and physical methods of metal nanoparticle synthesis are energy intensive, low yield and may produce high levels of hazardous byproducts that may have adverse medical effect. Biologically synthesizing nanoparticles (using microorganisms) is more effective and environmentally safe (2).

Microbial synthesis of nanomaterials utilizes of biological components, primarily prokaryotes. Since, microbes produce nanomaterials as part of their metabolism, they can be used for various applications. Metals nanoparticles such as silver and gold are receiving great interest due to their applications in diverse areas such as medicine, cosmetics, coatings, packaging, and in biotechnology research areas. Reducing the particle size of metals is an efficient and reliable tool for improving their biocompatibility. Furthermore, nanomaterials can be modified for better efficiency to facilitate their applications in different fields such as bioscience and medicine.

Flavonoids are a group of naturally occurring low-molecular-weight polyphenol compounds that have recently become the subject of therapeutic interest. Flavonoids have

good antibacterial activities against different gram negative and gram positive bacteria. Quercetin is one of the major plant flavonoids found in many fruits, vegetables, leaves, and grains, have antimicrobial potential against broad spectrum of bacteria and fungi (3, 4). Quercetin has antimicrobial and antiviral activity, antidiabetic activity and anti-mutagenic activity.

Metal nanoparticles are highly ionic and can be prepared with extremely high surface areas and can be engineered with other reactive surface sites. The antibacterial effects of silver nanoparticles are widely studied. Silver nanoparticles interact with outer membrane of bacteria, and arrest the respiration and other metabolic pathway that leads to the death of the bacteria. Silver nanoparticles have also been known to be a promising antimicrobial agent that acts on a broad range of target sites both extracellularly as well as intracellularly (5,6). It is also been reported that there is formation of pits on the cell surface, and there is accumulation of the nanoparticles on the cell surface **(Figure 1).** Silver nanoparticles shows very strong bactericidal activity against gram positive as well as gram negative bacteria including multi-resistant strains (7, 8).

Since overuse/ misuse of antibiotics has led to the development of multi-drug resistant bacteria, as a strategy to overcome these limitations of conventional synthetic antibiotics compounds, a nanotechnology based strategy has been presented as an alternative strategy in developing alternative antimicrobial agents that can efficiently kill bacterial cells and display immense potential (9).

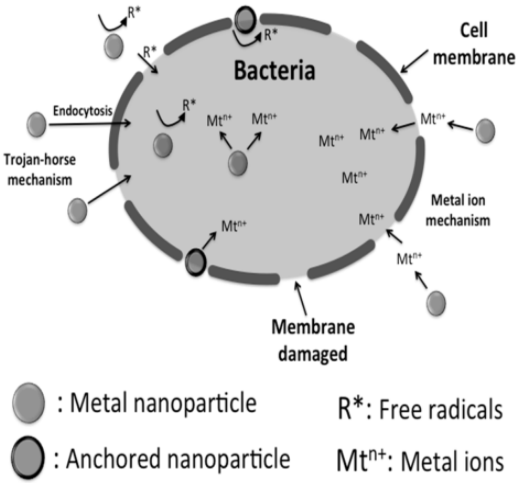


Figure 1. Silver ions inhibit the reproduction of microbes (adapted from *Int. J. Mol. Sci.* **2015**, *16*(1), 2099-2116; doi:10.3390/ijms16012099).

Based on this premise, in this study, we have investigated the synergistic activity of quercetin and biologically synthesized silver nanoparticles and compared their activity with and without conventional antibiotics - penicillin/streptomycin, and our results supports our hypothesis that quercetin along with silver nanoparticles can increase the potentiality of antibiotic and can also be used as an alternative way to treat antibiotic resistance threats. Our aim has been synthesize silver nanoparticles using microorganisms. The synthesized silver nanoparticles have been studied in combination with quercetin along with antibiotics to evaluate the bacterial inhibition.

