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Apoptotic Cascade Activation in Breast Cancercells by Holostemma Adakodien Root Extract

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ABSTRACT:

Background and objective: Holostemma adakodien, a plant root extract, has shown potential anticancer activity. The study aims to analyze the anticancer activity of Holostemma adakodien root extract using acetone extract and to determine the mechanism by which Holostemma adakodien induces cell death in cancer cells, specifically through apoptosis.

Materials and Methods: The breast cancer cells MDA MB 231 and MCF - 7 were used for the cytotoxicity assay. The cells were grown in a specific culture medium with fetal calf serum and antibiotics. H. adakodien Acetone fraction, an annexin staining kit, rabbit polyclonal antibodies, and Cytochrome C were purchased for the experiment. Different staining methods were used to characterize apoptotic cell death, including staining of condensed nuclei and annexin binding.Cell viability was assessed using the MTT assay and cell counting was performed using trypan blue dye exclusion assay.

Results: H.adakodien inhibits the growth of human breast cancer cells MDA MB231 and MCF-7 in a concentration-dependent manner. The degree of cell death induction is found to be dose-dependent, ranging from 37.5 to 300 pg/mL. The acetone extract of H.adakodien shows a clear concentration-dependent inhibition of growth in the breast cancer cells.

Treatment with H.adakodien results in morphological alterations, nuclear condensation, and translocation of phosphatidyl serine in the cancer cells.

The cytotoxic effects of H.adakodien involve apoptotic changes, as evidenced by nuclear condensation observed in the treated cells.H-adakodien induces apoptosis, not necrosis, in the breast cancer cells. H.adakodien causes nuclear fragmentation in the cancer cells, a characteristic feature of apoptosis.

Conclusion: The analysis indicates that the treatment of cancer cells with Holostemma adakodien root extract leads to cell death through the activation of the apoptosis cascade.

Keywords: Apoptotic Cascade, Breast Cancer Cells, Flow Cytometry, Cell Death Induction, Nuclear Fragmentation.

1. INTRODUCTION

Apoptosis takes an active part in the development of embryos, the right functioning of the immune system, and the natural turnover of cells during adulthood. However, the deregulation of apoptosis is a major factor in the pathophysiology of several illnesses, including cancer, autoimmune diseases, and neurodegenerative diseases (Thompson,1995). The majority of cancer chemotherapy treatments use extremely cytotoxic medications that specifically target cells that are actively proliferating. However, a lot of researchers have been interested in testing essential oils for their potential to treat cancer in recent years. It has been demonstrated that a range of dietary monoterpenes is highly beneficial for cancer chemotherapy and chemoprevention. For instance, monoterpenes are a novel class of therapeutic agents that have been shown to have both chemopreventive and chemotherapeutic effects in mammary tumor models in a previous report (Marisetti&venkatarathnamma, 2023)

It is commonly acknowledged that tumor cells have a variety of defense mechanisms that enable them to become highly sensitive to internal or external shocks and obstruct the apoptotic machinery's ability to function.
In-depth research is being conducted to identify the variables that may cause dysregulation of apoptosis and to create novel medications that target apoptosis as a therapeutic target. It is evident that there are comparatively small therapeutic windows in cancer treatment, and that tumor cells develop resistance to many chemotherapeutic medications after repeated administration.
The majority of anticancer drugs now on the market cause tumor cells to undergo apoptosis. Two main pathways can be activated to start the induction of the endogenous death machinery (Fang *et al*., 2023). One involves the recruitment of caspase-8 into the death signaling complex as a result of ligands binding to death receptors, such as CD95 and tumor necrosis factor-receptor. The other pathway is mostly regulated at the mitochondrion and is induced by various apoptotic stimuli, including radiation and anticancer drug (Ashkenazi& Dixit, 1998). It has been discovered that Holostemma effectively kills mouse cells. However, there are no studies on the mechanism of cell death induced by *Holostemma.* Recent studies on mouse embryonic fibroblast cells suggest that apoptosis may be involved in the cytotoxicity of certain drugs (Mallikarjuna *et al*.,2011).
The study aimed to investigate whether the cell death induced by *Holostemma adakodien* occurs through apoptosis by Flow cytometric analysis. The flow cytometric analysis indicates that the cell death induced by the treatment of *Holostemma adakodien* involves the activation of the apoptosis cascade.

