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Short communication

Synthesis, optimization and characterization of silver nanoparticles using the catkin extract of *Piper longum* for bactericidal effect against food-borne pathogens via conventional and mathematical approaches

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ABSTRACT

Inspired with an increasing environmental awareness, we performed an eco-friendly amenable process for the synthesis of silver nanoparticles (AgNPs) using the catkins of *Piper longum* as an alternative approach with the existing methods of using plant extracts. The fabrication of nanoparticles occurred within 10 min. This was initially observed by colour change of the solution. UV-visible spectroscopic studies (UV-Vis) were performed for further confirmation. The analysis elucidated that the surface plasmon resonance (SPR) was specifically corresponding to AgNPs. Fourier transform infrared spectrophotometry (FTIR) studies indicated that polyphenols could possibly be the encapsulating agents. The size and shape of the nanoparticles was analysed using Transmission electron microscopy (TEM). The nanoparticles were predominant spheres ranging between 10 and 42 nm at two different scales. The formation of elemental silver was confirmed further by X-ray photoelectron spectroscopy (XPS) and X-ray powder diffraction (XRD). GC-MS analysis was used to identify the possible encapsulates on the nanoparticles. The antibacterial effect of the biosynthesized AgNPs was tested against two gram-positive (*Bacillus cereus* and *Staphylococcus aureus*), and five gram-negative (*Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhi*) bacteria. Outcomes of the study suggest that these pathogens were susceptible to the AgNPs. This is the first ever international report on correlating the antibacterial effect of silver nanoparticles using mathematical modelling with a conventional antimicrobial assay. The results indicate that nanoparticles of silver synthesized using catkin extract of *P. longum* can be exploited towards the development of potential antibacterial agents.

1. Introduction:

Due to the gaining interest in studies of nano-range of 1–100 nm for biomedical applications, the biosynthesis of AgNPs using valuable medicinal plant extracts has considerably increased [1–3]. However, the synthesis of nanoparticles with the desired quality is one of the most important aspects of modern nanotechnological approaches [4].

There have been a number of studies describing the different

methods for metal nanoparticle synthesis involving reductions performed by chemical, electrochemical, photochemical and other means [5–7]. But it has been reported that biological methods are superior to chemical ones in terms of economic feasibility and environmental safety. Biosynthesis of AgNPs by microorganisms is a recently well thought exploitation [8]. Though it has levelled a considerable success, the use of plant-based materials has gained much attention rather than microorganisms for green synthesis of metal nanoparticles because of

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limited toxicity, less time and the added advantage of available natural capping agents. Moreover, it reduces the cost of isolation of microorganisms and enhancement of culture media for microbe assisted biosynthesis and uses several sources of reductants such as leaves, flowers and catkins [2,9–13].

P. longum (Piperaceae, long pepper) is cultivated almost all over India and is known to possess antimicrobial activities [14,15]. It is an active ingredient of Kabasura Kudineer, a traditional siddha formulation with non-toxic, drug-like properties and enhanced bioavailability. It is used to treat viral fevers and respiratory infections. A recent *in silico* study indicates that the binding efficacy of this formulation is significant with the spike protein of COVID-19 [16].

AgNPs have been reported for their antibacterial, anti-biofilm and antiviral properties at limited concentrations [17–21]. Management of infections in medicine to several other industries is critical and an alternative for chemical agents has become a necessity. Disinfection has become a critical parameter for pandemics such as COVID-19 and colloidal silver can be used in intensive care units of hospitals as novel standard for prophylactic treatment of ventilator acquired pneumonia. Silver nanoparticles alone or in combination with existing antibiotics are suitable and efficient antimicrobial agents [22–24]. Pharmaceutically valuable molecules could be retained after bioreduction by the biosynthesized AgNPs that might have potential application in therapeutics [25]. Silver nanoparticles are used widely in packaging industries to increase the shelf-life of food products pertaining to their antimicrobial effects [26–29].

Therefore, considering the importance of medicinal value of *P. longum*, its catkin extract was used to synthesize AgNPs by the reduction of silver ions and to exploit its antibacterial efficacy. The results indicate that AgNPs could be efficient antibacterial agents.

2. Experimental section:

Catkins of *P. longum* were collected and shade dried at room temperature for a period of 7 days. Silver nitrate (AgNO_3) was purchased from Qualigens Fine Chemicals, Mumbai, India (99.9% pure).

2.1. Preparation of catkin extract

Desired amounts of the finely ground catkin powder (5, 10 and 15 gm) were added to desired volume of menstruum used (100 mL of sterile deionised water) and stirred well. The resulting mixture was then heated at 60 °C for 10 min to produce a galenical. This solution was then allowed to cool and further filtered using Whatman paper No. 1. The hydrosol obtained was used for further experimentation following previous reports with slight modifications [30].

2.2. Synthesis of AgNPs

For the bioreduction process to occur, catkin broth was mixed with AgNO_3 and incubated at varying temperatures and varying concentrations of catkin and AgNO_3 . Initially, 5 mL of 5%, 10% and 15% catkin extracts were added to 95 mL of 10^{-3} M AgNO_3 solution individually and allowed for thermal reduction to occur at temperatures ranging from 30 ° to 95 °C. Later, the concentrations of AgNO_3 (1.5–2.5 mM) were varied, setting 5% catkin extract as the standard. After the reduction of 1.5 mM AgNO_3 by the 5% *P. longum* catkin extract, AgNPs were separated from the solution by centrifuging at 10000 rpm for 20 min. The pellet was re-dispersed in 25 mL of fresh deionised water and centrifuged at 10000 rpm for 20 min as before for three times after being replaced with fresh deionised water every time. The final pellet after centrifugation and resuspension in fresh water was used for further analysis [31]. The control nanoparticles were synthesized by treating glucose as a reductant with silver oxide (1 mM) as the precursor following the same procedure [32].

