Research Article

# Antioxidant, Cytotoxic and Apoptotic Activities of Crude Extract of *Alpinia purpurata* on Cervical Cancer Cell Line

**Enock Kiage Oirere[1], Palanirajan Anusooriya[2], Deivasigamani Malarvizhi[2], Chinthamony Arul Raj[2], Velliyur Kanniappan Gopalakrishnan[1,2*]**
[1]Department of Bioinformatics, Karpagam University, Coimbatore, Tamil Nadu, India.
[2]Department of Biochemistry, Karpagam University, Coimbatore, Tamil Nadu, India.
***Corresponding author's E-mail:** vk.gopalakrishnan@karpagam.ac.in

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**ABSTRACT**

*Alpinia purpurata* (Vieill.) K. Schum belongs in the Zingiberaceae family and is a very popular garden plant in India, it possesses moderate antibacterial and anticancer activities. To study the antioxidant, cytotoxic and apoptotic activities of n-hexane leaf extract of *A. purpurata*; antioxidant activity was determined by measuring (i) the scavenging effect of plant extract against 2, 2-diphenyl-1-picryl hydrazyl (DPPH) and 2, 2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS), hydroxyl radical scavenging, superoxide radical scavenging, nitric oxide radical scavenging, hydrogen peroxide radical scavenging, metal chelating activity and (ii) reducing power capacity and ferric reducing power (FRAP). Cytotoxicity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) and acridine orange/ethidium bromide staining to assess the anticancer activity of the crude extract on HeLa cells. The antioxidant activity of the plant extract was found to be close to that of standard ascorbic acid. DPPH radical scavenging activity $IC_{50}$ value was 495 ± 1.43µg/ml and 440± 1.39µg/ml for extract and ascorbic acid respectively. ABTS radical scavenging activity $IC_{50}$ value was 420± 2.26µg/ml and 400 ± 2.10µg/ml for extract and ascorbic acid respectively**.** The reducing power and ferric reducing activity of the extract increased with the increase of their concentrations. In MTT assay, leaf extract inhibited HeLa cells in a dose-dependent manner, showing cytotoxicity with $IC_{50}$ of 41.25µg/ml. Morphological changes observed by fluorescent miscroscopy on the leaf extract treated cells stained with acridine orange/ethidium bromide suggested an apoptotic activity. This study clearly demonstrated that n-hexane leaf extract of *Alpinia purpurata* is a good antioxidant and induces apoptosis in HeLa cells.

**Keywords:** *Alpinia purpurata*, n-hexane, HeLa cell line, antioxidant, cytotoxicity, apoptosis.

## INTRODUCTION

The oxidative stress is one of the major causative factors in the induction of various chronic and degenerative ailments which includes cancer.[1] Folk medicine is used in many places and plants contain a rich source of natural antioxidants that mostly serve as candidates for the development of novel drugs.[2] Among the countless naturally occurring antioxidants carotenoids, ascorbic acid and phenolic compounds have the best performance.[3] They inhibit lipid peroxidation, scavenge free radicals and active oxygen species by proliferating a reaction cycle and chelating heavy metal ions.[4] In the search for sources of natural antioxidants and compounds with radical scavenging activity during the recent years, some have been found such as echinacoside in *Echinaceae* root,[5] anthocyanin,[6] phenolic compounds,[7] water extracts of roasted *Cassia tora*,[8] whey proteins,[9] and thioredoxin *h* protein from sweet potato.[10]

Apoptosis or programmed cell death is a vastly organized process which leads to morphological and biochemical changes including nuclear pycnosis and cytoplasm condensation to produce membrane-bound apoptotic bodies that are phagocytosed by macrophages or adjacent cells. Compounds that suppress or inhibit the proliferation of tumor cells by inducing apoptosis have potential as antitumor agents.[11] Among most common neoplastic diseases affecting women, cervical carcinomais one of them, comes second after breast cancer which is the most prevalent, each year there is approximately half a million new cases worldwide.[12] Hence, the development of chemotherapeutic or chemopreventive agents against cervical cancer is very vital to minimize the cases caused by this disease.

