Advances In Multidisciplinary & Scientific Research
A Multidisciplinary & Interdisciplinary Journal
Volume 6, 2020 Edition

Antimicrobial Activities of Extracellularly Synthesized Silver Nanoparticles from *Aspergillus Flavus* and *Alternaria Alternata*

Kaka M. O.[1], , Ajayeoba T.A.[1], Oyebamiji A.K.[2], Adeosun I.J.[1], Olotu T.M.[1], Ekwonwa E.C.[3], Ogenma U.T.[3],Gbadeyan Adebayo M.[4], Owolabi S.O.[5] & *Oyawoye O.M[1]

[1]Department of Microbiology, Lab. of Mol. Biology, Bioinformatics and Immunology, Adeleke University, P.M.B. 250, Ede, Osun State, Nigeria
[2]Department of Basic Sciences, Adeleke University, P.M.B. 250, Ede, Osun State, Nigeria
[3]Department of Public Health, Adeleke University, Ede.
[4]Microbiology and Parastology Dept, Seventh-Day Adventist Hospital School of Nursing, Ile-Ife, Nigeria
[5]Department of Civil Engineering, Adeleke University, Ede.
***Corresponding Author E-mail**: oyawoyeom@yahoo.com
Phone: +2348034373651

ABSTRACT

This study was carried out to evaluate the extracellular biosynthesis of Silver Nanoparticles using *Aspergillus flavus* and *Alternaria alternata* isolated from fruit waste (Orange) samples using the vegetative and cell-free filtrate method. The antimicrobial activity was performed against *Escherichia coli* and *Streptocous fecalis*. Characterization of the silver nanoparticles was achieved using Color change, UV-Visible Spectrophotometry and Fourier Transform Infrared Spectroscopy (FTIR). The Nanoparticles showed UV-Visible absorbance peaks that correspond to the Plasmon resonance of silver nanoparticles. The FTIR spectra showed the presence of aromatic and aliphatic amines, confirming the presence of proteins as the stabilizing agent surrounding the silver nanoparticles. The silver nanoparticles showed higher antimicrobial activity against *Escherichia coli.* Silver nanoparticles (1mM) from *Aspergillus flavus* were not significantly different ($p<0.05$) from Nitrofurantoin. The use of these fungi for silver nanoparticles synthesis offers the benefits of eco-friendliness and amenability for large-scale production and shows that biosynthesized silver nanoparticles can be effective as an alternative therapy in solving antimicrobial resistance problems.

Keywords: *Aspergillus flavus; Alternaria alternata;* Silver nanoparticles; Antimicrobial Resistance; Green Synthesis.

Aims Research Journal Reference Format:
Kaka M. O. , Ajayeoba T.A, Oyebamiji A.K., Adeosun I.J., Olotu T.M., Ekwonwa E.C., Ogenma U.T.,Gbadeyan Adebayo M., Owolabi S.O. & Oyawoye O.M (2020): Antimicrobial Activities of Extracellularly Synthesized Silver Nanoparticles from Aspergillus Flavus and Alternaria Alternata. Advances in Multidisciplinary Research Journal. Vol. 6. No. 1, Pp 59–72.
Article DOI: dx.doi.org/10.22624/AIMS/V6N1P2

1. INTRODUCTION

There is an urgent need to develop new bactericides because of the emergence and increase of microbial organisms which are resistant to multiple antibiotics and have become an increasing public health problem [1]. Silver has been used for years in the medical field for antimicrobial applications, however, Nanosilver, being less reactive than silver ions, is expected to be more suitable for medical applications [2].

Humans have learned to harness fungi for the protection of human health in antibiotics, anti-cholesterol statins, and immunosuppressive agents [3], while industry has utilized fungi for large scale production of enzymes, acids, and bio surfactants [4]. With the arrival of contemporary applied science, fungi have remained important by providing a greener alternative to chemically synthesized nanoparticle [5]. Fungi have a number of advantages for nanoparticles synthesis in comparison with other organisms, as they are relatively easy to isolate and culture and they secrete large amounts of enzymes, particularly extracellular ones[4]. Nanoparticles biosynthesis occurs once the microorganisms grab target ions from their environment and switch the metal ions into the component metal through enzymes generated by the cell activities [6].

Antibacterial assays of photosynthesized Silver Nanoparticles have been assessed against human pathogenic Gram-positive and Gram-negative bacteria including *Staphylococcus epidermidis* and *Salmonella typhimurium*[7]. The antibacterial activity has also been evaluated using *Sphaerulina albispiculata* in synthesis of Silver nanoparticles [8]. Nanoparticles biosynthesis have been reported and established using the vegetative and/or the cell-free filtrate methods [2]. However, whether using myco-synthesized nanoparticles and their antibacterial activities both as an option to confront the transmission of and infection by human pathogenic bacteria and most importantly as a means of biological waste management remain to be established [5].

Thus this work is aimed at exploring the potentials of the metabolites of two fungal organisms (*Aspergillus flavus* and *Alternaria alternata*) isolated form fruit wastes from South-West Nigeria in the green synthesis of Silver Nanoparticles and evaluating the antimicrobial susceptibility of two major bacteria of clinical importance (*Escherichia coli* and *Streptococcus fecalis*) to the synthesized particles. To explore the synthesizing activities and eco-usefulness of a microorganism gotten from a fruit waste, two major fungal species were isolated and identified from Orange fruit waste gotten from Ede, Osun State and products of their metabolites were challenged with different concentration of silver nitrate for the reduction to silver nanoparticles, which were then further assayed for antibacterial activities against clinical pathogens of *Escherichia coli* and *Streptococcus fecalis*. This was done after characterization of established silver nanoparticles.