II. MATERIALS AND METHODS

Materials

Silver nitrate, nutrient agar (NA) nutrient broth medium (NB), quercetin were procured from Sigma Aldrich. Whatmann paper No 1, Dimethylsulphoxide (DMSO), commonly used antibiotic penicillin and streptomycin was used in the study. Bacterial Cultures used: *Escherihia coli; Staphylococcus aureus; Klebsiella pneumoniae.*

Methods

Preparation of silver nanoparticles using E.coli

E.coli strains were collected from St. Joseph hospital and were re-cultured on nutrient agar. Single colony of E.coli was collected from nutrient agar plate and was suspended in saline suspension. E.coli culture was added to freshly prepare nutrient broth and was kept on shaker at room temperature for 24 hours. Turbidity indicated the growth of organism in the broth. 0.1 M of silver nitrate was weighed and dissolved in deionised water. Freshly prepared silver nitrate solution was added to E.coli cultured nutrient broth. This was incubated for 24 hours on shaker incubator at 37°C. The broth color changes from pale yellow to brown indicating the synthesis of silver nanoparticles. A positive control (without the silver nitrate solution) and the negative control (pure silver nitrate solution without cell-free filtrate) did not show any change of color of the reaction mixture. The brown solution was centrifuged at 15000 RPM for 30 minutes at 10°C. Supernatant was discarded and pallet was collected and dried. The powdered silver nanoparticles were used for further experiments. The following representative microbes – Gram negative (*Escherichia coli, Klebsiella pneumoniae),* Gram positive (*Staphylococcus aureus*) were used to evaluate the anti-microbial and the synergistic activity of silver nanoparticles-quercetin with and without antibiotics.

Preparation of soluble silver nanoparticles, quercetin and silver nanoparticles-quercetin

Silver nanoparticles and quercetin were dissolved separately in 0.1% DMSO and both solutions were mixed in ratio of 1:1. These individual and mixed preparations were tested with all the three bacterial cultures mentioned above, along with/without conventional antibiotics. The following combinations were used for testing the antibacterial activity;

Table 1. Combinations of quercetin, silver nanoparticles and antibiotics

Sample 1	Control (no treatment)
Sample 2	Silver nanoparticles
Sample 3	Quercetin
Sample 4	Antibiotics
Sample 5	Silver nanoparticles + antibiotics
Sample 6	Quercetin +antibiotics
Sample 7	Silver nanoparticles + quercetin
Sample 8	Silver nanoparticles + quercetin+ antibiotics

Method for testing synergistic activity of test compounds for antimicrobial activity by disc diffusion method

To evaluate antimicrobial effect of silver nanoparticles against *E.coli, S.aureus*, and *K. pnuemoniae*, minimum inhibitory concentration was determined. Representative microorganisms of gram positive bacteria (*Staphylococcus aureus*) and gram negative bacteria (E.*coli*, *Klebsiella pneumoniae*) were used to evaluate the antibacterial activity of both silver nanoparticles, quercetin along with/without antibiotics and combination of solutions using disc diffusion method. Muller and Hinton (MH) agar plates was poured and allowed solidify. Two quadrants were drawn on the agar plates and spread plate method was performed by swabbing each of the microbial suspensions separately on the prepared MH agar plates using sterile cotton swabs. 1 mm discs of Whatmann paper number No 1 was were impregnated with of freshly prepared silver nanoparticles, quercetin (dissolved in 0.1% DMSO), and combination solutions were placed on MH agar plates. These plates were then incubated at 37°C for 18 - 28 hours. The zone of inhibition was measured after incubation period.

Determination of minimum inhibitory concentration and calculation of FIC index

Broth microdilution method in accordance with National Committee for Clinical Laboratory Standards guideline was used to determine the minimal inhibitory concentration (MIC). Silver nanoparticles ranging from 5-50 mg/mL and quercetin ranging from 10 - 100 mg/mL were used to determine the MIC. Briefly, bacterial cells were grown to mid exponential phase in in MH medium. Aliquots of the bacterial cells were then seeded in the wells of a 96-well microtitre plate at a density of $1x10^6$. Each of the serially diluted solutions of the test compounds and combinations was then added to the bacterial suspensions.

Combination assays

The MICs of each antimicrobial substance alone or in combination were determined by a broth microdilution method in accordance with CLSI standards using MH broth, modified for a broth micro dilution procedure. For the combination assays, a 96 well titre plate with combination of silver nanoparticles and quercetin was used to test the different combinations as follows. A 96 well titre plate with of silver nanoparticles and quercetin was set up as described above for the combined treatment**.** Growth control wells containing the medium were included in each plat**e.** For the first clear well in each row of the microtitre plate containing all antimicrobial agents, the fractional inhibitory concentration (FIC) was calculated as follows (10);

FIC of drug A = MIC drug A in combination/ MIC drug A alone, and FIC of drug B=MIC drug B in combination/ MIC drug B alone.

