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Green Synthesis of Nanomaterials with Phytochemicals for Treating Multidrug Resistant Bacteria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The Bacteria with Multidrug resistance and Extreme drug resistance are increasing at a rapid rate. Various methods have been employed to combat drug resistant bacteria. Major classes of antibiotics aren't effective against these bacteria. Alternative methods have been studied in recent years. Nanoparticles are used against multidrug resistant bacteria; The green synthesized nanoparticles are more reliable due to more shelf life and lesser toxicity relative to chemically synthesized nanoparticles. Multi drug resistant *E. coli* and *Staphylococcus aureus* was isolated from sewage samples. Green synthesized nanoparticles from various plants samples have been

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prepared with Zinc and Copper forming respective oxides with Neem, Nakara, Jatropha, Mango, Clove, Ginger, Cardamom, Cinnamon and Betel against multidrug resistant *Escherichia coli* and *Staphylococcus aureus*. Isolated *E. coli* was susceptible to Fluoroquinolone and Augmentin whereas *S. aureus* was susceptible to vancomycin. Green synthesized nanoparticles had more antimicrobial activity against *E. coli* and *S. aureus* than chemically synthesized nanoparticles and plant extracts. Green synthesized Nakara CuO nano particles had inhibition zone of 31 ±0.6mm and 30 ±0.7mm for *E. coli* and *S. aureus* respectively, ZnO nano particles of Nakara had 25 ±0.6mm inhibition zone for *E. coli* and *S. aureus*. Green synthesized Jatropha CuO nano particles had inhibition zone of 26 ±0.5 and 26 ±0.4mm for *E. coli* and *S. aureus*. ZnO nano particles of Jatropha had 31±0.7mm and 30±0.7mm inhibition zone for *E. coli* and *S. aureus* respectively. The Scanning electron microscopy studies revealed 26nm Jatropha ZnO and 25nm Nakara CuO nanoparticles. Nano materials were found to be non-toxic in cell line studies. The present study concludes to study the impact of green synthesized nanoparticles as an alternative to antibiotics to combat multidrug resistant bacteria.

Keywords: Phytochemicals; surface coating; green synthesis; resistant bacteria; metal oxide nanomaterials.

1. INTRODUCTION

Excessive use of antibiotics by human, as well as in agriculture and in aquaculture led to the rapid increase in multidrug resistant bacterial strains. IDSA (The Infectious Diseases Society of America) classifies antimicrobial resistance as one of the major significant global threats to human health [1]. Bacteria found resistant to majority of the known Antibiotics including those considered last-line treatments like vancomycin [2]. Due to development of resistance to several antibiotics, antimicrobial medicines lose their effectiveness, making the infections harder or impossible to treat, raising the risks of disease transmission, chronic illness and mortality.

Phytochemicals are active compounds naturally occurring in plants, recognized for their potential health benefits and contributions to human nutrition and medicine. They play major role in plant growth and defence mechanism against competitors, predators and pathogens. Numerous plants serve as significant sources of antimicrobial complexes that demonstrate potent activity against bacterial strains. More than 7000 species of wild consumable plants contribute nutrition in human being [3]. and most of the antimicrobial activity is yet to be studied [4,5].

Green synthesis of nanoparticles is carried out by producing nano particles through living cells through biological pathways, this method of synthesis is more efficient and higher yield compared to other methods. Green synthesis methods are recognized for their eco-friendly nature, non-toxicity, cost efficiency, and superior stability compared to alternative, physical, and chemical approaches [6]. Metal and metal oxide nanoparticles formed by green synthesis method are increasingly applied in the biomedical field, including disease treatment, wound healing, immunotherapy, dentistry etc [7].

It is planned in study to green synthesize nano materials and evaluate the antimicrobial activity against the extreme drug-resistant gram negative and gram-positive bacteria.

2. MATERIALS AND METHODS

Sample collection: 15 Sewage sample collected from different locations of Hyderabad Telangana, India during May, June 2024.

2.1 Isolation of *Escherichia coli* and *Staphylococcus aureus*

Pure *E. coli* and *S. aureus* were isolated from collected sewage samples. The samples were spread on the MacConkey agar plate and Mannitol Salt Agar (HIMEDIA, India) incubated at 37^{o}C for 24hrs. Colonies which were similar based on growth on specific media to *E. coli* and *S. aureus* were sub-cultured on nutrient agar plates.

