

Evaluation of Anti-Bacterial and Anti-Cancer Activity of Graphene Oxide-Loaded Magnetic Nanocomposites

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ABSTRACT

The emergence of antibiotic-resistant microbial strains presents a big challenge in healthcare sector which necessitate innovative approaches for effective antimicrobial therapies. In this context, the study explores the synthesis and biomedical application of graphene oxide (GO)-loaded magnetic nanoparticle (MNP) nanocomposites, heralding a novel strategy in the battle against bacterial infections and cancer. The unique physicochemical characteristics of GO including its extensive surface area, functionalization flexibility, biocompatibility, and high drug-loading capacity, are leveraged to enhance both antibacterial and anticancer efficacy. This research focuses on the fabrication of GO-MNPs hybrids, their surface characterization and the evaluation of their biological properties. The successful conjugation of MNPs to GO was confirmed by Fourier-Transform Infrared Spectroscopy (FTIR) analysis. The anti-bacterial activity of GO loaded MNPs and MNPs alone was assessed separately by Minimum Inhibitory Concentration (MIC) against different drug resistant Gram-positive *Bacillus subtilis*, *Bacillus muralis*, *Bacillus licheniformis* and Gram-negative bacteria (*Enterobacter asburiae*, *Escherichia coli*, *Pseudomonas aeruginosa*) while the cytotoxic effect was evaluated in HepG2 cells using neutral red assay. According to our results, GO loaded MNPs nanocomposites showed significantly enhanced anti-bacterial and ant-cancer activities as compared to MNPs when they were using lonely. The promising results of this research pave the way for further exploration of these nanocomposites in diverse biomedical applications.

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Key words

Graphene oxide, Magnetic nanoparticles, Nanocomposites, Anti-bacterial, Anti-cancer

INTRODUCTION

Nanotechnology has opened up exciting opportunities for the manipulation of materials at the nanoscale, specifically in biomedicine (Sukumaran *et al.*, 2018). Among other materials being explored, carbon and graphene have emerged as environment friendly options due to their abundance in nature. Graphene possesses remarkable properties, arranged in a hexagonal lattice as a single layer of carbon atoms. Graphene has many applications especially in diagnostics, drug delivery, therapeutics,

and even in biomedical device development and biosensing, due to its biocompatibility and ultrathin nanomaterial composition. Graphene oxide (GO), being an oxidized form of graphene is the most appropriate choice for biomedical applications along with the functional groups as -OH, -CHO, -CO, -COOH and epoxide. Such groups enable easy customization of GO with biomolecules and drugs (Garg *et al.*, 2017).

Different types of microbial infections have been found to be susceptible to nanostructures made up of Graphen. With an increase of multi-drug resistance in microbes, there is an increase in demand for new antimicrobial agents or alterations to enhance the effectiveness of existing ones. Due to the prevalence of infectious infections, traditional antibiotics are no longer as effective and are now required for a variety of medical procedures. Antibiotics are now necessary in all health systems and helped to bring about previously unheard-of advances in medicine and society. The researchers have extensively studied various material systems including magnetic nanoparticles for antimicrobial properties (Yao *et al.*, 2019). Additionally, graphene-based

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materials including graphene-based hybrids have been examined for their ability to combat bacteria and are thus suitable candidates for versatile antibacterial applications. GO is supposed to inhibit infections caused by bacteria because of its biocompatibility (Diez-Pascual, 2020). Nevertheless, their cytotoxicity and antibacterial mode of action has not been explained yet. It has been reported that entrapment has cutting effect of GO is affected by size of GO sheets (Yu *et al.*, 2020). Magnetic nanoparticles exhibit considerable promise as antibacterial agents due to their magnetic characteristic. These particles are highly valuable in nanomedicine for bacterial detection and therapeutic applications due to their higher surface area and biocompatibility. Furthermore, magnetic nanoparticles' stronger antibacterial activity can aid in the fight against problems like antibiotic-resistant bacteria, which continue to endanger people's lives all over the world (Xu *et al.*, 2019).

