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Original article

Chrysanthemum morifolium extract mediated Ag NPs improved the cytotoxicity effect in A549 lung cancer cells

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ABSTRACT

In this study, the anti-cancer compound was extracted from *Chrysanthemum morifolium* (*C. morifolium*) by soxhlet apparatus method. The available anti-cancer compounds of *Chrysanthemum morifolium* extract were clearly detected by LC-MS analysis based on the retention time, percentages of height and occupied area. The anti-cancer compounds were separately screened from mixed compounds by NIST Wiley library interpretation. Further, the silver nanoparticle (Ag NPs) was synthesized from *C. morifolium* extract, and their surface plasmon resonance effect of the extract was scanned by UV–vis spectroscopy at 410 nm. Morphological identification of size and shape of the Ag NPs was confirmed by scanning electron microscope and transmission electron microscope images. Further, the anti-cancer effect of synthesized Ag NPs was exhibited more cytotoxicity effect at the concentration of 200 μg/mL. Furthermore, the morphological damages of A549 cells were clearly confirmed by fluorescence microscope using AO/EB fluorescence stain. Finally, the results were suggested that the biosynthesized Ag NPs has potential anti-cancer agent against A549 lung cancer cells.

1. Introduction

Worldwide, the increasing mortality rate of lung cancer is increasing frequently due to the adaptation of cancer environment including continuous smoking, modernized culture, without exercise (Que et al., 2019). It is most dangerous disease for human for frequently increased death rate after blood cancer (He et al., 2016). It crossed at least 50,000 death per year compared with other cancer, and it is a leading cancer in developing and developed countries (Zhang et al., 2020). All the countries are struggling to prevent the lung cancer and their related infections. The current treatment methods are ineffective due to the routine use of unfavorable drug (Saravanakumar et al., 2019). The previous methods of surgical, chemotherapy and laparoscopy are not successful in control of lung cancer cells. These methods are also not effective for lifetime cancer patients due to the end stage. Due to these defects, all the countries are searching a new tool for improve the diagnosis of cancer in starting stage as well as new methods to improve the drugs against cancer cell cycles, cell signaling and target sites (Venugopal et al., 2017). Initially, if cancer cells enter into primitive stage, it will be difficult to eradicate due to the production of responsible gene expression. In medical field refereed that the initial stage of cancer cells inhibition is better choice to eradicate cancer cells completely. Recent years, all the developing and developed countries are motivated the research and development units for cancer cells to inhibit in initial stage (Tammina et al., 2017). More research activities, increased awareness program, decreased cancer environments, behavior changes are the best was to eradicate the cancer cells. Importantly, the alternative way is need to inhibit the cancer cells and it should be help to demolish the cancer cells in long term (Naveen kumar et al., 2018). Recent years, nanoparticles and their properties have enormous anti-cancer activities against cancer cell treatment long term

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with minimum toxicity. Also, this is one of the new routes to treat the cancer cells in initial stage and also prevent the cancer cells completely (Samuel et al., 2020).

