Antimicrobial potential of bioactive metabolites, Silver nanoparticle from *Bacillus* spp.
and some antibiotics against Multidrug Resistant *Salmonella* spp.

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Running Title: Antagonistic potential of metabolite and nanoparticles from *Bacillus* sp.

ABSTRACT

Objective: The synthesis of nanoparticles using microorganism and their metabolites are of increasing interest because they are potential producer of biocompatible and environmental friendly nanoparticles. Their nanoparticles can serve as potent alternatives to antibiotics against multidrug resistance bacteria (MDR). Antibacterial potential of *Bacillus* spp. metabolite, its silver nanoparticles and some antibiotics against multi drug resistant *Salmonella* spp. was evaluated.

Method: The antimicrobial potential of metabolites from *Bacillus* spp. was determined and SNPs biosynthesized using the metabolites was characterized. The effect of physicochemical parameters on SNPs biosynthesis, antimicrobial activity of the SNPs and combination of SNPs and antibiotics against Multidrug Resistant (MDR) *Salmonella* strains were evaluated.

Result: The bioactive metabolites of the *Bacillus* spp. exhibited varied antimicrobial potential against the tested MDR *Salmonella* spp. The metabolites were able to bio-reduced AgNO_3 to Ag^0 for SNPs biosynthesis. Change in colour from whitish to darkish brown and Surface Plasma Resonance (SPR) peak of 600 – 800 nm was observed. The SNPs were aggregated, rods and crystalline in shape and the sizes were 15 μm , 16 μm and 13 μm . Carboxylic acid, amino acid, alcohol, esters and aldehydes were the functional group found in the biosynthesized SNPs. The antibacterial activity of BAC1-SNPs, BAC7-SNPs and BAC20-SNPs against the MDRSA9 and MDRSA18 ranged

50 from 6.0 – 22 mm and 11 – 20 mm. SNPs biosynthesized at pH 7 and 10mM AgNO₃
51 had the highest antagonistic activity. Combination of SNPs and antibiotics exhibited the
52 best antagonistic potential.

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Conclusion: The metabolites and SNPs biosynthesized using *Bacillus* spp. Metabolites exhibited antagonistic effect against MDR *Salmonella* spp. The combined SNPs and antibiotics had better antimicrobial activity.

Keywords: *Bacillus* strains, metabolites, Antimicrobial, Silver nanoparticles, MDR *Salmonella* species, Synergistic effect.

TURKISH ABSTACT

Amaç: Mikroorganizma ve bunların metabolitlerini kullanan nanopartiküllerin sentezi, biyolojik olarak uyumlu ve çevre dostu nanopartiküllerin potansiyel üreticisi olmaları nedeniyle artan ilgi konusudur. Onların nanopartikülleri, çoklu ilaç dirençli bakterilere (MDR) karşı antibiyotiklere güçlü alternatifler olarak hizmet edebilir. *Bacillus* spp.'nin antibakteriyel potansiyeli metabolit, gümüş nanoparçacıkları ve çok ilaca dirençli *Salmonella* spp. Değerlendirildi.

Yöntem: *Bacillus* spp.'den metabolitlerin antimikrobiyal potansiyeli. belirlendi ve metabolitler kullanılarak biyosentezlenen SNP'ler tanımlandı. Fizikokimyasal parametrelerin SNP biyosentezi, SNP'lerin antimikrobiyal aktivitesi ve SNP'lerin ve Çoklu İlaç Dirençli (MDR) *Salmonella* suşlarına karşı antibiyotik kombinasyonu üzerindeki etkisi değerlendirildi.

Sonuç: *Bacillus* spp. Nın biyoaktif metabolitleri. Test edilen MDR *Salmonella* spp.'ye karşı çeşitli antimikrobiyal potansiyel sergiledi. Metabolitler, SNP biyosentezi için AgN03'ü Ag0'a indirgenebildi. Rengi beyazımsı ila kahverengimsi kahverengiye dönüşmüş ve 600 - 800 nm Yüzey Plazma Rezonansı (SPR) zirvesi gözlenmiştir. SNP'ler toplandı, çubuklar ve kristal şeklinde ve boyutları 15 µm, 16 µm ve 13 µm idi. Karboksilik asit, amino asit, alkol, esterler ve aldehytler, biyosentezlenmiş SNP'lerde bulunan fonksiyonel gruptu. BAC1-SNP'lerin, BAC7-SNP'lerin ve BAC20-SNP'lerin MDRSA9 ve MDRSA18'e karşı antibakteriyel aktivitesi 6,0 - 22 mm ve 11 - 20 mm arasında değişmiştir. PH 7 ve 10mM AgN03'te biyosentezlenen SNP'ler en yüksek antagonistik aktiviteye sahipti. SNP'lerin ve antibiyotiklerin kombinasyonu en iyi antagonistik potansiyeli göstermiştir.

Sonuç: Metabolitler ve SNP'ler *Bacillus* spp. Kullanılarak biyosentezlenmiştir. Metabolitler, MDR *Salmonella* spp.'ye karşı antagonistik etki göstermiştir. Kombine SNP'ler ve antibiyotikler daha iyi antimikrobiyal aktiviteye sahipti.

Introduction

Infections caused by harmful microorganisms affect many people globally and it's an important cause of morbidity and mortality with adverse effect on the healthcare economy ^{1, 2}. The treatment of these infections has become a public health concern as a result of development of resistance to antibiotics by these etiological agents ^{3, 4}.

Salmonella, the most commonly isolated food borne pathogens associated with fresh fruits and vegetables, is a gram negative flagellated rod-shaped facultative anaerobe bacterium ^{5, 6}. *Salmonella* is a group of bacteria that causes typhoid fever, food poisoning, gastroenteritis, enteric fever and other illnesses. *Salmonella* infections or *Salmonellosis*, are bacterial diseases of the intestinal tract.

