

Microbial Synthesis of Reduced Graphene Oxide Nanosheets by *Proteus mirabilis* MT378377 and Antibacterial Activity against Multidrug Resistant Bacteria

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Abstract

Graphene oxide contains different functional groups containing oxygen that can be removed through different processes used for the synthesis of reduced graphene oxide. The present study focused on the biosynthesis of reduced graphene oxide employing bacteria through optimization of conditions. Initially six bacterial isolates were screened for reduction of graphene oxide. Based upon 16SrDNA sequence homology GS3 showed 99% homology with *Proteus mirabilis* strain. The *Proteus mirabilis* MT378377 was further optimized at different temperature where a wavelength at 262nm and maximum absorbance of 2.422 was measured at 37°C. In the presence of pH 5 the absorbance further increased to 2.575 with peak at 250nm. Among all the metal ions studied maximum absorbance was obtained in the presence of sodium chloride (NaCl, 1mM) which was 2.346 at 252nm wavelength. Addition of Tween 80 (surfactant) further increased synthesis with 2.926 absorbance at 252nm wavelength. Optimal initial concentration of graphene oxide (GO) was 1mg/ml that proved effective for rGO synthesis with 2.989 absorbance at 252nm. rGO synthesis was efficient under dark condition obtaining 260nm wavelength with 2.972 absorbance. Upon characterization rGO had 91.28 nm size nanosheets and had some of the oxygen functionalities removed. The formed nanosheets had zeta potential of -31.5 mV and PDI of 1260. Comparison of antibacterial activity of graphite, graphene oxide and reduced graphene oxide nanosheets showed that graphene oxide showed highest antibacterial activity against MDR pathogens whereas these pathogens showed resistance towards rGO nanosheets. Due to their non-toxic nature and presence of functional groups they can be used for other purposes such as nanocarriers in drug delivery systems.

Introduction

Nanotechnology has become a promising field of known research in biomedicine. On the basis of morphology, dimensions (range) and chemical characteristics nanoparticles are classified into different types such as ceramic, lipids, metal nanoparticles and carbon based nanostructures. Among carbon based nanostructures three noteworthy classes of graphene-based nanostructures are pristine graphene, GO and rGO [20, 35].

NPs synthesized by biological methods show higher stability than those produced by chemical methods [9]. For production of reduced graphene oxide (rGO) in large quantity, thermal GO reduction [25], electrochemical reduction [38, 45, 2] chemical reduction methods are used [34, 42, 14]. It is well known that electrochemical reduction requires expensive platinum electrodes and consumes a lot of energy, while thermal reduction occurs in the presence of hydrogen gas along with high temperature which requires special reactors. Whereas, chemical methods require a concentrated alkaline solution and toxic or corrosives hydrazine as strong reducing agents. There is a need to develop and improve eco-friendly strategies for the large scale reduction of rGO [12, 11, 33, 42, 46, 1]. For microbial synthesis different bacteria such as *Shewanella*, *Escherichia coli* and baker's yeast were used for synthesis of reduced graphene oxide nanosheets (rGO) [15, 19]. The outer membrane c-type cytochromes or self-secreted electron mediators both have been described for graphene oxide reduction through extracellular electron

transfer mechanism. Direct electron transfer also has been reported as another possible mechanism for GO reduction [37]. *Lysinibacillus sphaericus* HJ-3 was also exhibited an efficient reduction of GO through direct cytochrome-mediated electron transfer and flavin-dependent shuttling mechanisms [46, 50].

Production of RGO by using three various bacterial strains – *Lactococcus lactis* (*L. Lactis*), *Lactobacillus plantarum* (*L. plantarum*) and *Escherichia coli* (*E. coli*) and examining the resulting RGO's toxicological properties for its safe application was also reported [49].

High antibacterial activity was measured against *P. aeruginosa* upon exposure to GO and rGO [13]. It has also been investigated that GO and rGO both influenced a potent antimicrobial activity against *Escherichia coli* in a time and dose dependent manner by involving oxidative pathways [14, 31]. Though the graphene interacts with bacteria through oxidization and disruption of vital biological structures, oxidative stress can be produced without ROS generation [22]. Reactive sharp edges in reduced graphene nanowalls (RGNWs) act as a good electron acceptor. The reduced graphene nanowalls show better antibacterial activity because of powerful interaction, in which charge from bacterial surface transfers to the reduced nanowalls. In comparison unreduced graphene oxide nanowalls show less antibacterial activity [3]. Bacterial cells from their microenvironment can be trapped and isolated by the aggregation of graphene sheets [4]. Large nanosheets can easily isolate the cells from the environment and shows highest antibacterial activity than the small nanosheets. As reported earlier, bactericidal activity of graphene oxide nanosheets against *E. coli* depends upon the sheet size too [21]. Other studies demonstrated that reduced graphene oxide (RGO) displayed different forms of microbiological behavior. Indeed, the antibacterial activity of RGO was registered only at high concentrations (200 and 400 µg/mL) against the model organism *Escherichia coli*. Meanwhile, no antibacterial activity could be detected at low concentrations (10 and 50 µg/mL) [48].

Another research showed MIC value of rGO/AgNC an excellent antimicrobial inhibition effect against some pathogenic bacterial strains viz. Gram-negative bacteria (*E. coli* and *Klebsiella pneumonia*) and Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) as well as *Candida albicans* was found for the pathogenic yeast [47].

The present study was intended towards bacterial synthesis of rGO nanosheets which is an eco-friendly method having antimicrobial potential against multidrug resistant pathogens.