2. MATERIALS AND METHODS

Study Area
Amala Cancer Research Centre and Rajiv Gandhi Centre for Biotechnology, 2007-2008

Cytotoxicity Assay of Breast Cancer Cells
MDA MB 231 and MCF - 7 breast cancer cells were used for the assays. In a humidified environment with 5% CO_2 at 37 ^{0}C, the cells were cultured in monolayer culture in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal calf serum (Sigma Chemical Company, St. Louis, MO) and antibiotics (100u/mL penicillin, 1000 microgram/mL streptomycin). The acetone portion of H. adakodien. Anannexin staining kit, rabbit polyclonal antibodies, and Cytochrome C (SC 7159 and AIF) (SC.5586) were

purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Staining of Apoptotic Nuclei

i) Apoptotic cell death was characterized by staining the condensed nuclei. Cells after treatments (300 pg/ mL *H. Adakolien* (HA) for 24 hours) were stained with acridine orange (50 ug/mL) and ethidium bromide (5 pg/mL) for 5 minutes at room temperature, and examined by an inverted fluorescence microscope. (LEICA (Wetzlar Germany))
ii) Cells were incubated a t37^{0}C for 15 minutes after staining with Hoechst dye (5pg/mL)

Annexin binding to apoptotic cells

For flow cytometric analysis, the cells were plated on a 60-mm culture dish at a density of 106 cells/dish, and exposed to the drug (300pg/mL HA) for 24 h. The cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS). The cells were stained with FITC-Iabelled annexin using Annexin V-FITC Apoptosis Detection Kit (Sigma) according to themanufacture's instruction, and a flow cytometric analysis was then carried out using a flow cytometer. (BD instrumentation)

Cell viability Assay-MTT

The MTT assay, (3, 4, 5-DimethyIthiazol-2-yl-2, 5-diphenyl tetrazolium bromide), is a commonly used colorimetric method to measure biological proliferation or growth. It is employed to determine the cytotoxicity of various hazardous substances and possible pharmaceutical agents. The test is an efficient and accurate way to measure drug sensitivity in human cells.

Cell counting and Trypan blue dye exclusion assay

Trypan blue dye exclusion assay is one of the methods to assess the viability of cells during counting. Dead cells will take up the dye and are stained blue, whereas viable cells will exclude the dye and fluoresce. By performing counting using trypan blue, a definite number of viable cells can be seeded in each well to get reliable and reproducible data.

Analysis of cell death (Apoptosis) using combined staining method (Acridine Orange/ Ethidium Bromide Staining)

Nuclear condensation is a characteristic phenomenon of apoptotic cell death. Combined staining of acridine orange and ethidium bromide is a widely used method to demonstrate apoptotic nuclei. Both viable and apoptotic cells take acridine orange and stain the cytoplasm green. Ethidium bromide will be taken up only by the apoptotic cells rapidly and stain the condensed nuclei whereas viable cells, due to high cell membrane integrity, do not take up ethidium bromide and are left with only acridine orange staining.

AnnexinVBindingAssay-toIdentifyApoptoticCells

Phospholipid- binding protein annexin V, measuring 35 kDa, binds to phosphatidylserine (PS), a membrane component that is often found on the inside face of cell membranes. PS molecules are translocated to the cell membrane's outer surface early in the apoptotic pathway, where Annexin V can easily bind to them. When coupled with biotin or a fluorochrome like FITC, PE, APC, Cy5, or Cy5.5, Annexin V can be easily identified by flow cytometry in cells that are in the early stages of apoptosis. Unlike assays based on nuclear alterations such as DNA fragmentation, this technique can detect apoptosis earlier. The loss of membrane integrity that follows the last stages of cell death brought on by either necrotic or apoptotic processes is preceded by the staining of Annexin V-FITC.

