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ORIGINAL ARTICLE

First report on rapid screening of nanomaterial-based antimicrobial agents against β-lactamase resistance using pGLO plasmid transformed *Escherichia coli* HB 101 K-12

M. Alpha Raj[1] · Y. Muralidhar[1] · M. Sravanthi[1] · T. N. V. K. V. Prasad[2] · M. Nissipriya[3] · P. Sirisha Reddy[4] · T. Shoba Neelima[5] · G. Dilip Reddy[1] · K. Adilaxmamma[1] · P. Anand Kumar[1] · T. Giridhara Krishna[2]

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Abstract Combating antibiotic resistance requires discovery of novel antimicrobials effective against resistant bacteria. Herein, we present for the first time, pGLO plasmid transformed *Escherichia coli* HB 101 K 12 as novel model for screening of nanomaterial-based antimicrobial agents against β-lactamase resistance. *E. coli* HB 101 was transformed by pGLO plasmid in the presence of calcium chloride (50 mM; pH 6.1) aided by heat shock (0–42–0 °C). The transformed bacteria were grown on Luria–Bertani agar containing ampicillin (amp) and arabinose (ara). The transformed culture was able to grow in the presence of ampicillin and also exhibited fluorescence under UV light. Both untransformed and transformed bacteria were used for screening citrate-mediated nanosilver (CNS), aloin-mediated nanosilver (ANS), 11-α-keto-boswellic acid (AKBA)-mediated nanosilver (BNS); nanozinc oxide, nanomanganese oxide (NMO) and phytochemicals such as aloin and AKBA. Minimum inhibitory concentrations (MIC) were obtained by microplate method using ρ-iodo nitro tetrazolium indicator. All the compounds were effective against transformed bacteria except NMO and AKBA. Transformed bacteria exhibited reverse cross resistance against aloin. ANS showed the highest antibacterial activity with a MIC of 0.32 ppm followed by BNS (10.32 ppm), CNS (20.64 ppm) and NZO (34.83 ppm). Thus, pGLO plasmid can be used to induce resistance against β-lactam antibiotics and the model can be used for rapid screening of new antibacterial agents effective against resistant bacteria.

Keywords pGLO plasmid · Transformation · *Escherichia coli* · Nanosilver · Nanozinc oxide · Aloin · Boswellic acid

✉ T. N. V. K. V. Prasad
tnvkvprasad@gmail.com

[1] College of Veterinary Science, YSR District, Proddatur, AP 516 360, India

[2] Nanotechnology Laboratory, Institute of Frontier Technology, Regional Agricultural Research Station, Chittoor District, Tirupati, AP 517 502, India

[3] Department of Microbiology, Kurnool Medical College, Kurnool, AP 518 002, India

[4] Division of Pathology, Veterinary Biological Research Institute, Shanti Nagar, Hyderabad, AP 500 028, India

[5] Department of Food Science, London Metropolitan University, London, UK

Introduction

Beta-lactam antibiotics account for almost 50 % of global use of antibiotics (Walsh 2003; Elander 2003). Such an extensive use of β-lactam antibiotics has led to the emergence of resistance globally (Thomson and Bonomo 2005; Rice 2012). The transfer of resistance genes is mostly mediated by plasmids and transposons (Bennett 2008; Touchon et al. 2012) and occurs by conjugation, transduction or transformation. Resistance to β-lactam antibiotics occurs by different mechanisms, (Wilke et al. 2005; Fisher et al. 2005) of which β-lactamase mediated resistance is important in Gram-negative organisms. (Li et al. 1994; Poole 1994) Multidrug-resistant bacteria producing β-lactamases exhibit resistance towards broad range of β-lactam antibiotics, including third-generation cephalosporins and pose serious challenge to clinicians and limits treatment options (Jacoby and Price 2005; Paterson and Bonomo 2005).

