IJPSR (2020), Volume 11, Issue 10 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 18 October 2019; received in revised form, 30 January 2020; accepted, 11 March 2020; published 01 October 2020

ANTI-PROLIFERATIVE AND APOPTOSIS INDUCING EFFECT OF *NARAVELIA ZEYLANICA* L. AGAINST LUNG CANCER CELL LINE A549

B. Umarani * and K. Saravanan

PG & Research Department of Zoology, Nehru Memorial College, Puthanampatti - 621007, Tamil Nadu, India.

Keywords:

Naraveliya zeylanica, Anticancer activity, MTT assay, Comet assay, and Mitochondria membrane potential

Correspondence to Author:
B. Umarani

Ph.D Scholar,
PG & Research Department of Zoology, Nehru Memorial College, Puthanampatti - 621007, Tamil Nadu, India.

E-mail: umamiraa86@gmail.com

ABSTRACT: Cancer is one of the most dangerous diseases globally, and it appears due to extreme free radical damage, some chemicals, and a lack of physical activity which dispute causes damage to the cellular DNA, protein, and lipids. A number of treatment methods are available, but they cause unwanted side effects. The use of plant-derived products in the treatment of cancer prominently reduces adverse and toxic side effects. The present study investigated the antiproliferative effects of *Naraveliya zeylanica* L. against the human lung cancer cell line A549. The cytotoxicity potential of ethanol extract of *N. zeylanica* was tested on A549 cell line by dose-dependent manner at concentrations ranging from 100-1000 µg/mL, and the viability of treated cells was evaluated by MTT assay. Different morphological changes in the cells and mode of cell death were analyzed by acridine orange/ethidium bromide (AO/EB) staining, single-cell gel electrophoresis (comet) assay and Hoechst staining methods. JC1 staining was adopted to check mitochondria-mediated apoptosis. Ethanol extract of *N. zeylanica* significantly inhibited the growth of lung cancer (A549) cells with induction of apoptosis. The IC_{50} concentration of (435.50 ± 0.05 µg/ml) the extract remarkably induced the A549 cell apoptosis and reduced the mitochondrial membrane potential. Taken together, our findings indicated that the ethanol extract of *N. zeylanica* possessed potent antiproliferative and apoptotic activities.

INTRODUCTION: From ancient times, herbal plants have been used for various medical purposes in several countries as a primary source. Because of their innate multipotential properties, it has been used for various diseases. Thus, several researches were undergoing to investigate the potential medicinal properties and uses of compounds in medicinal plant extracts for the preparation of drugs [1].

QUICK RESPONSE CODE
DOI: 10.13040/IJPSR.0975-8232.11(10).5053-58
The article can be accessed online on www.ijpsr.com
DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(10).5053-58

In Asian and African populations, various medicinal plants have been used for thousands of years in folk medicines, and several plants are consumed for their health benefits. According to the World Health Organization (WHO), some nations still rely on plant-derived treatment as their main source of medicine, and developing nations are utilizing the benefits of naturally sourced compounds for therapeutic purposes [2].

Compounds which have been identified and extracted from terrestrial plant source for their anticancer properties include polyphenols, brassinosteroids, and taxols. Plant-derived drugs are desired for cancer treatment as they are natural and readily available drugs. Traditionally, the plant naravelia (*Naravelia zeylanica*) is used for the

treatment of various diseases like pitta, helminthiasis, dermatopathy, leprosy *etc.* [3] Further, it shows multiple pharmacological activities like antibacterial, antioxidant, antiulcer and anti-helmintic [4]. Thus, the present study was carried out to evaluate the anticancer activity of ethanol extract of *N. zeylanica* against the A549 lung cancer cell line.

MATERIALS AND METHODS:

Collection and Identification of Plant: The leaves of *Naravelia zeylanica* were collected from Kolli hills, Tamil Nadu, India, and it were identified and authenticated (BU002) by The Rapinat Herbarium, St. Josephs College, Trichy.

Extraction: The *N. zeylanica* leaves were shade-dried and coarsely powdered using a pulverizer. Then, the powder was successively extracted with ethanol (80 ºC) using a Soxhlet apparatus. The solvent was removed by vacuum distillation in a rotatory evaporator at 60 ºC. The extract was filtered through Whatman no. 1 filter paper and concentrated on a water bath to a syrupy mass and stored in a desiccator for future use.

