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# BIOSYNTHESIS OF *PARMOTREMA RETICULATUM* EXTRACT INTO SILVER NANOPARTICLES: INDUCES APOPTOSIS AND WOUND HEALING

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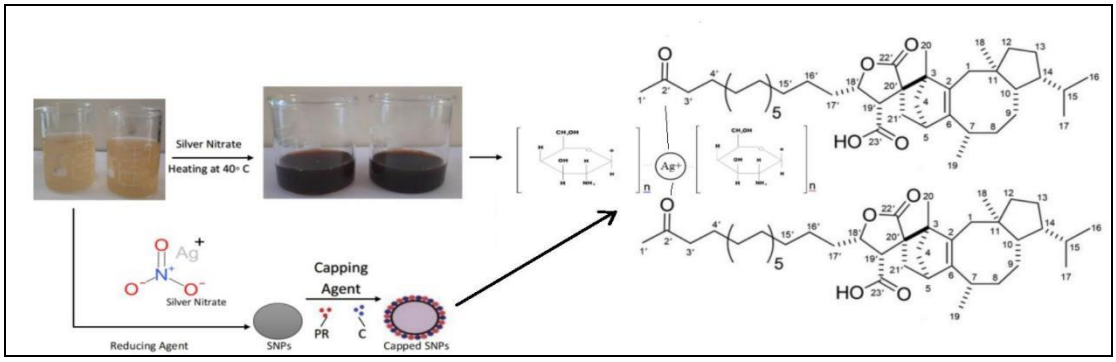
## ABSTRACT

Anticancer and antioxidant properties are present in *Parmotrema reticulatum*. It includes a number of bioactive compounds, including usnic acid, which has been demonstrated in multiple animal models to hasten collagen deposition, angiogenesis, and reepithelialization during wound healing. Here, we demonstrate a simple, one-step biosynthesis of silver nanoparticles using chitosan as a capping agent and hydro-alcoholic extracts of *Parmotrema reticulatum* as a reducing agent. The creation of silver nanoparticles (SNPs) was determined using UV-VIS spectroscopy. Smaller, less aggregated, irregular-shaped SNPs were seen, according to the SEM analysis. PC-SNPs PC-SNPs (50 mM) performed better than all other treatments, according to the MTT test, displayed $IC_{50}$ value of 62.07±9.1 and 56.11±4.2 at 24 and 48 hrs, respectively. KB-3-1 cells treated with optimized PC-SNPs (50 mM) at sub $IC_{50}$ and $IC_{50}$ values of 65.86 µg/ml and 34 µg/ml indicates apoptotic cells with nuclear chromatin condensation and the formation of nuclear fragments and apoptotic bodies. Furthermore, the KB-3-1 oral cell line was used in a scratch experiment to test the PC-SNPs' efficiency in promoting wound healing. It was found that after receiving the PC-SNPs treatment, the KB-3-1 cell line travelled more readily towards the artificial wound. The amount of ROS was found to have greatly decreased. All of the research results pointed to *Parmotrema reticulatum* loaded silver nanoparticles as a potential drug delivery method with anti-proliferative and wound-healing properties.




**Keywords:** chitosan; oral cancer; KB-3-1 cell lines

## 1. Introduction

Oral cancer is the sixth most common malignancy worldwide. The majority of oral cancer cases and one-third of the worldwide burden are found in India. Oral cancer presents a significant health risk to countries going through economic change [1]. Human papillomaviruses and Epstein-Barr viruses are two viral illnesses that are less commonly associated with oral cancer risk than behavioral risk factors including exposure to smoke and excessive alcohol usage [2].

Cancer sickness continues to be one of the world's major health issues despite substantial improvements in anticancer therapy [3, 4]. All known pathways of carcinogenesis have been identified as direct molecular targets for phytochemicals, the secondary metabolites of plants [5-9]. Based on several preclinical and clinical investigations, phytochemicals show tremendous potential for enhancing the clinical status in cancer patients. An important way to finish the traditional management of the disease in terms of either chemoprevention or therapy may be through the appropriate administration of plant-based natural compounds [10].

Lichens develop in challenging environments; as a result, they are repositories of a number of physiologically active compounds that offer them resistance to environmental stress [11].