Effective tackling of antimicrobial resistance requires better diagnostic techniques and novel drugs (Khanal et al. 2013). Traditionally, the discovery of new antimicrobial

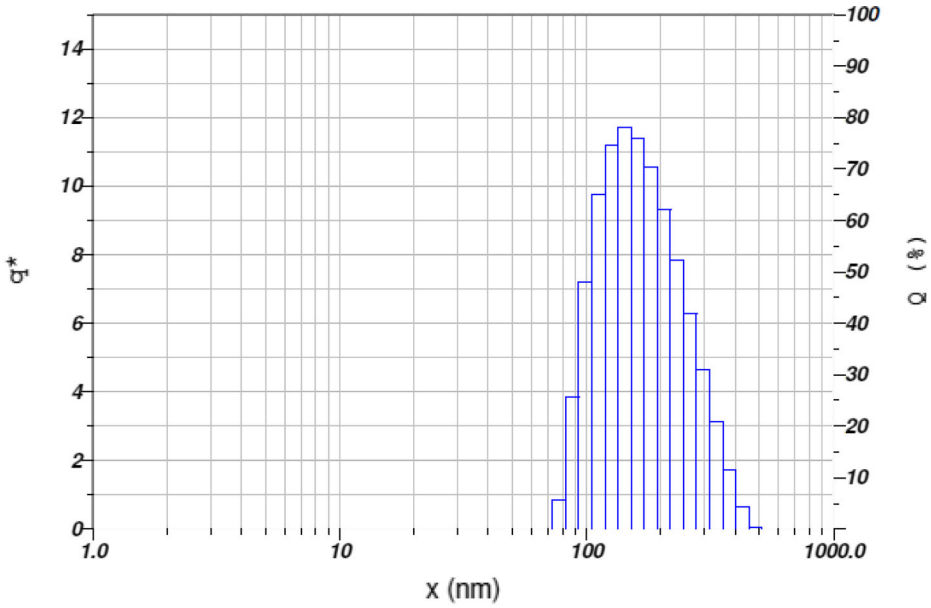

Fig. 1 Particle size distribution of aloin coated (ANS) silver nanoparticles (mean size 142.7 nm)

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	177.8 nm	71.1 nm	142.7 nm
2	—	--- nm	--- nm	--- nm
3	—	--- nm	--- nm	--- nm
Total	1.00	177.8 nm	71.1 nm	142.7 nm

agents effective against resistant bacteria involves the use of pathogenic resistant strains isolated from clinical setting. Further, the presence of resistance genes needs to be confirmed through PCR or other molecular techniques. Ideally, a bacterial model for screening new compounds effective against resistant forms should be non-pathogenic and allow for rapid high throughput screening. We propose pGLO plasmid transformed *Escherichia coli* HB 101 K 12 as a novel model for screening candidate drug molecules effective against β-lactam antibiotics. The model has the advantages of being non-pathogenic and provides visual confirmation for the presence of resistance genes. Further, as both resistant and non-resistant strains of the same bacteria are available, the pattern of susceptibility can be studied.

pGLO plasmid consists of genes encoding for either kanamycin resistance or ampicillin resistance, green fluorescent protein (GFP) genes and arabinose operon repressor (Bassitu 2011). The resistance to ampicillin is conferred through the production of β-lactamase enzyme, which destroys β-lactam antibiotics. GFP is a fairly small protein (27 kDa) isolated from the jellyfish, *Aequorea victoria* and requires no cofactors or substrates for its expression (Chalfie et al. 1994). GFP emits green fluorescence at 509 nm providing visual confirmation with a hand held ultraviolet (UV) lamp or non-invasively with UV spectrometry. The modified GFP gene is quite stable and is mainly used for educational purposes to demonstrate genetic engineering to students (Mosher 2002). Several applications for pGLO plasmid and GFP were reported. GFP tagging for fluorescence-based random mutagenesis of IS2 transposase for structure–function analysis was reported (Lewis et al. 2011). A high throughput microfluidic biosensor using *E. coli* HB 101 and pGLO plasmid for the detection of specific substrates and for screening of antibiotic drugs was developed (Sun et al. 2011). pGLO plasmid was used for evaluating survival of Salmonella spp. and *Yersinia enterocolitica* bacteria in pig slurry treated with urea and ammonia (Bolton et al. 2012) and to engineer a sensitive biosensor to screen for enzyme activity in *E. coli* producing 3, 4 dihydroxy benzoate (Jha et al. 2014). In the recent years, several reports have been cited in the literature on antimicrobial properties of a variety of nanoscale materials against an array of pathogens (Prabha shetty et al. 2014; Supraja et al. 2015).