Recent years, nanotechnology is entered into biomedical field and it given the solution to alternative drug choice in drug delivery process (Rajivgandhi et al., 2019aa, 2019bb, 2019bc). It is in anno size, so it is easy to success in biomedical field (Mortazavi-Derazkola et al., 2020). The nano sized particles are used in all the fields including soil, agriculture, food, pharmaceutical and biomedical with increased nature (Bello et al., 2017). Also, it has wide range of application in the entire field through the way of alternative agent (Sankar et al., 2013). In addition, the nano sized particles are used in environmental pollution control and material chemistry and astro physics (Jeyaraj et al., 2013). Usually, the nanoparticles have the sine of 1–1000 nm and recent years mostly used in biomedical field compared with other field (Esawy et al., 2019). It is an alternative agent for drug delivery, increased drug nature and inhibit the various infections effectively (Hashemi et al., 2020a, 2020b). Among the different nanoparticles, the most important and highly reported nanoparticle is silver nanoparticles (Ag NPs), and it is most exploited and effective biological characteristic agent compared with other nanoparticles (Rajasekharreddy and Usha Rani, 2014). Last ten years, the silver nanoparticles research is increased very high in the field of medical with enhanced bioactivities (Rao et al., 2020). It is synthesized form various routes such as chemical, physical and biological. Among the various route, the most reported biological routes are very efficient than other routes due to the unique properties with decreased toxicity (Korkmaz et al., 2020). Previously, more reports of biosynthesized Ag NPs has enormous biological activities like anti-microbial, anti-cancer, anti-viral, larvicidal and anti-biofilm activities (Manjunath Hulikere et al., 2019; Rajivgandhi et al., 2020a, 2020b; Bello et al., 2017; Sankar et al., 2013). At present, the biological mediated Ag NPs is very effective against various infections than other route of synthesis. In biological route, previously used plant, seaweed and microbes are best choice. Sometimes, the Ag NPs absorbed the source compositions, nutrients and other habitats and produced enhanced bioactivities compared with original one (Venugopal et al., 2017). Therefore, this study was concentrated on green synthesized nanoparticles using *C. morifolium* and their anti-cancer effect against A549 lung cancer cells which carried out by various invitro experiments.

2. Materials and methods

2.1. Needed chemicals

The Indian medicinal plant of *C. morifolium* was collected from Pottanam Village, Namakkal, Tamil nadu, India. Silver nitrate and nanoparticle synthesis materials were purchased from Suresh Scientific @ Co, Tiruchirappalli, Tamil Nadu, India. The cancer cells A549 were procured from King Institute, Guindy, Chennai, Tamil nadu, India. Other related chemicals and solutions were purchased from Ponmani @ Co, Tiruchirappalli, Tamil nadu, India.

2.2. Extraction of plant extract

The soxhlet apparatus was filled by 10 g of purely washed plant extract through powder nature and run the instrument continuously till solute fully. For the extraction, the methanol was acted as an effective solvent. After heating, the sample phases of the soxhlet were cooled 2 h and completely lyophilize after removal of the extract phases. The sample was lyophilized using lyophilizer for purify the active components of the extract (Rajivgandhi et al., 2020a, 2020b). Then, the available chemical compounds of the extract were scanned by LC-MS (Shimadzhu, Japan). Finally, the active biological components of the extract was purified separately and used to synthesis of Ag NPs for detection of anti-cancer activity.

2.3. Detection of anti-cancer compounds form LC-MS analysis

The presence of anti-cancer compounds in the *C. morifolium* was screened by using LC-MS instrument. This method was followed by previous report of Taskin et al. (2020). With some alteration. In LC-MS analysis, the instrument was attached with split ratio of 1:2 (Extract: dichloromethane) at 200 °C. The chemical components were purified by HP-4MS column of 20 m × 0.30 m m (dichloromethane + 5% phenyl). The 30S helium gas and injector temperature timing of start and end is 50–350 °C at 4 °C. It is oven temperature. The purified components materials were mixed with HCL and diluted with water with injected volume of 1:1 ratio.

2.4. Synthesis of Ag NPs

Sterile, clean100 mL test tube was taken and filled with 1:10 ratio of crude extract and silver nitrate. The test tube was maintained in the water bath, which previously set in 90 °C for proper heat. The test tube was allowed 1 h to change the color of the mixture samples. For adjustment of pH 1 N NaOH and 1 N H_3PO4 was used After 1 h, the mixture of the solution color was gradually changed to yellow or orange or light brown color. The color changes were indicated that the Ag NPs was synthesized in the mixture solution. Consequently, the synthesized Ag NPs was accurate or not was proved by result of UV-spectrometer analysis at the wavelength of 200–800 nm. After initial confirmation, the morphology, size and shape of the green synthesized Ag NPs was confirmed by SEM at 0.1-30kv of accelerating voltage (Shimadzhu, Japan) and TEM instruments at 120–200 KV accelerated (Scavenging, Germany). The entire method was followed by Rajivgandhi et al. (2019a), Rajivgandhi et al. (2019b), Rajivgandhi et al. (2019c).