There are several *Parmotrema reticulatum* in the Dima Hasao Hills area of Assam, North East, India. Numerous lichen species have received a lot of interest recently as sources of natural antioxidants. Anticancer and antioxidant properties are present in *Parmotrema reticulatum*. Six compounds were identified from lichen *Parmotrema reticulatum* in another investigation. They were shown to have the structural components reticulatin, zeorin, leucotylin, lupeol, betulinic acid, and dihydroreynosin. Reticulan is a novel C43-spiroterpenoid metabolite of lichen demonstrated potent α-glucosidase inhibition [12]. The preliminary antioxidant [13] and antibacterial properties of this species have been investigated against breast cancer cells, it demonstrated the ability to induce apoptosis and be cytotoxic [14]. Usnic acid is a dibenzofuran derivative that occurs naturally in the lichen *Parmotrema reticulatum* has potent antibacterial, anti-inflammatory, and antioxidant properties. It has received a great deal of interest as a potential bio-active agent for wound healing therapy because of its ability to promote tissue regeneration and reduce infection rates. According to various *in-vitro* and *in-vivo* studies, usnic acid has the capacity to accelerate wound healing, particularly in burns, diabetic ulcers, and chronic wounds [15].

The importance of nanoparticles (NPs) in contemporary medicine has repeatedly been recognised in recent years. Consequently, nanomedicine's long-term goal is to enhance the healthcare system by more effectively treating fatal illnesses. Consequently, the discovery of nanomedicine molecules has enormous promise for enhancing cancer treatment strategies [16-19].

Silver nanoparticles (SNPs) have received the most attention among the nanoparticles because of their bio-compatibility, antibacterial, anti-fungal, anti-inflammatory, and anticancer potential [20]. Due to its antiproliferative and apoptosis-inducing qualities, nanosilver has been established in recent years to be an effective anticancer drug in multiple investigations [21]. According to Iravani *et al.* SNPs may be created by chemical, physical, and biological processes. Researchers have been studying biological systems, such as plants, algae, bacteria, yeast, and fungus, for the last ten years [22]. These systems have the ability to oxidise inorganic metal ions into metal nanoparticles through the reductive abilities of the proteins and metabolites they contain. The traditional chemical and physical production of SNPs for use in anticancer therapy can be replaced by such environmentally friendly techniques [23]. Nanoparticles can be created utilising bio-synthetic techniques by reducing and stabilising them using natural substances. It has been proposed to use organisms for the biosynthesis of metal and semiconductor NPs as an environmentally and economically friendly alternative to chemical and physical methods [22].

In this work, silver nanoparticles made from *Parmotrema reticulatum* extract were produced, characterised, and tested for their ability to inhibit cell proliferation and promote wound healing.

## 2. Material

### *2.1 Chemicals and reagents*

Silver nitrate was purchased from Loba Chemie, Pvt. Ltd., Mumbai, India. *Parmotrema reticulatum* was procured from a local market at Koilaha, Kaushambi, U.P., India. Chitosan was obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All the other chemicals utilized for the study were of analytical grade and obtained from local commercial sources.

### *2.2 Cell line*

KB-3-1 cancer cell line was procured from the cell bank of the National Centre for Cell Science (NCCS), Pune, India. KB-3-1 cells were grown in high glucose Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 100 units/mL penicillin, 100µg/mL streptomycin, in a proper humidified atmosphere of 5% CO2 and air at 37°C. Before use, KB-3-1 cells were taken from fluid nitrogen and washed two times with fresh culture medium. After two passages, we prepared KB-3-1 cell for experiments and 1% penicillin-streptomycin arrangement (Sigma Chemical Co.) were utilized.

### *2.3 Preparation of parmotrema reticulatum (P) extract*

For around 15 days, the lichen was dried in the shade and powdered, then defatted using petroleum ether using soxhlet device. The defatted lichen was then dried before being thoroughly extracted for 15 days by maceration process using water and ethanol (80:20). The extract was allowed to concentrate by evaporation and then kept in a tightly closed container for future investigation [24, 25].

### *2.4 Preparation of Silver Nanoparticles (SNPs)*

SNPs were prepared by simple chemical reduction of silver nitrate using chitosan (C) and P extract as a reducing or stabilizing agent [26]. An equal volume of chitosan solution (prepared by dissolving chitosan (0.03g/ml in 1 % v/v acetic acid solution) and P extract was taken and mixed through continuous stirring on magnetic stirrer until mixture become homogenous. Prepared mixture was divided into two separate beakers containing 5ml each, then equal volume of silver nitrate solution (10 and 50 mM) was added to it. The chemical reduction reaction was allowed to occur through heating in the water bath at 40°C for 5 minutes. The light yellow color of the mixtures turned dark brown, which indicated the formation of SNPs, as per previously reported studies [27]. The resultant formulation was stored for further characterization.

