Available online on 15.04.2019 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

Open Access Research Article

Aglaia malabarica induced apoptosis evaluation in plant meristem, erythrocytes and human cancer cell lines

***Archana Elamkulam Ravindran and John Ernest Thoppil**

Cell and Molecular Biology Division, Department of Botany, University of Calicut, Malappuram, Kerala, India

ABSTRACT

Apoptosis forms the basis of the drugs targeting cancer cells. The present study aims to explore apoptosis inducing ability of the evergreen forest plant *Aglaia malabarica* in three model cell systems. *Allium cepa* root meristem treated with the plant extract showed considerable decrease in the mitotic index and also revealed increased cytological aberration in a dose dependent manner on treatment with the leaf extract. Evans blue staining of the meristem confirmed the apoptotic death induction by the plant extract in a dose dependent way. Erythrocytes were subjected to treatment with the plant extract and remarkable apoptotic signs like membrane blebs, membrane distortions *etc.* were observed. HeLa cells were also characterized by the formation of echinoid bodies, membrane blebs, nuclear disintegration, fragmented nucleus *etc.* after treatment with *A. malabarica* extract. The quantitative estimation of cell viability was done using MTT assay and morphological alterations was studied by AO/EB double staining. The plant is proved to be of high potential in inducing apoptosis, in rapidly dividing cells and thereby opens a new possibility in drug preparation against uninhibited cell proliferation.

Keywords: *Aglaia*, apoptosis, eryptosis, chromatin aberration, MTT, HeLa

Article Info: Received 19 Feb 2019; Review Completed 23 March 2019; Accepted 26 March 2019; Available online 15 April 2019

Cite this article as:

Ravindran AE, Thoppil JE, *Aglaia malabarica* induced apoptosis evaluation in plant meristem, erythrocytes and human cancer cell lines, Journal of Drug Delivery and Therapeutics. 2019; 9(2-s):118-124

http://dx.doi.org/10.22270/jddt.v9i2-s.2611

***Address for Correspondence:**

Archana Elamkulam Ravindran, Cell and Molecular Biology Division, Department of Botany, University of Calicut, Malappuram, Kerala, India

INTRODUCTION

Aglaia, belonging to the family Meliaceae, comprises of over 120 species of tropical evergreen trees distributed in south-east Asian region. The genus *Aglaia* is well marked for the presence of rocaglamides, as it is the only natural source of these flavaglines. A large number of species of *Aglaia* are used by the tribal community for its diverse properties. Some species are used for its edible fruits, scented flowers and medicinal or healing properties like treatment of inflammation, fever, diarrhoea and wounds. The extracts of some species are also used against infections as bactericides and insecticides[1]. *Aglaia malabarica*, locally known as 'Chuvannakil', is a sub-canopy wild tree endemic to the Western Ghats in India. The plant can be identified by its unique lepidote scale on the ventral side of the leaves and the golden brown young fronds. *A. malabarica* is one among the least explored species for its bioactivity and phytochemical constitution.

Apoptotic cell death in multicellular organisms has been associated with the elimination of unwanted cells, cell homeostasis, and response to biotic and abiotic stresses, differentiation and aging[2,3]. Dysregulation of cell homeostasis and controlling mechanism might lead to the disruption of the whole system and often results in diseases like cancer. Morphological features such as cytoplasmic condensation, nuclear DNA fragmentation, chromatin disintegration, membrane blebbing and formation of apoptotic bodies are exhibited in the apoptotic cells[4].

Almost every drug targeting the death of the malignant tissues are based on their ability to induce apoptosis in rapidly dividing cells. Nowadays, developing plant derived substitutes for chemotherapeutics is acquiring momentum as the plant based products are characterized by low toxicity against normal cells. The present study is a novel attempt to evaluate the cytotoxicity and apoptosis inducing ability of the extracts of *Aglaia malabarica* in rapidly dividing cells.

MATERIALS AND METHODS

Preparation of extracts: The leaves of *A. malabarica* were collected from Wayanad district, Kerala, India. The specimen was authenticated at the Department of Botany, University of Calicut, Kerala, India (CALI no. 123754). The leaves were shade dried for ten days followed by grinding and later stored in an air tight container. Methanolic extract was prepared using soxhlet apparatus for 6 h and the filtrate was evaporated to dryness under low pressure.