Materials and methods

Chemicals

Luria–Bertani (LB) agar, LB broth, Calcium chloride (50 mM; pH 6.1), Ampicillin (Amp) and L (+) Arabinose

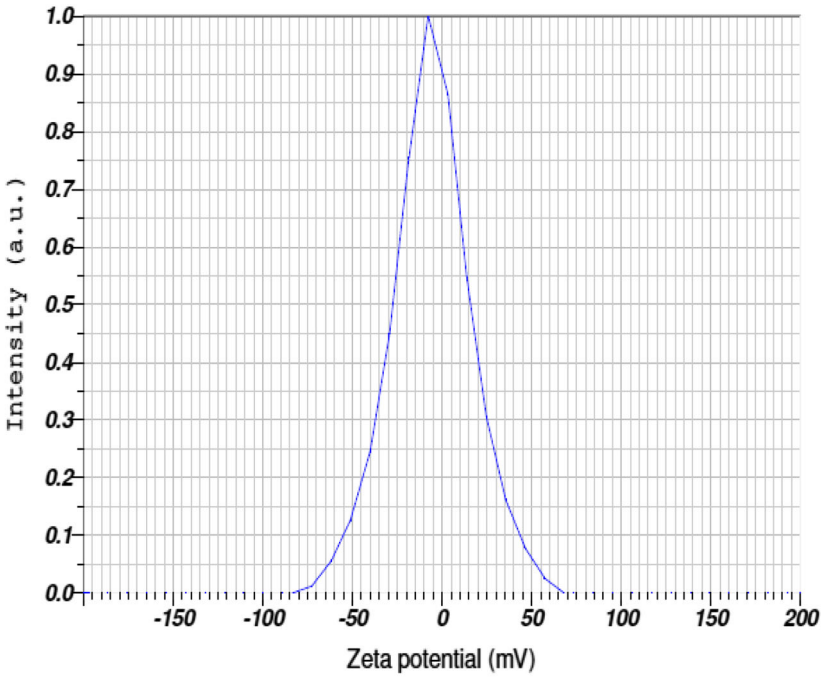

Fig. 2 Micrograph representing zeta potential of −5.8 mV of aloin coated silver nanoparticles (ANS)

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-5.8 mV	-0.000045 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : -5.8 mV
Electrophoretic Mobility mean : -0.000045 cm^2/Vs

(Ara) were obtained from Bio-Rad, USA. Mueller–Hinton (MH) broth, ρ-iodo nitro tetrazolium (INT) and dimethyl sulfoxide (DMSO) were obtained from Hi-Media, India. Aloin was from Sigma-Aldrich, USA, and Acetyl-11-α-keto-β-boswellic acid (AKBA) was obtained from Natural Remedies, India.

Preparation of the nanoparticles used in this study

Silver nitrate (>99 %) and trisodium citrate dihydrate (99.0 %) were purchased from Sigma-Aldrich. Sodium citrate and silver nitrate (5:1) were mixed in a conical flask and aged for about 2 h. The solution was heated to 100 °C on slow heating. During the heating process, 2–3 drops of 0.01 M sodium borohydrate was added to the solution. Then it has been observed that the colour of the solution was changed gradually to yellow within a few minutes, indicating the formation of Ag nanoparticles. The solution was kept boiling for an additional 6 min and was allowed to cool. Nanozinc oxide and nanomanganese oxide particles were prepared using oxalate decomposition method (Prasad et al. 2012). Aloin-mediated nanosilver particles and AKBA-mediated nanosilver particles were prepared by using the method described by Chaitanya et al.

Transformation of *Escherichia coli*

The process of transformation of *E. coli* HB 101 K 12 using pGLO plasmid was carried out as per manufacturer's instructions. Briefly, two to four colonies of the bacteria were suspended in 250 µL of 50 mM $CaCl_2$. 10 µL of pGLO plasmid was added and incubated on ice for 10 min. Heat shock was provided by quickly transferring to a water bath at 42 °C for exactly 50 s and then rapidly transferring onto ice. After an incubation of 2 min, 250 µL of LB broth was added and the culture was incubated overnight at 37 °C. 100 µL of the culture was spread each on LB/Amp/Ara agar, LB/Amp and LB agar and incubated at 37 °C. The procedure was repeated for *E. coli* culture without the addition of pGLO plasmid to obtain negative controls.

