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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(3): 397-401
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www.thepharmajournal.com
Received: 19-11-2018
Accepted: 23-02-2019

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2D carbon nano material based gentamicin nano composite for antibacterial activity against *Escherichia coli*: A novel strategy towards multidrug resistant bacteria

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Abstract
Multidrug resistance is one of the burning issues in medical sciences as it makes bacteria, able to stop antibiotic, working on it. These resistant strains of bacteria make treatment difficult, costly, with addition of prolonged hospital stay and increase rate of mortality. In the recent years 2D carbon nano composite based drugs has proved its potential, against microbial infections due to their high pay load, and site specific drug delivery. Novel nano composite: combination of-protein synthesis inhibitor: gentamicin, conjugated with efficient nano carrier: graphene could result better in antibiotic resistance, on topical infection caused by multi drug resistant *E. coli* 1614. This study was primarily based on two stages: conjugation of 2D carbon nano material based gentamicin nanocomposite and it's drug-carrier interaction studies, using FTIR as a tool. There resulted spectra of FTIR showed no significant interaction of the drug with the chosen nano-carrier used in the nanocomposite formulation. In second phase, screening for antibacterial activity, proved gentamicin loaded graphene nanocomposite as an excellent tool against topical infection caused by multidrug resistance strain of *E. coli* 1614.

Keywords: novel nano composite, 2D nano carbon, drug carrier interaction, multi drug resistance

1. Introduction
Multidrug resistance strains are one of the burning issues in medical sciences about which WHO is also thoughtful about [1] as drug resistance bacteria would lead us to post antibiotic era, where simple wound or infection would be able to kill us [2]. To get rid of this situation a wholesome set of new antibacterial drugs are required [3]. In the recent years the nano-composite based drugs has proved its potential, against microbial infections [4, 5]. Single layer of graphite: graphene [6] could be count as a competent weapon against multi drug resistant bacterial strains as it work in four headed arrow pattern: produce stress on membrane [7], wrap and isolate the cell, formation of electrical charge, formation of reactive oxygen species and eventually kills the cell [8]. While it is combined with bacterial protein synthesis inhibitor: gentamicin as drug of choice, would work as an active nanocarrier [9], and that would reduce toxicity as a consequence of preferential accumulation of dosage form at target site. This would show prolonged therapeutic effect because of the controlled delivery of drug from the carrier, with addition of lower the dose required for efficacy. This gentamicin loaded graphene nano composite would be expected to illustrate some extraordinary synergistic antibacterial activity against topical infection [10]. To achieve these objectives nano composite would be conjugated and it's drug-carrier interaction studies would be carried out, to demonstrate the conjugation of graphene and gentamicin nanocomposite, FTIR would be the tool. Antibacterial activity of the nanocomposite, against topical infection causing multidrug resistance strain of *Eschericia coli 1614* would be checked through zone of inhibition study.

2. Materials and Methods
2.1 Materials
Drug of choice, gentamicin sulphate was purchased from Yarrow Chem Products, Mumbai, India. Two dimensional carbon nanomaterial: graphene was obtained from Reinste Nano Ventures Pvt. Ltd., India. Other chemicals such as: Acetone, Hydrochloric Acid, Sodium Chloride, Sodium Hydroxide, Mannitol were purchased from CDH, India.

Along with these methanol, ethanol, Potassium Dihydrogen were bought from Qualigens Fine Chemicals, India and n-Octanol was from Lobe Chemicals, India. Bacterial culture media: nutrient broth and nutrient agar were procured from Hi- Media Pvt. Ltd., India. *Escherichia coli 1614* were obtained from Institute of Microbial Technology (IMTECH), Chandigarh.

2.2 Methods

2.2.1 Conjugation of Gentamicin sulphate to Graphene nano sheets

Simple physico absorption principle was followed while loading gentamicin sulphate onto graphene [11]. Here sonication was choice of method. Initially graphene suspension (0.145 mg/ml) was sonicated with gentamicin (1mg/ml) at pH 7 for 30 min, followed by stirring in dark at room temperature for overnight, with the expectation of gentamicin sulphate to be coupled with graphene nanosheets. Next morning the whole sample was ultracentrifuge at 15000 rpm for 1 h. and excess and uncoupled drugs i.e. supernatant were pipette out, leaving the gentamicin loaded graphene in precipitate.

