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In vitro Anticancer Activity of Silver Nanoparticles Biosynthesized from Seeds of *Sesamum indicum* Against Dalton's Lymphoma Ascites

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ABSTRACT

The objective of this study was to explore the anticancer activity of silver nanoparticles green synthesized from aqueous extract of *Sesamum indicum* (*S. indicum*) seeds in Dalton's lymphoma ascitic cells. The anticancer efficacy of biosynthesized silver nanoparticles from plant extract (S-AgNPs) was compared relatively with chemically synthesized nanoparticles (C-AgNPs), which were produced using trisodium citrate. The C-AgNPs were characterized using *UV-visible spectroscopy* and *X-ray diffraction*. Following this, MTT assay was done *in vitro* to evaluate the percent cell inhibition and half maximal inhibitory concentration (IC_{50}). Among the various treatments, S-AgNPs were found to have higher percent cell inhibition as 77.61 percent. Trypan blue dye test was done to assess the percent of cell viability and number of viable cells. The cell viability in percentage for S-AgNPs was low (43.8 percent), whereas for C-AgNPs, it was 57.48 percent. After this, cells after treatment at IC_{50} concentration, were subjected for Acridine orange / Ethidium bromide staining. This revealed the manifestation of cells in the late apoptotic stage, treated with S-AgNPs. Hence, it may be concluded that silver nanoparticles biosynthesized from sesame seeds induces cytotoxicity in cancer cells.

HIGHLIGHTS

- Silver nanoparticles from aqueous extract from seeds of *Sesamum indicum* and trisodium citrate were produced and characterized.
- Dalton's lymphoma ascitic cells were used for assessing anticancer activity.
- Cytotoxic activity was analyzed using MTT, trypan blue and Acridine orange / Ethidium bromide staining.

Keywords: *Sesamum indicum*, biosynthesized silver nanoparticles, Dalton's lymphoma, percent cell inhibition

Cancer is the leading cause of death among all the diseases that affect humans (Jeyaraj *et al*., 2013). Annual cancer cases are expected to rise from 14 million in 2012 to 22 million over the next two decades, according to the WHO. As a result, one of the most ardent targets are the establishment of potent and effective antineoplastic drugs (Al-Sheddi *et al*., 2018). Plants have long been used to treat a variety of diseases, like cancer, with over 60 percent of currently used anti-cancer drugs derived from natural sources. Most of the anticancer drugs that have been recommended for therapeutic purpose are derived from the natural products (Bhanot *et al*., 2011).

The majority of clinically used anticancer drugs have multitudinous remarkable limitations. Existing cancer treatments face common challenges such as low solubility

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in water, localization of therapy to tumour sites, drug resistance by tumour and short drug circulation times. Furthermore, it also causes serious complications such as occurrence of multidrug resistance, heart problems and low white blood cell counts (Cho *et al*., 2008). To overcome these constraints, plant based metallic nanoparticles are produced using green synthesis method. Nanoparticles, in cancer therapy, are known to have better pharmacokinetics, precise targeting of tumour cells, reduced side effects and drug resistance (Yao *et al*., 2020).

Sesame seeds from *Sesamum indicum* (*S. indicum*) is an oil seed crop, belongs to the family of Pedaliaceae. Many studies have been reported that oil from sesame seeds are known to have anticancer activity. The seeds and oil of *S. indicum* are reported to have lignans like sesamolin and sesamin. These lignans are revealed to have pharmacological activities like lipid-lowering, attenuating the free radicals, antihypertensive and anti-aging effect (Yokota *et al*., 2007; Anilakumar *et al*., 2010). Several lignans isolated from sesame, are found to be the precursors of mammalian lignans having protective effect against mammary tumour (Elleuch *et al*., 2011). Yokota *et al*. (2007) reported that impediment in the MCF-7 cancer cells growth was owing to the lignan, sesamin, which downregulated the protein, cyclin D1 expression. Ghani *et al*. (2012) reported the cytotoxic effect of defatted sesame seeds in Rhabdomyosarcoma (RD), Mammary adenocarcinoma of murine (AMN), Epidermoid carcinoma in Larynx of humans (Hep-2). Numerous studies have been reported that sesame display antineoplastic and cytotoxic activities in various cancer cells in both *in vitro* and *in vivo*.