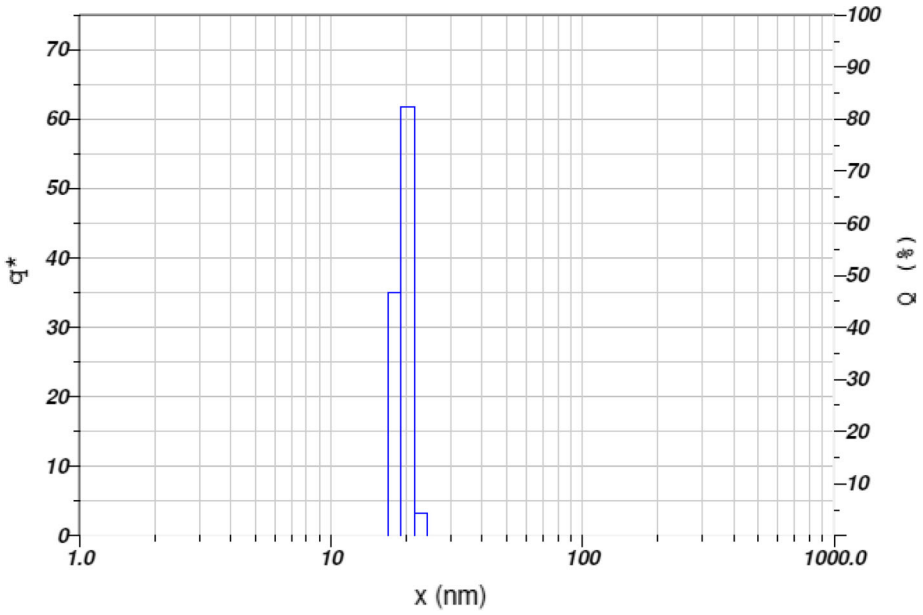

Fig. 3 Particle size distribution of *Boswellia ovalifoliolata* extract mediated silver nanoparticles (BNS)

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	19.5 nm	1.3 nm	19.6 nm
2	—	— nm	-- nm	— nm
3	—	— nm	-- nm	— nm
Total	1.00	19.5 nm	1.3 nm	19.6 nm

Transformation efficacy

The transformation efficacy was calculated by counting the number of colonies on LB/Amp/Ara plates for the transformed culture using the following formula:

Transformation efficiency
= Total no. of colonies on agar plate/
pGLO DNA spread on each plate.

Minimum inhibitory concentration by microdilution method

The minimum inhibitory concentration (MIC) of various compounds was evaluated as per Clinical and Laboratory Standards Institute (CLSI 2006). A twofold dilution of CNS, ANS, BNS and ampicillin was made in 100 µL of Mueller–Hinton (MH) broth in a microplate. NMO, NZO, Aloin and AKBA were dissolved in DMSO and similar two-fold dilutions were carried out in MH broth. To each well, 50 µL of 1:10 diluted 0.5 Mc Farland units of bacterial suspension was added to provide a final concentration of 5×10^5 cfu/mL per well. Positive and negative controls for culture and broth were also maintained. The plates were covered with parafilm to prevent drying and incubated at 37 °C for 18 h. One hour before the completion of incubation, 50 µL of p-nitro iodo tetrazolium (INT) (2 mg/mL in distilled water) (Eloff 1998) was added to each well and the plates were incubated at 37 °C for another hour. The minimum inhibition concentration was defined as the minimum concentration of the compound, which inhibited visible growth of bacteria, evidenced by lack of development of any colour.

Dynamic light scattering (particle size) and zeta potential analysis

Dynamic light scattering (DLS) technique is one of the widely accepted techniques to measure the hydrodynamic diameter (HDD) of the particles in a hydrosol. The particle size measurements were carried out using Nanopartica SZ-100 (HORIBA). Zeta potential was also measured using the same instrument using electrical conducting cell.

Results and discussion

Particle size and zeta potential analysis

Particle size and zeta potential measurements were carried out to reveal the hydrodynamic diameter and electrostatic