2.3. Characterization of the synthesized nanoparticles

UV–visible spectroscopic analysis was done by using Hitachi double beam equipment (Model Lambda 35) spectrophotometer. For FTIR measurements, Spectrum RX 1 instrument was used. TEM analysis was performed using Tecnai 10 instrument for characterizing the size and shape of the synthesized AgNPs at 100 and 200 nm scales. XPS analysis was performed with Omicron ESCA Probe spectrometer. GC-MS analysis was performed using Agilent GC 7890A / MS5975C instrument using Agilent DB5MS capillary column. The crystalline nature of AgNPs was studied using Phillips PW 1830 model XRD instrument.

2.4. Antimicrobial studies

Antibacterial activity of nanoparticles were tested against *Bacillus cereus* (MTCC 1272), *Escherichia coli* (MTCC 1687), *Klebsiella pneumoniae* (MTCC 530), *Proteus mirabilis* (MTCC 425), *Pseudomonas aeruginosa* (MTCC 1688), *Salmonella typhi* (MTCC 531) and *Staphylococcus aureus* (MTCC 96) cultures procured from the Microbial Type Culture Collection Centre (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Since disk diffusion is the common standard for bacterial sensitivity test, standard disks of 6 mm were purchased from HIMEDIA (Mumbai, India), impregnated with nanoparticles (50 μl), placed on LB agar medium, seeded with the test pathogens and incubated at 37 °C. The sensitivity of bacterial cultures was measured in triplicate after 24 h [33,34].

3. Results and discussion

3.1. Colour change

Colour change in solutions incubated with precursors and reductants could be considered an initial confirmation for the synthesis of nanomaterials [35]. A pale brown colour was observed after catkin extract and AgNO_3 were mixed and incubated. This is due to the excitation of SPR vibrations after incubation as indicated in Fig. 1A [36–38]. Although the colour change was observed at 30 °C and 60 °C, prominent changes were observed in 90 °C and 95 °C. Therefore, incubations at 90 °C and 95 °C were considered for further study for rapid synthesis.

3.2. UV–Vis analysis

3.2.1. Effect of temperature, reductant and precursor concentrations

The characteristic SPR of silver nanoparticles at 5% (434 nm), 10% (427 nm) and 15% (439 nm) catkin broth concentrations were observed

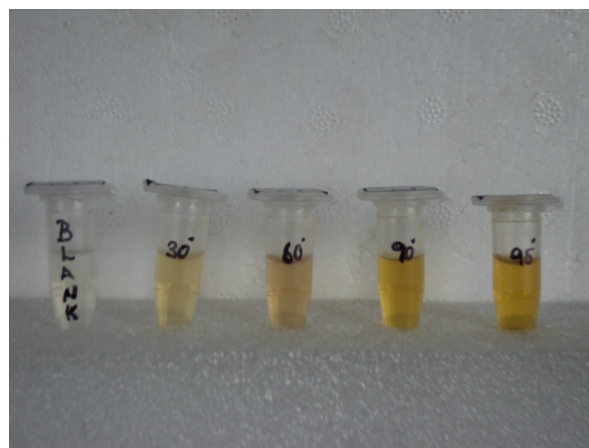


Fig. 1A. Colour change on incubating 1.5 mM AgNO_3 and 5% catkin broth together as a solution at varying temperatures (30 °C, 60 °C, 90 °C, 95 °C).

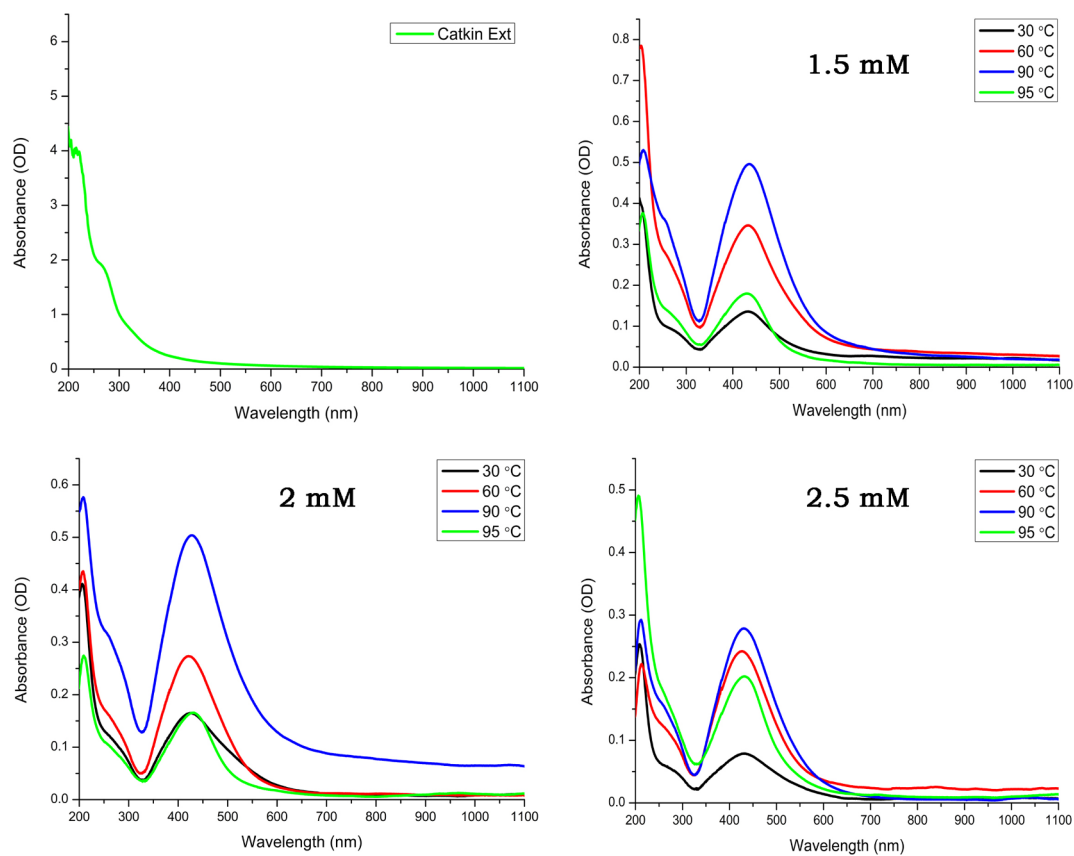


Fig. 1B. UV– visible spectra of the synthesized AgNPs at varying AgNO_3 and catkin broth concentrations incubated at 90°C and pH 7.