2. MATERIALS AND METHODS

2.1 Isolation of Fungal Cultures

Silver nitrate was obtained from Sigma Aldrich and used without further purification. All other reagents were of analytical grade with maximum purity and were all properly washed with distilled water and oven dried before use. Potato Dextrose agar was purchased from Oxoid and prepared according to manufacturer's instructions. Spoilt Orange samples were gotten aseptically from Ede, Osun state market; serial dilution and pour plate methods were carried out in the Microbiology Laboratory according to the method described by [5]. A volume of 1ml each of the dilution were transferred to Potato Dextrose Agar, incubated for five (5) days, a pure culture gotten after sub-culture. Macroscopic identification was done based on colonial morphology, color, texture and shape, while microscopic identification was done using Lactophenol blue as a staining agent according to the method described by [6]

2.2 Silver Nanoparticle Biosynthesis

2.2.1 Vegetative Method

According to the method described by [6] each fungal sample was grown in 200ml bottles each containing 100ml of potato dextrose broth and at 25-28^{o}C under continuous mixing condition by a magnetic stirrer(rotary shaker) at 120rpm for 72 hours.

The mycelial (vegetative part of the fungus) mass was then separated from the culture broth by sterile filter paper, and the settled mycelia were washed thrice with sterile distilled water. 10g of the harvested mycelial mass was mixed with a 100ml aqueous solution of 1mM silver nitrate solution ($AgNO_3$). Then the mixture was placed in a 100rpm rotating shaker at 28^{o}C for 72hours duration. In this process, silver nanoparticles were produced through reduction of the silver ions to metallic silver (Ag^{+} to Ag^{0}). Change in color of the fungal biomass incubated with silver nitrate solution was visually observed over a period of time.

2.2.2 Cell-free Filtrate Method

Fungal isolates were grown in Potato Dextrose Broth liquid medium. The flasks were inoculated with spores and incubated at 28^{o}C in static conditions for 72 hours. The biomass was harvested by filtration using Whatman filter paper No. 1 and washed with distilled water to remove any components of the medium. Biomass of 25g was placed in individual flasks containing 100ml Milli-Q water then the flasks were incubated under the conditions described above for 24 hours. The biomass was filtered, and the crude cell filtrate was collected and treated with 1mM silver nitrate solution at room temperature in the dark. Control containing cell-free filtrate without silver nitrate solution. Change in color was observed over a period of time.

2.3 Characterization of the Biosynthesized Silver Nanoparticle

Color Change

The color change in the reaction mixture was recorded through visual observation. The color change from yellow to dark brown indicated that the silver nanoparticles were synthesized.

UV-Visible Spectrophotometry

UV-Visible measurements Spectral analysis for the development of nanoparticles were observed using UV-Visible Spectrophotometer from (200-800nm) in 2ml quartz cuvette with 1cm path length at a resolution of 1nm at room temperature. Silver nanoparticles formed gave a sharp Plasmon band in the range of visible region of the electromagnetic spectrum.

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was carried out to identify the possible interactions between silver and bioactive molecules. A known weight of sample (1mg) was ground with 2.5g of dry potassium bromide and filled in a 2mm internal diameter micro-cup and loaded unto FTIR set at 26^{o}C. The samples were scanned using infrared in the range of 4000-500 cm-1 using Fourier Transform Infrared Spectrometer (SCHIMADZU) Model-6700). The spectral data were compared with the reference chart to identify the functional groups present in the sample.

2.4 Antibiotic Susceptibility Testing

The bacteria *Escherichia coli* and *Streptococus fecalis* were obtained from Adeleke University Medical Microbiology and Bacteriology Laboratory and inoculated into nutrient broth. Mueller Hinton Agar (MHA) was poured into sterile petri plates which werse left overnight at 37^{o}C and subsequently bacterial lawns were prepared using each bacterial strain. Agar wells were made on MHA plates using 6mm Cork borer, the plates were then loaded with synthesized silver nanoparticles and incubated at 37^{o}C. Commercial antibiotic discs were used as control. After incubation the plates were examined for zone of inhibition, and these methods were carried out according to the method standardized by the Clinical and Laboratory Standards Institute [9]. The sensitivity of the isolates to Silver Nanoparticles 1mM and 5mM were classified as 'resistant' and 'susceptible' according to their zones of inhibition.

Statistical Analysis

The antibacterial activities of silver nanoparticles (1mM and 5mM) against commercial antibiotics [Ciprofloxacin (5µg), Gentamycin (10 µg), Nitrofurantoin (300 µg)] were compared statistically with Statistical Analysis Software 92.2 (SAS) using Analysis of Variance (ANOVA). Statistical significance was defined by a *p* value less than 0.05.

3. RESULTS AND DISCUSSION

Biosynthesis of Silver Nanoparticles

A total of sixteen fungal isolates were detected on Potato Dextrose Agar (PDA). The purified fungal isolates were then grouped into four major genera, as shown in Table 1.0. Cell-free filtrate and silver nitrate solution mixture changed from almost colorless to light yellow after 24 hours and later to dark brown after 72 hours. The control sample, however, remained colorless. This is shown in Plate 1.0. This difference in the color intensity in the vegetative-agitated fungal biomass as compared to the cell-fee filtrate biomass could be due to the production of more metabolites by fungi through agitation [10], thus implying that agitation could be better than static conditions and vegetative rather than cell-free filtrate method.