The plants belonging to Zingiberaceae are known to contain medicinal properties.[13,14] A range of essential oils is found in the species of Zingiberaceae.[15] Rhizome extracts of some species of the medicinal Zingiberales are broadly used as food and traditionally as remedy.[16] Leaves of various Zingiberaceae are also used in food flavoring and as indigenous medicine.[17] *Alpinia* species are well known medicinal herbs that have been proven by previous researches to have several effects, namely anti-inflammatory,[18,19] antioxidant, antimicrobial,[20,21] antidermatophytic,[22,23] antinociceptive,[24] hepatoprotective,[25] immunostimulatory,[26] and anticancer[27,28] activities. *Alpinia* is the principal genus in ginger family in which *Alpinia purpurata* (Vieill.) K. Schum is a very popular garden plant in India.[29] The sharp odour of rhizome could enhance appetite, taste and voice. It is also used for treatment of renal diseases, rheumatism, sore throat and headache.[30] The MABA (Microplate Alamar Blue Assay) assay study of the crude ethanolic extract of the various parts of *A. purpurata* depicted the leaf extract to possess the highest activity, followed by the rhizome and flower extracts.[31] The plant has moderate antibacterial and anticancer activities which may be due to the rich phytochemicals in the leaves of *A.*

*purpurata*.[32] The objective of the present study was to assess the cytotoxic and apoptotic effect of n-hexane leaf extract of *Alpinia purpurata* on cervical cancer HeLa cell line and antioxidant activity of the plant.

## MATERIALS AND METHODS

### General Experimental Procedures

The plant powder was exhaustively extracted using soxhlet apparatus and the extract was evaporated to dryness using rotary flash evaporator (Buchi type). Human cervical cancer cell line (HeLa) was purchased from National Centre for Cell Science (NCCS, Pune, India) and were maintained in Dulbecco's modified eagles media (HIMEDIA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypsin-EDTA (HIMEDIA) and Fetal bovine serum (FBS) were purchased from Invitrogen (USA). Organic solvents, silica gels and $CO_2$ incubator were obtained from NEW BRUNSWICK SCIENTIFIC (NBS), EPPENDORF, GERMANY. DNA-binding dyes Acridine Orange and Ethidium Bromide (Sigma, USA). Optical density was read using a micro plate reader (ELISASCAN, ERBA). The stained cells were observed by a fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

### Plant collection and extraction

The leaves of the plant *A. purpurata* were collected from the natural habitats of Kanyakumari district, Tamil Nadu, India. The plant specimen was authenticated by Dr. G.V.S Murthy, Botanical Survey of India, Coimbatore, TNAU Campus, India. A voucher specimen was deposited in the laboratory for future reference (BSI/SC/5/23/10-11/Tech).[33] The voucher specimen was deposited at the herbarium of Karpagam University, Coimbatore. The leaves of *A. Purpurata* were washed thoroughly in tap water, shade dried and powdered. The powder (100g) was exhaustively extracted with n-hexane in the ratio of 1:5 (*w/v*) for 24 hr by using soxhlet apparatus. The extract was evaporated to dryness using rotary flash evaporator (Buchi type).

### Antioxidant Activity

The scavenging activity DPPH free radicals of the n-hexane leave extract of *Alpinia purpurata* was restrained according to the procedure described by Blois.[33] The Nitric oxide was generated by sodium nitroprusside and measured by the GriessIllosvoy reaction by the method of Green.[34] The superoxide scavenging activity of the n-hexane extract of *Alpinia purpurata* was measured by reduction of nitrobluetetrazolium (NBT) method of Fontana.[35] The hydroxyl radical scavenging activity was measured with a slight modification of Elizabeth and Rao.[36] The ability of the n-hexane leave extract of *Alpinia purpurata* to scavenge hydrogen peroxide was determined according to the method of Ruch.[37] The ABTS radical cation scavenging activity was performed with slight modifications described by Re.[38] The chelating of ferrous ions by the n-hexane leave extract of *Alpinia purpurata* was estimated by the method described by Dinis.[39] The Reducing power capacity was evaluated by the modified method of Oyaizu.[40] FRAP assay was used to estimate the reducing capacity of leave extract, according to the method of Benzie and Strain.[41]