### *2.5. In-vitro characterization of PC-SNPs*

#### *2.5.1. UV–visible spectroscopy*

UV-spectroscopy was employed to monitor the synthesis of SNPs. The wavelength range was set at 300-500 nm on UV–Vis spectrophotometer model LT-2910 (Labtronics, India).

#### *2.5.2. Particle size and PDI*

A particle size analyzer was utilised to evaluate the PDI and particle size of the prepared formulation using the accepted approach, employing technology based on laser light scattering [28].

#### *2.5.3. Morphological evaluation*

Scanning electron microscopy (SEM) was used to analyse the morphology and shape of the prepared PC-SNPs. The SEM sample was placed on top of a micro cover-slip, allowed to air dry, and then was sputtered with Au/Pd. Accordingly, produced samples were examined using a Jeol (JSM- 6490LV) electron microscope from Japan at 20kV accelerating voltage and 15,000X magnifications [29, 30].

### *2.6 Cell lines studies*

#### *2.6.1 Cell lines culture*

Firstly, before preparing the media, the laminar hood was cleaned with alcohol and was kept in UV for 10 minutes. For preparing the media, 0.9 g of DMEM powder and 0.74 g sodium bicarbonate powder was weighed and brought in the laminar hood and added in 200 ml of distilled water and mixed well until the powder dissolves. After the powder is completely dissolved, 28 ml of media was removed from the 200ml of media. In the 172 ml media, 24 ml of FBS and 4 ml of antibiotics were added and mixed. The media was filtered with the help of a 50 ml syringe and syringe filter and the pH was checked, which was 7.4. The media was preserved in the refrigerator until further use. KB-3-1 cell lines were bought from the National Center of Cell Culture Science (NCCS), Pune, Maharashtra. The cell lines bought were cultured in media in a T25 flask and were kept in an incubator at 35$^{o}$C and 5% $CO_2$. The cells were seen confluent after 3 days when observed under an inverted microscope. When 90% of confluent cells are seen, they are passed into one T25 flask and one T75 flask. For passing, the confluent T25 flask was placed in the laminar hood after cleaning the laminar hood with alcohol and the media present in the confluent flask was discarded and 1000 ml of trypsin was added. After adding the trypsin the flask was kept in the incubator for 1min and observed under the inverted microscope and when the adherent cells were seen observed moving. The flask was put back into the laminar hood and 500 ml of cells were transferred to a T75 flask and 250 ml of cells were transferred into another T25 flask and the remaining 250 ml of cells were kept in the primary flask.4 ml of media was added in both T25 flasks and 8 ml of media was added in the T75 flask.

#### *2.6.2 Cell Viability Assay*

Confluent flasks were observed under inverted microscope and the most confluent flask was treated with 1000 ml trypsin. After trypsinization, 700 ml cells were added in 20 ml of complete media and 300 ml of cells were kept in the flask by adding 4ml of media. A 96 well plate was taken and each well was filled with 200 ml from the 20ml of cell containing media. Another plate was prepared by the same method. The plate was labelled and kept in the incubator for 24 hrs. After 24 hrs the plate was treated with the 8 different concentrations like *100* mM*/ml, 50* mM */ml, 40* mM */ml, 30* mM */ml, 20* mM */ml, 10* mM */ml, 5* mM */ml, and 1* mM */ml* were given in a 96 well plate. Some wells were kept control. The 2 plates after giving the treatment were kept for 2 different time periods (24 and 48 hrs). After a specific time periods, 10 ml of MTT assay was added to each well and the plate was wrapped with silver foil and kept in the incubator for 3 hrs. The MTT assay measures the cellular metabolic activity of the cells and acts as an indicator for cell viability, proliferation and cytotoxicity. Later the plate was removed and each well was emptied by using micro-pipette and 100 ml of DMSO was added in each well and the plate was kept in the incubator for 10 minutes. After 10 minutes, the plate was analysed by the ELISA reader and data was obtained [31].

#### *2.6.3 Scratch assay:*

The most confluent flask was selected for colony-forming cell assay after observing under an inverted microscope. Media from this flask was first discarded and 500 µL trypsin was added to the flask and incubated in the incubator for 2-3 minutes and then observed under the microscope, as soon as the adherent cells were observed to be moving 50 µL of the cells were seeded in 6 well plate and fresh 4 ml media was added to each well. The plate was incubated for 48 hours. Next day, the cells were treated with prepared SNPs in two different concentrations like 65.86 µM/ml, 34 µM/ml, while the third and sixth wells were kept as control. The plate was again incubated for 24 hours. Following 24 hours, the media from the wells was discarded and cells were washed with 2 ml of 1X PBS. Fixation with 1 ml of chilled methanol was performed. Methanol was added carefully in order to not disrupt the colonies.

