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ORIGINAL ARTICLE

Assessment of antioxidant and antibacterial activities of Zinc Oxide nanoparticles, Graphene and Graphene decorated by Zinc Oxide nanoparticles

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Abstract

Zinc Oxide nanoparticles (ZnO-NPs) and graphene carbon material, due to lower drug resistance, can replace antibiotics, and by decorating of graphene with Zn-NPs, their properties can be greatly improved. The purpose of this study was to evaluate the antioxidant and antibacterial effects of ZnO-NPs biosynthesized using Crocus Sativus petal extract, graphene and graphene decorated by ZnO-NPs biosynthesized using Crocus Sativus petal extract (G-ZnO). Their physicochemical characterizations were performed by UV-Vis spectroscopy, Transmission electron microscopy (TEM) and field emission scanning electron microscopy (FE-SEM), revealing that ZnO-NPs with a mean size of 25 nm and spherical-shape were distributed uniformly on the surface of the graphene without aggregation. The antioxidant activities of ZnO-NPs, graphene and G-ZnO were evaluated using DPPH and ABTS assays. Antibacterial activities of three compounds were tested against Gram negative bacteria *Escherichia coli (E. coli)* and Gram positive bacteria *Staphylococcus aureus (S. aureus)* using macrodilution method. The results of this study showed that these three compounds have antioxidant and antibacterial effects. And it was show that but also antioxidant and antibacterial activity of G-ZnO was higher than ZnO-NPs and graphene. G-ZnO could be useful as a natural antioxidant and antibacterial in the pharmacy industry.

Keywords: *Antibacterial; Antioxidant; Graphene; G-ZnO, Pharmaceutical Industry; ZnO-NPs.*

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INTRODUCTION

Nanotechnology is an interdisciplinary approach to biochemical applications, and currently nanoparticles with antioxidant and antibacterial properties against degenerative diseases and cancer have been considered [1, 2]. Antioxidants play important role in the performance of all biological systems, which are caused by the interaction of biomolecules with molecular oxygen, free radicals and the destruction of bio-molecules [3]. Therefore, the production of natural antioxidants are necessary to prevent oxidative stress and their destructive effects [4]. Some nanoparticles play important role in the breakdown of these free radicals, thus destroying the oxidative damage of the human body [3]. In this regard, it is possible to use green nanoparticles that exhibit antioxidant properties. Among nanoparticles, ZnO-NPs can be mentioned that have been considered in scientific and industrial applications at recent decades [5]. In addition, ZnO-NPs are mitochondrial respiratory regulator that reduces the effectiveness of antioxidant enzymes such as glutathione transferase, which result in

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excessive release of free radicals. Previous studies have shown that ZnO-NPs have been reported for non-toxic human cells. This aspect makes use of them as antimicrobial agents, harmful to microorganisms and adaptation to human cells [6], but ZnO-NPs accumulate due to van der Waals forces and superficial effects, which leads to the weakening of unique chemical properties and loss of their antibacterial activities [7]. Therefore, the level of ZnO particles should be corrected with an organic or stable polymer reaction to reduce aggregation [8]. For this reason, many researchers are interested in creating distinct and effective antibacterial, anticancer, or cytotoxic molecules [7]. By decorating of surface graphene with Zn-NPs, their antibacterial and antioxidant properties can be greatly improved**.** Graphene, a 2D honeycomb-shaped carbon fiber sheet [9] with attractive properties such as high ratio of surface to volume, high optical brightness and electronic transport capability [8], is able to provide a building platform for the growth and stabilization of nanoparticles and prevents their accumulation [10, 11] , which ultimately can form a more stable composition with high antibacterial and anticancer activities [7]. Antimicrobial activity of graphene is due to physical and chemical interaction when exposed to bacterial cells [1]. Graphene shows antibacterial effects due to functional groups at the surface and edges. The sharp edges of graphene damage the cell membrane and increase the oxidative stress due to the oxidative nature and the release of reactive oxygen species [12]. The purpose of this study was to assessment the antioxidant and antibacterial activities of ZnO-NPs biosynthesized using Crocus Sativus petal extract, graphene and G-ZnO.