UV-Vis spectra for cell-free filtrates of *Aspergillus flavus* silver nitrate reaction mixtures (1mM,2mM, 3mM,5mM) alongside reaction mixtures containing Vegetative, agitated *Alternaria alternata* and *A. flavus*+ 1mM silver nitrate solution mixture after two weeks were recorded at 400-450nm after 72 hours as shown in Figure 1.0. The UV-Vis spectra for *A.flavus* (1mM and 2mM) showed absorbance peaks at 425nm, and 3mM at 429 nm, this is indicative of the wavelength at which Plasmon Resonance (excitation of conduction electrons) occurs in the silver nanoparticles[11]. UV-Vis spectra for 5mM however did not reveal a significant absorbance peak. UV-Vis spectra for *A.flavus* (1mM) after two weeks remained at 425nm indicating stabilization of the biosynthesized silver nanoparticles[11].

The UV-Vis spectra for the vegetatively synthesized silver nanoparticles from *A. alternata* also showed an absorbance peak at 440nm. The UV-Visible spectroscopy results suggest that the absorption band at a 420-440nm range is indicative of plasmon interactions under certain conditions, e.g light or dark. This is referred to as Surface Plasmon Resonance [2].

In addition, 5mM reaction mixture had lower absorption peak than the rest concentrations, implying that higher concentrations of silver ions in interaction with fungal cells could lead to aggregation or agglomeration of the reaction solution and thus, instability of the silver nanoparticles formed. This correlates with the results reported by [12].

Table 1.0: Morphological characteristics of fungal species isolated form spoilt Oranges.

Name	Colony/Color on PDA	Reverse	Conidia Head	Conidia Shape	Seriation
Aspergillus flavus	Yellowish green, flat and granular	Yellow	Radial	Globulose	Biseriate
Alternaria alternata	Grayish black, floccus	Brown	Branched, acropetal, elongated	Short, ovoid	Multi-celled
Penicillum notatum	Dense green,fluffy	Dark brown	Single cell	Flask-shaped	Chin philiades
Fusarium oxysporum	Creamy chalky	White	Fusiform, slightly curved with pointed tip	Short, single-celled	Non-septate, not in chain

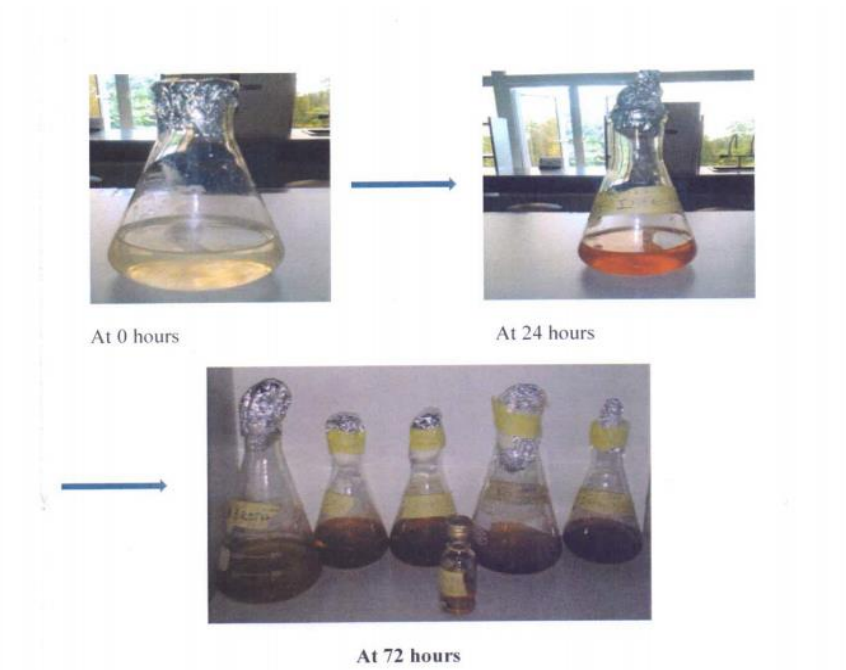
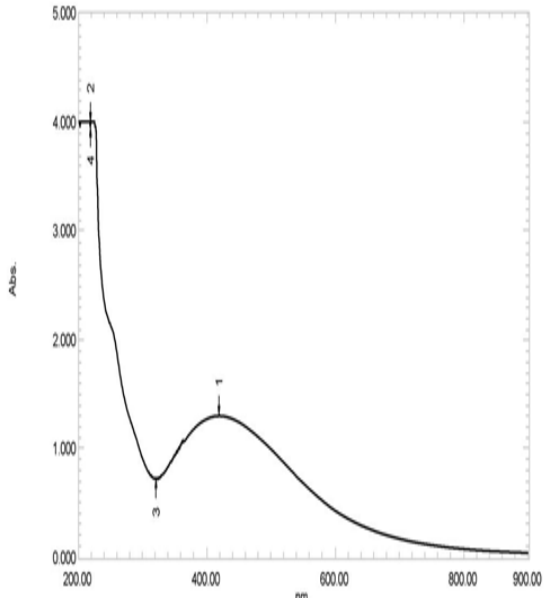
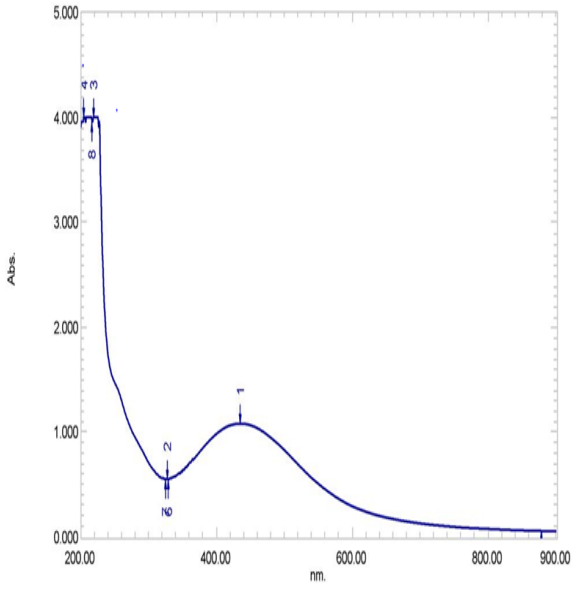