Silver nanoparticles (AgNPs) have a salient role in nanotechnology by means of their pre-eminent properties like conductivity, chemical stability, catalytic activity and biological activities such as anticancer, antifungal, antiviral, antibacterial and anti-inflammatory activities. AgNPs have been studied extensively in cancer research owing to their cytotoxic potential. With the silver nanoparticles green synthesized from aqueous extract of plants like *Curcuma longa* and *Zingiber officinale*, they showed significantly higher cytotoxic effect against colon carcinoma cells as reported by Venkatadri *et al*. (2020). Multitudinous research works have reported regarding the anticancer activity of biosynthesized nanoparticles from plant extract (Basak *et al*., 2018; Priya *et al.,* 2020; Sreeharsha *et al*., 2020).

Daltons Lymphoma Ascites (DLA), is a lymphoma of T-cells, which is transplantable in nature. It usually develops in murine thymus. These cells are widely used in research of cancer since it is easily available for appraising the effect of novel therapeutic compounds (Koiri *et al*., 2017).

So, the current study was done to evaluate the cytotoxic activity of silver nanoparticles, biosynthesized from the aqueous extract of seeds of *Sesamum indicum* (*S. indicum*) against Daltons Lymphoma Ascites (DLA) tumour cells.

MATERIALS AND METHODS

Chemicals

The present study used silver nitrate, trisodium citrate, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide), Dimethyl sulfoxide (DMSO), Acridine orange and Ethidium bromide and trypan blue dye.

Biosynthesis of silver nanoparticles from aqueous extract of *Sesamum indicum* (AESI)

The seeds of *S. indicum* were collected and aqueous extract were prepared in Soxhlet apparatus by hot extraction. Then, the extract was analyzed for the presence of phytochemicals. Following this, silver nanoparticles were prepared by mixing one mM solution of silver nitrate with aqueous extract. The colour change is indicative of the formation of nanoparticles. It was characterized by using UV- visible spectroscopy, X-ray diffraction analysis and field emission scanning electron microscopy. The preparation of aqueous extract, phytochemical analysis and green synthesis of silver nanoparticles from the seeds of *S. indicum*, followed by characterization were described in detail in Keerthika *et al*. (2021).

Synthesis of silver nanoparticles by chemically reduced method (C-AgNPs)

The silver nanoparticles can be produced chemically, using trisodium citrate. Silver nitrate solution of one mM concentration is boiled by covering with watch glass in a

hot plate. The solution is stirred by using magnetic stir bar. While boiling, 6 mL of 10 mM tri sodium citrate is added one drop per second. After removing from the hot plate, the solution is allowed to cool. The colour change can be noticed.

UV- Visible spectroscopy

The chemically produced nanoparticles were characterized by Perkin-Elmer, Lamda 25- UV- Visible spectroscopy at wavelength range of 300-700 nm.

X-ray diffraction (XRD analysis)

The study of crystal structure of C-AgNPs were explored at Centre for Materials for Electronics Technology (C-MET), Athani, Thrissur by using X-ray diffraction spectroscopy. They analysed at 20 mA current, 30 kV voltage with rate of 30 kV voltage.

Evaluation of cytotoxicity from the biosynthesized silver nanoparticles (S-AgNPs) against DLA cells