Materials and methods

Reduction of graphene oxide (GO) nanosheets

Graphene oxide was synthesized according to modified Hummer's method [40]. From soil sample different bacterial isolates were isolated on nutrient agar. Afterwards these colonies were screened for the synthesis of rGO nanosheets. Reduced graphene oxide (rGO) was synthesized by using two methods. Both methods consisted of three basic steps, first biomass preparation, then stock preparation of GO, later reduction of graphene oxide.

Biomass preparation

Method I: The culture (108 cells/ml) grown in LB broth was directly used for the reduction of graphene oxide (GO).

Method II: The culture was centrifuged at 6500 rpm/min for 15 minutes to separate cells and the supernatant was discarded. This pellet was used for reduction of graphene oxide [37].

Reduction of Graphene oxide

According to modified Hummer's method, graphene oxide was synthesized [40]. To 5ml of GO (0.5 mg/ml) dispersion in water, equal volume of inoculum was mixed and incubated for 72 h at 37°C. The stable black dispersion was formed after the reduction process. The black pellet was centrifuged separated and suspended in water.

Molecular identification of the bacterial isolate

On the basis of 16S rDNA sequence, identification of bacterial isolate GS3 was done through First Base Company (Singapore) using sequencing primers 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'. PCR primers used were 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'. The phylogenetic relationship was checked on the basis of homology sequence by Neighbor joining method [17]. The isolate GS3 was identified as *Proteus mirabilis* (NCBI Genbank Accession No. MT378377).

Optimization of condition for synthesis of rGO nanosheets

Effect of incubation temperatures (30°C, 37°C, 40°C, 45°C and 50°C) on the formation of rGO nanosheets was investigated. GO was added to bacterial cultures grown at different temperatures to observe rGO synthesis. Then GO was added to the culture and incubated for 72 hrs. Similarly effect of different pH (4–8) was also studied for rGO synthesis. For this the bacteria were grown in broth having different pH. After 24 hrs of incubation, GO was added to the culture and formation of rGO nanosheets was monitored. Likewise, the effect of various metal ions (1mM) such as (MgSO₄, NaCl, ZnSO₄, CuSO₄, and KH₂PO₄) and different surfactants (1mM) such as tween 20, tween 80 (0.2%), SDS and enhancer (PEG) were also investigated for reduction of GO nanosheets. The effect of different concentration of GO (0.1, 0.5, 1, 2.5, 5, 10 and 15mg/ml) was also investigated for the synthesis of rGO nanosheets. The reaction mixture was kept in both light and dark conditions to check their effect on formation of rGO nanosheets. rGO synthesis was timely monitored at different time intervals (1hr, 2hrs, 3hrs, 4hr, 5hrs, 6hrs, and then day 1 to day 4).

Structural and morphological characterization of rGO

Different techniques were used for the characterization of rGO such as, UV–visible spectroscopy analysis, FTIR spectrometer IRTracer-100 (SHIMADZU, NA), Zeta potential and Scanning electron

microscope EVO LS 10 (ZEISS, USA) [39, 29].

Antibacterial assay

Antibacterial activity of rGO nanosheets against multi drug resistant bacteria *Escherichia coli* BTCB201, *Staphylococcus aureus* BTCB203 and *Salmonella typhi* BTCB202 was checked by using well diffusion method [6]. For inoculum preparation, cell density of pathogens was adjusted (0.5 McFarland turbidity standards) by suspending in normal saline (10ml of 0.85% NaCl) [27]. Different concentration of graphene oxide, graphite (1, 2, 3,4,5,6 mg/ml) and rGO nanosheets (0.01mg/20ul, 0.02mg/40ul, 0.03mg/60ul, 0.04mg/80ul, 0.05mg/100ul and 0.06mg/120ul) were loaded in wells. After 24 hrs of incubation zones of inhibition were measured [6].

Statistical analysis

All the experiments were carried out in triplicates. Use of one way ANOVA was done for finding out the significance difference ($p \leq 0.05$) between means of different parameters and SPSS program was used along with application of Duncan's multiple range tests [8].

Results and discussion

Synthesis of Graphene oxide

Graphene oxide synthesis was indicated by change in colour i.e. yellow to brown (Fig. 1a) and was further confirmed through UV -Vis spectra which indicated that GO peak at 230nm with 0.668 absorption value with an arm at 300 nm with 0.312 absorbance value (Fig. 1b). In another study graphene oxide presented a similar peak at 230nm with an arm at 300 nm in UV-Vis spectra [40].

Biosynthesis of rGO

All the isolates were screened for the synthesis of rGO nanosheets. Biosynthesis of rGO from GO was further confirmed through UV-Vis spectrum analysis, the absorption peak of GO shifted from 230nm to absorption peak of rGO i.e. 260nm (Fig. 1e). The results of UV-Vis spectrum for method I showed maximum (2.154) absorbance at 260nm for GS3 strain (Fig. 1d). Other studies have shown method II for the synthesis of reduced graphene oxide and peak obtained was 267nm [15]. When compared, method I proved more effective for the synthesis of rGO. In method I presence of bacteria somehow stimulated the synthesis of rGO, might be due to direct interaction of GO with the bacterial cell membrane or cell membrane bound enzymes. Enzymes play a significant part in biosynthesis of nanoparticles. Nicotinamide adenine dinucleotide phosphate (NADPH) is an enzyme found in Baker's yeast act as a reducing and functionalizing agent in GO reduction process [19]. The outer membrane c-type cytochromes or self-secreted electron mediators both have been described for graphene oxide reduction through extracellular electron transfer mechanism. Direct electron transfer also has been reported as another possible mechanism for GO reduction [37, 46, 49].