The plate was then incubated for an additional 60 minutes. Methanol was aspirated and a sufficient 0.5 % crystal violet staining solution was added to cover the cells. The plate was incubated for 40 minutes at room temperature. The cells were washed with distilled water to remove excess dye.

Scratches were then counted manually using an inverted microscope. Wound is defined as a group of at least 50 cells [32, 33].

### *2.6.4 ROS generation assay*

ROS accumulation was evaluated by the deploying of cells to both the fluorescence microscopy and flow-cytometric methods. After cell-incubation for 24 h with Sub $IC_{50}$ and $IC_{50}$ concentrations of 65.86 µM/ml, 34 µM/ml PC-SNPs, cells were properly fixed and incubated in 10 µm DCHFDA (Sigma), for 20 min in the dark condition. Cells were then analysed using a fluorescence EvosFlc microscope (Invitrogen).

### *2.6.5 Apoptosis assay*

The KB-3-1 cells were cultured as per a standardized procedure as previously described [34, 35]. The cells were further treated with PC-SNPs formulation with their Sub $IC_{50}$ and $IC_{50}$ concentrations of 65.86 µM/ml, 34 µM/ml PC-SNPs and incubated for 24 h. Following the incubation, the cells were trypsinized and 100 µL of cell suspension was from each sample treated cells were transferred to separate tubes. After transfer, the cells, 100 µL of the Annexin V & amp; Dead Cell Reagent was added to each tube and mixed thoroughly by pipetting up and down. Following pipetting, the tubes were kept for incubation at room temperature for 20 minutes in dark. After incubation, the cells were analyzed for apoptosis using a fluorescence EvosFlc microscope (Invitrogen). The treated cells were gated against untreated control cells.

## 3. Results and Discussion

### *3.1 Preparation and in-vitro characterizations of PC-SNPs*

Chitosan and parmotrema extract were used to reduce silver nitrate in order to create PC-SNPs. As a reducing/capping agent, parmotrema extract and chitosan make a suitable choice since they are nontoxic, natural, and loaded with polar functional groups. When SNPs are ready, the colour changes from a pale yellow to a dark brown. The shift in colour is caused by the stimulation of Ag+ atoms' surface plasmon vibrations. The chitosan and phytochemicals in the parmotrema extract would attach to the silver nitrate when it was introduced to the parmotrema extract/chitosan solution, most likely through electrostatic interactions. It is anticipated that in an alkaline environment, -OH/=O groups in phytochemicals and the electron-rich oxygen atoms of -OH/-NH2 groups in chitosan will interact with electropositive metal cations. Silver ions are reduced to elemental silver as a result of this interaction. The graphical abstract also illustrates the possible process via which phytochemicals and chitosan interact with silver ions. The components are also in charge of preventing SNP aggregation by complexing with silver ions, which lends credence to the capping theory [27, 36].

UV-visible spectroscopy is an efficient method for investigating the biosynthesis of SNPs. The absorption maximum was evident at 430.2 nm, which signified the formation of SNPs [36]. The morphological evaluation of PC-SNPs through SEM displayed an irregular shape, as displayed in Figure 1.