### Cytotoxicity Assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] colorimetric assay was used to evaluate the anti-proliferative activity of n-hexane crude extract. This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable tumor cells into an insoluble (dark purple) color formazan product, which can be measured spectrophotometrically after dissolving in dimethylsulfoxide (DMSO). Cellular toxicity of the crude hexane extract from *A. purpurata* leaves on cultured cells was measured using this method.[42] Briefly, 200 µl of cells ($5\times10^4$ cells/ml) were seeded in 96 well microplates and incubated for 24 hr (37°C, 5% $CO_2$ air humidified). Then 20 µl of prepared concentrations of 6.25, 12.5, 25, 50 and 100µg/ml of the sample was added and the cells divided into 6 groups. The samples were initially dissolved in DMSO before being added to the culture media. Control group contained DMSO at the same concentration [0.5% (v/v)] of the treated group. After 24hr of incubation, 20 µl of MTT solution (5 mg/ml in phosphate buffer solution) was added and the plates incubated for another 3 hr. 150 µl of medium containing MTT were then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Optical density was read at 540 nm using DMSO as blank in a micro plate reader (ELISASCAN, ERBA) and the cell percent viability was calculated by the formula [% viability = (OD of Test/ OD of Control) X 100]. The 50% reduction in cell number relative to the control or $IC_{50}$ was established by extrapolation from linear regression of experimental data.

### Apoptosis studies with AO/EB staining method

DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EB) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells.[43] AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. HeLa Cells were added to a final concentration of $5\times10^4$/ml then 0.6ml were seeded at the 6-well plates and incubated for overnight. Cells were left untreated or treated with $IC_{50}$ concentration of 41.25 µg/ml of plant extract.

After being cultured for 24 hrs and 48 hrs, the cells were washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10min.

The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of

fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

### Statistical analysis

All experiments were conducted in triplicate and the data obtained were represented as mean ± standard deviations that were interpreted using Microsoft Office Excel 2007®.

## RESULTS AND DISCUSSION

### Free radical scavenging potential and antioxidant properties

Natural products play vital role in chemotherapy, having contributed a greater percentage in cancer chemotherapeutic pharmaceuticals.[44,45] Agents with capability of inhibiting cell proliferation, inducing apoptosis or modulating signal transduction are currently used for the treatment of cancer.[46] Flavonoids are potent water-soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity. Some of the research evidence suggest that the free radicals induce oxidative damage to biomolecules (lipids, proteins and nucleic acids) and the damage result to various diseases in humans for instance diabetes mellitus, atherosclerosis, ageing, inflammation, cancer, and AIDS.[47]

In this research, the antioxidant and anticancer activity of *A. purpurata* was investigated. There are various methods to determine the antioxidant capacities which differ in terms of their determination principles and experimental conditions.[48] For measuring antioxidant activity, the parameters such as DPPH radical scavenging activity, ABTS radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, nitric oxide radical scavenging activity, hydrogen peroxide radical scavenging activity, metal chelating activity, reducing power capacity and FRAP assay are used. In radical scavenging activity, all the above nine antioxidants show good scavenging activity in concentration dependent manner. The scavenging activity also increases with the increase in the concentration of the extract.

The reducing ability of a compound generally depends on the presence of reductants which have exhibit anti-oxidative potential by breaking the free radical chain, donating a hydrogen atom.[49] The plant extract reduces the most Fe3+ ions in a concentration dependent-manner. The high reducing power is the high absorbance at 700 nm. The reducing power of the extract is compared with Ascorbic acid (Figure 3A). From previous research it was found that plant-derived extracts containing antioxidant principles has also cytotoxicity activity toward tumor cells.[50]

Antioxidant properties, specifically radical scavenging activity, is very crucial due to the depletion and damaging effect to free radicals in living organisms.

The DPPH radical scavenging (%) activity of n-hexane leave extract of *Alpinia purpurata* , compared to standard ascorbic acid, is shown in Figure 1A. The $IC_{50}$ value of n-hexane leave extract of *Alpinia purpurata* and ascorbic acid were found to be 495 ± 1.43µg/ml and 440± 1.39µg/ml respectively.

The plant extract exhibited potent ABTS radical cation scavenging activity in concentration dependent manner with the $IC_{50}$ being 420 ± 2.26µg/ml and the $IC_{50}$ of the standard ascorbic acid was found to be 400 ± 2.10µg/ml (Figure 1B).

The nitric oxide radical scavenging activity of leaf extract of *Alpinia purpurata*, $IC_{50}$ value was 250 ± 1.83µg/ml and for ascorbic acid was 160 ± 1.45 µg/ml (Figure 1C). The superoxide scavenging activity of n-hexane leave extract of the *Alpinia purpurata* was increased markedly with the increase of concentrations (Figure 1D). The half inhibition concentration ($IC_{50}$) of leave extract was 400 ± 2.88µg/ml and ascorbic acid was 300 ± 1.85µg/ml.