Cytotoxicity evaluation using *Allium cepa* meristem: Pesticide free *Allium cepa* bulbs were procured from TNAU, Tamil Nadu, India and were grown on moist autoclaved sand. Sprouted bulbs were treated after the roots acquired a

length of approximately 2 cm. Various concentrations of plant extract were used 0.05, 0.1, 0.15, 0.2 and 0.3% for different time periods *viz*., 1 h, 12 h and 24 h. Malathion was taken as the positive control while distilled water to be the negative. Squash preparations of the healthy roots, after excision, were made using standard protocol[5] and viewed under Leica DM2000 microscope. The microphotographs were taken using the top mount camera Leica DMC2900.

***In-situ* apoptosis detection**: *Allium cepa* bulbs were treated with the different concentrations of the plant extracts *viz*., 0.1, 0.2 and 0.3% for 1 h. Controls were taken as same as above. The treated bulbs were soaked in Evans blue solution (0.25% w/v) for 15 min. The roots of equal length were then excised and macro-photographed after washing[6].

Eryptosis detection: Human blood was collected in a heparinised vial from the blood bank, Calicut, Kerala, India. This was centrifuged (8,000 rpm for 15 minutes) at room temperature and the filtrate was discarded in order to remove the plasma and buffy coat. RBCs were washed three times with normal saline (0.9% w/v), and resuspended in four times its volume[7]. 1 mL RBC suspension was incubated with equal volume of 0.1% leaf methanolic extract for 1h at room temperature. Cell suspension incubated along with normal saline was used as the control. Smear of the treated cells were prepared followed by staining with May-Grünwald-Giemsa method[8, 9] and the microphotographs were taken (Leica DMC2900).

MTT analysis: HeLa cells were cultured on modified Dulbecco's modified Eagle's medium (DMEM) up to 70% confluence. Various concentrations of plant extracts (6.25, 12.5, 25, 50 & 100 μg/ml) were prepared in DMEM and the cells were incubated in it, after the removal of the previous medium, for 24 h. Further observation of the treated cells was done using Olympus CKX41 and microphotographs were taken using Optika Pro5 CCD camera. Percentage viability was calculatedusing the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD of the samples}}{\text{Mean OD of control group}} \times 100$$

LD_{50} was also calculated from the values obtained using ED50 PLUS V1.0 software.

Double staining of HeLa cells: The cells were treated with the LD50 concentration and were subjected to double staining in order to detect apoptosis. These were washed with PBS and stained with a mixture of acridine orange and ethidium bromide (100 μg/mL w/v) for 15 min. under room temperature. Then the cells were washed again with PBS and observed under the fluorescent microscope (Olympus CKX41). Microphotographs were taken using Optika Pro5 CCD camera.

Statistical analyses: The data obtained from the experiments were subjected to one way ANOVA and in order to validate the results Duncan's multiple range test was performed. The results are expressed as mean ± SE and $P < 0.05$ was considered statistically significant.

RESULTS

Cytotoxicity evaluation using *Allium cepa* meristem: The dividing capacity of the cells was hindered by the treatment of the cells with the plant extract in a dose dependent manner. The mitotic index was found to be decreasing with the increasing concentration. The root meristem also showed aberrant cells as a result of treatment and the percentage aberration was found to be directly proportional to the concentration of the extract (Table 1). Meristem after the treatment was characterized by micronucleus formation, pulverized chromatin, chromatin erosion, disintegration *etc.* which are remarkable features of cells progressing towards apoptosis (Fig. 1).

