The Pharma Innovation Journal 2019; 8(11): 296-301

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(11): 296-301

www.thepharmajournal.com
Received: 04-05-2019
Accepted: 08-07-2019

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Design and development of gentamicin based novel nano composite against multi drug resistant bacteria

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Abstract
Drug resistance by bacteria makes treatment procedure expensive, difficult and time consuming. Countries where antibiotic could be purchased without prescription, there situations are deadlier and with the increased rate of travelling throughout the globe multidrug resistant bacteria have reached every nook and corner of civilization. That going to lead us to the age where a simple sore throat might cause life risk, like pre-antibiotic era. In the recent years 2D carbon nano composite based drugs have 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 with the improved pharmacokinetics at intended site, against topical infection caused by drug resistant *E. coli* 1614. This study was primarily based on two stages: preconjugation studies including FTIR analysis. There nine different conjugates of gentamicin loaded graphene have been developed and further solid state characterization showed no significant interaction of the drug with the chosen nano-carrier used in conjugate that would lead to cumulative antibacterial activity of drug and carrier. In second stage was to looking for LD_{50} determination through colony count method. LD_{50} determination study concluded that, gentamicin loaded graphene is an excellent antibacterial agent against *E. coli* 1614 with a LD_{50} of as low as 9.4 µg/ml.

Keywords: Novel nano composite, 2D nano carbon, gentamicin, preconjugation, LD_{50}

1. Introduction
Its been seventy long years of antibiotics are being used to treat bacterial infections [1, 2, 3]. In initial years this system received a huge success in reduction of mortality [4]. Nowadays due to overuse of antibiotics, natural biota of bacteria like: soli, human body [5], so that of animals are having antibiotics present throughout the time, which keeps on the selection pressure on bacteria [6], and eventually makes the bacteria resistant to that antibiotic [7]. On these resistant strains of bacteria treatment becomes difficult, costly, with addition of prolonged hospital stay and increased rate of mortality [8].
In the recent years the nano-composite based drugs has proved its potential, against microbial infections [9, 10]. Moreover the synergistic effects has been observed with low dimentional carbon based nano formulations [11], for the treatment of the bacterial infection [12]. In addition single walled nanotubes: (SWNTs) diameter close to one nanometer, when conceptualized by wrapping a one atom thick layer of graphite: graphene has proved its potential as an efficient carrier for drug delivery due to their high pay load, and site specific drug delivery [13].
Combination of different antibiotic therapy, conjugated with efficient nano carrier could result better in antibiotic resistance, so bacterial protein synthesis inhibitor: gentamicin was chosen as our drug of choice, which would work with an active nanocarrier [14], while loaded with graphene would improve the pharmacokinetics of therapeutic agents to intended site [15], with addition of lower the dose required for efficacy.

2. Materials and Methods
2.1 Materials
Gentamicin sulphate was brought Yarrow Chem Products, Mumbai, India. Graphene was purchased from Reinste Nano Ventures Pvt. Ltd., India. Sodium Bicarbonate, Phosphate Disodium Hydrogen, Phosphate Methylene Chloride, Calcium Chloride, Dehydrate Magnesium Chloride were purchased from S.D. Fine Chemicals, India. Bacterial culture media: nutrient broth and nutrient agar were procured from Hi- Media Pvt. Ltd., India. Escherichia coli 1614 was obtained from Institute of Microbial Technology (IMTECH), Chandigarh.

2.2 Methods

2.2.1 Preconjugation studies of gentamicin sulfate

Preformulation studies could be described as authenticating the drug by the determination of its physical and chemical properties, which are considered as prime factor in the conjugation of a stable, effective and safe nano composite [16].

Identification

The identification of gentamicin sulfates was carried out by different methods like appearance, odor, taste, followed by melting point determination along with chemical test for identification [17].

2.2.2 Conjugation of gentamicin sulphate to graphene nanosheets

Gentamicin sulphate was loaded onto graphene through simple physico absorption: sonication. In beginning graphene suspension (0.145 mg/ml) was sonicated with gentamicin (1mg/ml) at pH 7 for 30 min. Then it was kept overnight for stirring in dark at room temperature. 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 [20].