Purchasing of Cell Lines and Culture: Human lung cancer cell lines A549 was acquired from National Center for Cell Science (NCCS), Pune, India. The cells were cultured in DMEM medium supplemented with 10% FBS, and with Penicillin at 100 µ/mL and streptomycin at the concentration of 100 µg/mL were used as antibiotics (Himedia, Mumbai, India), at 37 ºC in a humidified atmosphere of 5% CO_2 in an incubator (Forma, Thermo Scientific, USA).

Cell Viability Assay: The MTT tetrazolium salt colorimetric assay [5] was performed to measure the cytotoxicity as revealed in cell viability. In this assay, mitochondrial enzyme converts soluble MTT into an insoluble colored formazan product in live cancerous cells, which may be measured spectrophotometrically [6]. The cells were seeded in 96 well plates at a density of 5000 cells/ well and kept for 24 h (37°, 5% CO_2). After 24 h, prepared concentrations of ethanol extract of *N. zeylanica* (100-1000 µg/ml) was added. The extract was dissolved in DMSO, and control cells contained DMSO at the equivalent concentration (0.02%) of treated cells. After 24 h of incubation, 20 µl of MTT solution (5 mg/ml in phosphate buffer solution) was added and kept the plate for 3 h at 37 ºC. To dissolve formazan crystals formed, medium containing MTT was gently replaced by DMSO. Absorbance was measured at 560 nm using an ELISA plate reader (Bio-Rad Hercules, California, USA). Data were collected for three times, each in triplicate values was used to calculate the means and the standard deviations. The percentage of inhibition was calculated from the following formula:

$$\%\ \text{of Inhibition} = \frac{\text{Mean OD of untreated cells (control)} - \text{Mean OD of treated cells}}{\text{Mean OD of untreated cells (control)}} \times 100$$

From the values, the obtained IC_{50} values for the ethanol extract of *N. zeylanica* were calculated by plotting percentage inhibition against different concentrations.

Acridine Orange (AO) and Ethidium Bromide (EB) Fluorescent Assay: Normally, cell division genetically regulated by programmed cell death called apoptosis, which controls the development of organisms, include organs and tissues by eliminating damaged cells, abnormal cells and physiologically functionless cells. The ongoing research on oncology mainly focused on the genes and signals capable of regulating apoptosis. Thus, the efficacy of the anticancer agent is checked by their ability to promote apoptosis in cancer cells [7]. AO and EB staining [8, 9] was performed against A549 lung cancer cells treated with IC_{50} concentration of ethanol extract of *N. zeylanica* for 2 h were cultured separately in 6-well plates, where DMSO (0.02%) was used as solvent control. The cells (25 µL of suspension containing 5000 cells) were incubated with acridine orange and ethidium bromide solution (1 part of 100 µg/mL each of acridine orange and ethidium bromide in PBS) and examined in a fluorescent microscope (Carl Zeiss, Jena, Germany) using a UV filter (450–490 nm).

Three hundred cells per sample were counted, in triplicate, for each time point and scored as viable or dead, and if dead whether by apoptosis or necrosis as judged from the nuclear morphology and cytoplasmic organization. The percentages of apoptotic and necrotic cells were then calculated. Morphological features of interest were photographed.

Hoechst 33528 Staining: A549 lung cancer cells were cultured in separate 6-well plates and treated with 24 h IC_{50} concentrations of ethanol extract of *N. zeylanica*. After the respective period of incubation time, treated and control cells were harvested and stained with Hoechst 33258 stain (1 mg/mL, aqueous) for 5 min at room temperature [10]. A drop of cell suspension was placed on a glass slide, and a coverslip was laid over to reduce light diffraction. At randomly 300 cells, in triplicate, were observed at 400X in the fluorescent microscope fitted with a 377–355 nm filter, and the percentage of cells that reflected pathological changes was calculated [11]. Data were collected for three replicates each and used to calculate the means and the standard deviations.

