Synthesis of Selenium Nanoparticles from neem seeds, and their antibacterial and anticancer activities against *Vibrio cholerae* and Breast cancer cell lines.

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Objectives:

1. Preparation of Neem seed extract.
2. Preparation of Selenium oxide Nanoparticles procedure.
3. Characterization of Selenium oxide Nanoparticles byultraviolet-visible spectroscopy (UV-vis), X-ray diffraction (XRD), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).
4. Anti-microbial (*Vibrio cholerae*) activity of Selenium oxide Nanoparticles.
5. To check anticancer activity of Selenium oxide Nanoparticles.

Abstract:

In recent years, the synthesis of nanoparticles using plant-derived extracts has garnered significant attention due to its eco-friendly nature and potential biomedical applications. This study investigates the synthesis of selenium nanoparticles (SeNPs) from *Azadirachta indica* (neem) seeds and evaluates their antibacterial and anticancer activities. Selenium nanoparticles were synthesized by reducing sodium selenite with the aqueous extract of neem seeds. The synthesis process was characterized by UV- spectroscopy, scanning electron microscopy (SEM), and energy dispersive X-ray (EDX) analysis, confirming the formation of spherical nanoparticles with an average size of 30-50 nm. The antibacterial potential of the SeNPs was assessed against *Vibrio cholerae*, a major causative agent of cholera, using disc diffusion and minimum inhibitory concentration (MIC) methods. The SeNPs exhibited significant antibacterial activity, with a clear inhibition zone and a low MIC, indicating their potential as a therapeutic agent for cholera. Furthermore, the cytotoxic effects of the SeNPs were evaluated on breast cancer cell lines (MCF-7) using MTT assays. The SeNPs showed a dose-dependent reduction in cell viability, suggesting potent anticancer activity. This study highlights the potential of neem seed-derived selenium nanoparticles as a promising candidate for antimicrobial and anticancer therapies, offering an environmentally sustainable alternative for treating bacterial infections and cancer.

Key words: *Azadirachta indica* (neem), *Vibrio cholerae,* (MCF-7) Michigan cancer foundation-7

Introduction:

Nanoparticle (NP) systems appear to be a promising alternative to per oral drug delivery as well as nutritional supplements. The effects of bioactive compound supplements – omega-3 and omega-6 fatty acids, probiotics, prebiotics, vitamins, and minerals – in nano-particulate preparations have already been dealt by numerous studies. Some applications of NPs in nutrition and medicine have already been approved for clinical use, and many others are at different stages of their development.Nile, (Shivraj Hariram, *et al.2020*) Nanoparticles are particles that exist on a nanometre scale (i.e., below 100 nm in at least one dimension). They can possess physical properties such as uniformity, conductance or special optical properties that make them desirable in materials science and biology (Srinivas *et al.*, 2010).

Traditional supplements of selenium generally have a low degree of absorption and increased toxicity. The traditional forms viz Se salts, amino acids, or selenium-enriched yeast supplements, next-generation selenium supplements, with lower risk for excess supplementation, are emerging. These are based on selenium forms with lower toxicity, higher bioavailability, and controlled release, such as zerovalent selenium nanoparticles (SeNPs) and selenized polysaccharides (SPs).(Diana, *et al 2018*).Therefore, it is imperative to develop innovative systems as transporters of selenium compounds, which would raise the bioavailability of this element and allow its controlled release in the organism. Nanoscale selenium has attracted a great interest as a food additive especially in individuals with selenium deficiency, but also as a therapeutic agent without significant side effects in medicine (Hosnedlova *et al.*, 2018).

Applications of nanoparticles in biology and medicine:

The nanomedicine has been generally defined as the medical application of nanotechnology. This application leads to a better repair, protection, and improvement of a great majority of human biological systems. The integration of nanotechnology in medicine, more commonly known as nanomedicine, allows new hope in the field of health. As an emerging discipline, nanomedicine is gradually being created by opening up new perspectives on key issues: optimizing drug delivery, specifically targeting tissues or cells, more optimal controlling the rate of release of the drug into the body, and providing early and accurate detection of diseases (Salata *et al.*, 2004).

Applications of Nano-materials to biology or medicine:

- Fluorescent biological labels
- Drug and gene delivery
- Bio detection of pathogens
- Detection of proteins
- Probing of DNA structure
- Tissue engineering
- Tumour destruction via heating (hyperthermia)

- Separation and purification of biological molecules and cells
- MRI contrast enhancement
- Phagokinetic studies

All three main categories of Se (inorganic, organic, and SeNPs) contain compounds with potential anticancer properties. For inorganic and organic Se compounds, research has found that they are both metabolized differently and have varied mechanisms of action in diverse bio-physiological processes, including their roles in cancer. Both forms of Se compounds can be readily absorbed by the human body, but only organic Se compounds, usually in the forms of amino acids (e.g., selenomethionine (SeMet) and selenocysteine), can be better retained and used. The cancer prevention ability of a range of inorganic and organic Se compounds has been supported by a large number of publications from a wide range of studies under different settings, including biochemical, epidemiological, clinical, and animal studies. However, toxicity risks accompanied by the use of these Se compounds have also been recorded. Although organic forms of Se may have lesser toxic effects than inorganic Se compounds, in reality, the toxic effects of Se are determined by multiple factors, with the forms of Se and dosage exposure being two of the most important parameters. Despite the greater toxic effects, inorganic Se compounds may have an advantage in certain aspects of cancer therapy

Neem plant and its medicinal applications:

Figure 1: Neem Plant

Taxonomical description of Neem Plant:

Kingdom - Plantae
Division - Magnoliophyta
Order – Sapindales
Family - Meliaceae
Genus – Azadirachta

The plant product or natural products show an important role in diseases prevention and treatment through the enhancement of antioxidant activity, inhibition of bacterial growth, and modulation of genetic pathways. The therapeutics role of number of plants in diseases management is still being enthusiastically researched due to their less side effect and affordable properties. It has been accepted that drugs based on allopathy are expensive and also exhibit toxic effect on normal tissues and on various biological activities. It is a largely accepted fact that numerous pharmacologically active drugs are derived from natural resources including medicinal plants (Zong *et al.*, 2012).

