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*International Journal of Current Research in Life Sciences*
*Vol. 07, No. 06, pp.2168-2172, June, 2018*

**ISSN: 2319-9490**

# RESEARCH ARTICLE

## FABRICATION OF GRAPHENE OXIDE USING PHYCOCYANIN - A NOVEL APPROACH FOR ANTIBACTERIAL AND ANTICANCER STUDIES

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Received 17th April, 2018; Accepted 25th May, 2018; Published 12th June, 2018

## ABSTRACT

Graphene is an allotrope of carbon consisting of a single layer of carbon atom arranged in an hexaGraphene oxidenal lattice. Graphene on conversion to Graphene oxide (GRAPHENE OXIDE) exhibits excellent physiochemical properties. GRAPHENE OXIDE can be prepared from the natural Graphite powder using chemical method, here GRAPHENE OXIDE was prepared using modified hummers method due its cost effectiveness. GRAPHENE OXIDE is rich with functional groups such as carboxyl (C=O), hydroxyl (-OH) and epoxide (Bridging Oxygen atom). The silver nanoparticles (AgNps) are synthesized from *Catharanthus roseus* and was incorporated into the prepared GRAPHENE OXIDE by incubation method and further loaded with phycocyanin. Phycocyanin is a light harvesting pigment isolated from *Spirulina platensis* and possess blue color which have a characteristic absorbance at visible light. The nanocomposite was further characterized using physiochemical methods such as UV-Vis Spectrophotometer, Fourier Transform Infra-Red (FT-IR) spectroscopy, X-ray powder diffraction (XRD) method and Scanning Electron Microscopy (SEM) study. From UV-Vis Spectroscopy study we observe that the peaks at 232nm(C=C) and a shoulder at 310nm(C=O) confirms the formation of GRAPHENE OXIDE. The GRAPHENE OXIDE was further confirmed by the FT-IR peak for carboxyl (C=O) at 1735cm$^{-1}$. The XRD spectra of GRAPHENE OXIDE shows a sharp diffraction peak at 2θ=11.95˚ with an interlayer distance of 0.77nm which confirms the crystalline form of GRAPHENE OXIDE. The formation of phycocyanin coated GRAPHENE OXIDE-AgNps nanocomposite was confirmed by the presence of peaks at 232nm (GRAPHENE OXIDE), 420nm (AgNps) and 620nm (Phycocyanin) measured using UV-Vis Spectrophotometer. Thus prepared nanocomposite material was evaluated for its antibacterial and anticancer studies.

***Key words:*** Graphene oxide, Silver nanoparticles, Phycocyanin, Antibacterial, Anticancer activity.



**Citation: Bernath Anusiya, J. and Chamundeeswari, M. 2018.** "Fabrication of graphene oxide using phycocyanin - A novel approach for antibacterial and anticancer studies" *International Journal of Current Research in Life Sciences*, 7, (06), 2168-2172.

## INTRODUCTION

Nanotechnology has been taking outstanding steps in all the scientific background that focuses mainly on carrying the solution for environmental and medical problems. Nanotechnology provides a different understanding when a nanoparticle is compared to their bulk counterparts (Balandin *et al.,* 2008).Carbon nanostructures have been extensively studied due to their properties and numerous applications. Single-layer transferable graphene nano-sheets were first obtained by both mechanical and chemical methods. Graphite on oxidation using strong oxidizing agents produce graphene oxide (Graphene Oxide) (Lerf *et al.,* 1998). In 1958, Hummers reported a method for the synthesis of GRAPHENE OXIDE by using $KMnO_4$ and $NaNO_3$ in concentrated $H_2SO_4$ (Hummers *et al.,* 1958). GRAPHENE OXIDE was prepared by this method in the present study and the AgNps was synthesised using biological methods by using plant extract such as *Catharanthus roseus* and then incorporated into the Graphene oxide.