2.2.2 Drug carrier interaction study through Fourier Transform Infrared Spectroscopy

FT-IR transmission spectra of gentamicin sulfate, graphene, gentamicin graphene nanocomposite obtained using Perkin Elmer FTIR Spectrophotometer (Model No. 91151), with Perkin Elmer Spectrum 2 software. There a total of 2% (w/w) of sample, with respect to the potassium bromide (KBr, Panreac) disc was mixed with dry KBr. The mixture was grounded into fine powder using an agate mortar before compressing into KBr disc under a hydraulic press at 10,000 psi. each KBr disc was scanned at 4mm/s at resolution of 2cm over a wave-number region 450-4000 cm^{-1} using IR software. The characteristic peaks were recorded for each samples [12, 13].

2.2.3 Antimicrobial activity

E. coli 1614 was chosen as bacterial strain to be tested on, as it is resistance against most of the commercially available antibacterial drugs [14].

Drug resistance and drug sensitivity of *E. coli* 1614

Table 1: Showing list of drugs *E. coli* 1614 resistant and sensitive of.

Bacterial strain	Drug resistance	Drug sensitivity
E. coli 1614	Ceftriaxone Ciprofloxacine Cefpime Ampicilline Amoxy / Clav Gentamicin Cefotaxim Levo	Amikacine Imipenum

Zone of inhibition study

In beginning the antibacterial activity was checked with zone of inhibition study. Nanocomposite with antibacterial activity would form zone of inhibition on agar plate. For that initially one loop full inoculated was added in nutient broth and was kept for overnight incubation at 37ºC. Next 1% inoculation was taken and added in nutrient broth and again kept for overnight incubation at 37ºC [15]. Then the culture was taken out during OD 0.6 at 640 nm and used for making lawn on plates having solidified nutrient agar [16]. After that, paper discs were used to place drugs of different concentrations and once again kept for overnight incubation at 37ºC. Finally zone of inhibition were measured after overnight (14 hrs.) incubation [17, 18]. There each plate had contained four discs with same doses of gentamicin loaded graphene nanocomposite as treatment, gentamicin and graphene ointment as positive controls, water as negative control respectively.

3. Results and discussion

To reach the increasing demand for efficient topical drug delivery systems, attempts have been made to develop drug loaded with nano carrier to achieve the objective.

3.1 Conjugation of Gentamicin sulphate to Graphene nano sheets

Gentamicin loaded graphene nanocomposite was prepared using pH 7 as the preferred pH, reason behind this was loading of gentamicin drug on to graphene oxide depended on how efficiently the environment was forming hydrogen bonds between the (–OH) and (–COOH) groups of graphene oxide and the (–OH) and ($–NH_2$) groups of gentamicin. In acidic environment, where pH was less than 7, due to presence of free H^+, ($–NH_2$) of gentamicin forms ($–NH_3^+$) with (H^+) and therefore could not participated in hydrogen bonding. Thus, at low pH only two kinds of hydrogen bonding could occur between (–COOH) and (–OH) of graphene oxide and the (–OH) of gentamicin. On the contrary, in basic condition, where pH was more than 7, -OH ions were numerous there, (–COOH) of graphene oxide exist as ($–COO^-$) and could not form hydrogen bonds with any group of gentamicin. Thereby hydrogen bonding could occur only between (–OH) of graphene oxide and the (–OH) and ($–NH_2$) of Gentamicin. That was why pH 7 was chosen to make gentamicin loaded graphene nanocomposite, as maximum hydrogen bonding were expected between graphene oxide and Gentamicin sulphate.