The GO itself possess inherent antimicrobial properties as it can disrupt the bacterial cell membrane, induce oxidative stress and can thus lead to the bacterial cell death. The antimicrobial effect can synergistically be enhanced when combined with magnetic nanoparticles as they can act as carriers, thereby delivering graphene oxide to the targeted microbial cells thus increasing the concentration of GO at the site of action and improving the overall antimicrobial activity. Using this hybrid enhances antimicrobial activity and can provide new dimensions in cancer research. The high-end super-paramagnetic characteristics of magnetic nanoparticles (MNPs) can help in targeted delivery of the drug to the tumor site (Zhang *et al.*, 2020). However, the hybrids of these particles can compensate the shortcomings while keeping their useful properties functional (Montoro-Leal *et al.*, 2020).

In this study, we designed a multifunctional GO loaded MNPs nanocomposites, characterized and investigated their antibacterial and cytotoxic activities through in vitro studies.

MATERIALS AND METHODS

Synthesis of MNPs and GO

Co-precipitation method was used to synthesize the MNPs (Majidi *et al.*, 2016). This process involved adding bases (sodium hydroxide, potassium hydroxide, or ammonium hydroxide) to a solution of iron (II) and iron (III) salts under varying temperature conditions (25°C, 50°C, 70°C). This method was chosen for its effectiveness in controlling the particle size and morphology. On the other hand, GO was synthesized by modified Hummer's method (Zaaba *et al.*, 2017). This modified approach involved a one-pot synthesis with three critical steps:

initial low-temperature pre-oxidation of natural flake graphite using a mixture of potassium permanganate and boric acid-stabilized potassium ferrate, followed by a secondary deep oxidation process, and finally hydrolysis and exfoliation into GO.

Synthesis of nanocomposites

The impregnation method was being used for the synthesis of nanocomposites. By dispersing GO in distilled water (1:1 w/v) and sonicated for half an hour. The MNPs were then added and then sonicated for about half an hour to obtain a homogenous suspension followed by centrifugation for 10 min at 10000. The pellet was lyophilized and GO nanocomposites were formed in the form powder.

FTIR analysis

The Fourier-Transform Infrared Spectroscopy (FTIR) analysis was used for the characterization of the surface chemical structures of GO nanocomposites and MNPs. Sample were analyzed in the range of 4000-400 cm^{-1} . For the FTIR measurements, the nanoparticles and nanocomposites were mixed with the potassium bromide (KBr) and pressed into pellets.

Antibacterial assays

Minimum inhibitory concentration (MIC) was used to analyze the antibacterial activity of MNPs and GO. These assays were designed to examine the antimicrobial activity against both Gram-positive (*Bacillus subtilis*, *Bacillus muralis*, *Bacillus licheniformis*) and Gram-negative bacteria (*Enterobacter asburiae*, *Escherichia coli*, *Pseudomonas aeruginosa*). LB-agar plate was used to sub-culture Gram-positive and Gram-negative bacterial strains. These were then incubated overnight. After that freshly prepared 10ml LB media was inoculated with discrete colonies of each strain and incubated for 24 h at 37° C. In order to determine the MIC value for tested bacterial strains, method described by National Committee for Clinical Laboratory Standards (NCCLS) was used with minor changes (Wypij *et al.*, 2021).

Neutral red assay

The anti-cancer activity of GO nanocomposites and MNPs was analyzed by neutral red assay. Seeding of HepG2 cells was done at 10,000/ cm^2 density in triplicate. After seeding the cells for 24 h, cells were treated with different concentrations (20-80 $\mu\text{g/ml}$) of MNPS and GO nanocomposites with fresh media. After 24 h of incubation, the culture media was changed with media containing neutral red and cells were left for about 2 h at 37°C and 5% CO_2 . After aspirating the media, the cells

were washed three times with 1X PBS and then incubated with 300 μ l of de-staining solution. After two min a typical pink color was observed. Then 150 μ l media containing cells were transferred in each well in 96-well microtiter plate. Absorbance was observed at 570nm. The average values of these observations were used to compare cell susceptibility and concentration of tested drugs by plotting graph. In order to analyze these cells, complete culture media were used as negative control while PBS containing media were used as positive control.