2.2.3 Characterization of gentamicin loaded graphene: Solid state characterization

Fourier transforms infrared spectroscopy (FTIR)

Perkin Elmer FTIR Spectrophotometer (Model No. 91151), with Perkin Elmer Spectrum 2 software were used to obtain FT-IR transmission spectra of gentamicin loaded graphene nanocomposite. There 2% of nano composite was mixed with potassium bromide (KBr, Panreac) in w/w ratio and discs were made only after grounding this mixture in fine powder. 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 sample [18, 19, 21].

2.2.4 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 [22].

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

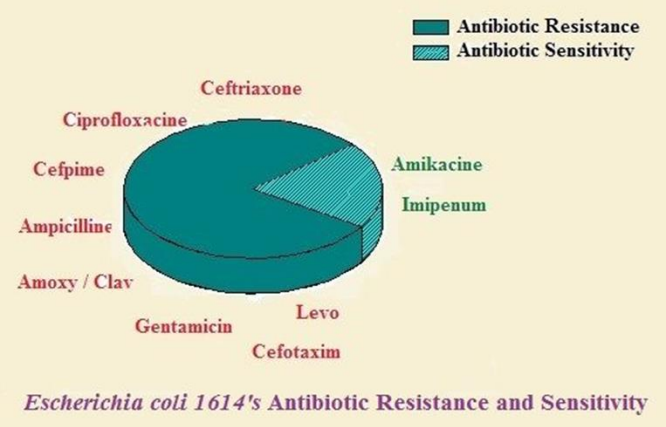


Fig 1: Showing list of drugs *E. coli* 1614 resistant and sensitive of.

2.2.4.1 Determination of working concentration for colony count assay

To start with colony count assay, firstly inoculum was done in nutrient broth and allowed it for overnight incubation [23]. From there 1ml of inoculate was taken and marked it as stock. And serial dilution was done, from starting with stock to 10^{-7} concentration [24]. After that 100µl of each concentration starting from stock, were spreaded over nutrient agar plate through spreader. Then those plates were kept for incubation at 37 ºC overnight. We have triplicated this experiment to reach precision [25].

Table 1: Shows results and observations in tabular form when *E. coli* 1614 stock was serially diluted upto 10^{-7} concentration.

Serial number	Concentration	Observation	Remarks (Figure 2.)
1	1	Too numerous to count	Clear lawn
2	10^{-1}	Too numerous to count	Lawn, with little gap between
3	10^{-2}	Too numerous to count	Lawn, with single colony
4	10^{-3}	465	More than the range to be studied for colony count assay
5	10^{-4}	378.33	Selected for further studies
6	10^{-5}	122	Too less to be studied for colony count assay
7	10^{-6}	46	
8	10^{-7}	7	

2.2.4.2 Colony count assay: determination of LD_{50}

In this assay, from 10^{-4} concentration of *E .coli* 1614 cell were evenly spreaded over nutrient agar plate with different doses of gentamicin loaded graphene oninments [26]. There quite a huge range of doses were covered - strating from zero (0) 15 µg/ml of dose to determine the LD_{50} [27].

3. Results and Discussion

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

3.1 Preconjugation studies

The preconjugation studies for the drug gentamicin sulphate had been performed to assure safety, stability and effectiveness of the nano conjugation. Apart from the preconjugation parameters studies (Table 1), the interactions of the gentamicin and graphene had been investigated.

The parameters investigated during preconjugation studies of drug gentamicin sulphate

Table 2: Showing preconjugation study of gentamicin sulphate.

S. No	Preformulation parameters	Gentamicin sulphate
1.	Physical appearance	White amorphous powder
2.	Odour	Odourless

3.	Taste	Bitter
4.	Melting point	221°C
5.	Solubility	Completely soluble in water and PBS pH 7.4, insoluble in acetone and ethanol
6.	Chemical test for identification	Passed (on heating with Ninhydrin for 4 min colour of the drug changes to violet)

The chosen model drug gentamicin was identified in laboratory by observing its physical appearance, melting point determination etc which were also seen to be concordant with the reported appearance.