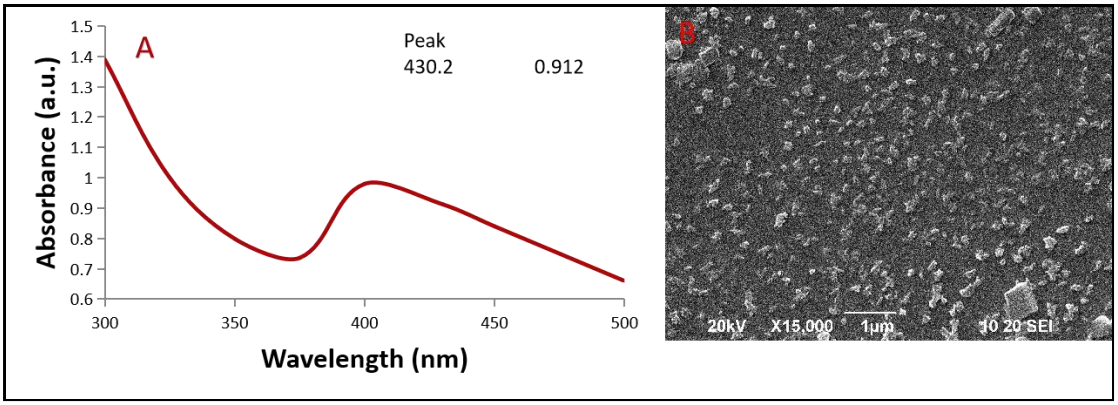



**Figure 1.** A. UV–Vis spectrum of PC-SNPs and B. SEM image of PC-SNPs

The average size of PC-SNPs (10 mM) and PC-SNPs (50 mM), as determined by particle size analysis, was found to be 23.7 nm and 51.5 nm, respectively, with PDI values of 0.48 and 0.40, respectively. The findings showed that the size of the nanoparticles grew together with the concentration of silver nitrate from 10 to 50 mM. Chitosan is used as a stabiliser for SNPs because it stops particles from growing after adhering to the surfaces of SNPs. Larger NPs developed as a result of less chitosan and extract being adsorbed onto the SNPs when the concentration of metal salt increased without changing the content of chitosan and parmotrema extract. Therefore, by varying the concentration of metal salt throughout the synthesis process, SNP particle size may be adjusted. Silver nanoparticles were out to be a superior option since they had a higher surface area than bigger nanoparticles, which may have a higher volume but a smaller surface area. Additionally, the produced metal particles were homogeneous since each preparation's PDI values were less than 0.5.

### *3.2 Cell line studies*

The formulations were then subjected to cytotoxicity assay with the help of MTT assay wherein the treatment with *8 different* concentrations like 100 *mM*/ml, 50 *mM* /ml, 40 *mM* /ml, 30 *mM* /ml, 20 *mM* /ml, 10 *mM* /ml, 5 *mM* /ml, and 1 *mM* /ml were given in a 96 well plate. Some wells were kept as controls. The controls had only cells and media. These plates were then kept at two different time periods, 24 and 48 hours [37]. The results of cytotoxicity assay indicated that PC-SNPs (50 mM) and P-SNPs (50 mM) showed much better results than P-SNPs (10 mM) and PC-SNPs (10 mM). $IC_{50}$ values for P-SNPs at two different time intervals were 64.07 ± 9.1 (µM/ml) and 60.11 ± 4.2 (µM/ml) whereas for PC-SNPs were 62.07 ± 9.1 (µM/ml) and 56.11 ± 4.2
(µM/ml) (Table 1, Figure 2 and 3). The most effective activity was shown by PCSNPs (50 mM) in comparison to other treatments. So, further tests were performed by taking PC-SNPs (50 mM).

**Table 1:** *In vitro* cytotoxicity evaluation using MTT Assay

| **Treatment** | **$IC_{50}$ values (µM /ml)** | |
|---|---|---|
| | 24H | 48H |
| | KB-3-1 | KB-3-1 |
| **P-SNPs (10 mM)** | 65.86 ± 5.1 | 61.34 ± 5.7 |
| **P-SNPs (50 mM)** | 64.07 ± 9.1 | 60.11 ± 4.2 |
| **PC-SNPs (10 mM)** | 66.56 ± 8.7 | 62.54 ± 3.6 |
| **PC-SNPs (50 mM)** | 62.07 ± 9.1* | 56.11 ± 4.2* |

Data is expressed as Mean ± SD (n=3) and (*) represents significant value.