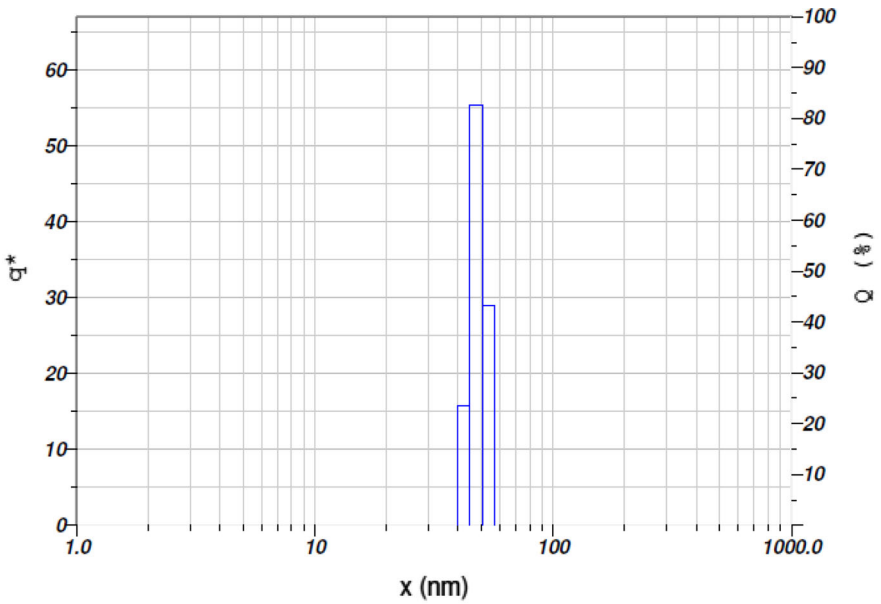

Fig. 4 Dynamic light scattering micrograph showing the particle size distribution of citrate coated (CNS) silver nanoparticles (mean size 48.2 nm)

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	48.5 nm	3.9 nm	48.2 nm
2	—	— nm	-- nm	— nm
3	—	— nm	-- nm	— nm
Total	1.00	48.5 nm	3.9 nm	48.2 nm

interaction of the prepared nanomaterials with their surrounding environment. The hydrodynamic diameter (HDD) of ANS (Fig. 1), BNS (Fig. 3) and CNS (Fig. 4) was recorded as 142.7, 19.6, 48.2 nm, respectively, which indicating the influence of bio-reducing agent on the size of the formed nanoparticles. Further, the zeta potentials of ANS and CNS particles were found to be −5.8 mV (Fig. 2) and −81 mV (Fig. 5), respectively, indicating the greater stability of CNS over ANS.

pGLO transformation

pGLO plasmid transformed *E. coli* exhibited an even lawn of growth on LB agar whereas 16 colonies were visible on LB/Amp and LB/Amp/Ara plates. A transformation efficacy of 100 cells/µg of pGLO plasmid was obtained. The colonies only on LB/Am/Ara plates showed fluorescence under long UV light (Fig. 6). The non-transformed *E. coli* showed growth only on LB agar.

Minimum inhibitory concentration

The MIC (ppm) of CNS, ANS, BNS, aloin, AKBA, nanomanganese, nanozinc oxide (NZnO), aloin, AKBA and standard ampicillin was determined for transformed and non-transformed bacteria (Table 1). Transformed *E. coli* was susceptible to CNS (20.64), ANS (0.32), BNS (10.32), NZO (34.83) and aloin (2.58); whereas resistance was observed against ampicillin (>666.7 ppm), NMO (>79.33) and AKBA (>73.33). Non-transformed *E. coli* was susceptible to all the compounds except nano manganese and AKBA. However, transformed *E. coli* (2.58) was more susceptible to aloin than untransformed *E. coli* (5.17).

The uptake of pGLO plasmid is enhanced in the presence of $CaCl_2$ aided by heat shock, which increases the competence of the bacterial cells to take up extraneous genetic material (Cohen et al. 1972; Bergmans et al. 1981). The competence of the bacteria is also reported to be increased by other methods such as electroporation, (Dower et al. 1998) plasmid artificial modification (Yasui et al. 2009) and micro-shock waves (Divya et al. 2011). Transformation with pGLO plasmid expresses β-lactamase enzyme. Hence, visible growth was observed on LB/Amp and LB/Amp/Ara agar plates only in +pGLO group (Fig. 6). However, fluorescence was not detected in +pGLO colonies on LB/AMP agar (Fig. 6) due to the absence of arabinose, which is required for switching on