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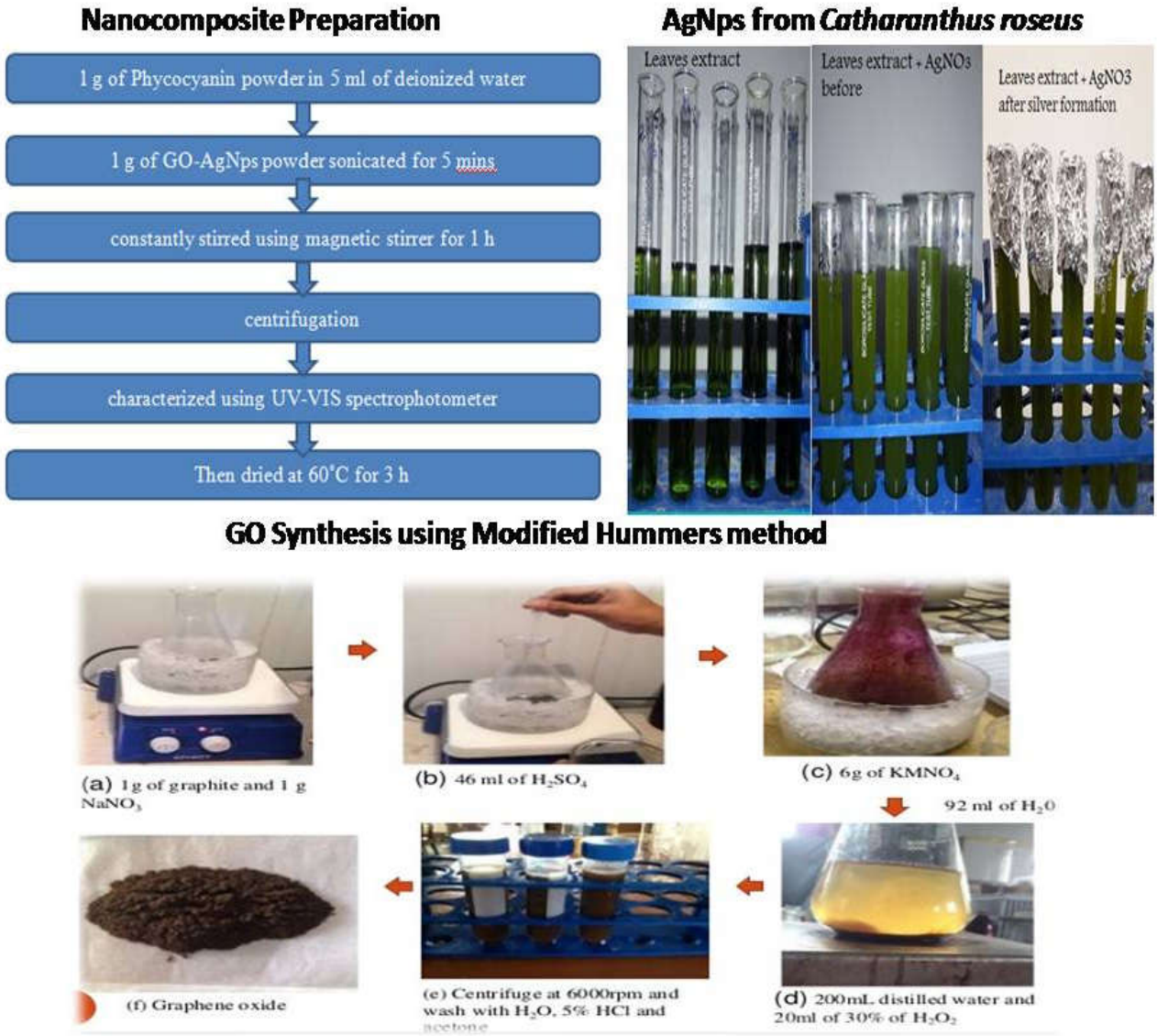
The Graphene oxide - AgNps was finally made to form a nanocomposite using phycocyanin isolated from *Spirulina platensis* (Shrivastava *et al.,* 2007). It is phycocyanin that gives many cyanobacteria their bluish colour and cyanobacteria are also known as blue-green algae. Phycocyanin is hydrophilic, strongly fluorescent and has antioxidant properties. Phycocyanin and related phycobiliproteins are manipulating in a number of applications in foods, cosmetics and medicine (Sekar *et al.,* 2008). It has also been observed that it can inhibit other enzyme activities and affects cell line (Madhyastha *et al.,* 2006). In our present study, a novel approach for antibacterial and anticancer studies were carried out by using light as a observing source for GRAPHENE OXIDE, AgNps and phycocyanin prepared as a nanocomposites by adopting a simple, ecofriendly and cost effective method shown below (Scheme 1).