2.1.1 Characterization

Colony morphology: Colony characteristics of the isolates were observed on specific media *E. coli* on MacConkey Agar, *S. aureus* on Mannitol Salt Agar.

Microscopy: Gram staining and microscopic observations were performed to confirm the organisms isolated.

2.1.2 Biochemical test

Several biochemical tests were carried out as Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test and Coagulase test were performed.

2.2 Antibiotic susceptibility Test

The Antibiotic susceptibility test of *E. coli* and *S. aureus* were carried out by placing HIMEDIA antibiotic disc on Mueller Hinton agar consisting of Antibiotics: Ampicillin Methicillin, Cephalosporin, Tetracycline, Monobactam, Carbapenem, Sulfonamide, Nitroimidazole, Macrolide, Rifamycin, Fluoroquinolone, Elfamycin, Ceftazidime, Cefepime, Norfloxacin, Levofloxacin, Chloramphenicol, Streptomycin, Augmentin, Kanamycin, Pencillin – G, Gentamycin and Vancomycin.

Augmentin antibiotic solution was prepared by adding 20mg Amoxicillin in 10mg potassium clavulanate and the solution was added in wells.

2.3 Molecular Analysis

16srRNA method was used to identify the bacterial cultures. Bacterial cultures were grown on nutrient broth for overnight to isolate the genomic DNA of *E. coli* and *S. aureus* by QIAamp DNA kits® Bacterial Genomic DNA Purification Kit (QIAGEN).

Extracted genome was amplified by Polymerase Chain Reaction. The universal primers with 16S rRNA gene, forward primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer (5′-CTGCTGCSYCCCGTAG-3′) were used for the amplification of the 16S rRNA gene fragment. The amplified PCR products were sequenced by ABI DNA sequencer (Applied Biosystem Inc).

The computation analysis of 16s rRNA gene sequence of *E. coli* and *S. aureus* isolates were compared with sequences in National Center for Biotechnology Information (NCBI) by BLAST [8]. Phylogenetic trees were made to understand the evolutionary relationships by ClustalW [9].

Selection of plant: The plant samples were collected from Anantagiri Hills forest, Vikarabad, Telangana, India specimens were identified by Dr. Vijaybhaskar Reddy, Taxanomist, Department of Botany Osmania university, Hyderabad.

Extraction of phytochemicals: Aqueous and ethanolic phytochemical extracts of Neem, Nakara, Jatropha milk, Mango seed, Clove, Ginger, Cardamom, Cinnamon and Betel leaves. were performed. Similar extracts were also prepared for spices.

Aqueous extracts were prepared by suspending 10 grams of samples in 90ml of phosphate buffer and grinded. The mixture was heated at 60^{o}C for 10mins in water bath. The collected solution was filtered through Whatman filter paper and filtrate was collected in sterile screw cap bottles and stored at 4^{o}C for further use.

Ethanolic extracts were prepared by suspending 10 grams of samples in 90ml of ethanol in sterile screw cap bottles, heated at 60^{o}C for 10mins, after cooling ethanolic fraction was separated. The filtrate was evaporated on rotatory evaporator at 65^{o}C until the ethanol is evaporated. The resultant powder was suspended in water, filtered through Whatman filter paper and stored at 4^{o}C for further use.

2.4 Synthesis of Nano Particles

Copper oxide and Zinc oxide nano particles were prepared by taking 0.02M of copper sulphate and Zinc acetate separately, dissolving each in 100ml water. The solutions were titrated against 1M NaOH dropwise 40ml for 10mins at 60^{o}C. Further stirring at 60^{o}C without NaOH was done until brick red and white colour precipitate was observed for Copper and Zinc respectively indicating the formation of copper oxide and Zinc oxide nano particles.

Green synthesis of Nano particles was performed by taking 3.1 grams of copper sulphate and 3.6 grams of Zinc acetate was taken separately and suspended in 100ml of Aqueous extracts samples. The solutions were titrated against 1M NaOH dropwise 4ml for 10mins at 60^{o}C. Further stirring at 60^{o}C without NaOH was done until brick red and white colour precipitate was observed for Copper and Zinc respectively indicating the formation of green synthesized copper oxide and Zinc oxide nano particles coated with aqueous extracts.