Assessment of Mitochondrial Membrane Potential (JC1 Staining): Mitochondrial membrane potential ($\Delta\psi m$) was assessed using the fluorescent probe JC-1, which produces orange-red fluorescence when accumulated in the mitochondria of healthy cells but fluoresces green when leached out into the cytosol due to loss of $\Delta\psi m$ resulting in a negative internal potential [12]. The A549 lung cells were grown in glass coverslips (22 × 9 × 22 mm) placed in the wells of 6-well plates and treated with the ethanol extract of *N. zeylanica* at the 24 h IC_{50} concentration, and 0.02% DMSO was used as solvent control. The cells were stained with JC-1 dye after 12 and 24 h exposure. The mitochondrial depolarization patterns of the cells were observed in the fluorescent microscope and the pathological changes in the cells were observed and recorded. Data were collected for three replicates each and used to calculate the means and the standard deviations.

Single Cell Gel Electrophoresis (Comet assay): DNA damage of individual cells was identified by using the comet assay [13]. The cells were treated with ethanol extract of *N. zeylanica* for 24 h. The harvested cells were suspended in low melting point agarose in PBS and pipetted out to microscope slides pre-coated with a layer of normal melting point agarose. The slides were chilled on ice for 10 min and then immersed in lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 m MTris, 0.2 mMNaOH [pH 10.01] and Triton X-100) was incubated overnight at 48 ºC in order to lyse the cells and permit DNA unfolding.

Thereafter, the slides were exposed to alkaline buffer (300 m MNaOH, 1 mM Na2-EDTA, [pH>13]) for 20 min to allow DNA unwinding. The slides were washed with buffer (0.4 M Tris, pH 7.5) to neutralize the excess alkali and to remove the detergents before staining with ethidium bromide (20 mL in 50 mg/mL). Photomicrographs were obtained using a fluorescent microscope. 150 cells from each treatment group were digitized and analyzed using CASP software. The images were used to determine the DNA content of individual nuclei and to evaluate the degree of DNA damage representing the fraction of DNA in the tail, so as to assign the cells among the five categories: dead, highly damaged, damaged, slightly damaged and intact. Data were collected for three replicates each and used to calculate the means and the standard deviations.

RESULTS AND DISCUSSION:

Effects of Ethanol Extract Treatment on the Viability of Lung Cancer Cells: The inhibitory effects of the ethanol extract of *N. zeylanica* using different concentrations of about 24 h on A549 lung cancer cells were investigated adopting MTT assay. The assay was aimed to determining the integrity of mitochondria so as to reflect on the viability or otherwise of the cells. It was recorded that the ethanol extract of *N. zeylanica* was cytotoxic to A549 lung cancer cell lines in a dose and duration-dependent manner. The IC_{50} of ethanol extract of *N. zeylanica* was calculated as 435.50 ± 0.05 µg/ml **Fig. 1**.

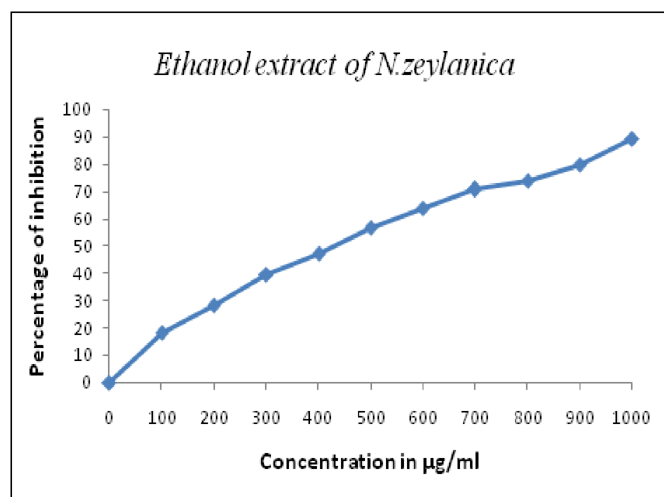

FIG. 1: INHIBITORY CONCENTRATION ON (IC_{50}) OF THE ETHANOL EXTRACT TO A549 LUNG CANCER CELL LINES

Morphological Changes in Lung Cancer Cells: AO & EB staining followed by fluorescent microscopy revealed apoptosis by fluorescence emission, which depends on the viability and membrane integrity of the cells. Uniformly green fluorescing nuclei with a highly organized cellular structure indicated the normal and viable cells **Fig. 2**. Apoptosis, or programmed cell death, is characterized by a number of characteristic features, which include chromatin fragmentation and condensation, membrane blebbing, internucleosomal DNA cleavage, caspase activation, and translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane [14]. Hence, the induction of apoptosis is one of the useful approaches in cancer therapies [15]. Liu *et al.,* 2009 proposed that AO penetrated normal and early apoptotic cells with intact membranes, fluorescing green when bound to DNA [16]. EB only entered cells with damaged membranes, such as late apoptotic and dead cells, emitting orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies [17]. Green fluorescing nuclei with perinuclear chromatin condensation were revealed in the bright green patches or fragments indicated cells in the early phase of apoptosis.