Table 1: Cytotoxicity induced by *Aglaia malabarica* extracts on *Allium cepa* meristem

Concentration	Mitotic index (%)	Aberration %
1 hr		
0.05	60.76 ± 6.2	55.33 ± 6.9
0.1	55.84 ± 3.9	57.99 ± 7.0
0.15	52.96 ± 3.1	65.44 ± 4.8
0.2	35.11 ± 3.8	87.96 ± 3.2
0.3	15.53 ± 2.1	92.62 ± 1.5
Positive	9.76 ± 1.3	92.92 ± 1.1
Negative	83.76 ± 2.0	1.06 ± 0.5
12 hr		
0.05	78.79 ± 3.3	23.18 ± 2.6
0.1	80.61 ± 3.6	33.90 ± 2.4
0.15	70.18 ± 1.3	63.07 ± 3.8
0.2	48.27 ± 4.2	60.25 ± 4.0
0.3	37.73 ± 2.5	77.65 ± 1.7
Positive	16.88 ± 2.2	94.55 ± 1.8
Negative	79.64 ± 0.9	2.41 ± 0.6
24 hr		
0.05	82.29 ± 2.7	23.25 ± 2.0
0.1	76.19 ± 0.7	37.83 ± 3.0
0.15	61.36 ± 0.9	57.51 ± 1.6
0.2	36.82 ± 3.6	68.73 ± 4.2
0.3	24.17 ± 2.5	84.88 ± 2.6
Positive	20.46 ± 2.2	89.86 ± 1.6
Negative	88.46 ± 2.3	0.71 ± 0.71