The most popularly known Neem tree is scientifically called as Azadirachta indica and is found in tropical regions indigenous to Indian subcontinents. Over two millennia, neem trees products have been utilized in India as traditional medicines due to their novel properties. Basically, neem is called arishtha in Sanskrit which means 'reliever of sickness' and in India, Neem is also called as the 'village pharmacy' for its properties to relieve from various infections, pains and fever. The biological activities of neem include anti-inflammatory, anti-pyretic, hypoglycaemic, anti-fungal, anti-gastric ulcer, diuretic, antibacterial, antitumour, antimalarial, anticancer, hypolipidemic etc. (Senthilkumar *et al.*, 2008)

Neem (Azadirachta indica) plants parts shows antimicrobial role through inhibitory effect on microbial growth/potentiality of cell wall breakdown. Azadirachtin, a complex tetranortriterpenoid limonoid present in seeds, is the key constituent responsible for both antifeedant and toxic effects in insects. Results suggest that the ethanol extract of neem leaves showed in vitro antibacterial activity against both Staphylococcus aureus and MRSA with greatest zones of inhibition noted at 100% concentration (Alzohairy *et al*., 2016).

- Neem plays role as free radical scavenging properties due to rich source of antioxidant. Azadirachtin and nimbolide showed concentration-dependent antiradical scavenging activity and reductive potential in the following order: nimbolide> azadirachtin > ascorbate.
- Neem ingredient shows effective role in the management of cancer through the regulation of cell signaling pathways. Neem modulates the activity of various tumour suppressor genes (e.g., p53, pTEN), angiogenesis (VEGF), transcription factors (e.g., NF-κB), and apoptosis (e.g., bcl2, bax).
- Neem also plays role as anti-inflammatory via regulation of proinflammatory enzyme activities including cyclooxygenase (COX), and lipoxygenase (LOX) enzyme.
- Treating scalp conditions, including dandruff, itchiness and head
- Treating acne
- Providing relief for skin disorders such as eczema and psoriasis
- Healing of wounds
- Treating and healing fungal infections, ringworm, infected sores and burns 6.treating athlete's
- Treating nail fungus and restoring brittle nails

The pharmacological application of nanodevices and systems is a rapidly emerging nanotechnology that is raising new possibilities in the diagnosis and therapy of different diseases. Nanotechnology has been providing promising tools for chemotherapy especially in cancer treatment. Nanotechnology deals with physical and biochemical properties of nanoparticles in the clinical application of drugs. Targeted nanoparticle drug delivery is intended to reduce the side effects of anticancer drugs with both decreases in consumption and treatment expenses, which are the major hurdles in conventional cancer treatment. The characteristic small size and special coating of nanoparticles facilitates the delivery of hydrophobic anticancer drugs to specific sites with reduced opsonization by defense mechanisms of the body. One of the vast applications of nanotechnology is the diagnosis, treatment, and prevention of breast cancers. Breast cancer is associated with high morbidity and mortality and is the second leading cause of death in Western countries. A large number of nanoparticles have been developed, specifically targeting metastasized tumors of the breast. The present review focuses on the application of different types of nanoparticles including gold, polymeric, and magnetic nanoparticles in the treatment of breast cancer (Hussain et al., 2018).

MATERIALS AND METHODS:

Methadology:

- **Plant material:***Neem Plant leaves and seeds*
- **Chemicals:** Selenium Dioxide, distilled water, Ethanol, LB Agar Media
- **Glassware's:** Chopping knife, conical flask, petri plates, beaker, spreader, Gel puncture, Tubes
- **Microbial Culture:**Organism: *Vibrio cholerae*; Strain: MTCC 3906.
- **Instruments:** Autoclave, centrifuge, spectrophotometer, hot plate, Transmission electron, Microscopy, Scanning Electron microscopy, laminar air flow, weighing machine

Preparation of selenium Dioxide precursor solution:

Materials of Sodium selenium Dioxide (SeO2, 99% purity, SDFCL Products in Mumbai). Preparation of 10Mm selenium Dioxide Solution 0.172g of selenium Dioxide was added into 100ml of distilled water and shake continuously for 1-2min to get selenium Dioxide stock solution /precursor.

Plant Leaf Sample Collection and preparation of *leaf and seed* Extract preparation: The Leaves and seedswere carried out the surface sterilization with Tween20 and washed thoroughly with distilled water, chopped small pieces using a sterile knife. Briefly, 10 g of small, chopped leaf was added in 100 ml of distilled water and kept in 60^0 c for 1 hour. The solution obtained was filtered through Whatman No. 1 filter paper, stored at 4°C and further used for synthesis of nanoparticles preparation.

Synthesis of SeNPs:

The complete understanding of the synthesis mechanism of nanoparticles using the biological agents has not been devised. The biological synthesis mechanism include both intra and extracellular of nanoparticles which are different for various biological agents and different biomolecules responsible for the synthesis of these nanoparticles. Biological agents used for nanoparticles synthesis represent mainly microbes including bacteria, fungi, algae and yeast and plants which react differently with metal ions. The extract was mixed with 10mM Selenium dioxide solution in water in the ratio of 1:9 and was

kept in the dark for 24h in room temperature in order to produce and settle selenium nanoparticles (Alagesan *et al.*, 2019, Sharma *et al.*, 2014 and Verma *et al.*, 2018) .

Characterization of synthesized SeNPs:

Characterization of Selenium nanoparticles is based on the size, morphology and surface charge, using such advanced microscopic techniques. Properties like surface morphology, size and overall shape are determined by electron microscopy techniques. The Preparation of Neem (Azadirachta indica) seed Extract extract and SeO2 solution mixture was then characterized using UV, XRD and TEM (Kapur and Soni. 2017).

UV - Spectroscopy: The formation of Selenium nanoparticles with Neem seed extract was monitored by observing the color change. After the formation of brick-red color, absorbance of the nanoparticles was measured at wavelength ranging between 200 and 800 nm by using UV–visible spectrophotometer.