3. RESULTS

H.adakodien induces apoptosis, not necrosis.

To assess whether the cell death induced by *H.adakodien* involves typical changes encountered during apoptosis, double labelling techniques using annexin V Flous/PI (propidiumiodide) were used to distinguish between apoptotic and necrotic cells. First, examined the cell membrane for alterations in phosphatidyl serine (PS). Annexin V tends to bind PS that is translocated onto the cell surface during the early phases of apoptosis at specific concentrations of calcium and salt. As a result, annexin V-labeled cells that had undergone apoptosis were identified and captured on camera-attached fluorescence microscopy. The addition of PI cannot enter the cells in the early stages of apoptosis when the membrane integrity is intact. MDAMB231 and MCF.7 clearly showed the apoptotic changes on treatment with *H.adakodien* root acetone fraction. The corresponding phase contrast microscopy of control and treated cells were shown in Fig. 1 and 2

Effect of *H.adakodien* root acetone fraction on nuclear fragmentation.

H.adakodien inhibits growth of MDAMB231 and MCF-7 cells (human breast cancer cells) in a concentration-dependent manner. The acetone extract caused a clear-concentration-dependent inhibition of growth of the breast cancer cells MDA MB231 and MCF.7 Cell viability was assayed by reduction of MTT at 48h (Fig. 3) after the addition of various concentrations (37.50 to 300 pg/mL) of *H. adakodien* acetone extract *H.adakodien* acetone extract induces Morphological Alterations, Nuclear Condensation and translocation of phosphatidylserine. The phenotypic characteristics of *H.adakodien* treated cells were evaluated by microscopic inspection of overall morphology.

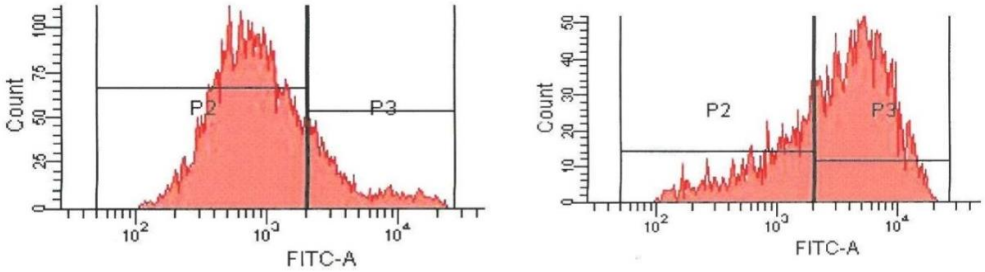
The nuclei of MDA MB231 and MCF.7 cells were stained Hoechst 3342 and assessed by microscopy. Fig.4 indicated that control cells had intact cell nuclei, while *H. adakodien* treated cells showed significant nuclear fragmentation, characteristic of apoptosis whereas the controlled cells showed a decrease in nuclear fragmentation.

The maintenance of complex biological systems' homeostasis and development depends heavily on the process of apoptosis. Apoptotic mechanisms that are dysregulated or fail to function properly can lead to cell transformation and give cancer cells an edge in growth. Cell shrinkage, chromatin condensation, DNA fragmentation, and the activation of certain cysteine proteases are its defining features (Jaiswal *et al*.,2002).

Treatment of cells with 300 pg/mL *H.adakodien* acetone root extract for 24 hours resulted in the formation of nuclear condensation, which was evident in the inverted fluorescent microscopy (Fig. 4). To confirm whether the cytotoxic effects induced by *H.adakodien* root acetone fraction in these cells involve apoptotic changes, cells were examined for characteristic apoptotic patterns.

Up on treatment of cells with different concentrations of *H.adakodien* root acetone fraction, nuclear condensation (examined by staining the cells with ethidium bromide and acridine orange) was visible in MDAMB231 and MCF.7 cells (Fig. 4).

