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Original article

Anti-cancer activity of biosynthesized silver nanoparticles using *Avicennia marina* against A549 lung cancer cells through ROS/mitochondrial damages

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ABSTRACT

The biosynthesized Ag NPs was synthesized by using marine mangrove plant extract *Avicennia marina*. The synthesized Ag NPs was confirmed by various physiochemical characterization including UV-spectrometer and XRD analysis. In addition, the shape and of the synthesized Ag NPs was morphologically identified by SEM initially and TEM finally. After confirmation, the anti-cancer property of synthesized Ag NPs was confirmed at 50 μg/mL concentration against A549 lung cancer cells by MTT assay. Further, the ability to stimulate the ROS generation and mitochondrial membrane at the IC_{50} concentration of Ag NPs was confirmed by fluorescence microscopy using DCFH-DA and rhodamine 123 dyes respectively. Finally, the result was concluded that the synthesized Ag NPs has improved anti-cancer activity against A549 cells at lowest concentration.

1. Introduction

Lung cancer is a dominant cancer and with high morbidity and mortality rates worldwide. It hikes day by day and estimated that, about 10,000 new cases annually (Malvin et al., 2020). About World Health Organization (WHO) statement, more than 3000 people will be died in 2019, due to the cigarette smoking, exposure to tobacco usage, physical inactivity and westernized diets (Rajivgandhi et al., 2018a). Also, it is a second most leading non-communicable disease accounted 50% in men and 30% in women (Rajesh kumar et al., 2018; Wong et al., 2017). The global death rate through lung cancer is increased frequently due to the cancer habitat including smoking, physical inactivity and modernized diet. It reported approximately 25,000 mortality rate in developing and developed countries. Recently, physical, chemical, radiation therapy chemotherapy and surgical methods of the treatments are not better suitable. Development of cancer cells by drug resistant is a major reason for failure of drugs (Vijayakumar et al., 2019). Discovery and development of advanced drugs against cancer cell proliferation is facing great challenges due to side effects, toxicity and more cost efficiency (Anna et al., 2017). Due to this defect, the emerging need to discover novel, cost effective and bio-compatible therapeutic approaches against cancer cells are needed.

Marine mangrove plant *Avicennia marina* is an important plant which grows in difficult environment condition such as high salt, pH, temperature, and excess carbon and nitrogen sources. It is used in pharmaceutical application and led the detection of variety of phytochemicals as effective chemo-preventive agents for different biological activities, particularly effective against cancer cells (Eswaraiah et al., 2020). It is an excellent reducing agents used as a reducing agents for various nanoparticle synthesis. It is an important plant material to apply in various nanoparticles synthesis and provide increased biological activities than other plants due to the various environmental stresses including pH, temperature, NaCl,

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humidity and etc (Swagat Kumar et al., 2018). Recently, Momtazi-borojeni et al. (2103) reported that the anti-cancer effect of *A. marina* by stimulating the apoptosis in various cancer cells such as MCF-7, MDA-MB 231, HeLa, A549 (Manilal et al., 2016)

Recently, the application of nanoparticle using plant material is heightened to revolutionize in various sectors such as drug delivery (Khalid et al., 2019), textiles (Mahmoodreza et al., 2019), biomedical (Arya et al., 2019), water treatment (Rupasree et al., 2019), agriculture (Diasa et al., 2019) and pharmaceutical industries (Ahmed et al., 2017). Between the nanoparticles types, Ag NPs is an important nanoparticle, excellently used in almost all the industries and health care settings (Pratik et al., 2019). It is used very widely due to the therapeutic perspective, such as anti-bacterial, anti-fungal, inflammatory response, anti-viral and anti-cancer activities (Amrithpal et al., 2019; Feizi et al., 2018; Rajivgandhi et al., 2019b; Dixit et al., 2018). Ag NPs are more beneficial compared with any other bulk materials because it has excellent structural arrangement, stability, and larger surface areas (Rufen et al., 2019). The physical, chemical routes of synthesis methods are very harmful compared with biological routes, because it is more eco-friendly environment, small investment and limited toxicity (Suganya et al., 2019). The more advantages of biological synthesis Ag NPs is more energy, more temperature and deadly chemicals. Sometimes the reducing agent of plant materials have great role to Ag NPs, the phenolic and phytochemicals are helped to improve the Ag NPs activity in biological activities. It extended to anti-oxidant activity role as they indicated less toxicity in living materials (Rajathi and Suja, 2017). It also acted as an excellent anti-cancer agent against various cancer cell lines because it acted as a selective target inhibitor in mitochondrial region, generation of ROS, ATP and caused intracellular DNA development (Adwin Jose et al., 2018).