The FIC index (FICI), calculated as the sum of each FIC, was interpreted as follows: FICI = 0.5, then this shows synergy quercetin and silver nanoparticles; FICI =1, then it reflects partial synergy; FICI greater than 1, then it has an additive effect; lastly if FIC =4, then it shows antagonism.

III. RESULTS

The biophysical properties of synthesized silver nanoparticles are important for their biological activity and efficacy. Therefore, characterization of AgNPs is important in order to evaluate the biological and functional aspects of the synthesized nanoparticles. Characterization was performed using a variety of analytical techniques, including UV-Vis spectroscopy, X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS).

Particle sizing of silver nanoparticles by Dynamic Light Scattering

The DLS pattern in our study revealed that the size of the synthesized silver nanoparticles showed an average size of 102 nm with a PDI value of 0.28 **(Figure 2).** The reason for slightly larger size of the particle could be due to particle aggregation.

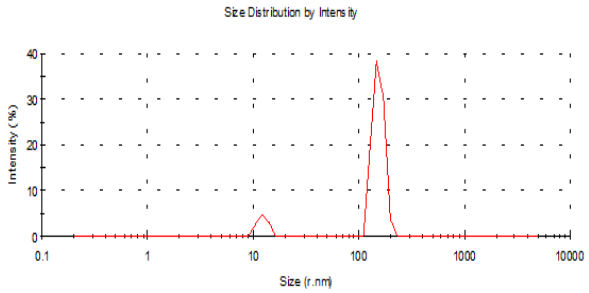


Figure 2. Size distribution analysis of biologically synthesized silver nanoparticles by dynamic light scattering (DLS).

Characterization of silver nanoparticles by UV Spectroscopy

The synthesis of silver nanoparticles was primarily confirmed by color change caused due to surface plasmon resonance of silver nanoparticles in the visible region. The absorbance intensity of the brown color increased steadily as a function of reaction time. The absorption maximum between 400 and 450 nm clearly indicates the formation of silver nanoparticles. The absorption spectra of nanoparticles showed a single broad -band absorption with peak maximum (Surface Plasmon Resonance, SPR) at the wavelength, 430 nm with steadily increased in intensity as a function of time of reaction without any shift in the peak (Figure 3).

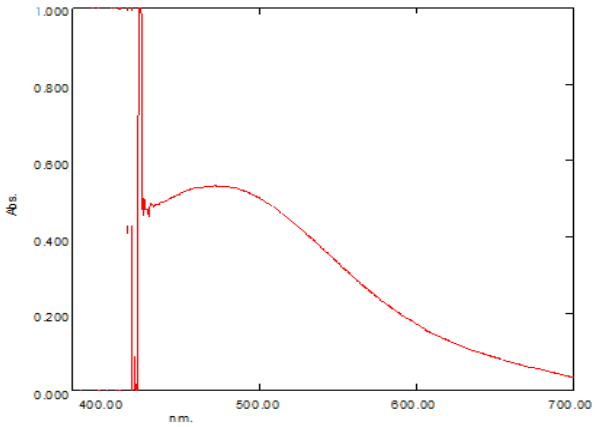


Figure 3. UV-Vis spectra of silver nanoparticle solution.

FTIR analysis

The functional groups of the synthesized silver nanoparticles were studied by using FTIR spectrometer (Perkin-Elmer LS-55- Luminescence spectrometer**)**. The lyophilized silver nanoparticles were characterized in the range of 4000-400 cm^{-1} using KBr pellets. FTIR spectrum showed absorption bands at 3442, 2923, 1636, 1391, 1079, and 530 cm^{-1} indicating the presence of capping agent with the nanoparticles **(Figure 4)**. The band at 3442 cm^{-1} in the FTIR spectra corresponds to O–H stretching vibration indicating the presence of alcohol and phenol. Tiny peak at 2923 cm^{-1} region arising from C–H stretching of aromatic compound were observed. The peak at 1636 cm^{-1} in the spectra corresponds to C–N and C–C stretching indicating the presence of proteins. The amide linkages between amino acid residues in proteins give rise to well-known signatures in the infrared region of the electromagnetic spectrum. The FTIR spectrum reveals two peaks at 1625^{-1} and 1391 cm^{-1} that correspond to the bending vibrations of the amide I and amide II bands of the proteins respectively.