Fig. 5.1 INDUCTION OF APOPTOSIS IN HUMAN BREAST CANCER CELLS BY H. *ADAKODIEN* (AF) MCF-7



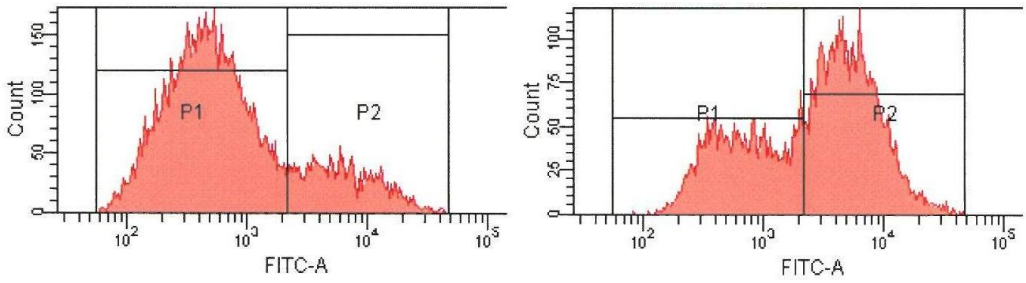
P2	81.50 %
P3	17.70 %

P2	28.7 %
P3	70.4 %

Translocation of phosphatidyl serine (PS) from inner to outer cytoplasmic membrane is a characteristic feature of apoptosis. This externalization exposes PS membrane for annexin binding (conjugated with fluorescein) and can be visualized through a fluorescence microscope or can be sorted using a flow cytometric instrument.

P2 represents annexin negative population and P3 represents annexin positive population. There was a significant increase in the apoptotic population, annexin positive population (from17to70%), after treatment with HA for 24 hours in MCF-7 cells.