RESULTS

The FTIR spectra of MNPs providing valuable insights into the chemical composition and functional groups present on the surface of MNPs (Fig. 1a). The stretching vibrations of the -OH band are represented by the peak at 3737 cm^{-1} in the spectrum, while the asymmetric C=O is responsible for the peak at 1701 cm^{-1} as shown in Figure 1a. In the same way, the distinctive Fe-O in the magnetic nanoparticles is represented by the absorption peak at 651 cm^{-1} .

The FTIR spectrum of GO nanocomposites, shown in the Figure 1b, reveals notable findings of successful integration of MNPs on to GO nano-sheets. It has been observed that new peaks were appeared, signifying

the MNPs and GO nano-sheets covalent bonding. The presence of a carboxyl group on the surface of magnetic nanoparticles and iron can generate a mono-dentate or bi-dentate complex, which can explain the two vibration peaks that occur at 1339 cm^{-1} and 1363 cm^{-1} . Similarly, a peak at 651 cm^{-1} represents the lattice absorption of magnetic nanoparticles. It suggests that ester O and nanoparticles have a strong connection.

All bacterial strains (*Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus muralis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterobacteriae asburiae*) appear to grow less as the concentration of magnetic nanoparticles increases (Fig. 2A). These bacterial strains have magnetic nanoparticles with MIC values ranging from 80 $\mu\text{g/ml}$ to 320 $\mu\text{g/ml}$. These results indicate the antimicrobial effectiveness of magnetic nanoparticles against a broad spectrum of bacterial strains, with higher concentrations exhibiting stronger inhibitory effects on bacterial growth. The MIC values for *Enterobacter asburiae* was found to be 80 $\mu\text{g/ml}$ while for *Bacillus subtilis* and *Escherichia coli* it was 160 $\mu\text{g/ml}$. Similarly, MIC value of 320 $\mu\text{g/ml}$ was found for *Bacillus licheniformis*, *Bacillus muralis*, and *Pseudomonas aeruginosa*. These findings, highlight the strain-specific variations in bacterial susceptibility to antimicrobial effects of MNPs.