In addition, biological synthesized Ag NPs reduced the time, temperature, size, shape and toxicity level (Inbatamizh et al., 2016). It also increased the yield, low energy supply and insufficient purification. Recently, green synthesized Ag NPs delivered the great attention in biomedical application and focused on very extensive research because of the ecofriendly nature. It removes the more purification process of the culture, easy scale-up, increased large scale synthesis and cost-effectiveness. Surprisingly, plant mediated Ag NPs involves mixing the aqueous extract with silver nitrate solution (Ganasekar et al., 2012).

To try to overcome this problem, marine mangrove plant *Avicennia marina* mediated Ag NPs has indicated as a promising alternative solution to fight against cancer cells, without any side effects (Behbahani et al., 2018). Previous reports also supported to the plant *A. marina* has excellent potential biomedical agent with anti-microbial, anti-tumor, cytotoxic, anthelmintic, analgesic, anti-inflammatory, hypertensive, immune stimulatory and anti-cancer activities (Manilal et al., 2016; Eswaraiah et al., 2020). Interestingly, there is no previous report on the biosynthesis of Ag NPs from marine mangrove plant *A. marina* against A549 human lung cancer cell lines (Momtazi-borojeni et al., 2103). Therefore, current study is focused on biosynthesize Ag NPs from *A. marina*, and to evaluate its anti-cancer properties for lung cancer of A549 cells through reactive oxygen species/mitochondrial damages.

2. Materials and methods

2.1. Needed materials and reagents

The fresh healthy *Avicennia marina* plant leaves were collected from Muthupet Mangrove system, Thiruvarur District, Tamil Nadu, India. For removal of infected leaves and surface contaminants, the samples were washed by tap water and followed by dis H_2O. The florescent dyes of AO/EB, DAPI, Rhodamine 123 were procured from Suresh Scientific & Co, Tamil Nadu, India. The A549 human lung cancer cells were procured from the King Institute of Medicine, ICMR institute, Chennai. The cell line was maintained in its respective media and supplemented with streptomycin, gentamycin antibiotics (100 mg/mL), and 10% FBS at 95% humidity with 5% CO_2.

2.2. Ag NPs synthesis

Avecinnia marina extract (10ML) and watery arrangement (90 mL) with currently made 1 mM of silver nitrate were taken in tube and incubated at 37 °C for 2 h for Ag NPs synthesis. Next, green color plant extract were changed to pale yellow color was indicated by naked eye observation. The formed solution was centrifuged at 5,000 rpm at 30 min at 4 °C to purify the nanoparticles, followed by dried and using sterile H_2O for washing. Finally the sample was maintained at incubator at 45 °C for 24 h. The collected powder was maintained in the refrigerator for further use (Yin Yin et al., 2010).

2.3. Characterization of Ag NPs

The synthesized silver nanoparticle was characterized by various spectroscopy UV–vis spectroscopy and XRD instrument (Shimadzhu, Japan), and followed by previous reports of Vijayan et al. (2017), Hileuskay et al. (2020).

2.3.1. Detection of Ag NPs by UV–Vis spectrometer

The preliminary confirmation of biosynthesized Ag NPs was calculated with 200–800 nm wave length of O.D value by UV–Vis spectroscopic analysis (UV, Nanotrop, 2000-r) and millipore water was used as a blank. The result was plotted by using Origin Software.