MTT assay

The MTT assay was done to analyze the maximum inhibitory concentration (IC_{50}) for the plant extract, biosynthesized silver nanoparticles and they were compared with standard drug, cisplatin. From the peritoneal cavity of Swiss albino mice, the cells of DLA were collected, washed with phosphate buffered saline, seeded in the wells having cell density of 5×10^3. Following this, the treatment compounds like S-AgNPs, aqueous extract (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL), cisplatin and C-AgNPs (500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL and 31.25 µg/mL) were added and incubated for 24 hours. After this, MTT of 10 µL (5 mg in one mL) was mixed and kept for 4 hours, at 37° C. Media along with MTT was removed and DMSO of 200 µL was added to dissolve purple coloured formazan crystals. Then, this was read in ELISA reader (Varioskan Flash, Thermofisher Scientific) at 570 nm. The percentage of cell inhibition was found by using the formula,

$$\frac{\text{Mean OD of Control} - \text{Mean OD of treated cells}}{\text{Mean OD of Control}} \times 100$$

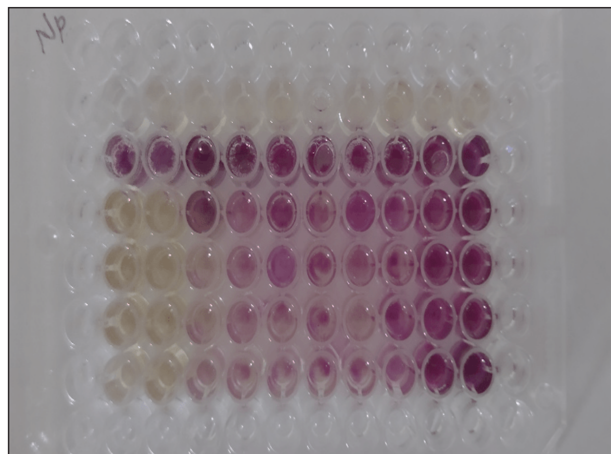

Fig. 1: MTT assay in 96 well plate after adding DMSO

Trypan blue dye exclusion test

This test was done by incubating the 5×10^5 DLA cells/mL in tubes with S-AgNPs, aqueous extract and cisplatin at their IC_{50} concentration. They were incubated at 37ºC for 3 hours. The viability of cells were assessed by adding trypan blue (10 µL) and cells (10 µL), together and analyzed in automated cell counter (Invitrogen Life technologies).

The viable cells in percent was calculated by,

$$\frac{\text{Total number of viable cells}}{\text{Total number of viable cells and non-viable cells}} \times 100$$

Acridine orange Ethidium bromide (AO/EB) staining

This was performed to determine the live, necrotic and apoptotic cells. It is proceeded by incubating DLA cells of the concentration, 5×10^5 cells along with IC_{50} of the treatment compounds for 24 hours. Both the stains (Acridine orange/ Ethidium bromide) at 10 µg/mL was mixed and added to 25 µL of the cell suspension. The cells were analyzed in Trinocular fluorescence microscope (DM 2000 LED) under blue excitation at 488 nm and emission at 550 nm.

STATISTICAL ANALYSIS

All results were expressed as Mean ± SE. All the statistical analysis were conducted using SPSS software version 24. The intergroup comparison was assessed by one

way analysis of variance (ANOVA) followed by Duncan Multiple Range test (DMRT) for pairwise analysis.

RESULTS AND DISCUSSION

Evidence of colour transformation

The formation of silver nanoparticles was found by the colour change from colourless solution to a colour of golden yellow. Similar colour change reaction was noticed by Suriati *et al*. (2014).

Fig. 2: Colour change from colourless to yellow coloured solution

UV- Visible spectroscopy

The chemical reduction method based synthesis of silver nanoparticles were found to have absorption peak at 425 nm. This was in accordance with the work done by Skiba *et al*. (2018), where they noticed peaks between 380 to 450 nm. Similar results were obtained by Rashid *et al*. (2013).

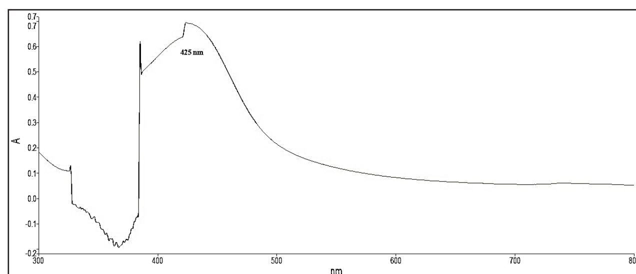

Fig. 3: UV- visible spectra for chemically produced silver nanoparticle

X-ray diffraction (XRD analysis)

The planes of diffraction from C-AgNPs gave rise to peaks at 38.1˚, 44.3˚and 64.5˚. Similar diffraction peaks were obtained by Sreelekha *et al*. (2021), where they compared the plant based and chemically synthesized nanoparticles. So this suggested that silver nanoparticles synthesized were cubic in structure.