## MATERIALS AND METHODS

**Materials:**Graphite (Gt) powder, Sodium hydroxide (NaOH), Potassium permanganate ($KMnO_4$) , Sodium nitrate ($NaNO_3$), Sulfuric acid ($H_2SO_4$), Hydrochloric acid (HCl) ,




Hydrogen peroxide ($H_2O_2$), Silver nitrate ($AgNO_3$) and deionized water was purchased from Sigma Aldrich, Chennai. *Catharanthus roseus* plant collected from our campus and Phycocyanin collected from Shibin exports, Chennai.

**Synthesis of Graphene Oxide (GRAPHENE OXIDE):** GRAPHENE OXIDE was synthesized from Gt powder using modified Hummers method. 1 g of Gt and 1 g of $NaNO_3$ were put into a 250 ml conical flask maintained at 0° C. The conc. $H_2SO_4$ of 50ml was added drop by drop into the flask containing the Gt and $NaNO_3$ under stirring at 5° C. The mixture was stirred continuously for 2 h and meanwhile 7.3g of $KMnO_4$ was added in small proportion to prevent the rapid temperature rise (<10° C). The temperature of the reaction was lifted to 35° C and was stirred for 2 h. After completion of the reaction, 90ml of deionized water was poured into the solution under viGraphene oxiderous stirring and dark brown suspension was obtained. The suspension was treated by adding the mixture of 30% $H_2O_2$ of 7 ml and water of 55ml to convert the residual permanganate and $MnO_2$ into soluble $MnSO_4$. The resulting suspension was in bright yellowish brown color. The GRAPHENE OXIDE powder was separated from the mixture by filtration. The GRAPHENE OXIDE powder was washed thrice with 3% dilute HCl and dried finally at 40° C for 4 h. The dried GRAPHENE OXIDE was then collected and characterized (Syed Nasimul Alam *et al.,* 2017).

**Incorporation of AgNps Into Graphene oxide**

To prepare a colloidal suspension, 12.5 mg of GRAPHENE OXIDE were dispersed in 20 mL of deionized water followed by sonication in an ultrasonic bath for 30 min. 8.2 mg of $AgNO_3$ was dissolved in 20 mL of deionized water and then mixed to the GRAPHENE OXIDE suspension. The mixture was sonicated for more 30 min and transferred to a volumetric flask. The silver nanoparticles were produced by modified Turkevich method using the plant extract from the leaves of *Catharanthus roseus* as stabilizing agent. The dispersion containing GRAPHENE OXIDE and $AgNO_3$ was heated in a reflux system and as soon as it began to boil, then 10 mL of a 1mM sodium citrate solution was added dropwise to the refluxed dispersion. The reaction was maintained for 30 min at the temperature of 130°C. The color of the solution slowly turned black-green, indicating the formation of the nanoparticle. The GRAPHENE OXIDE-Ag nanoparticle was centrifuged at 50,000 rpm for 2 h using an ultracentrifuge. The GRAPHENE OXIDE-Ag nanoparticle was washed three times, re-suspended in deionized water and dried by lyophilization.

**Preparation of nanocomposite**

1 g of Phycocyanin powder was dissolved in 5 ml of deionized water and it is stirred in a magnetic stirrer for some times to

gain complete solubilization. 1 g of GRAPHENE OXIDE-AgNps powder was suspended in 10 ml deionized water and sonicated for uniform distribution for 10 min. Both the solutions are constantly stirred using magnetic stirrer for 1 h. The solution was centrifuged at 5000 rpm for 20 min. After centrifugation, two layers were observed on the centrifuge tube. The above one is the supernatant and the bottom settled one is the pellet. Both the supernatant and pellet was characterized using UV-VIS spectrophotometer. Then the above suspension was dried at 60˚C for 3 h. The fine powdered nanocomposite was observed after drying.