Anti-cancer activity of Seed and Leaf extracts NaNps: Breast cancer cell line (MCF-7)

Cytotoxicity (MTT) Assay:

Principle:

The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

Materials:

1. Cell line- **Breast cancer cell line (MCF-7).**
2. Cell culture media – Dulbecco's Modified Eagle Medium with High Glucose (DMEM-HG) supplemented with 10% Foetal Bovine Serum (FBS).
3. 1X Dulbecco’s Phosphate Buffered Saline (DPBS), 0.25% Trypsin-EDTA solution, MTT reagent, Dimethyl Sulfoxide (DMSO) were all purchased from HiMedia, India.
4. Cell culture treated T-25 flasks from Biolite, Thermo Fisher Scientific Inc., USA.
5. 10mL serological pipettes and 96-well plates from Nunc, Thermo Fisher Scientific Inc., USA.
6. 5mL, 2mL and 1.5mL tubes, Tarsons, India.

Procedure:

1. Cells cultured in T-25 flasks were trypsinized and aspirated into a 5mL centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted, using DMEM HG medium, such that 200µl of suspension contained approximately 10,000 cells.
2. To each well of the 96 well microtitre plate, 200µl of the cell suspension was added and the plate was incubated at 37ºC and 5% CO_2 atmosphere for 24 h.

3. After 24 h, the spent medium was aspirated. 200µl of different test concentrations of test drugs were added to the respective wells. The plate was then incubated at 37ºC and 5% CO_2 atmosphere for **24 h**.
4. The plate was removed from the incubator and the drug containing media was aspirated. 200µl of medium containing10% MTT reagent was then added to each well to get a final concentration of 0.5mg/mL and the plate was incubated at 37ºC and 5% CO_2 atmosphere for 3 h.
5. The culture medium was removed completely without disturbing the crystals formed. Then 100µl of solubilisation solution (DMSO) was added and the plate was gently shaken in a gyratory shaker to solubilize the formed formazan.
6. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm. The percentage growth inhibition was calculated, after subtracting the background and the blank, and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) was generated from the dose-response curve for the cell line.

(Ref: MTT Cell Proliferation Assay Instruction Guide – ATCC, VA, USA www.atcc.org, and Alley *et al*., 1986)

Anti-Bacterial Activity: SeNPs were tested Againes Antimicrobial activity against - Organism: Vibrio cholerae; Strain: MTCC 3906. The microbial culture were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), a national facility established in 1986 is funded jointly by the Department of Biotechnology (DBT) and the Council of Scientific and Industrial Research (CSIR), Government of India. The MTCC, housed at the Institute of Microbial Technology (IMTECH), Chandigarh (Badreah et al., 2021). The SeNps nanoparticle synthesized using plant and seed extract. and tested for antimicrobial activity by LB - agar well diffusion method against pathogenic microbe that is Vibrio cholerae; Strain: MTCC 3906. The pure cultures of bacteria were subculture on LB broth. From the broth 50µL microliters were taken on LB Agar plate and homogeneously spread onto the individual plates using sterile glass spreader. Wells of 5 mm to 10 mm diameter were on Muller Hinton agar using gel puncture. Different concentration of SeNps nanoparticle 20 µL, 40 µL, 60 µL, 80 µL and 100 µL was poured on each well. After 48 hours incubation the various levels of zone of inhibition was measured (Rajeshkumar et al., 2014).

RESULTS AND DISCUSSION:

Characterization of SeNPs:

Visual observation on formation of SeNPs:

The seed and leaf extract was added to the 10mM of SeNPs solution at 60^0 C and observe about color changes from Light green colour to Brick red colour and kept for 24 hours to obtain the color change. The color changes showed in figure 2, which indicate the formation of Selenium nanoparticles.

Figure 2: Visual indication of synthesized Selenium nanoparticles

UV visible spectrometry:

UV visible spectrometry were used to preliminary confirmation and identify, characterize and analyze SeNps. For the formation of nanoparticles by the wavelength range between 200 – 800 nm. As per the review of literature shows Normal range of UV for nanoparticles will be between 200-800nm. Synthesized SeNPs with *seed and leaf*extracts shows UV ranges between 230-438 nm and preliminary confirmed by UV results that it successfully synthesized SeNPs with Neem seed and leaf extract can form Selenium nanoparticles the results were sowed in figure 3.

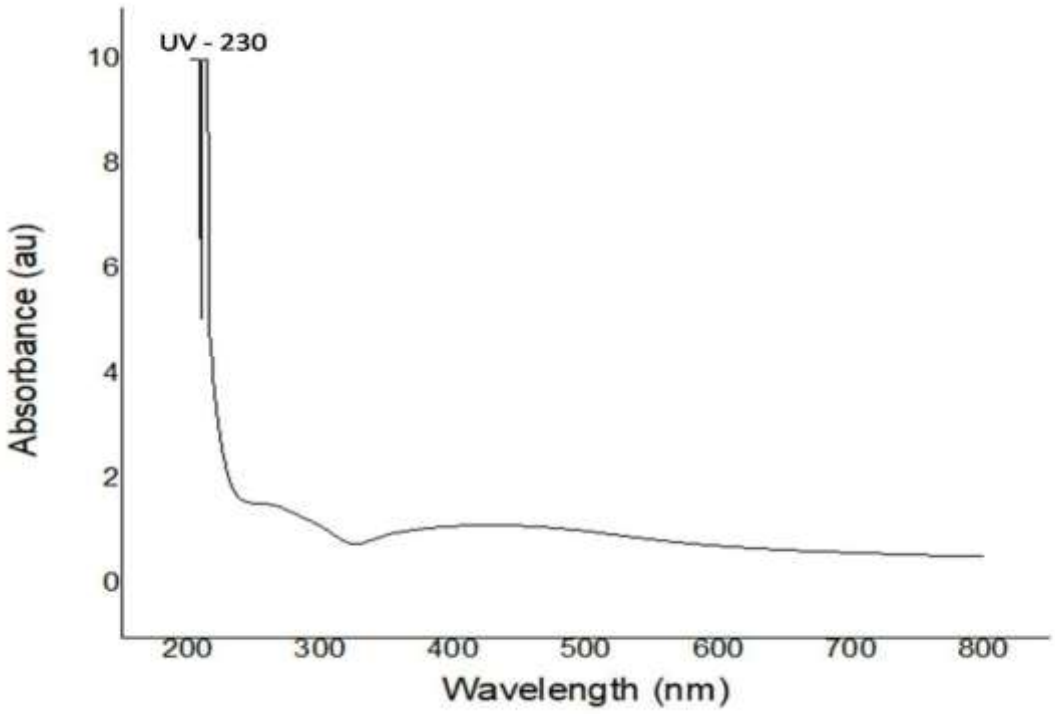

Figure 3:UV spectrophotometer range of SeNPs

XRD Analysis:

