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Ocimum gratissimum Capped Sulfur Nanoparticles and Antibacterial Efficacy against Multidrug-Resistant Microbes

Owolabi M. Bankole[1,2], Oludare Temitope Osuntokun[3*], Adedapo Adeola[2] and Afolabi Owoeye[1]

[1]Centre for Bio-Computing and Drug Development (CBDD), Adekunle Ajasin University, Akungba, Nigeria.
[2]Department of Chemical Sciences, Adekunle Ajasin University, Akungba, Nigeria.
[3]Department of Microbiology, Adekunle Ajasin University, Akungba Akoko, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author OMB designed the study, synthesized the nano-particles from Ocimum gratissimum Capped Sulfur. Author OTO perfumed the antibacterial analysis of Ocimum gratissimum nano-particles on selected clinical organisms. Authors AA and AO wrote the first draft of the manuscript. Authors OMB and OTO managed the analyses of the study and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

This manuscript reports for the first time synthesis of sulfur nanoparticles prepared from thiosulphatepentahydrate ($Na_2S_2O_3 \cdot 5H_2O$) using either oxalic acid alone ($SNP-1$), or mixture of oxalic acid and aqueous solution of *Ocimum gratissimum* ($SNP-2$). The synthesized sulfur nanoparticles were obtained in satisfactory yields, and characterized with techniques such asUV-Vis, XRD, SEM, EDX, TEM, and FT-IR. Presence of capping agents: Oxalic acid and biomolecule contents of *Ocimum gratissimum* were confirmed by FTIR. Crystallinity, morphology, shapes and elemental compositions of as-prepared nanoparticles were confirmed by XRD, SEM, TEM and EDX,

**Corresponding author: E-mail: osuntokun4m@gmail.com, osuntokun4m@yahoo.com;*

respectively. Antimicrobial activities of the prepared sulfur nanoparticles against five (5) multidrug-resistant microbes were used for this research work. This included *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa and Salmonella pullorum.* The zone of inhibition of the sulfur nanoparticles tested against selected clinical isolates. *Staphylococcus aureus* was observed to have the highest susceptibility to sulfur nanoparticles ($\mathrm{SNP}-2$) mediated with *Ocimum gratissimum* plant extract with diameter of 21.0mm; *E.coli* and *Salmonella P. aeruginosa* showed resistance. All tested clinical isolates were resistant to the other sulfur nanoparticles (SNP1) synthesized in the absence of *Ocimum gratissimum* plant extract.

Keywords: Sulfur nanoparticles; Ocimum gratissimum; antibiotics; antimicrobial activity, multidrug-resistant microbes.

1. INTRODUCTION

Nanotechnology which spans across all areas of science and engineering deals with the fabrication, development, characterization and application of small-sized devices or materials whose sizes ranging from 1 - 100 nm [1,2]. The small-sized materials otherwise known as nanoparticles have intriguing features that differ biologically, chemically, and physically from the bulk materials [2]. Due to their small sizes and large active surface areas, nanomaterials have been found useful as potent antimicrobial therapies and in other biological applications [2].

Manipulation of nanomaterials for enhanced treatment of pathogenic bacteria and other infectious diseases has continued to revolutionize global human health [2]. Metallic nanoparticles have been in the forefront ofextensively reported antibacterial agents for treating bacterial infections and other multi-drug resistant pathogens [3,4]. Although many successes have been recorded with the use of metal-based nanoparticles as antibiotics, they are, however, not only harmful to the target bacteria but are also toxic to normal human cells [5]. Thus, research attention has been shifted to the use of metal-free or non-metallic nanoparticles as therapies against bacterial infections.

Sulfur nanoparticles are non-metallic nano particles and have been reported to have wide potential applications in agriculture, biomedical, and medicine [6-8]. Rhim et al. [9], demonstrated that chitosan-capped sulfur nanoparticles exhibited strong antimicrobial activities against Gram-positive and Gram-negative bacteria and also prevented the proliferation of cancer cells with a minimal toxic effect on normal cells. In another report, enhanced bactericidal and bacterial static effects of biosynthesized sulfur nanoparticles using the extracts of different medicinal plants against Gram-positive and Gram-negative bacteria have also been reported [10]. This is an indication that the antibacterial activity of sulfur nanoparticles was enhanced in the presence of active biomolecules used as capping agents. Other biologically synthesized sulfur nanoparticles with significant bioactivities have been reported in the literature [7,8,11]. Scientists and biomedical experts are investing in the biosynthetic method of preparation of nanoparticles using leaf extract of medicinal plants. Biosynthetic method has advantages over conventional chemical methods because it is eco-friendly, cost-effective, requires less reaction time, and does not produce hazardous by-products. Herein, a biosynthetic method was employed to prepare sulfur nanoparticles using leaf extract of *Ocimmum gratissimum* as a reducing and capping agent. The *O. gratissimum,* also known as scent leaf or African Basil, is a perennial herbaceous plant belonging to the family of *Lamiaceae*, and native to Africa and Southern Asia [12]. Aqueous and alcohol extracts of leaves of *O. gratissimum* have medicinal values which has been reported to possess significant antibacterial and antifungal activities against different pathogens [12,13]. The use of leaf extracts of *O. gratissimum* for the synthesis of sulfur nanoparticles hasnot been reported before in literature. In this present work, sulfur nanoparticles are prepared using either only oxalic acid (abbreviated as $\mathrm{SNP}-1$), or a mixture of leaf extract of *O. gratissimum* and oxalic acid (abbreviated as $\mathrm{SNP}-2$), as reducing and capping agents. Antimicrobial activities of prepared nanoparticles against pathogenic microorganisms; Gram positive and Gram negative bacterial were carried out and the results compared.