**Characterization of nanocomposite**

The synthesized GRAPHENE OXIDE was sonicated for fine dispersion and characterized using UV-VIS spectroscopy (SYSTRONICS double beam UV-VIS spectrophotometer: 2202, B.W2.0nm, Scan mode: 200-800nm).The dried powders of GRAPHENE OXIDE and GRAPHENE OXIDE-AgNps were analysed using Fourier transform infrared (FTIR) spectroscopy (JASCO INTERNATIONAL CO. JAPAN FOURIER TRANSFORM INFRARED SPECTROMETER MODEL FTIR-6300, Crystal growth center, Anna university, Chennai) within a range of 500-4000 $cm^{-1}$ and X-ray diffraction (XRD) analyses were performed in PANalyticalX' Pert Powder XRD, Crystal Growth Center, Anna University, Chennai. The X-ray source was 3 kW with a Cu target; high-resolution XRD patterns were measured using a scintillation counter (λ=1.5406 Å). The XRD was run at 40 kV and 40 mA, and samples were recorded at 2θ values between 10° and 80°. Carl Zeiss MA15/ EVO 18 Scanning Electron microsope of resolution 3.0 nm at 30kV and SE detector was used to acquire SEM images. The solid samples were transferred to a carbon tape held in an SEM sample holder, and the analyses were performed at an average working distance of 6 mm.

**Optimization studies**

The concentration of nanocomposite was optimized using antimicrobial activity. The antimicrobial activity was done using both Broth dilution assay and Agar well diffusion method.

**Broth dilution assay for optimization of nanocomposite concentration**

After optimizing the concentration as 100mg/mL, it is further used for the antibacterial studies.12g of LB Broth was dissolved in 250 mL of distilled water and sterilized. The sterilized broth was poured into 100ml conical flask. About 1% *E.coli* culture was inoculated into the media and the different concentration of nanocomposite were added. The antibacterial study was observed in the LB broth medium. The various concentrations such as 10, 25, 50, 75 and 100 µg of phycocyanin loaded GRAPHENE OXIDE-AgNps was added to the medium and kept in the sunlight, LED and dark for 3 h and then incubated at 37˚C for 48 h.

**Agar well diffusion method for optimization of nanocomposite concentration**

The 4g of LB agar was dissolved in distilled water and sterilized at 121˚C for 15 min. Then the sterilized medium was poured in the petri plates. The solidified LB agar plates were swabbed with 100µL of *E.coli*. The well was prepared on the LB agar plate with the help of cork borer (10mm diameter). 100µL (100 µg) of phycocyanin loaded GRAPHENE OXIDE-AgNps (sample) and 100µL(100 µg) of ampicillin (positive control) was added to the well. Then plates were incubated overnight at 37℃ after exposed into sunlight for 30 min.

**Cytotoxicity assay on cancer cell lines**

MCF 7 cells obtained from NCCS (National Centre For Cell Science, Pune) were cultured in Rose well Park Memorial Institute medium (RPMI)**.** It is supplemented with 10% fetal bovine serum, penicillin/streptomycin (250 U/mL), Gentamycin (100µg/mL), and Amphotericin B (1mg/mL) were obtained from Sigma Chemicals, MO, USA. All cell cultures were maintained at 37◦C in a humidified atmosphere of 5% $CO_2$. Cells were allowed to grow to a confluency over 24 hr before use.

**Cell growth inhibition studies by MTT assay**

Cell viability was measured with the conventional MTT reduction assay, as described previously with slight modification. Briefly, MCF 7 cells were seeded at a density of $5\times10^3$ cells/well in 96-well plates for 24 hr, in 200ul of RPMI with 10% FBS. Then culture supernatant was removed and RPMI containing various concentrations of test samples was added and incubated for 48 hr. After treatment, cells were incubated with MTT (10µl, 5mg/mL) at 37 ˚C for 4 hr and then with DMSO at room temperature for 1 hr. The plates were read at 595nm on a scanning multi-well spectrophotometer.
Cell viability (%) = (Average test OD/Control OD) x 100.

## RESULTS AND DISCUSSION

**Synthesis of Graphene oxide and UV- visible spectroscopy analysis of Graphene oxide**

The Gt (black color) was converted to GRAPHENE OXIDE (yellowish brown color) which indicates the conversion of Gt to GRAPHENE OXIDE in the presence of strong oxidant such as$KMnO_4$ (Fig 1a).