The XRD pattern of synthesized SeNPs is shown in Figure 2A, where two different regions were observed. A broad shoulder between 10–30°, at low angles, was shown corresponding to the amorphous phase (a-SeNPs). This behavior also was observed by other authors who reported the predominance of the amorphous region in synthesized SeNPs. However, some peaks at higher diffraction angles 2θ (degrees) of 28.4, 34.6, 46.5, 55.3 and 56.6 corresponding to different crystalline planes of SeNPs, were observed. These results are in agreement with those reported by Mellinas et al., 2019. who ascribed peaks at similar angles to the 28.4, 31.6, 45.5, 50.3 and 56.6 crystalline planes figure 4.

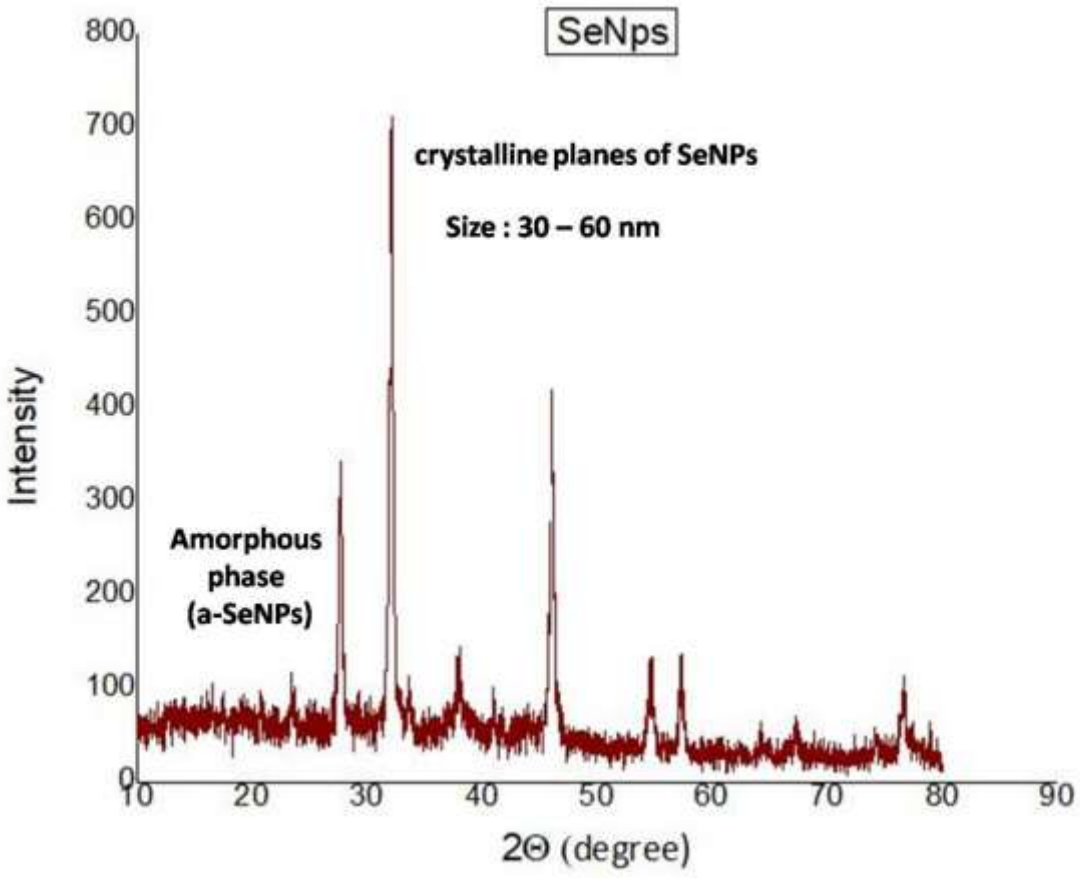

Figure 4: XRD analysis pf SeNps

TEM [Transmission Electron Microscopy] Analysis:

Transmission electron microscopy (TEM) allow surface and morphological characterization at both nanometer and micrometer scale. Transmission Electron Microscopy (TEM) uses an electron beam to interact with a sample to form an image on a photographic plate or specialist camera.

Analysis:-

TEM analysis give the information about the morphology of the Selenium nano-particles .generally Selenium nano particles are spherical or crystal structures. Tem also give average mean size of

Selenium nano particles. TEMmeasurements were performed on JEOL model JEM 2100 instrument operated at an accelerating voltage at 120 kV Results were showed in figure 5.