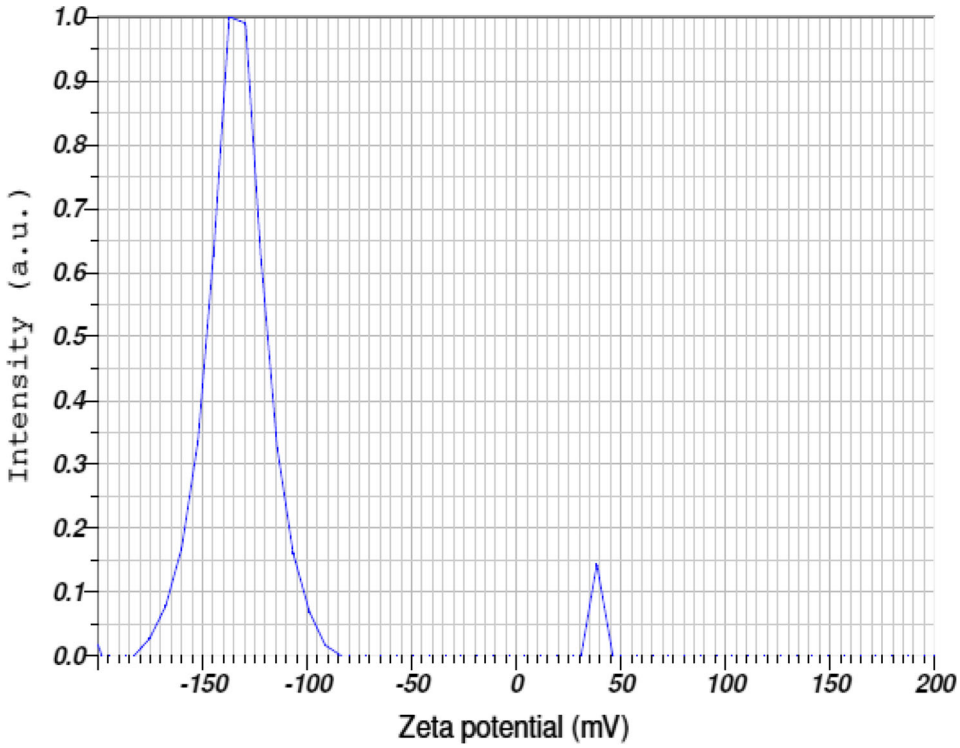

Fig. 5 Micrograph representing the zeta potential (−80 mV) of citrate coated silver nanoparticles (CNS)

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-133.4 mV	-0.001033 cm2/Vs
2	38.2 mV	0.000296 cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : -81.0 mV

Electrophoretic Mobility mean : -0.000627 cm^2/Vs

GFP expression. GFP is expressed only in the presence of sugar arabinose, as the genes ara A and D are replaced with GFP genes. In the LB/AMP/ARA agar, due to the presence of arabinose, fluorescence was exhibited by +pGLO colonies confirming transformation.

In microdilution method for MIC determination, MH broth is recommended as the medium of choice for susceptibility testing for aerobic or facultative organisms. The broth demonstrates good batch-to-batch reproducibility, is low in sulphonamide, trimethoprim, and tetracycline inhibitors and yields satisfactory growth of most pathogens and could be supplemented to support the growth of fastidious bacteria (Ericsson and Sherris 1971). The resistance of +pGLO *E. coli* to ampicillin is due to the presence of β-lactamase gene, which produces β-lactamase that cleaves the amide bond of β-lactam ring rendering ampicillin ineffective (Livermore 1995; Poole 2004). Among the compounds screened, ANS showed highest antibacterial activity against resistant bacteria followed by CNS and BNS. Similar observations of potent antibacterial activity of ANS against *Staphylococcus aureus* with an MIC of 21.8 ng/mL were reported (Kumar et al. 2013, 2014). Several authors evaluated the antibacterial activity of citrate mediated silver nanoparticles against *E. coli O157:H7* and observed a MIC of 12.43 ppm; (Petrus et al. 2011); *E. coli* (ATCC8739) with an MIC of 160 ppm (El-Kheshen and El-Rab 2012).

Apart from nanosilver particles, NZnO also exhibited antibacterial activity against transformed and non-transformed bacteria. Nanozinc oxide was shown to have a MIC of 0.1 ppm against *E. coli* K88 strain (Wang et al. 2012). Liu et al. (2009) observed complete inhibition of microbial growth at a concentration of 12 mmol/L with nanozinc oxide against *E. coli* O157:H7. Nano manganese oxide on the other hand was not effective against both transformed and non-transformed *E. coli.* However, earlier (Al-Hazmi