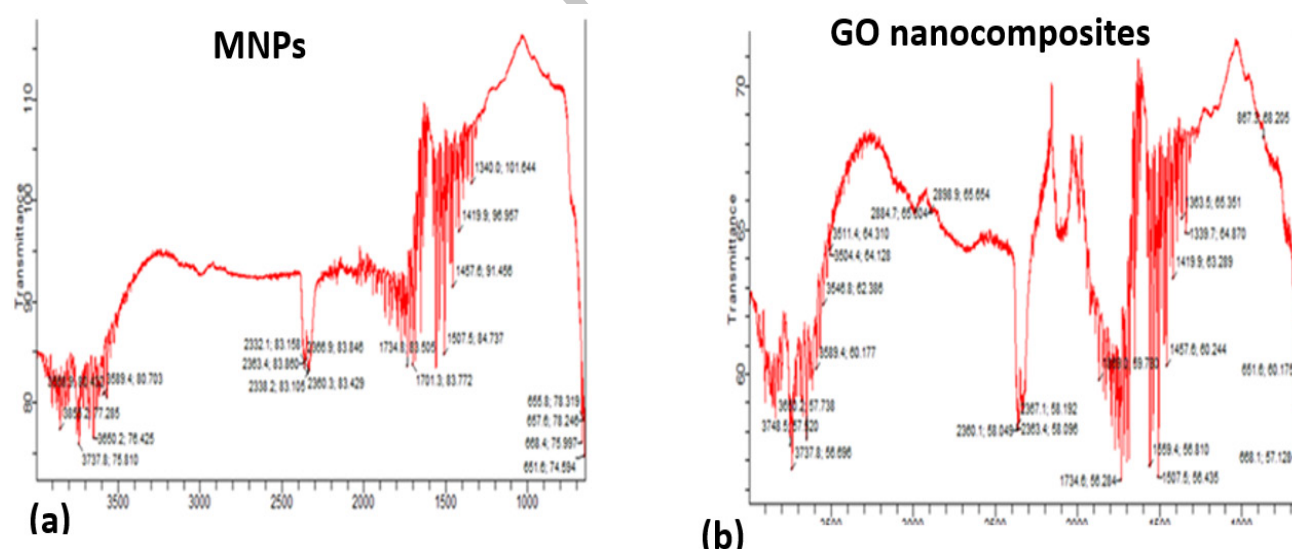


Fig. 1. FTIR analysis of MNPs (a) and GO nanocomposites (b). The spectrum in (a) exhibits distinct peaks, indicative of various functional groups present in the MNPs as the prominent peak at 3737 cm^{-1} indicates the presence of OH groups, other at 1701 cm^{-1} suggesting the presence of carbonyl functional groups and the absorption peak at 651 cm^{-1} confirming the presence of iron-oxide in the magnetic nanoparticles. The spectrum in (b) shows the emergence of peaks at 1339 cm^{-1} and 1363 cm^{-1} indicates the formation of mono or bi-dentate complex between iron and carboxyl groups while peak at 651 cm^{-1} corresponds to lattice absorption of magnetic nanoparticles. |

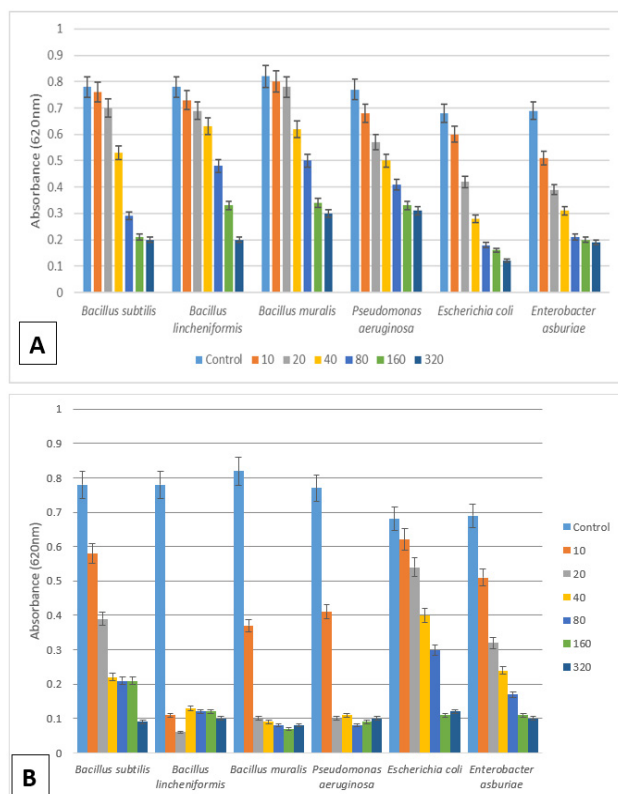


Fig. 2. Antibacterial activity of magnetic nanoparticles (A) and GO nanocomposites (B) against different bacteria. The figure shows concentration ($\mu\text{g/ml}$) dependent decrease in bacterial growth across all the tested strains.

The MIC assay reported decline in bacterial growth with increase concentration of GO nanocomposite as compared to the MNPs (Fig. 3). It was found that *B. licheniformis*, *B. muralis* and *P. aeruginosa* showed significant less MIC for GO nanocomposite as compared to the MNPs.