2.3.2. XRD analysis of Ag NPs

The powdered XRD of synthesized powdered nanomaterial was calculated by using Bruker AXS X-ray diffractometer with diffraction intensities at 2θ range from 10° to 80°.

2.3.3. SEM and TEM analysis of Ag NPs

The surface integrity and morphological confirmation of Ag NPs was viewed by SEM using sputter coated grid with 120 kV (JEOL Tokyo, Japan). In addition, the final confirmation of Ag NPs morphology was effectively analyzed by TEM (Shimadzhu, Germany). The morphological analysis of Ag NPs identification was followed by previous report of Siman et al. (2013).

3. Anti-cancer studies

3.1. Cytotoxicity assay

The cytotoxicity assessment of synthesized Ag NPs against A549 lung cancer cells was detected by MTT (dimethylthiazol-diphenyl tetrazolium bromide) assay (Venugopal et al., 2017). Briefly, one day old culture of cancer cells was seeded into the 96-well plate containing complete medium in the ration of $\sim 2 \times 10^4$ cells and incubated at 37 °C for 24 h with reduced pressure of 5% CO2 and 95% humidity. After incubation, various concentrations (5–50 μg/mL) of DMSO sonicated Ag NPs was added into the 96-well plate and without addition of Ag NPs containing well were used as a control. After incubation, 15 μl of MTT solution was added in tested wells and allowed to incubate at 37 °C for 4 h. After incubation, the formazan crystal formation was observed in the treated wells, and followed by diluted the samples using DMSO.

Finally, the formation of color intensity containing wells was measured by spectrophotometer (Shimadzu, Japan) at the O.D 540 nm. Based on the triplicate values, the result of the cytotoxicity effect of Ag NPs was obtained in percentages using following formula,

$$\text{Percentage of } IC_{50}\ [\text{Mean O.D}_{\text{Control}} - \text{Mean O.D}_{\text{Test}}] \times 100$$

Finally, the cytotoxicity IC_{50} value of the Ag NPs against proposed A549 cells was noted for further *invitro* and *invivo* experiments.

3.2. Detection of ROS damage

The oxidative stress responses of A549 cells creates intracellular ROS generation due to the treatment of Ag NPs was identified by florescence microscope using ′,7′-dichlorofluorescein-diacetate (DCFH-DA) dye (Baghbani-Arani et al., 2017). Briefly, cover slip containing well grown A549 cells was treated with IC_{50} dose of Ag NPs, and maintained at 37 °C for 24 h for complete treatment. After incubation, the cells were separated by using 1000 rpm centrifugation for 10 min, and followed by washed with 1x PBS. Next, the cells were stained with 10 μg/mL of DCFH-DA for observed the ROS generated cells. The excess stain was removed by using what man No. 1 filter paper using 1x PBS. After washing, the treated and untreated A549 cells were identified by fluorescence microscopy at 40× magnification (Carl Zeiss, Jena, Germany).

3.3. Mitochondrial damage ($\Delta_{\psi}m$)

The florescent images of mitochondrial membrane damaged ($\Delta\psi_m$) effect of Ag NPs treated A549 cells was detected by florescence microscopy using rhodamine 123 as a specific dye (Rajivgandhi et al., 2018b). After treatment with IC_{50} concentration of Ag NPs, the released cytochrome *c* from mitochondria to cytosol in the final stage of apoptosis was clearly indicated that the Ag NPs has anti-cancer effect. In the mechanism of apoptosis, mitochondrial membrane damage was initial confirmation between the Ag NPs and A549 cancer cells. Briefly, the well grown culture on the coverslip was treated by IC_{50} concentration of Ag NPs was incubated at 37 °C for 24. After incubation, the cells were received by centrifugation and followed by trypsinization. After trypsinization, the cells were washed with 1x PBS. After 15 min, 5 μg/mL of rhodamine 123 dyes was added on the cover slip containing cells for 1 h. After incubation, the excess stain was removed by wiping of what man No.1 filter paper using 1x PBS. Finally, the treated and untreated cells were examined under fluorescence microscope for detection of damaged and undamaged membrane morphology differentiation with $\Delta\psi_m$ parameter in the A549 cells.