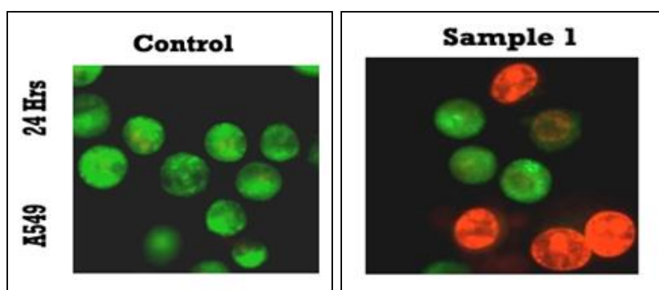

FIG. 2: MORPHOLOGICAL CHANGES OBSERVED FOR CONTROL AND ETHANOL EXTRACT OF *N. ZEYLANICA* TREATED A549 CELLS STAINED WITH ACRIDINE ORANGE AND ETHIDIUM BROMIDE

Orange to red fluorescing nuclei with highly condensed or fragmented chromatin indicated that the cells were in a late stage of apoptosis. Uniformly orange to red fluorescing nuclei with no indication of chromatin fragmentation, but the entire cells as well as nuclei were swollen to large size indicated necrotic cells **Fig. 2**. Data on cells indicating apoptotic and necrotic morphologies, produced on treatment with the ethanolic extract of *N. zeylanica* for 24 h through manual counting which are presented.

That is revealed the complex is highly efficient in bringing about apoptosis in a high manner, but necrosis was also produced to a certain extent. To detect apoptosis at a basic level, we adopted Hoechst staining, which revealed the changes in gross cytology of the cell, with special reference to cytoplasm and nucleus. The final stage of apoptosis is characterized by DNA degradation and cell damage into dense membrane-surrounded fragments (apoptotic bodies). Some apoptotic bodies contain fragments of the nucleus, while others contain only cytoplasm [18]. Cells that have died by apoptosis will generally display condensed DNA and fragmented nuclei; meanwhile, the healthy and necrotic cells do not [19]. After treatment with IC_{50} concentration of the ethanol extract of *N. zeylanica* for 24 h, the cells were observed for cytological changes. The observations revealed that the treatment about chromatin fragmentation, binucleation, cytoplasmic vacuolation, membrane blebbing, and late apoptosis indication of dot-like chromatin, although fairly good percentage of cells indicated features of necrotic death. Data collected from manual counting of cells with normal and abnormal nuclear features are shown in **Fig. 3**.

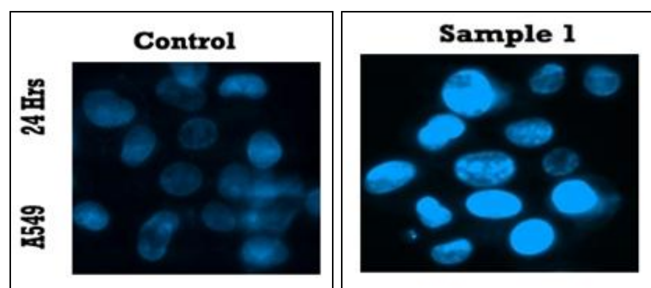

FIG. 3: MORPHOLOGICAL FEATURES OF NUCLEI OBSERVED FOR CONTROL AND ETHANOL EXTRACT OF *N. ZEYLANICA* TREATED LUNG CANCER CELLS STAINED WITH HOECHST 33258

Mitochondrial Membrane Potential (Δψm): This assay to detect the changes in mitochondrial function employs the fluorescent cation JC-1, which emits red fluorescence when sequestered in to the mitochondria of healthy cells with high Δψm. Cells Δψm undergoing apoptosis are no longer able to sequester the JC-1 cation into the mitochondria due to loss of Δψm and so cells fluoresce green. The **Fig. 4** shows the results of JC-1 staining of lung cancer cells treated with the extract at its 24 h IC_{50} concentration. The treatment led to a high proportion of apoptotic cells in A549 lung cancer cell line.