2.5. Anti-cancer activity

2.5.1. Cytotoxicity assay

Anti-cancer nature of green synthesized Ag NPs against A549 lung cancer cells were evaluated by 96-well micotitre plate assay for detection of cytotoxicity of the Ag NPs (Naveen kumar et al., 2018). Shortly, the fresh complete medium was filled by 24 h old ~ 2×10^4 cells of A549 through seeding method. The seeded plate was maintained at 37 °C for 24 h in CO_2 incubator under reduced pressure with 5% related humidity. This stage was used to attach the cells into the plate. After attachment, the cells were treated with different diluted concentration (5–250 μg/mL) of Ag NPs and put into the CO_2 incubator under reduced pressure with 5% related humidity at one day. Without addition of AgNPs containing A549 culture well was acted as a control. After, 100 μL MTT solutions were added into the wells and then incubate the plate at 37 °C for 4 h. Next, observe the formazan crytal formation in the treated wells and dissolved with 200 μL of fresh DMSO solution. Finally, the O.D of 590 nm was taken using microtitre reader plate (BioTek Instruments, Winooski VT) after the intracellular modified color changes wells. Finally, the color changes of the wells O.Ds were compared with control O.Ds and made to percentage of inhibition. Consequently, the IC^{50} concentration was noted by using following formula,

$$IC_{50\ value} = [(\text{Mean O.D contorl} - \text{Mean O.D}_{Treatment})/\ \text{Mean O.D control}] \times 100.$$

2.5.2. Detection of morphological variation (AO/EB) assay

The fluorescence absorption mediated morphological differentiation in the Ag NPs treated slide was proved by fluorescence microscope, with the following modification of Venugopal et al. (2017). The fresh 24 h old A549 culture was seeded into the 6-well adherent plate with inside the cover slide. Then, different concentration of Ag NPs was treated into the wells cultures and allowed to adherent or non-adherent the cells till 24 h. After incubation, the cells were trypsinized in all the wells for complete detachment of non-adherent cells. After, 10 μL of AO/EB was gradually added into all the wells including untreated control well. Then, mixed gently and excess stain was removed using Whatman No.1 filter paper. Finally, the morphological differentiation of treated or untreated cells were differentiated using fluorescence microscope analysis (Carl Zeiss, Jena, Germany) at magnification range of 40x.

3. Result

3.1. LC-MS report of Chrysanthemum morifolium extract

Totally, 55 peaks were exhibited in the extract of *C. morifolium* after take report of LC-MS (Fig. 1). All the peaks were carefully interpreted with Bharathidasan University using NIST Wiley Library for detection of available chemical derivatives in the extract. Also, the retention time, area of height, peak percentages were also discussed based on the NIST reports and identified 6 different anti-cancer compounds. Whether, the identified compounds were present in the plant materials or not were also checked in previous reported articles, and found that the articles have the identified compounds with anti-cancer compounds. The available peaks were effectively shown in Fig. 1. The available anti-cancer compounds of the extract and their retention time and occupied area, percentage of area were effectively screened such as Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3, 7,9-Di-*tert*-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, trifluoroacetoxy hexadecane, heptadecyl trifluoroacetate, BIS(2-ethylhexyl) phthalate. The retention time of 11.30, 22.45, 30.16, 11.90 and 20.10, occupied area of 12, 7866, 19, 8453, 22, 8790, 20, 8976 and 19, 8079, area percentages of 3.6, 2.9. 3.0, 2.8 and 2. 6. The result was good agreement with previous report of Sixto et al. (2019), and anti-cancer compounds were present in plant extract. The similar study of anti-cancer activity was reported by Peng et al. (2020) and *C. morifolium* was excellent plant materials for inhibition of cancer cells. Recently, Rajasekharreddy and Usha Rani, 2014 reported that the plant *C. morifolium* increased anti-microbial and anti-cancer activities. Sometimes, the effect of compounds were identified based on the tropical and subtropical regions and it's changed their potential activity also (Mighri et al., 2019). Some researchers are also reported that the plant *C. morifolium* was very effective anti-cancer compound producer and it alters the intracellular molecules in cancer cells (Ložnjak et al., 2020).