Fig. 5.2 INDUCTION OF APOPTOSIS IN HUMAN BREAST CANCER CELLS BY H. *ADAKODIEN* (AF) MDA MB 231



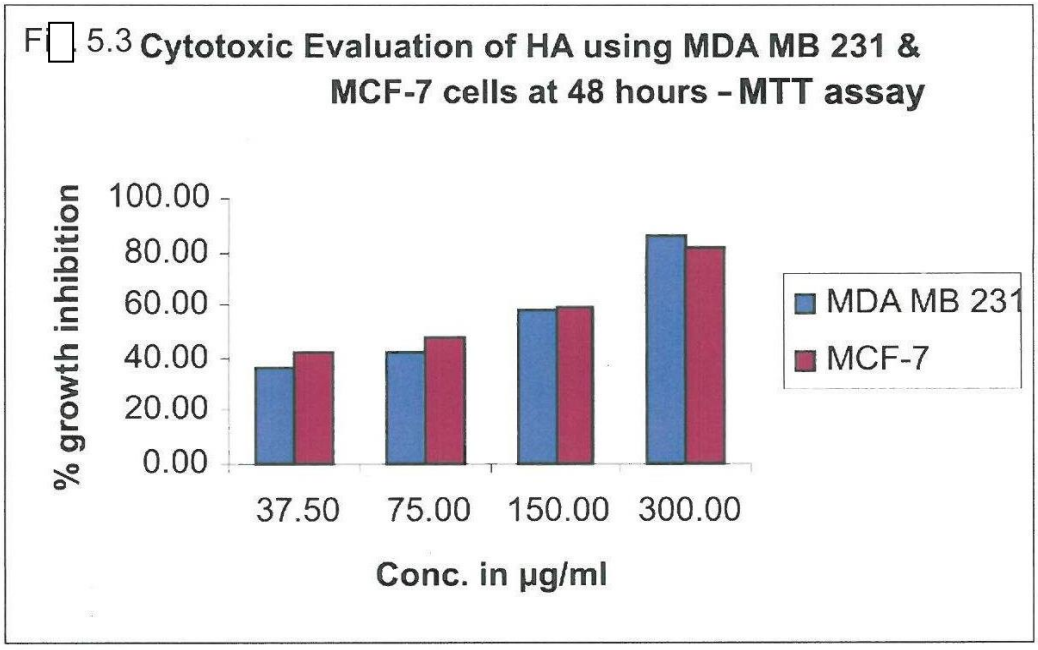
control

P1	80%
P2	20%

HA 300

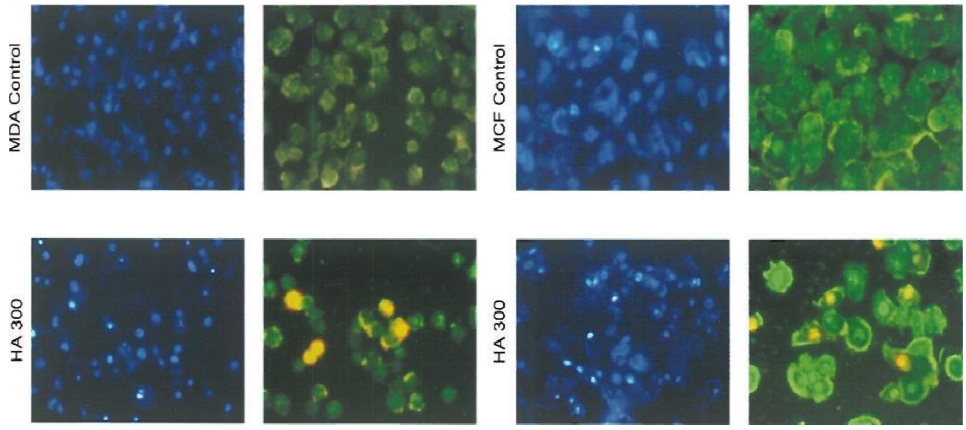
P1	39%
P2	61%

P2 represents annexin negative population and P3 represents annexin positive population. There was a significant increase in the apoptotic population, annexin positive population (from 20to 61%), after treatment with HA for 24 hours in MDAMB 231 cells.



A dose dependant growth inhibition is observed in both the celllines upon treatment with a HA (37.50to300yg/ml)

Fig.4: NDUCTION OF APOPTOSIS IN HUMAN BREAST CANCER CELLS BY H.ADAKODIEN (AF) (MCF-7,MDAMB231)



Staining of apoptotic nuclei using AO/Et Brand Hoechst dye

Hoechst3342AO/EtBrHoechst3342AO/EtBr

i) Cells after treatment with HA were stained with different dyes to evaluate apoptosis.Ethidium bromide is selectively taken up by the apoptotic cells and stained by the condensed nuclei (stained yellow) whereas the control cells have taken only acridine orange (stainedgreen).
ii) Hoechst staining could demonstrate condensed apoptotic nuclei (after treatment with HA300pg/mL) as observed as condensed white dots.

4. DISCUSSION

The present study was designed to investigate the cytotoxicity and antitumor effects of

Holostemma adakodien by determining whether its mechanism of action involves apoptosis. The present set of investigations was therefore initiated to study the apoptotic potential of the acetone fraction of *H.adakodien*.

Apoptosis, a crucial process for maintaining homeostasis and development in biological systems, can be dysregulated, leading to cell transformation and providing cancer cells with a growth advantage. The characteristic features of apoptosis include cell shrinkage, chromatin condensation, DNA fragmentation, and activation of specific proteases. The study findings support the potential of H. adakodien root extract in inducing apoptosis in breast cancer cells, highlighting its possible role in cancer therapy.

This study showed that acetone fraction markedly reduced the cell viability in a concentration-dependent manner, both in MDAMB 231 and MCF-7 cells at 48hrs (Fig. 3).

Fischer and Schulze (2005) suggested that the antioxidant and free radical scavenging potential of *Holostemma adakodien* root tubers through spectrophotometric assays using DPPH and nitric oxide radicals results in significant scavenging activity against these radicals, highlighting the plant's potential as a natural antioxidant source.