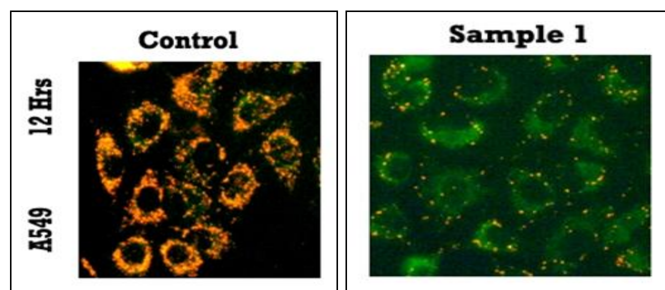

FIG. 4: PHOTOMICROGRAPHS OF A549 LUNG CANCER CELLS, JC1 DYE ACCUMULATED IN THE MITOCHONDRIA OF HEALTHY CELLS AS AGGREGATES (RED- ORANGE FLUORESCING); IN CELLS TREATED WITH ETHANOL EXTRACT OF *N. ZEYLANICA* DUE TO COLLAPSE OF MITOCHONDRIAL MEMBRANE POTENTIAL, THE JC-1 DYE RETAINED INTO THE CYTOPLASM IN ITS MONOMERIC FORM, WHICH FLUORESCED GREEN

Assessment of DNA Damage by Commet Assay: Single cell gel electrophoresis assay (comet assay) is considered to be rapid, simple, visual, and sensitive [20]. In the comet assay DNA of damaged single cells takes the appearance of a comet, with head and tail regions. The CASP image analysis software is of help in (i) analysis of a variety of geometric and densitometry parameters, and (ii) measuring the amount of DNA in the head (intact DNA) and the tail (DNA with strand breaks) regions **Fig. 5**. Since the tail length and density reflect the extent of strand breaks in DNA, the percentage of DNA in the tail provides a quantitative measure of the damaged DNA as a fraction of the total DNA. The results revealed that DNA damage was induced in a high percentage in lung cancer cells (A549) by the ethanol extract of *N. zeylanica*.

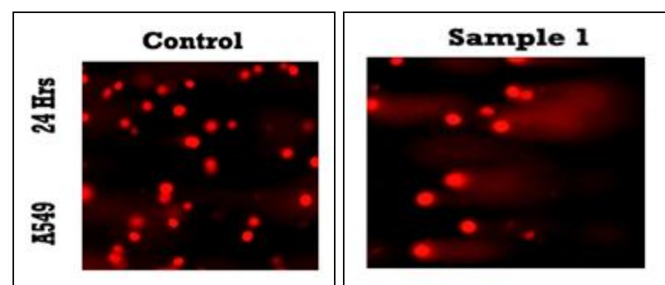

FIG. 5: DNA DAMAGE IN ETHANOL EXTRACT OF *N. ZEYLANICA* TREATED LUNG CANCER CELLS IS REVEALED IN THE COMET ASSAY

CONCLUSION: The present study unambiguously showed that the ethanolic extract of *N. zeylanica* has showed stronger antiproliferative effect on A549 lung cancer cell line in a concentration dependent manner.

The mechanism underlying morphological changes were also produced, nuclei alteration thus highly interrupt the nucleus structure of the cells, induction of DNA damage and alteration in mitochondrial transmembrane potential. Our findings also support the other results that the suggested ethanol extract of *N. zeylanica* leads to DNA damage, as well as mitochondrial membrane changing effect on A549 lung cancer cell line and is therefore worthy of further investigation needed in this plant.

ACKNOWLEDGEMENT: The authors thank the Management, the Principal, and Head of the Department of Zoology, Nehru Memorial College, Puthanampatti, Trichy for providing necessary facilities to do this research work successfully. The first author thanks the University Grants Commission, New Delhi, for awarding Rajiv Gandhi National Fellowship (RGNF-SRF) for financial support. The authors also thank Dr. R. Thirumurugan, Professor and Coordinator, National Centre for Alternatives to Animal Experiments, Bharathidasan University, Trichy for given permission to carry out *in-vitro* studies in the NCAAE and B. Gowthami, Research Scholar for her meticulous help in the *in-vitro* studies of anticancer activity.