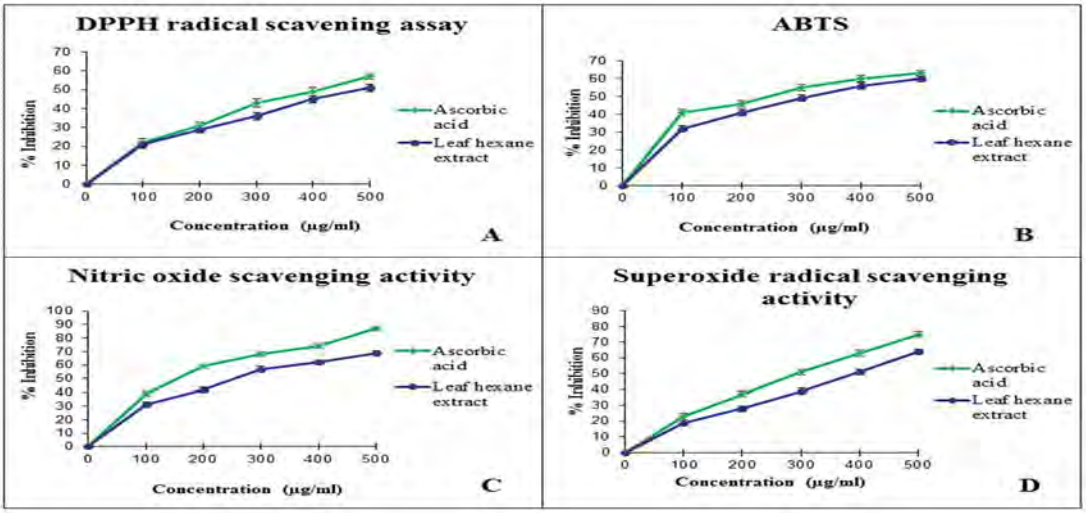


**Figure 1:** (A) DPPH radical scavenging assay; (B) ABTS radical scavenging activity; (C) Nitric oxide scavenging activity; (D) Superoxide radical scavenging activity.