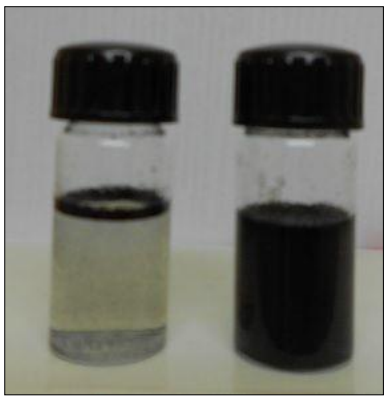

Fig 1: Digital photograph of Graphene nano sheets, not dispersed in water (left) and dispersed in water after drug conjugation (right)

3.2 Drug-carrier interaction studies: Solid state characterization of drug-carrier system: Fourier Transforms Infrared Spectroscopy (FTIR)

Gentamicin, graphene and gentamicin loaded graphene nanocomposites were analyzed for characteristic transmission bands, indicative of their interaction (Fig. 2). Perkin Elmer Spectrum 2 software revealed that, the peaks at the 1650-1540cm^{-1} region of the spectrum correspond to the N-H bending vibration of primary aromatic amines. The peak at

1653cm^{-1} in both the gentamicin and gentamicin loaded graphene nanocomposites spectra were due to the non-reacted NH_2 groups of gentamicin sulfate. Similarly, peaks observed between 3700-3584cm^{-1} in FTIR spectra of gentamicin and gentamicin loaded graphene nanocomposites represent non-reacted free hydroxyl groups of gentamicin sulfate. Thus the resulted spectra of FTIR (Fig. 2) showed no significant interaction of the drug with the chosen carrier: graphene, used in the gentamicin and gentamicin loaded graphene nanocomposites.

The drug-carrier interaction study was carried out by FTIR spectroscopy. The FTIR spectra of nano-conjugate: gentamicin loaded grap hene compared with the individual spectra of both to find out any interaction. The resulted spectra of FTIR showed no significant interaction of the drug with the chosen nano-carrier used in the nanoparticles formulation (Fig. 2).

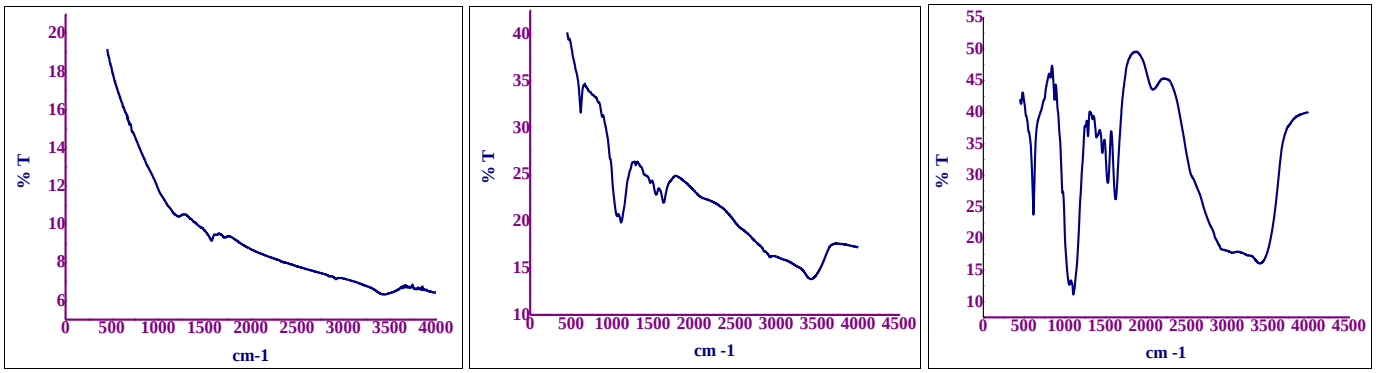

Fig 2: Shows a comparative picture of FTIR spectra of graphene, gentamicin and gentamicin loaded graphene nanoparticles.

3.3 Antibacterial activities

Results of antibacterial assay of *E. coli* 1614 via zone of inhibition were documented:

Once the zone of inhibition study was done, statistical analysis to understand the significance of gathered data was performed, using 'OriginPro 8' software. Here is the presentations of, result of zone of inhibition assay in graphical form.

