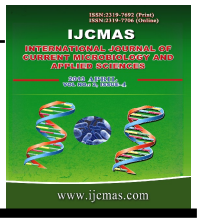
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 4 (2013) pp. 1-11**
http://www.ijcmas.com



Original Research Article

The Antibacterial Activity of Graphite Oxide, Silver, Impregnated Graphite Oxide with Silver and GO-Coated Sand Nanoparticles against Waterborne Pathogenic *E.coli* BL21

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K e y w o r d s

Antibacterial activity; Graphite oxide; GO-Coated Sand; Silver nanoparticles; *Escherichia coli* BL21.

A B S T R A C T

Microbial contamination of drinking water is an environmental problem around the world that poses a great threat to human health and effective treatments are needed to solve such water pollution problems. In the present study, Nanostructured Graphite Oxide (GO) and low-cost GO coated with silver or sand nanoparticles were developed and characterized. Antibacterial efficacy of these nanoparticles was investigated using waterborne pathogenic *E.coli* strain. Two methods were used to test the antibacterial effect of the prepared nanoparticles against the bacterium under test: (a) Plate Assay Method and (b) Shake Flask Test. The results revealed that both tests showed slight inhibition of pathogenic *E.coli* with sand and Graphite Oxide nanoparticles. 0.1 to 1.5 mol.L^{-1} of impregnated GO with silver and GO-Coated Sand showed a higher bactericidal effect against the bacterium under test. Furthermore, the results showed the highest bacterial removal efficiency (100%) by the Coated Graphite Oxide and the lowest by sand filters with 17.9 % to 88.9 % reduction rate from 0 to 24 hours, respectively. Therefore, this study suggests that the filter system with GO composite can be used as an effective filter for water disinfection and production of potable and pathogens free drinking water.

Introduction

Microbial contamination of surface, ground and drinking waters is an environmental problem around the world that poses a great threat to human health. The presence of coliforms in drinking water is considered to be a public health concern since detection of these microorganisms contributed either to faecal contamination or an exogenous contamination with enteric and other relevant microbial pathogens. It is paramount to protect water sources in our country from any source of contamination, and to construct water disinfection and delivery systems, as well as sewage

treatment facilities that can remove these microorganisms.

Several nanoparticles as activated carbons and graphene-based materials have proven to kill some waterborne bacteria, like *Aeromonas* spp., *Pseudomonas aeruginosa* and *Escherichia coli* from potable water supply systems (Quinlivan, *et al.,* 2005). Moreover, Graphite-based materials, mainly graphite oxide, have been shown to be strongly cytotoxic toward bacteria, and the antimicrobial actions caused by these nanoparticles because both membrane and oxidation stress (Shaobin, *et al.*, 2011).

Furthermore, several studies have shown that nanoparticles, mainly metal oxides, activated carbons and graphene-based materials, can disrupt and kill bacteria via the oxidation of glutathione, an important cellular antioxidant (Shaobin, *et al*., 2011). These nanomaterials act as conducting bridges that extract electrons from glutathione molecules thereby releasing them into the external environment, but the effect of membrane-disruption disappears after four hours of incubation. In addition, it has been shown that the physicochemical properties of graphene-based materials, such as the density of functional groups, size and conductivity can be tailored to either reduce environmental risks or increase application potential (Shaobin, *et al*., 2011).

Nanomaterials coated with silver nanoparticles are an important technology that could assist in providing a safe drinking-water supply. Silver ion (Ag^{2+}) is widely used as a nonspecific antibacterial factor and it acts against a very broad spectrum of bacterial species (Babu, *et al*., 2006). This action was proven to be due to the binding of the positive silver ions with the negatively charged microbial proteins preventing their replication, and via attachment to sulfhydryl groups preventing their proliferation (Babu, *et al*., 2006).

Moreover, Silver in the form of nanoparticles that release silver ions more effectively has a high bactericidal activity due to its high surface-area-to-volume ratio (Duran, *et al*., 2010) and the fact that it can be easily deposited on solid materials for the deactivation of microorganisms in water treatment (Nair and Pradeep, 2007). Various forms of silver nanoparticles coated on materials/substrates as impregnated silver with activated carbon or with graphite oxide have been used to test antibacterial properties (Bandyopadhyaya, *et al.*, 2008). It has been shown that the impregnation of silver with these nanoparticles is an important adsorbent used in water purification because they possess effective antibacterial properties that can be efficient for water disinfection (Bandyopadhyaya, *et al*., 2008).