MATERIALS AND METHODS

Synthesis of ZnO-NPs

ZnO-NPs were synthesized using green technique. In brief, it begins by adding 100 ml zinc acetate dehydrate solution to 50 ml of Crocus Sativus petal water extract under constant stirring for 2-3 hours at 70°C. The solution was centrifuged at 10000 rpm for 10 min and washing in distilled water. The powder was obtained after was drying at 100°C for 2 hours.

Preparation of G-ZnO

In this study, Zinc was coated with heat-induced oxidation on the surface of graphene, and after cooling, ZnO-NPs with a spherical structure on graphene surface with unchanged morphology were placed.

Characterization

Synthesized nanoparticles and G-ZnO were checked by recording UV-Visible spectra using spectrophotometer (Cary60 UV-VIS spectrophotometer). The surface and structural morphology of the samples were observed using Transmission electron microscopy (TEM) (JEM-2200FS microscope) and field emission scanning electron microscopy (FE-SEM, Model: AURIGA-39-50).

Antioxidant assays

The materials used in this study included DPPH (2, 2-Dipheny-1-Picrylhydrazyl), BHA (Butylated hydroxyanisole), solution, ABTS (2, 2'-Azino-bis (3-Ethylbenzothiazoline-6–sulphonic acid) from Sigma-Aldrich USA, ethanol and EDTA (Ethylenediaminetetraacetic acid) were purchased from the German Merck company. The antioxidant capacities of ZnO-NPs, graphene and G-ZnO were evaluated using DPPH and ABTS assays.

DPPH free radical scavenging assay

Different concentrations of nanoparticles were added to DPPH solution for 30 min at 37° C in the dark. BHA was used as standard antioxidant for comparison with antioxidant activity of nanoparticles. Change in color from violet to yellow was observed caused by antioxidant potential. Absorbance of the solution at a wavelength of 517 nm was measured using a spectrophotometer. The ability of test samples to scavenge DPPH radicals were calculated by the following equation [2]:

% Scavenging= (Absorbance of Control – Absorbance of Sample / Absorbance of Control) × 100

ABTS free radical scavenging assay

The ABTS radical cation was prepared by mixing ABTS solution and sodium persulfate at room temperature in the dark for 16 h. The solution was diluted with methanol. Absorbance of reaction mixture was measured at 734 nm. Scavenging activity was calculated using the given equation [3]:

% Scavenging = (Absorbance of Control - Absorbance of Sample / Absorbance of Control) × 100

Antibacterial assay

To confirm the antibacterial activities of nanoparticles, we were used two strains of bacteria including, gram-positive (*Staphylococcus aureus* ATCC25923) and gram-negative bacteria (*Escherichia coli* ATCC25922 were purchased from the Iranian Pasteur Institute. Antibacterial activities were determined by using macrodilution method. Different concentrations of the nanoparticles ranging from 0.15 to10 µg/ml were prepared by serial dilution in Muller Hinton broth medium, and then, 10 µl of bacterial suspension added in test tubes. Blank Muller Hinton broth was used as negative control. After that, the test tubes were incubated for 24 h at 37°C. The first tube in the series with no visible growth after incubation period was taken as the minimum inhibitory concentration (MIC). Minimum bactericidal concentration (MBC) can be determined by testing the MIC. For this purpose, the MIC test tube that did not show any bacterial growth, in Muller Hinton Agar culture medium was cultured. The plates were then placed in an incubator for 24 h. After the incubation periods, the lowest concentration of the nanoparticles that did not produce any bacterial growth on the solid medium was regarded as MBC value for these nanoparticles [13].

Statistical analysis

All samples were tested in three individual experiments and data expressed as mean ± standard deviation. Statistical analysis was analyzed using SPSS software v18.0 (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) was used for analysis of variance. The comparison of the meanings was performed with least significant differences (LSD). A value of $P < 0.05$ was considered statistically significant finding.