Effect of incubation temperature

The optimum incubation temperature i.e. 37°C was selected for rGO nanosheet synthesis by *Proteus mirabilis* MT378377 with maximum absorbance of 2.422, at 262nm. When compared significant ($p \leq 0.05$) reduction of graphene oxide was observed at 37°C than at other temperatures (Fig. 2a). In other studies, reduced graphene oxide nanosheets were also synthesized at 37°C by using different microbes [19, 15]. Mesophilic microbes cannot with stand high temperature therefore their optimum activity is at 37°C and not at elevated temperature [32, 36].

Effect of pH

In order to standardize the pH of reaction mixture for the synthesis of rGO nanosheets the experiment was performed at pH that ranged from 4 to 8. It was observed that *Proteus mirabilis* MT378377 at pH 5 showed significant ($p \leq 0.05$) reduction in graphene oxide with a peak at 250 nm and absorbance of 2.575 (Fig. 2b). pH has been known to show effects on the morphology and internal structure of reduced graphene oxide [5]. In a study it was reported that at acidic pH (pH 3), defected nano sheets were produced with smaller size (sheets of rGO with 1um size). However, under alkaline conditions the sheets produced reached tens of micrometers [5].

Effect of inorganic salts

Effect of various salts $MgSO_4$, NaCl, $ZnSO_4$, $CuSO_4$, and KH_2PO_4 were observed for the synthesis of reduced graphene. After 48 hrs the black dispersion at bottom was formed which indicated the time reduction for the synthesis of rGO. In the current study a 252nm peak with maximum absorbance of 2.346 was obtained in the presence of NaCl indicating significant ($p \leq 0.05$) reduction as compared to other salts (Fig. 2c). Addition of metal ions in current study not only increases the reduction of graphene oxide but also decreased the time for the synthesis of reduced graphene oxide from 72 hrs to 48 hrs. Metal ions increased the bacterial growth and eventually the enzyme production that led to decrease in nanoparticle size and time of formation. In another study stable silver nanoparticles were produced from *Penicillium fellutanum* (marine fungus) of 5 to 25nm size in the presence of 0.3% NaCl [18].

Effect of surfactants

Of all the surfactant studied Tween 80 proved effective in synthesis of rGO with a peak absorbance of 2.926 at 252nm (Fig. 2d). Different concentrations (0.2%, 0.5%, 1%, and 1.5%) of Tween 80 was further studied to find the effect on rGO synthesis. It was observed that 0.2% concentration proved effective for stabilization of rGO with a peak at 252nm and absorbance of 2.511, while at 0.5% and 1% concentration multiple peaks were obtained (Fig. 2e). Other studies showed that addition of other surfactants such as CTAB, SDBS, TBAOH Tween 20 and PDPA, also stabilized the graphene based nanostructures through intercalation and are easily dispersed in aqueous solvents of these surfactants [43]. In some studies, both PDPA and Tween 20 not only were used as a stabilizing agent for GO but also as a reducing and immobilizing agent for Au nanoparticles [24, 12]. Surfactants are also responsible for the size reduction,

and there are many surfactants reported which yields small sized nanoparticles including Sodium lauryl Sulphate, SDS, Triton X, Tween 80, EDTA and Tween 20 [26].

Effect of substrate concentration

In the current study the concentration of graphene oxide 1mg/ml proved efficient for the synthesis of rGO nanosheets. At this concentration the UV-Vis spectrum showed a peak at 252nm with maximum absorbance of 2.89 (Fig. 2f). From previous studies rGO was synthesized from graphene oxide with varying concentrations from *E.coli* (5-2mg/ml) and *Shewanella* (0.5mg/ml) [37, 15].

Effect of light and dark conditions

In the absence of light GO showed significant ($p \leq 0.05$) reduction with the maximum absorbance of 2.668 at 260nm as compared to reduction in the presence of light (Fig. 3a). Many other studies dealing with biosynthesis of rGO nanosheets did not mention whether the synthesis was achieved in the light or dark [15,19,37]. However, in one of the study syntheses of silver nanoparticles under dark conditions reduced the size of NPs and were more stable [28].

Effect of times

The results of UV-Vis spectra revealed that the rGO nanosheets were more synthesized at day 3 with significant ($p \leq 0.05$) reduction and a peak at 260nm with 2.972 absorbance (Fig. 3b). Similarly in one of the study effects of time on synthesis of rGO was observed with the addition of amino acid and L-ascorbic acid in which reduction of GO gradually increased with time [10, 42, 7].

Bacterial identification

Gram staining showed Gram negative rod shaped diplo bacilli (Fig. 1c). The molecular identification indicated bacterial isolate GS3 identified as *Proteus mirabilis* as it showed 99% similarity with *Proteus mirabilis* (Fig. 3c).

rGO characterization

SEM was used for the structural and morphological characterization of reduced graphene oxide and showed a relatively large number of sheet-like structures that indicated the successful formation of rGO nanosheets (Fig. 3f). The large number of reactive edges and a sheet-like structure with wrinkles were indicative of the successful formation of rGO nanoflakes [2, 23, 29, 30].