2. MATERIALS AND METHODS

2.1 Materials

Sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$, 99%), citricacid ($C_6H_8O_7$, R) were purchased from Sigma–Aldrich. Absolute ethanol was purchased from Pascal Scientific Limited, Nigeria, and used as received. Fresh leaves of *Ocimum gratissimum* were collected from the campus of Adekunle Ajasin University, Akungba, and used as a source of nanoparticle preparation. Deionized water was used throughout the experiments.

2.2 Methods

2.2.1 Preparation of leaf extract

The fresh leaves of *O. gratissimum* were removed from their stalks and washed thoroughly with running tap water to remove any attached particles ordebris, and finally washed with deionized water. Subsequently, the leaves were allowed to dry for 3-4 weeks at room temperature to remove the adsorbed moistures. The dried leaves were ground into fine powders in a clean agate mortal and stored in a tight container for further use. Biomolecule contents of the leave were extracted by adding 20 g of the powdered leaves to 100 mL deionized water in a 250 mL beaker and heated to $100℃$ for 60 min. The crude greenish extract was filtered through Whatman No. 1 filter paper and stored in the refrigerator at -10℃ prior to the preparation of sulfur nanoparticles.

2.2.2 Synthesis of Sulfur Nanoparticles ($SNP-1$ and $SNP-2$), Scheme 1

Synthesis of sulfur nanoparticles in the presence or absence of *O. gratissimum* plant extract is as follows: sodium thiosulfate pentahydrate (0.403 M) and 50 mL of *O. gratissimum* leaf extract were to 150 mL deionized water and allowed to stir on a magnetic stirrer at room temperature for 30 min. Then the aqueous solution of citric acid (2.42 M, 50 mL) was added drop wise under stirring to allow the precipitation of sodium thiosulfate as sulfur nanoparticles and SO_2,

Scheme 1 following a previous report [14]. The mixture was stirred for an additional 1 h and was allowed to stand undisturbed for 5 h for complete disproportionation of sodium thiosulfate. Biosynthesized sulfur nanoparticles were collected by centrifugation, washed with deionized water and EtOH, and dried in an oven at 50°C for 24 h. For comparison, sulfur nanoparticles in the absence of leaf extract were also prepared using $Na_2S_2O_3$and citric acid but without leaf extracts. Sulfur nanoparticles prepared in the absence and presence of plant extract are named $SNP-1$ and $SNP-2$, respectively.

$$Na_2S_2O_3(aq) + H^+(aq, \text{ citric acid}) \rightarrow SO_2(g) + S\downarrow + H_2O(l) \quad (1)$$

2.3 Material Characterizations

The solid reflectance spectra of prepared $SNP-1$ and $SNP-2$ were recorded on a Shimadzu UV-VIS-NIR Spectrophotometer UV-3100 with an MPCF-3100 sample compartment with samples mounted between two quartz discs which fit into a sample holder coated with barium sulfate. The spectra were recorded over the wavelength range of 800-250 nm, and the scans were conducted at a medium speed using a 20 nm slit width. Infra-red spectra were recorded on a Thermo Fisher Scientific FTIR spectro photometer, using pressed KBr pellets. The transmission electron microscope (TEM) image was recorded using a JEOL JEM-2010 electron microscope operating at 200 KV. Surface morphology and elemental composition of sulfur nanoparticles were analysed using a scanning electron microscope (SEM) equipped with energy dispersive analysis of X-ray equipment (EDAX) (XL 30 FEG ESEM).

2.4 Sample Collection of Clinical Isolates

Pure cultures of Five (5) selected clinical isolates; *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa and Salmonella pullorum* were collected from the Microbiology laboratory of Adekunle Ajasin University for this study. All the cultures were grown and maintained in nutrient broth at 37°C and used for further studies.