RESULTS AND DISCUSSION

Characterization of ZnO-NPs and G-ZnO

The absorption spectrum of the ZnO-NPs and G-ZnO in the UV-visible range was used to characterize the absorption spectrum at room temperature. As shown in Fig.1, characteristic absorption peak of the G-ZnO have increase compared to the ZnO-NP so that ZnO-NPs have a strong absorption maximum at a wavelength of 362 nm. The spectra of G-ZnO show an absorption peak at 369nm and indicate small redshift of the absorption edge, which can be due to the chemical bonding between ZnO-NPs and graphene that resulted in rapid electron transfer and increased transition energy, which agrees with observations of previous reports[7, 14].

The surface and structural morphology of ZnO-NPs and G-ZnO were confirmed by TEM and FESEM analyses. Fig. 2a show the TEM analysis of ZnO-NPs. The size nanoparticles range from 3 to 52 nm with an average of 25 nm. Fig. 2b show the presence of nanoparticles anchored to the surface of graphene. The majority of the G-ZnO were within the range of 30-55nm, with an average of 38nm.

FESEM images reveal the surface morphologies of ZnO-NPs and G-ZnO spherical which are presented in Fig. 3. When zinc ions react with graphene, nanoparticles with spherical morphology uniformly disperse on surface of the graphene without aggregation.

ZnO-NPs were non-uniformity with small particles sizes (3 to 52 nm), and the particles are close to each other. Whereas in the case of G-ZnO was observed improvement of the particle size of 30-55nm with an average of 38nm. Images clearly showed that the most of the ZnO-NPs with a mean size of 25 nm and spherical-shape were distributed uniformly on the graphene without

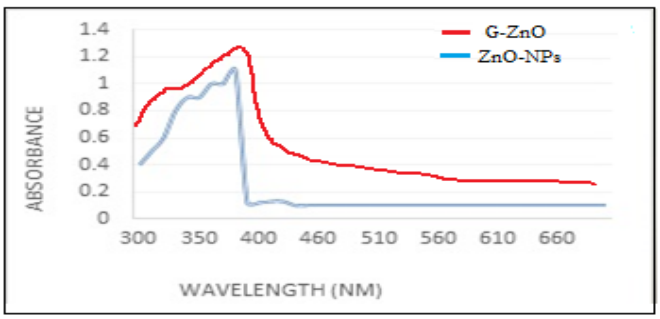

Fig.1. UV-visible absorbance spectra of the ZnO-NPs and G-ZnO.