4. Result and discussion

4.1. Characterization of Ag NPs

The absorption spectra of plant extract surfaces and *Avicennia marine* mediated Ag NPs was presented in Fig. 1a. The UV-spectrometer analysis is an important spectroscopy method to characterize the Ag NPs initially based on the size between 2 and 100 nm. The peak of 420–500 nm range of the peak was excellent absorption peak for Ag NPs. Between these peak, the nanoparticles was released the conduction band of the particle, and it is called as surface plasma resonance (Castro-Aceitunoa et al., 2017). Initially, yellowish to brown color transmission in the conducted reaction is considered as an Ag NPs synthesis. Based on the guidelines, the SPR band of 420 nm in *Avicennia marine* extract synthesized particle was confirmed as Ag NPs in our study. Whereas, there was no any other impurities and contamination was also received in that peak of any visible range. It indicated that the synthesized process was pure and confirmed the silver nitrate was reduced and formed as an Ag NPs (Khoshnamv et al., 2019).

The purity and crystalline nature of the plant mediated Ag NPs powder was used to analyze the XRD spectrum pattern and shown in Fig. 1b. After analysis, the XRD spectra were showed with distinct peak at 2θ range = 30.04°, 37.02°, 40.13°, 48.33° and 78.01° which can be indexed to respective diffraction planes of 120, 200, 230, 240, 301 and 410. Thus, result clearly indicated that the sharp and narrow diffraction peaks formed Ag NPs of our result was highly pure and crystalline nature (Deepika et al., 2020). The resulted XRD pattern was highly correlated with the JCPD file no (04-0783) of Castro-Aceitunoa et al. (2017).

The morphological structure of plant synthesized Ag NPs was identified by SEM image (Fig. 2a). The spherical shape morphology with extremely agglomerated medium sized particles was exhibited by SEM analysis (Fig. 2b). The spherical arrangement in shape and 10 to 20 nm size of the morphology was clearly shown with plant mediated synthesized Ag NPs. Further, the TEM is a powerful instrument used to detect the clear morphology and size distribution of the nanoparticles (Deepika et al., 2020). In particular, it exhibited the individual morphology of Ag NPs and the polydispersity of nanoparticles in defined nanometer. In our study, the synthesized Ag NPs was shown in spherical morphology with between the 10 to 100 nm sizes. In addition, it exhibited with large number of uniform sized nanoparticles at various magnifications (Fig. 3a, b).

4.2. Anti-cancer studies

4.2.1. Cytotoxicity effect of Ag NPs

After 24 h incubation, the concentration dependent inhibition of Ag NPs against A549 lung cancer cells was observed in cytotoxicity assay (Fig. 4). It revealed that the marine mangrove plant *Avecinnia marina* mediated Ag NPs was excellent anti-cancer agent against A549 lung cancer cells. It inhibited the cancer cells at very lowest concentration due to the unexplored nature of mangrove plant containing biochemical components, nutrients, pH, temperature and other stress responses factor (Naveen kumar et al., 2018ab; Rajivgandhi et al., 2018a). Recently, Eswaraiah et al., 2020 reported that the marine mangrove plant *Avicennia marina* has anti-cancer properties against various cancer cells. It enhanced the anti-cancer activity than other plant and chemical synthesized Ag NPs due to the fluctuated marine environmental conditions such as temperature, salinity, pH, carbon and nitrogen sources (Ramachandran et al., 2018). In our study, the 15% inhibition range was observed at 10 μg/mL and 94% inhibition was observed at 80 μg/mL concentration. When the concentration of Ag NPs was increased, the viability of the A549 cells was decreased gradually. The result was proved that the Ag NPs was concentration dependent inhibitor agent for A549 cells. In addition, 54% of inhibition was observed at 50 μg/mL and it was comparatively low than previous reports (Eswaraiah et al., 2020; Momtazi-borojeni et al., 2103). Previously, Momtazi-borojeni reported that the crude extract of *Avicennia marina* exhibited with excellent anti-cancer properties at 250 μg/mL. After addition of MTT solution, the formazan production was appeared in consecutive time interval and identified by changed color formation in the treated well. Whereas, the untreated control cells was showed no any color changes in the respective well. It concluded that the color changes was appeared due to the alteration effect of Ag NPs, and leads to more number of cell death.