The occurrence of enteric fever has been a major health problem in spite of the use of antibiotics and the development of newer antimicrobials. Resistance of *Salmonella* spp. to previously efficacious drugs like ciprofloxacin, ampicillin, ceftriaxone, and cotrimoxazole has been reported ⁷. Adabara et al. ⁸ reported the prevalence and antibiotic susceptibility pattern of *Salmonella typhi* among patients attending a military hospital in Minna, Nigeria. The isolates were resistant to all of the antibiotics tested which are the drug of choice routinely used in the study area for the treatment of typhoid fever.

MDR *Salmonella* strains are very common and are the causative agent of endemic and epidemic typhoid fever infections in many communities. MDR *Salmonella typhi* poses a serious threat because they have developed resistance to available antibiotics resulting in an increase in death rate and high capability for epidemic outbreaks of typhoid which may be difficult to manage.

The consequence of typhoid fever outbreak may be highly destructive most especially in developing countries where health facilities are very poor. *Salmonella* has been reported as the most common cause of bacterial food-borne illness in the U.S. and is estimated to annually cause over 1 million cases, 19,000 hospitalizations, 350 deaths, and \$2.6 billion in social costs ^{9, 10}. *Salmonella enterica* serovar typhimurium is one of the most common salmonellae in humans and livestock, and most of these strains are resistant to multiple antibiotics.

Bacillus species are Gram-positive, endospore-forming, chemoheterotrophic rod-shaped bacteria which are usually motile with peritrichous flagella; they are aerobic or facultative anaerobic and catalase positive and a member of the phylum *Firmicutes*.

Many *Bacillus* species are of remarkable importance because they are antibiotics producer. *B. subtilis*, *B. polymyxa*, *B. brevis*, *B. licheniformis*, *B. circulans*, *B. cereus* has been reported as an antibiotics producers. Antibiotics such as bacitracin, gramycidin S, polymyxin and tyrotricin produced by *Bacillus* spp. have found application in medical treatments ^{11 – 14}. The wide range of antimicrobial, anti-viral, anti-ameobocytic and anti-mycoplasma activities of *Bacillus* species have been reported ¹⁵⁻¹⁷.

The ability of *Bacillus* species to produce different metabolites with antimicrobial activity has been extensively used in medicine and pharmaceutical industry; one of its potential is on its application as a bio-control agent for animals, humans and plants diseases ¹⁸.

Silver and silver containing products are one of the most popular and efficient antimicrobiocidal agents commonly used against pathogenic bacteria ¹⁹. Since the ancient times silver has been in used as an antimicrobial agent but with the development of antibiotics, there has been a reduction in the medical applications of silver as an antimicrobial ²⁰.

Nano level manipulation and physicochemical changes of silver can lead to production of silver nanoparticles with an increase in antimicrobial potential. Silver nanoparticles are characterized with high surface-area-to-volume ratio and the unique chemical and physical properties which confer on them better antimicrobial properties ²¹.

Silver nanoparticles having size in the range of 10–100 nm showed strong bactericidal potential against both Gram-positive and Gram-negative bacteria ²². Silver in the form of SNPs is known to exhibit strong biocidal effects on different bacterial strains, including multidrug resistant bacteria ^{23, 24}. SNPs have little chance of drug resistance which provides a solution to multidrug resistance problems ²⁵. Drug resistance has been reported as one of the most serious threatening and widespread problems in all the developing countries ²⁶. Hence, diverse medical applications of AgNPs is necessary to avoid the effect of these drug-resistant pathogenic microorganisms by various methods, ranging from silver-based dressings and silver-coated medical devices such as

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biological labelling, water treatment, in optical devices and as antibactericidal²⁷⁻²⁹. Microbial resistance to antimicrobials is highly prevalent worldwide, particularly in developing countries and this has made therapeutic options to appear more limited than ever. As a result of the emergence of MDR pathogen research interest has been geared towards the development of efficient and safe antimicrobials. For these reasons, this research aimed at investigating the antimicrobial potential of metabolites from *Bacillus* spp., its SNPs and combination of the SNPs with some antibiotics against some selected MDR *Salmonella* spp.

Materials and Methods

Microorganisms

Culture of *Bacillus* spp. previously isolated from fermented locust bean (*Parkia biglobosa*) samples and 10 MDR *Salmonella* spp. was obtained from the culture collection of our previous work in Microbial Physiology and Biotechnology Unit, Department of Microbiology, University of Ibadan. The isolates was kept on Nutrient agar slants and kept in the refrigerator. Constant sub culturing was carried out to ensure the viability of the isolates.

Production of bioactive metabolites from *Bacillus* strains

Three *Bacillus* strains were used for the production of bioactive metabolites using sterile Nutrient broth (NB). The sterile NB was inoculated with the young culture of *Bacillus* sp. the inoculated broth was incubated at 35°C for 48 hrs. The supernatant obtained from the fermentation broth was used as bioactive metabolites.

Antagonistic potential of the bioactive metabolites produced by *Bacillus* spp. against some MDR *Salmonella* spp.

The antagonistic potential of the bioactive metabolites against some MDR *Salmonella* spp. was investigated using Agar well diffusion method³⁰. The inoculum suspensions of 24hr old culture of the *Salmonella* spp. was spread plated on Mueller Hinton agar (MHA). Uniform wells of 6 mm were bored on the agar. Each well was filled with 60 µl of

the bioactive metabolites from the *Bacillus* isolates. The plates were incubated at 37°C for 24 hrs. The incubated plates were observed for a zone of inhibition (ZOI) around the well. Formation of a clear zone around the well is an indication of antibacterial activity³¹. Results were considered positive if the diameter (mm) of the ZOI around the well was greater than 1mm. The bioactive metabolites of the best three *Bacillus* spp. were used for further studies.

Production of SNPS using the selected *Bacillus* strains

The bioactive metabolites the best three *Bacillus* spp. were used for the production of SNPs. 30 mL of the bioactive metabolites was added to 70 mL of 10mM AgNO₃ solution. The mixture was stored at room temperature (35°C) in a dark place for 24 – 72 hrs. After 12hrs incubation, the whitish solution turned brownish which confirmed the formation of SNPs.