2.5 Antibiotic Susceptibility Test for the Bacterial Isolates

The test was performed to determine the phenotypic resistance of the bacterial isolates to commonly used antibiotics. These tests were carried out following the Kirby-Bauer disc diffusion method [15]. Inoculum from a culture of bacterial isolates on nutrient agar slants were inoculated into test tubes containing sterilized nutrient broth and incubated at 37°C for 18h

which serve as the stock for the test. Mueller-Hinton agar was prepared and sterilized, then dispensed into sterilized Petri dishes. The plates were allowed to cool for about 15min to allow it to gel and excess surface moisture to be absorbed. The inoculum was introduced into plates by streaking before applying the antibiotics discs. Two types of discs were used; Cephalosporin antibiotic discs (Oxoid); Cefuroxime (30 µg), Ceftazidime (30 µg), Cefoxitin (30 µg), Cefpodoxime (10 µg), Cefepime (30 µg) and Multi test predetermined commercial Gram-negative and Gram-positive organisms which were applied to the surface of the inoculated agar plated aseptically using sterile forceps. The discs were then placed firmly by slightly pressing on the inoculated plates with the sterilized forceps to ensure complete contact with the agar. After 24h of incubation, each plate was examined, susceptibility to each antibiotic was indicated by a clear zone. The zone of inhibition was measured using a calibrated ruler which was held on the back of the inverted petri dish and was recorded [16].

3. RESULTS AND DISCUSSION

3.1 Material Characterization

The solid reflectance spectra of as-prepared of $\mathrm{SNP-1}$ and $\mathrm{SNP-2}$ are shown in Fig. 1. Formation of as-prepared bare and *O. gratissimum* stabilized sulfur nanoparticles were confirmed by the presence of primary (λ_{max}) and secondary absorption peaks around 269-272 nm, and 310-312 nm, respectively. The primary absorption peaks ($\mathrm{SNP-1}$; 272.5 nm, and $\mathrm{SNP-2}$; 269 nm, Table 1) are due to successful disproportionation of sodium thiosulfate to sulfur nanoparticles in the presence of organic acid [17,18]; while the secondary peaks ($\mathrm{SNP-1}$; 312 nm, and $\mathrm{SNP-2}$; 310 nm, Table 1)are attributed to $b_2 \rightarrow e_3$ electronic transitions [19]. Both primary and secondary peaks of $\mathrm{SNP-1}$ are up to 2-3 nm blue-shifted compared to $\mathrm{SNP-2}$, confirming that bare sulfur nanoparticlesis slightly smaller in size compared to *O. gratissimum* stabilized analogue, due to quantum confinements of the later.

The stabilization of sulfur nanoparticles in the absence (oxalic acid only; $\mathrm{SNP-1}$) and presence ($\mathrm{SNP-2}$) of bioactive molecules of *O. gratissimum* was confirmed by the FTIR spectra shown in Fig. 2A & B. The broad absorption band of $\mathrm{SNP-1}$ centered at $3363\ \mathrm{cm^{-1}}$ is associated with the $-\mathrm{OH}$stretching of oxalic acid; while the broad and split bands in $\mathrm{SNP-2}$ around 3201-3514 $\mathrm{cm^{-1}}$, are associated with the combined $-\mathrm{OH}$and $-\mathrm{NH}$ groupsof fatty and amino acids in the plant extract [20-22]. This is an indication that the bioactive molecules of *O. gratissimum* extracts act as capping agents on synthesized sulfur nanoparticles. In $\mathrm{SNP-2}$ spectrum, characteristic absorption bands at 2926, 2851 cm^{-1}, 1656 cm^{-1}, and 1616 $\mathrm{cm^{-1}}$, corresponding to $-\mathrm{CH_2}$, $-\mathrm{CH_3}$, CONH (amide), and $\mathrm{C=C}$, respectively, which are not present in $\mathrm{SNP-1}$, confirmed the interaction between the protein in the plant extract and the biosynthesized nano particles. Thus, FTIR spectrum of $\mathrm{SNP-1}$differs from that of $\mathrm{SNP-2}$. In $\mathrm{SNP-1}$, intense absorption peaks at 1650 cm^{-1} was assigned to carbonyl stretching $(\mathrm{C=O})$ of oxalic acid. The multiple strong bands in the range of 1315–1385 $\mathrm{cm^{-1}}$ are due to the overtones of C-O stretch (alcohol), C=S stretch(sulfide), and S-O stretch (sulfoxide) [11,23]. Successful formation of sulfur nano particles was confirmed by the presence of absorption bands ranging from $550-817\ \mathrm{cm^{-1}}$ in $\mathrm{SNP-1}$; this peak shifted to $553-840\ \mathrm{cm^{-1}}$ in $\mathrm{SNP-2}$, due to the interaction of sulfur nanoparticles with the biomolecule contents of *O. gratissimum* leaf extract [24,25, 26].

