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Therapeutic use of biologically produced sulfur nanoparticles from *Allium fistulosum* against antibiotic-resistant foodborne pathogens

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Abstract. *Saddiqua A, Ullah S, Khan MS, Rehman S, Abubakar AA, Safdar K, Ali S, Javaria S, Kanwal A, Batool SN, Bhatti F, Sucipto TH, Ansori ANM. 2024. Therapeutic use of biologically produced sulfur nanoparticles from* Allium fistulosum *against antibiotic-resistant foodborne pathogens. Biodiversitas 25: 2348-2354.* Food spoilage is a significant issue since foodborne illnesses affect millionsworldwide. The main root cause of food spoilage is the presence of microbes, some of which show resistance to commercially available antibiotic drugs. Therefore, it is essential to search for fresh and effective antibacterial medications. Nanotechnology can give solutions to fight against foodborne pathogens. Sulfur nanoparticles show many beneficial and effective results as antimicrobial agents. The aim of this study was to evaluate the efficacy of biologically synthesized sulfur nanoparticles from *Allium fistulosum* to inhibit antibiotic-resistant foodborne pathogens. The green synthesis of sulfur nanoparticles was done by combining plant leaf extract with sodium sulfide. This study used the plant *Allium fistulosum* to make sulfur nanoparticles. The characterization of SNPs was done through a scanning electron microscope, UV spectrophotometer, fourier transform infrared spectrometer, and nanoparticle tracking analysis. Then, the antimicrobial properties of the bioengineered sulfur-based nanoparticles against foodborne pathogens were checked alone and in combination with market-available antibacterial agents. *Aspergillus flavus* and *Salmonella typhi* were among the foodborne pathogens against which SNPs' in-vitro antibacterial activity was tested. Sulfur nanoparticles from *Allium fistulosum* had 291 nm absorption spectra showing to be almost 100 nm in size and had a spherical shape. The strongest antibacterial efficacy against *S. typhi* was observed by SNPs (24 mm). Synergistic antibacterial action was seen when nanoparticles were combined with antibiotics of commercial importance. A noticeable antifungal effect against *A. flavus* was observed by combining SNPs with amphotericin B. The findings of in vitro studies prove that novely designed sulfur nanoparticle have profound impacts on microorganisms. It was also observed that the effect of antibiotics like ampicillin and amphotericin B was enhanced when coupled with nanoparticles.

Keywords: *Allium fistulosum, Aspergillus flavus,* foodborne pathogens, *Salmonella typhi,* sulfur nanoparticles

Abbreviations: SNPs: Sulfur Nano-Particles; SEM: Scanning Electron Microscopy; FTIR: Fourier-Transform Infrared Spectroscopy; NTA: Nanoparticle Tracking Analysis; TTC: 2,3,5-Triphenyl Tetrazoliumchloride; MIC: Minimum Inhibitory Concentration

INTRODUCTION

In the US and around the globe, foodborne illness is still a major problem and challenge. In accordance with the report of the CDC (Centers for Disease Control and Prevention) of the US, forty eight million Americans, or 1-6 of the population, fall ill each year, 128,000 require hospitalization, and 3000 pass away from foodborne illnesses (Oliver 2019). There is a link between the food we eat and disease in people. Foodborne diseases are caused by foodborne pathogens, such as bacteria, viruses, and parasites. A foodborne disease outbreak is the occurrence of the same disease in more than one person, spreading by eating a common meal.

Foodborne disease arises through the spoilage of food established (often reproduces) in the body of human host or when a disease-causing pathogen is introduced in a food product and produces a toxic agent that the human host then absorbs. The two main categories under which diseases appear are foodborne diseases and foodborne infections.

Foodborne illnesses specifically take much longer time than foodborne intoxications to manifest symptoms after consumption due to the involvement of an incubation period (Bintsis 2017). World Health Organization (WHO), reported that 1.8 million children are estimated to die from diarrhea each year. A large portion of juvenile diarrhea is spurred on by germs that are frequently consumed through contaminated food or water. Humans can contract these infections in several ways, such as consuming infected food, touching live animals, or being exposed to a polluted environment. Foodborne illnesses can impact a large number of foods and products, and food has a major impact on the transmission of a considerable part of these infections. Over the past few decades, several nations have launched effective interventions to prevent and manage foodborne illnesses (Gupta et al. 2016).