3.3 Conjugation of gentamicin sulphate to graphene nanosheets

At lower pH (acidic environment), due to presence of free H^+, ($–NH_2$) of gentamicin forms ($–NH_3^+$) with (H^+) and therefore cannot participate in hydrogen bonding. In the other hand, at higher pH (basic condition) -OH ions are numerous, (–COOH) of graphene oxide exists as ($–COO^-$) and cannot form hydrogen bonds with any group of gentamicin. That is why pH 7, was selected for loading of gentamicin on graphene, as the loading is primarily dependent on efficiently the environment is forming hydrogen bonds between the (–OH) and (–COOH) groups of graphene oxide and the (–OH) and ($–NH_2$) groups of gentamicin [20].

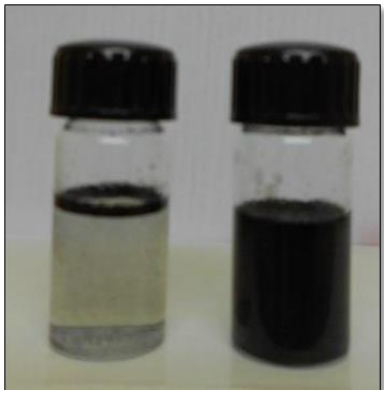
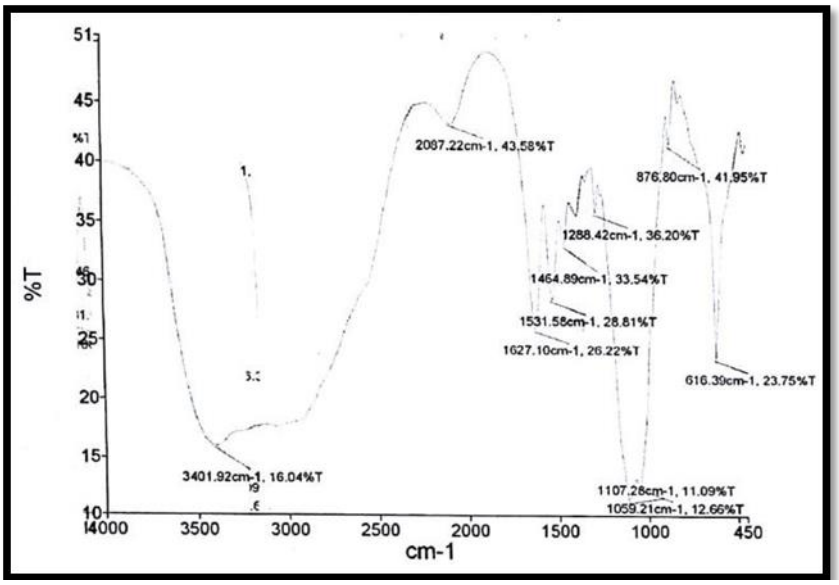


Fig 2: Digital photograph of Graphene nanosheets, not dispersed in water (left) and dispersed in water after drug conjugation (right) [20].

3.4 Solid state characterization of gentamicin loaded graphene nano composite: Fourier Transforms Infrared Spectroscopy (FTIR)

Gentamicin, graphene and gentamicin loaded graphene nanocomposites were analyzed for characteristic transmission bands, indicative of their interaction (Fig. 1). Perkin Elmer Spectrum 2 software reveals 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.

Fourier transform infrared spectroscopy of gentamicin loaded graphene nanoparticles



List of Peak Area/Height		
Peak Name	X (cm-1)	Y (%T)
1	3401.92	16.04
2	2087.22	43.58
3	1627.1	26.22
4	1531.58	28.81
5	1464.89	33.54
6	1288.42	36.2
7	1107.28	11.09
8	1059.21	12.66
9	876.8	41.95
10	616.39	23.75

Fig 3: FTIR spectra of gentamicin loaded graphene nanoparticles

3.5 Antibacterial activities

3.5.1 Determination of working concentration for colony count assay

Next was to work on colony count assay to determine the LD_{50} of gentamicin loaded graphene nano composite.