The MIC values in case of GO nanocomposite (Fig. 3A) was reported 20 $\mu\text{g/ml}$ for *B. licheniformis*, *B. muralis* and *P. aeruginosa* as compared to MIC value for MNPs as 320 $\mu\text{g/ml}$. While *E. asburiae* exhibits an MIC value of 160 $\mu\text{g/ml}$ for GO nanocomposite (Fig. 3B) as compared to 80 $\mu\text{g/ml}$ for MNPs. Furthermore, *B. Subtilis* an MIC value of 320 $\mu\text{g/ml}$ in case of GO nanocomposite and 160 $\mu\text{g/ml}$ for MNPs. demonstrating varying susceptibility of bacterial strains to GO nanocomposites, highlighting their potential as effective antimicrobial agents.

Anti-proliferative effect of magnetic nanoparticles and GO nanocomposites

Anti-proliferative effect of MNPs and GO nanocomposite was evaluated using neutral red assay in HepG2 cells. It was found that MNPs had no effect

on cell viability, while GO nanocomposite found to decrease cell viability at all concentrations. Furthermore, it was found that an increase in the concentration of GO nanocomposites, there is an evident proportional decline in cell viability (Fig. 4). For instance, at 20 $\mu\text{g/ml}$ cell viability decrease by 17% and at 80 $\mu\text{g/ml}$, cell viability decreases by 63%.

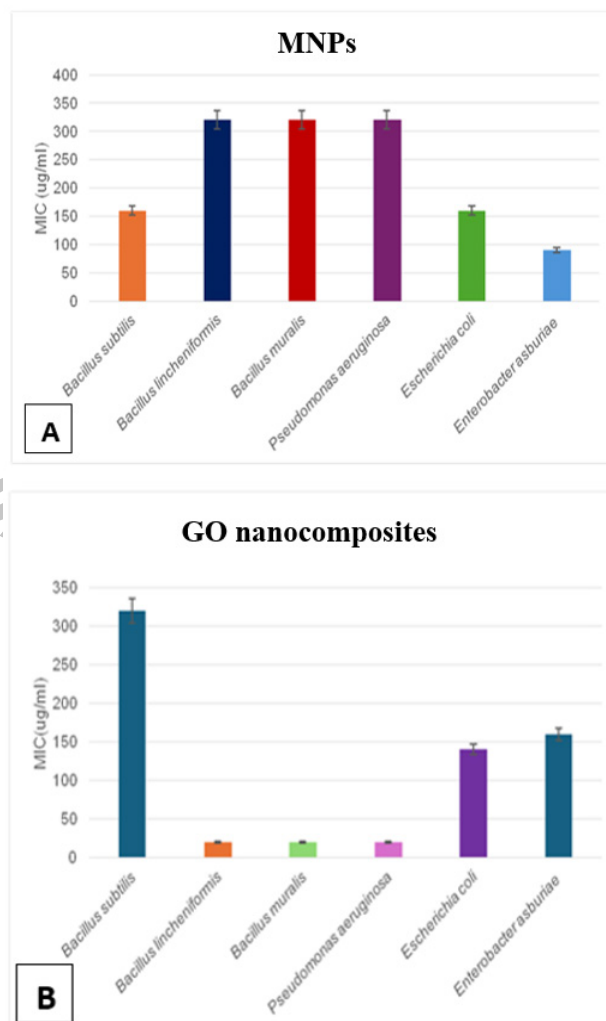


Fig. 3. MIC values of MNPs (A) and GO nanocomposites (B) against bacterial strains. *E. asburiae* exhibit lowest MIC value 80 $\mu\text{g/ml}$, 160 $\mu\text{g/ml}$ for *B. subtilis* and *E. coli* and 320 $\mu\text{g/ml}$ for *B. licheniformis*, *B. muralis* and *P. aeruginosa*. (B) shows the impact of GO nanocomposites on bacterial strains. The MIC values of GO range from 20 $\mu\text{g/ml}$ for *B. licheniformis*, *B. muralis* and *P. aeruginosa*, while *E. asburiae* exhibits an MIC value of 160 $\mu\text{g/ml}$ and *B. subtilis* an MIC value of 320 $\mu\text{g/ml}$, demonstrating varying susceptibility of bacterial strains to GO nanocomposites, highlighting their potential as effective antimicrobial agents.

