Indian Journal of Applied Microbiology
ISSN (Online): 2454-289X, ISSN (Print): 2249-8400

Volume 20 Number 2
July - December 2017, pp. 1-10

Microbial Reduction of Nanographene Oxide using Actinobacteria isolated from Mushroom Associated Rhizosphere Soil

K. Kavitha*, G. Priya, G. Banu Priya, R. Balagurunathan* and M. Sangeetha

Actinobacterial Research Laboratory, Department of Microbiology, Periyar University, Periyar Palkalai Nagar, Salem – 636 011, Tamil Nadu, India.

Abstract: Graphene is a captivated requirement in multiplicity in almost all the fields especially, solar, sensor, battery and biomedical applications due to its superior physcio-chemical and biological properties. Natural sources such as plants, micro and macromolecules and micro organisms are used as reducing agents in graphene nanoparticles synthesis to avoid the hazardous of conventional methods. This study focused isolation of actinobacteria from mushroom associated rhizosphere soil and its role in graphene oxide reduction and bioactive compound production from isolated actinobacteria and analyze its potential. The morphological, microscopical, field emission-scanning electron microscopical view and biochemical identification of isolated actinobaterial strain MAA-1 was observed as Gram-positive, grey color aerial mycelium, non-motile in nature with spiral spore chain morphology and belong to *Streptomyces sp*. A compound drived from agar surface fermentation of MAA-1 was subjected on methicillin- resistant *Staphylococcus aureus* and vancomycin resistant *Enterococci* and found diminutive activity. The intracellular content of *Streptomyces sp*. changes the brown colour graphene oxide to black colour graphene under specified condition which confirmed the reduction of oxide. and leads to formation of graphene. A disc diffusion method confirms the superior anti-fungal activity of microbial reduced graphene oxide among the prepared nanoparticles. This is the first report for environmental friendly reduction of graphene oxide using mushroom associated actinobacteria and bioactive compound production to control infections in immune depressive patients. The results suggested that the further optimization, purification, characterization and *in*

*Author for Correspondence. E-mail: kkavitha07@gmail.com, rbalaguru@yahoo.com

vitro analysis of bioactive compound and nano-graphene will be useful for assortment of biomedical and environmental applications.

Keywords: Actinomycetes; *Agaricus bisporus*, Bioactive Compound, Graphene, Natural products; Streptomycetes.

Introduction

Microorganism has ability to produce secondary metabolites, which have found application in combating a variety of human infections [1]. Among the microorganisms, marine bacteria particularly actinomycetes produce unique and novel secondary metabolites and it is useful for different biological activities, such as antifungal, antibacterial, antitumor, anticancer, anti-parasitic and immunosuppressive activities [1,2]. Soil dwelling, actionbacteria are more potent source of novel enzymes and secondary metabolites, including many antibiotics and bioactive compounds than the other microorganism. Nearly 80% of naturally occurring antibiotics have been isolated from different actinomycetes especially from *Streptomycetes*. *Streptomycetes* yielded many therapeutic agents such as *streptomycin*, erythromycin, tetracycline, amphotericin, chloramphenicol, daptomycin fosfomycin, lincomycin, neomycin, puromycin, anticancer drugs exemplified by adriamycin and the immunosuppressant tacrolimus [1,3]. There are more bioactive compounds such as avermectin (*Streptomyces avermitilis*), bleomycin (*Streptomyces verticillus*) and daunomycin (*Streptomyces peuceticus*) as antitumor compounds, Tacrolimus, is an binding protein formerly known as FK506 or fujimycin (*Streptomyces tsukubaensis*) as an immunosuppressant, and validamycin (*Stereptomyces hygroscopicus var. limoneus*) as a treatment of rice sheath blight disease [2,4]. .