Unconventional packaging manufacturing is a growing field of research. Advanced packaging of food can greatly reduce the chances of food spoilage by microorganisms and extend the retaining life of goods. Utilizing nanotechnology offers fresh chances to investigate how bactericidal nanoparticles with strong bioactivity work (Anvar et al. 2021). Nanotechnology is the knowledge and use of nano-size (ranging from 1 to 100 nm) materials and structures. The word "nano" approximates one billionth of anything (Sujithra and Manikkandan 2019). Nanotechnology provides total food management, from production to processing and packaging. Nanomaterials greatly enhance food's nutritional value, safety, and overall quality. In food science, numerous companies, academic institutions, and organizations are creating new methods, products, and strategies (Singh et al. 2017). Food shelf life has been extended by using nanocomposite and nanolaminates in packaging as a barrier against extreme heat and mechanical damage. Applications for nanoparticles go beyond antibacterial food packaging (Fatima et al. 2021).

Recently, sulfur nanoparticles have drawn much interest because of their distinctive functional characteristics. Sulfur Nanoparticles (SNPs) are mainly applied as photocatalytic and antibacterial agents. SNPs exhibit more particular characteristics than their bulk counterparts because of their nanoscale size and large area of surface. Although there are many ways to make SNP, the green synthesis approach is recommended since it is more environmentally friendly and has superior water solubility (Roy and Rhim 2022). SNPs produced with green technology are recognized for being non-toxic and biocompatible when used to create active packaging films (Dhuldhaj et al. 2012; Roy and Rhim 2022). Shankar and Rhim (2018) claimed that SNPs have not been used extensively in developing food packaging or packaging materials. As was demonstrated sulfur nanoparticles can be utilized in food antimicrobial packaging films. They produced chitosan- based nanocomposite films containing a variety of SNP types. In another study, they found that SNPs improved the chitosan film's hydrophobic, mechanical and water vapor barrier properties and antibacterial activity. SNPs can be used to make excellent food packaging materials to improve the food quality and the shelf life of packaged food items (Shankar et al. 2021).

The main focus of the this study was to establish a novel green method for the synthesis of sulfur nanoparticles using *Allium fistulosum* and to assess their efficacy against antibiotic-resistant foodborne pathogens in ecofriendly way.

MATERIALS AND METHODS

Material used

Sodium sulfide (Na_2S) was purchased from Sigma Aldrich, USA. Dow Chemical Company, Germany, used to prepare sulfur powder. None of the compounds were further purified and were all analytical grades. Hot air oven, UV-Visible Spectrophotometer (Shimadzu 1601, Japan), Scanning Electron Microscopy (SEM) (JSM-IT 100), Fourier-Transform Infrared Spectroscopy (FTIR) (THERMO NICOLET 730, USA), Nanoparticle Tracking Analysis (NTA) (LM-20, NanoSight Pvt Ltd, UK) were used during the experiments.

Preparation of extract

Fresh, mature leaves of *Allium fistulosum* (green onion) were selected and cleaned with water to make them dust-free. 10 g of the leaves were boiled with distilled water (100 mL), then kept at room temperature for cooling. The mixture was filtered through filter paper, and centrifuged at 4,000 rpm for 30 minutes. Next, sulfur nanoparticles were made using the filtrate that was produced.

Biosynthesis of sulfur nanoparticles using plant leaf extract

For the biosynthesis of sulfur nanoparticles, the powdered sulfur was properly crushed in a mortar and pestle. The powdered sulfur was transferred to a flask containing 50 mL sodium sulfuric acid (1 M). The temperature of the reaction mixture was up to 100°C, and it was stirred continuously until the sulfur dissolved. If the pale yellow color of the solution changed to reddish-orange, sodium polysulphide was formed. Following this, leaf extract was mixed in a 1:4 ratio with the sodium polysulphide solution while constantly stirring. Then, concentrated H_2SO_4 was added dropwise for the precipitation of sulfur nanoparticles. Centrifugation at 7,000 rpm for 45 min sedimented the suspended sulfur nanoparticles and then washed with distilled water to make it free of biological remnants. In a hot air oven, the final form of sulfur nanoparticles was dried at 80°C.