2.5 Purification of Nano Particles

Precipitated nanoparticles were collected in an Eppendorf and subjected to centrifugation at 10,000rpm for 5mins. The pellet was collected and washed with non-ionized distilled water. The washed pellet was dried in hot air oven for overnight at 80^{o}C.

Table 1. Plant Sample collection and part of samples

Samples	Scientific name	Part of Sample
Neem	*Azadirachta indica*	Leaf
Nakara	*Ximenia americana*	Latex
Jatropha	*Jatropha curcas*	Latex
Mango	*Mangifera indica*	Kernel
Clove	*Syzygium aromaticum*	Clove flower buds
Ginger	*Zingiber officinale*	Rhizome
Cardamom	*Elettaria cardamomum*	Seeds
Cinnamon	*Cinnamomum verum*	Bark
Betel	*Piper betle*	Leaf

Characterization of Nano particles: Nano particles characterization was carried out by the Scanning electron microscopy and antimicrobial activity.

The antimicrobial activity of phytochemicals, nano particles and green synthesized nano particles was tested against *E. coli* and *S. aureus*. The 100µl cultures *E. coli* and *S. aureus* were spread on Mueller Hinton agar separately. The 30µl liquid samples and solid samples of 3mg were added in wells and incubated at 37^{o}C for 24hrs.

Green synthesized CuO and ZnO nano particles were further characterized by Scanning electron microscopy. The samples were sterilized under UV light and placed on SEM stubs, the samples were then gold coated and scanning electron microscopy was performed.

2.6 Toxicity

The cell toxicity was measured by a MTT method which is a simple non-radioactive colorimetric assay [10]. A549 cells (Lung cancer) and A375 (Melanoma cells) cells were plated out at a density of $1X10^4$ cells/well in 96-well microtiter plates. After 24 h incubation, the cells were treated with nano materials (green synthesized Zinc oxide nanoparticles with Jatropha and Copper oxide nanoparticles with Nakara) upto 20ppm for 24 h. followed by incubation, the media were replaced with 20µl of MTT reagent (5 mg/ml) and incubated in 5% CO_2 at 37°C for 4 h. DMSO was then added to solubilize the MTT tetrazolium crystal. Absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The data were analyzed with 3 parallel experiments and were expressed as mean ± standard deviation.

Statistical Analysis: Experiments were repeated thrice in triplicate (n=9) and value with standard deviation is presented.

3. RESULTS

Colony on MacConkey agar morphology: *E. coli* strain had small round, smooth and pink colonies with depression in middle. *Staphylococcus aureus* had round, convex colonies with yellow colonies on MSA agar due to fermentation of mannitol.

Microscopic observations: *E. coli* was rod shaped, gram negative, non-sporing with peritrichous flagella. *Staphylococcus aureus* was coccus shape, gram positive and grape like clusters arrangements were observed.

Biochemical test: The following observations of biochemical tests were observed for *E. coli* and *Staphylococcus aureus*.

3.1 Antibiotic Susceptibility Test

The Antibiotic susceptibility tests were conducted by placing various HIMEDIA antibiotic disc and antibiotic susceptibility profiles were made by the observation of clearance zones formed.

Table 2. Biochemical tests for *E. coli* and *Staphylococcus aureus*

Biochemical test	*Escherichia coli*	*Staphylococcus aureus*
Indole test	Positive	Negative
Voges Proskauer	Negative	Negative
Citrate utilization test	Positive	Negative
Catalase test	Negative	Positive
Coagulase test	Negative	Positive