Morphology of as-prepared sulfur nanoparticles were verified using a scanning electron microscope (SEM), Figs. 3A&B. The SEM images show that oxalic acid capped sulfur nanoparticles are clusters of small-sized and spherically shaped with definite morphologies, Fig. 3A. Biosynthesized nanocrystals, on the other hand, are conspicuously aggregated with irregular particle sizes and shapes, Fig. 3B. The EDS spectrum of bare nanoparticles shows the presence of C, O, and Na alongside theS atom peak, Fig. 4A. Aside the S peak which confirms the formation of sulfur nanoparticles, the presence of Na,O and C peaksare from $\mathrm{Na_2S_2O_3.5H_2O}$ and oxalic acidused as precursors for the preparation of sulfur nanoparticles, Fig. 4A. EDX analysis of biosynthesized sulfur nanoparticles (Fig. 4B) shows the presence of an intense S peak, confirming the successful formation of sulfur nanoparticles. Other elements observed in the EDX image alongside the S peak areCa, Na, C, O, and Mg, Fig. 4B. Mineral content analysis of the leaves of *O. gratissimum* in previous literature reports hasshown that they are very rich in Ca, Mg, Na and K [26], hence the presence of Ca and Mg peaks in the EDX image of *O. gratissimum* stabilized sulfur nanoparticles.

Table 1. Absorption spectral data for $SNP-1$ and $SNP-2$ nanoparticles

Nanoparticles	Primary absorption peak (nm)	Secondary absorption peak (nm)
$SNP-1$	269	310
$SNP-2$	272.5	312

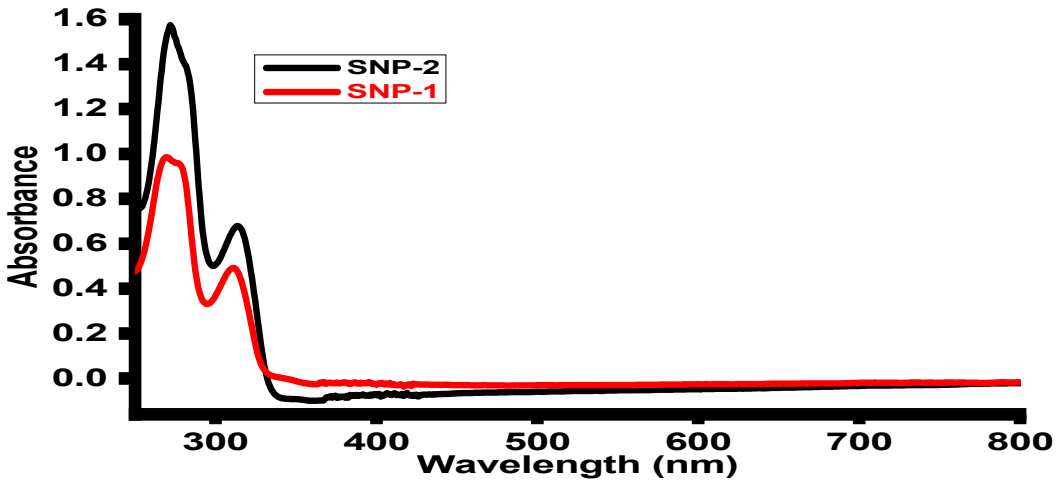

Fig. 1. UV-Vis absorption spectra of bare $(SNP-1)$and*O. Gratissimum*capplied sulfur $(SNP-2)$nanoparticles