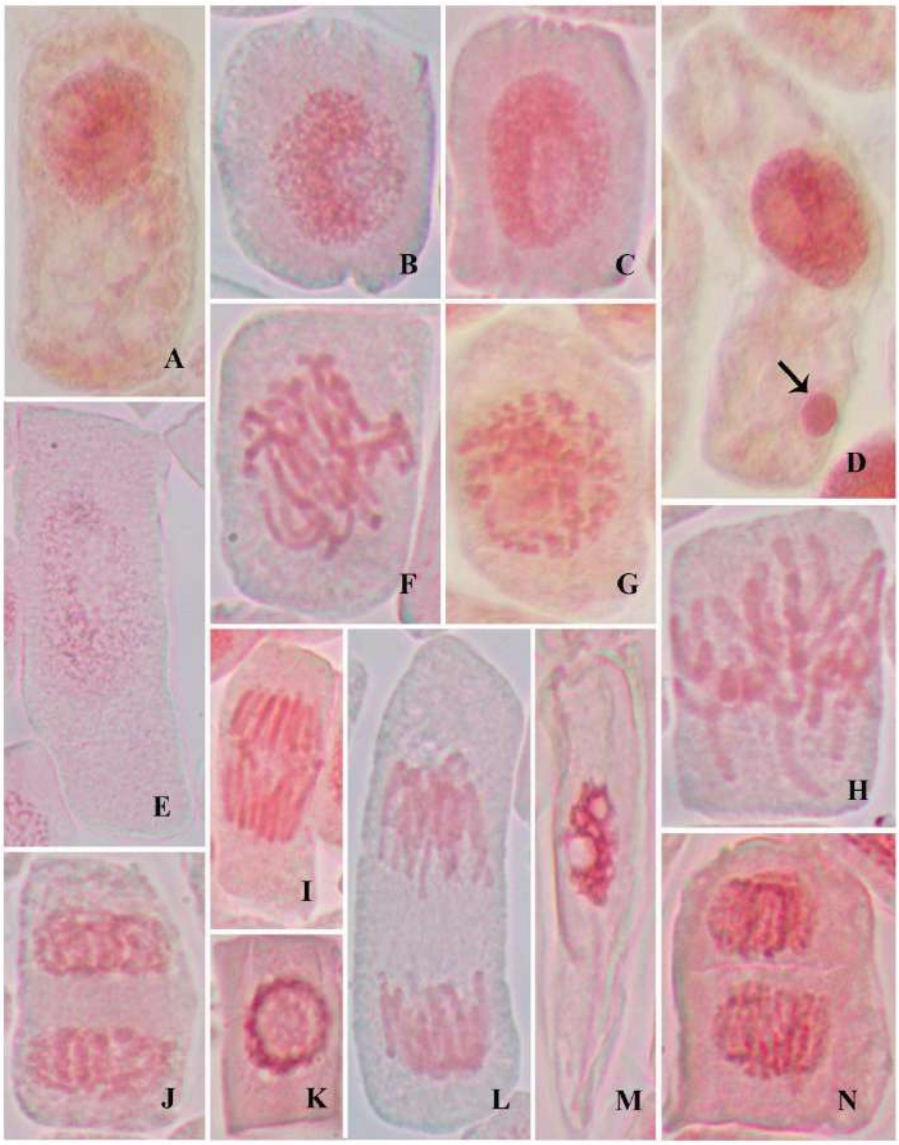

Figure 1: Apoptotic cells of *Allium cepa* meristem observed after their treatment with *A. malabarica* extract. **A.** Cytoplasmic lesion at interphase, **B.** Nuclear breakage at interphase, **C.** Nuclear lesion at interphase, **D.** Micronucleus at interphase, **E.** Pulverised chromatin at interphase, **F.** Chromosome fragmentation at early anaphase, **G.** Fragmented chromosomes at early metaphase, **H.** Chromosome dissolution at metaphase, **I.** Laggard fragments at anaphase, **J.** Chromatin erosion at cytokinesis, **K.** Chromatin disintegration and nuclear vacuolation, **L.** Pulverised chromatin at anaphase, **M.** Chromatin disintegration with multiple nuclear lesions and cytoplasmic shrinkage, **N.** Pulverised telophase with early cell plate formation.

***In situ* apoptosis detection**: The *A. cepa* root meristem treated with the plant extract took up the stain in an increasing order of the concentration of the extract. The positive control, where the meristem was treated with Malathion, stained almost completely thereby denoting high apoptotic death while, in the negative control, where it was treated with distilled water, minimal staining of the meristem was observed. Thus the apoptotic death in the meristem treated with the plant extract was found to be dose dependent (Fig. 2).

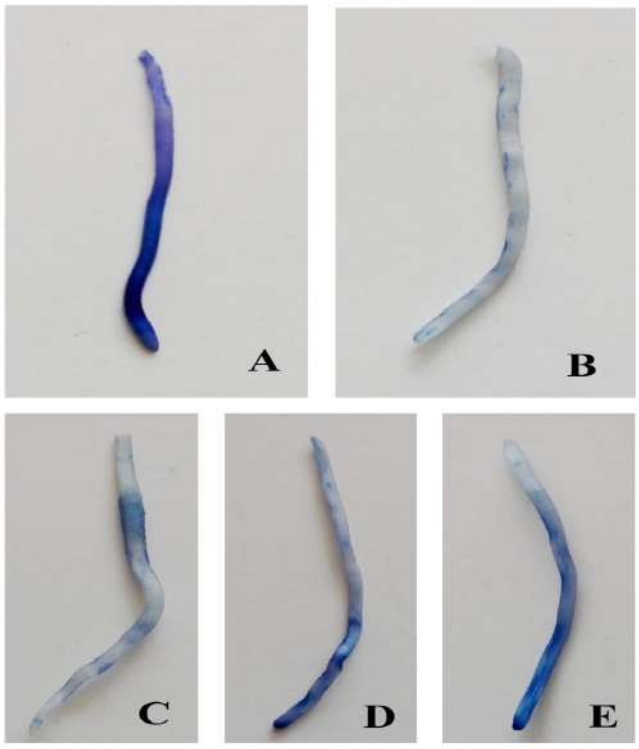

Figure 2. Apoptotic death of *Allium cepa* root meristem observed by Evans blue staining after treating with *A. malabarica* extract. **A.** Positive control, **B.** Negative control, **C, D & E.** Roots from the bulbs treated with various concentrations of *A. malabarica* methanolic extracts *viz.*, 0.1, 0.2 and 0.3 %, respectively.

Eryptosis detection: The RBCs after treatment with the plant extract were characterized by the apoptotic signs like membrane blebs, distorted membranes and apoptotic bodies, thereby confirming the potential of the plant to induce cell death (Fig. 3).