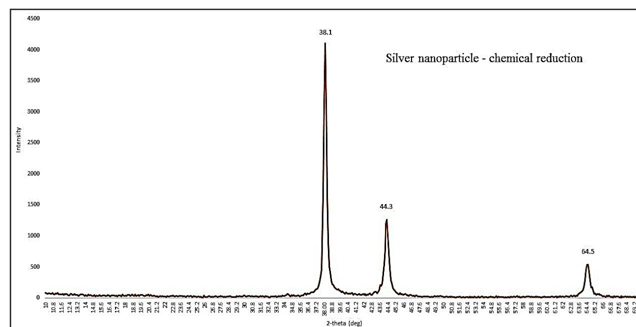

Fig. 4: X-ray diffraction planes for C-AgNPs

Evaluation of Cytotoxicity from the biosynthesized silver nanoparticles (S-AgNPs) against DLA cells

MTT assay

This was done to establish the viability of cells after treating with plant extract, nanoparticle and cisplatin. The results are listed in the table 1. The percentage of inhibition was calculated and half maximal inhibitory concentration (IC_{50}) was assessed by using AAT Bioquest and tabulated in table 2. The half maximal inhibitory concentration was found to be 114.21 and 149.44 µg/mL for cisplatin and C-AgNPs respectively. For the plant extract, the value of IC_{50} was 112.86 µg/mL, whereas for S-AgNPs, showed reduction to 71.47 µg/mL. The effect of S-AgNPs are compared with drug cisplatin and chemically synthesized nanoparticles. The results were in accordance with the work done by Mohanta *et al*. (2017) describing the anticancer activity of the silver nanoparticles in osteosarcoma cells, using aqueous extract made from leaves of *Erythrina suberosa*, where they explained that the enhanced activity from nanoparticles might be due to interrelation between phytochemicals found on the surface of nanoparticles. Similar results were obtained with silver nanoparticles synthesized by Sriranjani *et al*. (2016) from

Table 1: Percent viability of DLA cells (MTT assay)

Conc. µg/mL	Cell viability (%)		Conc. µg/mL	Cell viability (%)	
	Cisplatin	**C- AgNPs**		**AESI**	**S-AgNPs**
31.25	73.96 ^{d} ± 1.79	87.76 ^{e} ± 0.60	62.5	62.3 ^{c} ± 0.20	53.24 ^{d} ± 1.54
62.5	62.6 ^{cd} ± 2.5	78.16 ^{d} ± 0.46	125	55.55 ^{bc} ± 0.93	41.57 ^{c} ± 0.58
125	33.61^{b} ± 1.83	67.75 ^{c} ± 0.47	250	52.62 ^{abc} ± 1.20	38.97 ^{bc} ± 0.86
250	24.73 ^{ab} ± 1.71	61.15 ^{b} ± 0.56	500	47.23 ^{ab} ± 2.52	25.52 ^{a} ± 1.31
500	13.5 ^{a} ± 0.88	49.40 ^{a} ± 0.42	1000	42.34 ^{a} ± 2.18	22.39 ^{a} ± 0.78

Values expressed as mean ± SEM (n=3), Means bearing similar superscript (a-e) within each column do not differ significantly at $p<0.05$. C-AgNPs (Chemically synthesized nanoparticles), AESI- Aqueous extract of *S. indicum*, S-AgNPs (Silver nanoparticles from *S. indicum*).