Characterization of the Synthesized SNPs

The SNPs was characterized using Visual detection for change in colour, UV–Visible absorption spectra of the were analyzed at room temperature using UV–Vis spectrophotometer (a Lambda 25-Perkin Elmer, Waltham, MA, USA) with a resolution of 0.5 nm at the wavelengths of 200-800 nm. The FTIR spectra of the nanoparticles were analyzed using FTIR spectroscopy (Shimadzu) operated at resolution of 4 cm⁻¹. The size, shape and morphology of the biosynthesized SNPs were determined using SEM.

Antibacterial activity of the SNPS against some selected (MDR) *Salmonella* spp. and their synergetic effect with some selected Antibiotic

The antibacterial activity of the biosynthesized SNPs and the synergistic effect of SNPs along with some selected antibiotics (Ciprofloxacin, Zinnacef, Amoxicillin, Pocephin, Streptomycin, Ampliclox and Gentamycin) was tested using agar well diffusion method³⁰ against the selected MDR *Salmonella* spp. as previously described.

Determination of Minimum Inhibitory Concentration (MIC) of the Biosynthesized SNPs

Minimum inhibitory concentration was carried out using different concentration (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%) of the SNPs against the isolates³⁰. The lowest dilution of the SNPs at which zones of inhibition was observed against the *Salmonella* sp. is regarded as the MIC for each SNPs. Ciprofloxacin was used as positive control.

Effect of physiochemical parameters on the SNPs biosynthesis

The effect of temperature (25°C, 35°C and 40°C), pH (4, 7 and 9) and different concentration of AgNO₃ (2 – 10 mM) on the biosynthesized SNPs was evaluated.

Results and Discussion

The antimicrobial activity of the bioactive metabolites of the *Bacillus* spp. against some MDR *Salmonella* spp. is shown in Table 1. The antimicrobial activity of *Bacillus subtilis* BAC1, *B. licheniformis* BAC7 and *Bacillus subtilis* BAC20 bioactive metabolites against the 10 MDR *Salmonella* strains ranged from 3.0 – 17.0 mm, 4 – 14 mm and 14 – 2.0 mm. MDRSA9 and MDRSA20 had the highest susceptibility.

Table 1: Antimicrobial activity (mm) of the bioactive metabolites of *Bacillus* spp. against some MDR *Salmonella* spp.

Bioactive <i>B.</i> spp. Metabolites	MDR <i>Salmonella</i> strains / Zone of inhibition (mm)									
	SA23	SA15	SA32	SA20	SA32b	SA9	SA7	SA18	SA21	SA8
BSM (BAC1)	7.0±0.5 ^e	11.0±1.0 ^b	8.0±0.5 ^d	5.0±1.0 ^f	4.0±1.0 ^{fg}	17.0±0.5 ^a	4.0±1.0 ^{fg}	10.0±1.0 ^c	0.0±0.0 ^h	3.0±0.5 ^g
BLM (BAC7)	0.0±0.0 ^g	10.0±0.5 ^d	6.0±0.5 ^e	4.0±1.0 ^f	10.0±0.0 ^d	14.0±1.0 ^a	0.0±0.0 ^g	12.0±0.5 ^b	10.0±0.0 ^d	11.0±1.0 ^c
BMM (BAC20)	2.0±0.0 ^f	0.0±0.0 ^g	0.0±0.0 ^g	14.0±1.0 ^a	12.0±0.0 ^c	6.0±1.0 ^e	2.0±0.0 ^f	11.0±1.0 ^d	6.0±0.0 ^e	13.0±0.5 ^b

Values are Means $\pm$ Standard Deviations of triplicate observations. Means with different alphabets across the row respectively are statistically significant at $p \leq 0.05$.

The ability of the *Bacillus* spp. metabolites to inhibit the test pathogens may be as a result of the production of some bioactive metabolites. Several antimicrobial activities of *Bacillus* spp. against pathogens have been reported. Antagonistic activity of *Bacillus subtilis* and *Bacillus cereus* against *Staphylococcus* spp. has been reported by Perez *et al.*¹³ and Riley and Wertz³².

Biosynthesis of SNPs using *Bacilli* spp. broth

The 3 bioactive metabolites were able to bio-reduce the AgNO_3 to produce SNPs by the evident change in colour from whitish reaction mixture to darkish-brown which indicates the formation of SNPs.

The biosynthesis of silver nanoparticles was done using the bioactive metabolites of *Bacillus* spp. with the highest antagonistic potential from the previous screening. This is in accordance with the work of Kalishwaralal *et al.*³³ who biosynthesized SNPs from culture supernatant of *Bacillus licheniformis*. The culture supernatant was able to bio-reduce Ag^+ to Ag^0 . Kanmani and Lim³⁴ reported the bio-reduction of Ag^+ to Ag^0 using exopolysaccharides.

Changes in colour from white to brown indicate SNPs biosynthesis. A visible colour change from whitish yellow to brown was also reported by El-Batal *et al.*,³⁵. Changes in colour may be attributed to the Surface Plasmon Resonance³⁴.

SPECTROPHOTOMETRIC ANALYSIS OF THE BIOSYNTHESIZED SNPs

The biosynthesized SNPs were characterized with UV-Visible spectroscopy. The UV-absorption measurements in the range of 200 – 1000 nm were observed (Figure 1 a-c).

The UV – spectra of the biosynthesized SNPs using BAC1 metabolite incubated at 24, 48 and 72hrs had the highest Surface Resonance Peak (SPR) at 600 nm (Figure 1c). SNPs from BAC7 metabolite had the highest SPR peak at 800 nm, 600 nm and 400nm (Figure 1b). SNPs from BAC20 metabolites had the highest SPR peak 600

nm, 600 nm and 800 nm at 24, 48 and 72 hrs of incubation (Figure 1c) which corresponds to plasmon excitation of the SNPs. All the peaks obtained were in the range of 400 – 800 nm. There was variation in SPR peak of the SNPs incubated at different time interval.