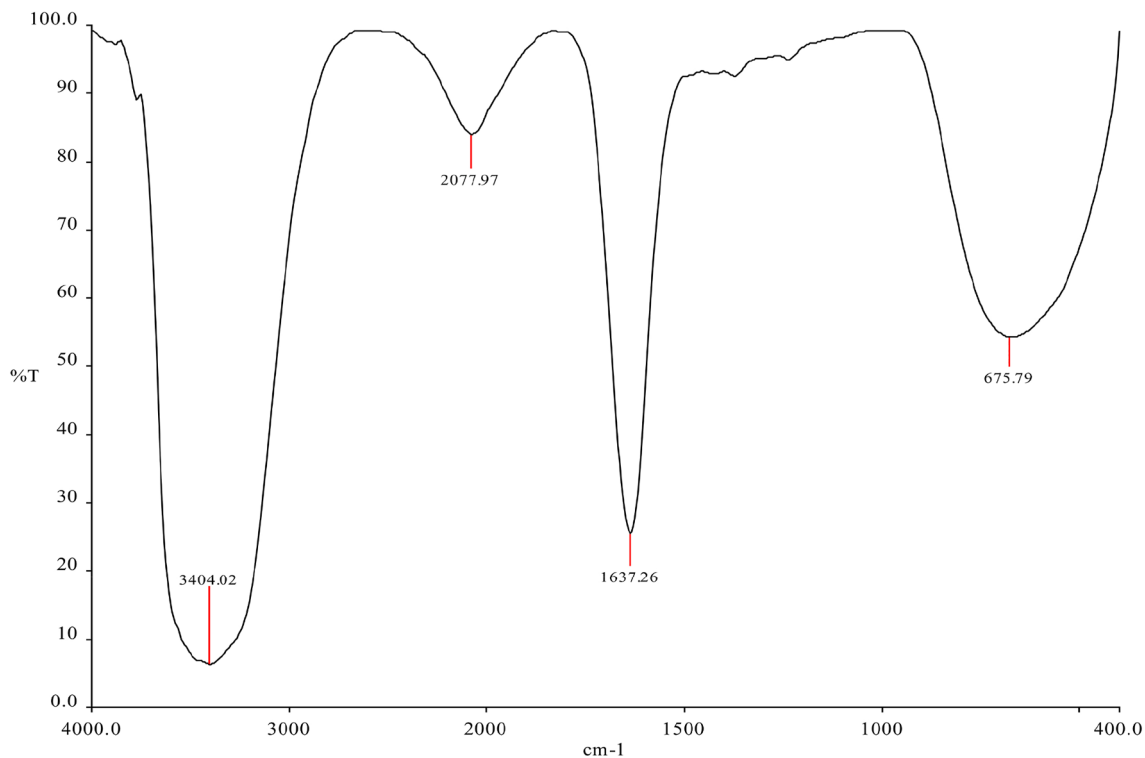


Fig. 2A. FTIR measurements of the catkin extract.

keeping 1.5 mM AgNO_3 concentration as a standard [39]. To involve minimal reactants, 5% catkin extract was used for further analysis. The SPR peaks for solutions tried using 5% catkin broths as standard with

varying concentrations of the precursor AgNO_3 in the range of 1.5–2.5 mM were 435 nm (1.5 mM), 427 nm (2 mM) and 430 nm (2.5 mM). The study also analysed the incubation of the precursor and

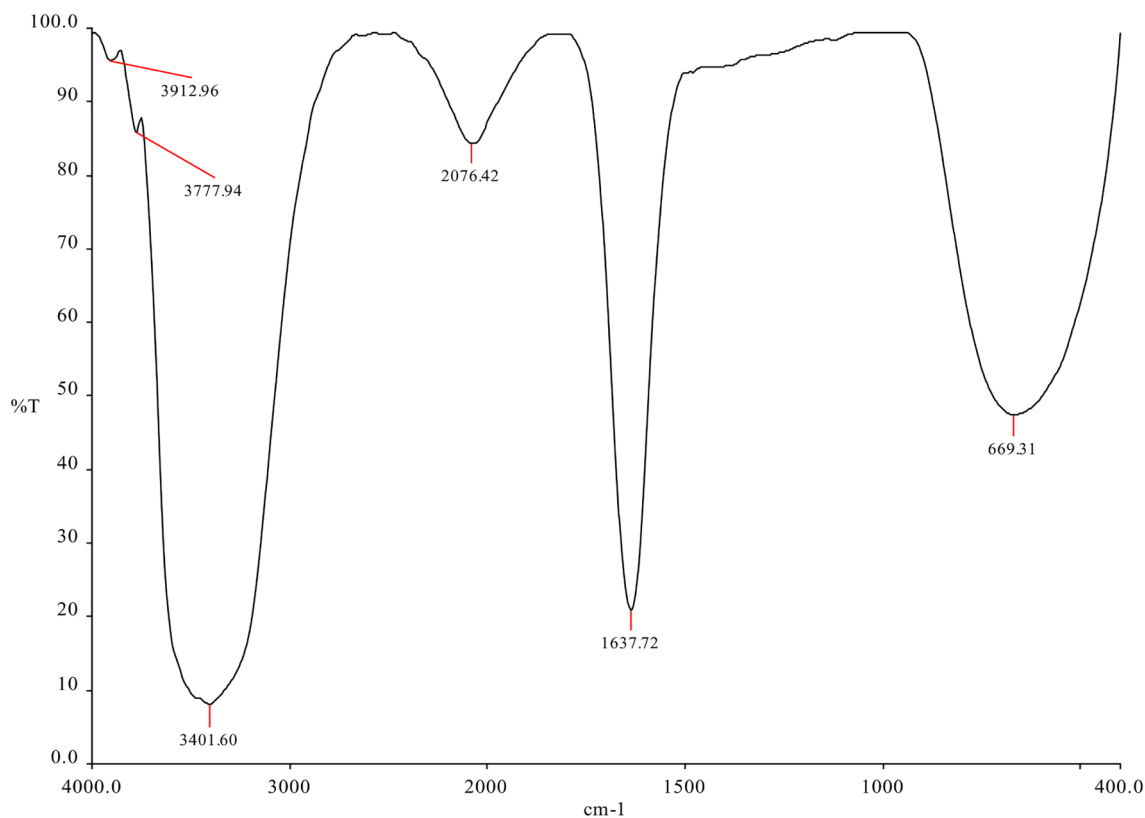


Fig. 2B. FTIR measurements of the AgNPs synthesized using 1.5 mM AgNO₃ and 5% catkin broth incubated at 90 °C and pH 7.