To understand which concentration of *E. coli* 1614 would be used for determining LD_{50}, serial dilution of stock to 10^{-7} concentration were done, and number of colonies formed after overnight incubation at 37 ºC are as follows:

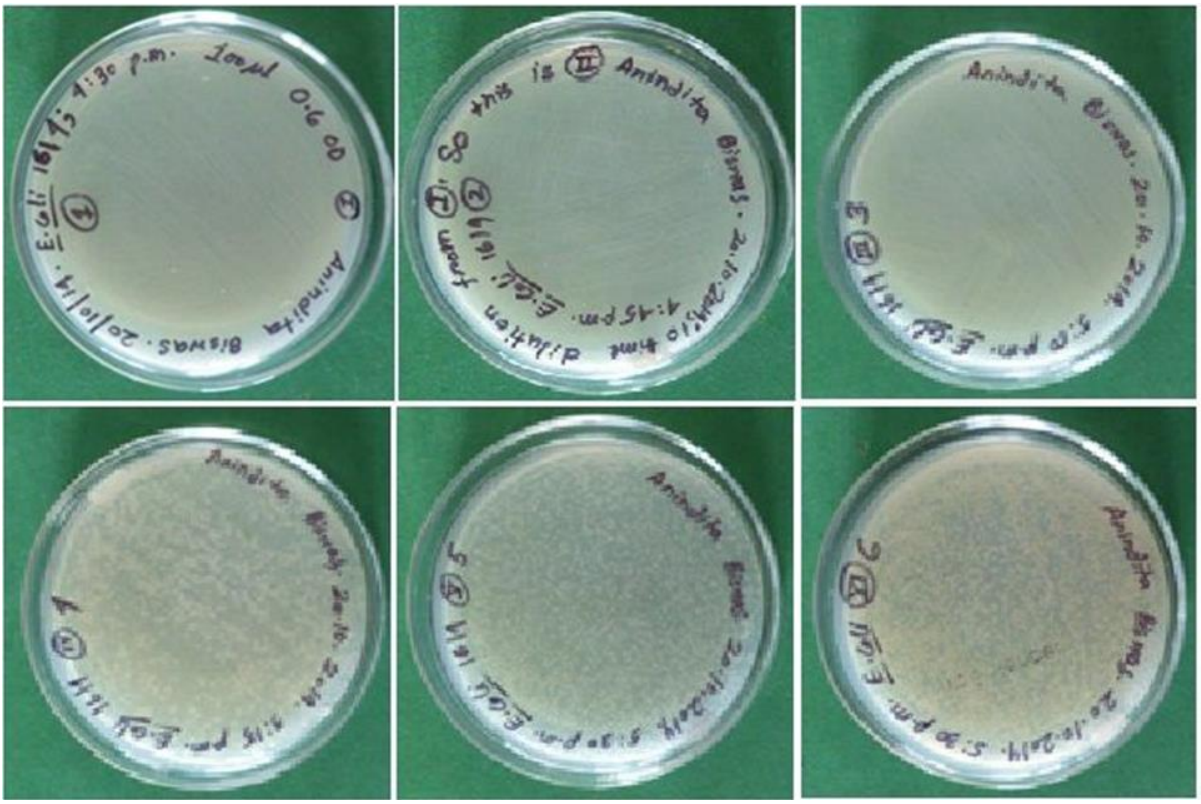


Fig 4: Shows formation of single colony from lawn as stock is serially diluted. Here stock is mentioned as 1 or I; 2 or II, 3 or III, 4 or IV, 5 or V, 6 or VI corresponds 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-6} respecively.

As per above study 10^{-4} concentration was selected to be worked on for further studies. There 100µl of inoculums had produces 378.3 colonies, the dose of gentamicin loaded graphene would be count as LD_{50}, where half of 378.3 colonies, i.e. 189.2 colonies would be produced. With this objective we went for our next set of experiments were planned.