Figure 1 a - c: UV-Visible spectra of the SNPs biosynthesized using metabolites of

265 *Bacillus* spp. (A) BAC1-SNPs, (B) BAC7- SNPs and (C) BAC20-SNPs at different
266 incubation time
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Primary confirmation of the silver nanoparticle biosynthesis was carried out by UV-visible spectroscopy in the range of 200 - 1000nm. A strong SPR peak was observed at 600 nm for the SNPs biosynthesized by the *Bacillus subtilis* BAC1 and BAC20 after 24 hrs of incubation indicates the formation of SNPs that varied in shapes and sizes. Kanmani and Lim,³⁴ reported a strong SPR peak at 400 – 550nm with a broad band. El-Batal *et al.*³⁵ reported a SPR band at 431nm of the SNPs synthesized with *Bacillus stearothermophilus* indicating the presence of spherical or roughly spherical SNPs. Valencia *et al.*³⁶ reported that solutions containing SNPs developed a yellow colour from initial whitish colour and presented SPR peaks between 411 - 414 nm.

Scanning Electron Microscopy

The biosynthesized SNPs were characterized by SEM. Figure 2 (a – c) shows the micrographs of the SNPs. The SEM images shows SNPs from BAC1, BAC7 and BAC20 were of varying shapes and sizes. BAC1-SNPs were aggregated, BAC7-SNPs were aggregated rods and BAC20-SNPs were crystalline in shape (Figure 2a – c). The sizes were 15µm, 13µm and 10µm respectively.

Figure 2c: SEM micrograph of SNPs biosynthesized using metabolites of *Bacillus* spp. (A), BAC1-SNPs, (B) BAC7- SNPs and (C) BAC20-SNPs

Scanning electron microscopy (SEM) is used for the morphological characterization at the nanometer to micrometer scale and can provide morphological information on the submicron scale and elemental information at the micron scale as reported by Schaffer *et al.*³⁷.

SEM studies revealed that the biologically synthesized SNPs are different in size and shape and they showed that they were aggregated, rod shaped and crystalline. The aggregation observed may be due to the drying process. This observation agrees with the work of Sadowski *et al.*³⁸ who observed aggregated SNPs biosynthesized using *Penicillium* strains isolated from the soil. They concluded that the drying can affect the shape and size of SNPs.

Rod shape observed in BAC7-SNPs is in accordance with the work of Gardea-Torresday *et al.*,³⁹ who reported rod shape from the SEM characterization of biosynthesis of gold nanoparticles from *Triticum aestivum* leaves.

Crystalline SNPs observed in this work agrees with the report of Vithiya *et al.*⁴⁰ who reported that the shape of the extracellular biosynthesized SNPs from *Bacillus* spp. was monodispersed.

FTIR Spectra of the SNPs

FTIR analysis of the SNPs was carried out to identify interactions between silver salts and protein molecules, which could account for the reduction of silver ions and stabilization of the SNPs formed.

The BAC1-SNPs characterized using FTIR shows 7 bands with transmission peaks at 3448.00, 2937.14, 1638.92, 1552.59, 1404.40, 1103.65 and 607.24 cm^{-1} (Figure 3a). The bands corresponded to O-H stretch of alcohol, C-H symmetrical stretch, presence of amide, NH bend, C=O stretch, C-O stretch of alcohol and acetylenic CH of alkynes respectively. The functional groups generally observed indicated the presence of amino acids, alcohol, aldehydes and carboxylic acid in the sample and this may be responsible for the reduction of silver nitrate to SNPs.

The FTIR analysis of BAC7-SNPs shows 9 bands and the peaks spectrum ranged from 3423.00 cm^{-1} and 352.84 cm^{-1} (Figure 3b). The peaks were attributed to O-H stretch vibration of alcohol, symmetrical C-H. C=O stretch of carboxylates and NH stretch of secondary amides, presence of O-H bends of esters, phenol and tertiary alcohol or C-C bend of aldehyde, C-O stretching vibration of carboxylic esters, C-O stretching vibration of secondary alcohol, presence of acetylenic CH of alkynes and aromatic benzene ring. From the FTIR spectrum, it was observed that the SNPs were surrounded by carboxylic acid, aldehyde, esters, protein and amino acids which may be responsible for the biosynthesis and stability of the SNPs (Figure 4b).

The FTIR analysis of BAC20-SNPs revealed 12 absorption peaks which ranged between 3760.00 cm^{-1} and 361.39 cm^{-1} . The peak indicated the presence of an O-H stretch free, strong alcohol, O-H stretch vibration of alcohol, C-H symmetrical stretching, carbonyl stretching of transition metals, NH bend of amide, NH stretch of secondary amides, C-O stretch of esters, carboxylic acids, acetylenic CH of alkynes and weak aromatic benzene. The functional groups corresponded to the FTIR spectrum generally indicated the presence of amino acids, alcohol, aldehydes and carboxylic acid which may be responsible for the reduction of silver nitrate to SNPs (Figure 3c). The presence of the intense pick at C=O stretching mode indicates the presence of carboxylic groups in the material bound to SNPs

Figure 3a - c: FTIR Spectra of the SNPs from *Bacillus* spp. (A) BAC1-SNPs, (B) BAC7- SNPs and (C) BAC20-SNPs

The Fourier Transform Infra-Red (FTIR) measurements were carried out to identify possible interactions between silver salts and protein molecule. FTIR is very useful for SNPs characterization as reported by Kanmani and Lim³⁴. El-Batal *et al.*³⁵ reported that the release of extracellular protein molecules from bacteria could possibly perform the function of formation and stabilization of SNPs in aqueous medium. Balashanmugam *et al.*³⁰ also suggested that from FTIR spectrum analysis, SNPs were surrounded by proteins and amino acids which may be responsible for the stability of the SNPs.

The functional groups such as carboxylic acid, amino acid, alcohol, esters and aldehydes present in the SNPs biosynthesized using the *Bacillus* metabolites were responsible for reduction, capping and stabilization of the nanoparticles. El-Batal *et al.*,³⁵ reported functional groups as the reducing agents responsible for SNPs formation and stabilization in their work.

Antibacterial activity of the biosynthesized SNPs from the *Bacillus* strains against MDR *Salmonella* spp.

The antibacterial activity of the biosynthesized SNPs from the *Bacillus* spp. against the selected MDR *Salmonella* spp. is shown in Figure 4.