Sand filters have been used in water purification to control microbiological contamination for over 150 years (Logsdon *et al*., 2002). Sand filters are an inexpensive, effective method of water treatment that can be self-constructed. However, its antibacterial and filtering property can be considerably enhanced when it is coated with a nanomaterial such as graphite oxide (GO) (Gao, *et al*., 2011).

The main objective of the present study was to evaluate the antibacterial efficacy of graphite oxide nanoparticles, graphite oxides impregnated with silver, and graphite oxides coated Sand against a waterborne pathogenic *E. coli*, where the combination of the graphite oxide and silver might offer an important antibacterial advantage, due to the strength

of these two nanoparticles and these materials used widely for application in water purification.

Materials and Methods

Microorganism

A standard *Escherichia coli* BL21 was kindly provided by MIRCENC (Microbiological Resource Center, Ain Shams University-Egypt).

Raw Materials

Nanostructured graphite oxide was prepared using a Modified Hummer Method (Hummers and Offeman, 1958) from purified natural graphite powder (Sigma Aldrich), Silver Nitrate, $AgNO_3$ (99%, Sigma Aldrich), Sodium Borohydride ($NaBH_4$, Sigma Aldrich), and Pure Sand (Sigma Aldrich).

Maintenance of the microorganisms

The Pathogenic bacterial strain under test was maintained on Nutrient Agar slants and stored at 4°C with regular transfers at monthly intervals. For long preservation, the slants were folded with 25% glycerol.

Preparation of seed culture

Transfers from single slant cultures (48 hours old) were taken into 50 ml aliquots of the seed medium containing (g/l): Beef extract, 1; Yeast extract, 2; Peptone, 5; Sodium chloride, 5 and 1L of distilled water. Dispensed in 250 ml Erlenmeyer flasks to initiate growth (OD<1). Standard inoculam of 2% (v/v) were taken from the latter liquid culture after growth for 18 hours at 30° C ± 2 on a reciprocal shaker to start growth in the fermentation flask which is equivalent to 3×10^8 colony forming unit/ ml (CFU/ml) according to McFarland scale 0.5

Preparation of silver nanoparticles

Silver nanoparticles were prepared according to the chemical reduction method adapted by Fang *et al*., (2005). 50 ml of 1×10-3 M silver nitrate solution was prepared, heated until boiling and then 5 ml of 1% tri-sodium citrate was added drop wise. The solution was mixed vigorously and heated until the colour changed into a pale brown followed by stirring until cooled to room temperature. The aqueous solution was air dried up to 4 days to obtain a powdered form of sliver nanoparticles.

Preparation of impregnated graphite oxide nanoparticles with silver

Graphite oxide (1g) was added to 20 ml of different $AgNO_3$ concentrations (a, 0.1; b, 1 and c, 1.5mol.L^{-1}). After 24 hour of impregnation in the dark, the powder samples were washed with water to remove loosely adsorbed $AgNO_3$, until the filtrate was free from $AgNO_3$. The powder samples collected after decantation were air-dried overnight. By adding 10 ml of 0.2 mol.L^{-1} $NaBH_4$ impregnated $AgNO_3$ was chemically reduced (over 24h) to form Ag particles. Washing with water was used to remove excess $NaBH_4$ followed by drying (Bandyopadhyaya, *et al*., 2008).

Preparation of GO coated Sand

Pure sand was washed with 10% HCl before use. Ten grams of clean sand were placed in a Petri dish, with 10 mL of GO/DI (0.35 wt %), and heated up to 150°C in a vacuum oven for two hours. The process was repeated to increase the GO-coating thickness on sand.

Antibacterial test

Graphite oxide, Graphite oxide coated sand, impregnated GO-Ag and silver nanoparticles were tested for their antibacterial effect against waterborne pathogenic *E.coli* under test. If this organism is killed, as a standard, all other borne-disease-causing organisms are assumed to be killed.