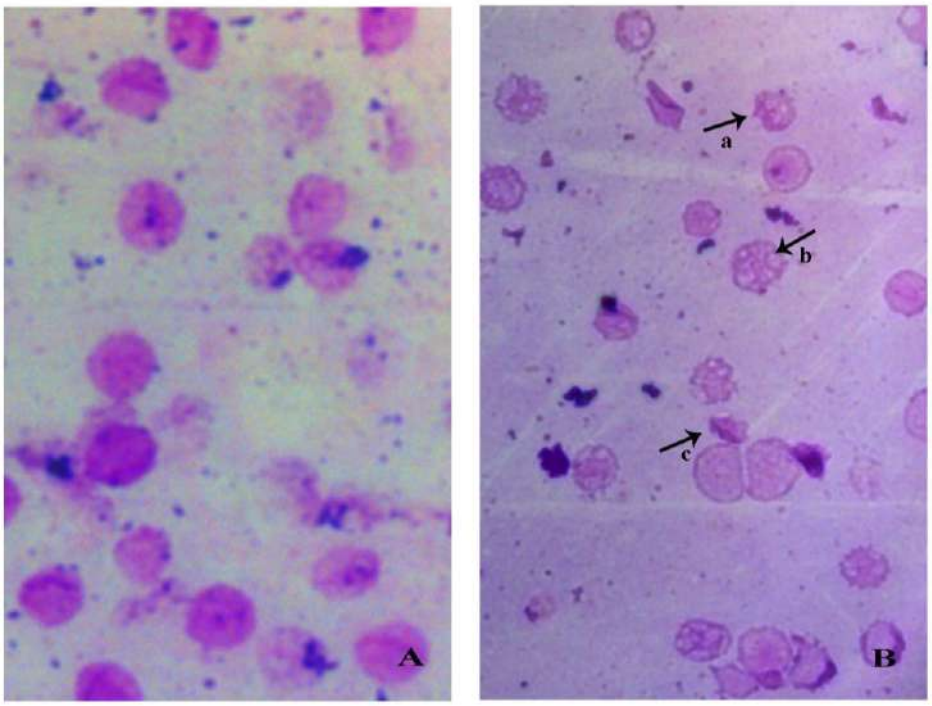

Figure 3: Apoptotic effect of *Aglaia malabarica* leaf extract on human erythrocytes. **A.** Untreated normal RBCs, **B.** Various membrane alterations, obtained in the treated cells like, **(a)** membrane blebs, **(b)** membrane distortions and **(c)** apoptotic bodies.

MTT analysis: HeLa cell lines treated with the plant extract showed death in an increasing rate according to increasing concentration. The apoptotic features like condensed nuclei, echinoid formation, nuclear fragmentation, blebs *etc.* were prominent in the treated cells. Apoptotic bodies were also formed. Cell adherence was considerably minimal in those treated with high concentration of the extract (Fig. 4). Percentage viability of the cells was found to be inversely proportional to the concentration of the extract (Table 2) and LD_{50} was found to be 42.137 μg/mL.

Table 2: MTT assay result of *Aglaia malabarica* leaf extract on HeLa cell lines

Sample Concentration (µg/mL)	Average OD at 540nm	Percentage Viability
6.25	1.021	81.25
12.5	0.812	64.62
25	0.715	56.95
50	0.613	48.79
100	0.534	42.55
Control	1.256	100