Characterization of synthesized sulfur nanoparticles

UV-Vis spectroscopy

UV-Vis spectrophotometer (200-800 nm range), was used to analyze the biologically reduced sulfur nanoparticles.

Fourier-transform infrared spectroscopy

The functional groups of bio-synthesized SNPs were analyzed using Thermo Nicolet 730 (USA) FTIR.

Nanoparticle tracking analysis

The size of sulfur nanoparticles was measured by using the NanoSight LM-20. In order to closely examine the liquid-

medium suspended particulates near to the optic component, the observing chambers of the LM-20 laser-based dispersion instrument were filled with them. A customized non-microscope light instrument (LM-20, NanoSight Pvt Ltd, UK) was used to examine the brownian movement of tiny particles in a given direction of the beam of laser light. After particulate shifts in the area of vision (100100 m) were observed at thirty frames per second, representative photos and movies were captured.

Scanning electron microscopy

Scanning Electron Microscopy (SEM) was performed using a Philips model CM 200 apparatus to determine the size and morphological structure.

Assessment of antimicrobial activity

The GCBB bacterial bank, Gomal University, D.I. Khan, Pakistan, supplied *Salmonella typhi* primary strain (ATCC 518812), which was utilized as a test microorganisms in foodborne illnesses. *Aspergillus flavus* was isolated from peanuts (DBT4).

In vitro evaluation of antimicrobial activity by well diffusion method

The Kirby-Bauer disc diffusion method was used to assess synthetic SNPs against *S. typhi* (ATCC 51812) (Khairan et al. 2019). The antibacterial potential of *S. typhi* was examined on a Muller-Hinton agar plate. After inoculation into Muller Hinton Broth, *S. typhi* was cultivated there for 24 hours at 37°C before streaking on the plates of agar media. To measure the effectiveness of sulfur nanoparticles, a primary pore was filled with 20 µL of the reaction mixture that is retained on the plates of agar and then incubated at 37°C for 24 hours. The antibacterial effect of synthesized nanoparticles in conjunction with antibiotics has also been studied. The millimeter-sized inhibitory zone analysis was carried out simultaneously.

The potato dextrose agar was used to evaluate the antifungal activity of nanoparticles against *A. flavus*. Colony-Forming Units (CFU) of seven days older fungus biomass, ranging from 0.4 to 104 CFU/mL, were inoculated to RPMI (Rosewell Park Memorial Institute media) 1640 (mammalian culture media) (CLSI 2019). The agar plates were spread with 20 milliliters of the spores suspension. The inhibition zones were assessed following a 48-hour incubation period at 28°C with the well on the agar surface. The synthesized nanoparticles were combined with drugs like fluconazole and amphotericin B to investigate their inhibitory abilities against agonist fungi. The experiments were repeated three times. The test was repeated three times.

Evaluation of Minimum Inhibitory Concentration (MIC)

The nano broth dilution approach was used to calculate the MIC of SNPs. The nanoparticle concentration range used to calculate the MIC value was 50 to 1000 mg/mL. Muller-Hinton culture was employed to determine the bacterium's Minimum Inhibitory Concentration (MIC) for *S. typhi*. On plates with 96 wells, 180 µL of Muller-Hinton broth was then poured into each well. Next, the identical well was filled with 20 µL of the nanoparticles mixture and 20 µL of every culture of bacteria. At 37°C, the dishes were left to incubate for 24 hours. Each of the wells was given 40 µL of Triphenyl Tetrazolium Chlorides (TTC) at a concentration of 0.2 mg/mL, and MIC values were then calculated. The Minimum Inhibitory Concentration (MIC) of microscopic particles versus *A. flavus* was determined using a plate with a microtiter and the micro dilute broth technique. Fungi were inoculated and cultured in RPMI medium for 7 days at 252°C. With a fungus load of 4-104 to 5-104 CFU/mL, the absorbance at 530 nm was measured using a colorimeter. By employing the RPMI substrate, the density of light of fungal suspension was adjusted to be between 0.15 and 0.17. The purpose of this solution was to gauge the nanoparticles' activity. Additionally, the MIC value of nanoparticles was calculated using 10-80 mg/mL range (Heithoff et al. 2023).