One of the leading causes of illness and mortality worldwide is cancer. The desire to discover and creates new, complementary, or synergistic anti-cancer drugs persists despite the enormous advancements in cancer therapies; researchers are beginning to turn more and more towards herbal medicine in light of the adverse effects of chemotherapy medications (Priya *et al*., 2015)

Purifying and analyzing a protease enzyme from *Holostemma adakodien schult* by using various analytical, biochemical, and molecular techniques to study this protease, which had not been previously reported. This study provides valuable information about the characteristics and properties of the protease from *H. adakodien.*

The induction of cell death may be the cause of the suppression of cell proliferation. It is critical to highlight the distinctions between apoptotic and non-apoptotic cell death in the context of cell death. Necrosis, as opposed to apoptosis, is a passive process brought on by acute damage that renders the cell incapable of carrying out its essential energetic processes. Apoptosis, on the other hand, is always a carefully controlled process that is brought on by physiological triggers or environmental harm (Nicotera *et al*., 1998).

Ethyl acetate extract of *Asclepias curassavica* induced apoptosis (programmed cell death) in human cancer cells by activating specific signaling pathways. This study contributes to a better understanding of the molecular mechanisms underlying the therapeutic properties of Asclepiascurassavica as a traditional medicinal plant for tumor treatment (Zheng *et al*., 2019).

In the early stages of apoptosis, while the cell membrane is still intact, phosphatidyl serine to which Annexin V binds specifically is translocated to the extracellular leaflet of the membrane. The flow cytometric data (Fig.1 & 2) revealed that the mode of cell death is apoptosis but not necrosis. This result indicated that the acetone fraction of *H.adakodien* root could induce apoptosis in human breast cancer cells(MDAMB231and MCF7cells).

The effects of the methanolic extract of *Calotropis gigantea* on human breast carcinoma cells. This study is the first to report on the induction of apoptosis in MCF 7 cells by the *Calotropis gigantean* methanolic extract. This study provides insights into the potential therapeutic applications of *Calotropis gigantea* in breast cancer treatment. (Kharat&Kharat, 2019).

pro-apoptotic and pro-autophagic properties of cardenolides from the aerial parts of

Pergularia tomentosa. The researchers suggest that these cardenolides have promising implications for cancer treatment due to their ability to induce programmed cell death and autophagy. This study highlights the potential therapeutic benefits of cardenolides derived from *Pergularia tomentosa* in the field of cancer research (Martucciello *et al*., 2022).

The prevalence of cardiac glycosides in the family's Apocynaceae and Asclepiadaceae within African medicinal plants emphasized the potential therapeutic benefits for the treatment of various conditions, including cancer. The study identifies several cardiac glycosides with known pharmacological properties, including cytotoxicity, antiviral effects, enzyme inhibition, anti-inflammatory and neurotoxicity (Akinwumi&Ambili, 2024).
Active compounds from Asclepias subulata, specifically cardenolides, have demonstrated anti-proliferative effects on human cancer cells. Extracts of A. subulata based on cardenolides, particularly calotropin showed anti-proliferative activity against various cell lines, with the most significant effect observed on the HeLa cell line (Dias- Silva *et al*., 2022).
In the present study, another significant change during apoptosis is the condensation of nuclei and fragmentation of the nucleus. The results with AO/ethidium bromide and also with Hoechst 3342 dye confirmed the induction of apoptosis in human breast cancer cells (MDA MB 231 and MCF.7)(Fig.4). It could be concluded from the above results that acetone fraction of *H.adakodien* root extract inhibits the growth of MDAMB231 and MCF.7 cells in a concentration-dependent manner. The cytotoxic and anti tumor effect of the acetone fraction of *H.adakodien* root is through the induction of apoptosis as demonstrated by the present data.