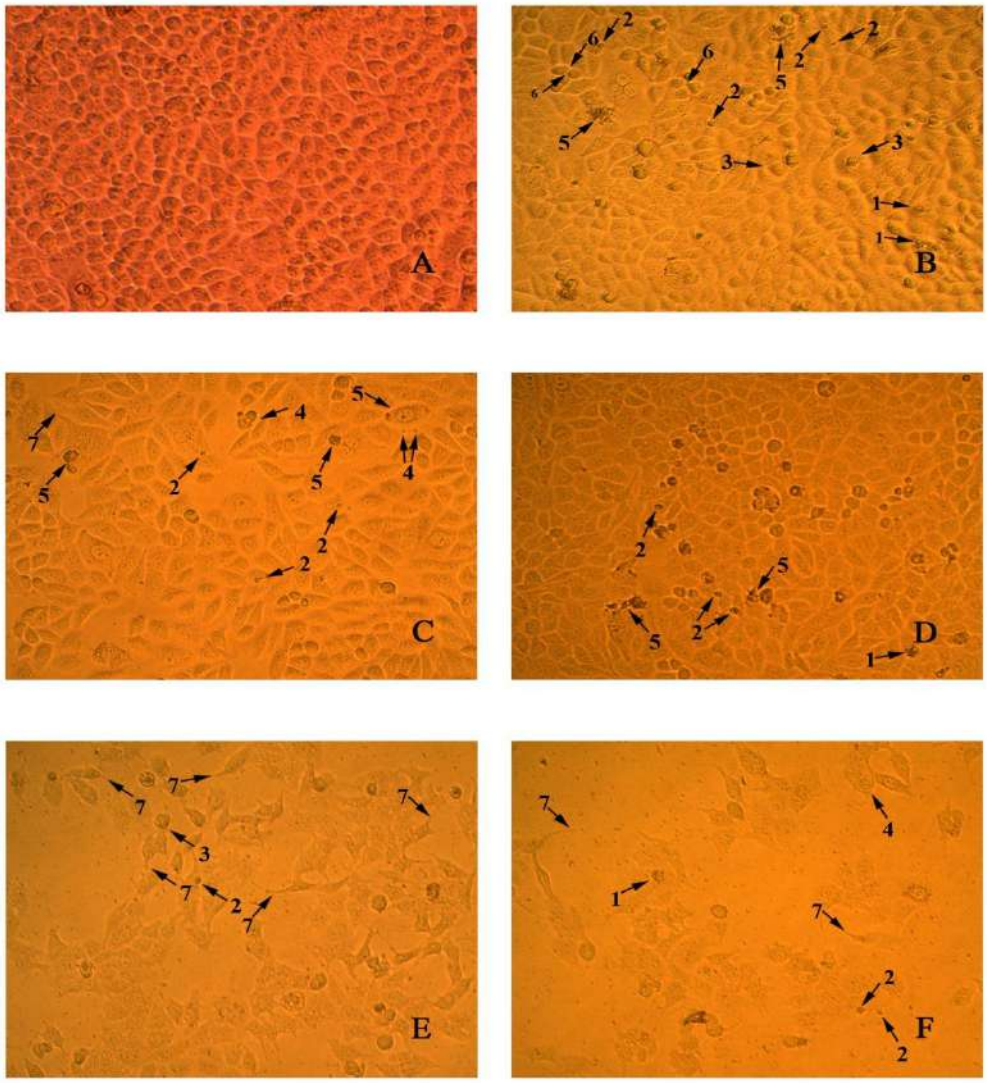

Figure 4: Apoptotis induction by various concentrations of *A. malabarica* extract in HeLa cell line as observed by MTT staining method. Various aberrations noticed - **1.** Nuclear fragmentation, **2.** Condensed nuclei, **3.** Cell shrinkage, **4.** Membrane blebbing, **5.** Apoptotic bodies, **6.** Budding, 7. Echinoid spikes. **A.** Control, **B.** 6.25 μg/ml, **C.** 12.5μg/ml, **D.** 25 μg/ml, E. 50 μg/ml, **F.** 100 μg/ml.

Double staining of HeLa cells: Apoptosis in the treated cells were well marked with apoptotic signs and almost half of the cells were stained orange denoting the dead cells and others green. In contrast, the untreated control cells were observed to be stained green completely denoting live cells and absence of apoptosis (Fig. 5).