Methicillin- resistant *Staphylococcus aureus* (MRSA) is a pathogen responsible for a wide range of infections such as boils, pneumonia, osteomyelitis, endocarditis, bacteremia, etc. and has developed resistance to the majority of conventional antibiotics [5]. Similarly, *Enterococcus* infections are caused mostly by vancomycin resistant *Enterococcus faecalis and E.coli* (VRE). Clinical pathogens such as *Staphylococcus aureus*, *Pseudomonas*, and *Enterobacteriaceae*, *Candida albicans*, *C. glabrata* and *Cryptococcus neoformans* plays vital role in many degenerative diseases [6-9]. Similarly, *Candida* species such as *C. albicans*, *C. glabrata* and *Cryptococcus neoformans* cause arthritis, infections in immune suppressed individuals and infections with bone rupture and cause degenerative diseases [6,8]. Pathogens often transform their genomic alignment against existing antibiotics and loss their sensitivity and became a drug resistant pathogens. The number of drug resistant pathogens are increasing day by day and become serious public health problems [6,8]. In addition, recent years new therapeutic agents have entered in the clinical area, unfortunately with some side effects [9,10].

Compare than the graphene oxide, reduced graphene oxide facilitates incredible properties such as high surface area, bacterial inhibition, enhanced mechanical property, bioactive nature [12]. Especially, nano-graphene possess unique reinforcing behaviour with a large surface area plays a key role in osteoinduction and osteoconduction which promotes cell adherence. Hence, graphene is used for orthopaedic and bone regeneration application vitally. In addition, the cancer cell

targeting and diagnosis through graphene attract us more towards the biomedical application [11, 12]. The most commonly used chemical reducing agents for graphene oxide are anhydrous hydrazine, hydrazine monohydrate, sodium borohydride, and hydroquinone. These reducing agents are highly toxic and harmful. Moreover, hydrazine-reduced graphene tends to agglomerate irreversibly and converts into graphite. Metal/hydrochloric acid reduction is another alternative; however, an impurity formed from the residual metal hinders further applications. Therefore, search towards the environmental friendly, green production of graphene is increased. Especially, microbial mediated syntheses are focused for owing to its simplicity, nill-toxicity, stability, viability, and scalability [13-17].

Hence, researchers are focused on potential microbial secondary metabolites to overcome the side effects of existing drugs and obstruction of drug resistant pathogens and microbial mediated nano-graphene oxide reduction for toxic free biomedical application. Actinomycetes are producers of reducing enzymes, metabolites with antimicrobial, anti-parasite, antiviral, antitumor activity, cytotoxic, etc; whose chemical structures are unique. Especially, S*treptomycetes* has a great potential for new and improved generations of antibiotics for multi-drug resistant pathogens and cancer treatments [7-10]. *Agaricus bisporus* is an edible basidiomycete mushroom have rich nutritional value especially, antioxidants and hydrazine, which are the main key of reduction activity. Mushroom contains hydrazine derivatives, including agaritine, gyromitrin poses no toxicological risk to humans when mushrooms are consumed in typical amount. In addition, mushrooms are well known for their antioxidant, antimicrobial, anti-inflammatory, anti-tumor and anticancer activities [14]. Muthoosamy et al., 2015 reports the graphene oxide reduction using mushroom and used for various technological application such as nano electronice, polymer nanocomposities, drug delivery sytems, biosensing, solar cell transistor devices, etc. [14]. In the present study, the production of bioactive compound and biological reduction of nano-graphene oxide through mushroom associated rhizosphere actinobacteria will be focused. In addition, the antibacterial activity of produced biocompound and nano-graphene tested against clinical pathogens are reported.

2. Materials and Methods

2.1. Sample Collection and Isolation of Actinobacteria

The mushroom samples were collected from the surrounding areas of Dharmapuri district, Tamil Nadu and were brought to the laboratory. Mushroom associated rhizosphere soil was serially diluted from 10^{-2} to 10^{-6} with 1 g of mushroom associated rhizosphere soil. Then the Starch casein plates were prepared (Soluble starch, Casein, KNO_3, $MgSO_4.7H_2O$, K_2HPO_4, NaCl, $CaCO_3$,$FeSO_4.7H_2O$ and agar, pH 7±0.2) and then 0.1 ml of sample from serial dilution was inoculated in starch casein medium using the L-Rod (spread plate technique) and incubated at 28°C for 4 to 7 days. After the incubation period, morphologically different colonies were isolated and sub-cultured in International Streptomycetes Project -2 (ISP2) medium [8] and named as MAA-1 and the subcultures were stored at 4°C.