4.2.2. Detection of ROS generation

In cancer cells inhibition, the ROS generation in the inside of the cancer cells due to the oxidative stress responses that stimulated

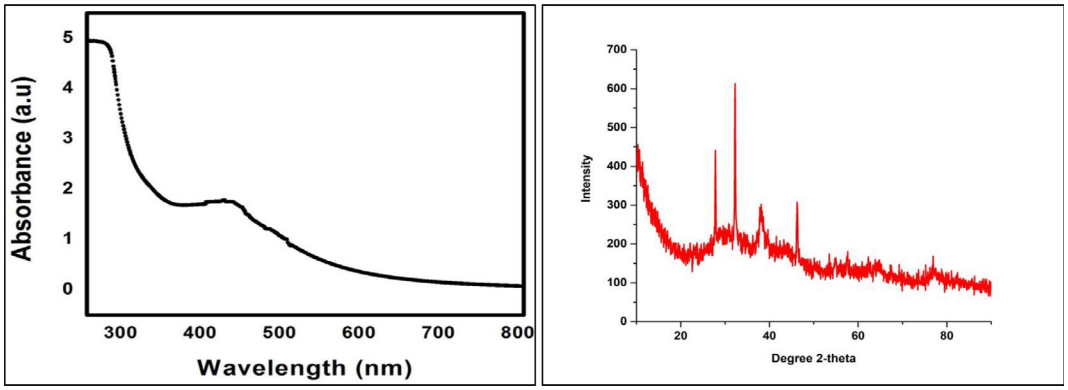

Fig. 1. Biosynthesized Ag NPs using marine mangrove plant *A. marinia* extract and confirmed by UV–visible spectrum (a) and X-ray diffraction pattern (b).

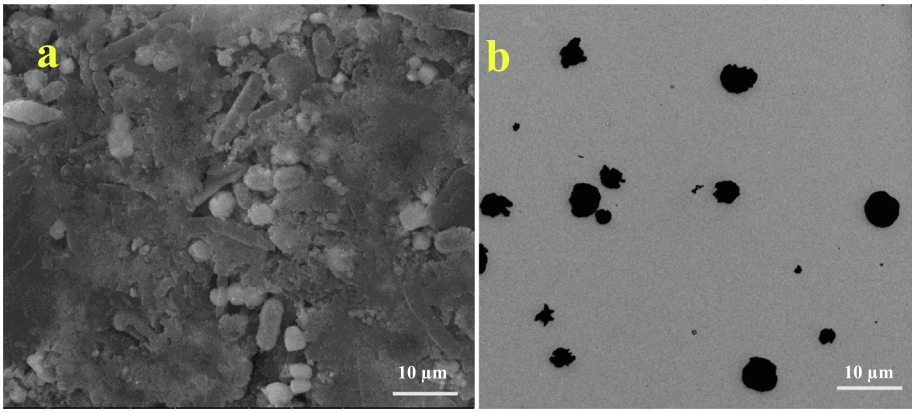

Fig. 2. Morphological observation of biosynthesized Ag NPs by SEM (a) and confirmation of Ag NPs by highest magnification (b).

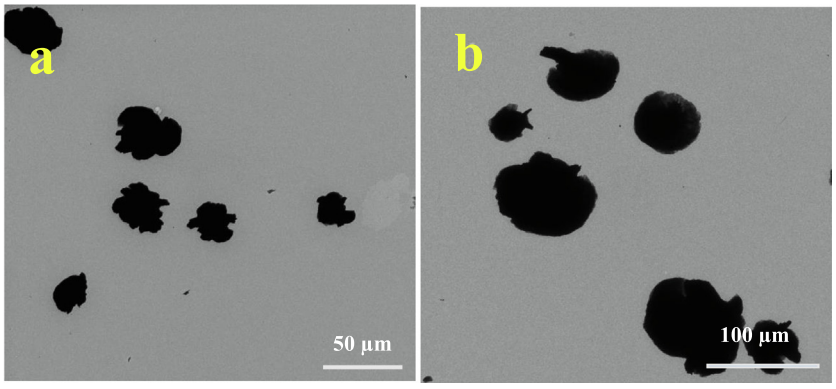

Fig. 3. The individual morphology and size of the biosynthesized Ag NPs at different magnification by TEM (a, b).

