Fabrication and Analysis of Nanofibrous scaffolds from Plumeria alba for biomedical application

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Abstract

Antibiotic resistance is rising to dangerously high levels and new resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. nanotechnology is one of the fast growing research areas in the field of biomedicine. The current studies discuss the synthesis of hydroxyapatite nanoparticles with various herbal extracts by wet synthesis technique, characterization of developed nanoparticles and final fabrication of nanofibrous scaffolds with PHB as the polymer. Fabrication of nanofibrous scaffolds from Plumeria alba latex serum extract was developed along with polymer polyhydroxy butyrate. Poly-β-hydroxybutyrate (PHB) is a biopolymer that belongs to polyester produced from micro-organisms. Polyhydroxy butyrate along with developed hydroxyapatite nanoparticles as three-dimensional scaffolds was developed by electrospinning procedure. The developed nanofibrous scaffolds were characterized for thickness, tensile strength, water contact angle (wetting ability), FTIR and SEM. Nanofibrous scaffolds of Plumeria alba were evaluated for cell proliferation by MTT cytotoxicity test against 3T3-L1 cell line on different concentrations.

Nanofibrous scaffold developed from Plumeria alba with hydroxyapatite nanoparticles has potent biomedical application for wound dressing and tissue regeneration.

Key words: Nanoparticles, hydroxyapatite nanoparticles, electrospinning, Nanofibrous scaffold and Plumeria alba

Introduction

Nanotechnology in research and innovation is concerned with building things like materials and devices on the scale of atoms and molecules. The technological inventions are tremendous in the field of nanoscience such as pharmaceutical, cosmetics, food science, chemical engineering, and biomedical engineering which are more potent and novel products and their application in biomedical fields are vast. Nanoparticles can be synthesized by physical, chemical, and biological methods (1). The biological synthesis of nanoparticles is used for its rapid and eco-friendly synthesis, also nanoparticles toxicity and size characterization can be controlled.

The efficiency of herbal drug solubility and localization in a specific site can be increased by the use of nanoparticles. Biocompatible nanomaterials that have optimal mechanical properties can be used as medical implants (e.g. dental restoratives and bone substitutes). Nanosized hydroxyapatite is the main component of mineral bone. Hydroxyapatite possesses exceptional biocompatibility and bioactivity properties with respect to bone cells and tissues (2) The popular and widely used technique is wet precipitation or chemical precipitation or aqueous precipitation. A relatively large amount of hydroxyapatite can be produced by precipitation technique in the absence of organic solvents at a reasonable cost (3).

Herbal medicines have been widely used worldwide for their better therapeutic values and fewer adverse effects as compared to modern medicines (4). Plants are the natural factories for the synthesis of nanoparticles (5). In the green synthesis of nanoparticles, biomolecules are reduced to metal ions in a single-step process.

The application of biopolymer (PHB) with the recent impact of various "green chemistry" strategies on the antimicrobial properties and different green pre-treatment technologies used for the surface modification of textile fibers (6). A nanocarrier is a nanomaterial mainly used as a transport module for another substance, such as a drug.

There are three basic techniques capable of generating nano-fibrous scaffolding: Electrospinning, Molecular self-assembly and Thermally induced phase separation. Cytotoxic effects of newly synthesized drugs investigated along with the cell viability. Numerous in-vitro assays in response to external factors rely on the quantification of cell viability and proliferation. An MTT assay is a colorimetric assay based on assessing the cell's metabolic activity. Cytotoxic effects of newly synthesized drugs can be investigated along with cell viability and cell proliferation.

Electrospinning has several advantages, including producing continuous fibers, applicability to various polymers and easy adjustability. The thickness of the mat can also be transformed by changing the collection time during the electrospinning.

MATERIALS AND METHOD

Fabrication of nanofibrous scaffolds

Preparation of electro-spinning solution from *Plumeria alba* hydroxyapatite nanoparticle with PHB.

Hydroxyapatite nanoparticles from *Wrightia tinctoria* latex serum and poly-β-hydroxybutyrate (PHB) were taken in a ratio of about 7:3 and mixed in the solution consisting of 7 ml of chloroform and 3 ml of dimethylformamide. Hydroxyapatite nanoparticles from *Plumeria alba* latex serum and poly-β-hydroxybutyrate (PHB) were taken in a ratio of about 7:3 and mixed in the solution consisting of 7 ml of chloroform and 3 ml of dimethylformamide (7). Both the mixture was then kept separately in a magnetic stirrer at 40-50˚C for 3 hours. The stirred mixture was then treated with 10% polylactic acid as a co-polymer and continuously stirred until the mixture gets completely dissolved.