Table 3. Antibiotic susceptibility test for *E. coli* and *S. aureus*

S.no	Antibiotics	*E. coli* (mm)	*Staphylococcus aureus* (mm)
1	Ampicillin	4±0.01	9±0.4
2	Cephalosporin	9±0.3	2±0.04
3	Macrolide	12±0.3	5±0.26
4	Monobactam	9±0.2	3±0.09
5	Carbapenem	8±0.2	4±0.08
6	Sulfonamide	7±0.3	7±0.3
7	Nitroimidazole	5±0.25	5±0.2
8	Rifamycin	7±0.4	2±0.03
9	Fluoroquinolone	23±0.9	6±0.1
10	Elfamycin	8±0.3	2±0.05
11	Ceftazidime	5±0.03	2±0.04
12	Cefepime	6±0.04	7±0.2
13	Norfloxacin	3±0.09	5±0.1
14	Levofloxacin	1±0.04	4±0.09
15	Chloramphenicol	6±0.03	8±0.2
16	Tetracycline	2±0.08	9±0.3
17	Streptomycin	9±0.3	1±0.04
18	Augmentin (Amoxicillin & Potassium Clavulanate)	26±0.6	4±0.03
19	Kanamycin	8±0.2	2±0.07
20	Pencillin – G	2±0.06	6±0.02
21	Gentamycin.	9±0.4	2±0.07
22	Vancomycin	7±0.02	26±0.665
23	Methicillin	9±0.4	8±0.3

The *E. coli* isolate was sensitive to Fluoroquinolone and Augmentin whereas *S. aureus* was resistant to every antibiotic except Vancomycin.

3.2 Molecular Analysis

The given organisms were identified as *E. coli* and *S. aureus* by 16srRNA analysis. The phylogenetic analysis was done by ClustalW [9]. and the tree were constructed by Neighbour joining method. The 16srRNA sequences of *E. coli* and *S. aureus* were submitted to NCBI. The accession numbers were given below:

E. coli Accession number: PQ084693
S. aureus Accession number: PQ084695

3.3 Antimicrobial Activity of Phytochemicals, Nano Particles and Green Synthesized Nano Particles

The aqueous and ethanolic extracts of Neem, Nakara, Jatropha milk, Mango seed, Clove, Ginger, Cardamom, Cinnamon and Betel were tested for antimicrobial activity. The Copper oxide and Zinc oxide nano particles and the green synthesized nano particles formed by respective phytochemicals were also tested for antimicrobial activity.

The antimicrobial activity results of were as shown in the Table 4, Table 5 and Table 6.

The green synthesized nano particles with greater activity were characterized by Scanning electron microscopy.

3.3.1 Toxicity

The nano materials were found to be non-toxic up to 20ppm concentration on cell lines tested. Green synthesized Zinc oxide nanoparticles with Jatropha and Copper oxide nanoparticles with Nakara's soluble MTT OD is less than the control Mitomycin C and Doxorubicin.

4. DISCUSSION

Extreme drug resistant bacteria were isolated from sewage samples collected in Hyderabad, Telangana, India. Among 24 antibiotics tested *S. aureus* was found to be sensitive to one antibiotic (vancomycin) whereas *E. coli* was found to be sensitive to two of the antibiotics (Fluoroquinolone and Augmentin). Similar findings of extreme drug resistance bacteria were reported in the literature. High resistance to the antibiotics i.e. cephalosporins, fluoroquinolones, trimethoprim-sulfamethoxazole, and tetracycline by Naziri Z et al. [11]. Nearly all antibiotics including frequently using beta lactam combination antibiotics were found resistant [12]. Out of the 73 isolated strains of *S. aureus* by Sadat SS et al. [13] 32 were found to be methicillin-resistant *S. aureus* (MRSA). Among methicillin-resistant *S. aureus* isolates, 96.8 and 12.5% were multi-drug resistance and extreme drug resistance, respectively. All the methicillin resistant strains were found to sensitive to vancomycin [13]. Iqbal Z et al [14] showed high resistant bacteria against cefuroxime, co-amoxiclav, cefixime, ceftazidime, cefotaxime, ceftriaxone, nalidixic acid, ciprofloxacin, pepedemic acid, norfloxacin, and co-trimoxazole. Nanomaterials particularly Silver (Ag), Copper (Cu), Zinc (Zn) were researched as potent antimicrobials. Copper nanoparticles shown the highest sensitivity for *E. coli* and *E. faecalis* [15]. The minimum inhibitory concentration of Cu/Zn nanomaterials for the *E. coli* and *S. aureus* strains were of 3.75 and 2.50 mg/ml [16]. 40 different isolates of subclinical mastitis are recovered from the milk samples were sensitive to zinc oxide nanoparticles [17]. The study of Abbas ZM et al. [18] showed that the conjugation of copper and zinc nanoparticles with the classical antibiotics has a great antibacterial activity 9 Plants aqueous extracts were used for antimicrobial activity and among them Jatropha and Nakara plants aqueous extracts were showing more antimicrobial activity against gram positive and gram-negative bacteria. These aqueous extracts were used for green synthesis of copper and zinc oxide nanomaterials.