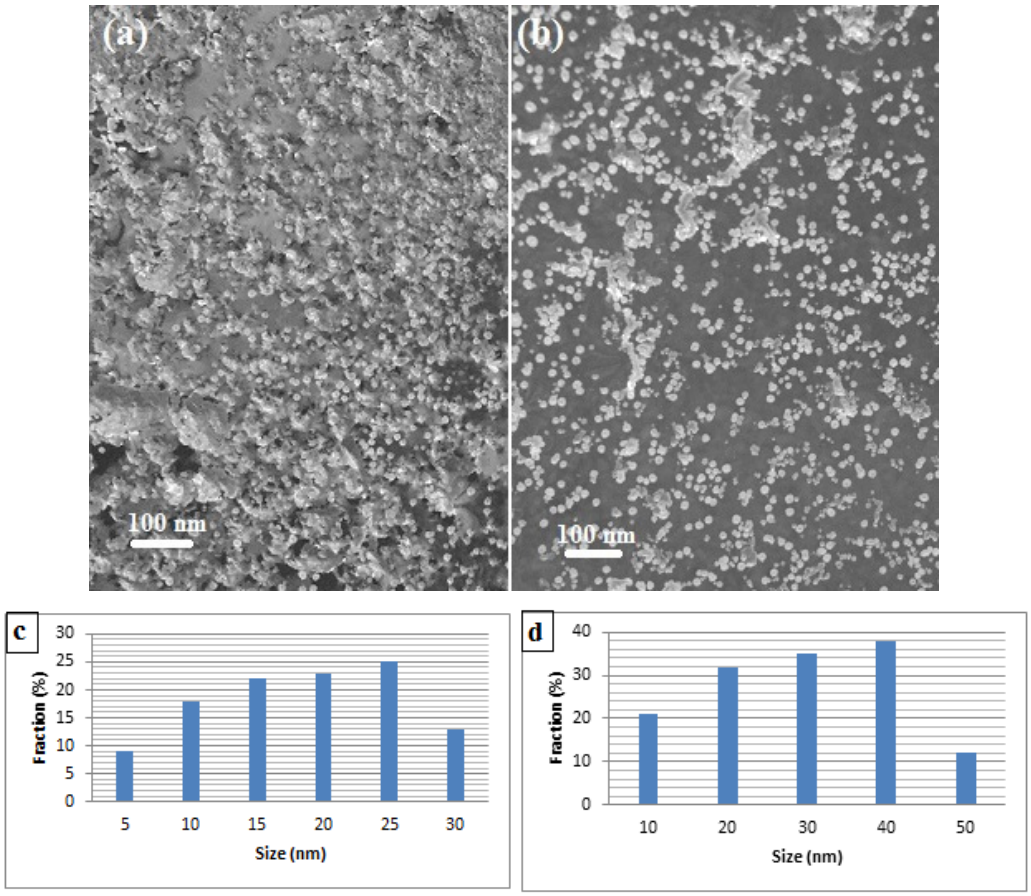

Fig.2. TEM images of a) ZnO-NPs, b) G-ZnO and particle size distribution of c) ZnO-NPs, d) G-ZnO.

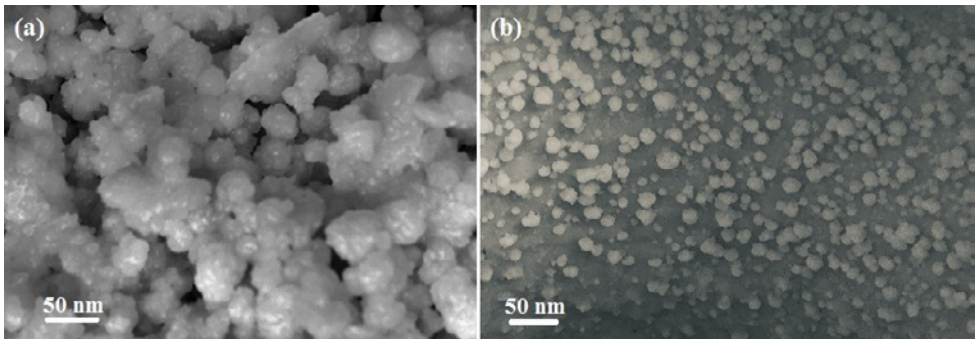

Fig. 3. FESEM images of a) ZnO-NPs and b) G-ZnO.

aggregation. There is not difference between surface morphologies of G-ZnO and ZnO-NPs, except a very small exchange in the particle growth. Previous studies showed that sheet like structure with wrinkles surface on the graphene can be attributed to the deposition of ZnO-NPs on the surface of the sheets [15]. In another study, GO-Ag nanocomposite exhibited a smooth and thin surface due to the presence of nanoparticles [16]. Biologically nanoparticles synthesized stabilized by formation of protein layer of active constituents for example, kaempferol and flavonoids on the surface of nanoparticles and play important role for binding to graphene materials [14]. FESEM images showed that the surface morphologies of GO, ZnO nanoparticles and ZnO/

rGO nanocomposite were hollow sphere [17]. Our findings are consistent with previously studies that suggested strong interaction between the metal nanoparticles and graphene materials[18]. Moreover, G-ZnO exhibited a flat surface and its structure showed ZnO-NPs deposited evenly on the graphene. The graphene has facilitated the infiltration of nanoparticles (with better loading), and then the formation of the smooth surface with an improvement in surface morphology of the nanocomposite [19]. These data confirmed that the surface of graphene decorated by the ZnO-NPs. Altogether, it was concluded that most of the ZnO-NPs absorbed on the graphene exhibited spherical shape and can be uniformly anchored and distributed on the surface of the graphene without aggregation.