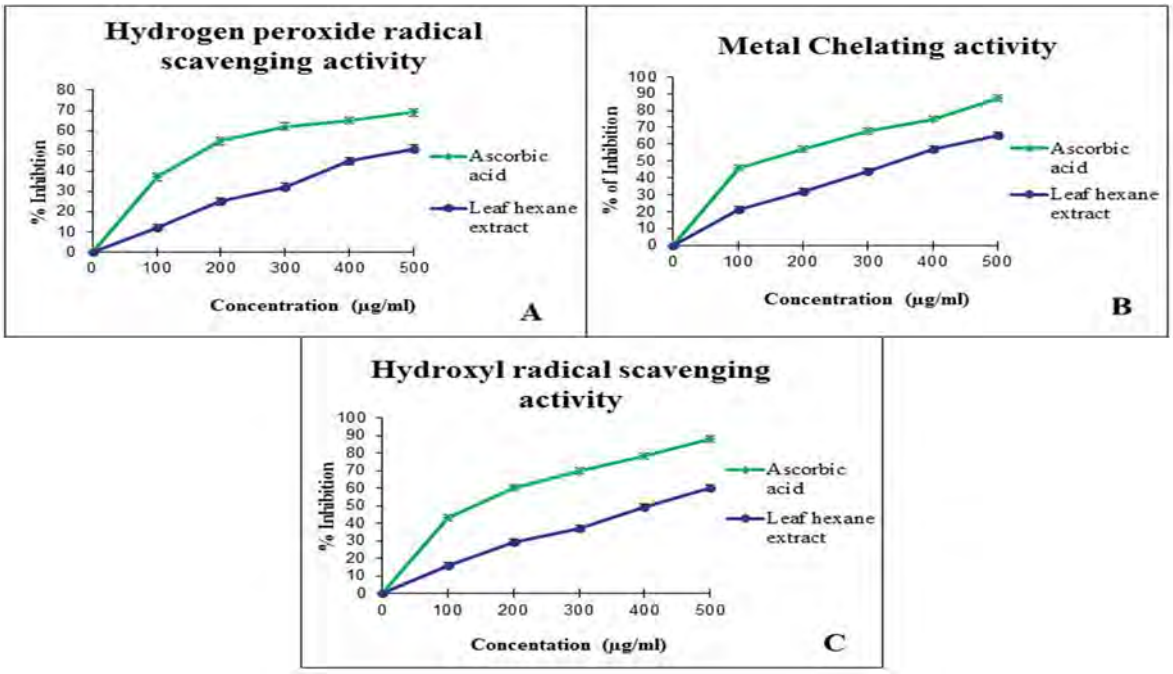


**Figure 2:** (A) Hydrogen peroxide radical scavenging activity; (B) Metal chelating activity; (C) Hydroxyl radical scavenging activity.

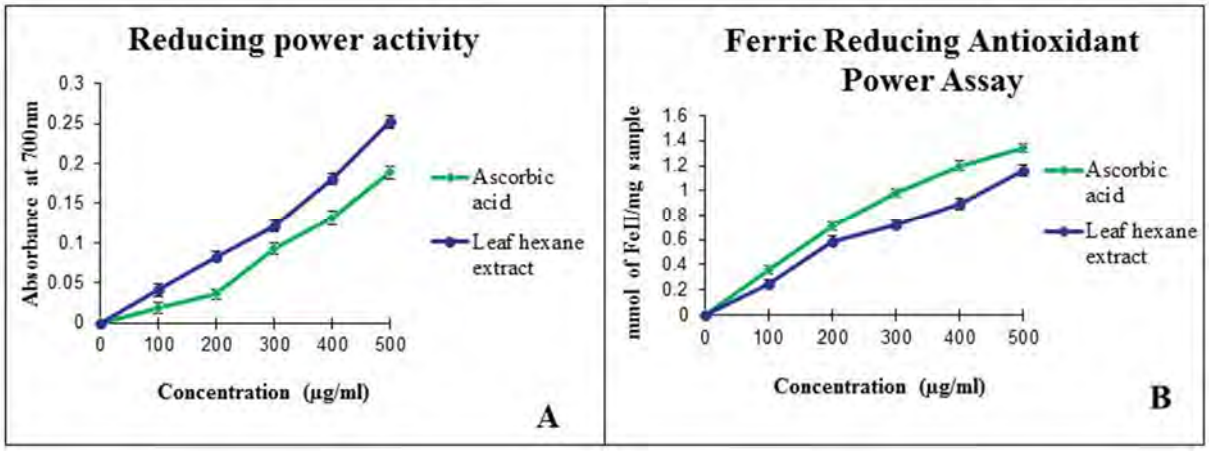


**Figure 3:** (A) Reducing power activity; (B) Ferric reducing antioxidant power assay.

Hydrogen peroxide radical scavenging activity of n-hexane leaf extract of *Alpinia purpurata* increased in dose dependent manner in comparison with the standard ascorbic acid. The $IC_{50}$ value of leaf extract and ascorbic acid were found to be 450± 2.86µg/ml and 350 ± 2.27µg/ml respectively (Figure 2A). The metal chelating activity is shown in figure 2B. The results are expressed as percentage metal chelating activity. Extract exhibited dose dependent metal chelating activity with an $IC_{50}$ value of 355 ± 2.81µg/ml and the $IC_{50}$ of standard ascorbic acid was found to be 265 ± 1.92µg/ml. The hydroxyl radical scavenging activity of the n-hexane leaf extract of *Alpinia purpurata* was found to be effective with the $IC_{50}$ of 410 ± 2.87µg/ml and the standard ascorbic acid was found to be 285± 2.16µg/ml (Figure 2C).

The reducing power potentials of the n-hexane leave extract of *Alpinia purpurata* in comparison with the standard ascorbic acid at 700 nm is explained in figure 3A.

The tendency for ferric ion reducing activities of *Alpinia purpurata* and ascorbic acid is shown in figure 3B. The result indicate that the reducing power and ferric reducing activity of the leaf extract increased with the increase in their concentrations.