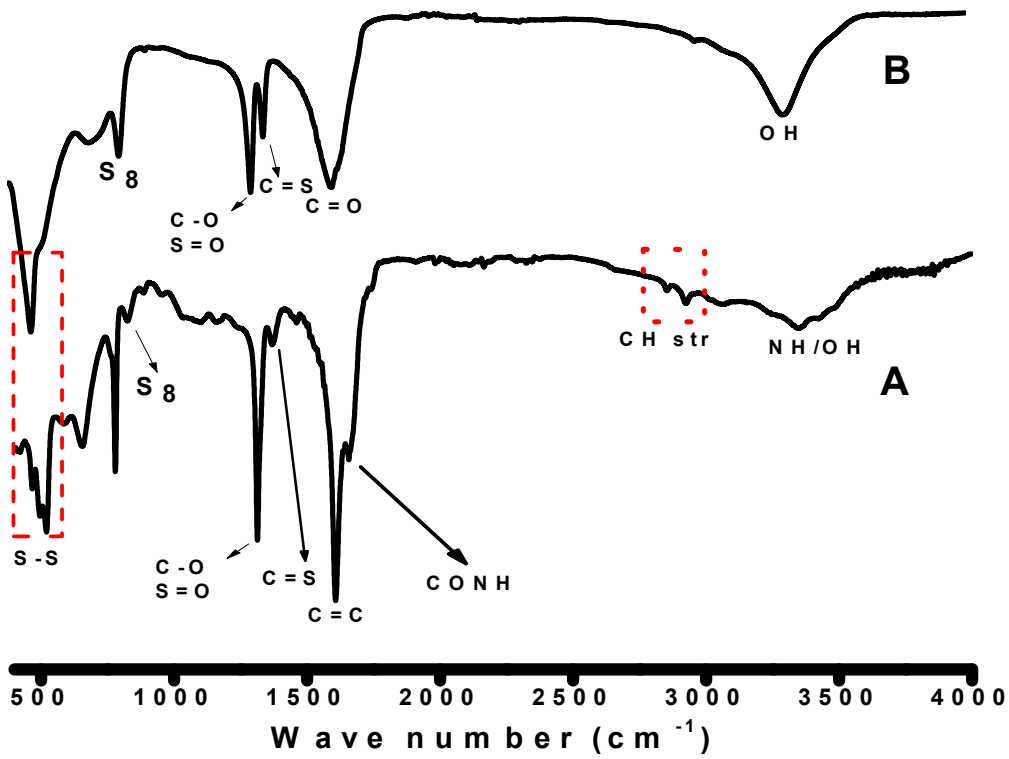

Fig. 2. Fourier transform infrared (FTIR) spectroscopy analysis of *O. gratissimum*capped sulfur nanoparticles (A) and bare (B) sulfur nanoparticles

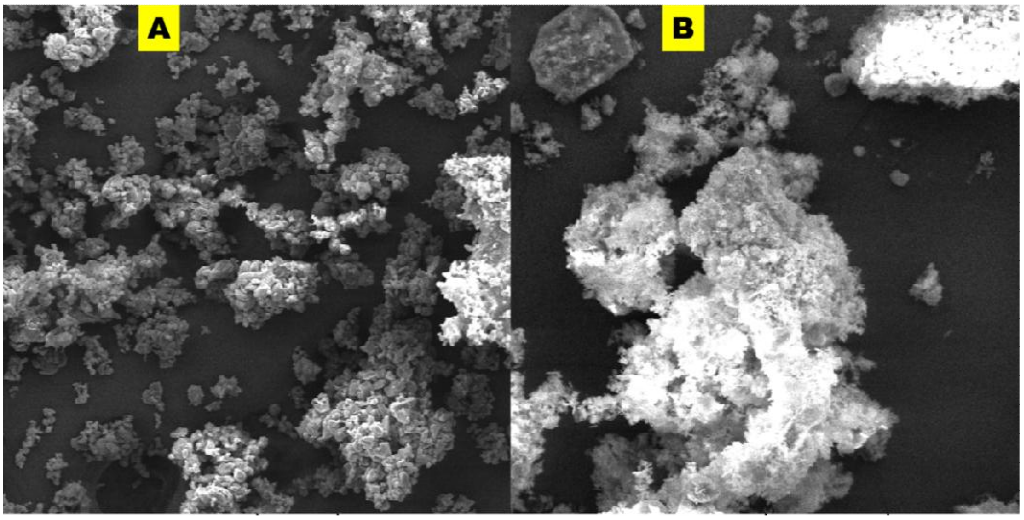

Fig. 3. Scanning electron microscope images of (a) bare$(\mathbf{SNP-1})$, and (b) biosynthesized sulfur $(\mathbf{SNP-2})$nanoparticles

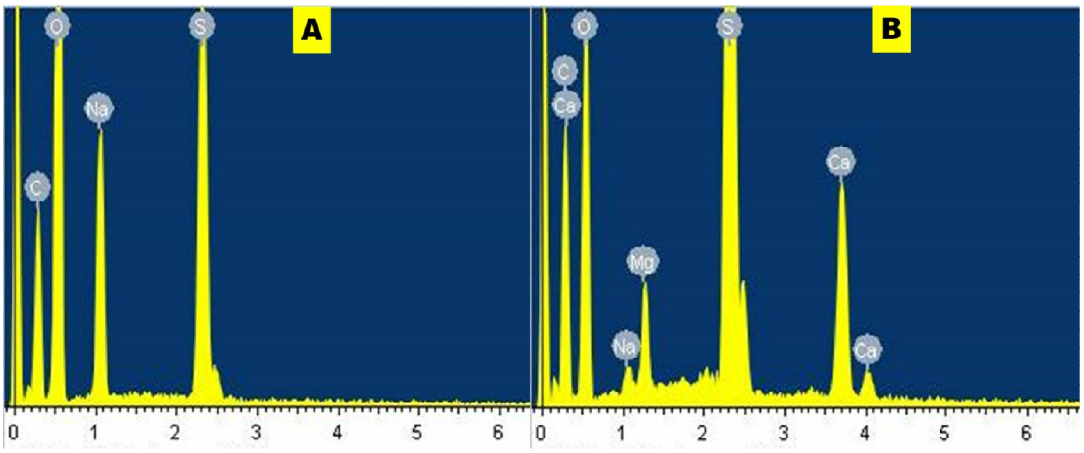

Fig. 4. Energy dispersive spectra of the synthesized (A) bare sulfur $(\mathbf{SNP-1})$ nanoparticles and (B) biosynthesized sulfur $(\mathbf{SNP-2})$ nanoparticles