The FTIR spectra of reduced graphene oxide showed that the oxygen functionalities present at peaks in 1226.73 cm^{-1} and 1492.90 cm^{-1} disappeared. Although many oxygen-functionalities residual on GO still existed on the rGO surface (Fig. 3d). The spectrum of GO showed peak at 3329.14 cm^{-1} which confirmed a water O-H stretch. The peak at 2345.44 cm^{-1} also confirmed C-N stretch. The carbonyl functional groups were also shown at 1658.78 cm^{-1} as C = O stretching and a peak at 1492.90 cm^{-1} is

indicating stretching vibration of C-OH groups. At 1226.73 cm^{-1} and 1064.71 cm^{-1} the peaks were assigned to stretching vibrations of C-O- epoxy, whereas the peak at 597.93 cm^{-1} also confirmed as C-Cl group (Fig. 3e). FTIR spectra clearly shows the oxygen-containing functional groups present in GO that are moderately removed in rGO [2, 23, 29, 30, 39].

Zeta sizer indicated size of rGO nansheets to be 91.28 nm with PDI of 1260 and with 99.3% intensity (Fig. 4a). Results indicated nano-sized and monodispersed reduced graphene oxide nanosheets. Reportedly, the average size distribution of rGO nanosheet was 122.4 nm [16]. rGO nanosheets exhibited negative zeta potential -31.5 mV . The magnitude of zeta potential value showed stability of nanoparticles. A previous study showed zeta potential of $-17.7 \pm 7.99\text{ mV}$ for the rGO synthesized [16].

Antibacterial assay

Graphene oxide at concentration of 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml, 5mg/ml and 6mg showed zone of inhibition of 10mm, 14mm, 15mm, 16mm, 17mm and 24mm against *Salmonella typhi* BTCB202, respectively (Fig. 5a). GO also showed different zones of inhibition of 11mm (1mg/ml), 13 mm (2mg/ml), 14 mm (3mg/ml), 18 mm (4mg/ml), 19 mm (5mg/ml), 23 mm (6mg/ml) against *Escherichia coli* BTCB201 (Fig. 5b). Furthermore, against *Staphylococcus aureus* BTCB203 zones of inhibition obtained was 5mm, 8mm, 11mm, 12mm, 14mm and 17mm at the concentration of 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml, 5mg/ml and 6mg/ml, respectively (Fig. 5c). In case of *Salmonella typhi* BTCB202, highest zone of inhibition obtained was 24 mm at 6mg/ml while the lowest zone observed was against *Staphylococcus aureus* BTCB203 17mm (6mg/ml). It was also observed that by increasing the concentration of graphene oxide its antibacterial activity against multi-drug resistant pathogens also increased. In previous studies it was proved that the dispersibility and size of materials also affect the antibacterial activity of graphene based materials. GO can form stable dispersions with small nanosheets that increased their antibacterial activity against MDR pathogens through membrane and oxidative stress mechanism [22, 48].

Whereas in case of Graphite, zone of inhibition obtained was 13mm (3mg/ml), 15mm (4mg/ml), 17mm (5mg/ml) against *Salmonella typhi* BTCB202 (Fig. 4c) and at 1mg/ml, 2mg/ml and 6mg/ml no zones were formed. Both *Staphylococcus aureus* BTCB203 and *Escherichia coli* BTCB20 were resistant towards graphite at all the concentrations studied (Fig. 4d, e). Other studies show that the antibacterial mechanism of Graphite is membrane and oxidative stress. It was proved that graphite has low antibacterial activity as compared to other graphene materials. The reason is that graphite nanosheets are unstable and contain large particles [22].

Antibacterial activity of reduced graphene oxide was also tested against MDR *Staphylococcus aureus* BTCB203 (Fig. 5f), *Salmonella typhi* BTCB202 (Fig. 5d) and *Escherichia coli* BTCB201 at different concentration but no zones of inhibition were formed (Fig. 5e). It indicated that MDR pathogens were resistant towards rGO. Among all GO showed highest antibacterial activity. In another study comparison of antibacterial activity of different graphene based materials such as graphite, graphite oxide, graphene

oxide and reduced graphene oxide was studied which showed that graphene oxide had the highest antibacterial activity [22]. In general, graphene materials, which contain a higher density of functional groups, and are smaller in size, have more chances to interact with bacterial cells, resulting in cell death. It was observed that thin layers of nanosheets form GO dispersion, while rGO dispersion mainly contains large aggregated particles. The different behavior of GO and rGO suggests the aggregation/dispersion of graphene-based materials may play an important role in their antibacterial activities. In another study the mechanism that is involved in antibacterial activity against *E.coli* was DNA fragmentation through the generation of intracellular ROS [14].

Conclusion

Conclusively, the approach proved effective, environmentally friendly and economical for the synthesis of reduced graphene oxide nanosheets from *Proteus mirabilis* MT378377. Although MDR pathogens including *Escherichia coli* BTCB201, *Staphylococcus aureus* BTCB203 and *Salmonella typhi* BTCB202 showed resistance towards rGO nanosheets but because of their oxygen containing functional groups they can also be used for synergistic antibacterial activity with other nanoparticles against several multidrug resistant bacteria. The rGO large surface area can also be used for other purposes such as nanocarriers for drug and gene delivery systems. They also play an important role in lubrication in machine elements, especially at macroscopic contacts etc. and therefore have promising future.