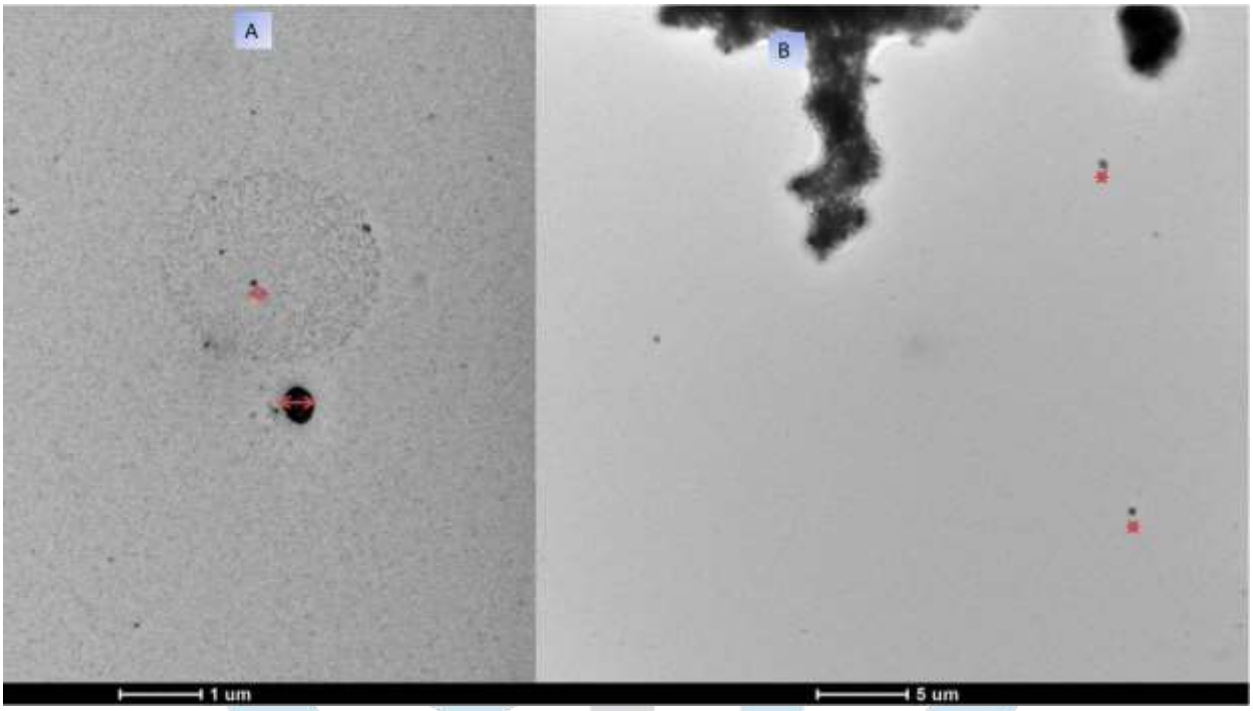

Figure 5 :TEM analysis results of *Persea Americana* leaf and seed extract ***of*SeNps. 1nm magnification Particle size ranges between 20nm - 170nm.**

Test details
Test equipment: Transmission Electron Microscopy
Maker : FEI
Model : Technai G2 Biotwin 120 KV
Testing Laboratory: Election Microscopr Facility ,Division of Biological Science,IISC

SEM Analysis: The SEM image of SeNPs were synthesized plant extracts and were assembled on to the surface due to the interaction such as hydrogen bond and electrostatic interaction between the bio-organic capping molecules bound to the SeNPs. The synthesized SeNPs were formed with size ranging from 46 nm to 168 nmFigure 6(Sowndarya et al., 2017).

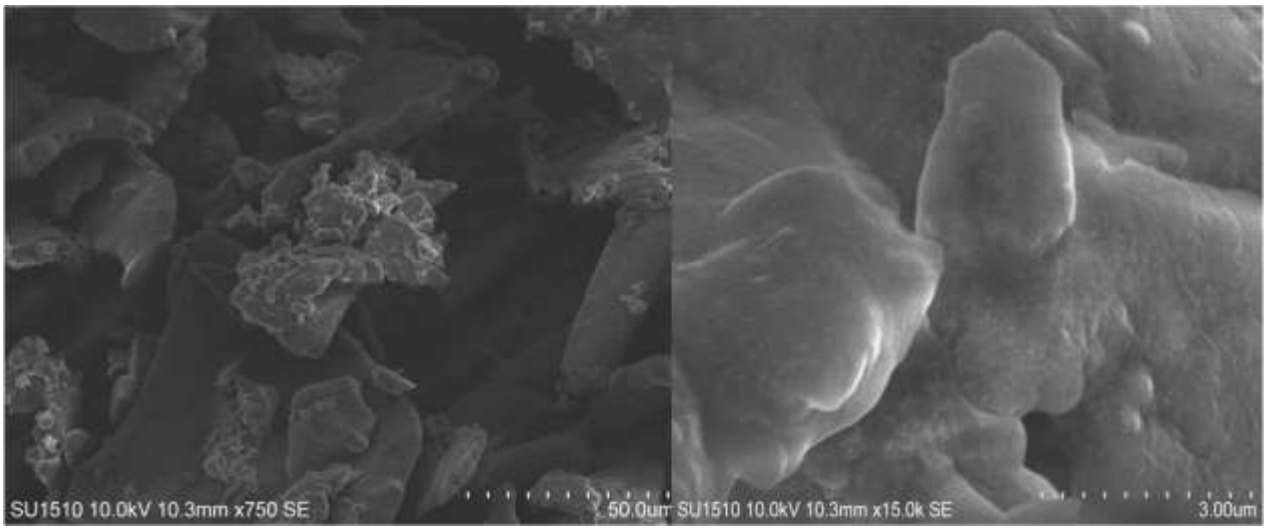

Figure 6:**SEM Analysis of SEED and Leaf extracts of SeNps**

Anti - Cancer Activity: Breast cancer is the most frequent malignancy in females. Due to its major impact on population, this disease represents a critical public health problem that requires further research at the molecular level in order to define its prognosis and specific treatment. Basic research is required to accomplish this task and this involves cell lines as they can be widely used in many aspects of laboratory research and, particularly, as in vitro models in cancer research. MCF-7 is a commonly used breast cancer cell line, that has been promoted for more than 40 years by multiple research groups (Şerban Comşa et al., 2015). The IC_{50} values of the test samples for 24 hour treatment were found to be very high toxicity at tested concentrations. The detailed results were showed in table and figure 7 and 8.