Elemental distributions as obtained from the EDX spectra (Fig. 4 above) showed the weight and atomic % of S atoms obtained from $\mathrm{SNP-1}$ and $\mathrm{SNP-1}$ (Fig. 5). Interestingly, at the same approximate concentrations of $\mathrm{Na_2S_2O_3.5H_2O}$ and oxalic acid, weight, and atomic % of S atom in sulfur nanoparticles of $\mathrm{SNP-2}$(prepared from plant extract, right) is more than two times larger than S atom in $\mathrm{SNP-1}$ (left) prepared from oxalic acid alone. Also, the total weight or atomic % of other mineral ions presence in $\mathrm{SNP-2}$ is far less than that of S atom, suggesting that the prepared nanoparticles composed solely of sulfur, Fig. 5.

Transmission electron microscope (TEM) micrograph confirm the synthesized sulfur nanoparticles with oxalic acid ($\mathrm{SNP-1}$) are monodispersed, and spherically shaped particles with an average particle size of $18 \pm 4\ \mathrm{nm}$. The TEM image of $\mathrm{SNP-2}$, on the other hand, showed that the spherically shaped sulphur nanoparticles are aggregated with undefined sizes. The observed dark grey areas in the TEM micrograph of $\mathrm{SNP-2}$ are due to the presence of bioorganic molecules from the *O. gratissimum* leaf extract.

The crystalline nature of the as-synthesized sulfur nanoparticles, in the presence or absence

of *O. gratissimum* leaf extract was analyzed using X-ray diffraction patterns in Fig. 6. Both sulfur nanoparticles showed similar XRD patterns with ten distinct diffraction peaks located at θ values of ca. 15.4°, 22.12°, 23.19°, 25.37°, 26.10°, 28.11°, 29.18°, 31.7°, 37.04°, and 43.11°, corresponding to crystal facets of 111, 220, 222, 040, 422, 206, 313, 044, 422, and 319, respectively. The positions, intensities, and crystalline phases of as-synthesized sulfur nanoparticles were similar and consistent with the reported standard diffraction patterns of the Joint Committee on Powder Diffraction, standard (JCPDS No. 08247). Average particle sizes of synthesized sulfur nanoparticles are determined by Debye–Scherrer formula, Eqn. 2.

$$D = \frac{(0.9\lambda)}{\beta \cos\theta}$$

Where D is mean particle size, λ is X-ray wavelength, β is the full width of half maximum of diffraction lines, and $\theta$is the diffraction angle. The average particle size of SNP – 1 and SNP – 220 and 34 nm, respectively.

Element	Weight%	Atomic%
C K	26.35	34.81
O K	54.46	54.01
Na K	8.58	5.92
S K	10.61	5.25
Totals	100.00	

Element	Weight%	Atomic%
C K	38.67	52.97
O K	30.62	31.48
Na K	0.48	0.34
Mg K	1.45	0.98
S K	23.54	12.07
Ca K	5.25	2.15
Totals	100.00	

Fig. 5. Elemental distributions of sulfur nanoparticles synthesized in the absence (SNP – 1; left) and presence (SNP – 2; right) of aqueous leaf extract of *O. gratissimum*