XRD Analysis

The phase variety and grain size, crystalline nature of synthesized silver nanoparticles was determined by X-ray diffraction spectroscopy (Philips PAN analytical). **Figure 5** shows a representative XRD pattern of the synthesized nanoparticles after the reduction of $AgNO_3$. The synthesized silver nanoparticles were studies with CUKα radiation at voltage of 30 kV and current of 20 MA with scan rate of 0.030/s. Different phases present in the synthesized samples were determined by X′ pert high score software with search and match facility. The particle sizes of the prepared samples were determined by using Scherrer′s equation as follows;

$$D \approx 0.9\lambda\beta\cos\theta$$

Where D is the crystal size, λ is the wavelength of X-ray, Θ is the Braggs angle in radians and B is the full width at half maximum of the peak in radians.

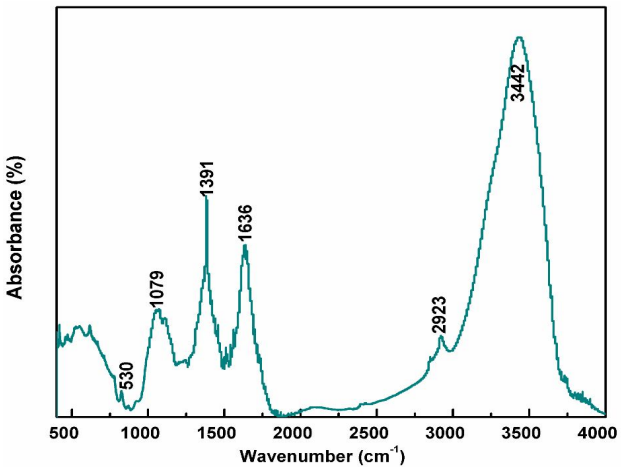


Figure 4. FTIR adsorption spectra of synthesized silver nanoparticles

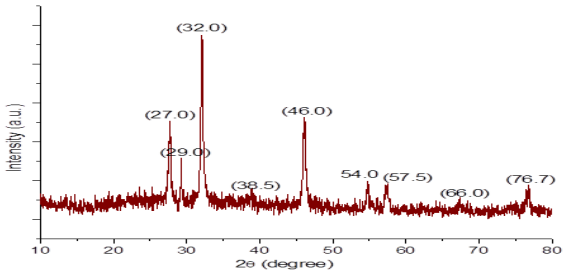


Figure 5. XRD analysis of Silver Nanoparticles

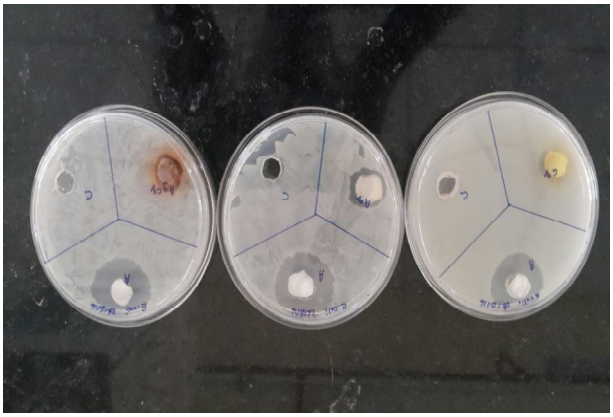
The analysis revealed cubic structure of silver nanoparticles. The high peaks in the analysis indicated the active silver composition and crystalline nature of the silver nanoparticles and from the angle value it is clear that compound is stable. The diffracted intensities were recorded from 2θ angles and values were used to calculate the grain size by using the Scherer's formula. $D= (0.9\lambda)/\beta\cos\theta$;

Where,
D is the average crystallite domain size perpendicular to the reflection planes.
λ- Is the X- ray wavelength
β- Is the full width at half maximum (FWHM) and
ɵ- Is the diffraction angle