MDA-MB-231 cell line vs leaf	**Test concentrations (%v/v)**						
	Blank	**Untreated**	**3.125**	**6.25**	**12.5**	**25**	**50**
Reading 1	0.001	0.832	0.33	0.638	0.174	0.24	0.071
Reading 2	0.002	0.825	0.34	0.596	0.224	0.208	0.429
Mean OD	0.0015	0.8285	0.335	0.617	0.199	0.224	0.25
Mean OD-Mean Blank		0.827	0.3335	0.6155	0.1975	0.2225	0.2485
Standard deviation		0.0049	0.0071	0.0297	0.0354	0.0226	0.2531
Standard error		0.0035	0.005	0.021	0.025	0.016	0.179
% Standard error		0.4232	0.6046	2.5393	3.023	1.9347	21.644
% Viability		100	40.326	74.4256	23.881	26.904	30.048

Table: Leaf SeNps MTT Assay Results

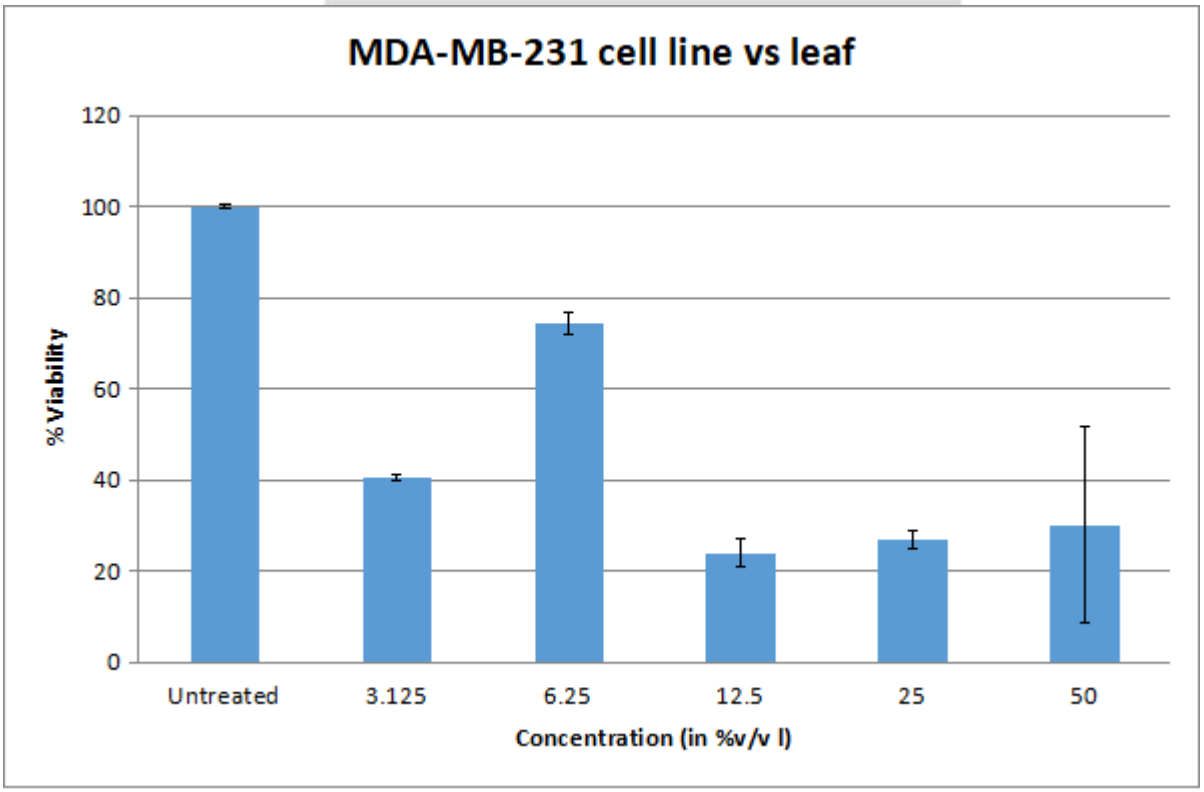

Figure 7: Cell viability of Leaf SeNps

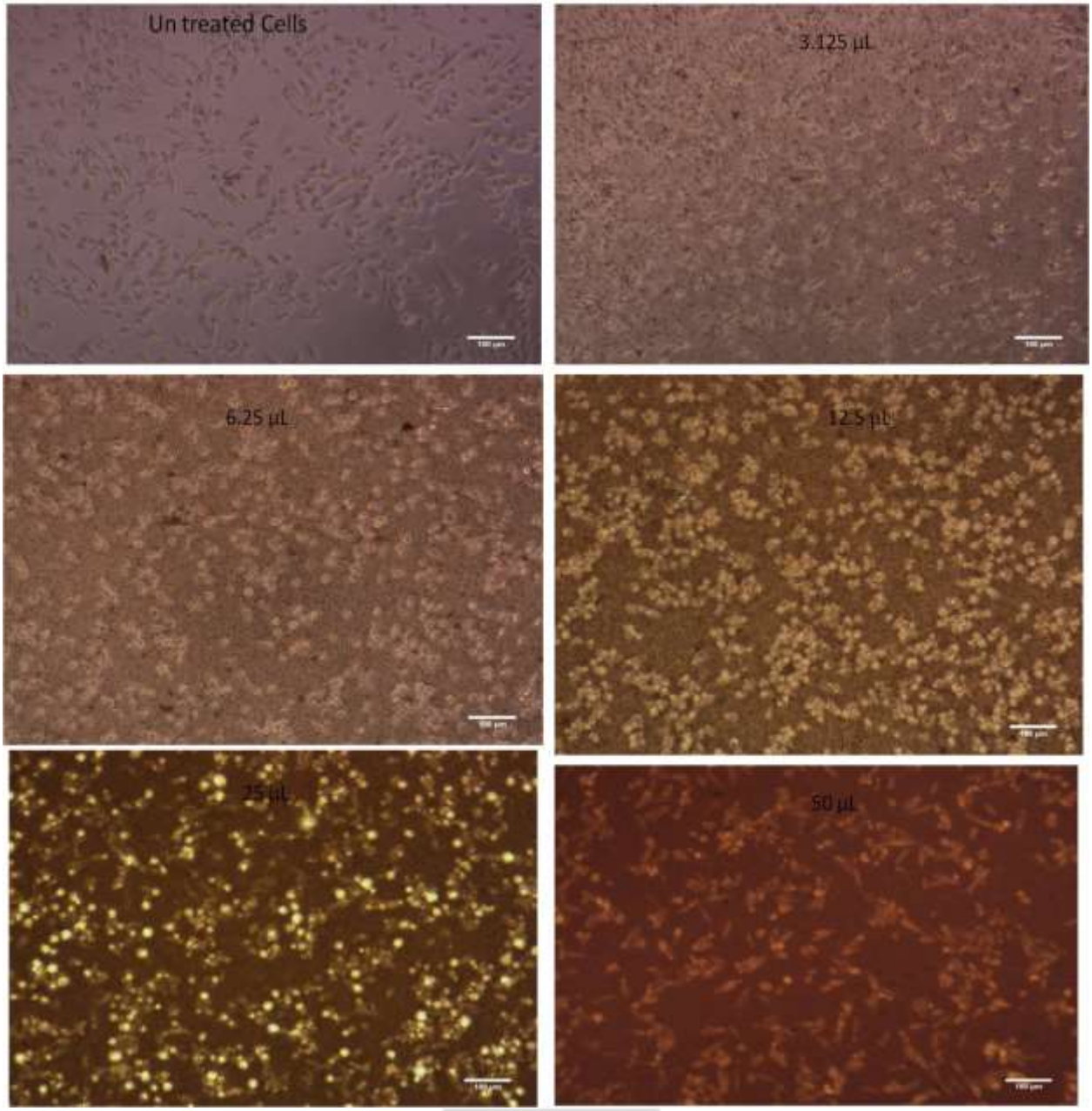

Figure 8: Leaf extract of SeNps of MTT Assay viability on Breast Cancer Cell lines - MCF - 7 cell line treated and untreated cell.

Table: Seed SeNpsMTT Assay Results

MDA-MB-231 cell line vs seed	Test concentrations (%v/v)						
	Blank	**Untreated**	**3.13**	**6.25**	**12.5**	**25**	**50**
Reading 1	0.001	0.832	0.17	0.161	0.112	0.2	0.14
Reading 2	0.002	0.825	0.22	0.144	0.081	0.17	0.127
Mean OD	0.0015	0.829	0.19	0.153	0.097	0.18	0.134
Mean OD-Mean Blank		0.827	0.19	0.151	0.095	0.18	0.132
Standard deviation		0.005	0.04	0.012	0.022	0.02	0.009
Standard error		0.004	0.03	0.009	0.016	0.02	0.007
% Standard error		0.423	3.2	1.028	1.874	1.81	0.786
% Viability		100	23.2	18.26	11.49	21.9	15.96