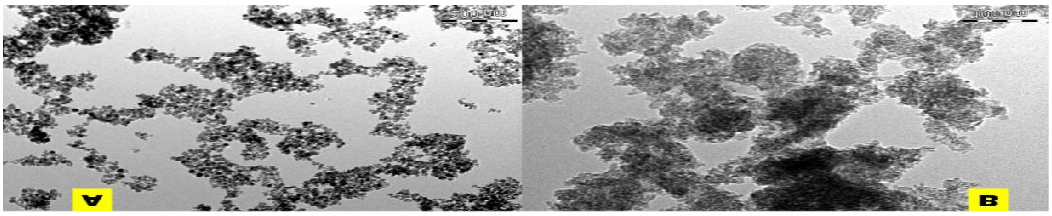

Fig. 6. Transmission electron microscope of (A) SNP – 1 and (B) SNP – 2

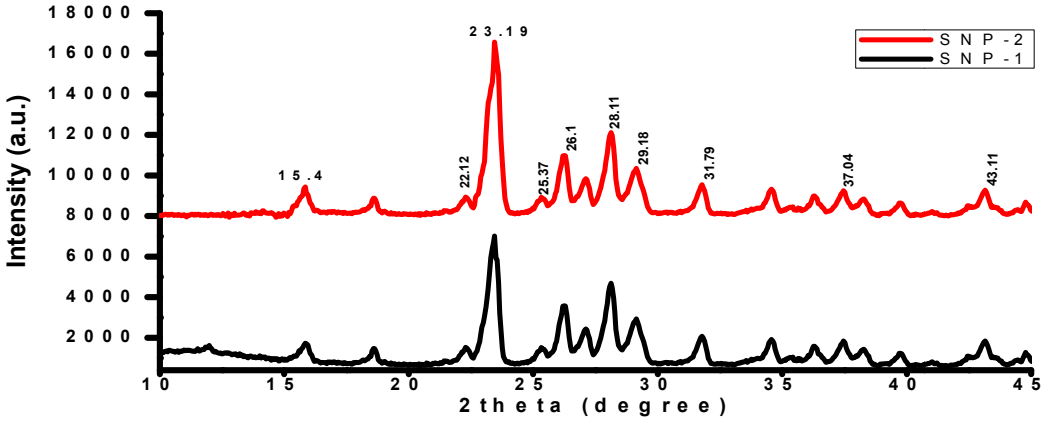

Fig. 7. X-RD analysis of sulfur nanoparticles synthesized in the absence (SNP – 1) and presence (SNP – 2) of aqueous leaf extract of *O. gratissimum*

The diameter (in mm) of the zone of inhibition of the sulfur nanoparticles was tested against selected clinical isolates. *S. aureus* was observed to have the highest susceptibility to sulfur nanoparticles (SNP – 2) mediated with *O. gratissimum* plant extract with a diameter of 20.00 mm; *E. coli* and *S. pullorum* showed the susceptibility diameter zone of 18.00mm; *K. pneumoniae* and *P. aeruginosa* showed resistance. All tested clinical isolates were resistant to the other sulfur nanoparticles (SNP – 1) synthesized in the absence of *O. gratissimum* plant.

For the clinical isolates; Percentage Frequency Distribution of Multidrug-resistant *Microbes against *Ocimum gratissimum* Capped Sulfur Nanoparticles SNP-2. Salmonella pullorum (32%), Staphylococcus aureus (36%), *Escherichia coli* (32%), *Pseudomonas aeruginosa* (0%) and *Klebsiella pneumonia* (0%) respectively

The result obtained also showed that *Ocimum gratissimum* extract increased the efficacy of sulfur nanoparticle as an antimicrobial agent. The antimicrobial properties of elemental sulfur have long been recognized, and the use of sulfur nanoparticles (SNPs) as antimicrobial agents was first proposed by Lawson [27]. Since then, Schneider et al. [28] demonstrated that SNPs of about 150 nm in size exhibited antimicrobial activity against various microorganisms. Two sulfur nanoparticles were used in this study (Table 1); SNP-2 was mediated with *Ocimum gratissimum* plant extract while SNP-1 was synthesized in the absence of *O. gratissimum* plant extract. *O. gratissimum* is commonly called 'Efinrin nla' and is widely cultivated in Nigeria for its medicinal use [29], *Ocimum gratissimum* phytochemical screening has indicated the presence of various phytoconstituents which are active against several bacteria including *Staphylococcus aureus, Listeria mono cytogenes, Escherichia coli* [30,28]. SNP-1 showed no antibacterial effect on all the bacteria (endophytic and clinical isolates) used in this study while SNP-2 showed potential antibacterial effects on three of the five selected clinical isolates; *Staphylococcus aureus* was observed to have the highest susceptibility with diameter of 20.00 mm; *Escherichia coli* and