3.2. Characterization of Ag NPs

Based on the Mi theory, the exhibited Ag NPs was shown at the 410 nm, and the exhibited peak was confirmed that the Ag NPs was synthesized form *C. morifolium* extract (Fig. 2). The color changes were clearly observed in the *C. morifolium iextract* after 1 to 2 h time interval. Likewise, the Ag NPs formed solution was shown with greenish yellow color and it revealed the surface plasmon

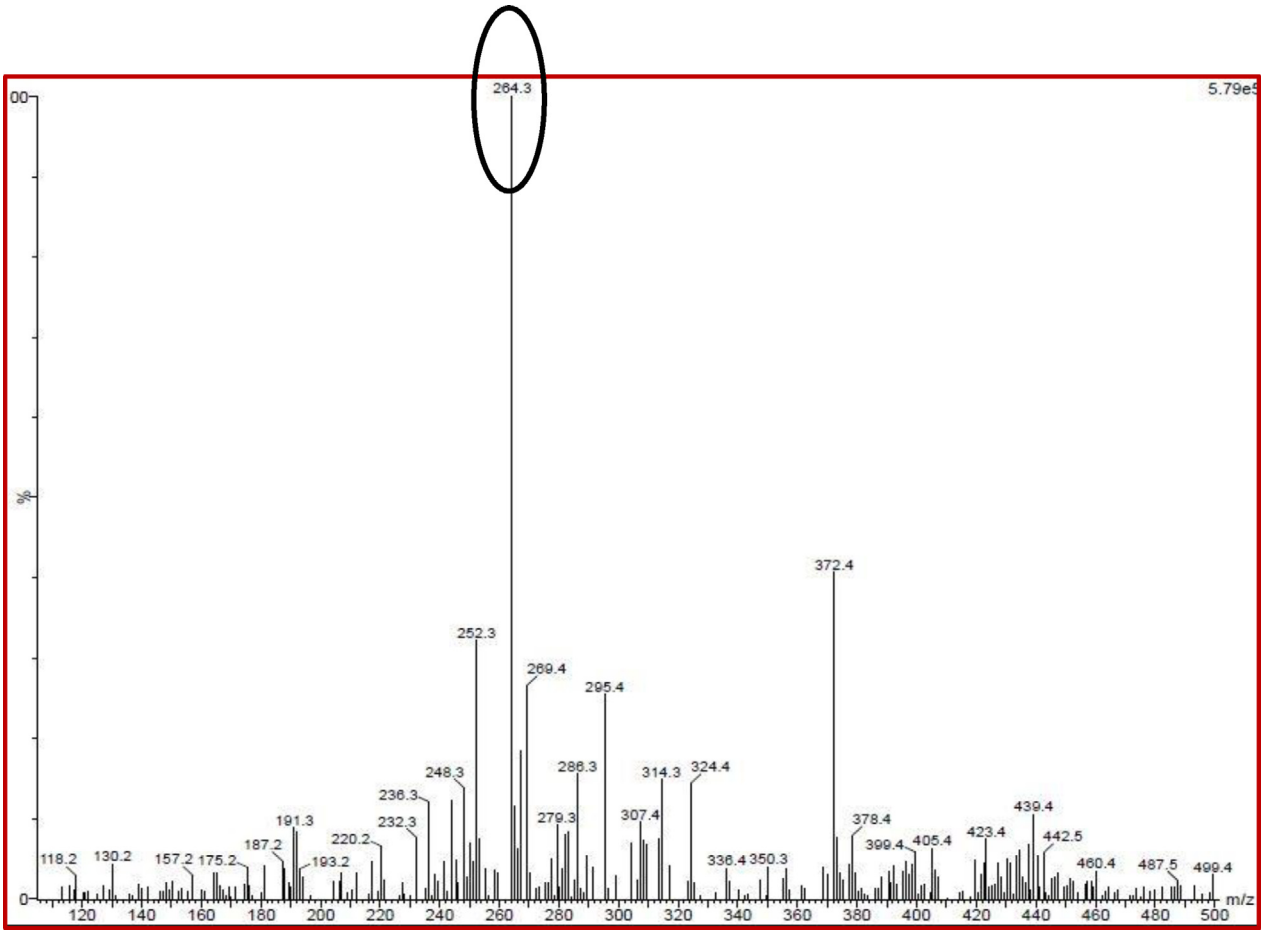

Fig. 1. Available anti-cancer compound of *Chrysanthemum morifolium* extract by LC-MS analysis.

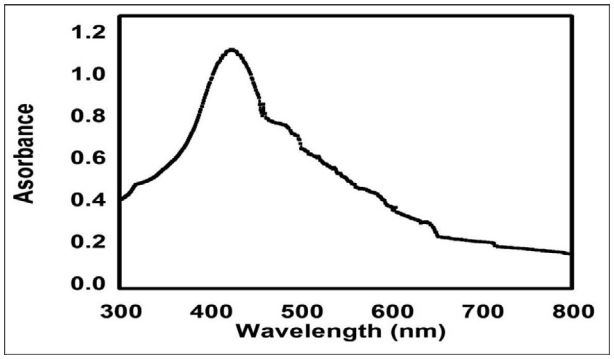

Fig. 2. UV–vis spectroscopy analysis of *Chrysanthemum morifolium* extract mediated Ag NPs synthesis.

resonance effect of *C. morifolium* extract. After the O.D value of spectrophotometer result was also drawn between the 200–800 nm and it confirmed that the synthesized material was Ag NPs. The confirmed result was indicated that the extract has no any impurities and transferred the Ag NPs through the process of Ag + ion transferred into plant components containing extract for synthesis of Ag NPs. In addition, the spherical shaped morphology and separate colonies were also clearly observed in the SEM images. Exact Ag NPs was found with agglomerated spherical shape morphology by SEM morphology (Fig. 3a). Also, original Ag NPs size and shapes were clearly shown in the SEM and supported by TEM results. Further, the size and shape confirmations of TEM images were also effectively supports the result and it confirmed that the morphology was Ag NPs morphology. The SEM and TEM morphological results of Ag NPs size and shapes were shown in the Fig. 3b. The size and shape based SEM images were used to confirm the Ag NPs morphology. Previously, the spherical shaped morphology of Ag NPs was effectively indicated in reported evidence of Vimala et al. (2015). Recently, Rajivgandhi et al. (2020a), Rajivgandhi et al. (2020b) was also agreed the result, and green synthesized Ag NPs images were shown spherical morphology with agglomerated images. Finally, the characterizations of UV, SEM and TEM results were indicated that the plant of *C. morifolium* was important plant and used for synthesis of high volume ration and high surface area of Ag NPs.