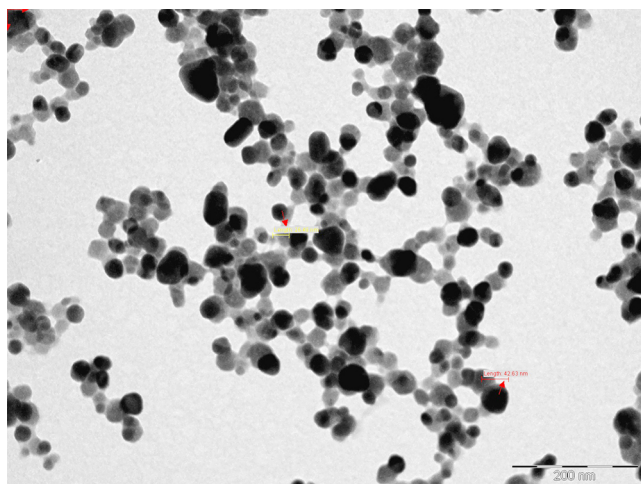


Fig. 3. TEM image of the biosynthesized AgNPs at 200 nm scale.

reductant at temperatures ranging between 30 °C, 60 °C, 90 °C and 95 °C. The peaks were consistent in the range between 420 nm and 440 nm (Fig. 1B). According to previously published reports, the nanoparticles in the range of 410–450 nm have been known to be spherical [40,41]. The particles with the SPR range of around 450 nm have been known to be in the size range of 2 to 100 nm [42,43].

3.2.2. Optimization of synthesis and characterization by UV-Vis analysis

Though the synthesis was observed at other catkin extract and AgNO₃ concentrations as predicted by all UV-Vis observations in this study, they were not characterized further because, the above-mentioned optimized concentration and pH provides an eco-friendly approach to the maximum, with minimal reactants (Fig. 1B). Furthermore, it is of interest to note that the reduction of silver ions completed

within 10 min of incubation, indicating the rapid biosynthesis of AgNPs. Therefore, to use limited reactant conditions such as temperature and also involve rapid synthesis, the hydrosol prepared by incubating 1.5 mM AgNO₃ and 5% catkin extract at 90 °C was considered to be optimized and used for further studies rather than 95 °C.

Since the particle sizes were miniature and the usage of harmful reactants were avoided, 5% catkin extract and 1.5 mM AgNO₃ concentration were preferred at pH 7 and 90 °C for further characterization and antimicrobial studies. Regarding stability, the synthesized nanoparticles were kept for three months at 4 °C in a refrigerator for stability analysis. The AgNPs were stable for more than three months. This was proven as the UV-vis peak remained the same reflecting the SPR for AgNPs when tested even after the period of three months. The antibacterial effect also remained the same after the described period.

3.3. FTIR analysis

FTIR analysis performed to identify the functional groups for determination of capping agents indicates that sharp absorption peaks were located at 3404 cm⁻¹, 2077 cm⁻¹, 1637 cm⁻¹ and 675 cm⁻¹ in the catkin extract (Fig. 2A). Similar peaks were retained at 3401 cm⁻¹, 2076 cm⁻¹, 1637 cm⁻¹ and 669 cm⁻¹ were observed in the hydrosol containing nanoparticles obtained at 90 °C (Fig. 2B). Similar peaks were observed for hydrosols at 60 °C and 95 °C (Supplementary Information 1). The new peaks observed for samples at 90 °C around 3912 cm⁻¹ and 3777 cm⁻¹ could be due to overtones and combination bands in the mid IR region and the moisture content in the sample [44]. The peaks at 3401 and 3404 cm⁻¹ in both solutions indicates free O–H stretches related to alcohols and phenols [45]. The absorption peaks at 1637 cm⁻¹, 667 and 669 cm⁻¹ are assigned close to that of native proteins [46]. The peaks around 2076 and 2077 cm⁻¹ could be attributed to alkynes [47]. These results indicate that the polyphenols and proteins in the catkin extract have acted as reducing, capping and stabilizing agents, for the nanoparticles, as predicted by the FTIR peaks.