362 Antibacterial activity of BAC1-SNPs, BAC7-SNPs and BAC20-SNPs against MDRSA9

363 ranged from 6.0 – 22 nm. MDRSA9 had the highest susceptibility to BAC1-SNPs. The

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BAC7-SNPs and BAC20-SNPs exhibited higher activity against the MDRSA9 compare to AgNO₃. Ciprofloxacin exhibited the highest activity against MDRSA9 compare to the SNPs.

Antibacterial activity of BAC1-SNPs, BAC7-SNPs and BAC20-SNPs against MDRSA18 ranged from 11 – 20 nm. All the biosynthesized SNPs had antagonistic activity against MDRSA18. Comparatively, the BAC7-SNPs exhibited highest antagonistic activity against MDRSA18 than BAC1-SNPs and BAC20-SNPs.

Comparatively, BAC1-SNPs had the antagonistic potential against MDRSA9 while BAC7-SNPs and BAC20-SNPs had highest antagonistic potential against MDRSA18. Ciprofloxacin exhibited the highest activity against MDRS18 compare to the SNPs.

Figure 4: Antibacterial activity of the synthesized SNPs against the selected (MDR) *Salmonella* spp.

Guzman *et al.*⁴¹ reported the possibility of SNPs acting similarly to the antimicrobial agents used for the treatment of bacterial infections, which show four different mechanisms of action including; interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis and inhibition of metabolic pathway. Sharma *et al.*⁴² also noted that SNPs not only interact with the surface of the membrane, but can also penetrate inside the bacteria. It is reported that the bactericidal effect of silver nanoparticles decreases as the size increases and is also affected by the

387 shape of the particles. Dhanalakshmi and Rajendran ⁴³ reported the interaction of

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nanoparticles with the DNA inside a bacterium thus losing its ability to replicate which may lead to the cell death.

Antibacterial activities of the SNPs produced from *Bacillus* spp. Metabolites against multi-drug resistant *Salmonella* spp. is in agreement with the work of Natarajan and Selvaraj ⁴⁴ who reported the antibacterial activity of the SNPs produced from *Bacillus subtilis* against pathogens like *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus coagulase* positive, *Serratia* spp. and *Salmonella typhi*. They also stated that the SNPs showed the most activity against *Salmonella typhi*. This is not in accordance with Kanmani and Lim ⁴⁵ that reported that Gram negative bacteria were more susceptible to SNPs than the Gram positive bacteria.

MIC of the SNPs against the MDR *Salmonella* spp.

The MIC of the SNPs against the MDR *Salmonella* spp. is shown in Table 2. BAC1-SNPs had MIC of 3.25 µg/mL on SA9 and 6.25 µg/mL on SA18. BAC7-SNPs had MIC of 3.25 µg/mL on SA9 and of 6.25 µg/mL on SA18. The BAC20-SNPs had MIC of 6.25 µg/mL on SA9 and 3.25 µg/mL on SA18. The *Salmonella* sp. SA9 was resistant to BAC20-SNPs at concentration of 3.25 µg/mL while *Salmonella* sp. SA18 was resistant to BAC1-SNPs and BAC7-SNPs at 3.25 µg/mL concentration.

Table 2: Determination of MIC of *Bacillus* spp. SNPs on the selected MDR *Salmonella* spp.

MIC Concentration (µg/mL)	Zones of Inhibition (mm)					
	MDRSA9			MDRSA18		
	BAC1-SNPs	BAC7-SNPs	BAC20-SNPs	BAC1-SNPs	BAC7-SNPs	BAC20-SNPs
50	13±0.0 ^{ab}	14.0±0.2 ^a	12.0±0.7 ^b	10.0±0.0 ^b	12.0±1.0 ^a	8.0±0.5 ^c
25	12±0.4 ^b	14.0±0.5 ^a	10.0±1.0 ^c	12.0±0.2 ^a	10.0±0.0 ^b	10.0±0.3 ^b
12.5	9.0±1.0 ^b	12.0±0.6 ^a	12.0±0.0 ^a	9.0±0.6 ^a	8.0±1.0 ^b	6.0±1.0 ^c
6.25	6.0±1	8.0±0.0	5.0±0.3 ^c	5.0±1.0	4.0±0.1	4.0±0.1

	3.25	4.0 ± 0.2^b	5.0 ± 0.5^a	0.0 ± 0.0^c	0.0 ± 0.0^b	0.0 ± 0.0^b	2.0 ± 1.0^a
Ciprofloxacin		12.0 ± 0.5^b	14.0 ± 0.0^a	4.0 ± 0.2^c	10.0 ± 0.8^a	10.0 ± 0.0^a	1.0 ± 0.5^b

Values are Means $\pm$ Standard Deviations of triplicate observations. Means with different alphabets across the row respectively are statistically significant at $p \leq 0.05$.

The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the SNPs that can have antimicrobial effect on the test organisms. 10mM was used to prepare the different concentrations of the SNPs for the MIC evaluation, different MICs were recorded for the two (MDR) *Salmonella* spp. Balashanmugam *et al.*³⁰ also reported the MIC of their SNPs after testing on some pathogens with *Escherichia coli* and *Bacillus subtilis* having the highest values.

EFFECT OF TEMPERATURE ON SNPs BIOSYNTHESIS

The effect of temperature (25 – 40°C), pH (4 – 9) and different concentration of AgNO_3 (2 – 10Mm) on the biosynthesis of SNPs was evaluated and the SNPs was characterized.

UV-Visible spectra of BAC1-SNPs had a sharp SPR peak at 400 and 800 at 25°C and 35°C. At 40°C, a sharp SRP was observed at 600 nm. SNPs synthesized at 35°C had the highest SPR peak. (Figure 5a). BAC7- SNPs biosynthesized at 25°C, 35°C and 40°C, the sharp SPR peak was observed at 400 nm and 800nm (Figure 5b). For BAC20-SNP, at 25°C and 40 °C, the UV-Visible spectra showed a sharp SPR peak at 400 nm and 800 nm while at 35°C, the sharp SPR peak was observed at 600nm (Figure 5c). Highest absorbance values were obtained at 35°C followed by 40°C for all the SNPs. The optimum temperature for the production of the SNPs was 35°C.