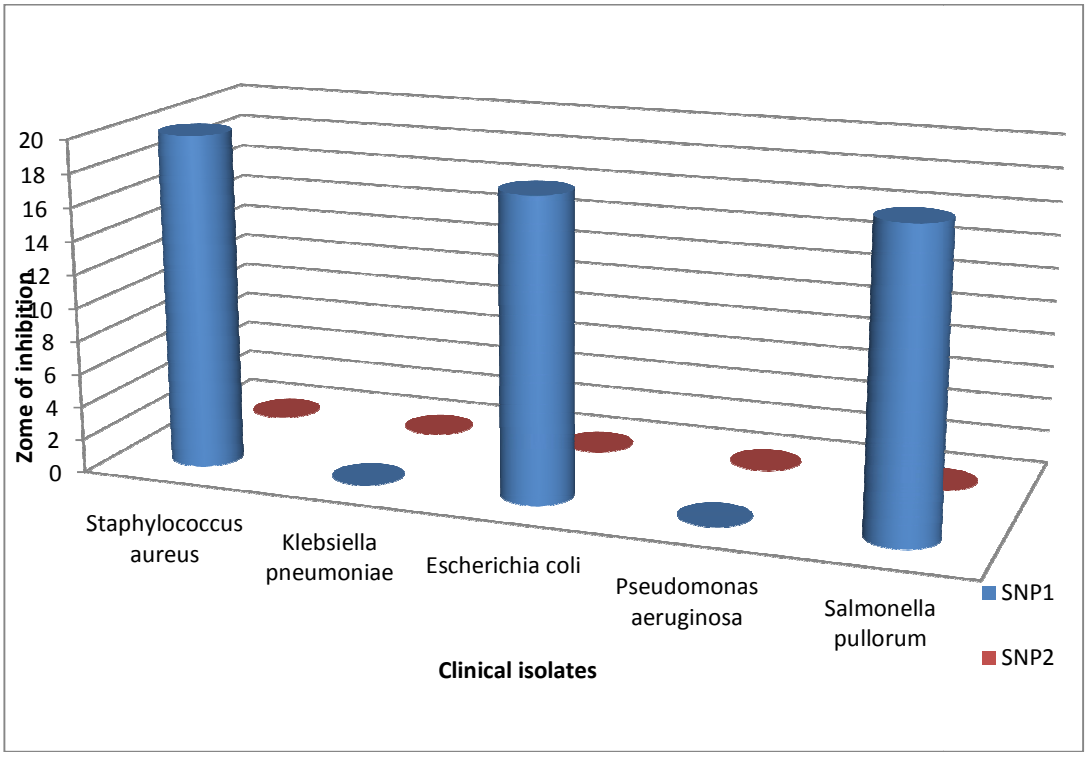

Fig. 8. Antibacterial efficacy of *Ocimum gratissimum* capped sulfur nanoparticles against multidrug-resistant microbes(SNP1 &SNP2)

Key: SNP 1 - Sulfur Nanoparticle 1; SNP s 2 - Sulfur Nanoparticle 2

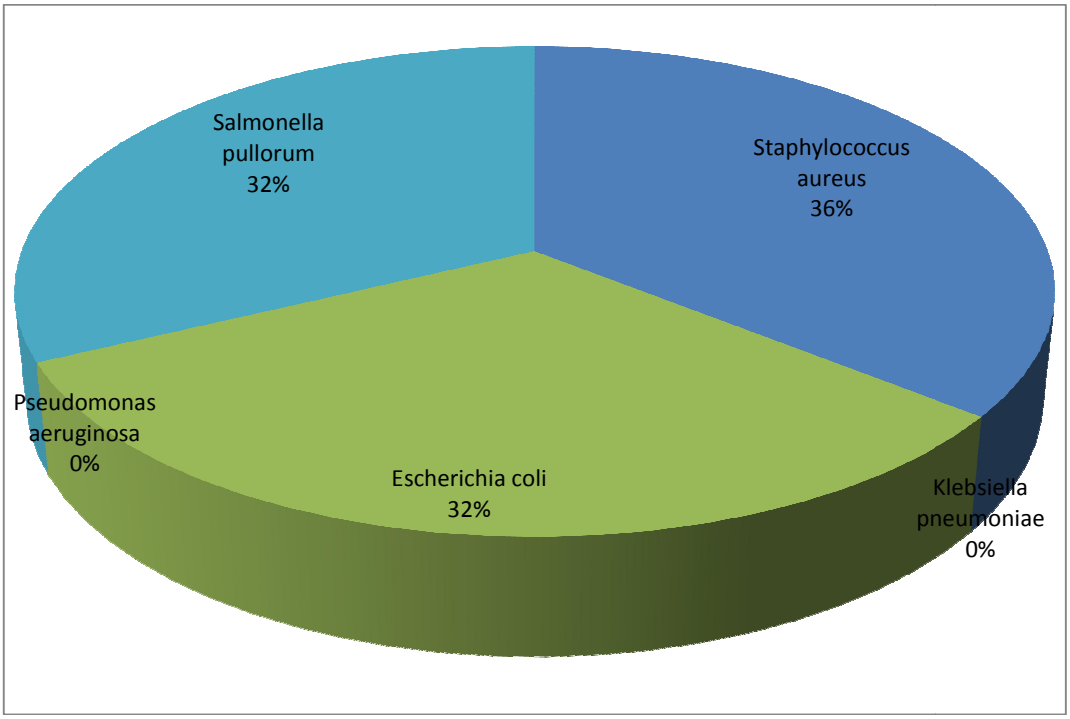

Fig. 9. Percentage frequency distribution of multidrug-resistant microbes against *Ocimum gratissimum* capped sulfur nanoparticles SNP-2

Salmonella pullorum showed the susceptibility diameter zone of 18.00mm. The endophytic bacterial isolates were also susceptible to the nanoparticles with lower diameter zones ranging from 9mm-14mm. The sulfur nanoparticle (SNP-2) showed potential antibacterial activity, the amount of nanoparticle used maybe increased to achieve an even better outcome. Schneider *et al*. [31] demonstrated that SNPs has no toxicity to human cells, thereby making them more suitableas an alternative antimicrobial agent.

4. CONCLUSION

In conclusion, multidrug resistant microbe will soon be a thing of the past, The era has come to stay where scientific knowledge has choosing a new means to battle multidrug resistant microbe and this scientific magical bullets is *Ocimum gratissimum* Capped Sulfur Nanoparticles, which has shown a drastic against clinical isolates and a revelry potential over conventional antibiotics.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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