Table 2: Percent cell inhibition of DLA cells (MTT assay)

Conc. µg/ml	Cell inhibition (%)		Conc. µg/ml	Cell inhibition (%)	
	Cisplatin	**C- AgNPs**		**AESI**	**S-AgNPs**
31.25	26.04 ^{c} ± 1.79	12.24 ^{e} ± 0.60	62.5	37.7 ^{c} ± 0.20	46.76 ^{e} ± 1.54
62.5	37.4 ^{c} ± 2.5	21.84 ^{d} ± 0.46	125	44.45 ^{bc} ± 0.93	58.43 ^{d} ± 0.58
125	66.39 ^{b} ± 1.83	32.25 ^{c} ± 0.47	250	47.38 ^{abc} ± 1.20	61.03 ^{c} ± 0.86
250	75.27 ^{ab} ± 1.71	38.85 ^{b} ± 0.56	500	52.77 ^{ab} ± 2.52	74.48 ^{b} ± 1.31
500	86.5 ^{a} ± 0.88	50.60 ^{a} ± 0.42	1000	57.66 ^{a} ± 2.18	77.61 ^{a} ± 0.78

Values expressed as mean ± SEM (n=3), Means bearing similar superscript (a-e) within each column do not differ significantly at $p<0.05$. C-AgNPs (Chemically synthesized nanoparticles), AESI- Aqueous extract of *S. indicum*, S-AgNPs (Silver nanoparticles from *S. indicum*).

aqueous extract of leaves of *Clerodendrum phlomidis,* which manifested greater cytotoxic effect in HT 29 and Ehrlich Ascites Carcinoma cells. It was previously reported that the enhanced anticancer activity could be attributed to the size-dependent infiltration of silver nanoparticles into cancer cells (Shawkey *et al*., 2013), interaction of silver ions with intracellular proteins and DNA (Satyavani *et al*., 2012), production of free radicals causing damage to the cancer cells (Venugopal *et al*., 2017).

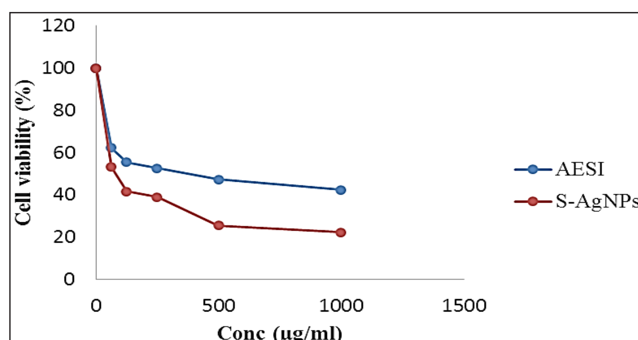

Fig. 5: Dose response curve for percent cell viability of DLA cells against ***Sesamum indicum*** and S-AgNPs

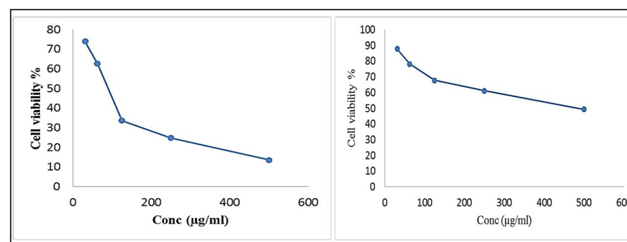

Fig. 6: Dose response curve for percent cell viability of DLA cells against cisplatin and C-AgNPs

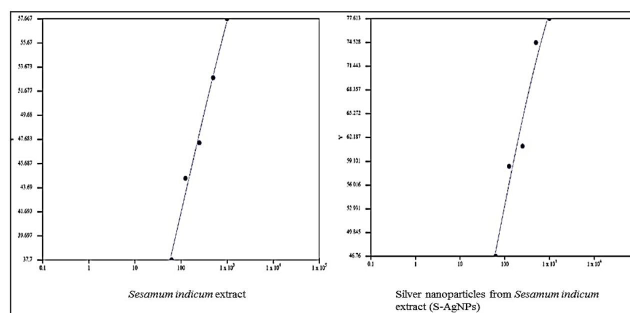

Fig. 7: Dose-response curve of the per cent cell inhibition of DLA cells against *Sesamum indicum* extract and silver nanoparticles from sesame seed extract