Electrospinning of *Plumeria alba* hydroxyapatite nanoparticle with PHB

The polymer mixture was loaded into a 10 ml syringe consisting of stainless steel blunt needle of a pore size of 0.7mm in diameter. A high voltage of about 12kV was supplied to the syringe needle and the flow rate of the polymer solution was maintained at 2500 µl/hr. Fibres produced from the syringe were collected on an aluminium foil sheet placed at a distance of 8 cm from the needle tip (8). Deposition of fibres was performed for 8 hours to obtain a nanofibrous scaffold layer.

Characterization of developed nanofibrous scaffolds (Rajzer, 2014)

Thickness (ASTM D 1777-96)

The thickness gauge Tilmet (ASTM D 1777-96) was used to determine the thickness of the developed nanofibrous scaffold of *Plumeria alba* [8].

Tensile Strength (Zwick/Roell)

Developed nanofibrous scaffolds of *Plumeria alba* sample of 10 cm long and 2 cm wide were used to analyse the Mean breaking strength, mean breaking elongation, and stress/strain percentage by Zwick Roell testing machine at a crosshead speed of 5 mm/min[8].

Wetting ability by water contact angle

Drop shape analysis was performed to measure the water contact angle of the developed nanofibrous scaffold of *Plumeria alba.* About 20µl of a single droplet of double distilled water was applied to the developed scaffold surface and the contact angles were measured at room temperature [8] to indicate the wetting ability of the scaffolds.

Fourier Transform Infra-red Spectroscopy analysis of developed nanofibrous scaffolds.

The presence of functional groups and chemical bonds present in the developed nanofibrous scaffolds using *Plumeria alba* hydroxyapatite nanoparticle was determined by the Shimadzu-single reflection ATR accessory Fourier to transform infrared spectrometer (FTIR) [9]. The spectrums were observed in the range of 400-4000 cm^{-1}.

Scanning Electron Microscopy analysis of developed nanofibrous scaffolds (

The diameter and morphology of the developed nanofibrous scaffolds prepared from *Plumeria alba* hydroxyapatite nanoparticles with PHB was analyzed using a Scanning Electron Microscope [8]. A length of about 3X3 cm processed scaffold was then visualized under SEM.

Cell proliferation and cytotoxicity.

Cell proliferation and cytotoxicity were determined by MTT assay colorimetrically[10]. The reduction of yellow-colored water-soluble tetrazolium dye to purple color in MTT to formazan crysKCs was measured spectrophotometrically at 570nm. The intensity of formazan crysKCs was proportional to the number of viable cells.

Determination of Cell proliferation and cytotoxicity

Cell line 3T3-L1 was used to study the cell proliferation and cytotoxicity of *Plumeria alba*. About 200µl cell suspension of 3T3-L1 was seeded in a 96-well plate at the required cell density (20,000 cells per well), without the test agent. Cells were treated with different concentrations of the compounds (Table 1) and incubated for about 24 hours. Sample scaffold materials were sterilized by washing with DPBS 5-6times and kept in UV light in the Laminar hood for 2-3hrs to ensure sterility [11]. Further, the scaffold materials were dissolved in 2ml of DMEM-high glucose media for 24hrs. After 24hrs, appropriate concentrations of the test agent were added to the sample materials. Then the plates were incubated for 24 hrs at 37°C in a 5% CO2 atmosphere. After the incubation period, the spent media was removed and an MTT reagent was added to make a final concentration of 0.5mg/ml of total volume. Light exposure to plates were avoided by wrapping the plates with aluminium foil. The plates were incubated again for about 3 hours. The MTT reagent was removed and then about 100µl of solubilization solution (DMSO) was added. Gentle

stirring in a gyratory shaker and pipetting occasionally completely dissolved the MTT formazan crystals for dense cultures. The absorbance was read on a spectrophotometer at 570nm and 630nm.

Preparation of electrospinning solution from *Plumeria alba* hydroxyapatite nanoparticle with PHB.

Electrospinning polymer solution prepared from hydroxyapatite nanoparticle developed latex serum extract of *Plumeria alba* and the produced PHB (7:3) treated with chloroform and dimethylformamide under magnetic stirrer and stored in room temperature before fabricating as scaffolds. Shahrooz *et al.,* 2009 reported that polycaprolactone and chitosan (7:3) were dissolved in polyvinyl alcohol solution serve as an electrospinning solution for the development of nanofibrous scaffold [12]. Similarly, polycaprolactone with the developed hydroxyapatite treated with 50:50 (v/v) dichloromethane and dimethylformamide solvent prepared as an electrospinning solution was reported by Patcharaporn *et al.,* 2007.