(a)Plate Assay Method (Qualitative test)

Melted M-Endo Agar medium was fortified with 3×10^8 CFU/ml medium of *E.coli* BL21 equivalent to 0.5 Mcfarland. About 20 ml of the previously prepared seeded agar was then dispensed in petridishes, solidified by refrigerating for 4-6 hours. Four mm diameter holes were made in the seeded agar using a sterilized cork borer. 25 mg of different nanoparticles under test were added one at a time in the holes, using one to two drops of sterilized distilled water, the plates were left at 4 □C for 1 hr then incubated at 37 □C for 24 hours and the antibacterial effect was measured referring to the zone diameter.

(b)Shake flask test in saline (Quantitative test)

For the shake flask test, 50 ml of sterile saline (0.9% NaCl) was inoculated with 1 ml bacterial suspension (3×10^8 CFU/ml) equivalent to 0.5 Mcfarland. 50 mg of different nanoparticles were added to the flasks one at a time, and the contents were stirred on a rotary shaker at ambient temperature.

The samples were drawn periodically (0, 1, 3 and 24 hours) from the flask and tested for the number of surviving *E.coli* by plate count method on M-Endo Agar, using standard procedures.

The percentage reduction of *E. coli* counts, were obtained after treatment with several nanoparticles according to this formula:

% Reduction of bacterial count=
((Viable count at $time_0$ -Viable count at $time_x$)/ Viable count at $time_0$)×100

Results and Discussion

Antibacterial test

(a) Plate Assay Method

Results in table 1 showed the inhibitory effect of graphite oxide, silver, GO-coated Sand and different concentrations of impregnated GO-Ag nanoparticles against the growth of *E.coli* BL21.

It was revealed that GO-Ag at 1.5 $mol.L^{-1}$ showed the greatest effect with a zone diameter of 17mm followed by silver nanoparticles (16mm) greater than the impregnated GO-Ag at a concentration of 1 $mol.L^{-1}$ (15 mm). Graphite Oxide showed the lowest inhibition effect with 8 mm inhibition zone (Fig 1), which was also reported by Hu *et al*., (2004), where graphite oxide nanoparticles exhibited the lowest inhibition zone compared to the impregnated GO with Silver, as reported previously by Qi, *et al*., (2011). Other researchers proved that the gram-negative *Escherichia coli* bacteria with an outer membrane were more resistant to the cell membrane damage caused by the graphite oxide than the other pathogens lacking the outer membrane (Akhavan and Ghaderi, 2010).

Table.1 Inhibitory effect of the nanoparticles under test against *E.coli* BL21, using plate assay method.

Nanoparticle used	Inhibition zone diameter (mm)
Graphite Oxide	8 .00
Silver Nanoparticle	16 .00
GO-Ag (a)	10 .00
GO-Ag (b)	15 .00
GO-Ag (c)	17 .00
GO-Sand	14 .00

(b) Shake flask technique

In a trial to test the antibacterial effect of nanoparticles under test against *E.coli* BL21 using shake flask fermentation technique, it was revealed that the total viable count of bacterial cells reduced slightly within one hour of contact with graphite oxide, sand and GO-Coated Sand, one at a time.

However, after shaking for 3 hours, the total viable *E.coli* count decreased to zero in the presence of GO and GO-Coated Sand, but some bacterial colonies remained in the presence of sand alone. After contact for 24 hours, all nanoparticles killed all bacterial cells, proving their bactericidal effect against pathogenic *E. coli*.

Figure. 2 showed the differences in the percent reduction of *E. coli* cells after treatment with the different nanoparticles under test, where 100% reduction in *E.coli* count was observed after 24 hours of contact with GO and GO-Coated Sand, as reported previously by Shaobin, *et al.*, (2012).

However, sand showed 88.9% reduction in bacterial count after 24 hours of contact (Fig. 2) as reported by Tor, *et al*., (2004). For both materials, a large fraction of cell death occurred in the first three hours of incubation. GO coated sand showed higher antibacterial effect than GO nanoparticle at all incubation time intervals (Fig. 2). Therefore, the coated graphite oxide with sand have more bactericidal effect against *E. coli* than the uncoated nanomaterial as reported previously by Gao, *et al*., (2011).