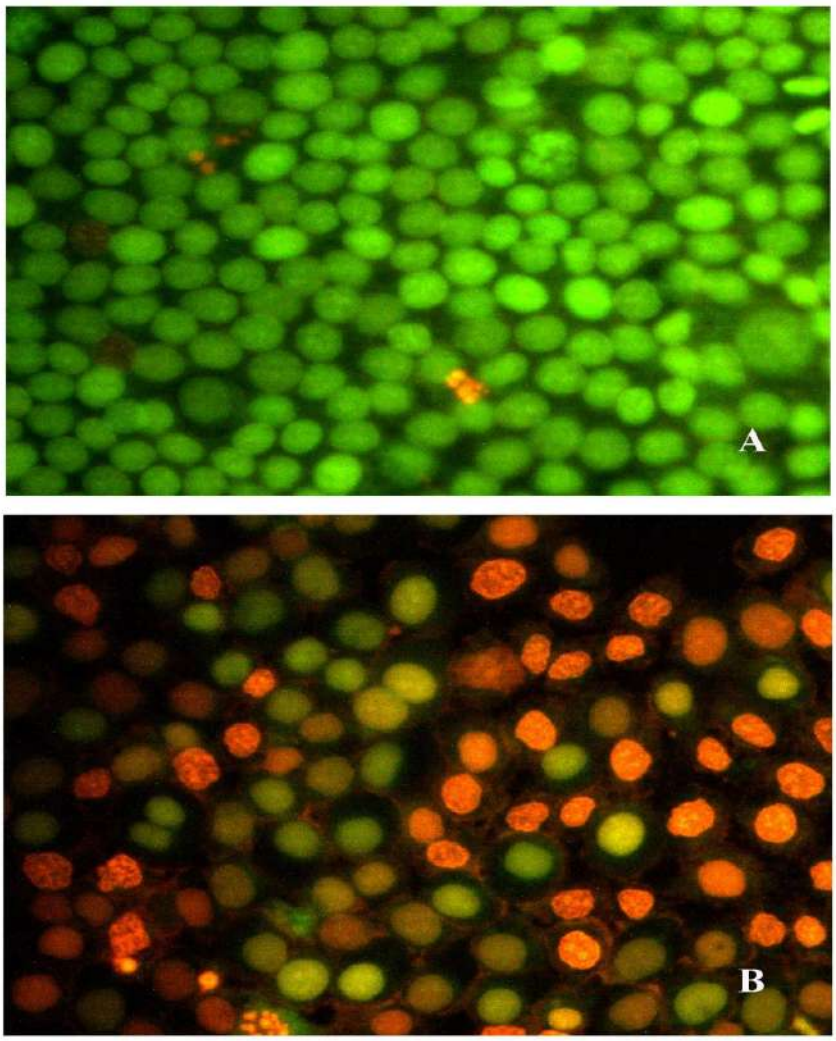

Figure 5: Determination of apoptosis in HeLa cells by the double staining method. **A**. Untreated control cells. **B.** Cells after the treatment with *Aglaia malabarica* extract for 24 h.

DISCUSSION

Apoptosis is an important process of cell death in a programmed manner that has a vital role in metabolism, development and even during stress responses. Dysregulation of this process leads to uncontrolled cell division. Thus, induction of apoptosis forms the basis of drugs against cancer development and an effective method to suppress tumor progression[10]. As the plant products are characterized by minimal side effects and negligible hindrance to normal metabolism, researchers are focusing on them for drug discovery against deadly diseases including cancer. Plants and plant products that are potent apoptosis inducers are gaining interest in anticancer drug discovery.

Many species of *Aglaia* has been reported for its cytotoxic potential against rapidly dividing cells. Rocaglamides isolated from *Aglaia crassinervia*[11], *Aglaia argentea*[12], *Aglaia abbreviata*[13], *Aglaia edulis*[14], *Aglaia elliptica*[15] and *Aglaia elliptifolia*[16] are reported to have cytotoxic activity against various human cancer cell lines. This potential of the plant is attributed to the presence of flavaglines and benzofurans in the leaf and bark extracts which forms the trigger for apoptosis, thereby down regulating tumorigenesis. The present study revealed the apoptosis inducing ability of *Aglaia malabarica* extract on different rapidly growing cell systems. The treated cells showed prominent apoptotic symptoms like loss of substrate adhesion, formation of apoptotic bodies, chromatin condensation, membrane blebbing and nuclear fragmentation. Several reports show the effect of flavonoids in the extracts as they are capable of influencing several cell functions by cell signal modulation[17], altering proliferation and cytotoxicity in cancer cell lines[18]. The plant *Aglaia malabarica* is having considerable amount of flavonoids in its leaf methanolic extract and hence, the apoptosis inducing ability of plant can be attributed these flavonoids. Thus, the plant owes interest as a potential apoptotic inducer and a possible anticancer agent and thereby becomes the promising part of drug discovery in future.