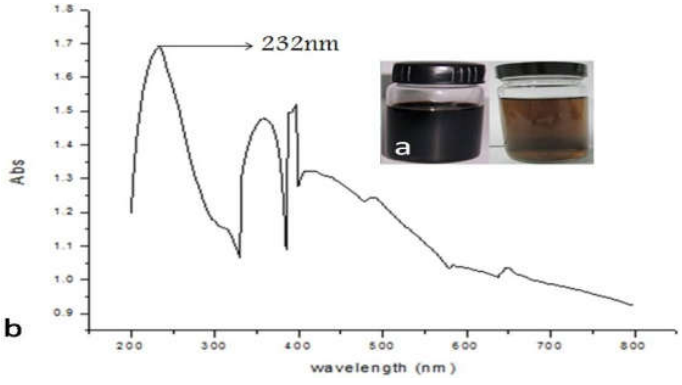



**Fig. 1a. indicates the Synthesis of GRAPHENE OXIDE and Fig. 1b. indicates the UV- visible Spectroscopy analysis of GRAPHENE OXIDE**

The organic molecules are generally known to have maximum absorption peak between 200-700 nm (Systronics Double beam UV-VIS Spectrophotometer: 2202). In fig 1b, the peak observed at 232 nm indicates the presence of aromatic C=C ($\pi$-$\pi^*$ transition) and a shoulder peak at 310 nm represents the presence of C=O (n-$\pi^*$ transition) bonds respectively which also confirms the formation of GRAPHENE OXIDE from Gt.

### FT- IR analysis of Graphene oxide

The FT- IR (Fig 2a) peaks at 1630 $cm^{-1}$, 1735 $cm^{-1}$ and 740 $cm^{-1}$ represents the stretching vibration of carboxylic acid (C=C) and carbonyl groups (C=O) present at the edges of GRAPHENE OXIDE. The absorption peaks at 1385 $cm^{-1}$ and 1110 $cm^{-1}$ corresponds to the stretching vibration of C-O of carboxylic acid and C-OH of alcohol, respectively. The absorption peaks at 2930 $cm^{-1}$ and 2850 $cm^{-1}$also represents the symmetric and anti-symmetric stretching vibrations of -$CH_2$ -. From fig 2b the presence of these oxygen-containing groups reveals that the Gt has been oxidized and hydrophilic in nature. FT-IR spectrum of the GRAPHENE OXIDE–AgNps, the peaks at 1,270, 1,320, 1,720, and 3,430 $cm^{-1}$are relatively weak compared to those of GRAPHENE OXIDE. Furthermore, the absorption peak at approximately 1,270 $cm^{-1}$is attributed to the C–OH bond, and the new absorption band at 1,720 $cm^{-1}$is attributed to the skeletal vibration of the graphene sheets. These results clearly demonstrate that GRAPHENE OXIDE was successfully bonded graphene and that strong interactions may exist between AgNps and the remaining surface hydroxyl groups.

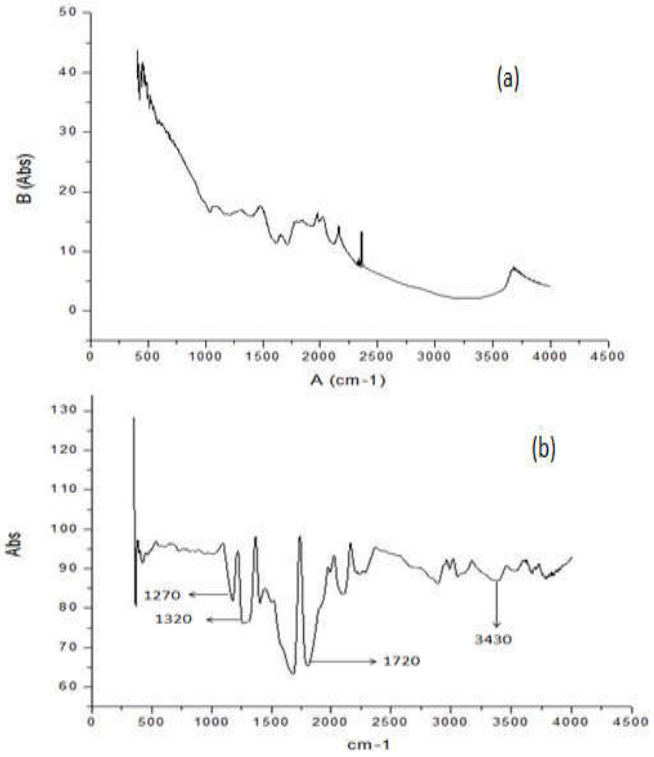



**Fig. 2. FTIR analysis of GRAPHENE OXIDE and GRAPHENE OXIDE-AgNps**

### X-RAY Diffraction (XRD) study of Graphene oxide

For the study, the data obtained by UV-Vis spectroscopy GRAPHENE OXIDE, GRAPHENE OXIDE–Ag were further characterized using XRD. The XRD patterns of GRAPHENE OXIDE is shown in Figure 3, which confirms the crystalline nature of the GRAPHENE OXIDE. GRAPHENE OXIDE exhibits a reflection at a low angle (2θ =11.95˚) compared with the pattern of pristine Gt (26.42˚). The reflection at 2θ =11.95˚ in GRAPHENE OXIDE disappears and a new reflection emerges at 2θ=26.42°, indicating the reduction of GRAPHENE OXIDE.