2.2. Morphological and Taxonomical Identification of Isolated Strain

Based on the results obtained, potential actinobacterial strain was selected for further investigations. Isolated colony was taken from the agar plate and smeared on a clean glass slide for microscopical view and Gram staining [14-16]. In addition to the aerial staining, motility, mycelia formation and spore chain morphology, and taxonomic identification of the strain was carried out based on Nonomura's key and Bergey's Manual [15,16].

2.3. Bioactive Compound Production from Actinomycetes Biomass

The ISP2 broth was prepared and sterilized at 121°C with 15 lbs for 20 minutes. After sterilization, broth was cooled and the loop of isolated actinobacterial (MAA-1) culture was inoculated. Then the broth was placed on the incubator at 28°C for 7 days. After incubation period, biomass is observed in the broth. The broth was centrifuged at 4°C, 5000 rpm, for 20 mins to separate the supernatant MAA-1S [17]. Potential actinobacteria strain was inoculated in Yeast Extract-Malt Extract (YEME) agar medium (20 ml of medium/ plate) with 50% seawater and incubated for 5-7 days at 28°C. After removing the mycelia aseptically from the agar surface, the antibiotic containing agar medium was cut into small pieces and extracted using organic solvent-ethyl acetate for overnight at room temperature. The solvent extract was concentrated under reduced pressure, quantified and named as MAA-1BAC.

2.4. Biomass Production and Intracellular Reduction of Nano-graphene Oxide

The ISP2 broth was prepared and sterilized at 121°C for 15 lbs. After sterilization, broth was cooled and loopful of isolated MAA-1 strain was inoculated in sterile ISP2 broth and incubated in the shaker at 28°C for 7 days. After incubation period biomass are observed in the broth. The broth was centrifuged at 4°C, 5000 rpm, for 20 mins to separate the pellet MAA-1P [17-19]

For the intracellular mediated actinobacterial nano-graphene oxide reduction, nano-graphene oxide was prepared by modified Hummer's method [12]. The phyisco-chemical characterisation of graphene oxide was thoroughly analysied as given by Kavitha *et al.*, 2013 and stored in desiccators for further use. Instead of chemical reducing agent (hydrazine), intracellular content of mushroom associated actionbacteria (*Streptomyces sp*.) was used in this study to reduce the nano-graphene oxide to nano-graphene. For that, 100 mL of prepared MAA-1P along with 2.5mg of nano-graphene oxide was added in sterile conical flask and incubated in shacking incubator at 200 rpm for 7 days at 25°C. After incubation period, the colour change will be observed. The reduction of graphene was monitored by visual inspection of the flask for change in colour of the culture medium [17-19].

2.5. Antimicrobial Activity

Common clinical pathogens such as gram-negative bacteria (vancomycin resistant *Escherichia coli* and drug sensitive *Klebsiella pneumoniae*), gram-positive bacteria; methicillin resistant *Staphylococcus aureus* and drug sensitive *Bacillus subtilis*) and drug sensitive *Candida albicans* and *Cryptococcus neoformans* were collected from the Actinobacterial Research Laboratory (Department of Microbiology, Periyar University, Salem, Tamil Nadu, India) were grown in LB

broth. For the antibacterial activity study, the stock cultures of the above bacteria were subcultured in a nutrient broth and stored for 6-10h at 37°C [7, 8]. The antibacterial activity of the prepared bioactive compound (200µL from 50 mg ml^{-1}concentration of MAA-1C stock) was examined by disc-diffusion method on Muller Hinton Agar (MHA) with freshly grown bacterial smear (24 hrs at 37°C) [7].

Similarly, the extracellular synthesized nanographene was tested against most affecting fungal pathogens such as *Candida albicans* and *Cryptococcus neoformans*. 200µL from 50 mg ml^{-1}concentration of each nanosample was loaded on the disc and incubated on Sabouraud's dextrose agar (SDA) agar for 24-48 hrs at 37°C [12]. After 24-48 hrs of incubation, the inhibition zone around the disc was measured and compared with the control. The antimicrobial assessment for bioactive compound and nano-graphene was performed in triplicate, and the mean value with standard deviations was reported in table 2 [7-9].