Acknowledgement: The authors are grateful to the University of Calicut for providing the facilities and Council for Scientific and Industrial Research (CSIR) for providing financial support in the form of Junior Research Fellowship.

Conflict of interest: The authors hereby declare that they have no potential conflict of interest.

REFERENCES

1. Satasook C, Isman MB, Ishibashi F, Medbury S, Wiriyachitra P, Towers GHN, Insecticidal bioactivity of crude extracts of *Aglaia* species (Meliaceae), Biochemical Systematics and Ecology, 1994; 22(2):121-127.

2. Danial NN, Korsmeyer SJ, Cell death: critical control points, Cell, 2004; 116(2):205–219.

3. Green DR, Apoptotic pathways: ten minutes to dead, Cell, 2005; 121(5):671–674.

4. Golstein P, Kroemer G, Cell death by necrosis: towards a molecular definition, Trends in Biochemical Sciences, 2007; 32(1):37-43.

5. Sharma AK, Sharma A, Chromosome techniques: theory and practice, Butterworth-Heinemann (2014).

6. Baker CJ, Mock NM, An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue, Plant Cell, Tissue and Organ Culture, 1994; 39(1):7-12.

7. Bhagyanathan NK, Thoppil JE, Pre-apoptotic activity of aqueous extracts of *Cynanchum sarcomedium* Meve & Liede on cells of *Allium cepa* and human erythrocytes, Protoplasma, 2016; 253(6):1433-1438.

8. Junqueira LC, Carneiro J, Junqueira LC and Carneiro J, Aparelho respiratório, Histologia Básica, 2004; 8:285-300.

9. Maiworm AI, Presta GA, Santos-Filho SD, Paoli SD, Giani TS, Fonseca AS and Bernardo-Filho M, Osmotic and morphological effects on red blood cell membrane: action of an aqueous extract of *Lantana camara*, Revista Brasileira de Farmacognosia, 2008; 18(1):42-46.

10. Brown JM, Attardi LD, The role of apoptosis in cancer development and treatment response, Nature Reviews Cancer, 2005; 5(2):231-237.

11. Su BN, Chai H, Mi Q, Riswan S, Kardono LB, Afriastini JJ, Santarsiero BD, Mesecar, AD, Farnsworth NR, Cordell GA, Swanson SM and Kinghorn AD, Activity-guided isolation of cytotoxic constituents from the bark of *Aglaia crassinervia* collected in Indonesia, Bioorganic Medicinal Chemistry, 2006;14(4):960-972.

12. Omobuwajo OR, Martin MT, Perromat G, Sevenet T, Awang K, Pais M, Cytotoxic cycloartanes from *Aglaia argentea*, Phytochemistry, 1996; 41(5):1325-1328.

13. Zhang F, Wang JS, Gu YC, Kong LY, Triterpenoids from *Aglaia abbreviata* and their cytotoxic activities, Journal of Natural Products, 2010; 73(12):2042-2046.

14. Kim S, Chin Y W, Su BN, Riswan S, Kardono LB, Afriastini JJ, Chai H, Fransworth NR, Cordell GA, Swanson SM, Kinghorn AD, Cytotoxic flavaglines and bisamides from *Aglaia edulis*, Journal of Natural Products, 2006; 69(12):1769-1775.

15. Cui B, Chai H, Santisuk T, Reutrakul V, Farnsworth NR, Cordell GA, Pezzuto JM, Kinghorn AD, Novel cytotoxic 1H-cyclopenta [b] benzofuran lignans from *Aglaia elliptica*, Tetrahedron, 1997; 53(52):17625-17632.

16. Wu T, Liou MJ, Kuoh CS, Teng CM, Nagao T, Lee KH, Cytotoxic and antiplatelet aggregation principles from *Aglaia elliptifolia*, Journal of Natural Products, 1997; 60(6):606-608.

17. Musonda CA, Chipman JK, Quercetin inhibits hydrogen peroxide (H2O2)-induced NF-kappaB DNA binding activity and DNA damage in HepG2 cells, Carcinogenesis, 1998; 19(9):1583-1589.

18. Kuntz S, Wenzel U, Daniel H, Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines, European Journal of Nutrition, 1999; 38(3):133-142.