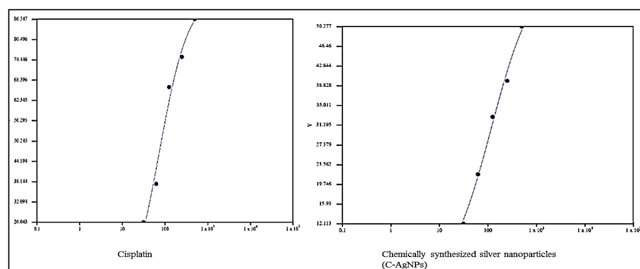

Fig. 8: Dose-response curve of the per cent cell inhibition of DLA cells against Cisplatin and chemically synthesized silver nanoparticles (C-AgNPs)

Trypan blue dye exclusion test

The viable cell count and cell viability in percentage was determined by incubating the DLA cells with cisplatin, C-AgNPs, aqueous extract and S-AgNPs for three hours at the IC_{50} concentration. The results were tabulated in table 3. After three hours of incubation, the average cell viability was found to be 46.28 percent. Johnson (2015) reported that trypan blue dye will penetrate inside the dead cells as it had lost the permeability of membrane. Similar results were seen in the findings of Raajshree and Durairaj (2018), where they found 55 percent cell viability after treating DLA cells with zinc oxide nanoparticles biosynthesized from *Turbinaria conoides* hydroethanolic extract. Also, Suresh *et al*. (2018) observed viability of DLA cells as 52 percent after treating with zinc oxide nanoparticles green synthesized from *Costus pictus*.

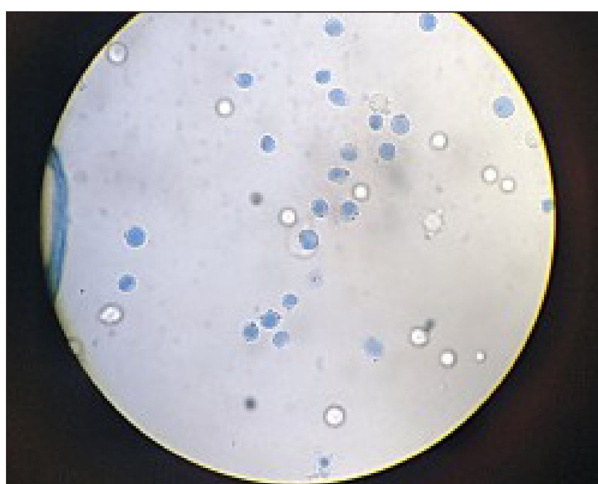

Fig. 9: Microscopical view of DLA live (unstained) and dead (blue) cells with trypan blue dye

Table 3: Trypan blue dye exclusion test for counting number of viable cells

Test compounds	Number of viable cells after 3 h ($\times 10^5$)	% Cell viability
Control	$4.9^{a} \pm 0.01$	$96.45^{a} \pm 0.30$
Cisplatin	$2.6^{c} \pm 0.02$	$51.18^{c} \pm 0.56$
AESI	$2.51^{c} \pm 0.04$	$49.40^{c} \pm 0.45$
S-AgNPs	$2.22^{d} \pm 0.02$	$43.8^{d} \pm 0.81$
C-AgNPs	$2.92^{b} \pm 0.01$	$57.48^{b} \pm 0.30$

Values expressed as mean ± SEM, n=3, Means bearing similar superscript (a-d) within each column do not differ significantly at $p<0.05$. AESI- Aqueous extract of *S. indicum*, S-AgNPs (Silver nanoparticles from *S. indicum*), C-AgNPs (Chemically synthesized nanoparticle).