3. Results

3.1. Isolation and Identification of Actinobacteria from Mushroom Associated Rhizosphere Soil

Mushroom associated rhizosphere soil actinobacteria were isolated from starch casein plates after 5 days of incubation (Fig. 1). The total numbers of population and CFU values were tabulated in table 1. Isolated colonies were streaked on ISP2 plates and incubated at 28˚C for 7 days. After incubation period, the organisms were grown well and shown similar morphological characters on ISP2 medium confirms the single actinobacteria colony from the mushroom rhizosphere soil and named as MAA-1.

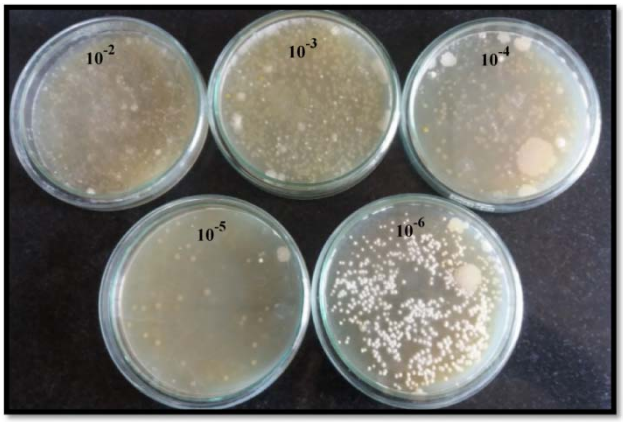
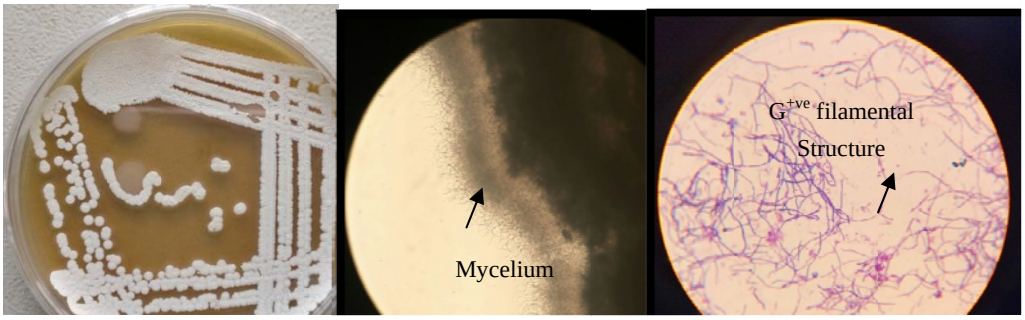


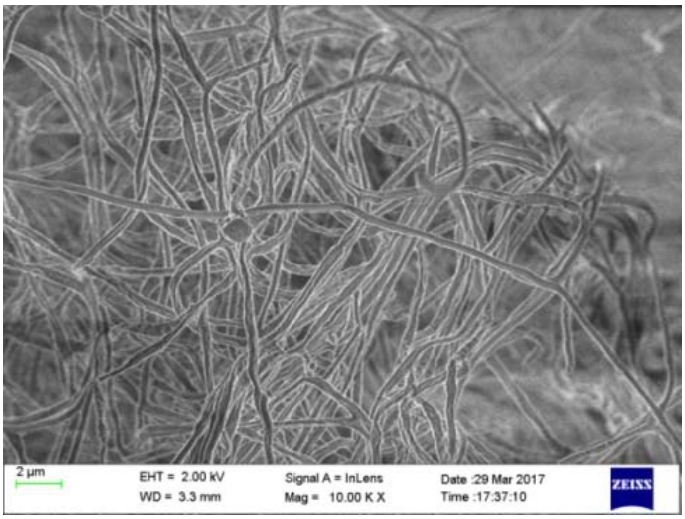
Fig. 1 Isolation of actinobacteria from mushroom associated rhizosphere