Furthermore, the antibacterial effect of silver nanoparticles and impregnated graphite oxide with silver nanoparticles were tested against pathogenic *E. coli* BL21. Results in table 2 and Figure 3 showed that no bacterial growth was observed after one hour of incubation with impregnated graphite oxide with silver at different concentrations, while after 3 hours of incubation with GO, 100% reduction in the bacterial count was reported.

Therefore, the change after 1 hour of incubation with the nanoparticles under test is due to the fact that the loss of cell viability has approached the complete inactivation (Shaobin, *et al.*, 2012). It was clear that the amount of silver particles on the graphite oxide seemed to be the main factor causing the complete reduction in the bacterial count and was responsible for the effective antimicrobial activity (Qi, *et al*., 2011; Ma, *et al*., 2011).

Moreover, other researchers showed that GO-Silver composite have a superior antibacterial activity towards *E. coli* due to the synergistic effect of GO and silver nanoparticles (Sreeprasad and Pradeep, 2012).

Figure.1 Plate assay method for the detection of the Inhibitory effect of nanoparticles under test against *E.coli* BL21. **a:** Graphite Oxide, **b:** GO-Coated Sand, **c:** GO-Ag (a), **d:** GO-Ag (b&c) and **e:** Silver nanoparticles.

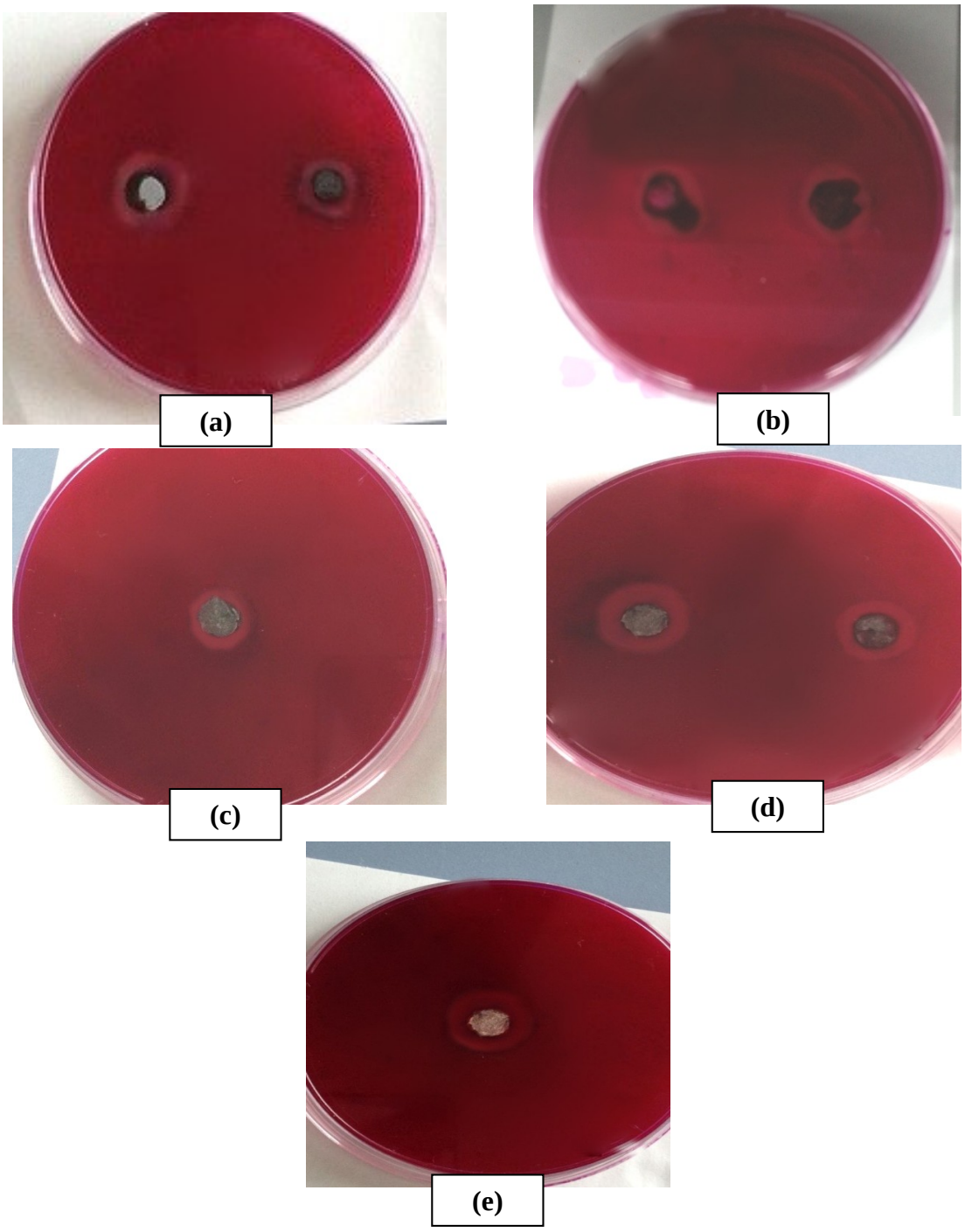


Table. 2 Total viable count as affected by the time exposure to different nanoparticles under shake flask technique.

Nanoparticles	Contact time (Hours)	CFU/ml $\times 10^4$
Graphite Oxide	0.0	68.0
	1.0	29.0
	3.0	0.0
	24.0	0.0
Sand	0.0	96.0
	1.0	52.0
	3.0	24.0
	24.0	13.0
GO-Coated Sand	0.0	56.0
	1.0	36.0
	3.0	0.0
	24.0	0.0
Silver	0.0	15.0
	1.0	4.0
	3.0	0.0
	24.0	0.0
GO-Ag (a)	0.0	21.0
	1.0	0.0
	3.0	0.0
	24.0	0.0
GO-Ag (b)	0.0	0.0
	1.0	0.0
	3.0	0.0
	24.0	0.0.
GO-Ag (c)	0.0	0.0
	1.0	0.0
	3.0	0.0
	24.0	0.0

In general, Most of the bacterial reduction and inactivation took place during the first three hours of incubation, and the mortality rate increases continuously with the increase of nanomaterial concentration and their antibacterial activities are time and concentration dependent, as reported by Shaobin, *et al.*, (2012). Thus, Silver nanoparticles showed efficient antibacterial activity against pathogenic *E. coli* BL21 (Fig 3) that was similar to that found by Sondi and Salopek-Sondi (2004).

Fig 2. Reduction percentage of the total viable count of *E.coli* BL21 as affected by the exposure time to **a:** GO, **b:** Sand and **c:** GO-Coated Sand under shake flask technique.