Electrospinning of *Plumeria alba* hydroxyapatite nanoparticle with PHB.

The polymer solution of *Plumeria alba* latex serum extract hydroxyapatite nanoparticle with poly-β-hydroxybutyrate was electrospun to fabricate as a scaffold collected on an aluminium foil (Figure 52). Nanofibrous scaffold using polycaprolactone and hydroxyapatite mixed with chloroform and methanol and electrospinned with 0.7 mm distance connected to a high voltage supply of 30kV[8].

Characterization of developed nanofibrous scaffolds

Thickness of developed nanofibrous scaffold (ASTM D 1777-96)

The thickness of the developed nanofibrous scaffold was determined using the thickness gage Tilmet (ASTM D 1777-96). The thickness of the developed nanofibrous scaffold from *Plumeria alba* hydroxyapatite nanoparticle with PHB determined as 0.12

mm. Similarly Rajzer, 2014 reported that the thickness of polycaprolactone with hydroxyapatite nanofibrous scaffold was determined as 0.11 mm - 0.32mm.

Tensile Strength of developed nanofibrous scaffold (Zwick/Roell)

The tensile properties of the developed nanofibrous scaffold were analyzed by Zwick- Roell testing machine at a crosshead speed of 5 mm/min. The rectangular sample was 10 cm long and 2 cm wide were taken for analysis to determine the mean breaking strength, mean breaking elongation and stress/strain percentage. Mean breaking strength and mean breaking elongation of the developed nanofibrous scaffold were determined along with the strain percentage (fig 1). *Plumeria alba* scaffold's mean Breaking Strength Fmax was found to be 1.43 higher flexibility and tensile strength than the other microfibrous or hybrid scaffold developed from polycaprolactone and hydroxyapatite respectively.

Wetting ability for developed nanofibrous scaffold by the Water contact angle

The water contact angle of the developed nanofibrous scaffold measured using drop shape analysis. A single droplet of double distilled water 20µl applied to the scaffold surface and contact angle measurement taken at room temperature indicates the wetting ability of the scaffold. *Plumeria alba developed* nanofibrous scaffold has 105.30 degrees on the right side, 105.13 degrees on the left side and has an average contact angle of 105.22 degrees.

Fourier Transform Infra-red Spectroscopy analysis of developed nanofibrous scaffolds

FTIR analysis represents the biomolecules and functional groups present in the developed nanofibrous scaffolds. There were several peaks found in the FTIR result represent the presence of numerous compounds in the developed nanofibrous scaffold (Figure 2). The strong peaks at 3500.31 cm^{-1} to 3100.28 $cm^{-1}$and 3541.31cm^{-1} represents the presence of the alcoholic functional group of *Plumeria alba* The peaks at 1400.18 cm^{-1}, 800.26 cm^{-1} and 1458.18cm^{-1} represents the presence of the carbonate group and peaks at 1080.85 cm^{-1} and 1041.50cm^{-1} represents the presence of the phosphate group which is the essential compounds present in the hydroxyapatite nanoparticles of *Plumeria alba*. The

presence of polyester from PHB biopolymer was confirmed by the presence of a peak at the range of 1200.30 cm^{-1}. The other peaks represent the presence of secondary metabolites of latex serum extracts because they are the precursors for hydroxyapatite nanoparticle production and act as a templating agent. The result conformed the incorporation of nano-hydroxyapatite with PHB biopolymer for the formation of the nanofibrous scaffold. The absorption band at 3571 cm^{-1} attributed to the presence of the alcohol functional group and the peak at 1457 cm^{-1},1414 $cm^{-1}$and 1457cm^{-1} were the main characteristics of the hydroxyapatite nanoparticles carbonate group. Similarly, FTIR the peaks at 462 cm^{-1}, 565 cm^{-1}, 605 cm^{-1}, 961 cm^{-1}, 1032 cm^{-1} and 1108 cm^{-1} spectrum represents the presence of phosphate group of hydroxyapatite nanoparticle represents the vibrational modes of peak and alcohol group presence at 3569 cm^{-1} .