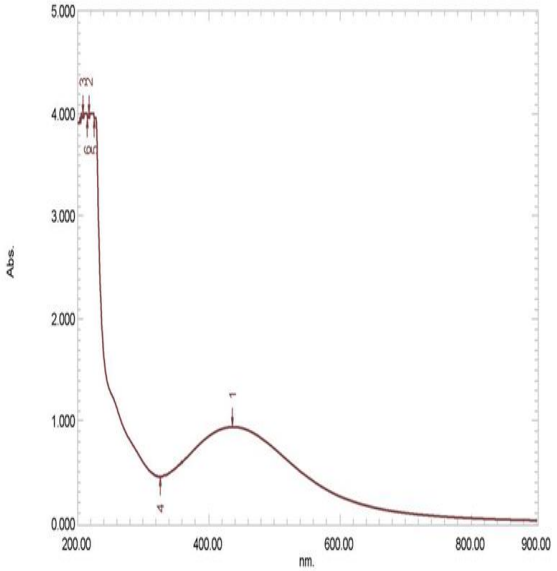
Plate 1.0: Reaction Mixture of *Aspergillus flavus* and *Alternaria alternata* biomass and Slver Nitrate Solution at Different Time Intervals


5.000
4.000
3.000
2.000
1.000
0.000
Abs.
200.00
400.00
600.00
800.00
900.00
nm.

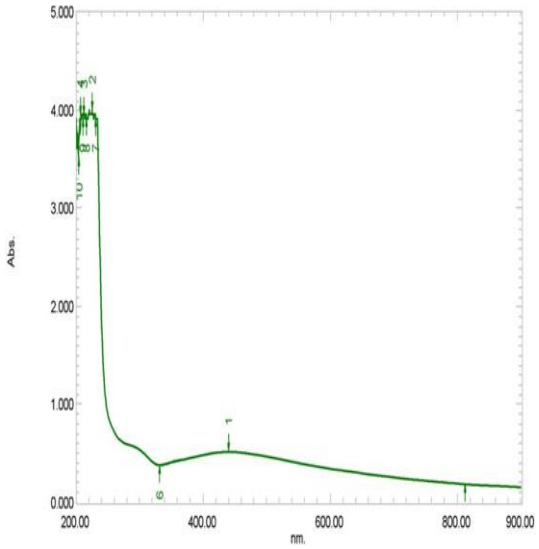
[a]

[b]

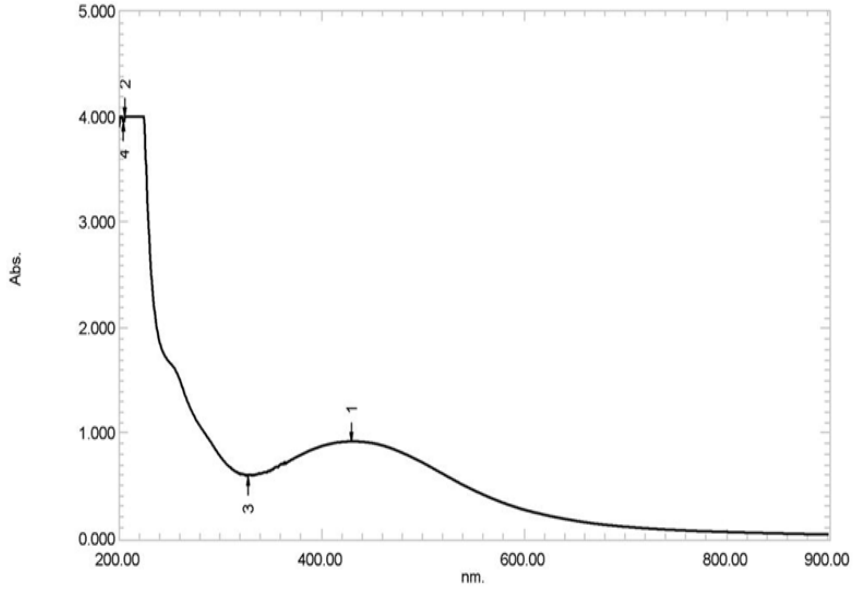

5.000
4.000
3.000
2.000
1.000
0.000
Abs.
200.00
400.00
600.00
800.00
900.00
nm.