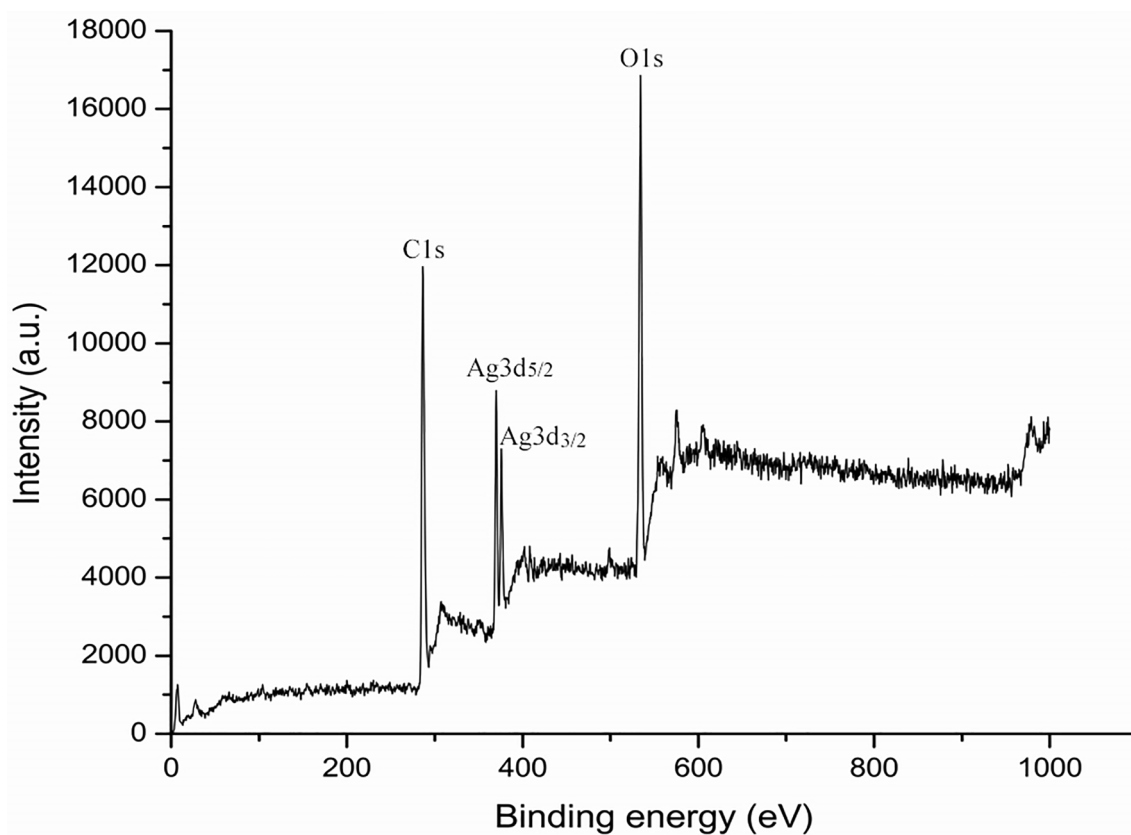


Fig. 4. XPS survey spectra of the synthesized AgNPs.

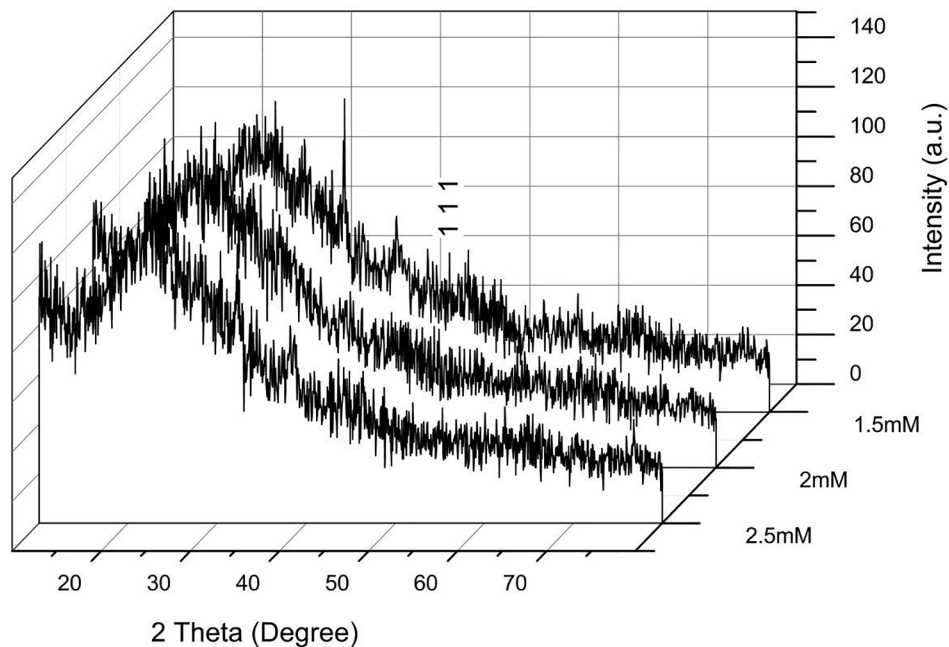


Fig. 5. XRD pattern of the synthesized nanoparticles at varying concentrations of silver nitrate (1.5, 2, 2.5 mM) and 5% catkin broth concentration.

They might also have acted as agents for elevated antimicrobial activity as compared to the nanoparticles synthesized by chemical means [48–53].

3.4. TEM analysis

TEM is a widely used microscopic technique for the characterization

of nanomaterials based on their size and morphology [54]. It was inferred from TEM analysis that the catkin extract derived nanoparticles were predominantly spherical in shape with varying sizes ranging from 26 to 42 nm at the 200 nm scale (Fig. 3). Previous reports using electron microscopy indicate that the sizes of biosynthesized silver nanoparticles range between 20 and 50 nm [55–58].