A comparative study on zone of inhibitions with all different doses

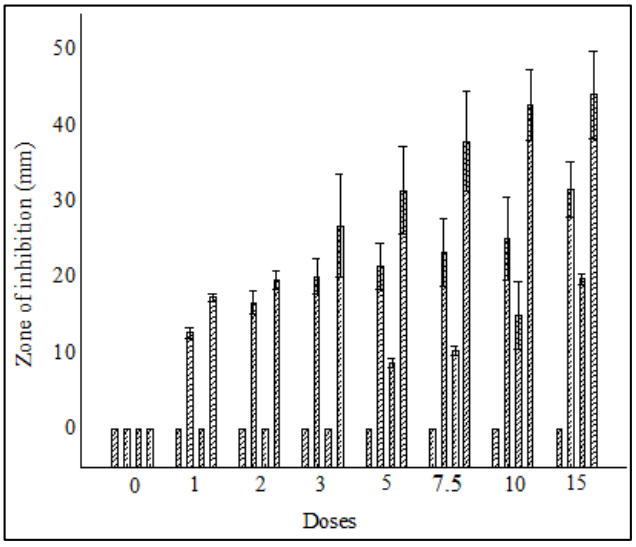

Fig 3: Showing the zone of inhibition and their standard deviation observed in water, gentamicin, graphene and gentamicin loaded graphene nanocomposite on *E. coli* 1614 lawn on nutrient agar plates. In each segment, first bar presents result of water, followed by gentamicin, graphene and gentamicin loaded graphene nanocomposite respectively, where the doses of choice are 1 µg/ml, 2 µg/ml, 3 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml and 15 µg/ml.

Fig. 3 implicate that, there were no observable zone of inhibition found in any dose of water against *E. coli* 1614 lawn speeded over nutrient agar plates. In support of Fig. 4 this result could be counted as, water has no antibacterial activity against *E. coli* 1614. So the zone of inhibition observed in gentamicin, graphene and gentamicin loaded graphene nanocomposite could be measured as intrinsic quality of gentamicin, graphene and gentamicin loaded graphene respectively.

In Fig. 3 it has been seen that gentamicin i.e. second bar from each segment initially shows 12.8 mm of diameter of zone of inhibition, with standard deviation (SD) of 0.68 at dose of 1 µg/ml, this diameter increases with increase of dose, and reach upto 31.7 mm with SD of 3.9 at 15 µg/ml dose.

In Fig. 3 it also has been demonstrated the result of zone of inhibition study with graphene i.e. third bar in each segment against *E. coli* 1614 lawn speeded over nutrient agar plates. There one could see there were no observable zones of inhibition in initial doses like- 1 µg/ml, 2 µg/ml or 3 µg/ml (Fig. 4). It was only dose of 5 µg/ml, where gentamicin produce 8.7 mm diameter of zone of inhibition with SD of 0.6, whereas at the dose as high as 15 µg/ml, gentamicin produce 19.8 mm diameter of zone of inhibition with SD of 0.8 against *E. coli* 1614 lawn speeded over nutrient agar plates.

Regarding gentamicin loaded graphene nanocomposite i.e. forth bar in each segment in Fig. 3 shows that it starts producing zone of inhibition with diameter of 17.3 with SD of 0.6 in dose of as low as 1 µg/ml, higher than zone of inhibition of graphene produced in dose of 10 µg/ml (15 mm with SD of 4.5). In Fig. 3 it could also be seen that gentamicin loaded graphene nanocomposite produce zone of inhibition with diameter of 42.8 mm with SD of 4.6 at dose of 10 µg/ml, higher than total of zone of inhibition produce by gentamicin 25.2 mm with SD of 5.4 and zone of inhibition produce by graphene 15 mm with SD of 4.5 at the same dose of 10 µg/ml. At highest dose of 15 µg/ml gentamicin loaded graphene nanocomposite shows zone of inhibition with diameter of 44.2 mm with SD of 5.8, which is higher than zone of inhibition produced by any treatment at any dose.