Antioxidant activity

The results of antioxidant activities show that with increasing concentrations of ZnO-NPs, graphene and G-ZnO, the absorption of DPPH and ABTS radicals increase. As shown in Fig. 4, the IC_{50} (50% inhibition of free radicals) of ZnO-NPs (Fig. 4a), graphene (Fig. 4b) and G-ZnO (Fig. 4c) to inhibit DPPH radicals were respectively 60, 700 and 58 µg / ml. Also, the IC_{50} content of ZnO-NPs (Fig. 5a), graphene (Fig. 5b) and G-ZnO (Fig. 5c) to inhibit ABTS radicals were 31.2, 700 and 22 µg / ml, respectively. The investigated nanoparticles have more effective inhibitory effect on free radicals ABTS than DPPH. G-ZnO had lower IC_{50} than ZnO-NPs and graphene, so it was more potent antioxidant.

The results of DPPH and ABTS scavenging activity in the order were: G-ZnO> ZnO-NPs>graphene. The enhanced antioxidant activity of G-ZnO compared

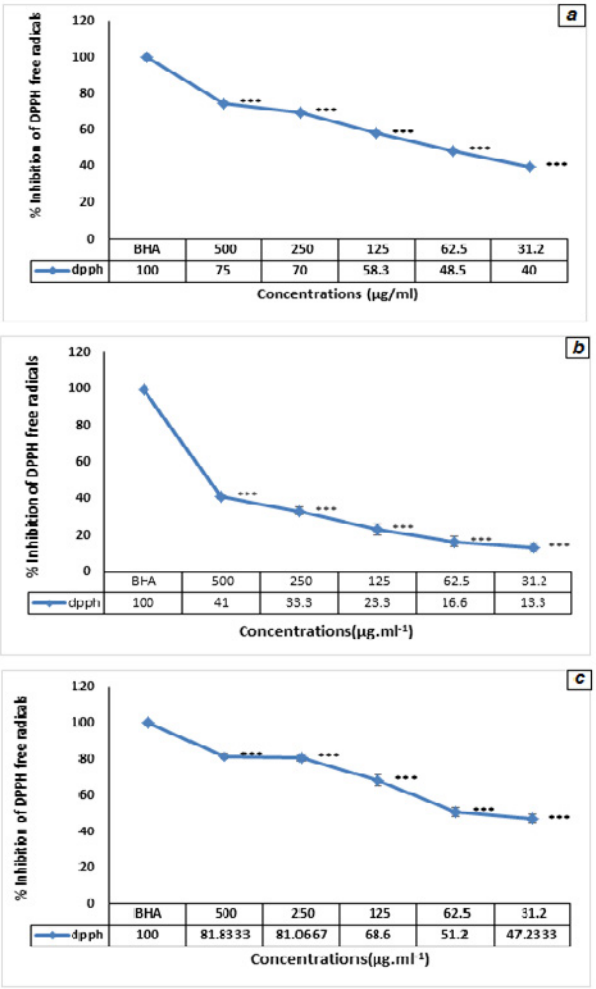

Fig.4. Antioxidant activities of: a) ZnO-NPs, b) Graphene and c) G-ZnO using DPPH method. *** P < 0.001 and ** P < 0.01.