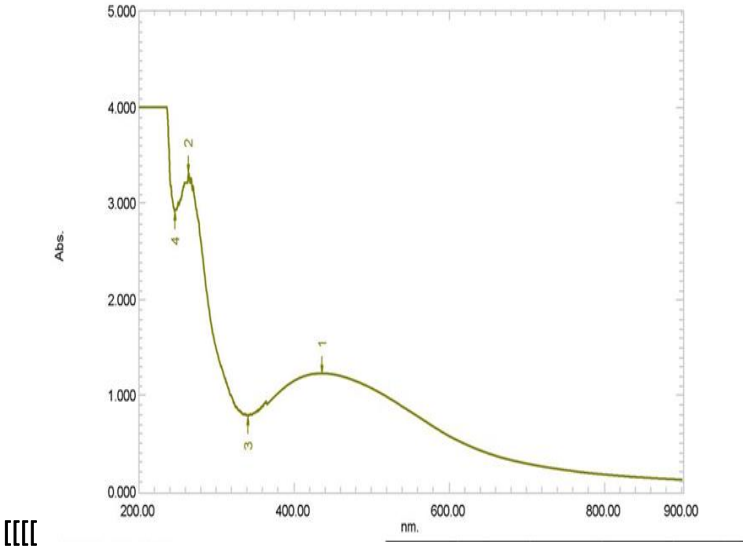

5.000
4.000
3.000
2.000
1.000
0.000
Abs.
200.00
400.00
600.00
800.00
900.00
nm.

[c]


5.000
4.000
3.000
2.000
1.000
0.000
Abs.
200.00
400.00
600.00
800.00
900.00
nm.

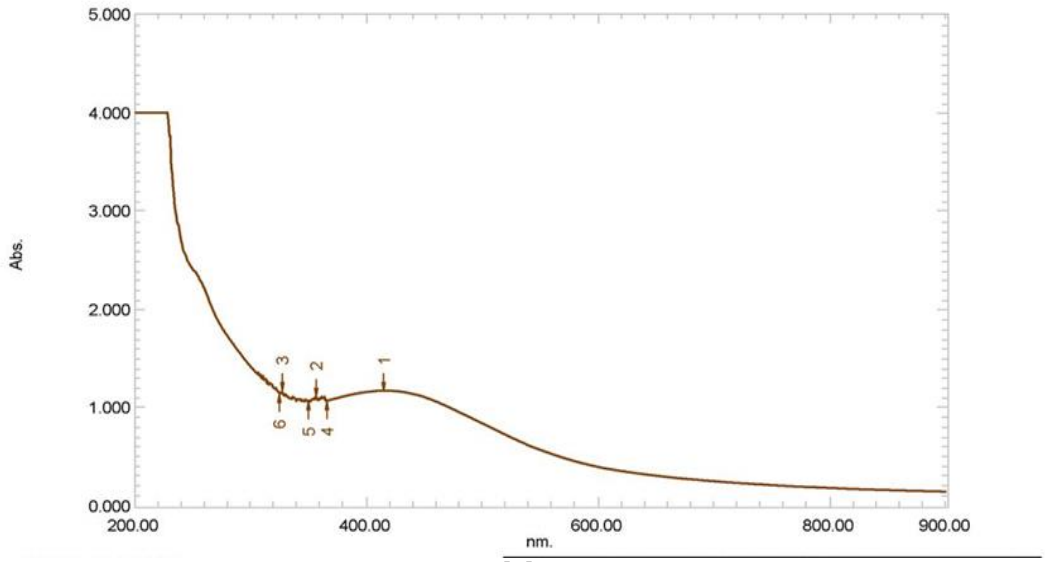
[d]


5.000
4.000
3.000
2.000
1.000
0.000
Abs.
200.00
400.00
600.00
800.00
900.00
nm.



[[[[[e]

[f]



[g]

Fig.1; UV-VIS Spectra for AgNPs (a-e) 1,2,3 and 5mM Silver Nitrate+*Aspergillus flavus* reaction mixture (f) 1mM Silver Nitrate+*Alternaria alternata* cell-free filtrate reaction mixture (g) Reaction mixture after 2 weeks

For vegetative, agitated *Alternaria alternata*, FTIR spectra revealed the presence of 8 bands at 3343.05, 2945.4, 2426.53, 2096.62, 1635.69, 1384.94, 1080.17, 1039.87 cm^{-1} as shown in

figure 8.0. The bands at 163.69 and 3343.05 correspond to the binding vibrations of Amide 1 band of protein with N-H stretching. The bands observed at 1384.94 and 1080.17 can be assigned to C-N stretching vibrations of aromatic and aliphatic amines respectively. The other bands however, are fingerprint regions.