Table 1 shows the total number of population such as number of bacterial and actinobacterial colonies and colony forming unit (CFU/ml) which revealed the actinobacteria was grown

gradually while increasing the dilutions rate from 10^{-2} to 10^{-6} (Fig. 1). Moreover, Fig.1 revealed that the increasing dilution from 10^{-2} to 10^{-5} gradually reduces the all the bacterial colonies. This is confirmed that the bacterial growth was high in initial dilutions and suppressed subsequently by the growth of actionobacteria and its domination. The dilution 10^{-2} and 10^{-3} showed mat like formation of bacterial colonies. 10^{-4} is taken for further bacterial studies because it shows individual colonies without any contamination. Total number of population in 10^{-4} is 120 and CFU/ml value is 1.2 X 10^{7}.



a. Quadrant streak Microscopic view Gram Staining



d. FE-SEM

Fig 2. Morphology of MAA-1 (Actinobacteria)

Table: 1 Total microbial population of mushroom associated rhizosphere

S. No	Serial dilution	Number of microbial population			
		Total number of colonies	CFU/ml	Different bacterial colonies	Different actinobacteria
1.	10^{-2}	200	$2.0X10^{4}$	20	0
2.	10^{-3}	150	$1.5X10^{5}$	15	0
3.	10^{-4}	120	$1.2X10^{6}$	10	1
4.	10^{-5}	70	$0.7X10^{7}$	8	1
5.	10^{-6}	300	$3.0X10^{8}$	5	1

The microscopic view (Fig. 2b) of the MAA-1showed well formed mycelia growth, which conformed the isolated organism is actinobacteria. In the Gram staining result (Fig.2c), the purple colour long mycelium supports that the isolated microorganism MAA-1 is actinobacteria. In addition to the above, the FE-SEM image (Fig. 2d) also confirmed the above state. In addition, biochemical analysis such as oxidative and catalase test, and taxonomical identifications are confirmed the isolated strain is *Streptomyces sp.* [15-17].

3.2. Biomass Production and Nano -Graphene Oxide Reduction

The obtained actinobacterial (MAA-1) biomasses in ISP2 broth were centrifuged at 5000 rpm for 30 min to separate the intra and extracellular contents. The obtained pellet from actinobacterial biomass was name as MAA-1P and stored at refrigerator for further analysis. The reduction of graphene oxide was analyzed using above microbial biomass under defined condition. The visual inspection of the flask for change in color from a clear light brown to black colour was observed and confirmed the reduction of graphene oxide (Fig.3) [17-19].

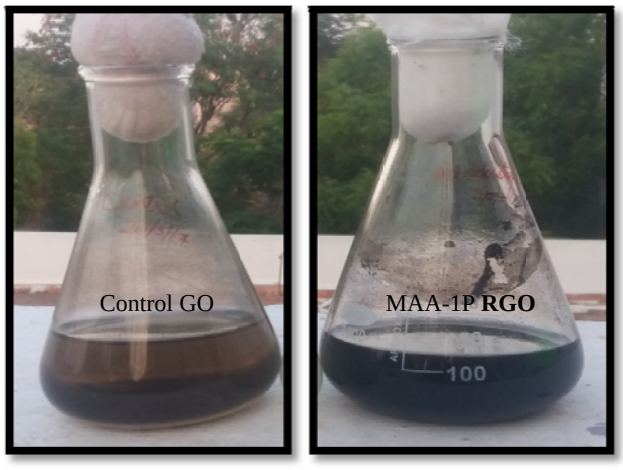


Fig. 3 Reduction of graphene oxide to graphene by intracellular actinobacteria

3.3. Antibacterial and Antagonistic Activity

Mushroom associated rhizosphere soil collected from Dharmapuri, India exhibited no antagonistic activity against Gram negative VRE and *K. pneumoniae* and gram-positive MRSA and *B. subtilis* strains. However, the bioactive compound isolated from MAA-1 shows 12 mm zone of inhibition against MRSA and 10 mm zone of inhibition against VRE. In our results, Gram-positive bacteria (*S. aureus*), shows higher reactivity with the bioactive compound than that of Gram-negative bacteria *(E. coli*), which is due to the positive–negative interaction thereby controlled cellular process [8,19] (Table 2). It was suggested that secondary metabolites produced by mushroom associated actinobacteria have an ability to kill the multi drug resistant pathogens at a certain level. Further optimization in extraction solvent, inoculated medium and on other clinical pathogens may give better result. The anti-fungal activity shows microbial reduced graphene oxide has higher activity than the chemically synthesised graphene oxide and reduced graphene oxide. The activity against *C. albicans* and *C. neoformans* of microbial reduced graphene oxide may be due to the environment friendly reduction without any chemical hazards [16-18]. Kavitha *et al.,* 2013 reported the cell wall play a key role in antimicobial activity ie., the component existing in the cell wall kill the pathogens habitually [7, 20-22].