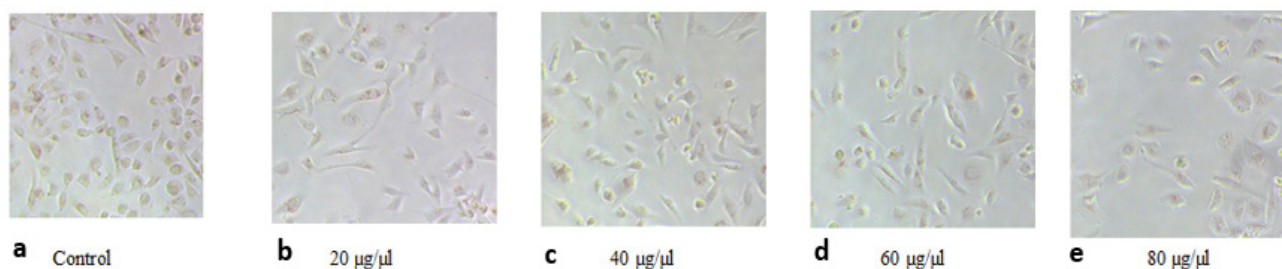


Fig. 4. HepG2 cells treated with GO nanocomposites in different concentrations.

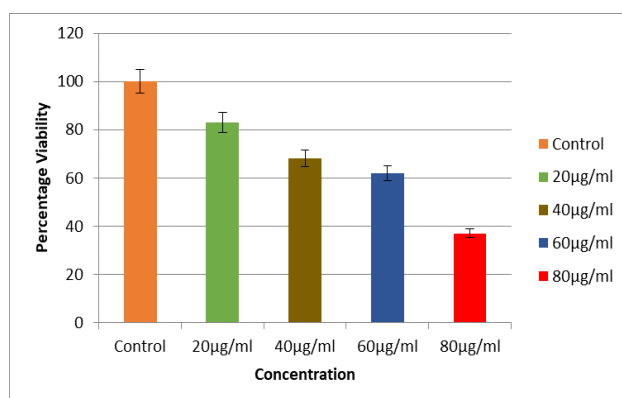


Fig. 5. Cytotoxicity of GO nanocomposites (in various concentration) against HepG2 cells.

Table I. MIC of MNPs and GO nanocomposite against different bacterial strains.

Bacterial strain	MIC of nanoparticles	
	MNP	GO
<i>Bacillus subtilis</i>	160±1.7	320±2.2
<i>Bacillus lincheniformis</i>	320±2.1	20±0.8
<i>Bacillus muralis</i>	320±1.8	20±0.7
<i>Pseudomonas aeruginosa</i>	320±1.9	20±0.5
<i>Escherichia coli</i>	160±1.8	140±1.2
<i>Enterobacter asburiae</i>	90±0.9	160±0.9

GO, graphene oxide loaded magnetic nanoparticle; MNP, magnetic nanoparticle.

A graph representing the relationship between percentage cell viability and different GO nanocomposites concentrations was created using the average results obtained from the neutral red assay. The percentage of cell susceptibility to GO nanocomposites is shown in Figure 5, where a decline in cell viability is observed at 20 µg/ml, 40 µg/ml, 60 µg/ml, and 80 µg/ml, respectively, by 17%, 32%, 38%, and 63%.

DISCUSSION

Nanotechnology, possessing the ability to manipulate the matter at nanoscale, have emerged as a promising technology of modern era with applications in various fields. Thus, bio-nanotechnology has gathered significant attention regarding its significant potential in diagnosis and disease treatment (Kinnear *et al.*, 2017). Extensive research have been made in order to explore the use of nano-composites in drug delivery, diagnosis, therapy and molecular imaging (Weissig *et al.*, 2014).