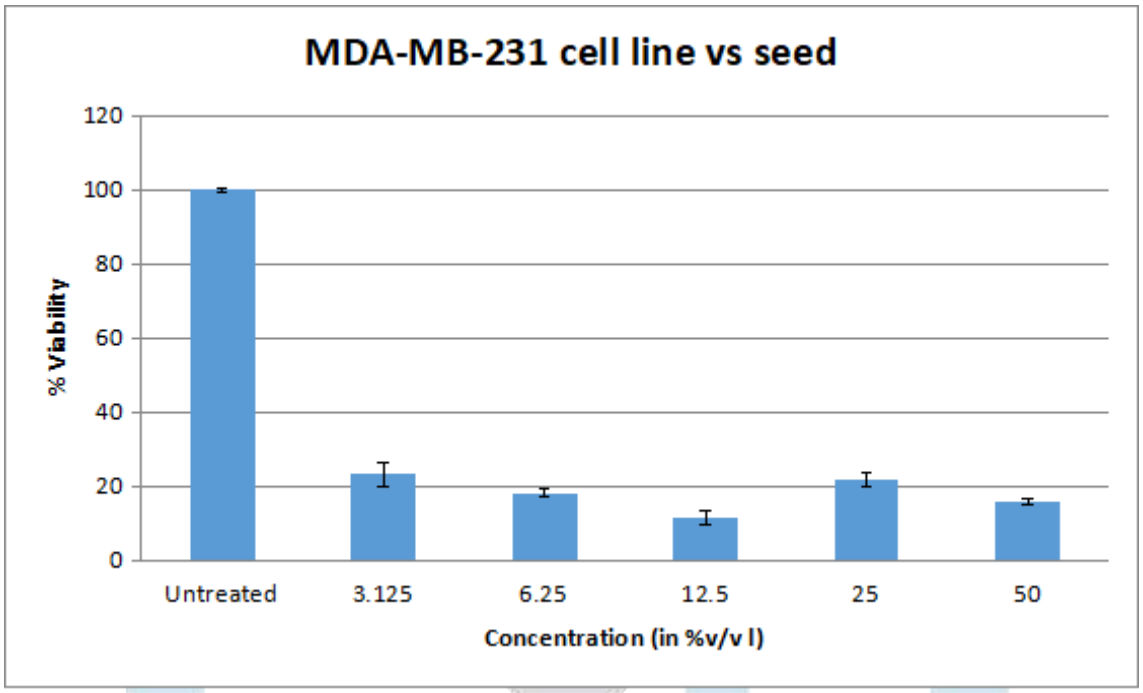

Figure 9: Cell viability of Seed SeNps

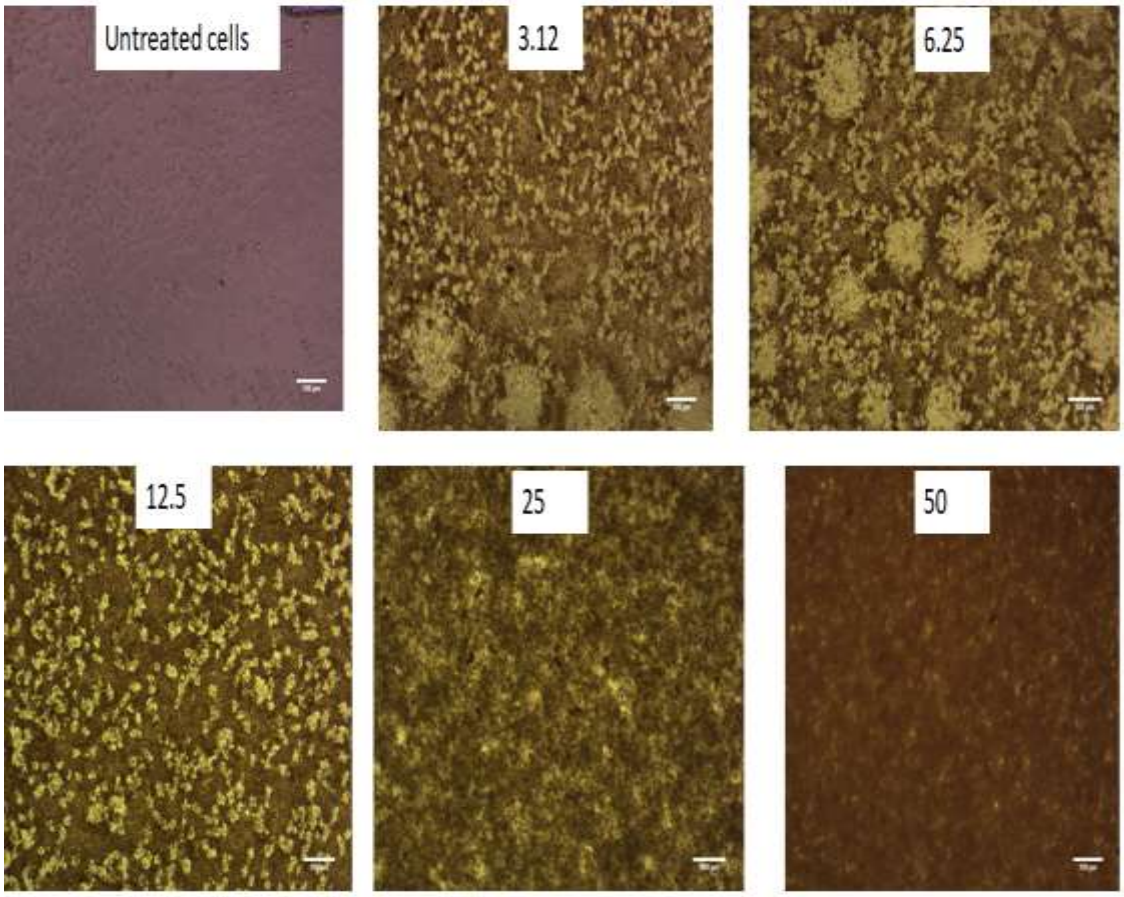

Figure 10: Cell viability of Seed SeNps

Antimicrobial Activity: The inhibitory zone values of SeNps (µL) and Inhibition Zone in CMs are 20 µL (1.6), 40 µL (1.8), 60 µL (2.5), 80 µL (2.6), 100 µL (2.9). The graphical representation were shoed in figure.

Figure 11: Growth of *Vibrio cholerae* (MTCC 3906) on LB -Agar medium after 48 hrs of incubation.

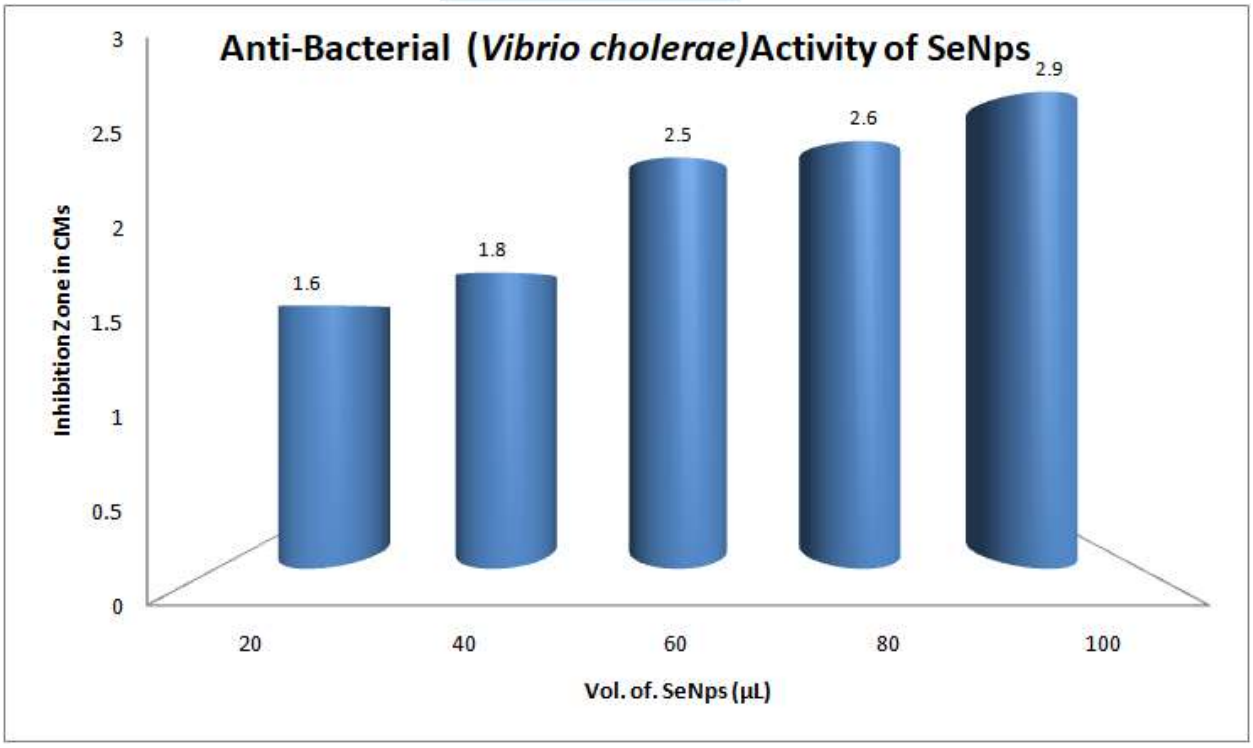

Figure 12: Anti-Bacterial (*Vibrio cholerae)*Activity of SeNPs:

Conclusion:

In nano-biology, synthesis of selenium nanoparticles is in the shining star. In this current study a simple and systematic approaches for the synthesis of selenium nanoparticles was established using the Neem leaf and seed extract. SeNPs which were characterized by different physicochemical techniques such as UV–Vis spectrophotometry, XRD, and TEM. Based on medicinal value oriented tree plant it is expected that plant seed extract synthesized for selenium nanoparticles finds promising medicinal uses. Furthermore, this could be a potent candidate for breaking the limitation in medical field. Moreover, serves as a potential anti-proliferative agent signifying the immense growth control against cancer cell. The combination of phytocompounds and nanoparticle resolve the unique platform for the drug and gene delivery in cancer therapy.

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