Scanning Electron Microscopy analysis of developed nanofibrous scaffolds

Scanning Electron Microscope (SEM) performed for electro-spun nanofibrous scaffold of *Plumeria alba* for the observation of morphological appearance. The SEM images with 5000X, 10000X, 20000X and 50000X magnification represent the developed hydroxyapatite nanoparticle [8]. The developed nanofibrous scaffolds of *Plumeria alba* was in the size ranging from 0.5-5µm with a variety of morphologies. Figure 3 represents the SEM image of developed nanofibrous scaffolds of *Plumeria alba*.

Determination of cell proliferation and cytotoxicity for *Plumeria alba* scaffold

Nanofibrous scaffolds of *Plumeria alba* were evaluated for cell proliferation by MTT cytotoxicity test against 3T3-L1 cell line. The observation of cell cytotoxicity study by ELISA reader against 3T3-L1 cells showed significant cell proliferating potential properties compared to the standard drug [11]. Nano scaffold had significant Non-cytotoxic potency against Mouse normal fibroblast cells and Direct Microscopic observations of drug-treated images of cell lines captured with the magnification of 10X shown in figure 4.

With the progress of cell incubation, the cell began to proliferate on the scaffolds on different concentrations of 6.25 %, 12.5%, 24%, 50% and 100% on the 3T3-L1 cells(table 2). Viability of cells on the scaffold was nearly the same at the first day. The end

results of 6.25% concentration showed higher efficiency in cell viability of 99.19 % and in 100% concentration 92.72% viability (fig 5).

The observations strongly suggest the test compound nanofibrous scaffold has non-cytotoxic potential against Mouse fibroblast cells based on the dosage of the drug after the incubation period of 24 hours and further studies need to be performed to evaluate the molecular mechanism of action behind the cell proliferating potential of the Nano scaffold against the fibroblast cells in in-vitro conditions [12]. The cytotoxicity of nano HAp-Cu/PEG 400 determined using MTT assay with rat primary osteoprogenitor cells and these biomaterials found to be non-toxic. The needle- and plate-shaped nano HA induced the most significant cell-specific cytotoxicity and IL-6 expression but showed the least particle–cell association.

CONCLUSION

In the present study, nanofibrous scaffolds were fabricated from hydroxy apatite nanoparticles of *Plumeria alba* by electrospinning technique. Cytotoxicity test and cell proliferation assay also developed a significant advantage of using these scaffolds for biomedical application. Hence, the nanofibrous scaffolds generated by electrospinning may be considered as a promising tool for drug release studies and multitude of tissue engineering applications.

Table 1 Concentrations of cell lines used for the cytotoxicity study

S.No.	Test Compounds	Cell Line	Concentration treated to cells

1	Untreated	3T3-L1	No treatment
2	Standard (Allantoin)	3T3-L1	12.5uM
3	Blank	-	Only Media without cells
4	Nano scaffold	3T3-L1	5 (6.25%, 12.5%, 25%, 50%, 100%)