3.5.2 Colony count assay: determination of LD_{50}

There 100µl of inoculum from 10^{-4} concentration of *E. coli* 1614 cell was spreaded over nutrient agar plate with different doses of gentamicin loaded graphene oninment. There quite a huge range of doses- strating from zero (0) to 15 µg/ml of doses range was covered. And it was observed that 7.5 µg/ml of gentamicin loaded graphene nano composite was producing 212.7 with colonies on plates after overnight incubation at 37 ºC. And this number was reduces to 182.3, when 10 µg/ml gentamicin loaded graphene nano composite was applied. Then it was clear that the LD_{50} of gentamicin loaded graphene nano composite lies between these two doses. Similar experiments were done with same doses of base, gentamicin, graphene nano composite in same environment to see their effect on *E. coli* 1614 (Fig. 3). Experiments were done in triplication to reach precision.

Once colony count assay was finished, it has been decided to analyze results using 'Origin Pro 8' software. Colony count assay's result are presented in graphical form, which were obtained via above mentioned software.

Fig. 3 Showing the number of *E. coli* 1614 colony and their standard deviation observed on nutrient agar plates where the doses of choice were 1 µg/ml, 2 µg/ml, 3 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml and 15 µg/ml of graphene nano composite.

Colony count assay using all four treatments using different their doses

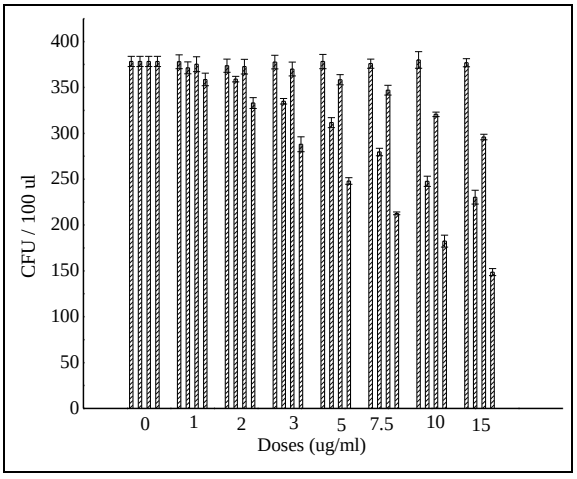


Fig 5: Showing number of *E. coli* 1614 colony and their standard deviation observed on nutrient agar plates when water, gentamicin, graphene and gentamicin loaded graphene nano composite are used. In each segment, first bar presents result of water, followed by gentamicin, graphene and gentamicin loaded graphene nano composite 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.

In colony count assay (Fig. 3), had showed that there was no significant change in number of *E. coli* 1614 colonies while negative control-water is used in different concentration on nutrient agar plate. There it could be seen that number of *E. coli* 1614 colonies hang between 373 to 380, while doses were used in range of zero (0) to 15 µg/ml. So it was proved that, the antibacterial activity observed in gentamicin, graphene and gentamicin loaded graphene nano composite should be measured as antibacterial activity of gentamicin, graphene and gentamicin loaded graphene respectively.

In Fig. 3 it could be seen that gentamicin nano composite reduce the number of *E. coli* 1614 colonies up to 230.3 with SD of 7.6, in a dose of 15 µg/ml, where the number of colonies found in 1 µg/ml dose was 371.3 with SD of 6.5.

There was not much reduction of colony numbers till 3 µg/ml of dose (Fig. 3). Significant reduction of colony number starts only when 5 µg/ml dose was used. It was found that 358.3 with SD of 5.5 *E. coli* 1614 colony, which reduced up to 296 with SD of 3 while maximum dose i.e. 15 µg/ml was used.

Some extraordinary results were obtained in case of gentamicin loaded graphene nano conjugate. The number of *E. coli* 1614 colony was as much as 378.3 with SD of 5.5 in zero (0) mg dose (Fig. 3). So as it had been discussed in previous section the study was to see 189.2 *E. coli* 1614 colony forming dose. That would be LD_{50} of gentamicin loaded graphene nano composite. In Fig. 3 it could be seen that number of *E. coli* 1614 colony reduced to 182.3 with SD of 6.5 while 10 µg/ml dose was used, whereas it was found 212.3 colonies with SD of 1.5, when 7.5 µg/ml of gentamicin loaded graphene was in action (Fig. 4). So it is clear that the LD_{50} of gentamicin loaded graphene nano composite it is between the above mentioned two doses. Using 'origin pro 8' software it has been measured as LD_{50} of gentamicin loaded graphene nano composite as low as 9.4 µg/ml.