RESULTS AND DISCUSSION

Conformation of sulfur nanoparticle

The biological sulfur nanoparticles showed a color change. After the synthesis, the Spring Onion's colorless solution turned yellowish turbid. The yellowish-colored precipitates were SNPs produced by reacting hydrochloric acid with sodium thiosulphate solution.

$$Na_2S_2O_3 + 2HCl \longrightarrow 2NaCl + S + SO_2 + H_2O$$

UV spectrophotometer

An optical analytic technique utilized for SNP preliminary detection is UV spectroscopy. The highest absorption was reached at 291 nm for the green-derived SNPs (Figure 1).

FTIR spectrophotometer

The significant absorption spectrum of small particles of *Allium fistulosum* was at 3371.01 cm-1, indicating a bending spectrum of hydroxyl (-OH) bending H-bonded alcohol. The large peak values of absorption at 2136.31 cm-1 were caused by asymmetrical stretching of the alkyne functional groups. The presence of the amide containing a carbonyl group was indicated by a sharp peak at 1636.90 cm-1. The amide linkage's N-H bending modes of resonance gave rise to this amide spectrum. The additional peaks at 552 cm-1 were due to the bending modes of aromatic molecules (Figure 2).

Nanoparticle Tracking Analysis (NTA)

The produced nanoparticles were analyzed using Nanoparticle Tracking Analysis (NTA) (LM20 Limited, UK). This characterization was done to determine their concentration and average size. The SNPs were discovered to be 3-106 particles/mL and 53±49 nm in size (Figure 3).

Scanning electron microscope

Results of SEM showed that SNPs had a 100 nm size and were spherical (Figure 4).