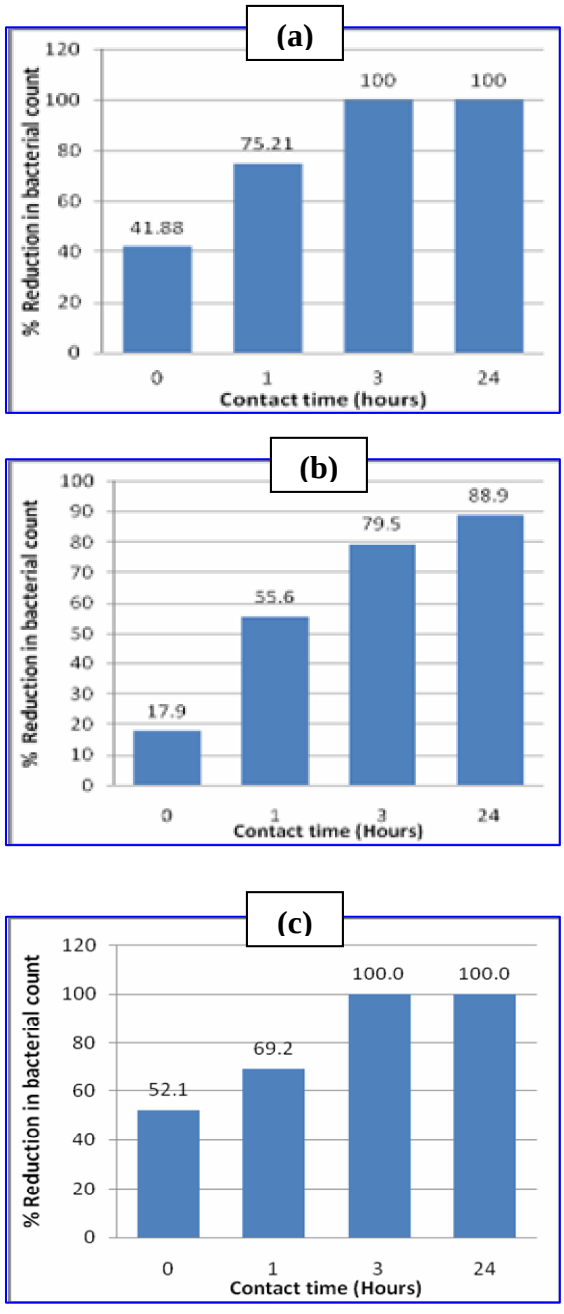
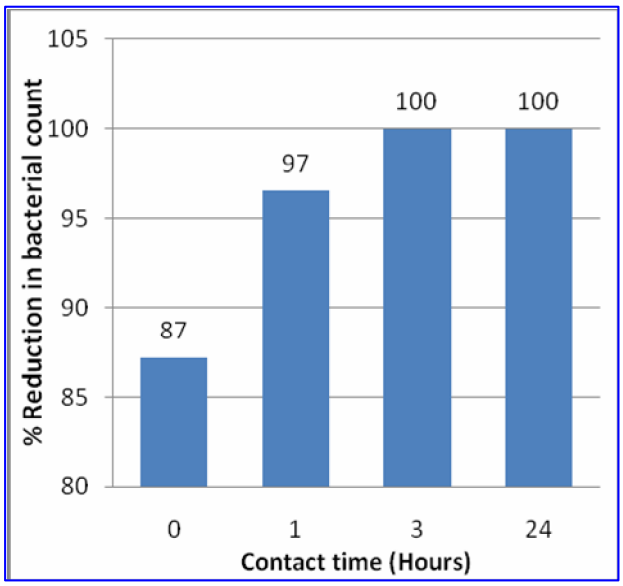
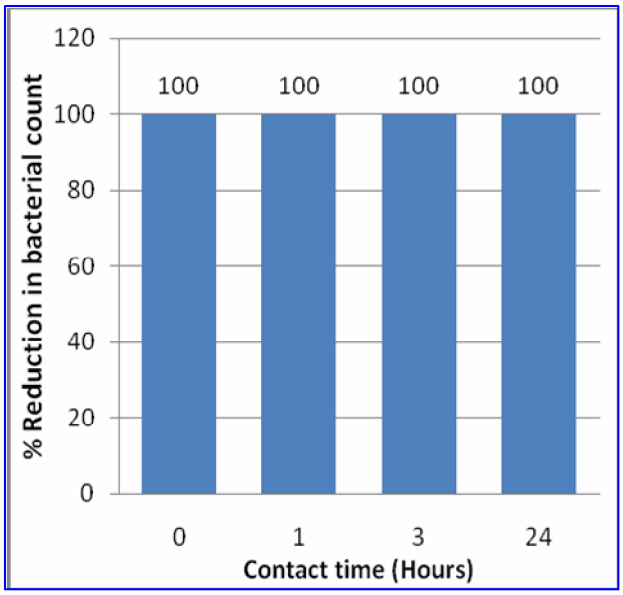