by treated material is an important study (Hu et al., 2015). The IC50 concentration of Ag NPs treated A549 cancer cells in our study was also proved that the cancer cells were produced the ROS in more amounts. Based on the observed result, the synthesized Ag NPs has anti-cancer properties due to the ROS stimulation. It is an excellent target site to prevent the growth cells in inside of the cancer cells. If the Ag NP has the ability of ROS production gene stimulation, it won't be allowed the further process in cell cycle including maturation, attachment, cellular proliferation, and enhance the genes of apoptosis and necrosis (Selvi et al., 2016). After respective time interval, the Ag NPs was generated the ROS in A549 cancer cells due to the oxidation process, and it accumulate on the DNA granules. Next, the DNA lost their transferring ability and blocked the polymerase enzyme production (Bhakya et al., 2016). In outside, the surrounding membrane was completely damaged and immature colonies were observed. To detect the oxidation potential, the fluorescent dye of DCFH-DA was used. After damaged the DNA ability, the condensed form of immature DNA was leaked out and bind with specific DCFH-DA dye and showed sparsed cell morphology. Compared with untreated control, the treated cells were showed with highly condensed morphology and necrotic structure (Fig. 5a). Whereas, the smooth morphology of untreated cells was showed with clumped colonies (Fig. 5b). It was clearly confirmed that the ROS was generated intracellularly in A549 cells due to the Ag NPs influences (Rajivgandhi et al., 2019b). In addition, the continuous ROS production of the cells

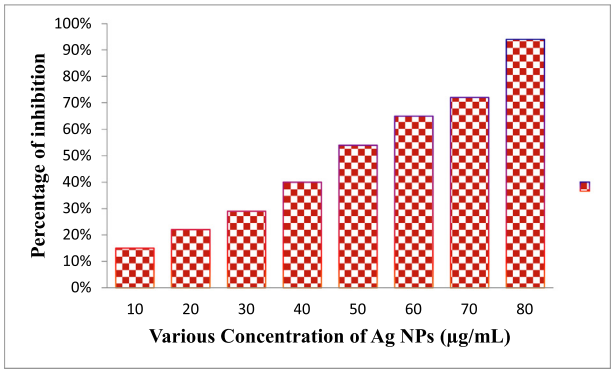

Fig. 4. Detection of IC_{50} concentration of Ag NPs against A549 lung cancer cells by cytotoxicity assay.

was gone to decline phase and leads to death due to the suppressed activator genes. Therefore, the current result was prove, Ag NPs has more ability to inhibit the cancer cells formation due to the oxidative stress mediated ROS generation. The recent report of Naveen kumar et al. (2018a,b) reported that the ROS is a major target to inhibit the cancer cells, and performed Ag NPs has stimulated the oxidative stress responses vigorously in A549 cells. Recently, Bhakya et al., (2016) was reported similar result against A549 cells by treating the Ag NPs at increased concentration. Also, the oxidative stress response went to down regulating process in apoptotic genes and leads to programmed cell death due to the intracellular leakages in mitochondrial membrane.