to ZnO-NPs and graphene may be due to synergetic effects between ZnO-NPs and graphene. Due to the properties of ZnO-NPs and graphene, G-ZnO could produce reactive oxygen species, Therefore, in activating transcription factors, changes in cytokines and intracellular calcium concentration could be involved. The main objective was to increase the antioxidant activities of nanoparticles in this study, because by decorating of graphene with Zn-NPs, the antioxidant activity of G-ZnO was higher than pure ZnO-NPs and graphene. Our results are consistent with other studies. DPPH scavenging activity by silver nanoparticles, graphene oxide, and silver nanoparticle-graphene oxide nanocomposite were 74.81 ± 2%, 48.66 ± 2% and 84.76 ± 2%, respectively and enhanced antioxidant property of silver nanoparticles-graphene oxide nanocomposite may due to the interaction between silver nanoparticles and graphene oxide [14].

ZnO-NPs biosynthesized using Crocus Sativus petal extract, due to the presence of proteins and amino acids in the ingredients (Crocus Sativus petal extract), scavenged ABTS and DPPH radicals in concentration-dependent manner. Nanoparticles showed the enhanced scavenging activity with the increase in DPPH scavenging potential of ZnO-NPs of *Cassia fistula* plant extract [20], *M. harantia* fruit extract [21] and *M. oleifera* and *T. indica* plant extracts [3] has been reported. The radicals scavenging activity by ZnO-NPs synthesized from ethanol extract of *M. harentia* fruit was recorded maximum inhibitory to 82.78% and IC_{50} value was 42.31 µg / ml, indicating the high antioxidant activity of ZnO-NPs [21].

In our study, graphene at high concentrations was able to release DPPH and ABTS free radicals and has weak antioxidant activity. We believe

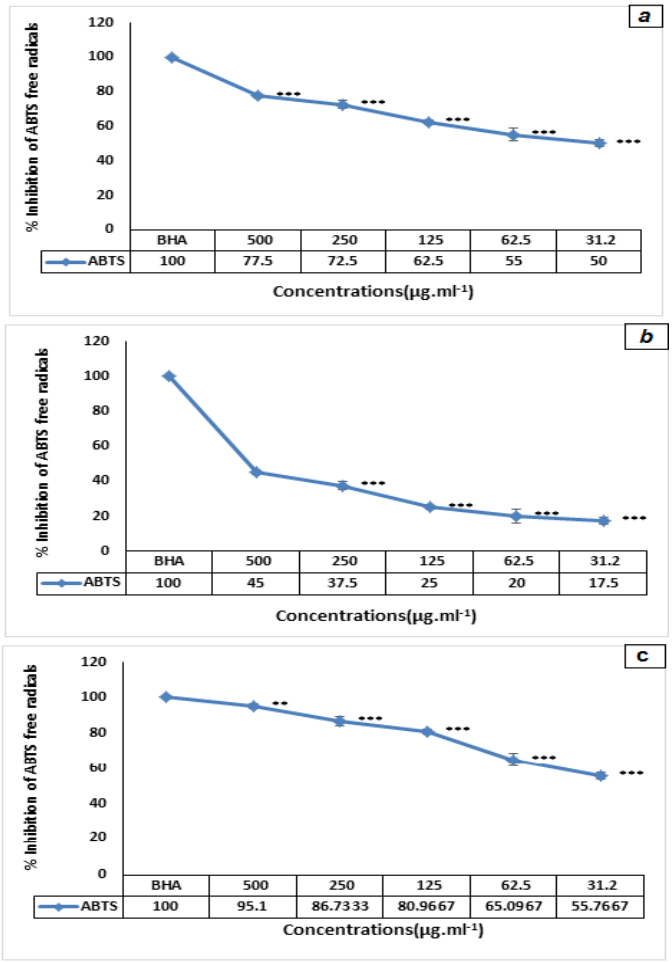

Fig. 5. Antioxidant activities of: a) ZnO-NPs, b) Graphene and c) G-ZnO using ABTS method. *** P <0.001 and ** P<0.01.

that the antioxidant activity of graphene can depend on chemical structure of graphene. Many antioxidants are phenolic compounds that fix their radical forms after hydrogen donation by resonance structures, in which the unpaired electron can be placed on an oxygen atom or urea or para carbon on the adjacent aromatic ring [22]. Graphene is widely aromatic in the form of sp^2-carbon domains, but is not very active as an H-donor. Therefore, it is not expected to exhibit strong antioxidant activity. Similar to our results, in the study found that graphene materials may also have pro-oxidant activity and show ROS inhibition and the antioxidant activity pattern can depend on the structure of graphene oxide or few-layer graphene [23].