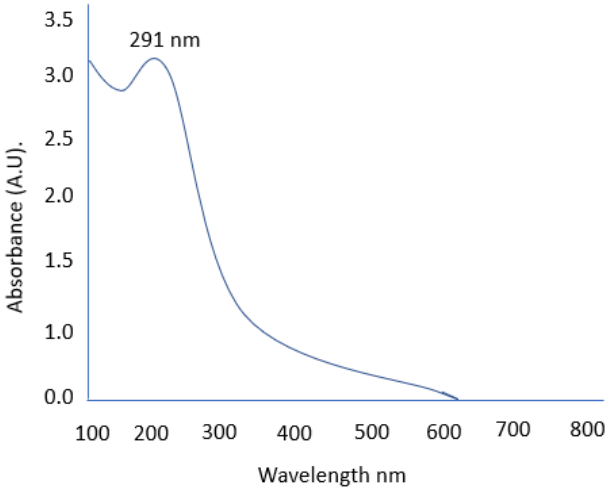

Figure 1. UV of SNPs of *Allium fistulosum*

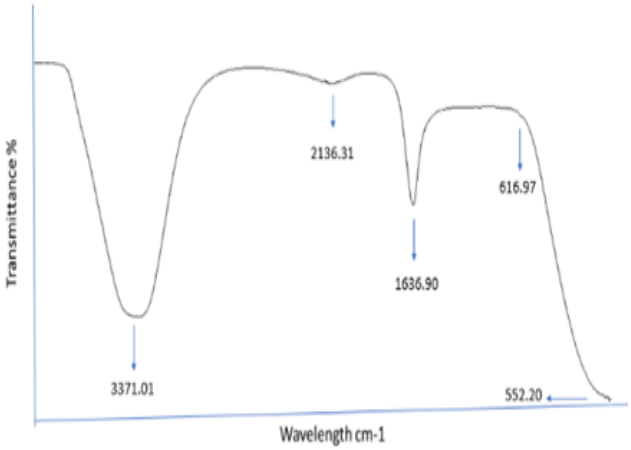

Figure 2. FTIR spectra of SNPs of *Allium fistulosum*

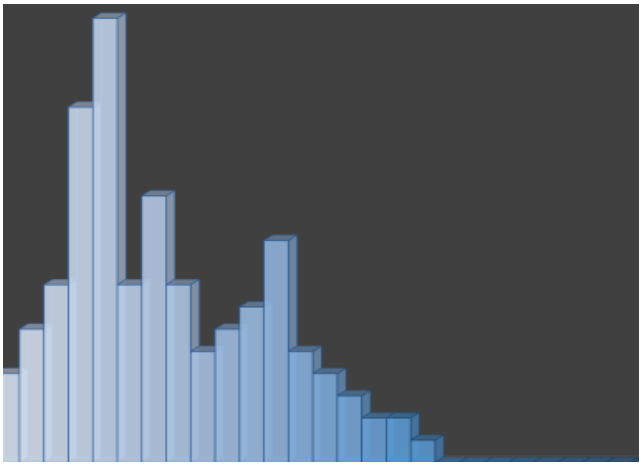

Figure 3. NTA of SNPs *Allium fistulosum*

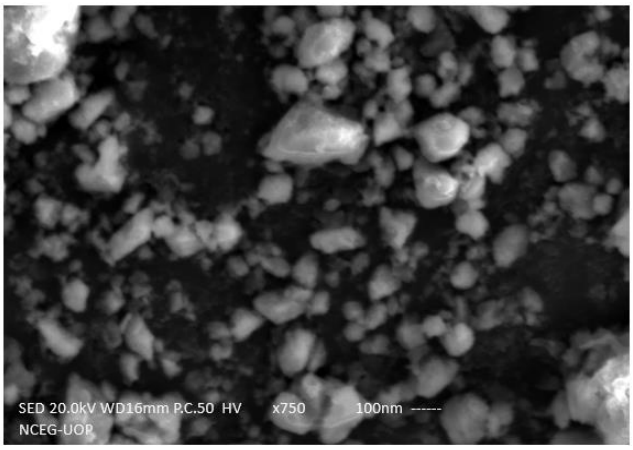

Figure 4. SEM of SNPs of *Allium fistulosum*

Antimicrobial and antifungal activity of nanoparticles

SNPs were examined for their antibacterial effectiveness against *S. typhi* and *A. flavus*. Results revealed that SNPs significantly affected both test microorganisms. SNPs were similar to certain *A. flavus* in action versus *S. typhi* (21 mm was the region of resistance). SNPs' antibacterial efficacy was also assessed in combination with antibiotics that were readily accessible on the market, like amaciline and amphotericin B. SNPs combined with amphotericin B had the strongest antifungal effect against *A. flavus* (Figure 5.A). It was observed that the existence of SNPs to inhibit *S. typhi* can improve the antibacterial activity of commercially available medicines (Figure 5.B). Synergistic antibacterial effect was shown when nano-particles were used with all currently accessible antibiotics.

TTC (triphenyltetrazolium chloride- a dye) was inserted into a 96-well plate after the MIC of the bacterium was determined. The microtiter plate's pink appearance was caused by a decrease in TTC, and the development of red color complex formazan indicated that bacteria were alive and actively growing there. The wells became discolored and appeared colorless due to inhibition of bacterial growth, this may be due to the presence of nanoparticles. The lowest SNP MIC was determined in *S. typhi* (250 mg/mL) (Figures 6.A and 6.B).

Discussion

Many health hazard within the human body are caused by disease-causing pathogens arising through spoilage of food or after the introduction of these pathogens in food products. These pathogens show antibiotic resistance towards many conventional antibiotic agents. Therefore, to overcome this issue, there is a need to develop novel outline of antibiotic components in the emergning era of nanobiotechnology. Keeping in view the effectiveness of sulfur nanoparticles against different bacterial and fungal strains, recent research idea was initiated to synthesize sulfur nanoparticles biologically from *Allium fistulosum* and then evaluate their efficacy against foodborne pathogens.