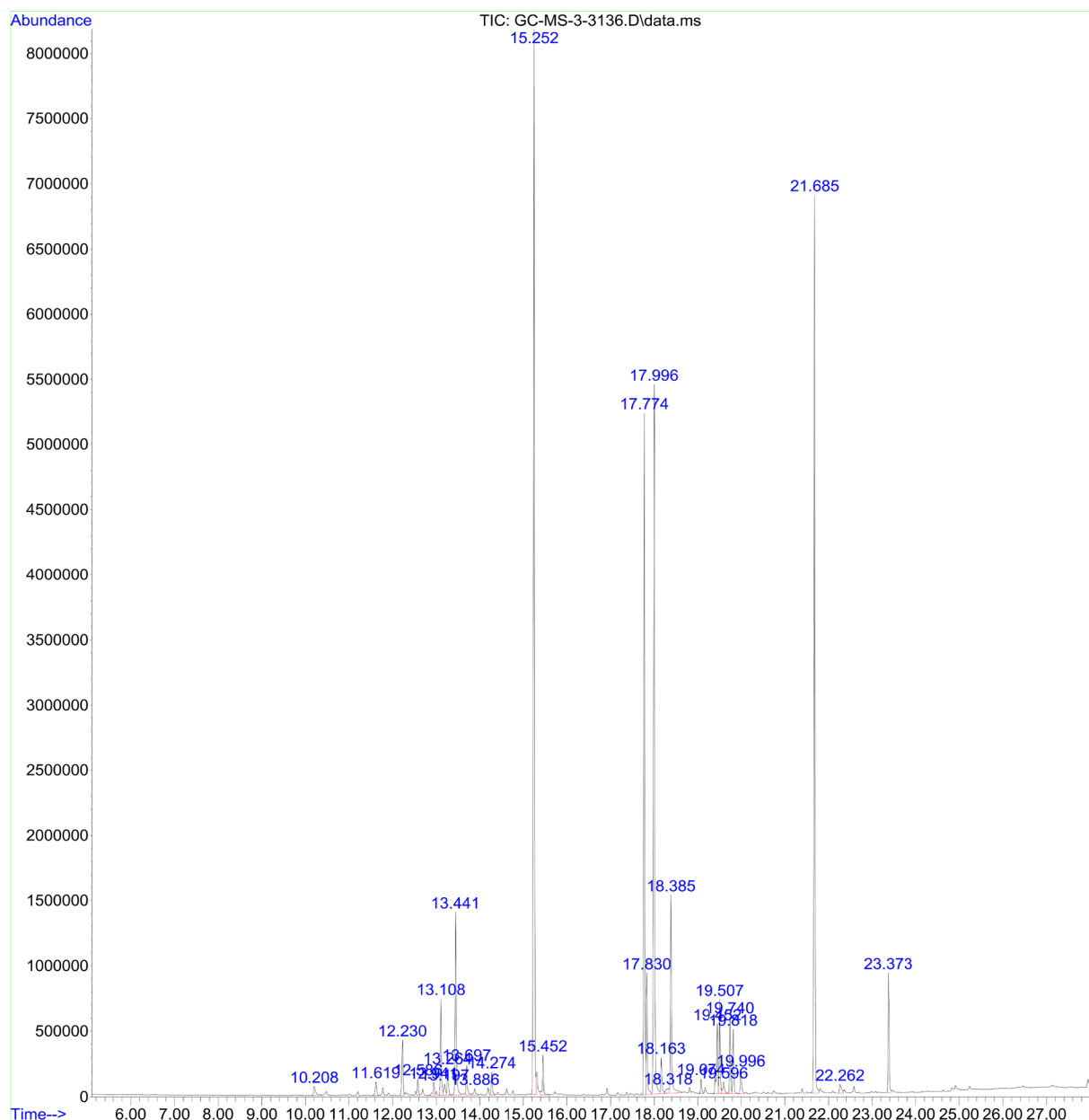


Fig. 6A. GC-MS chromatogram of the catkin extract.

3.5. XPS spectra

Scan survey denoted strong peaks of C1s, O1s and Ag3d core levels. The major C1s peak occurred at 286.2 eV. This corresponds to α -carbon. The O1s value was centred at 529.8 eV [59]. Correspondingly, the two spin-orbit components Ag3d_{5/2} and Ag3d_{3/2} were observed at the binding energies of 368.22 and 374.28 eV, separated by 6 eV [60] (Fig. 4). The core values for metallic Ag lies in this range as established by previous studies [61–63].

3.6. XRD

Comparing the hydrosols containing nanoparticles prepared at different concentrations, 1.5 mM clearly represents the main peak at 38.34 which corresponds to plane 111 (JCPDS file no: 89-3722). From the determination of width and using Debye-Scherrer's equation, the average particle size measured was 7 nm [64]. Additionally, a variety of peaks were produced due to the hindrance of various bioorganic agents

or bioactive compounds possibly from the catkin extract present on the surface of AgNPs [56,65]. Therefore, the XRD pattern of AgNPs synthesized using *P. niruri* reveals that the particles are crystalline in nature (Fig. 5).

3.7. GC-MS analysis

The comparative analysis of peak numbers indicates that both the catkin extract and hydrosol with nanoparticles constituted several similar active components (Figs. 6A, 6B). The structures of these compounds are presented in Fig. 6C. Although, several peaks were lost in this comparison, no novel peaks with established biological activity were observed in nanoparticle containing hydrosol. Among these constituents, benzohydrazide is a lead compound with significant therapeutic effects on cancer, killing of microbial pathogens and treatment of neurological disorders such as epilepsy [66]. Cinnamate derivatives are organic aromatic melatonin scavenger with potent antibacterial agents [67–70]. Fatty acids such as hexadecanoic acid are effective