3.3. Anti-cancer studies

3.3.1. Cytotoxicity assay of cancer cells

After time interval of one day, the quantity of A549 cells were decreased and confirmed by O.D value calculation. The viability of the A549 cells was decreased at increasing concentration of Ag NPs. It shown more turbidity at the concentration of 300 μg/mL (Fig. 4). This result was suggested that the Ag NPs was transferred to surface of the cancer cells and extended the proliferation ability. Therefore, the cells were gone to minimum quantity and undergone to decline phase. In result, the 53% of cell viability was observed at the concentration of 200 μg/mL and it suggested the concentration dependent cell decrease. Initially, the decreased viability was started at the concentration of 25 μg/mL only. At this concentration was used for initial inhibition study and extended up to 300 μg/mL. All other *invitro* experiment, the IC_{50} concentration of 200 μg/mL was fixed. Based on the time and concentration, the inhibition concentration of 200 μg/mL was very effective compared to previous study of plant (Venugopal et al., 2017). This result was agreed by Hashemi et al. (2020), Hashemi et al. (2020b) and plant mediated Ag NPs is an excellent anti-cancer agent against A549 lung cancer cells. The inhibition was stimulated by morphology and mitochondrial region due to the effect of plant was reported by Khorrami et al. (2020). The more precipitation and increased concentration was observed against A549 cells after used plant and their color was changed due to the effect of formazan production. Our result was agreed by previous report of Naveen Kumar et al. (2018), the plant extract of *C. morifolium* is excellent plant for synthesis of Ag NPs with potential anti-cancer activity. Also, the phytochemical and bioactive derivatives were play a major role for improve the bioactivity of Ag NPs. Finally, our result was proved that the *C. morifolium* mediated Ag NPs was very effective anti-cancer agent for A549 lung cancer cells.