Acridine orange /Ethidium bromide (AO/EB) staining

This staining was done to differentiate live, dead and necrotic cells following treatment with various test compounds. The photographs for different groups were obtained and illustrated in Fig. 9. The principle of staining was narrated by Kasibhatla *et al*. (2006). They explained that acridine orange stains live as well as dead cells, whereas ethidium bromide would be able to stain the cells that lost their membrane integrity. The live cells stain uniformly green. The early apoptotic cells appeared to have bright green dots in their nuclei and stain green owing to condensation of chromatin and fragmentation of nuclei. The ethidium bromide stains late apoptotic cells as orange colour. Necrotic cells appear to be red with nuclear morphology as that of viable cells without condensed chromatin.

The untreated cells were live with green fluorescence possessing circular nucleus. The cells treated with S-AgNPs were found to be in late apoptotic stage with orange stained, asymmetrical condensed nucleus. Some of the cells were red coloured indicating cells in necrotic stage. The cisplatin treated cells exhibited earlier apoptosis. Cells under C-AgNPs treatment were live and emitted green fluorescence along with few cells emitting orange fluorescence.

The results of this study were in correspondence with the work by Dolai *et al*. (2012), where they treated cells of Daltons Lymphoma Ascites with *Anthocephalus cadamba* plant extract. They found live cells with green nucleus,

apoptotic cells with orange coloured condensed nuclei and necrotic cells with red stained nuclei. Similar reported were obtained by Mani *et al*. (2019) and Soltani and Darbemamieh (2021).

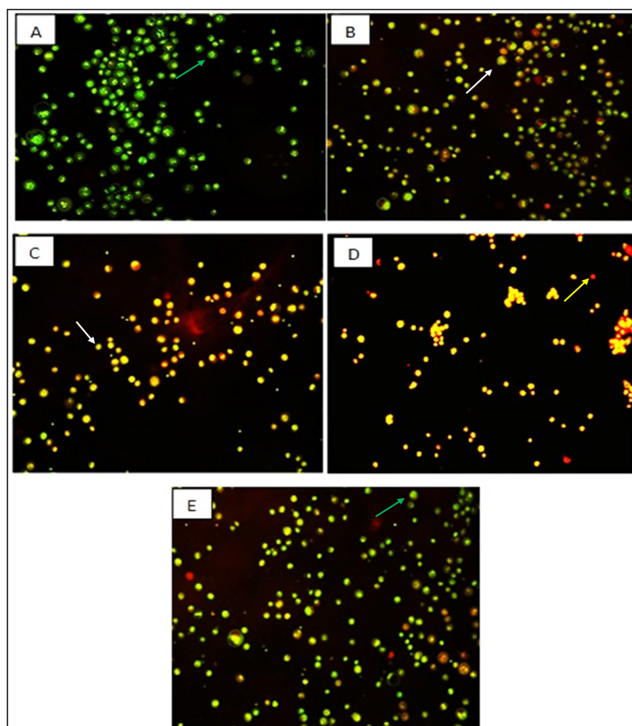

Fig. 10: Staining of DLA cells with AO/EB (20X magnification); **(A)** cells without treatment (control cells); **(B)** Cisplatin; **(C)** AESI treated; **(D)** S-AgNPs and **(E)** C-AgNPs. Green arrow- live cell, white arrow- early apoptotic cell, yellow arrow- late apoptotic cell

CONCLUSION

The aqueous extract from the seeds of *Sesamum indicum*, biosynthesized and chemically synthesized silver nanoparticles exhibited cytotoxic activity against DLA cells. The anticancer activity was greater with the biosynthesized silver nanoparticles than chemically produced silver nanoparticles. The latter were characterized and found to be in nano scale having 425 nm from UV-visible spectroscopy with distinct XRD peaks indicating their cubic structure. The IC_{50} value for S-AgNPs was 71.47 µg/mL, which is lower than the plant extract (112.86 µg/mL) and C-AgNPs (149.44 µg/mL). The lower value of IC_{50} indicated that the green synthesized silver nanoparticle compound is relatively effective at lower concentration than C-AgNPs. It is evident from the *in vitro* assays that biogenic nanoparticles produced greater inhibitory effect on the growth of cancer cells than the plant extract.

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