5. CONCLUSION

In conclusion, the findings of this study indicate that the acetone fraction of Holostemma adakodien root extract has cytotoxic and antitumor effects on human breast cancer cells through the induction of apoptosis. This is evidenced by cell death, nuclear condensation, and fragmentation of the nucleus in treated cells. These findings suggest that the acetone fraction of Holostemma adakodien root extract may have potential as a therapeutic agent for breast cancer treatment. Further research is needed to fully understand the detailed mechanism of action and evaluate its effectiveness in vivo.

Conflict of interest
There is no conflict of interest regarding the publication of this study. The research was conducted with the sole purpose of investigating the anticancer activity of *Holostemma adakodien* root extract and understanding its mechanism of action. No financial or personal relationships with any organization or individual that could potentially bias the results or interpretation of the findings were involved. The study was carried out in an unbiased and objective manner, adhering to ethical guidelines and scientific principles.

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6. REFERENCE

1. Akinwumi, I. A., &Ambali, O. A. (2024).Cardiac Glycosides from African Medicinal Plants as Promising Therapeutics. Tropical Journal of Phytochemistry and Pharmaceutical Sciences, 3(2), 158-167. https://doi.org/10.26538/tjpps/v3i2.1
2. AshkenaziA.and Dixit V. M;1998.Science.281:1305-1308
3. Dias-Silva, M., Velazquez, C., &Vilegas, W. (2022).Antiproliferative activity of standardized herbal phytopreparation from Asclepiassubulata [version 2; peer review: 2 approved. F1000research, 11.https://doi.org/10.12688/f1000research.111181.2
4. Fang, Z., Zhu, Z., Zhuang, Z., Li, Z., Yan, C., Yang, M., ...& Gong, A. (2023). Cascade biomimetic intelligent nanotheranostic agents for imaging-guided tumor synergistic therapy. Nanomedicine, 18(1), 35-52.
5. Fischer, U., & Schulze-Osthoff, K. (2005).Apoptosis-based therapies and drug targets.CellDeath&Differentiation, 12(1),942-961.
6. JaiswalA.S,BloomL.B,NarayanS;2002.Oncogene,21: 5912-592
7. Kharat, K. R., &Kharat, A. S. (2019). The Calotropisgiganteamethanolic extract induces apoptosis in human breast carcinoma cells. Iranian journal of medical sciences, 44(6), 483.
8. Mallikarjuna, B., Nagalakshmi, U. R., Gayathri, D., &Gopal, R. G. (2011). In Vitro Antioxidant And Free Radical Scavenging Potential Of Holostemma Ada-Kodien K. Schum., An Important Rare Medicinal Plant. International Journal of Pharmaceutical Sciences and Research, 2(9), 2413.
9. Marisetti, A. K., &Venkatarathanamma, V. Invitro Anticancer Activity Of Medicinal Plants. IJRDO – journal of applied science, 5(2).
10. Martucciello, S., Paolella, G., Romanelli, A. M., Sposito, S., Meola, L., Cerulli, A., ...& Caputo, I. (2022). Pro-apoptotic and pro-autophagic properties of cardenolides from aerial parts of Pergulariatomentosa. Molecules, 27(15), 4874.
11. Nicotera, P., Leist, M., &Ferrando-May, E. (1998). Intracellular ATP, a switch in the decisionbetweenapoptosisandnecrosis.Toxicologyletters,102,139-142.
12. Priya, V., Jain, P., Vanathi, B. M., Raj, P. V., Kamath, B. V., Rao, J. V., ... &Sivagurunathan, N. (2015). Methanolic root extract of Calotropisgigantea induces apoptosis in human hepatocellular carcinoma by altering Bax/Bcl-2 expression. Am J PharmacolSci, 3, 13-7.
13. ThompsonC.B;1995.Science.267: 1456-62
14. Zheng, X., Xu, Y., Liu, B., Qi, Y., Zhang, Y., Li, X., ...& Wan, C. (2019). Ethyl acetate extract of Asclepiascurassavica induced apoptosis in human cancer cells via activating p38 and JNK MAPK signaling pathways. Evidence-Based Complementary and Alternative Medicine, 2019.