Table: 2 Antibacterial and antifungal activity of mushroom associated rhizosphere actinobacteria

S.NO	Organisms	Zone of inhibition (mm)			
		Bioactive compound from MAA-1C	GO	Chemically syntheised RGO	Intracellular syntheised (MAA-1P) RGO
1	*E. coli* (VRE)	10±1.5	-	-	-
2	*S. aureus* (MRSA)	12±1	-	-	-
3	*C. albicans*	-	13±1.5	0	18±0.5
4	*C. neoformans*	-	12±0.5	0	17±1

4. Conclusion

Actinobacteria isolation from mushroom associated rhizosphere soil was successfully achieved. The isolated actinobacterium was predicted as rod shaped chain, Gram positive and non-motile bacterium. The antibiotic susceptibility test for the bioactive compound (MAA-1C) tested against VRE and MRSA shows diminutive activity. Optimization in medium may give better bioactive compound. Moreover, it needs further optimization in different bacterial, fungal and anti-cancer cells. Variation in concentration of samples as well as quality of dispersion may also give better activity. The present study reported the capability of mushroom associated actionobacteria to synthesis graphene intracellularly is the first time by using environmental friendly method. The further processing such as purification of graphene from intracellular actinobacterial medium and toxicity study may be a great remedy for cancer related orthopaedic replacements without toxic in nature and side effects. The present study concludes that, the isolated *Streptomyces sp.* are the next and best alternative source for the synthesis of environment friendly graphene nanoparticles, and produce bioactive compound, which will be utilized for various post orthopaedic and biomedical treatments.

Acknowledgment

The author Dr. K. Kavitha acknowledges the financial support provided by Dr. D.S. Kothari Postdoctoral Fellowship (Ref. no: No.F.4–2/2006 (BSR)/BL/15–16/0225, UGC, New Delhi. Author also acknowledges DST-FIST (SR/FST/LSI-640/2015(c) dt. 30/05/2016), New Delhi for the instrumentation facilities.

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

References

1. Azerang, P and Sardari, S., 2017, "Bioactive Compound Produced from Actinomycetes-Streptomyces", Nov. Appro. Drug Des. Dev., 1(3), pp.555565.
2. Janardhan, A. Kumar, A.P. Viswanath, B., Saigopal, D.V.R. and Narasimha, G., 2014, "Production of Bioactive Compounds by Actinomycetes and their Antioxidant Properties", Biotechnol. Res. Intern., 2014, pp.1-8.
3. El-Shatoury, S.A., El-Shenawy, N.S. and Abd El-Salam, I.M., 2009, "Antimicrobial, antitumor and in vivo cytotoxicity of actinomycetes inhabiting marine shellfish," World J. Microbiol. Biotechnol, 25(9), pp. 1547–1555.
4. Zengler, K., Paradkar A., and Keller, M., 2005, "New Methods to Access Microbial Diversity for Small Molecule Discovery", In: Zhang L., Demain A.L. (eds) Natural Products. Humana Press: pp. 275–293.
5. Bibb, M. J., 2005, "Regulation of secondary metabolism in Streptomyces," Curr. Opin. Microbio., 8(2), pp. 208–215.
6. Atta H.M., 2015, "Production, Purification, physico-chemical characteristics and biological activities of antifungal antibiotic produced by *Streptomyces antibioticus* AZ-Z71". American-Eurasi. Journal of Scientific Research 5(1), pp. 39-49.
7. Kavitha, K., Sutha, S., Prabhu, M., Rajendran, V., Jayakumar, T., 2013, "In situ synthesized novel biocompatible titania–chitosan nanocomposites with high surface area and antibacterial activity", Carbohydr. Polym., 93(2), pp. 731–739.
8. Kavitha, K., Navaneethan, D., Vinoth, A., Balagurunathan, R., 2017, ''Scavenging free radicals and soaring osteoinduction by extra cellular matrix protein–based nanocomposites on degenerative bone treatments". Mat. Sci Eng. C., 77, pp. 1189–1195.
9. Kandiah, K. Venkatachalam, R. Wang, C. Valiyaveettil, S. Ganesan, K., 2014, "In vitro and preliminary in vivo toxicity screening of high-surface-area TiO2–chondroitin-4-sulfate nanocomposites for bone regeneration application", Colloids.Surf. B, 128, pp. 347–356.
10. Hancock, R.E.W., Sahl, H.G., 2006, "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies", Nature Biotechnology, 24, pp. 1551 – 1557.