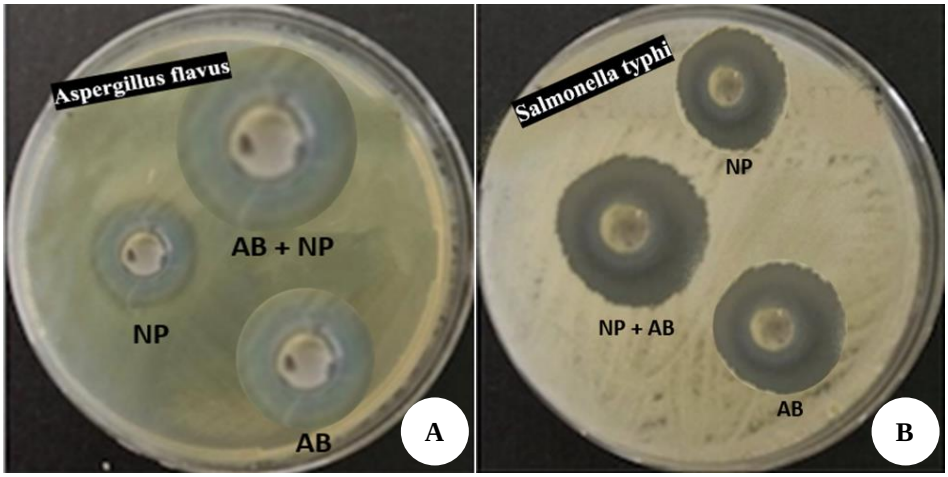

Figure 5. A. Antifungal effect of SNPs of *Allium fistulosum* against *Aspergillus flavus*; B. Antimicrobial activity of SNPs of *Allium fistulosum* against *Salmonella typhi*

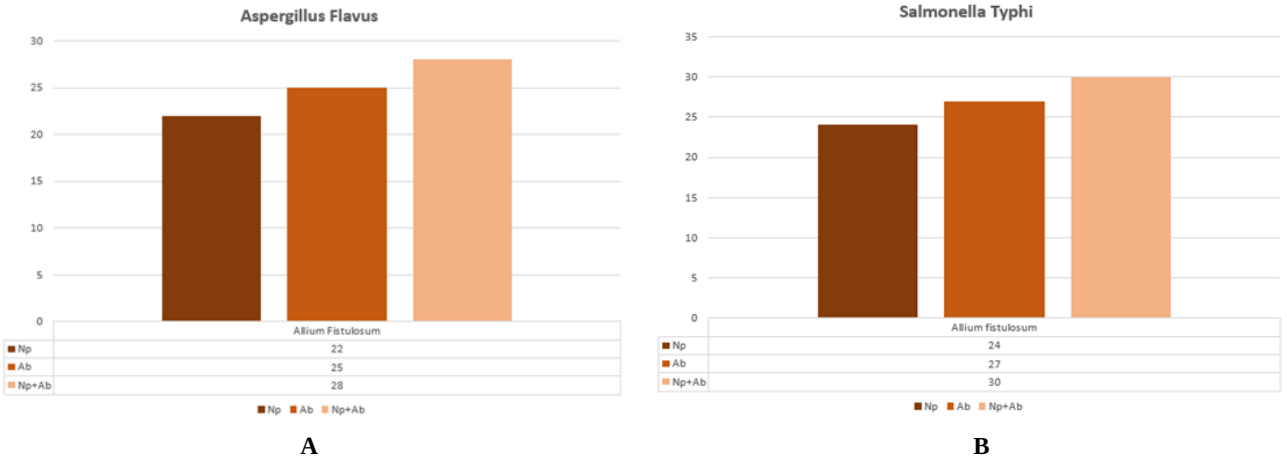

Figure 6. A. Antifungal activity of SNPs *Allium fistulosum* against *Aspergillus flavus*; B. Antimicrobial activity of SNPs of *Allium fistulosum* against *Salmonella typhi*

Converting the colorless solution of spring onion into a yellowish turbid confirmed the biological production of sulfur nanoparticles. The results of this study are found to be similar to Awwad et al. (2015) findings, they used the fruity ingredient of *Albizia julibrissin* to generate SNPs. When hydrochloric acid was used to treat sodium thiosulphate solution, the reaction combination experienced an excessive amount of reactivity, which caused SNPs to precipitate and sulfonic acid to be produced.