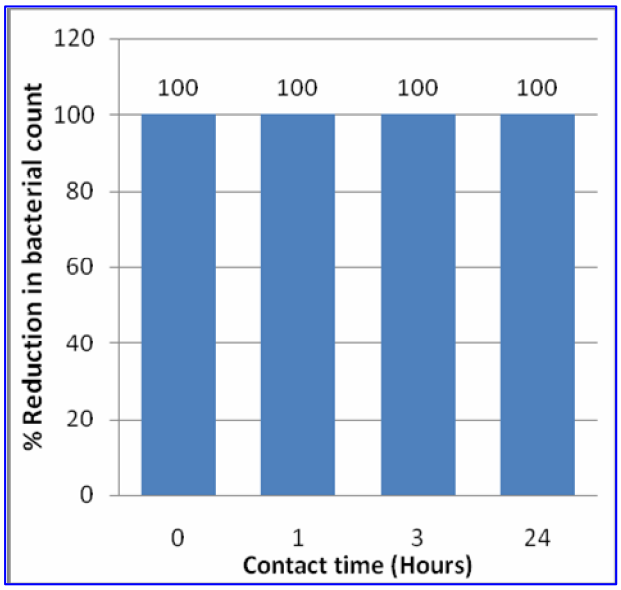
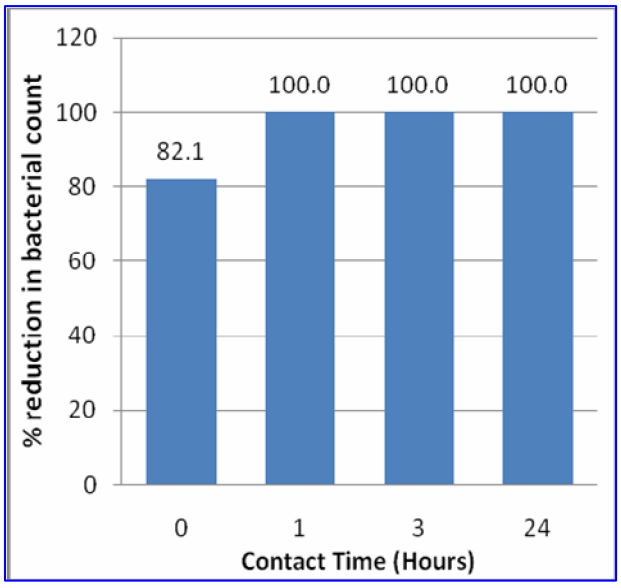