### Cytotoxicity and Apoptosis study

Apoptosis induction is the preferred approach in the development of anticancer drugs as it is the most effective way of treating cancer. In this study, the aim was to investigate the cytototoxic effects and if the apoptotic mechanism on HeLa cells is induced by *Alpinia purpurata* extract. The cytotoxicity of *Alpinia purpurata* extract on HeLa was evaluated by MTT assay based on percentage of cell viability. N-Hexane-leaf extract was found to have cytotoxic effect against HeLa, showing cell proliferation inhibition in a concentration-dependent manner. The $IC_{50}$ for *A. purpurata* crude extract was found to be 41.25µg/mL (Figure 4).

The results obtained from the present study showed that *A. purpurata* has an anticancer activity. Apparent hallmark morphologies of apoptosis were observed in treated HeLa cells in dual acridine orange and ethidium bromide staining (Figure 5). Control HeLa cells which were elongated and spindle in shape became disfigured after *Alpinia purpurata* extract treatment. Cell shrinkage with irregular shape, cytoplasmic condensation, nuclear pycnosis and blebbing followed by appearance of apoptotic bodies led to a decrease of the number of viable cells after treatment with the extract for 24 and 48 hrs respectively. Nutrient depletion in growth media or contact inhibition resulting to natural death of cells may be the cause for the appearance of few apoptotic cells in the control HeLa.

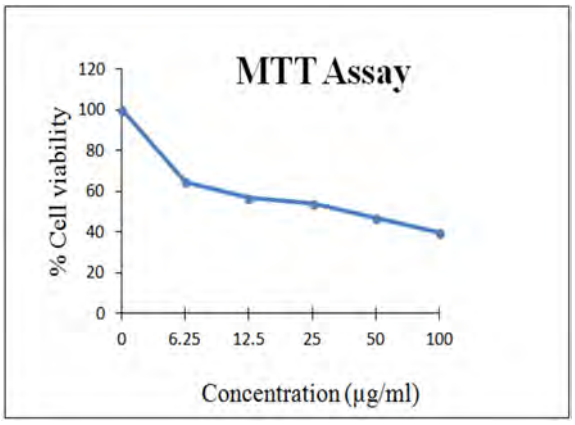


**Figure 4:** MTT analysis, shows n-hexane-leaf extract inhibited HeLa cells in a dose-dependent manner, with cytotoxicity of $IC_{50}$ (41.25µg/ml).

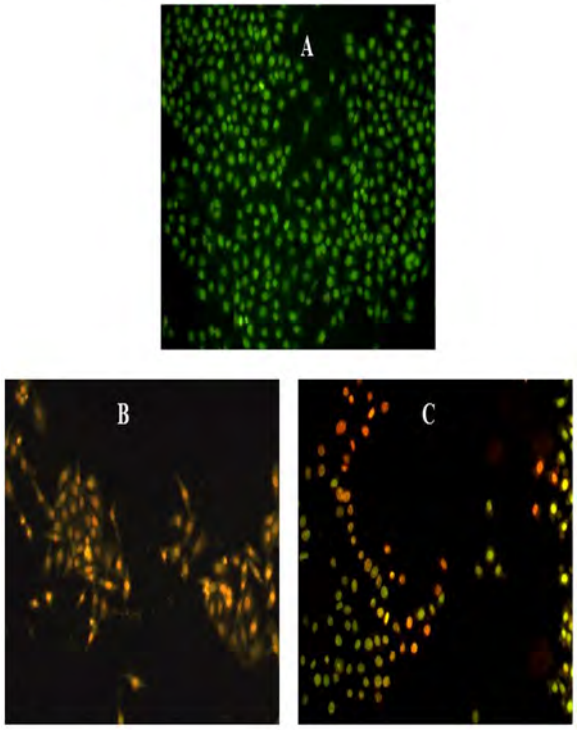


**Figure 5:** AO/EB Staining. For HeLa cells A. Shows the control. B. Treated with extract (41.25µg/ml) for 24hr C. Treated with extract (41.25µg/ml) for 48hr.

## CONCLUSION

In conclusion, this study clearly demonstrated that n-hexane-leaf extract of *Alpinia purpurata* is a good antioxidant and induces apoptosis in HeLa cells. These results suggest that *Alpinia purpurata* that act as an apoptotic inducer could become a potential anticancer agent in development of the drug. Future prospects of this study involve identification and isolation of cytotoxic and apoptotic inducer bioactive compounds from the leaf extract of this plant with further extrapolation on their molecular structures and mechanism of action. More studies can be carried out to determine the cytotoxic and apoptotic effects on several cell lines followed by *in vivo* studies.

### Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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