Figure 1: Tensile strength and strain% of the developed nanofibrous scaffold

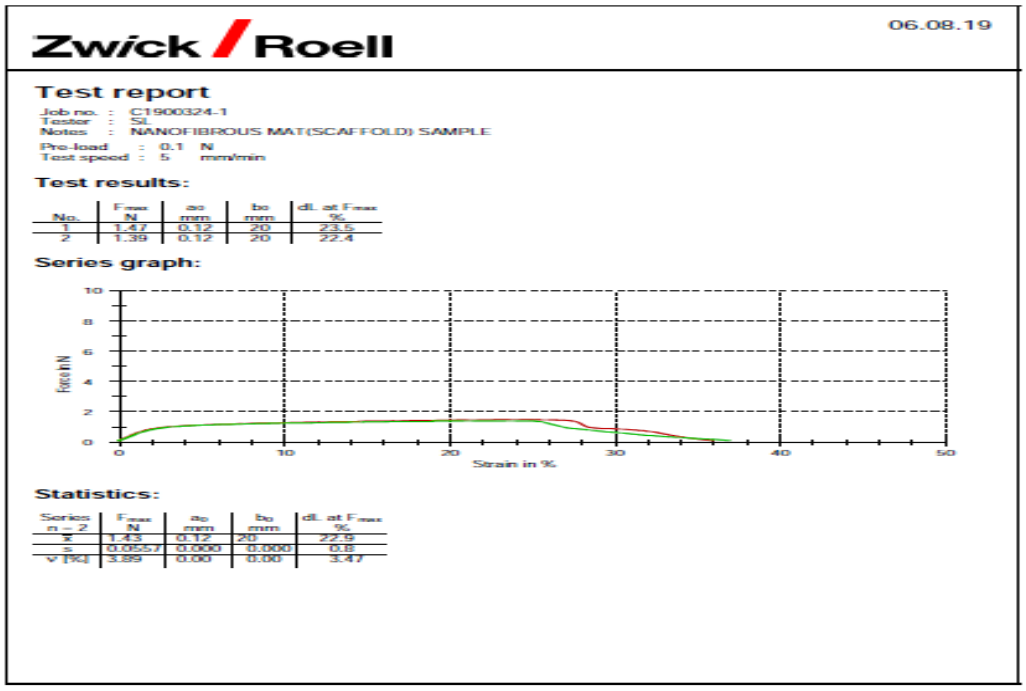


Figure 2: Fourier Transform Infra-red Spectroscopy analysis of developed nanofibrous scaffold of *Plumeria alba*