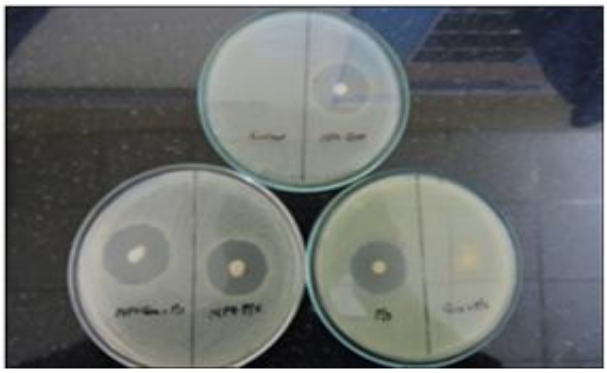
The average of grain size of the silver nanoparticles formed in the bio reduction process was determined using Scherer's formula and was estimated as 32 nm. The lattice constant and lattice constant calculated from this pattern was 4.085 A°.

Synergistic effect of silver nanoparticles and quercetin compared with antibiotics

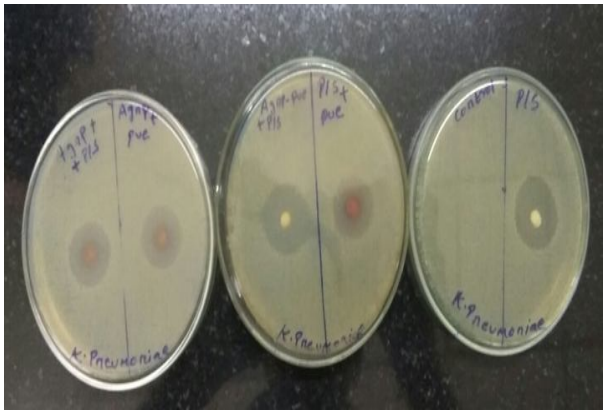
To evaluate antimicrobial effect of silver nanoparticles against *E.coli*, *S.aureus*, and *K. pnuemoniae*, minimum inhibitory concentration was determined. The synergistic effect of each test combinations as mentioned in Table 1. Growth of bacteria was inhibited with synthesized silver nanoparticles, silver nanoparticles-quercetin, quercetin, quercetin-antibiotics silver nanoparticles-antibiotics and silver nanoparticles–quercetin-antibiotics. Appropriate control without any treatment was used in all the chosen bacteria. The zone of inhibition in all the plates were observed and recorded. The combinatorial zone of inhibition of silver nanoparticles-quercetin-antibiotics was observed more in Gram positive *S.aureus (36 mm)* when compared to *E.coli* and *Klebsilla pneumoniae (27 mm and 29 mm).* The ratio based on potency for the three combinations were slightly different, as seen in Table 2.



A)Inhibition zone in E.coli by AgNPs, quercetin, pen/strap and synergism between AgNPs and quercetin.



B) Inhibition zone in *Stap. Aureus* by AgNPs, quercetin, pen/strap and synergism between AgNPs and quercetin.



C) Inhibition zone in *Klebsiella pneumoniae* by AgNPs, quercetin, pen/strap and synergism between AgNPs and quercetin.

Zone of Inhibition (mm)

Figure 6 A.B & C. Zone of inhibition in all the three chosen bacteria

The antimicrobial activity of quercetin and silver nanoparticles synthesized showed positive results in inhibiting

the gram-positive as well as gram-negative bacteria and it is presented in **Table 2**. Silver nanoparticles along with quercetin also increase the antimicrobial activity of conventional antibiotics as shown in **Table 2**. The highest synergistic activity of quercetin, silver nanoparticles along with the antibiotics was observed in *Escherichia coli.* The activity of antibiotics and silver nanoparticles, quercetin along with antibiotics was almost the same in E.coli. *Staphylococcus aureus* showed maximum inhibition with silver nanoparticles and quercetin **(Figure 7).** However, antibiotics in combination with silver nanoparticles and quercetin had enhanced inhibition in S. aureus followed by *Klebsiella pneumoniae.*

Table 2. Inhibitory zone of silver nanoparticles, quercetin and penicillin-streptomycin