3.3.2. AO/EB stain usage of morphological differentiation

Consecutively, the confused morphological observation was observed in the IC_{50} dose of Ag NPs treated and untreated A549 cells by fluorescence microscope after using dual stains of AO/EB (Fig. 5). In this experiment, the emission of light intensity was shown in damaged cells compare with normal untreated cells. The condensation and necrotic cells were shown highly in the treated cells. In addition, the apoptosis was processed continuously and it leads to cell death. All the cells were undergone to decline phase and it confirmed more apoptosis was shown. The activator genes were processed successfully and induced more proliferation for damage. The intracellular membranes were shown more orange color compare with green color of control cells. Also, the necrotic cells were exhibited with orange color cells in the treated images and also shape and size of the cells were also changed. Instead, the smooth, normal and original morphology with A549 cells were viewed. AO is a dye which binds only in the normal cells, whereas, EB is a dye which has the ability to bind in damaged cells. In our result, the normal cells were observed the AO stain and it emitted green color intensity appearance. Whereas, the more amount of

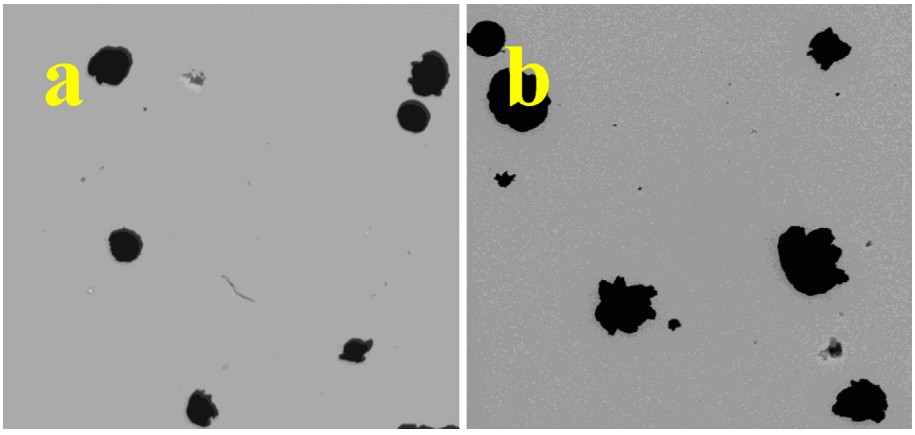

Fig. 3. Morphological Differentiation of Ag NPs using SEM and TEM analysis of *Chrysanthemum morifolium* extract.