Figure.3 Reduction percentage of the total viable count of *E.coli* BL21 as affected by the exposure time to: **a:** Silver, **b:** Impregnated GO-Ag (c), **c:** Impregnated GO-Ag (b) and **d:** Impregnated GO-Ag (a) under the shake flask technique.









The mechanism of inhibitory action caused by silver nanoparticles against *E.coli* BL21 is partially known, where some researchers reported that silver nanoparticles inhibit bacterial growth through binding to the thiol group leading to bacterial inactivation (Yan, *et al*., 2012). Moreover, the Gram negative bacteria have a layer of lipopolysaccharides at the exterior that are composed of covalently linked lipids and polysaccharides; they lack strength and rigidity (Guzman, *et al*., 2008).

Negative charges on the lipopolysaccharides are attracted towards the positive charges available on silver nanoparticles (Michael, *et al*., 2005). The opposite charges attract each other due to electrostatic forces. So once the nanoparticle comes in contact with the bacterial cell, it either inhibit the cell wall synthesis, damage the cytoplasmic membrane, inhibit nucleic acid and protein synthesis or inhibit specific enzyme systems which result in the complete bacterial inhibition (Tripathi, *et al*., 2010). The mechanism by which the nanoparticles are able to penetrate the bacteria is not understood completely, but previous studies suggested that when *E.coli* treated with silver, changes took place in its membrane morphology that produced a significant increase in its permeability affecting proper transport through the plasma membrane, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane, resulting into cell death (Morones, *et al*., 2005). Moreover, these studies showed that bacterial inhibition caused once silver nanoparticles penetrated inside the bacteria and caused damage by interacting with phosphorus and sulfur containing compounds such as DNA (Morones, *et al*., 2005).

The antibacterial effect of GO, GO-Coated Sand, Silver nanoparticles and impregnated GO with silver against waterborne pathogenic *E.coli* BL21 was obtained and compared. Plate assays and shake flask methods showed that Graphite Oxide has lowest antibacterial activities compared to the other prepared nanoparticles. However, impregnated GO with Silver at the highest concentration (1.5 $mol.L^{-1}$) showed the maximum inhibitory effect against *E.coli* BL21. Therefore, higher concentrations of silver ions causes' greater bactericidal effect and their antibacterial activities are time and concentration dependent. Most of the bacterial cell number reduction occurred after three hours of incubation, and the reduction rate was greatly influenced by the increase of silver ion concentrations and contact time. The present study demonstrated the potential of Graphite Oxide composites for use in water purification and that silver impregnation is very effective in producing potable drinking water.

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