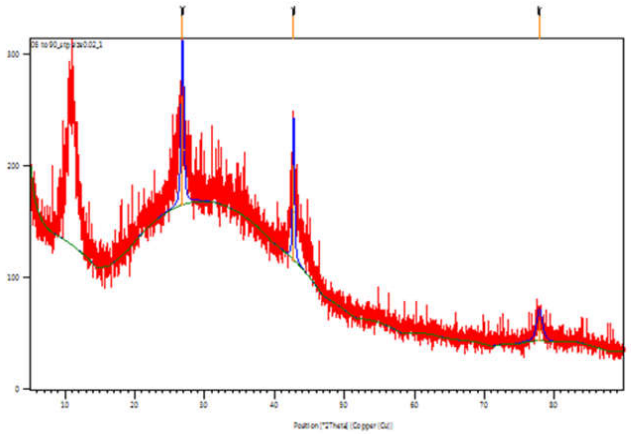


**Fig. 3. X-ray diffraction (XRD) Analysis of GRAPHENE OXIDE**

### Scanning electron microscope (sem) analysis of Graphene oxide

The figure 4a &b shows that the GRAPHENE OXIDE has a two-dimensional sheet-like structure. The films are stacked one above the other and also show wrinkled areas. Lamellar structures having a length of upto 1.29 nm and width of 239 µm could be seen in the SEM images. The individual GRAPHENE OXIDE sheets were found to have a thickness of 1-2 µm and are found to be much larger than the thickness of single layer graphene.

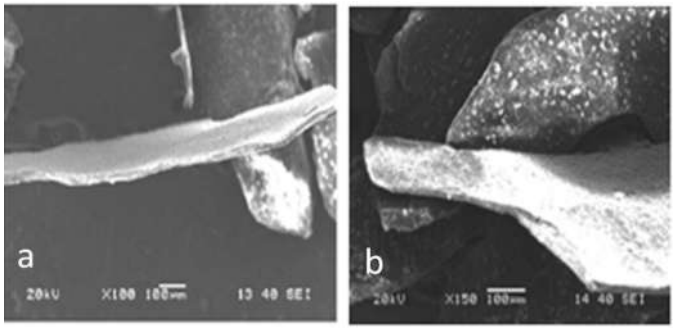



**Fig. 4a & b. Scanning Electron Microscope (SEM) analysis of GRAPHENE OXIDE**

### Optimization of nanocomposite concentration

The concentration of nanocomposite was optimized using antimicrobial activity. The antimicrobial activity was done using both Broth dilution assay and Agar well diffusion method

### Broth dilution assay

From the antibacterial study using LB Broth dilution assay, the final concentration of phycocyanin loaded GRAPHENE OXIDE-AgNps was fixed to 100mg/mL. In both broth dilution assay control shows high turbidity which indicates Graphene oxideod viability rate of the *E.coli* cells when compared to the Amp and nanocomposite treated with 10-50µg (10 -50µl) concentration. A decrease in viability rate indicates a high inhibition which was observed in 75 & 100 µg (75 & 100 µl) of nanocomposites particularly in the samples exposed to sunlight for 3 h and then incubated at 37˚ C for 48 h when compared to LED and dark exposure. This clearly indicates that activity is mainly due to the light absorbing phycocyanin activity.