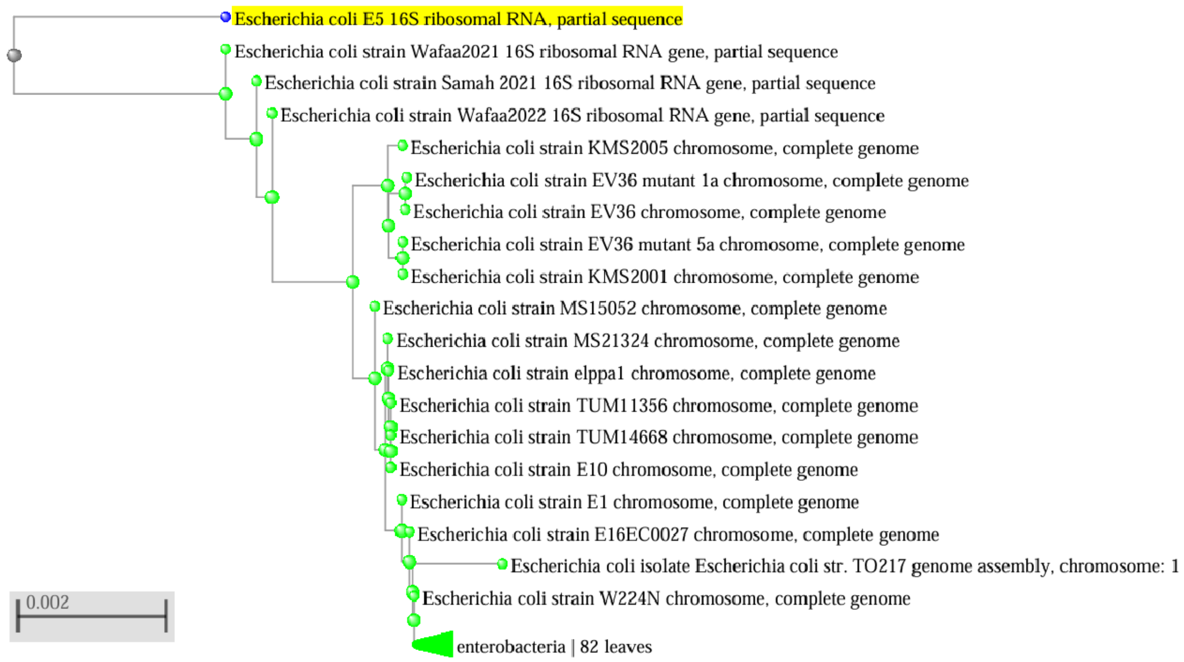

Fig. 1. Neighbour joining tree of *E. coli* containing 16srRNA gene sequence

Table 4. Antimicrobial activity of Aqueous and ethanolic extracts of phytochemicals collected from plant samples against *E. coli* and *S. aureus*

Sample	*E. coli* (mm)		*S. aureus* (mm)	
	Aqueous	Ethanolic	Aqueous	Ethanolic
Neem	22 ±0.4	18 ±0.4	20 ±0.6	19 ±0.4
Nakara	23 ±0.3	20 ±0.5	22 ±0.5	19 ±0.3
Jatropha milk	24 ±0.6	19 ±0.4	23 ±0.4	20 ±0.5
Mango seed	15 ±0.2	14 ±0.3	16 ±0.3	12 ±0.1
Clove	14 ±0.1	12 ±0.2	16 ±0.2	15 ±0.1
Ginger	13 ±0.1	11 ±0.1	14 ±0.2	11 ±0.1
Cardamom	17 ±0.3	15 ±0.2	17 ±0.3	16 ±0.2
Cinnamon	15 ±0.2	12 ±0.1	14 ±0.3	11 ±0.2
Betel	16 ±0.2	12 ±0.2	17 ±0.4	15 ±0.3