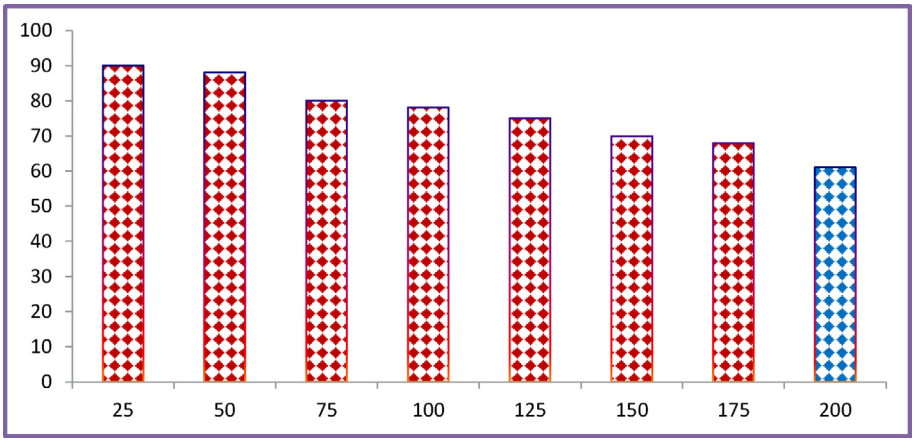

Fig. 4. Cytotoxicity assay of Ag NPs treated A549 lung cancer cells by MTT microtitre model.

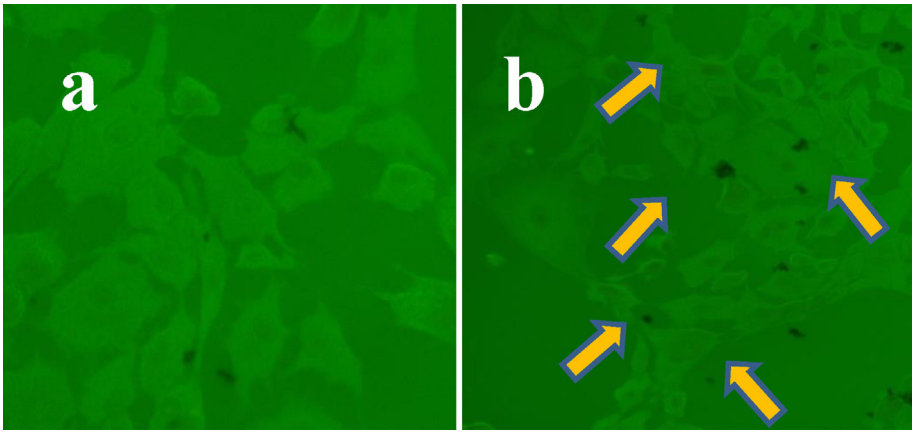

Fig. 5. Detection of intracellular morphological damages of A549 lung cancer cells using AO/EB fluorescence dye by fluorescence microscope.

red color intensity with high proliferation ability of EB stain indicated that the cells were damaged due to the Ag NPs. Therefore, the AO/EB result was confirmed that the Ag NPs was very efficient against A549 lung cancer cells. In addition, the Ag NPs was acted as a building block against A549 cells in the cell cycle process, because, all the cells were undergone to decline phase. The more nutrient depletion and growth cell receptor damages were shown Saravanakumar et al. (2019). Recently, complete death cells due to the stimulation of responsible factors activation by biosynthesized Ag NPs reported by Venugopal et al. (2017). The nucleus leakages materials were observed the fluorescence dyes were clearly confirmed the Ag NPs ability against A549 lung cancer cells (Jeyaraj et al. (2013). This statement was agreed by Esawy et al. (2019), and biosynthesized Ag NPs has effective anti-cancer agents against various cancer cells and it proved against A549 cells previously. Previously, Lee et al. (2014) and Ukiya et al. (2002) were reported that the plant extract was shown anti-inflammatory, antiadipogenic and anti-cancer activity against various cancer cells. These are most supported to anti-cancer activity of *C. morifolium* extract. Therefore, the present result was suggested that the biosynthesized Ag NPs using marine algae *C. morifolium* as an important source for synthesis of potential anti-cancer agent.

4. Conclusion

This study was concluded that the plant *C. morifolium* extract has excellent plant for synthesis of Ag NPs for inhibit the A549 lung cancer cells. Also, the phytochemical derivatives and their chemical components were influenced the Ag NPs activity against A549 cancer cells. Additionally, the IC_{50} concentration of the Ag NPs against A549 cells was shown at 250 μg/mL concentration. Further, the damaged morphology of A549 cells after influence of IC50 concentration was confirmed that the biosynthesized Ag NPs has excellent anti-cancer activity. Altogether, the biosynthesized Ag NPs is an excellent anti-cancer agent for 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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