Antibacterial activity

MIC results of ZnO-NPs, graphene and G-ZnO (Table 1) against *Staphylococcus aureus*, respectively, 125, 62.5 and 62.5 µg / ml, and MIC results of ZnO-NPs, graphene and G-ZnO against *Escherichia coli* were 250, 250 and 125 µg / ml, respectively, indicating a stronger antibacterial effect of G-ZnO compared to ZnO-NPs and graphene. The results showed that the three compounds tested against gram-positive and gram-negative bacteria had significant antibacterial activities. Although gram negative bacteria appeared to is more resistance to three compounds tested than gram positive bacteria (Fig. 6).

The antibacterial activities of tested samples in the order were: G-ZnO > graphene > ZnO-NPs. Our results show that graphene has inhibitory effect on *Staphylococcus aureus* and *Escherichia coli*. High sensitivity of graphene is due to the interaction π between a thick atomic layer, electron bands with close distance and a large surface. Hu *et al.* [24] found that graphene oxide have strong antibacterial activity against *Escherichia coli* and it reduced bacterial viability to 98.5%. SEM images of previous studies reported that direct contact with graphene nanostructures can break the cell membrane of the bacteria [25]. Antibacterial mechanism for graphene-based materials, including early graphene deposits on the cell, bacterial DNA damage, membrane stress due to direct contact with sharp nanostructures and the production of reactive oxygen species.

The weak antimicrobial activity of ZnO-NPs is due to the fact that ZnO-NPs accumulate easily in the solution due to van der Waals forces and superficial effects. Therefore, the level of ZnO particles should be corrected with an organic or stable polymer reaction to reduce aggregation. Graphene has a layered structure in which carbon atoms twist to form tetrahedrons that cause wrinkles on the surface. Chemically activated groups on the surface π-conjugated to the graphene surface allow nanometer and micrometer particles to be connected covalently onto graphene [26].

Table 1. MIC and MBC of ZnO-NPs, graphene and G-ZnO against *Escherichia coli* and *Staphylococcus aureus*.

Staphylococcus aureus		Escherichia coli		Tested nanoparticles
MBC(µg.ml^{-1})	MIC(µg.ml^{-1})	MBC(µg.ml^{-1})	MIC(µg.ml^{-1})	
500	125	500	250	ZnO-NPs
500	62.5	1000	250	Graphene
125	62.5	500	125	G-ZnO

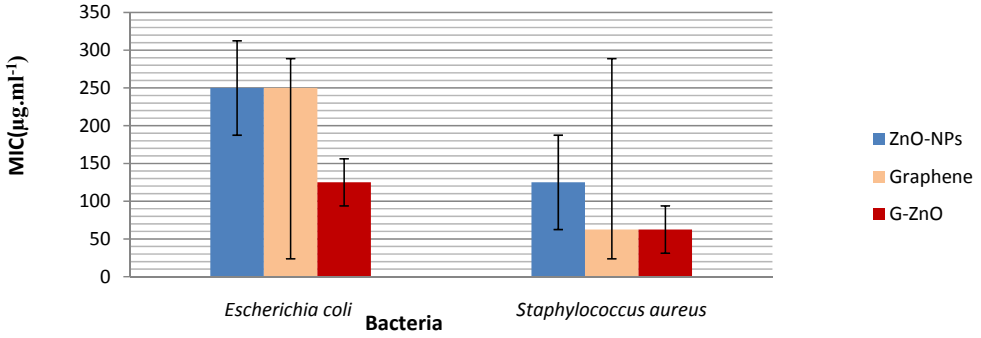

Fig.6. MIC of ZnO-NPs, Graphene and G-ZnO against *Escherichia coli* and *Staphylococcus aureus*.