Declarations

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Author Contribution

Prof. Dr. Mehwish Iqtedar: Designed the study and reviewed the manuscript
Sana Rashid: Did the research work and wrote the manuscript
Dr. Roheena Abdullah: Provided lab facility
Dr. Afshan Kaleem: Statistical analysis
Dr. mahwish aftar: Did Molecular Analysis

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Figures

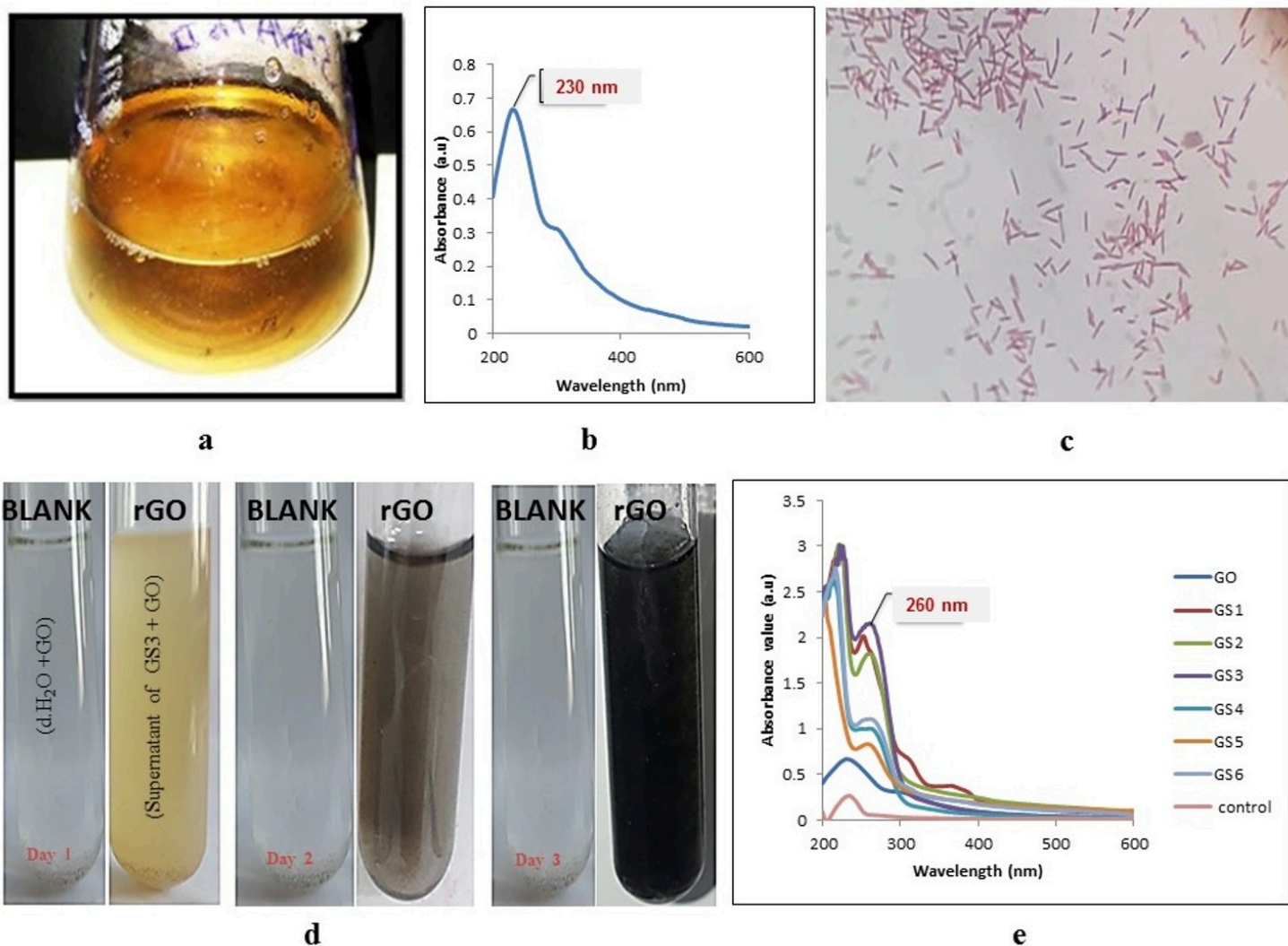


Figure 1

Yellow to brown solution of Graphene oxide (GO) (a) UV-Vis spectrophotometer analysis of Graphene oxide with absorption peak at 230nm (GO) (b) Gram negative rods of *Proteus mirabilis* MT378377 at 100X oil immersion (c) Bacterial isolate GS3 producing black colour indicating synthesis of rGO nanosheets (d) UV-Vis spectrophotometer analysis of all bacterial isolates (e).