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434 Figure 5a: UV-Visible spectra of BAC1 SNPs at different temperature

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437 Figure 5b: UV-Visible spectra of BAC7 SNPs at different temperature

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440 Figure 5c: UV-Visible spectra of BAC20 SNPs at different temperature

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The effect of temperature on SNPs synthesis was evaluated and the best temperature for the production of SNPs was 35°C. This is not in accordance to the work of Muhammad Amin *et al.*⁴⁵ who observed that with increase in temperature from 25°C to 45°C, the SPR peaks became sharper, an absorption band at 406nm was obtained which suggested the formation of SNPs.

Annadurai *et al.*,⁴⁶ also reported that the synthesis of silver nanoparticles from leaf extract of *Coleus aromaticus* increases when the reaction temperature was increased and they concluded that higher temperature (70°C) was best for nanoparticles synthesis. Muhammad Amin *et al.*,⁴⁵ observed that the best temperature required for the completion of reaction was investigated to be 45 °C.

EFFECT OF pH ON SNPs BIOSYNTHESIS

The UV – spectra of the effect of different pH (4, 7 and 9) on SNPs biosynthesis is shown in Figure 6 (a –c). The UV-Visible spectra of BAC1- SNPs produced at different pH is shown in Figure 6a. at pH 4 and 7, a sharp peak was observed at 600nm while at pH 9, sharp SPR peak was observed at 400 nm. The highest absorbance was observed at pH7. The UV-Visible spectra of BAC7-SNPs at pH4, pH 7 and pH 9 had a broad spectrum between 400nm and 800nm. BAC7-SNPs at pH7 had a sharp peak at 200 nm (Figure 6b). The UV-Visible spectra of BAC20-SNPs biosynthesized at different pH is shown in Figure 5c. BAC20-SNPs had highest sharp SPR peak at 600nm. The SNPs biosynthesized at pH 4 and 7 has a broad band between 600 nm and 800 nm.

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467 Figure 6(a- c): UV-Visible spectra of SNPs biosynthesized at different pH by *Bacillus*
 468 spp. A), BAC1-SNPs, B) BAC7-SNPs and C) BAC20 SNPs.

469 The optimum pH for the production of SNPs by *Bacillus* spp. fermentation broth was at
 470 pH 7. Goldie Oza *et al.* [46] reported the optimum pH for extracellular fabrication of
 471 silver nanoparticles using *Pseudomonas aeruginosa* was pH10. Annadurai *et al.* (47)
 472 reported that the alkaline pH was more suitable for SNPs synthesis by *Coleus*
 473 *aromaticus*. Amin *et al.* (45) reported more SNP biosynthesized under basic conditions

474 while the formation of SNPs was repressed by acidic conditions and at lower pH (pH 5),
475 larger nanoparticles were formed, whereas, at higher pH (pH 9), smaller and highly
476 dispersed nanoparticles were formed.

478 Antimicrobial potential of AgNO₃ and SNPs biosynthesized at different pH against MDR-
479 *Salmonella* spp.

480 The antimicrobial activity of the SNPs biosynthesized at different pH against the MDR-
481 SA9 is shown in Table 3a. All the AgNO₃ and the SNPs had antimicrobial activity
482 against MDR-SA9.

483 The antibacterial activity of BAC1-SNPs, BAC7-SNPs and BAC20-SNPs at pH 4, pH7
484 and pH 9 against MDR-SA9 ranged from 8 -16 mm, 4 -14 mm and 4- 14mm. BAC1-
485 SNPs produced at pH 9 and BAC7-SNPs and BAC20-SNPs produced at pH7
486 exhibited the highest antagonistic activity against MDR-SA9.

487 Antimicrobial activity of the SNPs biosynthesized at different pH by *Bacillus* spp. against
488 MDR-SA18 is shown in Table 3b. The SNPs had antimicrobial activity against MDR-
489 SA18 at different pH. The antimicrobial activity of BAC1-SNPs, BAC7-SNPs BAC20-
490 SNPs against MDR-SA18 ranged from 4.0 – 9.0 mm, 4.0 – 9.0 mm and 3.0 - 10 mm.
491 The highest activity was recorded at pH 7. There was no zone of inhibition at pH 9 by
492 BAC1-SNPs and BAC7-SNPs against MDR-SA18 at 24 hrs of incubation.

493 Table 3a: The antimicrobial potential of AgNO₃ and SNPs biosynthesized at different pH
494 against MDR-SA9.

Samples	Antimicrobial activity (mm)					
	BAC1-SNPs SA9		BAC7-SNPs		BAC20-SNPs	
	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs
SNPs pH4	10.0±1.0 ^a	8.0±1.0 ^b	4.0±0.0 ^d	4.0±0.0 ^d	7.0±0.3 ^c	4.0±1.0 ^d
SNPs pH7	12.0±0.5 ^b	10.0±0.0 ^b	14.0±1.0 ^a	12.0±0.5 ^b	14.0±0.5 ^a	11.0±0.0 ^{bc}
SNPs pH9	14.0±0.2 ^b	16.0±0.2 ^a	12.0±0.3 ^c	9.0±0.4 ^d	6.0±0.0 ^f	8.0±0.5 ^e

495 Values are Means ± Standard Deviations of triplicate observations. Means with different
496 alphabets across the row respectively are statistically significant at p≤0.05.

497 Table 3b: The antimicrobial potential of AgNO₃ and SNPs biosynthesized at different pH
498 against MDR-SA18.

Samples	Antimicrobial activity (mm)					
	BAC1-SNPs		BAC7-SNPs		BAC20-SNPs	
	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs
SNPs pH4	4.0±0.0 ^c	6.0±0.5 ^b	7.0±0.2 ^a	4.0±0. ^c	2.0±0.2 ^d	6.0±0.2 ^b
SNPs pH7	9.0±0.4 ^b	8.0±0.00 ^c	6.0±0.0 ^d	9.0±0.2 ^b	6.0±0.50 ^d	10.0±0.4 ^a
SNPs pH9	0.0±0.0 ^d	3.0±0.2 ^c	0.0±0.0 ^d	4.0±0.0 ^b	5.0±0.2 ^a	3.0±0.27 ^c

Values are Means ± Standard Deviations of triplicate observations. Means with different alphabets across the row respectively are statistically significant at $p \leq 0.05$.