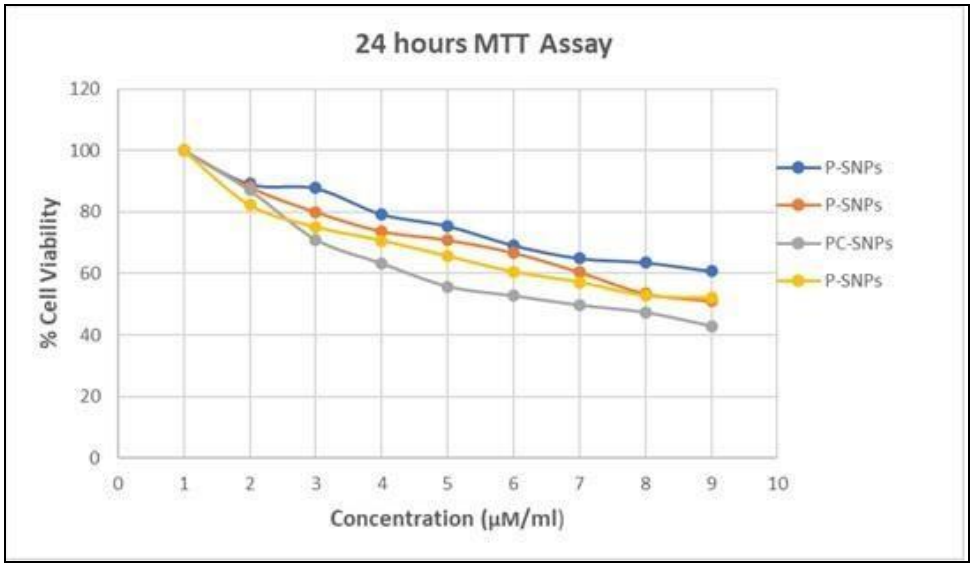



**Figure 2.** Cells after 24 hours of treatment.

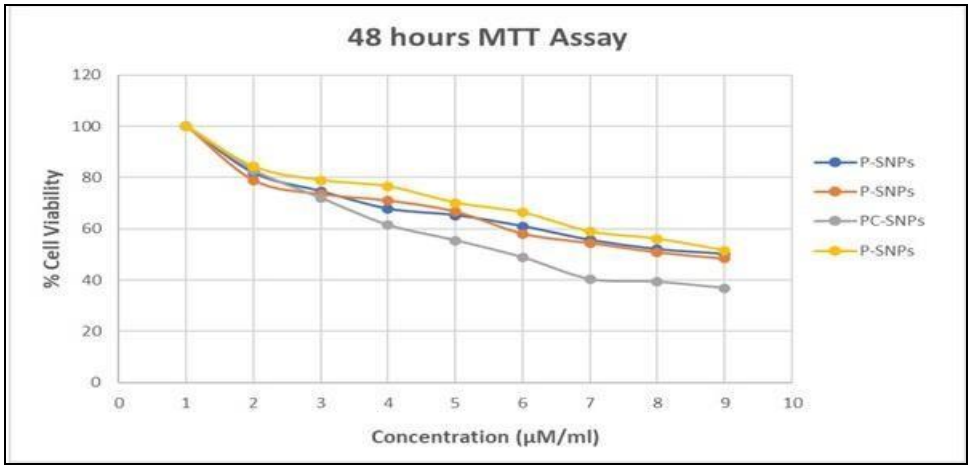



**Figure 3.** Cells after 48 hours of treatment.

Since apoptosis is a plausible mode of controlling cancer cell proliferation by an anticancer agent; we next studied the role of PC-SNPs (50 mM) formulation in inducing apoptosis in KB-3-1 cell line. Microscopic analysis was performed with cells treated with the $IC_{50}$ value 65.86 µg/ml and Sub $IC_{50}$ value 34 µg/ml PC-SNPs for 24 hr (Figure 4 and 5). In KB-3-1 cell, both treatments induced more than 3 folds augmentation in apoptosis. The increase in apoptosis in KB-3-1 cells was found to be 2.7 folds (120%) (65.86 µg/ml) and 11 folds (86%) (34 µg/ml), respectively.

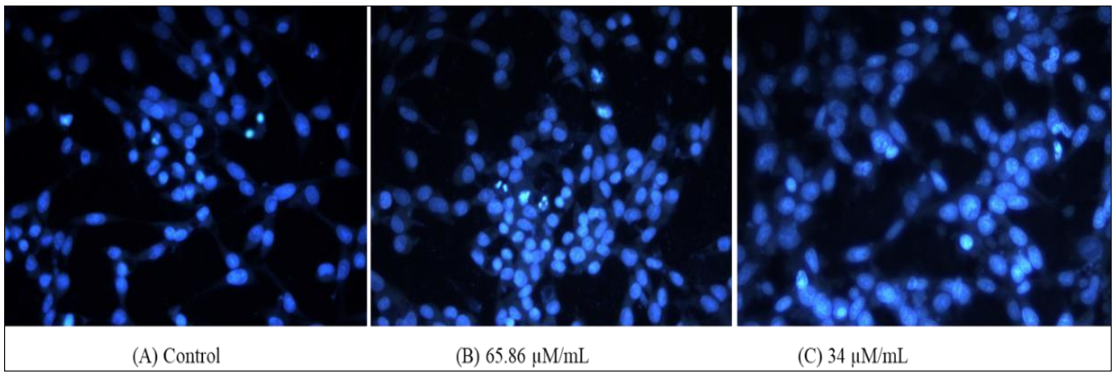



**Figure 4.** KB-3-1 cells treated with optimized PC-SNPs (50 mM) at sub $IC_{50}$ and $IC_{50}$ values of 65.86 µg/ml and 34 µg/ml indicates apoptotic cells with nuclear chromatin condensation and the formation of nuclear fragments and apoptotic bodies.