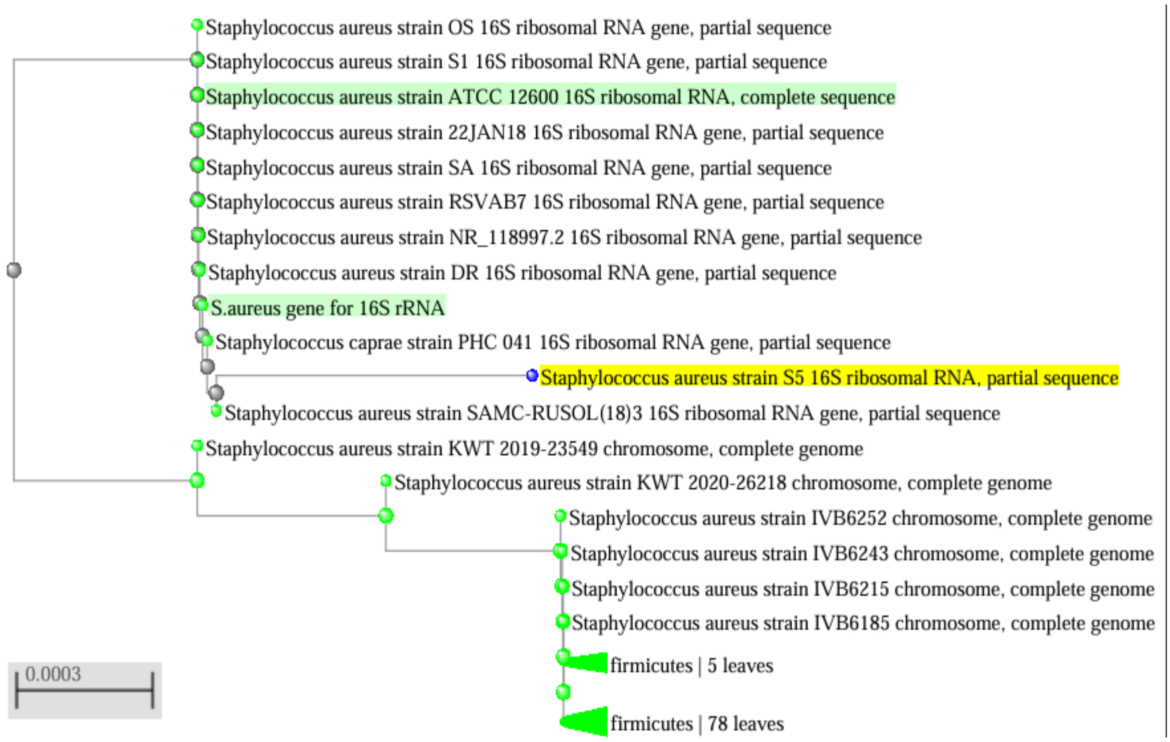

Fig. 2. Neighbour joining tree of *S. aureus* containing 16srRNA gene sequence

Table 5. Antimicrobial activity of Chemically synthesized nano particles against *E. coli* and *S. aureus*

Nano particles	*E. coli* (mm)	*S. aureus* (mm)
CuO nano particle	19 ±0.4	20 ±0.3
ZnO nano particle	21 ±0.5	20 ±0.4

Table 6. Antimicrobial activity of Green synthesized nano particles against *E. coli* and *S. aureus*

Green synthesised nano particles	*E. coli* (mm)		*S. aureus* (mm)	
	ZnO synthesized	CuO synthesized	ZnO synthesized	CuO synthesized
Neem aq	26 ±0.5	24 ±0.6	26 ±0.7	26 ±0.5
Nakara aq	25 ±0.6	31 ±0.6	25 ±0.6	30 ±0.7
Jatropha milk aq	31 ±0.7	26 ±0.5	30 ±0.7	26 ±0.4
Mango seed aq	18 ±0.4	20 ±0.3	19 ±0.4	21 ±0.3
Clove aq	18 ±0.3	20 ±0.4	19 ±0.2	20 ±0.4
Ginger aq	19 ±0.4	18 ±0.3	18 ±0.3	20 ±0.3
Cardamom aq	18 ±0.4	19 ±0.2	20 ±0.6	20 ±0.4
Cinnamon aq	20 ±0.5	21 ±0.6	19 ±0.4	19 ±0.5
Betel aq	19 ±0.3	20 ±0.4	19 ±0.3	21 ±0.6

The green synthesized nanomaterials sizes were with an average of 26nm for ZnO with Jatropa and 25nm for CuO with Nakara. Similar studies of Pradheesh G et al. [19] reported Ag_2O nanomaterials of different sizes which are below 100nm. The nanomaterials of zinc acetate and zinc nitrate were of spherical shape with the average size of 21.49 and 25.26 [20]. Vishveshvar K et al. [21] reported SEM studies of green synthesized CuO with average size of 300nm.