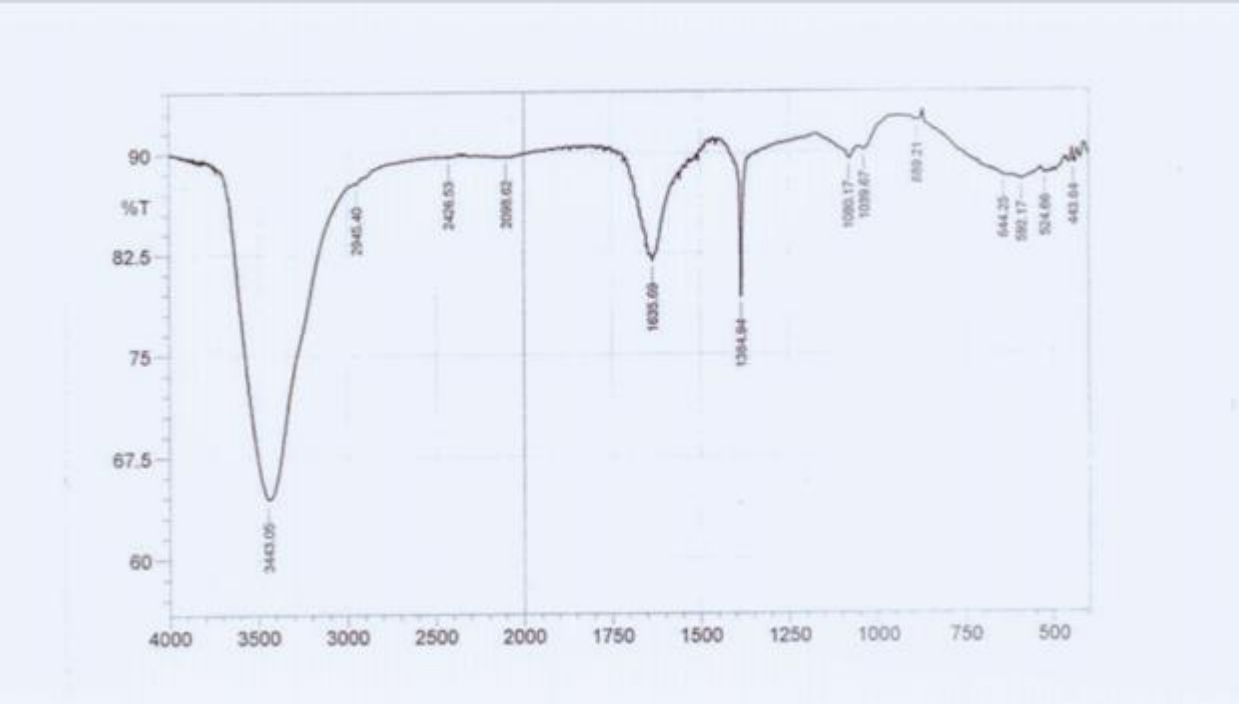


Fig. 8.0: FTIR Analysis of *Alternaria alternata* silver nanoparticles

For *Aspergillus flavus*, the FTIR revealed the presence of 6 bands at 3343.05, 2360.95, 1635.69, 1384.94,1114.89 and 1085.96 as shown in Figure 9.0. The other bands are fingerprint regions. The interpretations are similar to that of *A. alternata*.

The FTIR results were suggestive of the fact that the surrounding biological molecules (proteins and other metabolites) could possibly perform the dual functions of forming and stabilizing the nanoparticles in aqueous medium [13].

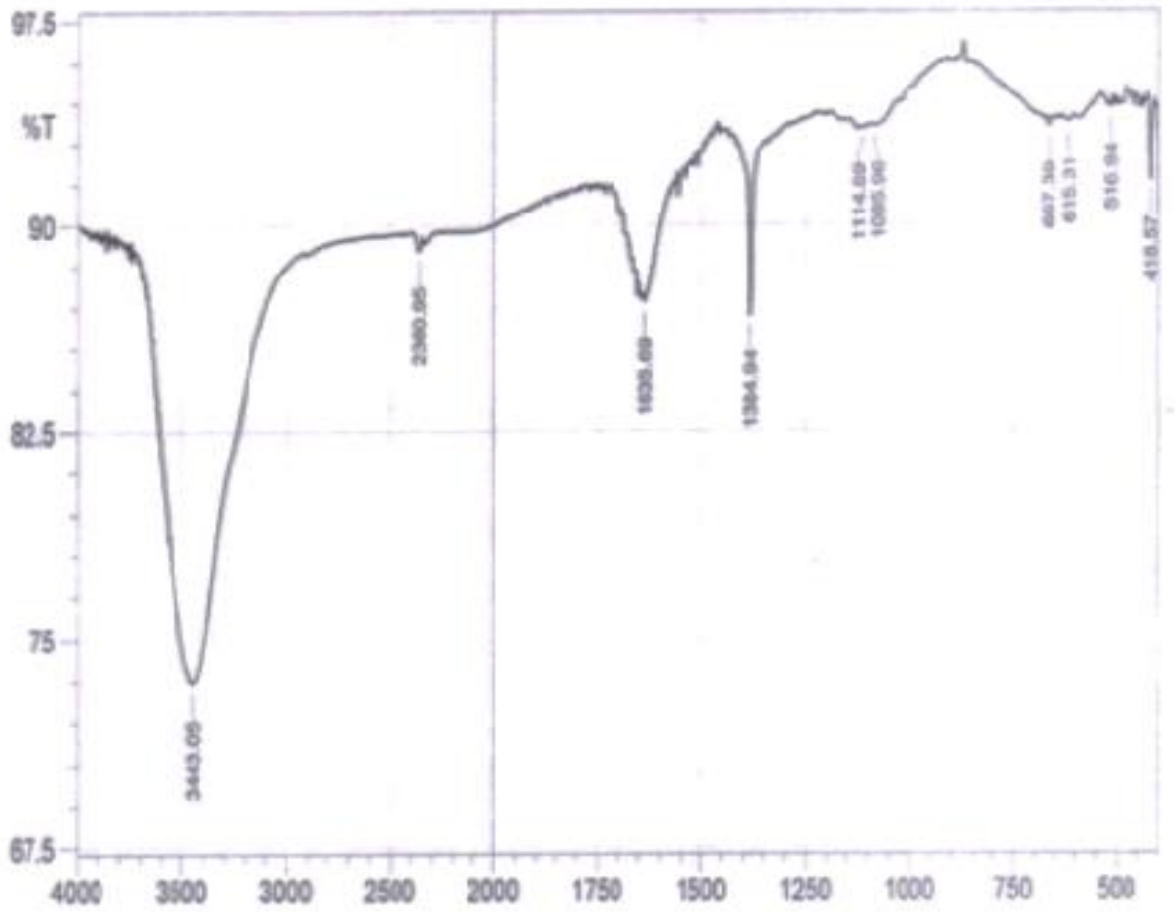


Fig. 9.0: FTIR Analysis of *Aspergillus flavus* silver nanoparticles

3.1 Antibacterial Activities of Biosynthesized Silver Nanoparticles

The antibacterial activities of 1mM and 5mM silver nanoparticles on Escherichia coli and *Streptococcus fecalis* in comparison with commercial antibiotics are as shown in Figure 8.0. The zones of inhibition of the silver nanoparticles vary with each concentration of silver nitrate and commercial antibiotics used.