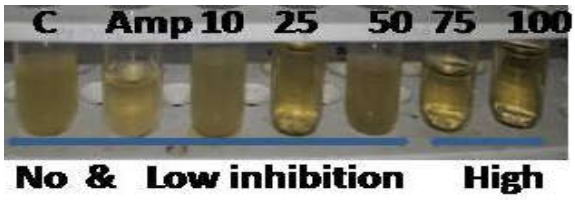



**Fig. 5. Broth dilution assay C - control(Untreated), Amp - Ampicillin and 10-100ul (100mg/ml)**

### Agar well diffusion method

In figure 6 the agar well diffusion method was shown. Amp acts as a positive control and shows a very Graphene oxideod antibacterial activity when compared to nanocomposite. The nanocomposite shows 0.7mm diameter zone of inhibition after exposure to sunlight 30min. From this observation the concentration of phycocyanin loaded GRAPHENE OXIDE-AgNps of 100mg/ml was fixed and the fixed concentration was further used for anticancer activity.


**Fig. 6. Agar well diffusion method Amp- Ampicillin; NC- Nanocomposite**

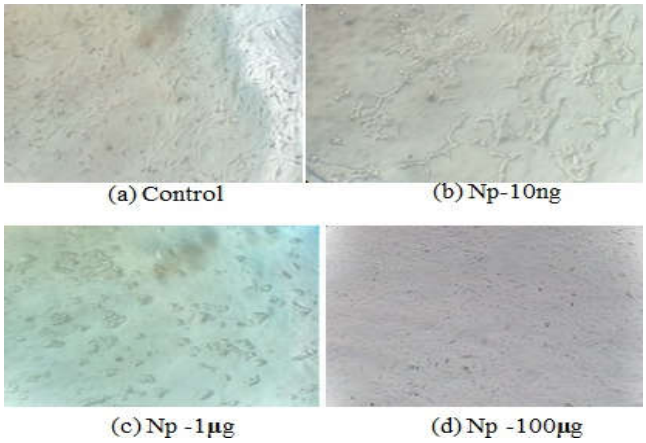



**Fig. 7. Cell growth inhibition studies by MTT assay; a-Control (untreated); b-d (treated with nanocomposite)**

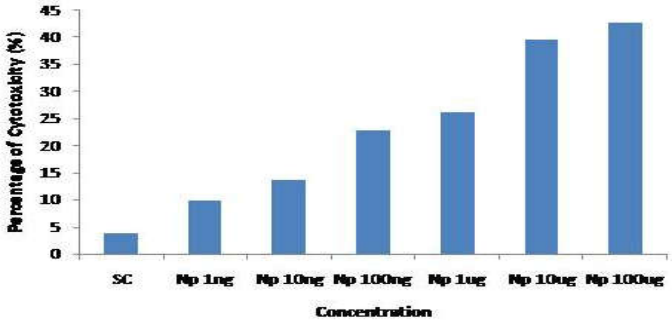



**Fig. 8. Percentage of Cytotoxicity**

### Cell growth inhibition studies by MTT assay

Figure 7 shows the phase contrast microscopy images of both untreated and treated cells. Control shows a Graphene oxideod confluency of the cells when compared to the treated cells of 10ng, 1µg and 100µg respectively. The change in morphology of the cells was well observed in the nanocomposite treated cells and finally the viability rate percentage was indicated below as a supplement data. Figure 8 shows the viabiliy rate i.e., concentation vs percentage of cytotoxicity effect of the prepared nanocomposite. The control - 3.8%, 10 ng - 13.5%, 10µg - 39.4% and 100 - 42.5% of viability respectively. The nanocomposite of 10µg itself shows almost of 40% of cytotoxity which is a positive indication that indicates a less quantity of sample is more than enough to provide higher efficiency. This anticancer activity of the nanocomposite was mainly due to the induced oxidative stress under influence of light observation by the nanocomposite, which may result in cell damage and apoptosis via DNA damage as reported in the earlier studies.

### Conclusion

Graphene oxide (GRAPHENE OXIDE) was profitably prepared by using modified Hummer's method and the nanocomposite was prepared by loading phycocyanin with GRAPHENE OXIDE-AgNps by simple incubation methods. The prepared nanocomposite (75µg) almost showed 40% of antibacterial and anticancer activity under the influence of light when compared to dark and LED light treated samples. Thus the prepared phycocyanin loaded GRAPHENE OXIDE-AgNps nanocomposite is a novel formulation which may aid the development of better antimicrobial and anticancer therapeutics that could provide novel ideas for new treatments for the dreadful diseases such as cancer, in order to provide a targeted photo thermal therapy under the influence of sunlight. In future, we have planned to formulate as a novel product for photothermal therapy with high therapeutic efficacy for the better mint of people who underGraphene oxidees radiation therapy.

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