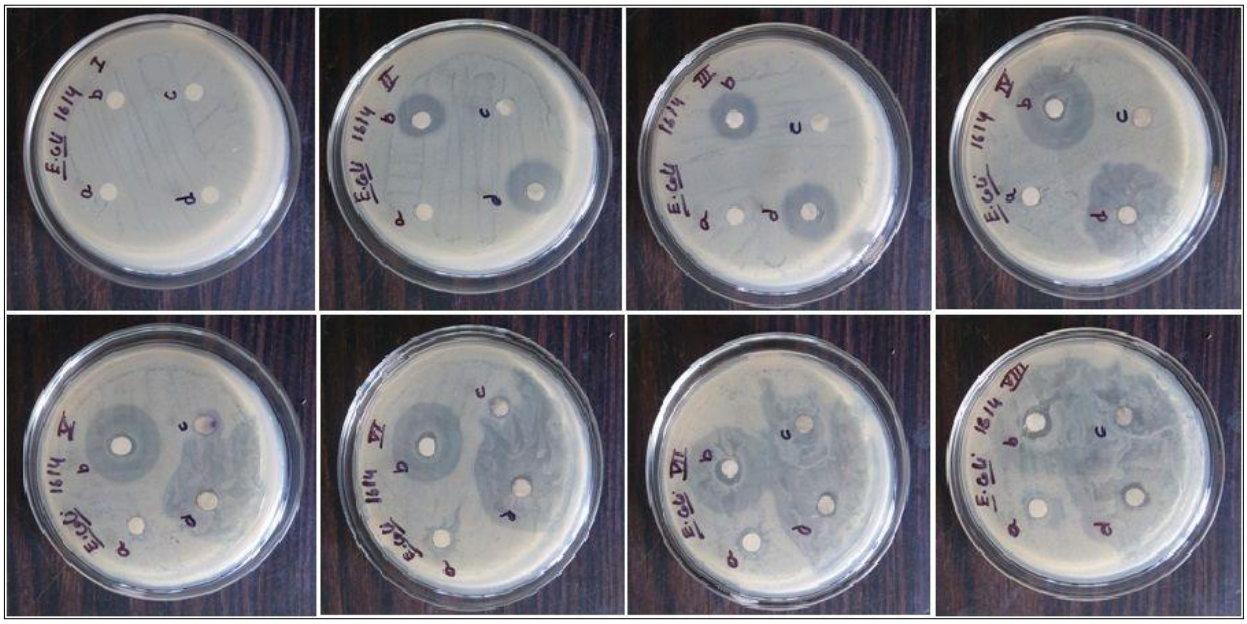

Fig 4: Depicts the of inhibition produce by different treatments- where a = water, b, c and d are gentamicin, graphene and gentamicin loaded graphene nanocomposite respectively against *E. coli* 1614 lawn speeded over nutrient agar plates, and I = 0 µg/ml, II = 1 µg/ml, III = 2 µg/ml, IV = 3 µg/ml, V = 5 µg/ml, VI = 7.5 µg/ml, VII = 10 µg/ml, VIII = 15 µg/ml.

With these first phase of antibacterial study was finish off. And this study concluded that, gentamicin loaded graphene nanocomposite was a good choice of drug against *E. coli* 1614 lawn speeded over nutrient agar plates, it has showed larger zone of inhibition produced by total zone of inhibition produced by gentamicin and graphene separately.

Conclusions

In this study a novel nano composite: gentamicin loaded graphene nanocomposite was prepared to combat against topical infection caused by drug resistant *E. coli* 1614. This study was primarily based on two stages: conjugation of gentamicin loaded graphene nanocomposite and looking for antibacterial activities of the conjugated nanocomposite.

In first phase of work, conjugation was done through ultrasonication, and its drug carrier interaction study showed that, gentamicin and graphene are conjugated without significant interactions. That is why the nanocomposite showed synergistic effect in antibacterial studies while carried out.

During antibacterial activities, the nanocomposite was checked for zone of inhibition study. There the study concluded that, gentamicin loaded graphene nanocomposite was a good choice of drug against *E. coli* 1614 lawn speeded over nutrient agar plates, it has showed larger zone of inhibition produced by total zone of inhibition produced by gentamicin and graphene separately.

Acknowledgements

The authors are thankful to Department of Biotechnology (DBT), New Delhi, India for funding. Authors would also like to thank Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, India for conferring necessary facilities to carry out this novel work.

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