Effect of different concentrations of AgNO₃ on the SNPs biosynthesis

The effect of different concentrations (2 - 10mM) of silver nitrate (AgNO₃) solutions on biosynthesis of SNPs was evaluated and was monitored with UV-Visible spectrophotometer. Figure 7a shows the spectra of BAC1-SNPs produced at different concentrations of silver nitrate. SPR peak was observed at 400nm for *Bacillus* sp. BAC1 SNPs produced with 8mM. The SNPs produce with 2mM and 4mM of AgNO₃ had abroad band between 400nm and 800nm. The SNPs produced with 10mM AgNO₃ a strong SPR peak at 600nm.

The spectra obtained for BAC7 SNPs produced with different concentrations of AgNO₃ was shown in Figure 7b. A broad band spectrum between 400nm and 600nm was observed for the SNPs synthesized with 2mM, 6mM and 8mM concentrations of AgNO₃. The SNPs produced with the 10mM AgNO₃ has a strong peak at 600nm. The 10mM AgNO₃ concentration had the highest OD.

The effect of concentration on biosynthesis of *Bacillus* sp. BAC20 SNPs was presented in Figure 7c. The SNPs produced with 2Mm to 10mM AgNO₃ concentration all had a broad band spectrum between 400nm and 800nm. A SPR peak of 600nm was observed for 2mM, 4mM and 6mM. The highest OD was shown by the SNPs produced with 10mM at 500nm.

Effect of different concentrations of AgNO₃ on the SNPs biosynthesis

522

523

524 Figure 7a: UV-Visible spectra of BAC1 SNPs at different concentrations of AgNO_3

525 Figure 7b: UV-Visible spectra of BAC7 SNPs at different concentrations of AgNO_3

526

527 Figure 7c: UV-Visible spectra of BAC20 SNPs at different concentrations of AgNO_3

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530

531 **AgNO₃**

532 The antimicrobial activity of the SNPs biosynthesized by *Bacillus* spp. using different
533 concentration of AgNO₃ is shown in Table 4a - b.

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All the SNPs produced by *Bacillus* spp. using different concentration of AgNO₃ had antagonistic activity against the isolates. The antimicrobial activity of BAC1-SNPs, BAC7-SNPs and BAC20-SNPs against MDR-SA9 ranged from 6-24 mm, 2 -25 mm and 2 – 20 mm at 24, 48 and 72 hrs of incubation. 10mM AgNO₃ had the highest antagonistic activity (15 mm) against SA9.

The antimicrobial activity of BAC1-SNPs, BAC7-SNPs and BAC20-SNPs against MDR-SA18 ranged from 2.0 – 15 mm, 2.0 -17 mm and 2.0 -21 mm. The SNPs from the *Bacillus* spp. had the highest activity against MDR-SA18 at 10mM concentration of AgNO₃.

Table 4a: Antimicrobial potential of SNPs biosynthesized using different concentration of AgNO₃ against MRD-SA9 at different incubation time

Zone of inhibition (mm) / Incubation time (hrs) against MDRSA9									
Concentrations of AgNO ₃ .	BAC1-SNPs			BAC7-SNPs			BAC20-SNPs		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hr	72hrs
2mM	6.0±0.5 ^e	8.0±0.0 ^d	8.0±0.1 ^d	4.0±0.2 ^f	8.0±0.0 ^d	14.0±0.0 ^a	12.0±0.3 ^b	6.0±0.0 ^e	10.0±1.0 ^c
4mM	8.0±0.0 ^f	20.0±1.0 ^a	12.0±0.0 ^d	14.0±0.45 ^c	10.0±1.0 ^e	12.0±1.0 ^d	18.0±0.0 ^b	10.0±0.0 ^e	8.0±0.0 ^f
6mM	12±0.53 ^d	17.0±0.3 ^b	16.0±1.0 ^c	2.0±0.0 ^g	10.0±0.5 ^e	16.0±0.5 ^c	4.0±0.10 ^f	10.0±0.0 ^e	18.0±0.5 ^a
8mM	6.0±0.6 ^f	16.0±0.0 ^c	18.0±0.53 ^b	6.0±0.2 ^f	12.0±0.2 ^e	14.0±0.3 ^d	2.0±0.0 ^g	20.0±1.0 ^a	12.0±1.0 ^e
10mM	10.0±0.0 ^g	20.0±1.0 ^d	24.0±0.25 ^b	6.0±0.0 ^h	22.0±1.0 ^c	25.0±0.0 ^a	17.0±0.6 ^e	15.0±0.6 ^f	20.0±1.0 ^d

Values are Means ± Standard Deviations of triplicate observations. Means with different alphabets across the row respectively are statistically significant at p≤0.05.

Table 4b: Antimicrobial potential of SNPs biosynthesized using different concentration of AgNO₃ against MRD-SA18 at different incubation time

Concentrations of AgNO ₃ .	Zone of inhibition (mm) / Incubation time (hrs) against MDRSA18								
	BAC1-SNPs			BAC7-SNPs			BAC20-SNPs		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
2mM	3.0±0.0 ^d	4.0±0.3 ^c	0.0±0.0 ^e	4.0±1.0 ^c	4.0±1.0 ^c	7.0±0.2 ^a	0.0±0.0 ^e	6.0±0.0 ^b	0.0±0.0 ^e
4mM	5.0±0.5 ^c	0.0±0.0 ^f	5.0±0.0 ^c	2.0±0.6 ^e	3.0±0.0 ^d	9.0±0.4 ^b	10.0±0.0 ^a	10.0±0.2 ^a	9.0±0.0 ^b
6mM	0.0±0.0 ^e	10.0±1.0 ^b	12.0±1.0 ^a	0.0±0.0 ^e	8.0±0.5 ^c	10.0±0.5 ^b	2.0±1.0 ^d	0.0±0.0 ^e	0.0±0.0 ^e
8mM	7.0±0.0 ^e	12.0±0.2 ^a	9.0±0.4 ^c	8.0±1.0 ^d	4.0±0.1 ^f	4.0±0.1 ^e	8.0±0.3 ^d	12.0±0.0 ^a	10.0±1.0 ^b
10mM	13.0±0.6 ^e	15.0±0.0 ^c	14.0±1.0 ^d	3.0±0.0 ^h	12.0±1.0 ^f	17.0±0.0 ^b	8.0±1.0 ^g	12.0±0.0 ^f	21.0±0.3 ^a

Values are Means ± Standard Deviations of triplicate observations. Means with different alphabets across the row respectively are statistically significant at p≤0.05.