To manufacture nanoparticles with desired morphologies, sizes, and chemical characteristics, two main methods are used: top-down and bottom-up (Ijaz *et al.*, 2020). In past few years magnetic nanoparticles especially iron oxide nanoparticles have been extensively studied due to their inherent properties and their ability to load drugs. These nanoparticles have shown potential as analytical imaging agents and carriers for drug delivery (El-Boubbou, 2018). Similarly, GO and its functional derivatives have attracted scientific interest due to their low toxicity and impressive biocompatibility.

The rising concern of bacterial drug resistance necessitates the development of alternative antibacterial therapies. Magnetic nanoparticles and graphene oxide have demonstrated antibacterial potential, making them efficient tools for combating bacterial infections.

As the leading cause of mortality worldwide, Cancer presents significant challenges in treatment. Current approaches (chemotherapy, surgery, and radiation therapy) have limitations together with non-specific drug distribution and inadequate drug delivery to tumor sites. In the current research, GO nanocomposites were prepared by incorporating MNPs onto GO. The co-precipitation method was employed in order to synthesize iron oxide-based MNPs, and Hummer's method was employed in synthesis of GO. MNPs were conjugated to GO using impregnation technique. A powder form of particles was obtained and undergone for the confirmation. FTIR analysis confirmed the presence of characteristic Fe-O

bonds through the absorption peak at 651 cm^{-1} . Moreover, MNPs conjugation over GO was verified by peaks at 1339 cm^{-1} and 1363 cm^{-1} .

MIC was used to assess the antimicrobial activity of the MNPs and GO nanocomposites. The findings were quite consistent with the research studies conducted (Ge *et al.*, 2019), who reported the enhanced removal of *E. coli* with GO nanocomposites. MIC values of GO nanocomposite were found to be lower than MNPs. In addition, the GO nanocomposites and MNPs both inhibited the growth of bacteria at varying concentrations, but GO nanocomposite were found to have more anti-bacterial activity as compared to MNPs. Similarly, another study demonstrated an 82% decline in cell viability of *E. coli* through the use of GO nanocomposite (Szunerits and Boukherroub, 2016).

Gram positive bacteria were found to be more susceptible to GO nanocomposite (Barani *et al.*, 2021). This high antimicrobial activity of such hybrids is due to the fact that they release hydroxyl radicals which causes bacteria to die. These findings were postulated in accordance to the results of MIC.

In neutral red assay, MNPs showed no effect on cellular viability as demonstrated by Paramo *et al.* (2023) while GO nanocomposites exhibited toxicity to Hep G2 cells at various concentrations, resulting in a significant decrease in cell viability by 63% at an $80\text{ }\mu\text{g/ml}$ concentration. This suggests that the GO-nanocomposite hybrids hold more potential as compared to MNPs as nano-carriers for targeted drug delivery to tumor sites as supported by the research work of Wang and coworkers (Wang *et al.*, 2017).

CONCLUSION

The synthesis and characterization of GO and MNPs based GO-nanocomposite hybrids have shown great potential in the field of antimicrobial and anti-cancer activity. The combination of these two materials allows for enhanced properties and improved performance against various microorganisms. The process of hybridization creates nanocomposites that possess unique characteristics such as increased stability, biocompatibility, and targeted delivery.

The synthesis techniques employed in creating these hybrids are relatively simple and cost-effective. This makes them manageable to a wide range of researchers and industries interested in developing effective antimicrobial solutions. With continued advancements in this field, we can anticipate the development of innovative strategies to combat microbial infections more effectively with minimal side effects on human health.

RECOMMENDATIONS

The findings of this study are essential for investigating this hybrid's antibacterial mechanism in more harmful microbes. Moreover, research on GO nanocomposites may be done to find out how they might be used in the biomedical industry.

DECLARATIONS

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Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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