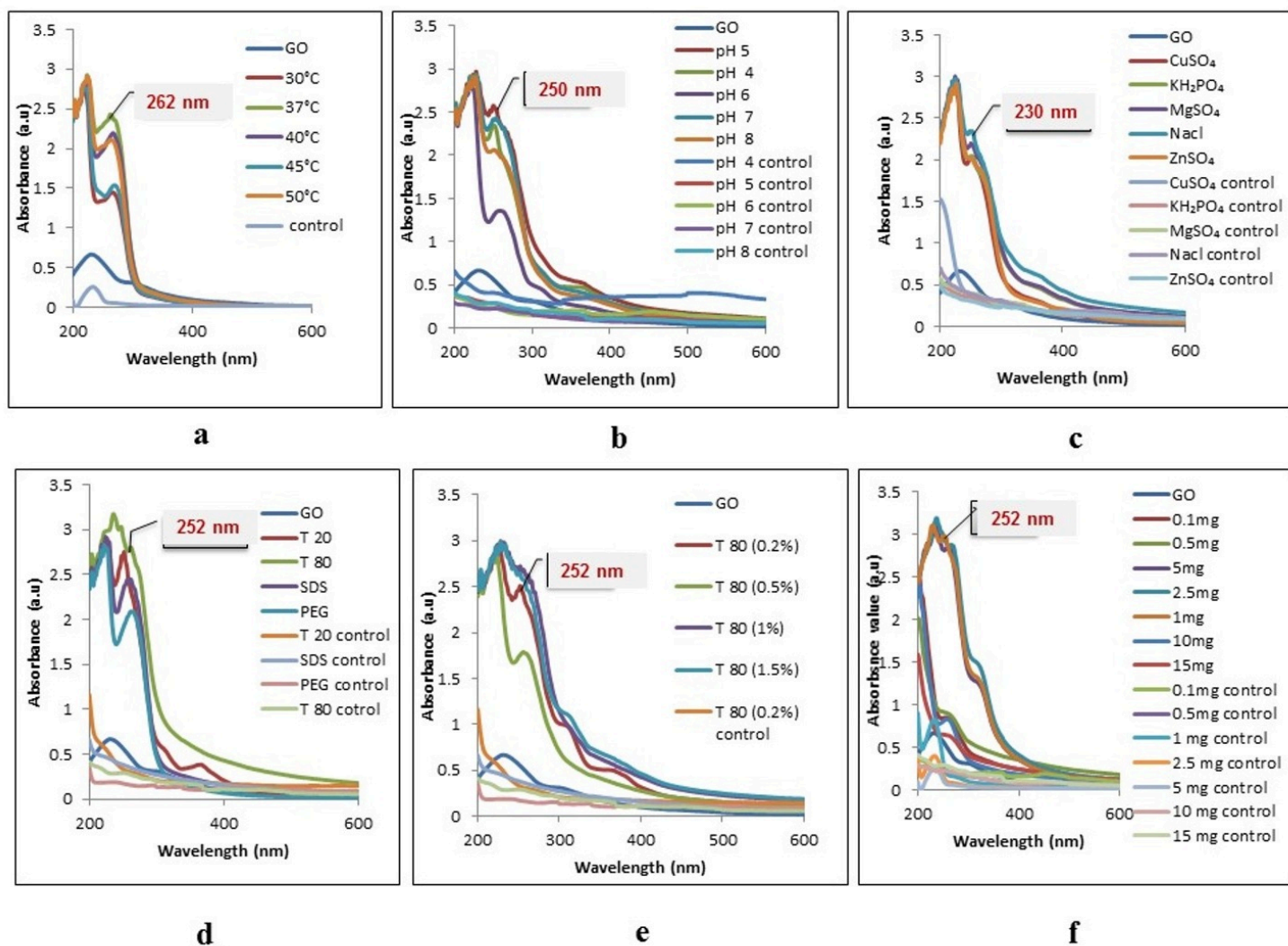


Figure 2

UV-Vis spectrophotometer analysis of rGO nanosheets synthesized by *Proteus mirabilis* MT378377 at different temperatures (a) UV-Vis spectrophotometer analysis of rGO by *Proteus mirabilis* MT378377 after incubating graphene oxide solution with bacteria at different pH (4-8) (b) UV-Vis spectrophotometry analysis of rGO synthesized by isolate GS3 using different metal ions (c) UV-Vis spectrophotometer analysis of rGO synthesized by *Proteus mirabilis* MT378377 with different surfactants (d) UV-Vis spectrophotometer analysis of rGO synthesized by *Proteus mirabilis* MT378377 under different concentration of TWEEN 80 (e) UV-Vis spectrophotometer analysis of rGO synthesized by *Proteus mirabilis* MT378377 at different graphene oxide concentration (f).