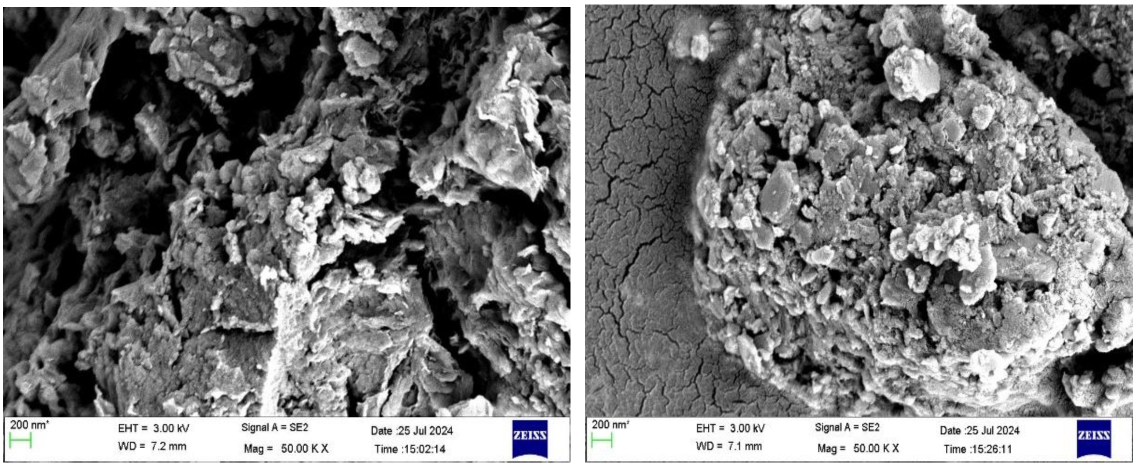

Fig. 3. SEM images green synthesized Zinc oxide nanoparticles with Jatropha and Copper oxide nanoparticles with Nakara

Antimicrobial activities of these green synthesized nanomaterials were more when compared with phytochemical, chemical synthesized nanomaterials and 30mg chloramphenicol. Antimicrobial activities were in accordance to the reports of the nanoparticles the inhibition for gram positive and negative bacteria with the minimal concentration of 12.5mg/ml. 20±0.7 and 16±0.5 diameter was observed as the highest inhibition against the *S. aureus* and *E. coli* strains [22]. Different concentrations of $CuSO_4$ (0.1 and 0.01M) shown antimicrobial effect for the strains of *E. coli* and *S. aureus* (33±0.57 and 6±2mm) whereas complete absence of growth is seen in case of *S. aureus* in study by Taran M et al. [23]. 98.8 and 99.7 percent efficiency reported against gram positive and negative bacteria with CuO/Ag nanoparticles whereas 91.7 and 89.3 percent efficiency reported with ZnO/Ag nanoparticles against *E. coli* and *S. aureus* observed by Asamoah RB et al. [24]. Green synthesis of nanomaterials is in acceleration due to ecofriendly process, non-consumption of toxic chemicals, safer synthesized metals etc.

Nanomaterials are generally regarded as toxic as reported in the studies by Hussain SM et al. [25] and Sakhtianchi R et al. [26] against mouse fibroblast cells Hence toxicity studies to chemically synthesized and green synthesized nanomaterials were carried out significantly less toxic when compared with chemical synthesized nanomaterials.

Green synthesized nanomaterials could be alternative to antibiotics to control the extreme drug resistance bacteria.

5. CONCLUSION

The present study concludes that the green synthesized nano particles are effective in treating multidrug resistant bacterial infections over antibiotics and chemically synthesized nano particles due to more shelf life, lesser side effects and had effective bactericidal properties, in turn reducing environmental wastage produced. Green synthesized nano particles retain their activity for longer periods than chemically synthesized nano particles. The usage of green synthesized nano particles can be cost effective and efficient compared to other conventional methods.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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