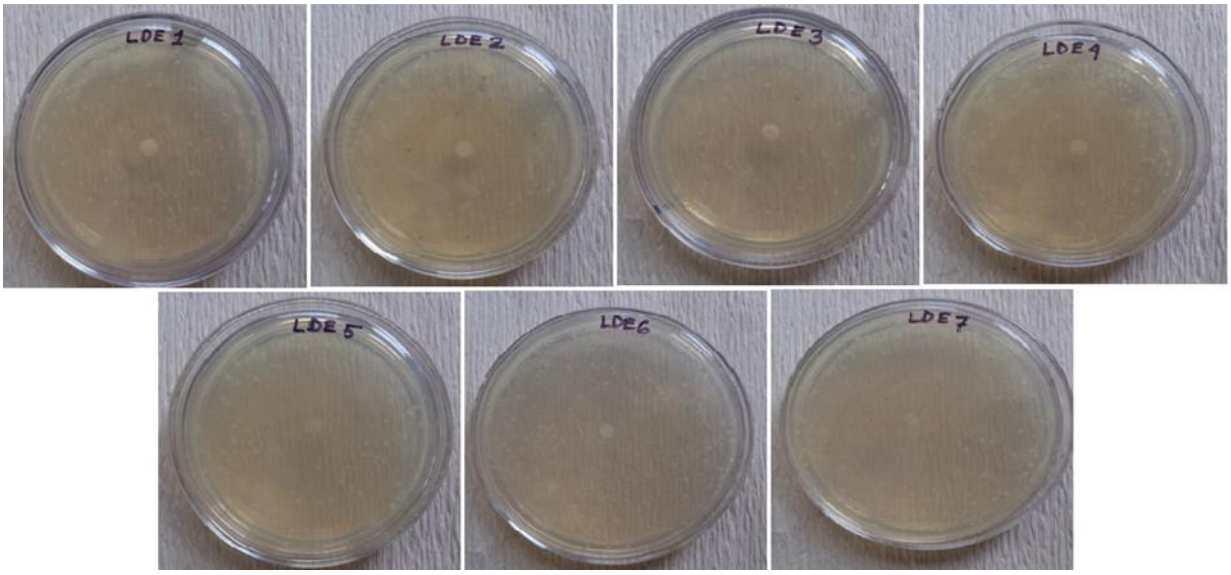


Fig 6: Depicts *E. coli* 1614 colony on nutrient agar plate, when gentamicin loaded graphene ointment is used in different concentrations. Here 1 = 1 µg/ml, 2 = 2 µg/ml, 3 = 3 µg/ml, 4 = 5 µg/ml, 5 = 7.5 µg/ml, 6 = 10 µg/ml, 7 = 15 µg/ml of gentamicin loaded graphene ointment is used on respective plate.

With these it could be concluded that, gentamicin loaded graphene is an excellent antibacterial agent against *E. coli* 1614 with a LD_{50} of 9.4 µg/ml. This shows more antibacterial activity than the total of gentamicin and graphene ointment separately. So these could be counted as one of the promising agent for infections caused by this above mentioned fatal bacterium.

4. Conclusions

In this study a novel nano composite: gentamicin loaded graphene nano ointment was prepared to combat against infections caused by multi drug resistant *E. coli* 1614. This study was primarily based on two stages: preconjugation studies, followed by solid state characterization of the nano conjugate, second portion were to looking for antibacterial activities progressed with LD_{50} determination.

In first phase of work, during preformulation studies drug of choice gentamicin and nano carrier graphene were authenticated successfully. Nano conjugation was carried out by simple physic absorption method with sonication. There FTIR analysis of nano composite had confirmed no interactions between its components: gentamicin and graphene.

LD_{50} determination study concluded that, gentamicin loaded graphene is an excellent antibacterial agent against *E. coli* 1614 with a LD_{50} of 9.4 mg. This shows more antibacterial activity than the total of gentamicin and graphene nano composite separately.

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