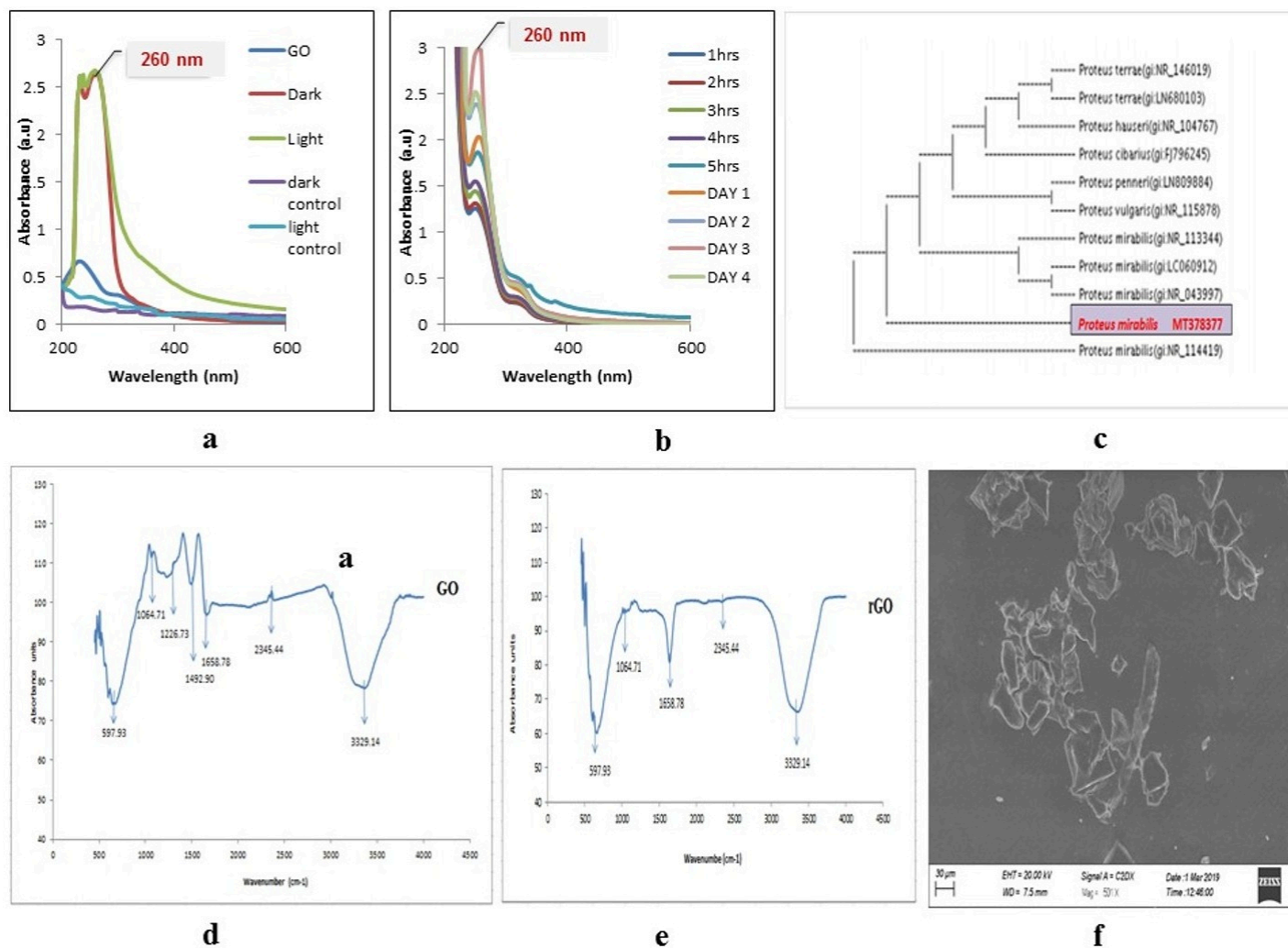


Figure 3

UV-Vis spectrophotometer analysis of reduced graphene oxide synthesized by *Proteus mirabilis* MT378377 under light and dark conditions (a) UV-Vis spectrophotometer analysis of reduced graphene synthesized by *Proteus mirabilis* MT378377 at different time interval (b) Phylogenetic tree of *Proteus mirabilis* MT378377(c) FTIR spectra of GO (graphene oxide) (d) FTIR spectra of rGO (reduced graphene oxide) (e) SEM image containing nanosheets of rGO (f).