In addition, Graphene offers a suitable platform for the development of nanocomposites, which provides the combination of nanomaterial with different properties to improve new materials or to provide new performance. In particular, graphene is used for binding to ZnO-NPs due to its high level, which acts as a supportive agent for the growth and stabilization of nanoparticles and prevents their accumulation [8]. In past studies, have also reported the antibacterial activities of ZnO-NPs of *Cassia fistula* plant extract [20] and *Trifolium pratense* flower extract [27]. Other studies have reported similar results, and gram-negative bacteria were more resistant to gram-positive bacteria, and the antibacterial activity of ZnO-NPs increases with decreasing particle size and increasing concentration, and the antibacterial effect of ZnO-NPs was time-dependent and gradually affected [28].

In this study, antibacterial activity of G-ZnO was stronger than graphene and ZnO-NPs. The mechanism of G-ZnO antibacterial activity is: a) the transfer of electron between graphene and the ZnO-NPs leads to adsorption of electrons by graphene, and the production of reactive oxygen species [8], b) Adhesion of graphene sharp edges and surface abrasion of ZnO-NPs leads to physical damage to the bacterial membrane. Positive-charged Zn ions interact with double-layer phospholipids, which can change the permeability of the cell membrane and increase the formation of reactive oxygen species in the solution [29]. ROS production in cell membrane causes the phenomenon of denaturation, fragmentation and cell death. Based on our results, it was found that the antibacterial effects of the three samples tested on *Staphylococcus aureus* is more pronounced, which is agrees with Hieu and Vi results [30], in which MIC value of ZnO/GO nanocomposite 1 : 1 is 80 µg/mL for *S. aureus* and 160 µg/mL for *E. coli* and have more active against gram positive than against gram negative bacteria. The sensitivity of the Gram-positive bacteria in comparison with the Gram-negative bacteria to the nanoparticles is not only related to the cell wall structure, but also to the physiology and metabolism or the degree of contact of the bacterial cell. Since gram-positive bacteria have more peptidoglycan than gram-negative bacteria and are more negatively charged due to peptidoglycan, most G-ZnO may be trapped in the gram-positive bacteria through peptidoglycan in comparison with the gram-negative bacteria. Also, the gram-positive bacteria have thicker cell wall compared to the gram-negative bacteria, which results in the permeability of more free-radicals with negative charge, such as superoxide anions and hydroxyl radicals, into the cell, and the death of the bacterial cell [29]. Zhang *et al.* [31] coated the bamboo with GO-ZnO nancomposites found excellent antibacterial activity for both gram-positive and gram negative bacteria for the treated bamboo compared to the untreated one. The antibacterial activity of ZnO/graphene quantum on *E. coli* was markedly enhanced under UV photo-irradiation as compared to ZnO and GQD separately, which was ascribe to the enhanced generation of ROS under the UV photo-irradiation with minor contributions from membrane damage [18]. The present study is best to investigate the antioxidant and antibacterial activities of ZnO-NPs, graphene and G-ZnO. Due to the potent antibacterial and antioxidant properties of G-ZnO, this compound can be used as a valuable natural source in the pharmaceutical industry and a suitable alternative for inhibiting microbism in clinical and food applications.

CONCLUSION

Based on the observations in this study, ZnO-NPs, graphene and G-ZnO showed antioxidant and antibacterial activities. From the comparison of the three tested samples, it was found that G-ZnO had significant antioxidant activity due to the synergistic and cross-linking effects of ZnO-NPs and graphene, as well as G-ZnO had stronger antibacterial activity than ZnO-NPs and graphene against gram-negative and gram-positive bacteria. In the future, G-ZnO can be used as a natural antioxidant and antibacterial in the pharmacy and food industry.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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