CONFLICTS OF INTEREST: The authors declare that there is no conflict of interest regarding this study.

REFERENCES:

1. Sivaraj R, Rahman PKSM, Rajiv P, Vanathi P and Venckatesh R: Biosynthesis and characterization of acalyphaindica mediated copper oxide nanoparticles and evaluation of its antimicrobial and anticancer activity. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 2014; 129: 255-58.
2. Wealth of India, (Publication and information directorate) CSIR, New Delhi, 1998.
3. Harsha VH, Hebbar SS, Shripathi V and Hegde GR: Ethnomedicobotany of Uttrakanada district in Karnataka, India – Plants in treatment of skin diseases. Journal of Ethnopharmacology 2003; 84:37-40.
4. Manasa B and Venugopal Y: Naravelia zeylanica: a review. International Journal of Pharmacy 2013; 3: 241-46.
5. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 1983; 65: 55-63.
6. Sylvester PW: Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. Methods Mol Biol 2011; 716: 157-68.

7. Taraphdar AK, Roy M and Bhattacharya R: Natural products as inducers of apoptosis: implication for cancer therapy and prevention. Current Sci 2001; 80: 1387-96.
8. Spector DL, Goldman RD and Leinwand LA: Cell: A laboratory manual. Culture and biochemical analysis of cells. CSHL press, New York 1998.
9. Umar RS, Arunachalam S, Periyasamy VS, Preethy CP, Riyasdeen A and Akbarsha MA: Synthesis, DNA binding and anti-tumour activities of some novel polymer cobalt III complexes containing 1, 10-phenanthroline ligand. Polyhedron 2008; 27: 1111-20.
10. Latt SA, Stetten G, Juergens LA, Willard HF and Scher CD: Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechest fluorescence. Journal of Histochemistry and Cytochemistry 1975; 23: 493-05.
11. Rajendiran V, Karthik R, Palaniandavar M, Stoeckli Evans H, Periyasamy VS, Akbarsha MA, Srinag BS and Krishnamurthy H: Mixed- ligand copper II- Phenolate complexes: effect of co-ligand on enhanced DNA and protein binding, DNA cleavage and anticancer activity. Inorganic Chemistry 2007; 46: 8208-21.
12. Reers M, Smith TW and Chen LB: J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 1991; 30: 4480-86.
13. Singh NP, Mccoy MT, Tice RR and Schneider EL: A simple technique for quantification of low levels of DNA damage in individual cells. Experimental cell Research 1988; 175: 184-91.
14. Talib WH and Mahasneh AM: Antiproliferative activity of plant extracts used against cancer in traditional medicine. Scientia Pharmaceutica 2010; 78: 33-45.
15. Reed JC: Apoptosis-based therapies. Nature Reviews Drug Discovery 2002; 2: 111-21.
16. Liu S: Radiolabeled cyclic RGD peptides as integrin r v 3 -targeted Radiotracers. Bioconjug Chem 2009; 20: 2199-13.
17. Ribble D, Goldstein NB, Norris DA and Shellman YG: A simple technique for quantifying apoptosis in 96-well plates. BMC Biotechnol 2005; 5: 12.
18. Kalinichenko SG and Matveeva NY: Morphological characteristics of apoptosis and its significance in neurogenesis. Neurosci Behav Physiol 2008; 38: 333-44.
19. Crowley LC, Marfell BJ and Waterhouse NJ: Analyzing cell death by nuclear staining with Hoechst 33342. Cold Spring Harb. Protoc 2016; 9.
20. Moller P, Knudsen LE, Loft S and Wallin H: The comet assay as rapid test in biomonitoring occupational exposure to DNA-damaging agents and effects of confounding factors. Cancer Epidermiol Biomarkers Prevent 2000; 9: 1005-15.

How to cite this article:
Umarani B and Saravanan K: Anti-proliferative and apoptosis inducing effect of *Naravelia zeylanica* L. against lung cancer cell line A549. Int J Pharm Sci & Res 2020; 11(10): 5053-58. doi: 10.13040/IJPSR.0975-8232.11(10).5053-58.

All © 2013 are reserved by the International Journal of Pharmaceutical Sciences and Research. This Journal licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License.

This article can be downloaded to **Android OS** based mobile. Scan QR Code using Code/Bar Scanner from your mobile. (Scanners are available on Google Playstore)