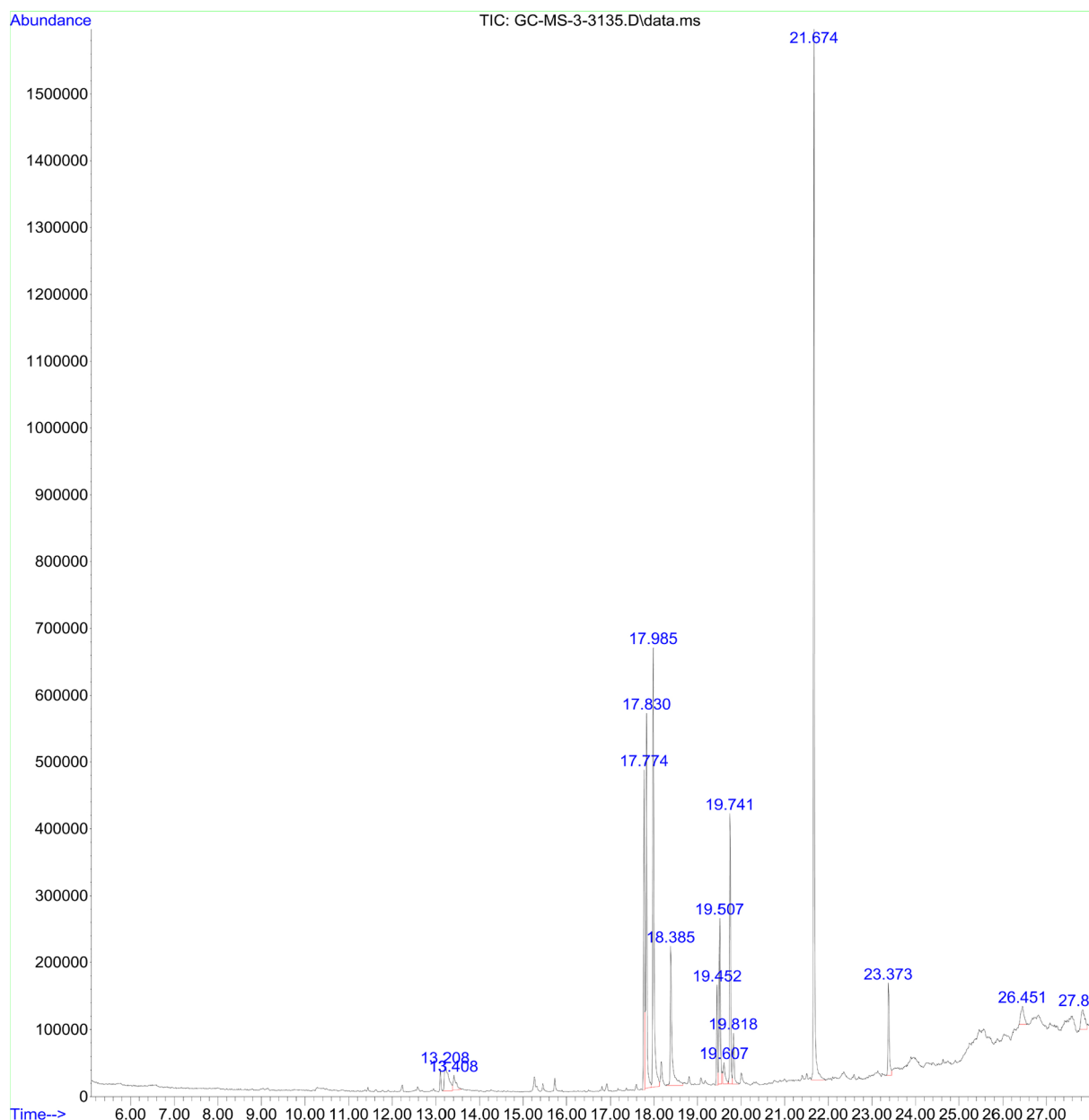


Fig. 6B. GC-MS chromatogram of the silver nanoparticles containing hydrosol.

against inflammatory disorders and microbial pathogens [71–73]. Extracts that constitute octadecadienoic acid, butenoic acid, propenoic acid and silane derivatives are known to possess antibacterial activities [74–78]. This analysis indicates that these constituents observed in the catkin extract has most probably reduced the precursor and likewise encapsulated the nanoparticles leading to enhanced antimicrobial activity compared to the chemically synthesized nanoparticles.

The mechanism of green synthesis of nanoparticles are attributed to the secondary metabolites, enzymes such as aspartic proteases, phytochelatin and metallothioneins which are considered to be metal-binding ligands presented in the plant extracts. Derivatives of phenols such as tannins are identified to be more efficient in reduction of silver salts into silver nanoparticles. Concentration of reducing agents and the silver salt used, pH, temperature and duration of incubation are critical parameters for the biosynthesis to occur [79–84]. Therefore, the phytochemicals identified by GC-MS, in this study, could be possible reductants and/or encapsulates on the nanoparticles from the catkin extract.

3.8. Conventional disk diffusion assay

Bacillus cereus, *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhi* are reported to be food borne pathogens [85–87]. Biosynthesized nanoparticles with its large surface area were tested for their antibacterial activity against both gram-negative and gram-positive microorganisms. The diameter of the inhibition zone (mm) around the disks impregnated with AgNPs against test strains was measured after the incubation period.

Comparative analysis of the overall zone of inhibition indicates a percentage increase of 16.4% between the test nanoparticles and control nanoparticles. *P. mirabilis* was the most and *S. aureus* the least susceptible to the test nanoparticles. The control nanoparticles showed increased activity against *K. pneumoniae* and minimal activity against *P. aeruginosa* (Fig. 7). Existing data for sensitivity to vancomycin, the most effective antibiotic against multiple drug resistant microbes, was used to compare with test nanoparticles [33]. The sensitivity of these

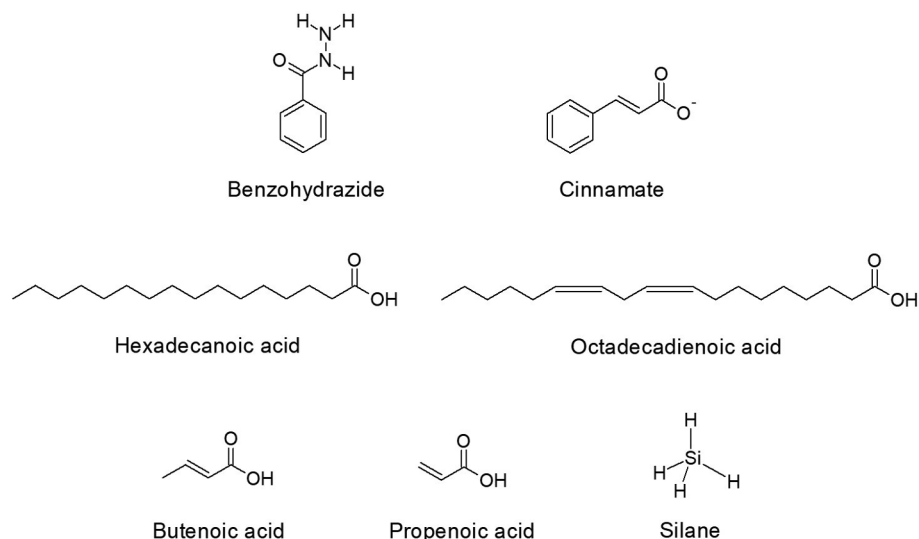


Fig. 6C. Structures of compounds identified in common between catkin extract and silver nanoparticles containing hydrosol using GC-MS.