Peak absorption was observed at 291 nm for SNPs by UV spectroscope. Jaiswal et al. (2019) demonstrated that SNPs' highest absorbance occurred from 280 to 300 nm. Again, Paralikar and Rai (2018) documented that SNPs from different medicinal plants (*P. longifolia, A. indica, M. indica,* and *C. roseus*) showed absorption peaks at 294, 292, 296, and 292 nm, respectively (Paralikar and Rai 2018).

The major peak values of *Allium fistulosum* extract coincide with those of *Albizia Julibrissin* fruit substance in the FTIR spectrum of SNP (Awwad et al. 2015). The formation of SNPs of *Allium fistulosum* was confirmed by comparing the major peaks with Nivetha *Allium cepa* FTIR (Thampi and Shalini 2015).

Both the concentration and mean size of the generated tiny particles were ascertained using Nanoparticle Tracking Analysis (NTA). The mean size of the SNPs was 53±49 nm and 3-106 particles/mL. Brownian's motion and size dispersion of the particulate as observed by the apparatus are necessary for NTA analysis (Tiwari et al. 2017).

The form as well as the patterns of the spherically shaped and 100 nm-sized SNPs were also determined by SEM. These findings closely resembled the data recorded by Paralikar and Rai (2020), research demonstrated that sulfur nanoparticles made from *Ficus benghalensis* plant leaves had a sphere-like form and particle sizes that vary from around 25 to approximately 120 nm (Paralikar and Rai 2020).

The antibacterial efficacy of SNPs was found to be more effective against *S. typhi* and *A. flavus* than the commercially available antibiotics. Suleiman et al. (2015) reported that SNPs targeting *S. aureus* had a significant

degree of action, with *E. coli*, *P. aeruginosa*, and *Aspergillus* sp. SNPs are highly efficient versus a variety of Gram-positive and Gram-negative bacteria that are important in both medical and agricultural fields (Suleiman et al. 2015). Recently, Kim et al. (2020) also reported the antibacterial activity of SNPs with MIC at 10 mm for *S. aureus.*

SNPs' antibacterial efficacy was also assessed in combination with antibiotics that are readily accessible on the market, like amaciline and amphotericin B. SNPs combined with amphotericin B had the strongest antifungal effect against *A. flavus*. It was discovered that the presence of sulfur nanoparticles improved the antibacterial activity of commercially available medicines that have activity against *S. typhi*. Synergistic antibacterial action was seen when nanoparticles were combined with antibiotics of commercial importance. The findings of this study are consistent with Suryavanshi et al. (2017), who reported that SNPs showed excellent efficacy towards *Salmonella typhi*, monocytogenes of *Listeria*, and *Chromobacterium violaceum* when evaluated for antibacterial effects of sulfur nanoparticles. With a concentration threshold of around 21 mM against *S. typhi* and little effect versus *C. violaceum*, SNPs have antimicrobial effects. However, their effectiveness against *Listeria monocytogenes* is significantly lower, with an inhibitory zone of exactly 13 mM. Additionally, they found that a citrus medical oil comprising SNPs and nano-functionalized *Eucalyptus globulus* leaves had the best antibacterial efficacy against *S. typhi*, followed by *C. violaceum* and *L. monocytogenes*. Commercially accessible medicines, such as tetracycline, oxytetracycline, and gentamicin were used to assess the antibacterial effects of SNPs. In comparison to the effectiveness against bacteria when administered alone, the antimicrobial effects of easily accessible antibiotics against *S. typhi*, *C. violaceum*, and *L. monocytogenes* improved with the addition of SNPs. Combining conventional medicines with nanoparticles showed a synergistic antibacterial effect.

In conclusion, the results of this study showed that extracts of plants have the potential to create sulfur nanoparticles. Sulfur nanoparticles demonstrated significant antibacterial activity against foodborne pathogens such as *S. typhi* and *A. flavus*. Additionally, SNPs showed synergistic efficacy with commercially sold antibiotics, such as amphotericin B and fluconazole. In addition to antibiotics, biologically produced SNPs can be utilized as innovative antimicrobial agents to aid in preventing foodborne infections. Additionally, it can be a powerful technique to improve bioavailability. This is the first report on an eco-friendly, green method for producing sulfur nanoparticles by combining sodium polysulphide with plant leaf extract.

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