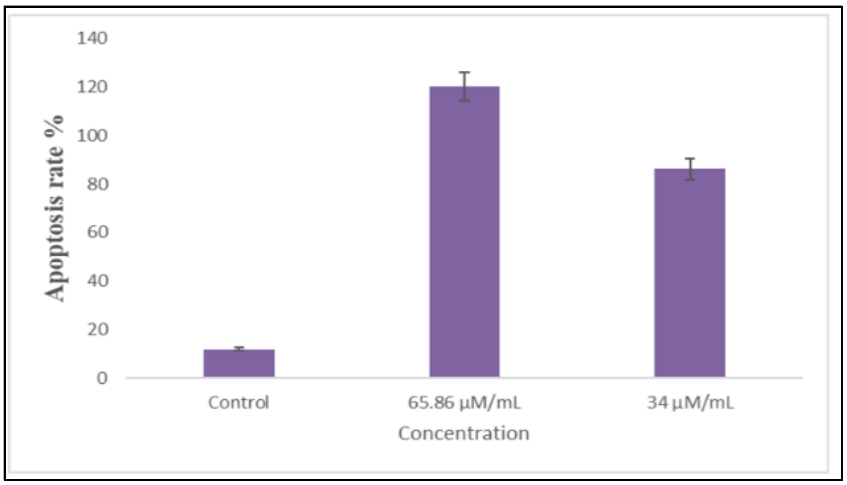



**Figure 5.** The Bar Graph represents the % change in apoptosis in KB-3-1 cells in the absence or presence of optimized PC-SNPs (50 mM) formulation treatment at sub $IC_{50}$ and $IC_{50}$ values of 65.86 µg/ml and 34 µg/ml. All values represent the mean ± SE.

On the other hand, elevated ROS levels may lead to oxidative DNA damage. Given that PC-SNPs formulation induced DNA damage in KB-3-1 cells, we decided to detect the effect of PC-SNPs (50 mM) formulation on ROS level in KB-3-1 cells. The ROS level was determined by DCFH-DA staining via a fluorescence microplate reader. As revealed in Figure, compared to the control cells, the intracellular ROS was significantly increased in the KB-3-1 treated cells in a concentration-dependent manner The $IC_{50}$ value 65.86 µg/ml and Sub $IC_{50}$ value 34 µg/ml PC-SNPs for 24 hrs (Figure 6 and 7).

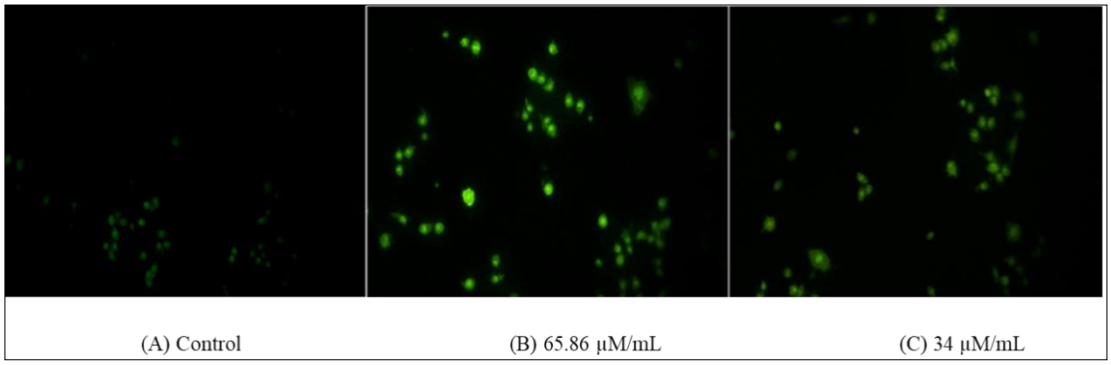



**Figure 6.** ROS generation after 48 hours, (A) Control cells (B) $IC_{50}$ value of PC-SNPs (50 mM), (C) Sub $IC_{50}$ value of PC-SNPs (50 mM).