Fig. 10.0 shows the graphical representation of the antibacterial activity of both concentrations of silver nanoparticles against the bacterial isolates in comparison with the commercial antibiotics used. It shows similar activity between Silver nanoparticles (1mM) and Ciprofloxacin. The organisms were also susceptible to Gentamycin, Nitrofurantoin and Ciprofloxacin.

It is indicated that Silver nanoparticle (1mM) has the same activity as Nitrofurantoin and *Escherichia coli*, there was similar activity between Silver nanoparticles (1mM and 5mM), Gentamycin and

Nitrofurantoin against *Streptococcus fecalis.* According to [11], antibacterial activities shown by the nanoparticles might be by oxidative stress generated by reactive oxygen species, and that it is possible that the nanoparticles, other than interact with the surface of the membrane, also penetrate the insides of the bacteria.

The result showed that the inhibition zone diameters of *Escherichia coli* were wider than those of *Streptococcus fecalis*, implying that the nanoparticles of 1mM rather than 5mM had higher antibacterial activity against human pathogenic Gram-negative rods. [14] however reported higher inhibition zones in a Gram-positive organism, *Staphylococus aureus*.

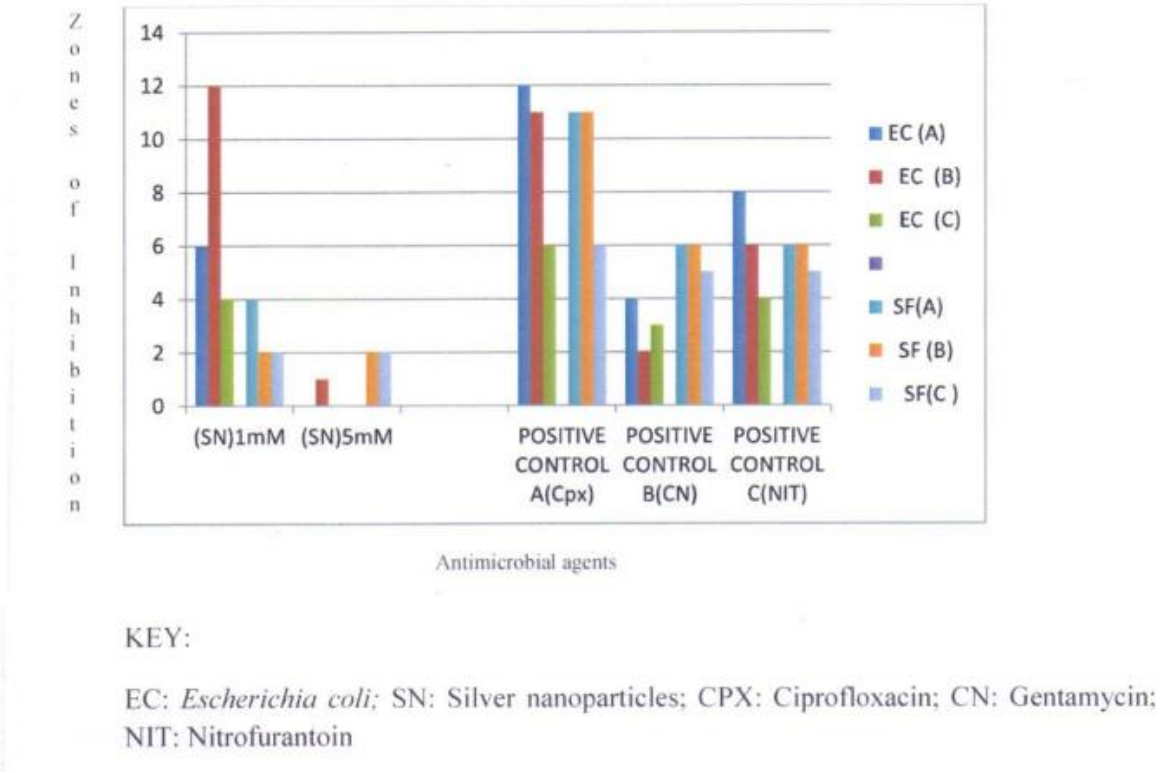


Fig 10.0; Zones of inhibition (mm) of Silver Nanoparticles (1mm and 5mm) and Control Antibiotics against Escherichia coli and *Streptococcus fecalis*.

4. CONCLUSION

This research work was aimed to throw more light on the importance of microorganisms not only for nanoparticles production but also indicates the use of nanoparticles as antibacterial agents. The suggested mechanism for the extracellular biosynthesis of silver nanoparticles by fungi is thought to occur with the involvement of carboxylic group or through nitrate-dependent reductase [12]. The fungus mycelium, upon exposure to the metal salt solution, produces metabolites for its own survival, leading to the reduction of the toxic metal ions to a non-toxic nanoparticle through the catalytic effects of the extracellular enzymes and metabolites of the fungus [15].