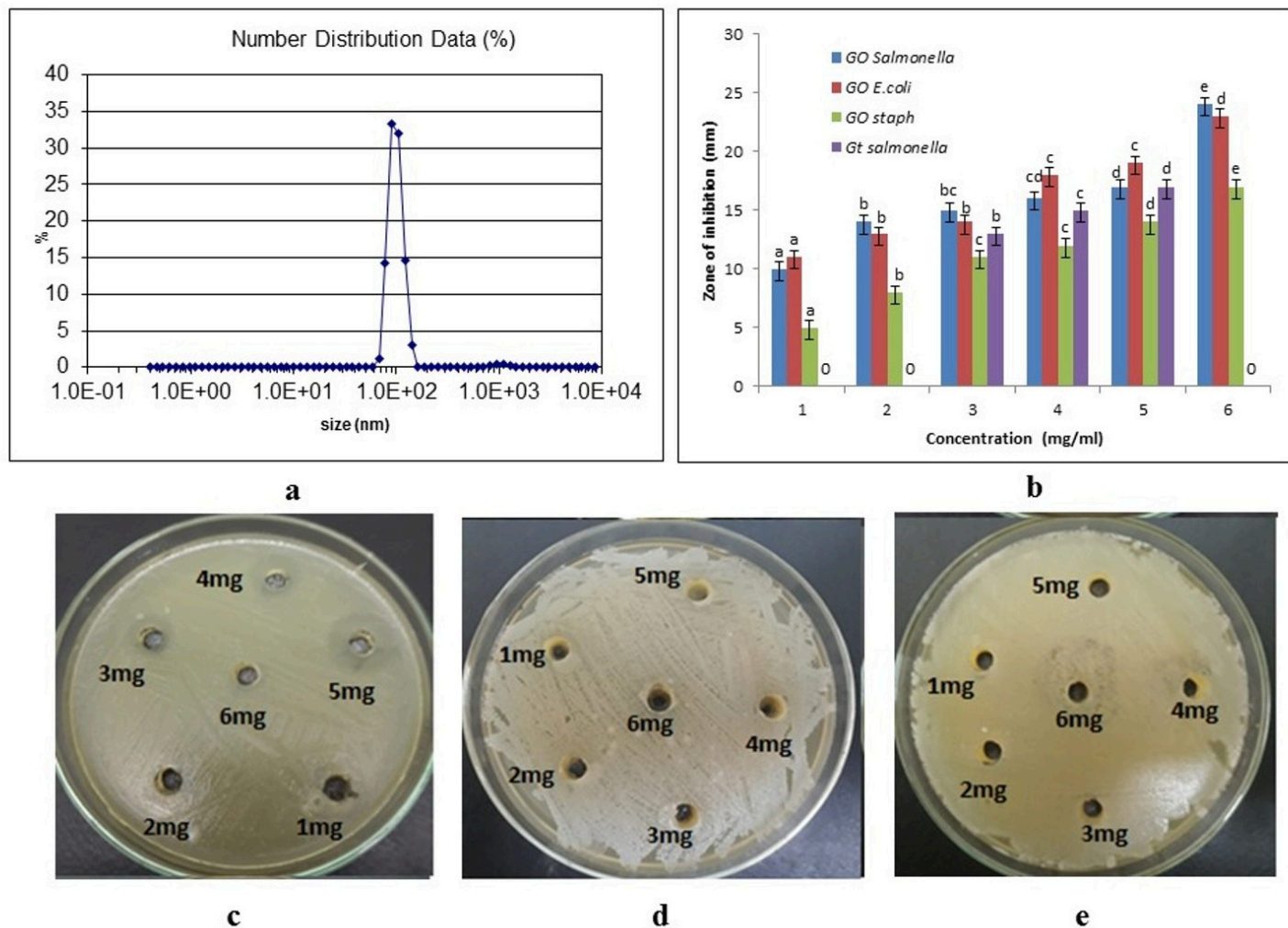


Figure 4

Zeta size of rGO nanosheets biosynthesized by *Bacillus cereus* MN181367 (a) antibacterial activity of graphene oxide (GO) and Graphite (Gt) against *Escherichia coli* BTCB201, *Staphylococcus aureus* BTCB203 and *Salmonella typhi* BTCB202 at different concentration (b) Zones of inhibition produced by Gt against *Salmonella typhi* BTCB202 (c) No zones of inhibition produced by Gt against *Staphylococcus aureus* BTCB203 (d) No zones of inhibition produced by Gt against *Escherichia coli* BTCB201 (e).

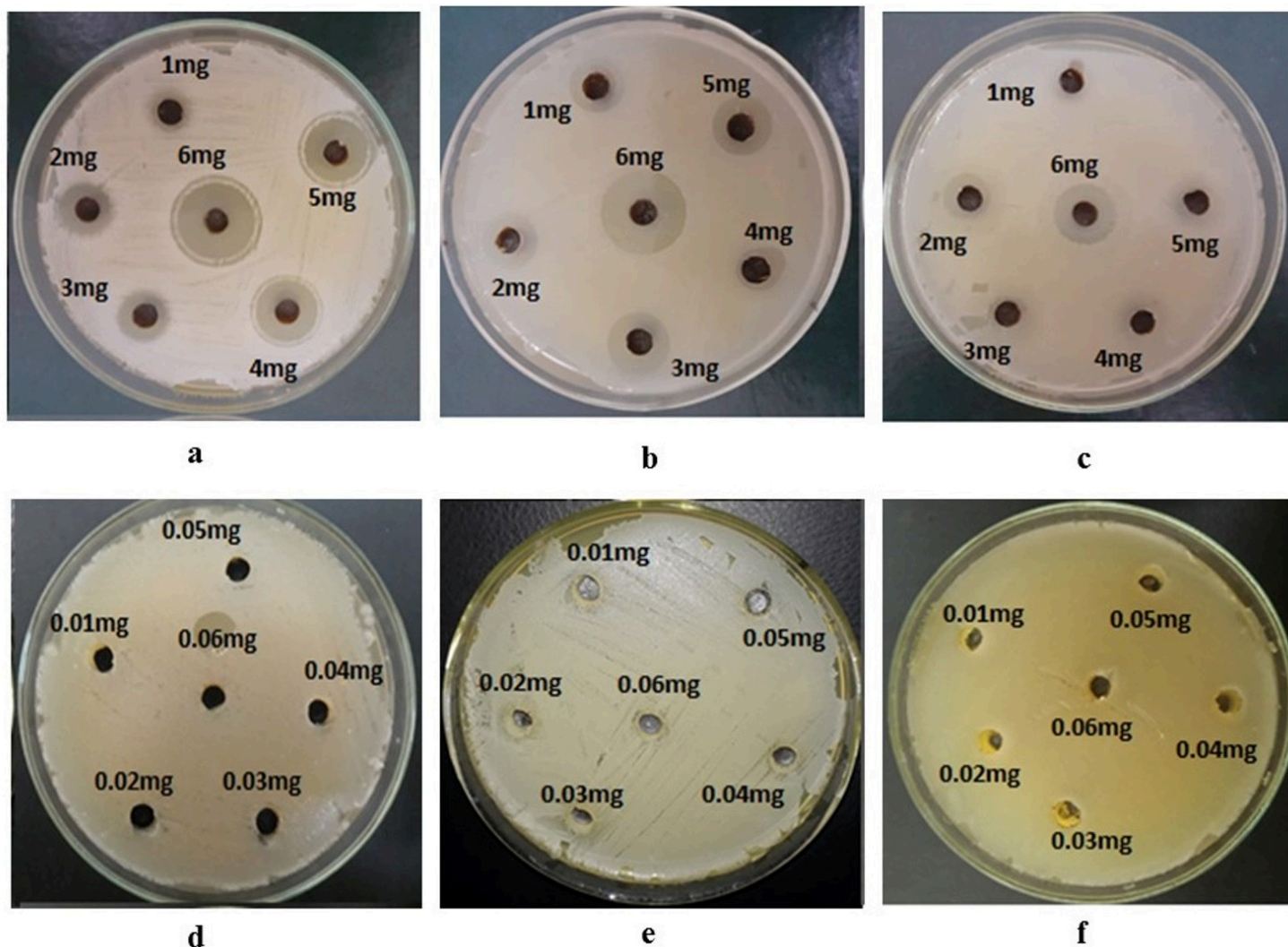


Figure 5

Antimicrobial activity Zones of inhibition produced by GO against *Salmonella typhi* BTCB202 (a) Zones of inhibition produced by GO against *Escherichia coli* BTCB201 (b) Zones of inhibition produced by GO against *Staphylococcus aureus* BTCB203 (c) No zones of inhibition produced by rGO against *Salmonella typhi* BTCB202 (d) No zones of inhibition produced by rGO against *Escherichia coli* BTCB201 (e) No zones of inhibition produced by rGO against *Staphylococcus aureus* BTCB203 (f).