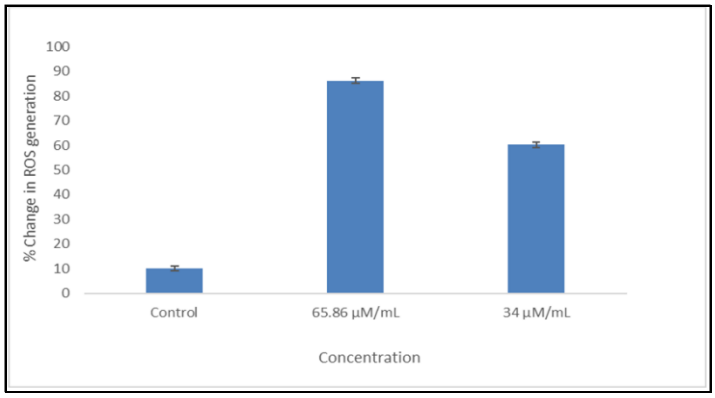



**Figure 7.** The Bar Graph represents the % change in ROS generation in KB-3-1 cells in absence or presence of optimized PC-SNPs formulation treatment with $IC_{50}$ and Sub $IC_{50}$ value for 24 h in KB-3-1 cells. All values represent the mean ± SE.

The Scratch assay measures a single cell's ability to heal a wound. It basically examines each cell in a population's ability to divide indefinitely. The results indicated that wound healing is increased by the treatments given to the wells. It indicated that after 48 hours PC-SNPs (50 mM) displayed increased inhibition compared to control wells (Figure 8 and Table 2).

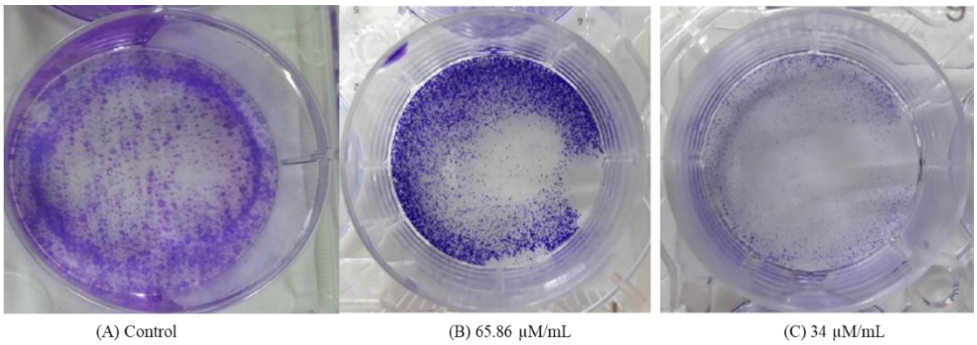



**Figure 8.** Scratch formation, observed after 48 hours, (A) Control cells (B) $IC_{50}$ value of PC-SNPs , (C) Sub $IC_{50}$ value of PC-SNPs .

**Table 2:** *In-vitro* scratch formation assay

| TREATMENT | DOSE | CELLS PLATED | COLONIES OBTAINED | PLATING EFFICIENCY | SURVIVING FRACTION |
|---|---|---|---|---|---|
| PC-SNPs (50 mM) | 0 | 1000 | 57 | 0.057 (57%) | 1 (100%) |
| | 100 µL | 1000 | 31 | 0.031 (31%) | 0.543 (54.3%) |
| | 200 µL | 1000 | 22 | 0.022 (22%) | 0.385 (38.5%) |

## 4. Conclusion

PC-SNPs formulations were developed and evaluated successfully. Formulations were screened for the antiproliferative and wound healing studies based on the cytotoxicity demonstrated on KB-3-1 cells in a time-dependent manner and the Scratch test, respectively. The findings demonstrated that *Parmotrama reticulatum* silver nanoparticles may be a valuable tool to anticipate wound healing, neoplastic damage, and dysregulation of proliferation markers. An excellent place to start for the development of alternative medicines based on silver nanoparticles from such lichens is our study's provision of preliminary data for the creation of novel antiproliferative and healing candidates and comprehension of their anti-cancer processes.

Furthermore, additional research must be done, including *in-vivo* and clinical trials, to look at the impacts on the human body, including safety and anti-cancer properties, before silver nanoparticles may be widely used in industry.

## Author Contribution

AP wrote the main manuscript text and prepared it with the help of MV. RT did extraction of *Parmotrema reticulatum*. AP and HT conducted the experimental activities. AP did experimental activities, related to pharmaceutics and HT did experimental activities related to cell lines. MA conceptualized, coordinated and supervised the activities. All authors reviewed the manuscript.

## Disclosure

No potential conflicts of interest were reported by the authors.

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