4.2.3. Assessment of mitochondrial damage ($\Delta_\psi m$)

After 24 h incubation, the mitochondrial membrane from leakages of cytochrome *c* materials was observed in florescence microscope after absorption of florescence dye Rhodamine 123 (Fig. 6). Previously, reported that the Rhodamine 123 is an excellent dye to detect the damaged membrane morphology in cancer cells. It interfere the intrinsic pathway in cancer cell cycle process. It has the ability to bind damaged mitochondrial membrane and clearly attached with damaged cells. When the cells were gone to damage, the apoptosis and necrotic cells were formed continuously and showed with orange color to green color appearance with necrotic cells. It is an important factor to monitor the depolarized membrane of mitochondria due to the dysfunction of responsive genes. When the mitochondrial membrane was damaged, the suppressive genes of BCL-2, caspases are activated and decrease the production of cancer cell responsive genes. After entry of the Rhodamine 123 into the mitochondrial membrane, the intracellular leakage materials were lost and changed their color from red to green that indicates apoptosis (Momtazi-borojeni et al., 2103). Our result was more evident the guidelines and inhibit the A549 cells at IC50 concentration of Ag NPs. Compared with control, the more number of apoptotic and necrotic cells were observed in treated cells (Fig. 6a, b). It activated the casecade of apoptotic cell receptor in A549 cells and frequent arrest of life cycle due to the increase of responsive genes activation. It is the primary step to activate the apoptosis. The result of Fig. 6b shows rough, loosely associated apoptotic cells were observed with highly necrotic nature. Whereas, Fig. 6a shows with smooth, tightly packed and clumped morphological colonies were observed. Our result was agreed by recent report of Rajivgandhi et al. (2019b) and loss of red color intensity is refereed as notable reduction in mitochondrial membrane stability. Similarly, loss of mitochondrial membrane damage by destabilization and functional alteration leads to high mortality was most effective in treatment of cancer cells. Our result agrees with Du et al. (2017) who reported that the mitochondria is involved in the stimulation of cell signaling genes, cellular differentiation, apoptosis and control of cell cycle growth.

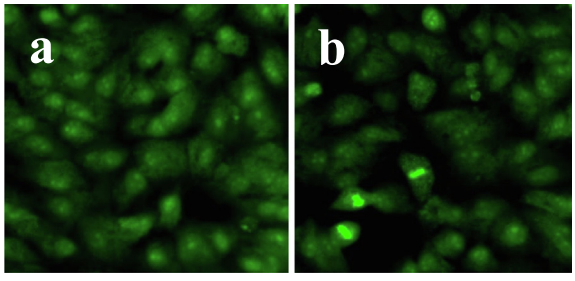

Fig. 5. ROS Production of biosynthesized Ag NPs against A549 damaged (a) and control (b) cells by fluorescent microscope using DCFDH-CA fluorescent dye.

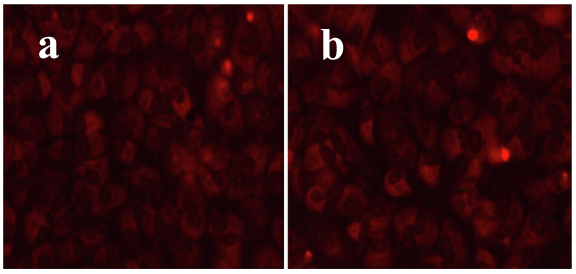

Fig. 6. Intracellular mitochondrial membrane damage (a) and control (b) cells of biosynthesized Ag NPs against A549 by fluorescent microscope using Rhodamine 123 fluorescent dye.

5. Conclusion

In current study, the marine mangrove plant *A. marina* is an excellent reducing agent for synthesis of Ag NPs, showing excellent anti-cancer properties against A549 cells. The plant extract of synthesized Ag NPs has various functional groups and leads to helped as a reduction of Ag metal ion. All the result of UV, XRD, SEM with EDX and TEM was efficiently supported to the nanomaterial transformation. Interestingly, Ag NPs has excellent anti-cancer activity against A549 cells and influencing high damages in mitochondrial membrane. In addition, the production of ROS level was very high and confirmed by florescent microscopic analysis with the help of DCF dye. All the *invitro* studies were confirmed that the Ag NPS very effective against A549 cells at concentration dependent manner. Furthermore, the increased toxicity of Ag NP treated *A. franciscana* exhibited significant mortality rate at increasing concentration. Therefore, our result was suggested that the Ag NPs has promising anti-cancer properties and effective against A549 lung cancer cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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