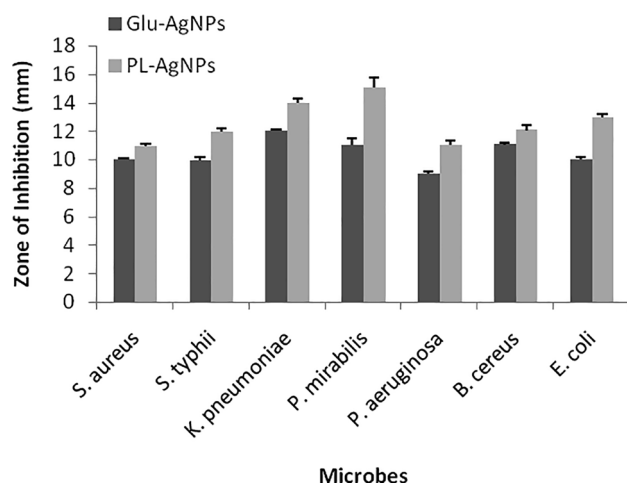


Fig. 7. Mean zone of inhibition (mm) of seven different pathogens generated by AgNPs synthesized using glucose and catkin extract of *Piper longum* (5%). The disc diameter was 6 mm. All experiments were performed in triplicate. The results were expressed as mean \pm SEM.

microorganisms to vancomycin (30 μ g) does range between 7 and 25 mm, according to previous studies. In our study, the nanoparticle solution loaded at 50 μ l per disk were one half equivalent to activity of vancomycin according to existing reports [88–91]. The zone of inhibition is significantly similar to the previously published reports [92,93].

The elevated antibacterial effect of nanoparticles against gram-negative bacteria in comparison to gram-positive bacteria could be

Table 1

Sensitivities of food-borne pathogens to silver nanoparticles. Mean zone of inhibition (mm) of AgNPs synthesized using catkin extract of *P. longum* (5%) against seven different pathogens. The disc diameter was 6 mm. The average of percentage increase in antibacterial activity among the 7 tested strains was 16.4%. The table also represents the percentage of increase in activity and the coefficient of mean deviation of the nanoparticles. The coefficient of mean deviation was calculated by dividing the mean deviation using the average.

Organism	PL-AgNPs	Coefficient of Mean Deviation	Glu-AgNPs	Coefficient of Mean Deviation	% of increase in activity
<i>S. aureus</i>	10.97 \pm 0.17	0.0101	10.03 \pm 0.08	0.01107	8.56
<i>S. typhii</i>	11.97 \pm 0.26	0.00928	9.97 \pm 0.18	0.01114	16.70
<i>K. pneumoniae</i>	13.97 \pm 0.35	0.00795	12.03 \pm 0.09	0.009233	13.89
<i>P. mirabilis</i>	15.07 \pm 0.70	0.0147	11.03 \pm 0.43	0.01611	26.80
<i>P. aeruginosa</i>	11.03 \pm 0.29	0.0161	9.03 \pm 0.15	0.01968	18.13
<i>B. cereus</i>	12.07 \pm 0.35	0.0184	11.07 \pm 0.12	0.01405	8.29
<i>E. coli</i>	12.93 \pm 0.24	0.01202	10.03 \pm 0.15	0.01771	22.43

attributed to the thickness of gram-positive bacterial cell wall (30 nm). The cell wall thickness of gram-negative bacteria lies in between 3 and 4 nm [94]. Another rationale behind enhancement of antibacterial effect of biologically synthesized AgNPs could be the particle size. AgNPs of size less than 50 nm are considered effective antibacterial agents as elucidated in this study [95,96].

Although the chemically synthesized nanoparticles indicated considerable zones of inhibition, the biologically synthesized nanoparticles inhibited the microbial growth better at the same volume of the hydrosol. Alkaloids, phenols, flavonoids and tannins are major constituents of piper fruits and could be probably responsible for the antibacterial efficacy of the same [97]. The possible encapsulation of phytochemicals of the catkins on AgNPs might be responsible for the enhanced antimicrobial activity compared to glucose derived AgNPs [98].

This antibacterial effect elucidates that larger surface area of the nanoparticles acts better on the bacterial cells and this is by membrane and its charge related aspects [99,100]. Finding its use in thousands of products for human appliances, silver nanoparticles penetrate bacterial cell wall, interact with their genetic material and impair cell division. This causes changes in biological processes such as respiration, thereby leading to cell death. According to the inhibition zones observed in this study, these nanoparticles could be considered effective bacteria-inhibitory agents. Therefore, the mechanism of antibacterial effect of AgNPs primarily relies on the release and electrostatic attraction of silver cations to the negative charge on the bacterial cell wall surface and permeate through it based on the thickness of peptidoglycan layer. This can result in interactions of these nanoparticles with sizes less than 100 nm with DNA and protein leading to denaturation which can

eventually lead to cell death by the production of reactive oxygen species [101–110].

3.9. Mathematical modelling:

The Karl Pearson's Co-efficient of Correlation, which usually exists between +1 and -1, among the tested nanoparticles was 0.7064. The calculated Pearsonian R value was indicative of a positive correlation (0 to +1) between the activity of biological and chemically synthesized nanoparticles [111]. *P. mirabilis* was the most deviated for both chemical and biosynthesized nanoparticles as calculated by the percentage of increase in activity between the both test agents and therefore, identified to be the most susceptible organism. Co-efficient of mean deviation determines that catkin derived AgNPs was most effective against *B. cereus*, whereas, chemically derived nanoparticles were most effective against *P. aeruginosa* in comparison to the other microbes (Table 1). Therefore, the mathematical modelling supports the interpretation that the biosynthesized nanoparticles were more efficient in comparative antimicrobial effect with glucose derived nanoparticles.

4. Conclusion

Food products become unsuitable for consumption after spoilage by microorganisms. Silver nanoparticles are used widely in food-packaging industries to prevent food-spoilage. In the present study, the catkin extract of *P. longum* was used for the optimized synthesis of AgNPs. The synthesized nanoparticles were characterized using UV, FTIR, TEM, XPS, GC-MS and XRD. Since, this study reports the antibacterial activity of the biosynthesized AgNPs against seven different food-borne gram-negative and gram-positive microorganisms, this study seems significant for the antibacterial testing of nanoparticles intended for antimicrobial applications. All the tested microorganisms were susceptible to the synthesized nanoparticles even after three months of synthesis and storage. The catkin-derived nanoparticles are economical and have an additional benefit of the natural capping agents compared to the nanoparticles synthesized using glucose. Hence, this rapid, eco-friendly and cost-effective method for the synthesis of AgNPs could be exploited for the development of beneficial medical appliances in food-packaging industries as evidenced by conventional disk diffusion and mathematical approaches.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2020.104230>.

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