Synergistic effect of the biosynthesized SNPs and some antibiotics against MDR *Salmonella* spp.

The synergetic effect of the biosynthesized SNPs and some antibiotic against MDR *Salmonella* sp. SA9 and MDR *Salmonella* sp. SA18 is shown in Figure 8 a-b.

The MDRSA9 shows susceptibility to antibiotics with antagonistic potential ranged between 3.0 – 20mm. Colistin had the lowest antagonistic activity while chlorotetracycline had the highest activity.

The synergetic effect of the biosynthesized SNPs and some antibiotic against MDR-SA9 is shown in Figure 8a . The antagonistic potential of BAC1 SNPs + the antibiotics ranged from 10 – 33 mm. BAC1 SNPs + Erythromycin had the highest antagonistic activity against MDR-SA9 while BAC1 SNPs + Oxytetracycline had the least activity.

564 The antagonistic potential of BAC7-SNPs + antibiotics against MDR-SA9 ranged from
565 6.0 – 30 mm. BAC7-SNPs + Gentamicin had the highest activity while BAC7 SNPs +
566 Colistin had the lowest antagonistic activity. The antagonistic activity of BAC20-SNPs +
567 antibiotics against MDR-SA9 ranged from 5.0 – 31 mm. BAC20 + Chlorotetracycline
568 had the highest antagonistic potential while BAC20-SNPs + Colistin had the least
569 activity.

570 The synergistic effect of the SNPs and the antibiotics against the MDRSA18 is shown in
571 Figure 8b. The antagonistic activity against MDR-SA18 ranged from 2.0 – 18 mm.
572 Colistin had the least activity while antibiotics Gentamicin had the highest activity
573 against MDR-SA18

574 The antagonistic activity of BAC1 SNPs + the antibiotics against MDR-SA18 ranged
575 from 9.0 – 20 mm showing a high susceptibility. MDR-SA18 had the highest
576 susceptibility to BAC1 SNPs + Ciprofloxacin while the least susceptibility was to BAC1
577 SNPs + colistin.

578 The BAC7 SNPs + antibiotics had antibacterial activity ranged from 10 – 24mm against
579 MDR-SA18. BAC7 SNPs + Gentamicin have the highest activity against MDR-SA9. The
580 antagonistic activity of BAC20 SNPs + antibiotics against MDR-SA18 ranged from 6.0 –
581 26 mm. BAC20 + Erythromycin had the highest antagonistic potential while BAC20
582 SNPs + Colistin had the least.

583 The antagonistic potential of the combined BAC1- SNPs and the antibiotics against the
584 MRDSA9 and MDRS18 is shown in Table 4a -b. The antagonistic activity against the
585 MRDSA9 and MRDSA18 ranged from 3.0 -33 mm and 2.0 -20 mm. MRDSA9 was
586 highly susceptible to BAC1-SNPs +Erytromycins

Figure 8a: Diameter of Zones of Inhibition in (mm) of the of the SNPs, antibiotic and combination of SNPs + antibiotics against the MDR-SA9

Figure 8b: Diameter of Zones of Inhibition (mm) of the SNPs, antibiotic and combination of SNPs + antibiotics against the MDR-SA18

596 It was found that the silver nanoparticles produced from *Bacillus* sp. enhanced the
597 reaction rates of the antibiotics in a synergistic mode as well as in its own way on these

598 pathogens. In both the cases of *Salmonella* spp. (SA9) and *Salmonella enterica sub*
599 *enterica* (SA18) the highest zone of inhibition was found in the combination of antibiotics
600 + silver nanoparticles. This result is in agreement with the findings of Krishna *et al.* ⁴⁸
601 who mentioned increasing efficacies (percentage) of antibiotics like streptomycin and
602 ceftriaxone when used in combination with silver nanoparticles against *Bacillus subtilis*,
603 *S. aureus*, *E. coli* and *Proteus vulgaris*.

604 Shahnaz and Anima ⁴⁹, reported that *in vitro* antibacterial activity of silver nanoparticles
605 synthesized from *Penicillium* species were carried out in combination with the
606 Sparfloxacin and Ofloxacin antibiotics against clinically isolated pathogens
607 *Staphylococcus aureus*, *Bacillus cereus*, *E. coli*, and *Proteus vulgaris*. It was found that
608 the SNPs produced by *Penicillium* species enhanced the reaction rates of the antibiotics
609 in a synergistic mode on the clinically isolated pathogens.

610 The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of
611 the SNPs that can have antimicrobial effect on the test organisms. 10mM was used to
612 prepare the different concentrations of the SNPs for the MIC evaluation, different MICs
613 were recorded for the two (MDR) *Salmonella* spp. Balashanmugam *et al.* ³⁰ also
614 reported the MIC of SNPs after testing on some pathogens with *Escherichia coli* and
615 *Bacillus subtilis* having the highest values.

616 CONCLUSION

617 The bioactive metabolites from the *Bacillus* spp. had antibacterial activity against the
618 selected MDR *Salmonella* spp. The bioactive metabolites were able to bio-reduced
619 AgNO₃ for SNPs biosynthesis. These biologically synthesized SNPs exhibited good
620 antibacterial activity and the combination of SNPs with some antibiotics equally
621 enhances the antibacterial potential of the antibiotics against the tested isolates. Thus,
622 these nanoparticles can be used as antibacterial agents alone or in combination with

623 antibiotics to improve their antibacterial potential.

624

625 **Disclosure of potential Conflict of Interest**

626 The authors declared no conflict of interest.

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Conflict of Interest

None

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