11. Poornima, P., Kumar, J.D., Zhao, Q., Blunder, M. and Efferth, T., 2016, "Network pharmacology of cancer: From understanding of complex interactomes to the design of multi-target specific therapeutics from nature", Pharmacological Research, 11, pp. 290-302.
12. Kavitha, K., Chunyan, W., Navaneethan, D., Rajendran, V. Valiyaveettil, S. and Vinoth, A., 2014, "In vitro gene expression and preliminary in vivo studies of temperature-dependent titania–graphene nanocomposites for bone replacement applications", RSC Advances, 4, pp. 43951-43961.
13. Gurunthan, S., Han, J.W., Eppakayala V., Jin-hoikim., 2013, "Microbal reduction of graphene oxide by Escherichia coli: A green chemistry approach", Int J Nanomedicine, 102, pp. 772-777
14. Muthoosamy, K., Bai R. G., Babangida, I., Mudavasseril, S., Lim, H. N., Loh, H.S., Huang, N.M., Chia, C.H., Manickam, S., 2015, "Exceedingly biocompatible and thin-layered reduced graphene oxide nanosheets using an eco-friendly mushroom extract strategy', Int J Nanomedicine,10, pp.1505-1519.
15. Hayakawa, M. And Nonomura, H., 1989, "A new method for the intensive isolation of actinomycetes from soil", Actinomycetol., 3, pp. 95-104.
16. Zhou, Y., Zhu, H., Fu, S.and Yao, Q., 2017, "Variation in Soil Microbial Community Structure Associated with Different Legume Species Is Greater than that Associated with Different Grass Species", Front Microbiol., 8, pp. 1007.
17. Shanmugasundarama, T., Radhakrishnan, M., Gopikrishnana, V., Pazhanimurugana, R. and Balagurunathan R., 2013, "A study of the bactericidal, anti-biofouling, cytotoxic and antioxidant properties of actinobacterially synthesised silver nanoparticles", Colloids and Surfaces B: Biointerfaces, 111, pp. 680– 687.
18. Rajan, B.M. and Kannabiran, K., 2014, "Extraction and Identification of Antibacterial Secondary Metabolites from Marine Streptomyces sp. VITBRK2", Int J Mol Cell Med. 3(3), pp.130–137.
19. Gurunthan, S, Han, J.W., Eppakayala, V., Jeyaraj M. and Kim, J.H., 2013, "An environmentally friendly approach to the reduction of graphene oxide by Escherichia fergusoni", Nanoscience and Nanotechnology. International Journal of Nanomedicine, 13, pp.2091-2098.
20. Tejada, G.M., Sánchez-Gómez, S., Kowalski, I., Kaconis, Y., Andra, J., Schürholz, T., Hornef, M., Dupont, A., Garidel, P., Gutsmann, T., David S.A. and Brandenburg, K., 2012, "Bacterial cell wall compounds as promising targets of antimicrobial agents I. Antimicrobial peptides and lipopolyamines", Curr Drug Targets., 13(9), pp. 1121–1130.
21. Gupte, S. and Kaur, M, 2017, "Novel Approaches to Developing New Antibiotics", J Bacteriol Mycol, 4(2), pp.00089.
22. Chung, Y. C., Su, Y. P., Chen, C. C., Jia, G., Wang, H. L., Wu, J. C. G., 2004, "Relationship between antibacterial activity of chitosan and surface characteristics of cell wall", Acta Pharmacologica Sinica, 25(7), pp. 932–936.