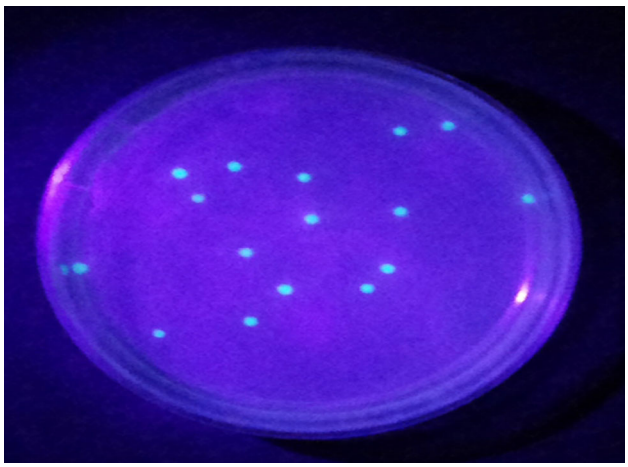

Fig. 6 pGLO transformed *Escherichia coli* HB 101 K 12 exhibiting fluorescence under UV light

et al. 2012) nano magnesium oxide wires were shown to possess bacteriostatic effect on *E. coli* and Bacillus spp. at concentrations above 100 ppm. As low concentration of MNO (79.33 ppm) was used in this study, the antibacterial activity of MnO could not be detected.

Aloin exhibited higher antibacterial activity against both transformed and non-transformed bacteria. Similar results have been reported previously (Tian et al. 2003; Minale et al. 2014) against *E. coli*. Aloin had an MIC of 10 ppm against *E. coli* CD/99/1, K88, K99, LT 37, ROW 7/12, 3:37 C, 306, and 872 strains. In this study, transformed *E. coli* exhibited enhanced susceptibility to aloin than non-transformed *E. coli*. The increased susceptibility could be explained by 'negative cross-resistance' or 'collateral sensitivity', where the induction of resistance to one compound enhances the toxicity to other compounds (Li et al. 2002; Palmer et al. 2010). Previously, similar phenomenon was observed through the increased susceptibility of *E. coli* to fusaric acid consequent to development of resistance against tetracyclines by modification of efflux pumps (Bochner et al. 1980).

Another phytochemical, AKBA showed no antibacterial activity against both transformed and non-transformed *E. coli*. This is due to the presence of lipophilic outer membrane in Gram-negative bacteria, which acts as a hydrophilic permeability barrier for the entry of hydrophobic compounds such as AKBA (Raja et al. 2011; Hancock 1997; Helander et al. 1998). Similar observations were made in studies dealing with antibacterial activity of other hydrophobic compounds against Gram-negative bacteria (Trombetta et al. 2005; Gallucci et al. 2009).

Table 1 Susceptibility of transformed and non-transformed *Escherichia coli* HB 101 K 12

Compound	Minimum inhibitory concentration (MIC) (ppm)	
	Transformed *E. coli* (+pGLO)	Untransformed *E. coli* (−pGLO)
CNS	20.64 ± 1.2	10.32 ± 0.4
ANS	0.32 ± 0.0	0.16 ± 0.0
BNS	10.32 ± 0.9	20.64 ± 0.8
NMO	>79.33 ± 2.6	>79.33 ± 2.2
NZO	34.83 ± 1.5	17.42 ± 0.9
Aloin	2.58 ± 0.2	5.17 ± 0.3*
AKBA	>73.33 ± 2.9	>73.33 ± 2.6
Ampicillin	>666.67 ± 3.8	10.42 ± 0.8

Each value is the ±SE of three replications

AKBA acetyl 11-α-keto β-boswellic acid, *ANS* Aloin mediated nanosilver, *BNS* AKBA mediated nanosilver, *CNS* citrate mediated nanosilver, *NMO* nano manganese oxide, *NZO* nanozinc oxide

* Indicates negative cross resistance

Conclusion

Antibiotic resistance is an expanding menace and this needs immediate attention by the scientific community. Nanoscale materials are the new generation of materials which exhibit novel antimicrobial properties, noble nanoscale materials in particular, against an array of microbes. Consequently, methods need to be developed for the rapid assessment of antimicrobial efficacy of materials with different size, shape and surface coatings. We reported for the first time that pGLO plasmid transformed *E. coli* HB 101 K 12 serves as a novel model for screening of new antimicrobial compounds including nanomaterial based antimicrobials which were effective against β-lactam resistance. The model has the advantages of being non-pathogenic and provides visual confirmation for the presence of resistance genes.

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