Representative microorganism	Silver Nanoparticle	Quercetin	Antibiotics	Silver nanoparticles & antibiotics	Quercetin & antibiotics	Silver nanoparticles + Quercetin	Silver nanoparticles + Quercetin + Antibiotics
Escherichia coli	10±0.2	17±0.4	23±0.4	23±0.1	21±0.3	25±0.2	27±0.1
Staphylococcus aureus	30±0.5	26±0.1	25±0.4	28±0.2	30±0.2	29±0.1	36±0.5
Klebsiella pneumonia	13±0.2	17±0.3	21±0.1	24±0.3	23±0.1	20±0.3	29±0.4

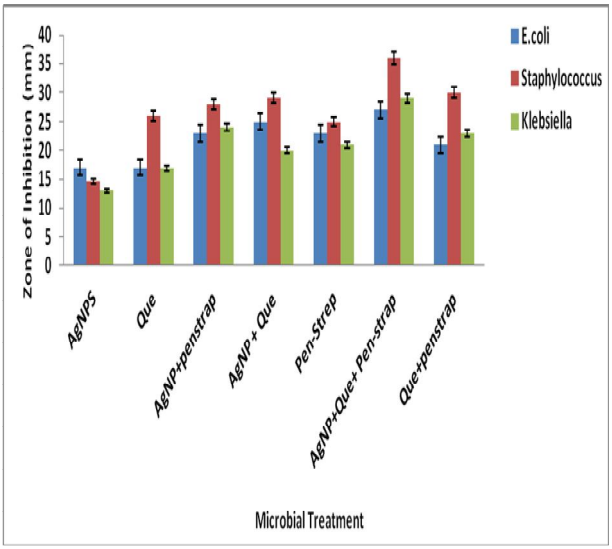


Figure 7. Microbial inhibition in all the three bacteria and synergism between silver nanoparticles, quercetin and antibiotics.

Table 3: MIC of AgNPs and Quercetin against different represented organisms

Representative Organisms	MIC of AgNPs (in mg)	MIC of Quercetin (in mg)
E.coli	20	30
S.aureus	50	30
K.pneumoniae	25	40

Table 4: FIC of AgNPs and Quercetin against different represented organisms.

Representative organisms	FIC of silver nanoparticles	FIC of Quercetin	FIC index
E.coli	0.25	0.66	0.91
S.aureus	0.6	0.5	1.10
K.pneumoniae	0.4	0.75	1.15

IV. DISCUSSION

In this study, we have evaluated the synergy between biologically synthesized silver nanoparticles, abundantly available plant flavonoid- quercetin and compared that with and without conventional antibiotics. Owing to the fact that, over and improper use of antibiotics has led to the rise of resistance in bacteria against certain antibiotics, has formed the premise of this study. Hence, as an alternative approach, the combinatorial effect of antimicrobial agents (quercetin + silver nanoparticles) showed additive effect and also increased the efficacy of conventional antibiotics (11). This effect could be due to interaction of active groups chemical such as, hydroxyl and amide group present in the antibiotic molecules which chelates antibiotic silver nanoparticles interaction Further more studies can be done with broad spectrum of pathogenic bacteria's that can tell us about the synergistic effect of various nanoparticles and other flavonoids. Synergism between drugs usually indicates that they have two different modes of antimicrobial action enhancing the antimicrobial effect, whereas antagonism can indicate two drugs competing for the same target which decreases the overall antimicrobial effect.

Given the relative observation of efficacies of the compounds on activity against *E. coli* and *S. aureus*, our combination test results would also be consistent with the findings from Alvarez (2008) and allow us to also conclude that quercetin may be a useful and effective synergist when used in combination with other antimicrobial agents, including traditional antibiotics.

From these studies, we would like to conclude that quercetin is on the operative agent involved in increasing susceptibility of the representative bacteria in the combination test. As mentioned earlier, the mechanism of action of quercetin is to target the porins located in the membrane of the bacteria. The porins which act as selective transmembrane channels are known to retard the diffusion of larger molecules including traditional antibiotics into the periplasm of the bacterial cell. The channels possess charged amino acids which are situated on the interior of the channel that induce an electrostatic field which blocks the larger molecules. Alvarez *et al* (2008), strongly suggest that quercetin has the ability to neutralize this electrostatic field and therefore allow passage of bulk antimicrobial compounds into the interior volume of

the bacterial cell (12). As a way of reinforcing this supposition, results showing unsatisfactory inhibition on the Gram-positive *S. aureus* were seen.

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