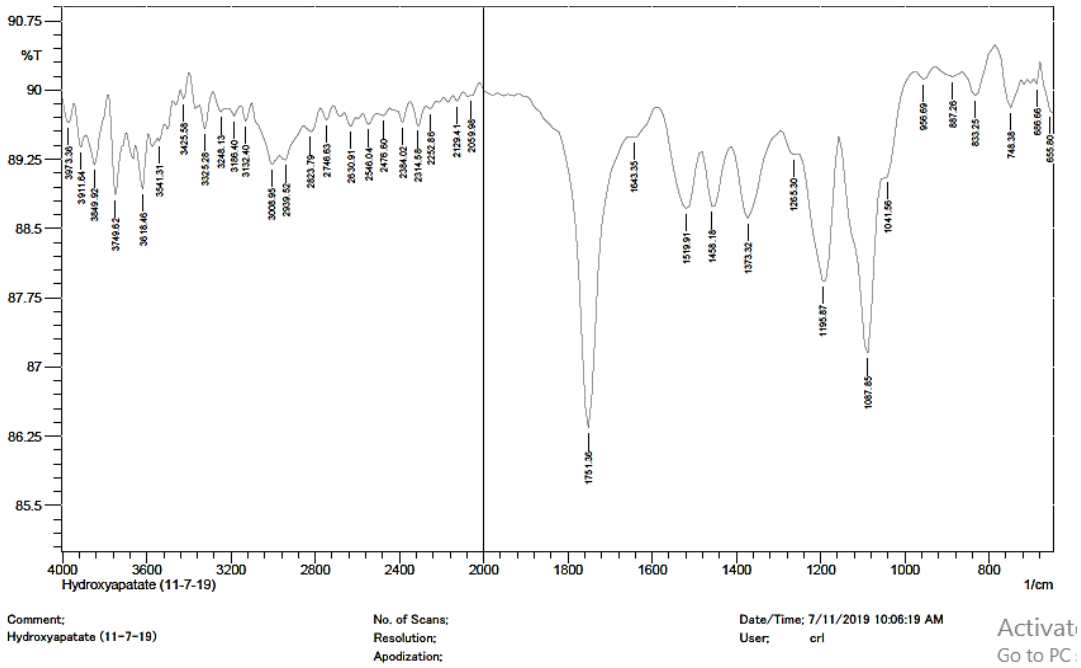


Figure 3: SEM Analysis of Electrospun nanofibrous scaffolds

(a)Plumeria alba

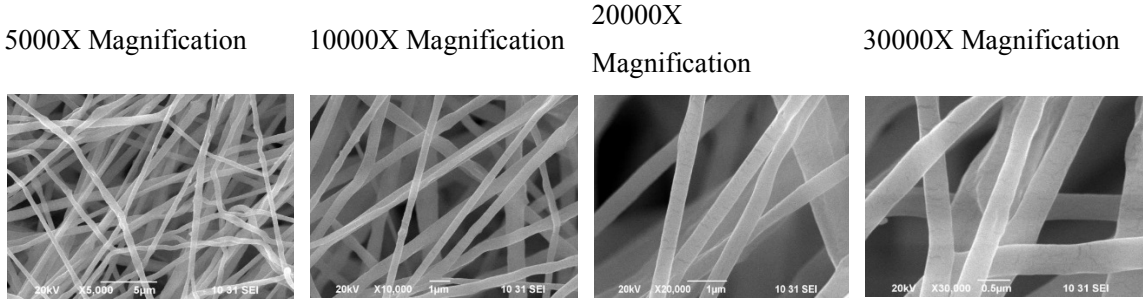


Figure 4: Bar graph depicting the percentage of cell viability on 3T3-L1 cells

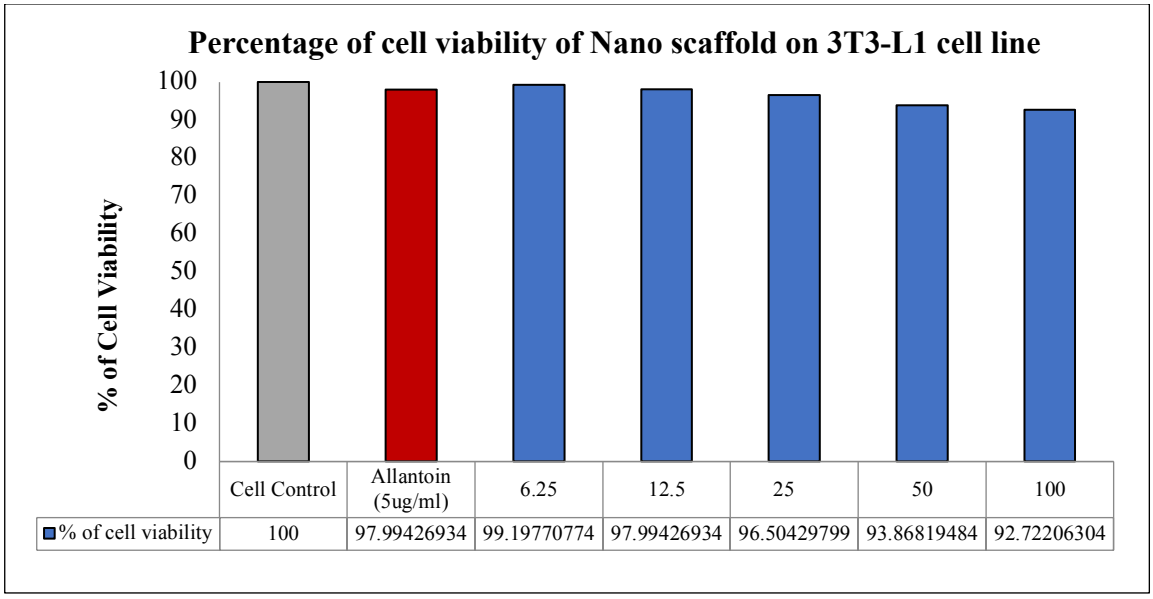
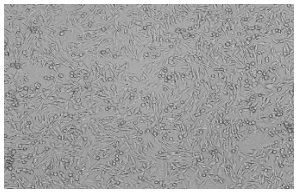


Table 2Percentage of cell viability of Nano scaffold against the 3T3-L1 cells

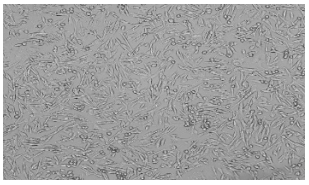
% of Cell viability vs 3T3-L1		
S. No	**Concentration (%)**	**% of cell viability**
1	Untreated	100
2	Allantoin (5ug/ml)	97.99
3	6.25	99.19
4	12.5	97.99
5	25	96.50
6	50	93.86
7	100	92.72

Figure 5: Direct Microscopic observations of drug treated images of cell lines under 10X magnification

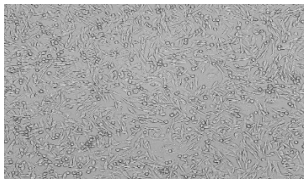
6.25% Concentration



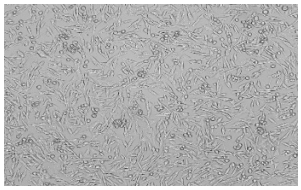
12.5 % Concentration



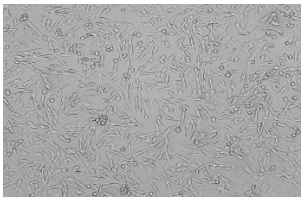
Cell control- Untreated



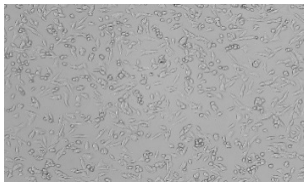
25% Concentration



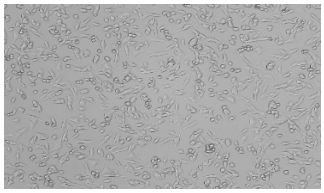
50 % Concentration



100 % Concentration



Allantoin- 5ug Concentration



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