The findings of this study thereforedemonstrate the simple, safe, cost-effective and eco-friendly preparation of silver-nanoparticles using the fungi *Aspergillus flavus* and *Alternaria alternata*. The antibacterial activity of the synthesized nanoparticles was exhibited more at lowest concentrations against Gram-negative bacteria. Thus, application of biosynthesized silver nanoparticles may lead to the development of suitable pharmaceutical and other industrial products.

4.1 Recommendation

It is recommended that further studies be done on the synergistic application of nanotechnology and bioinformatics tools in vaccine prediction for tackling antimicrobial resistance.

Acknowledgements

The authors would like to appreciate Prof. Dare Enock Olugbenga of the Faculty of Chemistry and Pharmacy, University of Regensburg, Germany for his support.

REFERENCES

[1] Ghorbani H, Safekordi A, Attar H and Rezayat S. 2011. Biological and non-biological methods for the silver nanoparticles synthesis. *Chem. Biochem. Eng. Q.* Volume 25, Isuue 3, pp.317-326.

[2] Xiangqian L, Huizhong X, Zhesheng C, and Guofang C. 2011. Biosynthesis of nanoparticles by microorganisms and their applications. *J. Nanomat*., Vol.2011, Article ID 270974, pp.16.

[3] AbdelRahim K, Mahmoud S. Y., Ali A. M., Almaary K. S., Mustafa M. A., andHusseiny S. M. 2017. "Extracellular biosynthesis of silver nanoparticles using *Rhizopus stolonifer*," *Saudi J Biol. Sci*., vol. 24, no. 1, pp. 208– 216.

[4] Natarajan K, Selvaraj S, Murty V. 2010. Microbial production of silver nanoparticles. *Dig J Nanomater Biostructures.* 5:135-140.

[5] Kumar D, Palanichamy V, and Roopan S. 2014. Green synthesis of silver nanoparticles using *Alternanthera dentara* leaf extract at room temperature and their antimicrobial activity. Spectrochimica Acta Part A-*Mol. Biomol. Spec.,* Vol.127, pp. 168-171.

[6] Sarsar V., Selwal M. K., and Selwal K. K. 2016. "Biogenic synthesis, optimisation and antibacterial efficacy of extracellular silver nanoparticles using novel fungal isolate *Aspergillus fumigatus* MA," *IET Nanobiotechnol.*, vol. 10, no. 4, pp. 215–221

[7] Prabakaran M, Subha V, and Merinal S. 2012. Biosynthesis of silver nanoparticles using *Sphaerulina albispiculata* and evaluation of antibacterial activity. *Euro J. Exp. Biol*., Vol.2, Issue 1, pp. 297-303.

[8] Lateef A, Azeez MA, Asafa TB, Yekeen TA, Akinboro A, Oladipo IC, Azeez L, Ajibade SE, Ojo SA, Gueguim-Kana EB, Beukes LS. 2016. Biogenic synthesis of silver nanoparticles using a pod extract of *Cola nitida*: antibacterial and antioxidant activities and application as a paint additive. *J Taibah Univ Sci* 10(4):551–562. https ://doi.org/10.1016/j.jtusc i.2015.10.010

[9] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 28th ed.2018 Volume 28, Number 3.

[10] Chengzheng W., Jiazhi W., Shuangjiang C. (2018). "Biogenic synthesis, characterization and evaluation of silver nanoparticles from *Aspergillus niger* JX556221 against human colon cancer cell line HT-29," *J. Nanosci. Nanotechnol.,* vol. 18, no. 5, pp. 3673–3681.

[11] Adegoke EA, Abel M, Agbaje L, Abayomi A, Opeyemi D, Itunu P, Deborah O, Taiwo V, Badmus J, Asafa T. B., Beukes L. S., Gueguim-Kana E. B., Abbas S. H. 2019. Biosynthesis of silver, gold and silver–gold alloy nanoparticles using *Persea americana* fruit peel aqueous extract for their biomedicalproperties. *Nanotechnol. Environ. Eng.* 4:13

[12] Korbekandi H., Ashari Z., Iravani S., and Abbasi S. 2013. "Optimization of biological synthesis of silver nanoparticles using *Fusarium oxysporum*," *Iran. J. Pharm. Res*. : *IJPR*, vol. 12, no. 3, pp. 289–298,.

[13] Dare E, Adesuji T, Oseghale C, Omojola J, Labulo A, Bodede O, Akinsiku A. 2016. Biosynthesis of silver nanoparticles using Garcinia kola and its antimicrobial potential. *Afri. J. Pure. Appl. Chem*. Vol. 10(1), pp.1-7. ISSN:1996-0840

[14] Fayaz AM, Balaji K, Girilal M, Yadaz R, Kalaichelvam PT, Venketesan R. 2010. Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against grampositive and gram-negative bacteria. *Nanomed Nanotechnol Biol Med* 6:103–109. https ://doi.org/10.1016/j.nano.2009.04.006

[15] Elegbede JA, Lateef A, Azeez MA, Asafa TB, Yekeen TA, Akinboro A, Oladipo IC,Azeez L, Ajibade SE, Ojo SA, Gueguim-Kana EB, Beukes LS. 2018. Fungal xylanases-mediated synthesis of silver nanoparticles for catalytic and biomedical applications. *IET Nanobiotechnol*. https://doi.org/10.1049/iet-nbt.2017.0299